Europ. J. Cancer Vol. 12, pp. 1–8. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research. Autologous Marrow Support and Intensive Chemotherapy in Cancer Patients

J. S. TOBIAS*† and M. H. N. TATTERSALL‡
*Sidney Farber Cancer Center, 35 Binney Street, Boston, Mass.
‡Charing Cross Hospital, Fulham Palace Road, London W6

THE chemotherapy of cancer has improved considerably in the last 10 years. Advances have chiefly resulted from the discovery of new and active agents, more effective use of drugs already available, or by employing new techniques in supportive care which permit more intensive treatment. All anticancer drugs suffer from a lack of selectivity and are toxic to normal as well as malignant cells. The bone marrow is a particularly vulnerable host tissue and myelosuppression is the dose-limiting toxicity for many anticancer drugs. Despite good evidence from experimental animal tumour systems that the dose–response curves of many active anti-neoplastic agents are steep [1, 2] the danger of myelosuppression has prevented attempts to confirm these observations in man.

Bruce *et al.* demonstrated that following administration of many antitumour agents, survival of murine lymphoma cells *in vivo* was inversely proportional to dosage [3]. Cytotoxic drugs could be classed as cycle-specific, phase-specific, or cycle-non-specific, depending upon the type of survival curve in the spleen colony assay (Fig. 1). For cycle-non-specific and cycle-specific drugs there was no limitation to the number of tumour cells killed with increasing dosage. For phase-specific drugs, a dose-response correlation was demonstrated only within certain limits; when the curve was no longer linear, the cytotoxicity of the drug was not increased by higher doses. Many clinically useful drugs, including the alkylating agents, fall into the category of cycle-specific drugs and it is therefore likely that the higher the dose of these compounds, the greater the tumouricidal effect.

In man there is some evidence that high dose alkylating agent therapy is more effective than low dose treatment. In multiple myeloma, treatment with large intermittent doses of melphalan is superior to small daily doses of the same drug [4]. In ovarian and testicular cancers, repeated large doses of alkylating agents are very active in advanced disease, perhaps more so than conventional low dose treatment [5,6]. The value of triethylene thiophosphoramide (thiotepa) in maintenance therapy for patients with ovarian carcinoma appears to be dose-related [7].

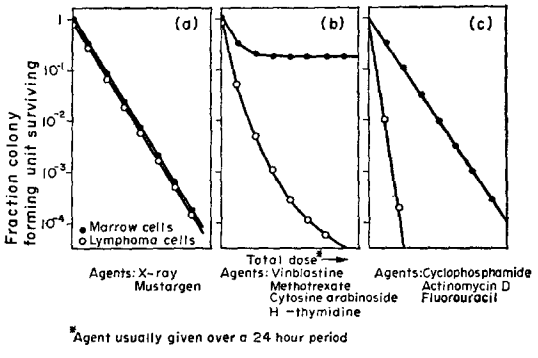


Fig. 1. Dose–survival curves for both normal haematopoietic and transplanted lymphoma colony forming cells.

Infection is the commonest cause of death in cancer patients [8, 9]. The risk of infection

†Present address: Department of Medical Oncology, St. Bartholomew's Hospital, London EC1.

is inversely related to the number of circulating granulocytes and the duration of granulocytopaenia [10, 11]. A number of recent reports have suggested that granulocyte transfusions using cells obtained either with the IBM cell separator or by filtration leukopheresis may improve the prognosis of infected granulocytopaenic patients [12. 13] but prolonged granulocyte support creates severe problems because of difficulty in obtaining donors and the development of leukocyte alloimmunization. A further problem is that granulocytes cannot be stored at present. An alternative approach in protecting granulocytopaenic patients from infection has been to reduce the risk of infection by the use of oral non-absorbed antibiotics and patient isolation in laminar air flow rooms or "life islands". Although these methods appear to be of value [14, 15], the procedures are too expensive and difficult to apply on a large scale.

Reinfusion of stored autologous marrow following intensive chemotherapy is an attractive alternative approach to supportive care for cancer patients. Large numbers of bone marrow cells can be obtained before chemotherapy and in contrast to other methods of patient support with blood products there is no immunological barrier to engraftment. The purpose of this report is to review the present status of autologous marrow support in cancer patients and to outline the problems and potential benefits of this approach.

MARROW PROCUREMENT AND PRESERVATION

Marrow procurement is straightforward and has been described many times [16 18]. In most studies, general anaesthesia has been used and marrow aspirated from the posterior iliac spines and crests. After filtration to remove spicules, marrow cells have been concentrated by centrifugation, the fat removed, and the cells stored either at −80°C or at −150°C. The use of cell separating techniques may allow a more concentrated population of mononuclear cells to be preserved [19]. This offers a theoretical advantage since granulocyte rupture during freezing may lead to lysozyme release and increased cell clumping on thawing.

The discovery of effective cryopreservation agents has made long-term marrow storage feasible. The agents most widely used have been dimethylsulphoxide (DMSO) and glycerol, and although DMSO damages cells kept at room temperature [20], it is probably the agent of choice because of rapid and uniform transport across the cell membrane and because glycerol is hypertonic and must be washed thoroughly from the stored cells before reinfusion. Although DMSO at high dosage causes cataracts in experimental animals, a recent study showed no adverse ophthalmological effects in man [21]. Ashwood-Smith showed that mouse bone marrow preserved in DMSO at −79°C for one month remained capable of rescuing lethally irradiated mice, and that frozen marrow was as efficient as fresh [22]. O'Grady and Lewis [23] compared 12% DMSO with 12% glycerol as cryopreserving agents for murine bone marrow. The marrow was frozen to −100°C and immediately thawed and reinfused into irradiated recipients. The cryopreservative used made no difference to repopulation as measured by the spleen colony technique [24], and the efficiency of fresh and frozen cells was equal. More recently, marrow was stored for lengthy periods either in 12% glycerol or 10% DMSO at −196°C [25]. Proliferative ability was tested by colony assay, and after a storage time of 2 years the marrow remained 80% viable. At very low temperatures, there was no significant difference between glycerol and DMSO, but at the higher temperature of −80°C, glycerol was superior. Buckner *et al.* [26] showed that monkey marrow preserved in 10% DMSO for 6–13 days at −80°C or −170°C was able to repopulate irradiated animals successfully; animals given supportive therapy, but no marrow, died of marrow aplasia within 13 days of irradiation whereas 14 out of 20 animals given autologous marrow survived beyond 60 days with recovery of white cell and platelet counts.

There is less experience with cryopreservation of human bone marrow. Clearly, the kind of experiment just described cannot ethically be performed in man, and the most reliable index of viability of cryopreserved marrow cells employs the colony assay system in artificial media [27]. Other methods such as exclusion of vital dyes and morphological appearances are less reliable [28]. Although human marrow stored in liquid nitrogen retains a normal morphological appearance for at least 2 years [29], no functional assays have been performed on marrow cells stored for this length of time. Gray and Robinson demonstrated that storage up to 14 days was not accompanied by significant loss of colony forming units (CFU-C) [30]. Ragab *et al.* [31] noted a substantial reduction in CFU-C after cryopreservation of leukaemic marrow for periods up to 16 weeks. Extended storage of normal human marrow

appears to result in a rather unpredictable fall in CFU-C [32]. In addition to the choice of cryopreservative and the temperature of preservation, the speed of dilution and pH of the diluting medium after thawing may also be important [19, 33].

EARLY ANIMAL MODELS AND HUMAN EXPERIENCE

Over 20 years ago it was reported that splenic shielding of lethally irradiated mice permitted haematopoietic recovery [34]. In rodents, fresh bone marrow was shown by Lorenz *et al.* [35] to protect against otherwise fatal doses of irradiation, and subsequently, it was shown that fresh marrow could protect against lethal doses of nitrogen mustard [36]. However, it soon became clear that the use of allogeneic marrow in the recipient rendered immuno-incompetent by total body irradiation or large doses of cytotoxic drugs, usually led to graft versus host disease (GVH) which itself was frequently fatal. The twin problems of GVH and marrow preservation dampened enthusiasm for marrow transplantation for the next 10 years.

Despite these difficulties, there were a number of early attempts to use autologous marrow in overcoming drug-induced myelosuppression in patients with cancer. McFarland *et al.* [37] reported on 5 patients with lymphoma who were treated with high doses of nitrogen mustard. Marrow was aspirated before therapy and 2×10^9 nucleated cells were re-infused 4 hr after treatment. Two patients died from acute infection, and in the other 3, there was no evidence that haematopoietic recovery was more rapid than would have been expected. There was a gratifying but transient tumour response in these 3 patients. A further series of patients with head and neck tumours were also treated with intensive nitrogen mustard therapy followed by autologous marrow infusion [38]. In this series, 3 patients given 2 mg/kg of nitrogen mustard but no marrow re-infusion all died of infection during the period of granulocytopaenia. Three patients given the same dosage but receiving autologous marrow all survived.

A much larger series of patients was reported by Kurnick [39]. Bone marrow was stored from 82 patients who were to be treated with chemotherapy or radiation. Re-infusion was carried out whenever leukopaenia or thrombocytopaenia was thought to be life threatening. Eight re-infusions were followed by survival for more than 14 days; at the time of re-infusion, the median peripheral white cell count was 1100/mm^3. Of 9 patients in whom re-infusion was followed by survival for less than 14 days, the median white cell count at re-infusion was 550 cells/mm^3. The patients who survived probably owed their survival to their greater baseline leukocyte count.

Pegg, Humble, and Newton [40] described 19 patients in whom autologous marrow was given after high doses of a variety of cytotoxic agents, including mannomustine, mustine, cyclophosphamide and melphalan. The rapid spontaneous haematological recovery following cyclophosphamide was not improved by re-infusion of autologous marrow, but 3 patients given marrow following large doses of mustine recovered a few days earlier than expected. The nadir of myelosuppression with mannomustine occurs later than cyclophosphamide, recovery taking approximately 2–3 weeks from the point of maximum myelosuppression [41]. Four patients given large doses of mannomustine recovered more rapidly with re-infusion of autologous marrow; recovery of these patients took one week from the point of maximal leukopaenia. There was a suggestion that the recovery time correlated with the number of marrow cells infused, but not with the drug dosage.

In an attempt to confirm that rapid repopulation with transfused marrow cells occurred following cytotoxic chemotherapy, Mayer *et al.* [42] labelled autologous marrow *in vitro* or *in vivo* with tritiated thymidine. In no instance was there clear evidence of early reconstitution when cytotoxic therapy was followed by cryopreserved autologous marrow infusion, nor were radioautographs of the recipient bone marrow or peripheral leukocytes ever positive. Striking rebound leukocytosis and thrombocytosis following treatment was seen in some patients who received no marrow reinfusion. It is now known that marrow kept at $-80°C$ is much less viable than that stored at lower temperatures, so the cryopreservation was clearly less than optimal [26]. At the same time that these human studies were performed, investigations in animals were carried out [43]. Following reinfusion of autologous radiolabelled marrow cells in the rat, a large number of labelled cells was found in the lungs at 1 hr, and labelled cells appeared later in the marrow and spleen. By 30 hr, labelled granulocytes were observed. In dogs, autologous marrow transfusion permitted recovery after a supralethal dose of radiation, though it was not possible to confirm that

the marrow had been repopulated with labelled cells. Presumably, cells which had incorporated radioactive label were either no longer capable of division, or had been destroyed during the freezing process. The ability of transplanted marrow to repopulate the recipient marrow is no longer in doubt; many studies using allogeneic marrow from a donor of the opposite sex or a different red cell type have confirmed chimerism in bone marrow or peripheral blood [44–46].

RECENT STUDIES

Recent studies have tended to concentrate on problems of allogeneic transplantation, since the possiblity of curing patients with aplastic anaemia and refractory acute leukaemia using this technique has shifted interest away from the use of autologous grafting. This shift of emphasis has been amplified by the increasing success of other methods of support for patients with transient myelosuppression. Recent reports on autologous marrow grafting have come from Thomas and his colleagues at Seattle [47] and Santos at Johns Hopkins University. Santos demonstrated that syngeneic transplantation of marrow cells in the rat could convert an LD_{90} dose of cyclophosphamide to an LD_{10} dose or less [48]. Sixty days after transplantation there were no deaths in a group of 10 animals whereas 18 out of 20 animals not given a marrow transplant survived less than 3 weeks; transplantation with allogeneic cells also resulted in significant numbers of deaths. Epstein *et al* [49] also used autologous marrow in dogs made hypoplastic with cyclophosphamide; 10 dogs receiving only supportive therapy all died within 14 days of treatment. However, of 10 dogs given stored autologous marrow 24 hr after the cyclophosphamide, 8 showed rapid haematopoietic recovery and these animals all survived. Studies from the same group using a primate host were less successful [50]. Twenty-five monkeys were given cyclophosphamide in various dose schedules, but no dosage was found which was invariably lethal unless supported by autologous marrow. Spontaneous recovery occurred when less than 200 mg/kg cyclophosphamide was given, whereas beyond 240 mg/kg animals died despite autologous marrow support, as a result of non-haematologic toxicity. Consistent grafting, with evidence of chimerism, could be obtained when animals were prepared with 180 mg/kg cyclophosphamide and then treated with allogeneic marrow, but at this dose, autologous cells were able to "take" but not to hasten spontaneous marrow recovery.

The clinical counterpart of this work was a study by Buckner *et al.* [51] who gave large doses of cyclophosphamide (60–240 mg/kg) to patients with disseminated malignant disease. Stored autologous marrow was re-infused in 6 patients following 120 mg/kg of cyclophosphamide. The marrow was stored at −170°C, using DMSO as a cryopreservative. Patients who received marrow transfusions did not have significantly more rapid recovery than other patients given the same dosage. However, a number of patients had tumour responses and further studies were carried out using high dose cyclophosphamide in patients with carcinomas of the ovary and testis [5, 6]. No marrow support was given in these latter studies, and although tumour responses were encouraging, the degree of response was not unequivocally superior to that seen with more conventional chemotherapy.

In cases of prolonged granulocytopaenia, the possibility of demonstrating early haematopoietic recovery with autologous bone marrow is much greater. Such granulocytopaenia typically occurs with extensive radiotherapy, and patients exposed accidentally to total body irradiation do benefit from transfusion of isologous marrow cells [52]. A recent series by Saenger *et al.* [53] demonstrated a relatively clear-cut improvement in marrow recovery in a large group of patients with advanced cancer given total body irradiation followed by autologous marrow re-infusion. Drugs with extended nadirs of leukopaenia include: BNCU, melphalan, bulsulfan, dimethylmyleran and adriamycin. Autologous marrow re-infusion following dimethylmyleran has been shown to improve haematological recovery in the dog [54]. A recent study has re-examined the value of autologous marrow support in patients undergoing intensive adriamycin/cyclophosphamide therapy [55]. Of 17 patients thus treated, 9 patients were given a very intensive dosage (adriamycin 100 mg/M^2 and cyclophosphamide 1000–2000 mg/M^2) with marrow re-infusion 72 hr following treatment. The study was uncontrolled, although three patients received this dosage without marrow support. Granulocytopaenia was relatively transient and most patients spent only one week with less than 1000 cells/mm^3. In one patient, there was good evidence of a reduction in the duration of granulocytopaenia following autologous marrow re-infusion. In the other patients, the duration of granulocytopaenia following this high dosage with autologous marrow support

was no greater than the duration of granulocytopaenia following more moderate but unsupported treatment with adriamycin (70 mg/M^2) and cyclophosphamide (750 mg/M^2) [56]. Although 75% of the patients became febrile during the intensive course, there were no documented cases of septicaemia and no drug-related deaths. Tumour response was encouraging but there was no clear evidence that partial responses could be converted into complete responses by the administration of one very intensive course of therapy.

A final potential use of cryopreserved autologous marrow is the treatment of drug-resistant leukaemia, when very intensive chemotherapy and/or radiotherapy may be followed by re-infusion of marrow obtained during remission (or during a stable phase of the disease, in the case of chronic granulocytic leukaemia). Buckner *et al.* recently reported two patients with blastic transformation of chronic granulocytic leukaemia treated in this manner [57]. One of these patients achieved engraftment of all but the lymphoid elements of the marrow. Treatment using isogeneic marrow from healthy identical twin donors may be more effective [58].

PROSPECTS FOR STEM CELL SUPPORT

A small number of stem cells are known to circulate in the peripheral blood of animals and man [59, 60]. A number of attempts have been made to use such cells either instead of or as a supplement to marrow stem cells for marrow support. Storb *et al.* cross circulated lethally irradiated dogs with normal dogs. Consistent marrow grafts could be demonstrated after a week of daily cross circulation [61]. More recently, peripheral blood stem cells have been re-infused into animals made aplastic with busulfan and cyclophosphamide, and 6 out of 7 dogs supported in this way survived an LD_{100} [62]. Further studies using the CFU assay system confirm that stem cells from the peripheral blood of dogs and man could be successfully cryopreserved in DMSO for 1 month [63]. In studies by Bruch *et al.*, dogs were leukopheresed for 5 hr, using the IBM cell separator. Mononuclear cells were separated, cryopreserved and reinfused into irradiated animals. The haematological recovery was superior to that of the control group receiving irradiation alone [64]. In man it has been shown that the number of circulating stem cells increases dramatically following chemotherapy [65]. In this study the Haemonetics semicontinuous flow cell separator was used to remove and concentrate mononuclear cells which were then assayed for CFU-C content. In two patients, the number of committed stem cells in the peripheral blood rose at least eight-fold over the 3 weeks following the administration of adriamycin/cyclophosphamide.

CONCLUSIONS

The problems facing the aggressive chemotherapist have not changed over the past 20 years. For most human cancers, it has not been shown that intensive treatment with massive doses of cytotoxic drugs leads to improved cure rates. Nor has it been demonstrated that stored autologous bone marrow can effectively support the patient myelosuppressed by chemotherapy. Autologous marrow infusion suffers from adherent disadvantages quite apart from the doubt regarding its value in reducing the degree or duration of granulocytopaenia. The speed of haemopoietic recovery is proportional to the number of fresh marrow cells re-infused [22], and it has recently been demonstrated that this is also true of cryopreserved cells [66]. In the rat, re-infusion of 1×10^7 mononuclear cells/kg is insufficient to improve recovery following total body irradiation, whereas a dose of 1×10^8 cells/kg significantly reduces the period of granulocytopaenia. In man, the dosage requirement is probably greater [18], although autologous grafting may require fewer marrow cells than allogeneic grafting [67]. It is likely that early studies failed to demonstrate rapid haemopoietic recovery because the dose of cells re-infused was too small. The total number of marrow cells obtained at one harvest is barely adequate for successful re-infusion on one occasion, and although bulk marrow can be aspirated more than once, this has only rarely been done. The number of CFU-C reinfused may well be more crucial than the absolute number of marrow cells. It is possible that pre-treatment of the donor with chemotherapy may increase the number of CFU-C at the time of the stem cell harvest, leading to a greater yield of CFU-C.

The possibility of obtaining large numbers of circulating stem cells by relatively simple pheresis measures makes the concept of autologous stem cell support attractive once more. There is no reason why such support would have to be restricted to a single course of chemotherapy, and this represents a major advantage. If it can be demonstrated that the use of these cells (possibly in conjunction with

marrow stem cells) permits the safe administration of more intensive chemotherapy, then predictions of greater efficacy from aggresive treatment can be more adequately tested in man. As this and other support measures become more sophisticated, there is reason to hope that the more aggressive drug treatment of cancer will prove to be fruitful.

Acknowledgement—J. S. T. received grants from the Cancer Research Campaign and Mental Health Research Fund, and a Berkeley Travelling Fellowship from the University of Cambridge.

REFERENCES

1. H. E. Skipper and L. H. Schmidt, A manual on quantitative drug evaluation in experimental tumor systems. *Cancer Chemother. Rep.* **17,** 1 (1962).
2. A. Goldin, Factors pertaining to complete drug-induced remission of tumor in animals and man. *Cancer Res.* **29,** 2285 Dec. (1969).
3. W. R. Bruce, B. E. Meeker and F. A. Valeriote, Comparison of the sensitivity of normal haematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*. *J. nat. Cancer Inst.* **37,** 233 (1966).
4. D. E. Bergsagel, An assessment of massive dose chemotherapy of malignant disease. *Canad. med. Ass. J.* **104,** 33 (1971).
5. C. D. Buckner, R. Briggs, R. A. Clift, A. Fefer, D. D. Funk, H. Glucksberg, P. E. Neiman, R. Storb and E. D. Thomas, Intermittent high dose cyclophosphamide treatment of Stage III ovarian carcinoma. *Cancer Chemother. Rep.* **58,** 697 (1974).
6. C. D. Buckner, R. A. Clift, A. Fefer, D. D. Funk, H. L. Glucksberg, P. E. Neiman, A. Paulsan, R. Storb and E. D. Thomas, High dose cyclophosphamide for the treatment of metastatic testicular neoplasms. *Cancer Chemother. Rep.* **58,** 709 (1974).
7. M. Hreshchyshyn, Single-drug therapy in ovarian cancer: Factors influencing response. *Gynecol. Oncology* **1,** 220 (1973).
8. V. Rodriguez, G. P. Bodey and E. J. Freireich, Bacterial and fungal infections during cancer chemotherapy. In: *Complications of Cancer Chemotherapy.* (Edited by G. Mathé and R. K. Oldham). Springer-Verlag, New York (1974).
9. E. M. Hersh, G. P. Bodey, B. A. Nies and E. J. Freireich, Causes of death in acute leukemia. A continuous study of 414 patients from 1954 to 1963. *J. Amer. med. Assoc.* **193,** 105 (1965).
10. G. P. Bodey, M. Buckley, Y. S. Sathe and E. J. Freireich, Quantitative relationships between circulating leukocytes and infection in patients with leukaemia. *Ann. int. Med.* **64,** 328 (1966).
11. G. P. Bodey and E. M. Hersh, The problem of infection in children with malignant disease. In: Neoplasia in Childhood. Proceedings of the 12th Annual Clinical Conference at the University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, Texas. Chicago, Edited by Year Book Publishers, Inc. (1969).
12. R. G. Graw, G. Herzig, S. Perry and E. S. Henderson, Normal granulocyte transfusion therapy. *New Eng. J. Med.* **287,** 367 (1972).
13. D. J. Higby, J. W. Yates, E. S. Henderson and J. F. Holland, Filtration leukophoresis for granulocyte transfusion therapy. *New Engl. J. Med.* **292,** 761 (1975).
14. A. S. Levine, S. E. Siegel, A. D. Schreiber, J. Hauser, H. Preisler, I. M. Goldstein, F. Siedler, R. Simon, S. Perry, J. E. Bennett and E. S. Henderson, Protected environments and prophylactic antibiotics. *New Engl. J. Med.* **288,** 477 (1973).
15. S. C. Schimpff, W. H. Greene, V. M. Young, C. L. Fortner, L. Jepsen, N. Cusack, J. B. Block and P. H. Wiernick, Infection prevention in acute nonlymphocytic leukaemia. *Ann. int. Med.* **82,** 351 (1975).
16. D. E. Pegg and N. H. Kemp, Collection, storage, and administration of autologous human bone marrow. *Lancet* **ii,** 1426 (1960).
17. R. E. Wilson, Techniques of human bone marrow procurement by aspiration from living donors. *New Engl. J. Med.* **261,** 781 (1959).
18. E. D. Thomas and R. Storb, Technique for human marrow grafting. *Blood* **36,** 507 (1970).
19. R. S. Weiner, J. S. Tobias and R. A. Yankee, *Biomedicine* (in press).

20. T. R. Henderson, R. F. Henderson and J. F. York, Effects of dimethyl sulfoxide on subunit proteins. *Ann. N.Y. Acad. Sci.* **243,** 38 (1975).
21. R. D. Brobyn, The human toxicology of dimethyl sulfoxide. *Ann. N.Y. Acad. Sci.* **243,** 500 (1975).
22. M. J. Ashwood-Smith, Preservation of mouse bone marrow at −79°C with dimethyl sulfoxide. *Nature (Lond.)* **190,** 1204 (1961).
23. L. F. O'Grady and J. P. Lewis, Stem cell renewal in frozen marrow. *Cryobiology* **5,** 250 (1969).
24. J. E. Till and E. A. McCulloch, A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res.* **14,** 213 (1961).
25. L. O'Grady and J. P. Lewis, The long-term preservation of bone marrow. *Transfusion* **12,** 312 (1972).
26. C. D. Buckner, R. Storb, L. A. Dillingham and E. D. Thomas, Low temperature preservation of monkey marrow in dimethyl sulfoxide. *Cryobiology* **7,** 136 (1970).
27. K. A. Dicke, M. C. G. Platenburg and D. W. van Bekkum, Colony formation in agar: *in vitro* assay for haemopoietic stem cells. *Cell Tiss. Kinet.* **4,** 463 (1971).
28. M. J. Ashwood-Smith, The preservation of bone marrow. *Cryobiology* **1,** 61 (1964).
29. T. I. Malinin, D. E. Pegg, V. P. Perry and C. E. Brodine, Long-term storage of bone marrow cells at liquid nitrogen and dry ice temperatures. *Cryobiology* **7,** 65 (1970).
30. J. L. Gray and W. A. Robinson, *In vitro* colony formation by human bone marrow cells after freezing. *J. lab. clin. Med.* **81,** 377 (1973).
31. A. H. Ragab, E. Gilkerson and S. C. Choi, The cryopreservation of colony-forming cells from the bone marrow of children with acute lymphocytic leukaemia. *Cancer Res.* **34,** 942 (1974).
32. J. M. Goldman, Personal communication.
33. K. A. Dicke and U. W. Schaefer, Preservation of hemopoietic stem cells in primates, in *Cryopreservation of Normal and Neoplastic Cells,* (Edited by R. S. Weiner, R. K. Oldham and L. Schwarzenberg). INSERM Paris (1973).
34. L. L. Jacobsen, E. K. Marks, E. L. Simmons, E. O. Gaston and R. E. Zirkle, Effective spleen protection on mortality following x-irradiation. *J. Lab. clin. Med.* **34,** 1538 (1949).
35. E. Lorenz, C. Congdon and D. Uphoff, Modification of acute irradiation injury in mice and guinea pigs by bone marrow injections. *Radiology* **58,** 863 (1952).
36. T. B. Loc, G. Mathé and J. Bernard, Essais de protection par les cellules hématopoietiques isologues ou homologues contre la toxicité de la méthyl-bis-chloroéthylamine chez la souris. *Rev. franç. Etud. clin. biol.* **III,** 461 (1958).
37. W. McFarland, N. B. Granville and W. Dameshek, Autologous bone marrow infusion as an adjunct in therapy of malignant disease. *Blood* **14,** 503 (1959).
38. P. Clifford, R. A. Clift and J. K. Duff, Nitrogen mustard therapy combined with autologous marrow infusion. *Lancet* **i,** 690 (1961).
39. N. B. Kurnick, Autologous and isologous bone marrow storage and infusion in the treatment of myelosuppression. *Transfusion* **2,** 178 (1962).
40. D. E. Pegg, J. G. Humble and K. A. Newton, The clinical application of bone marrow grafting. *Brit. J. Cancer* **16,** 417 (1962).
41. A. M. Barlow, J. T. Leeming and J. F. Wilkinson, Mannomustine in treatment of leukaemias, polycythaemia and malignant disorders. *Brit. med. J.* **ii,** 208 (1959).
42. L. M. Meyer, T. D. Fliedner and E. P. Cronkite, Autologous bone marrow transfusion following chemotherapy. *Ann. N.Y. Acad. Sci.* **114,** 499 (1964).
43. T. D. Fliedner, E. D. Thomas, L. M. Meyer and E. P. Cronkite, The fate of transfused H^3-thymidine labeled bone marrow cells in irradiated recipients. *Ann. N.Y. Acad. Sci.* **114,** 510 (1964).
44. E. D. Thomas, C. D. Buckner, R. Storb, P. E. Neiman, A. Fefer, R. A. Clift, S. J. Slichter, D. D. Funk, J. L. Bryant and K. E. Lerner, Aplastic anemia treated by marrow transplantation. *Lancet* **i,** 284 (1972).
45. R. Storb, E. D. Thomas, C. D. Buckner, R. A. Clift, F. L. Johnson, A. Fefer, H. Glucksberg, E. R. Giblett, K. G. Lerner and P. Neiman, Allogeneic marrow grafting for treatment of aplastic anemia. *Blood* **43,** 157 (1974).
46. G. Mathé and L. Schwarzenberg, Bone marrow transplantation in France, 1958–73. *Transplant. Proc.* **6,** 335 (1974).

47. E. D. Thomas, R. Storb, R. A. Clift, A. Fefer, F. L. Johnson, P. E. Neiman, K. G. Lerner, H. Glucksberg and C. D. Buckner, Bone marrow transplantation. *New Engl. J. Med.* **292,** 832 (1975).
48. G. W. Santos, Effect of syngeneic and allogeneic marrow transfusion on cyclophosphamide-induced lethality in the rat. *Exp. Hemat.* **10,** 8 (1966).
49. R. B. Epstein, R. Storb, R. A. Clift and E. D. Thomas, Autologous bone marrow grafts in dogs treated with lethal doses of cyclophosphamide. *Cancer Res.* **29,** 1072 (1969).
50. R. Storb, C. D. Buckner, L. A. Dillingham and E. D. Thomas, Cyclophosphamide regimens in rhesus monkeys with and without marrow infusion. *Cancer Res.* **30,** 2195 (1970).
51. C. D. Buckner, R. H. Rudolph, A. Fefer, R. A. Clift, R. B. Epstein, D. D. Funk, P. E. Neiman, S. J. Slichter, R. Storb and E. D. Thomas, High dose cyclophosphamide therapy for malignant disease: toxicity, tumor response, and the effects of stored autologous marrow. *Cancer (Philad.)* **29,** 357 (1972).
52. E. D. Thomas, R. H. Rudolph, A. Fefer, R. Storb, S. Slichter and C. D. Buckner, Isogeneic marrow grafting in man. *Exp. Hematol.* **21,** 16 (1971).
53. E. L. Saenger, E. B. Silberstein, B. Aron, H. Horwitz, J. G. Kereiakes, G. K. Bahr, H. Perry and B. I. Friedman, Whole body and partial body radiotherapy of advanced cancer. *Amer. J. Roentgenol.* **117,** 670 (1973).
54. H. J. Kolb, R. Storb, P. L. Weiden, H. D. Ochs, H. Kolb, T. C. Graham, G. L. Floersheim and E. V. Thomas, Immunologic, toxicologic, and marrow transplantation studies in dogs given dimethylmyleran. *Biomedicine* **20,** 341 (1974).
55. J. S. Tobias, R. S. Weiner, C. T. Griffiths, C. M. Richman, L. M. Parker and R. A. Yankee, Unpublished data.
56. J. S. Tobias, R. S. Weiner, C. T. Griffiths, C. M. Richman, L. M. Parker, M. C. Bowers and R. A. Yankee, Autologous bone marrow transplantation following high dose chemotherapy. *Clin. Res.* **23,** 344a (1975).
57. C. D. Buckner, R. A. Clift, A. Fefer, P. E. Neiman, R. Storb and E. D. Thomas, Treatment of blastic transformation of chronic granulocytic leukemia by high dose cyclophosphamide, total body irradiation and infusion of cryopreserved autologous marrow. *Exp. Hematol.* **2,** 138 (1974).
58. C. D. Buckner, R. A. Clift, A. Fefer, R. Storb and E. D. Thomas, Human marrow transplantation—current status. In: *Progress in Hematology*, Vol. VIII. (Edited by E. B. Brown) New York, Grune & Stratton, 1973.
59. J. W. Goodman and G. S. Hodgson, Evidence for stem cells in the peripheral blood of mice. *Blood* **19,** 702 (1962).
60. P. A. Chervenick and D. R. Boggs, *In vitro* growth of granulocyte and mononuclear cell colonies from blood of normal individuals. *Blood* **37,** 131 (1971).
61. R. Storb, R. B. Epstein, H. Ragde, J. Bryant and E. D. Thomas, Marrow engraftment by allogeneic leokocytes in lethally irradiated dogs. *Blood* **30,** 805 (1967).
62. R. Catchatourian. K. M. Debelak-Fehir and R. B. Epstein, Hematopoietic function assayed by colony-forming units in fresh and cryopreserved peripheral blood. *Abstracts of the Amer. Assoc. of Hematol.*, December (1974).
63. K. M. Debelak-Fehir, R. Catchatourian and R. B. Epstein, Hemopoietic colony-forming units in fresh and cryopreserved peripheral blood cells of canines and man. *Exp. Hematol.* **3,** 109 (1975).
64. C. Bruch, E. Herbst, W. Calvo, P. Huget, H. D. Flad and T. M. Fliedner, Freezing of blood leukocytes for transplantation in lethally irradiated dogs, in: *Cryopreservation of Normal and Neoplastic Cells* (Edited by R. S. Weiner, R. K. Oldham and L. Schwarzenberg), INSERM. Paris (1973).
65. C. M. Richman, R. S. Weiner and R. A. Yankee, Increased circulating stem cells following chemotherapy in man. *Proc. Amer. Soc. clin. Oncology* **16,** 263 (1975).
66. R. S. Weiner, J. S. Tobias, C. M. Richman, B. Brown and R. A. Yankee, Cryopreserved bone marrow transfusion: kinetics of reconstitution. *Proc. Amer. Ass. Cancer Res.* **16,** 161 (1975).
67. D. W. van Bekkum, The double barrier in bone marrow transplantation. *Semin. Hematol.* **11,** 325 (1974).

Europ. J. Cancer Vol. 12, pp. 9–12. Pergamon Press 1976. Printed in Great Britain

Growth of Morris Hepatoma No. 7794A with and without Vitamin B6. Effect of Inoculation Time*

GEORGE P. TRYFIATES
Department of Biochemistry, West Virginia University Medical Center, Morgantown, West Virginia 26506, *U.S.A.*

and

H. P. MORRIS
Department of Biochemistry, Cancer Research Unit, College of Medicine, Howard University, Washington, D.C. 20001, *U.S.A.*

Abstract—*The effect of time of inoculation of tumor cells on the growth of the highly differentiated transplantable Morris hepatoma 7794A under vitamin B6 deficient and control conditions was studied. Groups of weaning Buffalo strain rats were fed* ad libitum *a diet lacking pyridoxine or were pair-fed the same diet supplemented with the vitamin. They were then inoculated intramuscularly in both hind legs with 7794A hepatoma cells at the beginning (to) and after* 7, 15, *and* 31 *days on their respective diets. Animals were sacrificed* 39–49 *days after inoculation, tumors were excised and after removal of connective tissue they were weighed. The average weight of hepatomas grown in animals fed the vitamin deficient diet for the same length of time was always similar and in all cases was less than the average weight of those hepatomas grown in pair-fed (control) animals. The difference between average tumor weights was not statistically significant* ($P < 0{\cdot}5$) *except when inoculation was performed after* 31 *days. The average weight of tumors from pair-fed controls, in this case, was* 3 *times higher over that of tumors grown in animals fed the vitamin deficient diet. The significance level was* $P < 0{\cdot}05$. *The data are interpreted to show that caloric restriction (pair-feeding) allows hepatoma 7794A to grow as during* ad libitum *conditions provided inoculation takes place after* 31 *days of feeding period.*

INTRODUCTION

RECENT studies in our laboratory have shown that lack of dietary pyridoxine brings about reduction in the rate of growth of several Morris hepatoma lines [1] including the highly differentiated, slow-growing transplantable hepatoma 7794A [2]. Results from preliminary tests had previously indicated that this hepatoma could grow normally as during *ad libitum* conditions provided inoculation with tumor cells was performed after pair-feeding for 31 days the pyridoxine sufficient diet. These preliminary observations were further verified with additional experimental data which are reported in this communication with some previous results for comparison. The results show: (*a*) average tumor weights from deficient animals were similar provided the length of the growth period was the same, i.e. time of inoculation was without effect in animals fed the deficient diet; (*b*) average tumor weights from vitamin B6 deficient and pair-fed control

Accepted 15 September 1975.

*This study was supported by USPHS grants CA10729 from the National Cancer Institute, and also by a general research support grant 5SO1 RRO534–13 from the School of Dentistry, (WVU) and award IN-76K from the local branch of the American Cancer Society to one of us (GPT).

animals are not significantly different ($P < 0{\cdot}5$) regardless of whether inoculation was performed at weanling (zero time) or after 7 and 15 days on the respective diets; (*c*) average tumor weights were significantly different ($P < 0{\cdot}05$) when inoculation was performed after 31 days on the respective diets; and (*d*) animal age has no effect on the growth of this hepatoma provided animals are given the deficient diet starting at weanling (time zero). These results verify previous findings regarding dependence of tumor growth on dietary pyridoxine availability [1, 2] and demonstrate further the essentiality of an extended pair-feeding period to the growth of this hepatoma.

MATERIAL AND METHODS

Buffalo strain male weanling rats were fed *ad libitum* pyridoxine deficient or this diet supplemented with the vitamin on a pair-feeding basis [2]. Tests began upon the arrival of animals to Morgantown (time zero). Animals were inoculated intramuscularly in both hind legs with hepatoma 7794A cells, generation 41–1 (1, 2, 3]. At sacrifice, tumors were removed and weighed after careful removal of connective tissue. Additional experimental details have been reported [1–5].

RESULTS

The results are presented in Table 1 and Figure 1. Average tumor, liver and body weights at sacrifice, food consumed, duration of tumor growth, animal weight at inoculation, time of inoculation, type of diet and other details are recorded. A statistically significant difference ($P< 0{\cdot}05$) between average tumor weights from vitamin B6 deficient and pair-fed controls was obtained only when inoculation with tumor cells was performed after feeding the respective diets for 31 days. In all other cases the difference between average tumor weights was not statistically significant ($P < 0{\cdot}5$). Further, regardless of time of inoculation average tumor weights from animal groups fed the deficient diet were similar. The same was found for pair-fed controls except for the group inoculated after 31 days on the supplemented diet.

Figure 1 shows the growth pattern of groups of animals which received the vitamin B6 deficient diet *ad libitum* and the sufficient diet on a pair-feeding basis for 31 days prior to inoculation with tumor cells. As shown, at 31 days (arrow), the deficient group also received pyridoxine for 3 days (see Table 1). This allowed completion of the test by preventing animals from dying as a result of vitamin deficiency. The growth pattern of animals fed the vitamin B6 (+) diet *ad libitum* is also shown for comparison. The growth patterns of the other animal groups (Table 1) were similar to respective patterns reported previously [2]. As shown, a spurt in animal growth (i.e., increase in body weight) resulted upon feeding pyridoxine for three days. In spite of this, however, a plateau in weight gain was subsequently reached indicating that the continued absence of dietary pyridoxine was basically the limiting factor causing the animals to stop growing. Animals, on the other hand, given the vitamin B6 (+) diet *ad libitum* continued to grow. This observation further supports the concept that animals fed the deficient diet need dietary pyridoxine to prevent weight loss and eventually premature death.

It should be mentioned that hepatomas from animals of groups C and D were allowed to grow for only 39 days while those from the other animals were allowed to grow for 47–49 days. This shorter period of tumor growth accounts for the observed lower weight of these hepatomas.

DISCUSSION

The results presented here show that a statistically significant difference between average tumor weights from animals fed the vitamin deficient diet and from pair-fed controls was obtained only if inoculation with

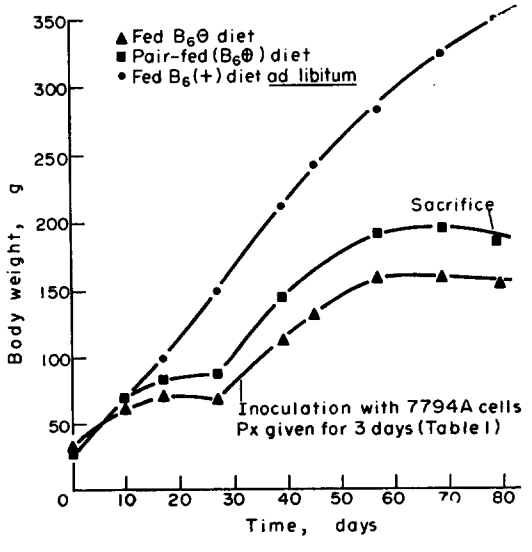


Fig. 1. Growth curves of Buffalo weanling rats fed the pyridoxine deficient diet ad libitum *or the pyridoxine supplemented diet on a pair-feeding or* ad libitum *basis.*

Table 1. *Effect of extending the period of feeding pyridoxine free diet prior to inoculation on the growth of Morris hepatoma 7794A**

Group	Number of animals	Diet†	Days fed before inoculation	Tumor growth (days)	Average weights at death (g)‡			Tumor weight/ body weight	Average diet consumed (g/rat/ 24 hr)	P§
					Body	Liver	Tumor			
A	5(50)‖	I	0	47	94 ± 14(5)	4·0 ± 0·2(5)	0·73 ± 0·2(4)	0·008	8·1 ± 2	—
B	4(50)	II	0	47	123 ± 5·8(4)	3·5 ± 0·5(4)	0·91 ± 0·1(10)	0·007	8·1 ± 2	< 0·5
C	6(64)	I	7	39	98 ± 9(6)	2·9 ± 0·1(6)	0·38 ± 0·1(12)	0·004	8·4 ± 2	—
D	6(64)	II	7	39	123 ± 7(6)	2·7 ± 0·1(6)	0·44 ± 0·1(11)	0·003	8·4 ± 2	< 0·5
E	4(88)	I	15	49	91 ± 6(4)	3·1 ± 0·6(4)	0·71 ± 0·3(6)	0·008	8·2 ± 2	—
F	5(96)	II	15	49	141 ± 7(5)	3·3 ± 0·5(5)	0·81 ± 0·1(9)	0·006	8·2 ± 2	< 0·5
G¶	4(80)	I	31	48	154 ± 7(4)	4·7 ± 0·5(4)	0·78 ± 0·5(5)	0·005	8·4 ± 2	—
H	5(106)	II	31	48	185 ± 1(5)	4·8 ± 0·4(5)	2·4 ± 0·5(8)	0·013	8·4 ± 2	< 0·05

* ± values are se.
†Diests: I is vitamin B6 deficient, ad libitum; II is pyridoxine supplemented [2].
‡Numbers in parentheses are animal, liver, or tumor weights averaged.
§Determined by *t* test. (Ave. tumor wt from B6 (—) group vs. same from pair-fed group).
¶Numbers in parentheses in this column are average body weights at the time of inoculation with tumor cells.
‖After 31 days on the B6 (—) diet, pyridoxine was given for 3 days. Each animal consumed approximately 360 μg of pyridoxine during this period.

tumor cells was performed after 31 days of feeding the animals the respective diets. If inoculation was performed at time zero (start of test), at 7 or 15 days the difference between average tumor weights from pair-fed controls and deficient animals was not significant (Table 1).*

The results of Figure 1 shows clearly a leveling off in average body weight gain of both the group fed the deficient diet and the pair-fed group. This observation is true for the period following feeding the deficient animals with pyridoxine for three days. Although the animals of the control pair-fed group were fed the same diet containing the same amount of pyridoxine as that which the deficient group of animals received for the three days, as mentioned above, the average body weight of these animals also leveled off. It is submitted that the observed plateaus of the growth curves are likely due to lack of sufficient dietary pyridoxine since (*a*) upon feeding pyridoxine for three days following 31 days of depletion a spurt in growth is seen and (*b*) animals fed *ad libitum* the sufficient diet continued to grow. It could be argued that caloric restriction causes the pair-fed animals not to grow. In view of the spurt in growth upon feeding additional pyridoxine for three days, however, it seems that lack of pyridoxine is very likely the cause. Since, tumor growth was not retarded in the pair-fed control group of animals (inoculation after 31 days) but was retarded in the other pair-fed groups (inoculation prior to 31 days) it seems that the limiting factor controlling tumor growth could not be caloric restriction. That availability of dietary pyridoxine is likely the case is also supported by the observation that hepatoma 7777 accumulates as much as 5× pyridoxal phosphate as host liver two weeks after inoculation at which time host liver is practically depleted [6]. Furthermore, it was recently reported [4] that Buffalo strain pair-fed control animals continued to lose liver pyridoxal phosphate up to 50 days old at which time this minimal level is maintained. At this age liver pyridoxal phosphate was less than 40% that found for 29 days old animals [4]. These observations indicate that during early development liver pyridoxal phosphate is probably mobilized and utlized in essential metabolic processes. Therefore, it appears reasonable that a period of 31 days of caloric restriction (pair-feeding) to be necessary for the animal to satisfy its own needs for vitamin B6 and for this hepatoma to grow.

The average tumor weights from animal groups C and D is lower than the average tumor weights of the other animal groups which, correspondingly, were treated in similar manner (Table 1). However, the hepatomas of these two groups grew for only 39 days while all other hepatomas grew for 47–49

*Results from another test (other project) where 11 animals were fed the vitamin B6 deficient diet for 3 weeks, then inoculated at both hind legs with No. 7794A hepatoma cells (av. body wt. 118g) and sacrificed (av. body wt. 183g) 49 days later were: Average weight of 20 hepatomas = 1·2g ± 0·05 (S.E.).

days. Nevertheless, this shorter growth period did not alter the effect of inoculation time of tumor cells on the pattern of tumor growth under pair-fed or deficiency conditions. As was mentioned previously a significant difference between the average hepatoma weights of groups C and D was not observed. Such a difference between average tumor weights was only found when inoculation with tumor cells was performed after 31 days of deficiency (groups G and H, Table 1).

The possibility of considerable impairment of metabolic function resulting from the 31 day depletion period should also not be overlooked [4, 5]. Therefore, in the case of this slow-growing, well differentiated hepatoma it is very probable that impairment in general animal metabolism as a result of deficiency also contributes to the retardation in the growth of this tumor [2]. This would not be the case, however, for fast growing tumors since depletion is of much shorter duration [1, 6].

REFERENCES

1. G. P. Tryfiates and H. P. Morris, Effect of pyridoxine deficiency on tyrosine transaminase activity and growth of four Morris hepatomas. *J. nat. Cancer Inst.* **52,** 1259 (1974).
2. G. P. Tryfiates, J. K. Shuler, M. J. Hefner and H. P. Morris, Effect of B6 deficiency on hepatoma 7794A growth rate: Activities of tyrosine transaminase and serine dehydratase before and after induction by hydrocortisone. *Europ. J. Cancer,* **10,** 147 (1974).
3. G. P. Tryfiates, J. K. Shuler and H. P. Morris, Enzyme activities in vitamin B6-deficient, normal and tumor-bearing animals: Effect of hydrocortisone. *Proc. Soc. exp. Biol. Medicine* **145,** 1363 (1974).
4. G. P. Tryfiates and F. L. Saus, Hormonal induction patterns of tyrosine aminotransferase in normal and cofactor depleted animals. *Cancer Biochem. Biophys.* **1,** 63 (1975).
5. T. Puskar and G. P. Tryfiates, Induction of tyrosine transaminase activity by hydrocortisone in vitamin B6 deficient rats. *J. Nutr.* **104,** 1407 (1974).
6. G. P. Tryfiates, F. L. Saus and H. P. Morris, Hormonal induction of tyrosine aminotransferase activity in host liver and hepatoma #7777 of normal and cofactor-depleted animals. *J. nat. Cancer Inst.* **55,** 839 (1975).

Europ. J. Cancer Vol. 12, pp. 13–18. Pergamon Press 1976. Printed in Great Britain

Cellular and Humoral Response to Immunization with Semi-Allogeneic Hybrid Cells in the Mouse

JACQUES JAMI,* NAZARIO RUBIO and EVELYNE RITZ
Institut de Recherches Scientifiques sur le Cancer, Boîte Postale n°. 8, 94800 Villejuif, France.

Abstract—*Efficiency of semi-allogeneic somatic cell hybrids to induce transplantation immunity against the syngeneic parental tumor has been previously demonstrated in mice. DBA/2 mice inoculated with YC semi-allogeneic cell hybrids (derived from L1210 leukemia cell line of DBA/2 mouse origin) are resistant to subsequent L1210 cell challenge; 129/Sv mice immunized with SIL semi-allogeneic hybrids (derived from a teratocarcinoma cell line of 129 mouse strain origin) become resistant to teratocarcinoma cell graft* [1]. *The aim of the present experiments was to determine whether a syngeneic cellular response would be detected in ^{51}Cr cytotoxic tests using the immunizing conditions sufficient to obtain tumor rejection. The kinetics of cellular and antibody responses of DBA/2 mice inoculated with YC hybrids and the kinetics of 129/Sv mice inoculated with SIL hybrids using hybrid and parental cells as target cells in ^{51}Cr release tests are described. Cellular cytotoxic activity for hybrid and allogeneic parental target cells was found in the two experimental groups, but by contrast, no activity for syngeneic L1210 target cells and a very weak (8%+1) and transient activity for syngeneic teratocarcinoma target cells were demonstrated. This finding suggests that either transplantation immunity tests are more sensitive than ^{51}Cr cytotoxic tests, or that syngeneic tumor rejection requires in addition to the effector cells involved in the immune cytolysis generally detected in ^{51}Cr tests interference of other factors. In sera of the DBA/2 mice examined for immune cellular activity, complement-dependent cytotoxic antibodies against both allogeneic and syngeneic target cells were found.*

INTRODUCTION

IMMUNE prevention of tumor growth in mice can be obtained by inoculating somatic cell hybrids having the related tumor cells as one parent, and allogeneic cells as the other parent. YC somatic cell hybrids derived from fusion of L1210 leukemia cells (from a DBA/2 mouse) and L cells (from a C3H mouse), are rejected upon inoculation into DBA/2 mice; these pre-immunized animals become resistant to subsequent inoculation of the highly malignant L1210 syngeneic cells [1]. Rejection of YC hybrids was expected since hybrid cells express histocompatibility antigens of both parental cells [2]. Rejection of L1210 cells suggested that some L1210-leukemia-associated antigens were also expressed in YC hybrids. In the same way, 129 mice were immunized against a syngeneic teratocarcinoma by 129-teratocarcinoma X C3H-L hybrids [1]. Protection of mice against Ehrlich ascites tumor and against a polyoma-induced tumor were also reported [3–6]. In many experimental systems, an immune response to weak cellular iso-antigens was obtained only immunizing animals with cells bearing, in addition, strong antigens [7–10], e.g., H-2 alloantigens. In the same way, efficiency of hybrid cells in immunizing mice against syngeneic tumors from which hybrids were derived is possibly correlated with the presence of allogeneic histocompatibility antigens contributed by the other parental cell.

Accepted 12 August 1975.

*Chargé de Recherche à l'Institut National de la Santé et de la Recherche Médicale.

There is considerable evidence that sensitized lymphoid cells participate in allograft rejection. Similar mechanisms are thought to be also involved in tumor immunity (see references 11 and 12). Release of ^{51}Cr from labeled target cells incubated in the presence of lymphocytes from recipient animals is a simple and reliable system to show easily direct cytotoxic activity of lymphocytes implicated in allograft rejection [13], as well as in syngeneic tumor rejection [14, 15].

Since mice immunized with semi-allogeneic somatic cell hybrids subsequently reject the related syngeneic parental tumor, they were examined for cellular cytotoxic activity against this tumor. This system allows us to compare cytotoxic activity of lymphoid populations *from the same animals* against syngeneic, allogeneic, and semi-allogeneic target cells, i.e., hybrids and their parental cells. While allogeneic cellular response was readily demonstrated, it was not possible to detect clear syngeneic cellular activity correlated with the resistance of immunized mice to syngeneic tumor cells.

MATERIAL AND METHODS

Cells

The origin and description of Cl1D fibroblastic cells, of L1210 leukemic cells, of SIKR teratocarcinoma cells, and of SIL (Cl1D × SIKR) and YC (Cl1D × L1210) hybrid cells were reported elsewhere [16, 17]. Cl1D cells were derived from a C3H mouse ($H2^k$), L1210 cells from a DBA/2 mouse ($H2^d$), and SIKR from a 129 strain mouse ($H2^b$).

Culture media

All cells were propagated in culture and inoculated to mice in Dulbecco's modified Eagle's medium with 10% calf serum. For cell cytotoxic assays, 10% fetal calf serum was employed. Assays for antibody-mediated cytotoxicity were made with Hanks' solution supplemented with 4% fetal calf serum free of gamma-globulin (HIPT). For inoculation and cytotoxic assays, cells were dispersed in 0·02% Versene (except L1210 cells which grow in suspension cultures) and rinsed in Dulbecco's phosphate buffered saline.

Mice

Experiments were performed with 3-month-old male and female DBA/2 mice obtained from the Centre de Sélection et d'Elevage d'Animaux de Laboratoire du Centre de la Recherche Scientifique, 45 – Orléans, France. 129/Sv mice (18) were bred in our Institute.

Immunization

DBA/2 mice were inoculated subcutaneously (sc) with $2{\cdot}5 \times 10^6$ Y2C hybrid cells, and 129/Sv mice with 3×10^6 SIL1 hybrid cells (day 0). Control groups of both DBA/2 and 129/Sv mice were injected sc with growth medium. Other DBA/2 recipients were inoculated sc with $2{\cdot}5 \times 10^6$ L1210 cells X-irradiated with 3000 R.

Blood and spleens from 3 mice in each group were taken daily. Sera were heat-inactivated 30 min at 56°C and stored at −80°C until tested.

Preparation of lymphoid cells

Spleens from sensitized and control mice were removed, finely minced in medium with scissors and gently pressed through two layers of 15 μm nylon filter cloth to obtain single cell suspensions. Cells were washed, counted and tested for viability by trypan blue exclusion, and adjusted to 1×10^7 cells/ml growth medium.

Labeling of target cells

Cl1D, SIKR, L1210, SIL1 and Y2C cells were washed in 0·02 Versene solution. Suspensions of 5×10^6 cells/ml of Versene solution containing 100 μCi of radioactive sodium chromate (^{51}Cr specific activity = 100–300 μCi/μg chromium, 2000 μCi/ml) were incubated 30 min in a water bath at 37°C with frequent shaking. After centrifugation, cells were washed twice in 30 ml medium, counted and adjusted to 1×10^5 viable cells/ml.

Assays

Cellular cytotoxic activity was determined following the technique of Brunner *et al.* [13, 19] slightly modified. 1×10^7 lymphoid cells from immunized and control animals and 1×10^5 ^{51}Cr-labeled target cells were mixed in 2 ml medium in plastic tubes and incubated in a 5% CO_2 atmosphere for 24 and 48 hr. Assays were made in triplicate. The tubes were centrifugated at 800 ***g*** for 10 min. The radioactivity of 1 ml of the supernatants and that remaining in the original tubes were counted separately in a well-type gamma counter. The percentage of ^{51}Cr released in the supernatant in each tube was calculated. Results were expressed as cytotoxicity (%) in experimental tubes minus cytotoxicity (%) in control tubes, and were corrected for maximal release of ^{51}Cr (100%). Maximal release was calculated from counts

after 3 cycles of freezing and thawing of aliquots of labeled cells. All experiments were repeated at least twice. Cytotoxic titers of sera were determined by incubating 0·1 ml of serial 2-fold dilutions of heat-inactivated mouse serum with 0·05 ml of ^{51}Cr labeled target cells ($2{\cdot}5 \times 10^4$ cells) and 0·1 ml of 1:10 dilution of rabbit serum as a source of complement, for 45 min at 37°C. Then 1·8 ml of cold HIPT was added to each tube, the cells were centrifuged at 800 *g* for 10 min, and the radioactivity of 1 ml of the supernatants and that remaining in the original tubes were counted to determine the percentage of ^{51}Cr released in each tube. Complement controls were made in triplicate. Cytotoxic index was expressed as cytotoxicity (%) in experimental tubes minus cytotoxicity (%) in complement control tubes, and corrected for maximal ^{51}Cr release by freezing and thawing cells 3 times. The titer of the serum is the reciprocal dilution that produced 50% of the maximum release above complement control with that antiserum.

RESULTS

1. *Immune spleen cell cytotoxicity of DBA/2 mice at different times after immunization*

DBA/2 mice were inoculated sc with $2{\cdot}5 \times 10^6$ Y2C cells (day 0). Spleen lymphocytes were tested daily for their cytotoxicity against Y2C hybrid, allogeneic Cl1D, and syngeneic L1210 parental cells. Each determination was performed using a pool of cells from 2 (experimental) or 3 (control) mice. Results of one experiment are shown in Fig. 1(A). A peak of cytotoxicity was found with Y2C target cells (38% ± 3) as well as with Cl1D target cells (52% ± 2) between 8–10 days, with a maximum by day 9. Cytotoxicity levels for Y2C cells were always slightly below those for Cl1D cells (Fig. 1(A)). Cytotoxicity for Y2C and Cl1D cells declined rapidly, but clear activity remained until day 17. No detectable activity for L1210 cells was demonstrable (3% ± 3). Thus, after 1 immunizing inoculation, an immune response was detected only against allogeneic or semi-allogeneic cells. Similarly, immunization of DBA/2 mice with X-irradiated L1210 cells did not provide a clear response either (4% ± 2) (Fig. 1(B)).

DBA/2 mice immunized by 6 inoculations of Y2C cells, and having rejected subsequently a challenge dose of 5×10^4 L1210 cells, were boostered 3 months later with 3×10^6 Y2C cells injected intraperitoneally (i.p.). Spleens were removed 3, 7, and 25 days after and lymphocytes tested for their cytotoxicity. A slight activity was found against allogeneic cells after 3 and 7 days, but again clear activity for syngeneic cells was absent (Table 1).

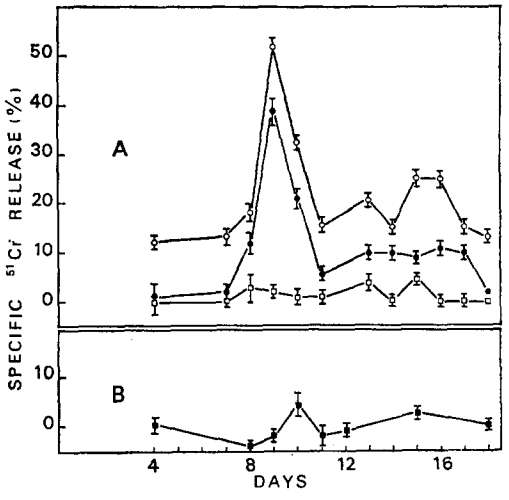


Fig. 1. Evolution of spleen lymphoid cell cytotoxicity of DBA/2 mice immunized s.c. at day 0 with: A: $2{\cdot}5 \times 10^6$ Y2C hybrid cells B. $2{\cdot}5 \times 10^6$ X-irradiated L1210 cells. Target cells: ● Y2C hybrids, ○ Cl1D and □ L1210 parental cells. ■ L1210 cells.

2. *Kinetics of cell cytotoxicity of 129/Sv mice immunized with SIL1 hybrid cells*

Spleen lymphocytes from 129/Sv mice inoculated sc with SIL1 cells were tested against ^{51}Cr-labeled SIL1 hybrid, Cl1D and SIRK parental cells. Results of one experiment are shown in Fig. 2. A cytotoxic activity was found against SIL1 and Cl1D target cells by day 7 after inoculation, with a peak at day 9. A slight activity against SIKR syngeneic cells (8% ± 1) was present by day 7, and disappeared by day 9.

Eight SIL1-immunized mice that had resisted to SIKR tumor challenge inoculation were boostered 8 months later with 5×10^6 SIL1 i.p. Ten days later, two determinations were made, each with spleens pooled from 4 mice. Allogeneic activity was found higher (50% ± 3) than after a single immunization, and a slight syngeneic activity (8% ± 1) was present.

3. *Humoral response after immunization of DBA/2 mice*

The sera of the DBA/2 mice used in the above experiments (§1) were examined for complement-dependent cytotoxic antibody against Y2C, Cl1D and L1210 target cells. Following a single s.c. immunization with $2{\cdot}5 \times 10^6$ hybrid

Table 1. *Lymphocyte cytotoxicity* (%) *of DBA/2 mice successfully immunized against* 5×10^4 *L1210 cells* and having been boostered with* 3×10^6 *YC2 i.p.*

Day after booster		Target cell		
		L1210	Cl1D	Y2C
3	experiment 1	1(± 2)	10(± 1)	28(± 2)
	experiment 2	3(± 1)	11(± 3)	14(± 1)
7	experiment 1	7(± 3)	6(± 1)	25(± 1)
	experiment 2	3(± 1)	7(± 1)	15(± 2)
25		0(± 2)	0(± 2)	1(± 1)

*1 month after the first s.c. immunization, mice were inoculated i.p. with 3×10^6 Y2C cells 4 times at 8-day intervals, and then were challenged s.c. with 5×10^4 L1210 cells 7 days later. 50% mice developed tumors and died. Those mice without tumors 90 days after the challenge inoculation were boostered with 3×10^6 Y2C cells i.p. and spleens were collected for lymphoid cell cytotoxic assays.

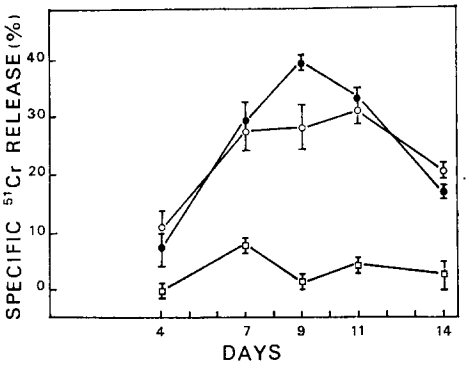


Fig. 2. *Evolution of spleen lymphoid cell cytotoxicity of* 129/*Sv mice immunized s.c. at day* 0 *with* 3×10^6 *SIL1 hybrid cells. Target cells:* ● *SIL1 hybrids,* ○ *Cl1D and* □ *SIKR cells.*

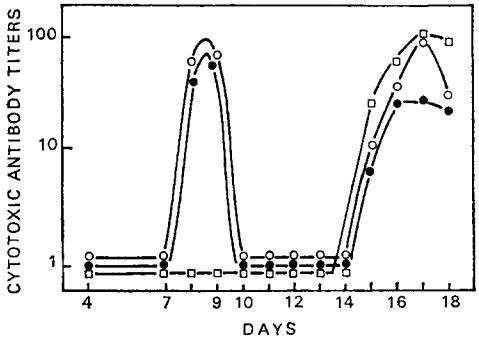


Fig. 3. *Kinetics of humoral complement-dependent cytotoxic antibodies of DBA/2 mice immunized s.c. at day* 0 *with Y2C hybrid cells. Target cells as in Fig.* 1.

cells, cytotoxic antibodies for Y2C and Cl1D cells were first detected at day 8 to peak titers of 100, fell rapidly to undetectable levels at day 10 and rose again by day 15 to reach a peak on day 17. For L1210 syngeneic cells, no cytotoxicity was detectable until day 14. On day 15, a sharp peak appeared and titers remained high at day 18 (Fig. 3).

DISCUSSION

Efficiency of semi-allogeneic hybrid cells to protect hosts of the inbred line of mice against challenge inoculation of the syngeneic parental tumor has been demonstrated [1–6]. In the experiments reported here, mice inoculated with hybrid cells were examined for spleen lymphoid cell cytotoxicity and cytotoxic antibodies against hybrid and parental cells, particularly the syngeneic tumor. Allogeneic response with hybrid and allogeneic parental target cells was found [14, 15, 19–21] but by contrast, no clear syngeneic response was demonstrated within 18 days after a single immunizing inoculation in DBA/2 mice with L1210 target cells, and a very weak (8% ± 1) and transient cellular activity in 129/Sv with SIKR target cells. Spleen cells from immunized DBA/2 mice selected by their survival to challenge inoculation of L1210 cells did not show any clear syngeneic cytotoxic activity either. However, L1210 cell-antigenic components were actually present in Y2C hybrids, as indicated by the demonstration of H-2^d antigens on Y2C hybrid cells [22] and of antibodies cytotoxic for L1210 target cells in sera of the DBA/2 mice examined for cellular immunity (see Fig. 3). Therefore, though immunizations were performed across a strong histocompatibility barrier, cytotoxic action against syngeneic tumor cells *in vitro* was very weak or absent, as found in syngeneic systems by others [23–25].

Inability to detect *in vitro* the direct cellular cytotoxic action on syngeneic tumor cells is probably not the result of a technical failure, since the cytotoxic action on the allogeneic parent and on the hybrid cells was readily

demonstrated in the same experiments. On the other hand, specific resistance can be transferred to untreated mice by spleen and lymph node cells from mice rendered specifically immune to syngeneic tumor cells [23, 25]. The discrepancy between the ability of immunized mice to reject syngeneic tumor cells and the absence of direct cytotoxicity of their lymphoid cells *in vitro* suggests that syngeneic tumor transplantation immunity could involve in addition to the lymphoid cells explored in our ^{51}Cr release tests interference of other factors. Experiments reported by Evans and Alexander [25] suggest that specific resistance requires to be expressed interaction between immune lymphoid cells and macrophages.

REFERENCES

1. J. Jami and E. Ritz, Expression of tumor-specific antigens in mouse somatic cell hybrids. *Cancer Res.* **33,** 2524 (1973).
2. R. A. Spencer, T. S. Hauschka, D. B. Amos and B. Ephrussi, Co-dominance of isoantigens in somatic hybrids of murine cells grown *in vitro*. *J. nat. Cancer Inst.* **33,** 893 (1964).
3. L. Chen and J. F. Watkins, Evidence against the presence of H2 histocompatibility antigens in Ehrlich ascites tumour cells. *Nature (Lond.)* **225,** 734 (1970).
4. R. Favre, J. Filipczyk, M. Berebbi and G. Meyer, Pouvoir immunoprotecteur d'une souche hybride contre l'inoculation de la souche tumorale parentale. *Ann. Immunol. (Inst. Pasteur)* **125C,** 491 (1974).
5. G. Klein and H. Harris, Expression of polyoma-induced transplantation antigen in hybrid cell lines. *Nature (Lond.)* **237,** 163 (1972).
6. J. F. Watkins and L. Chen, Immunization of mice against Ehrlich ascites tumour using a hamster/Ehrlich ascites tumour hybrid cell line. *Nature (Lond.)* **223,** 1018 (1969).
7. D. B. Amos and F. D. Day, Passive immunity against four mouse leukoses by means of isoimmune serum. *Ann. N.Y. Acad. Sci.* **64,** 851 (1957).
8. P. A. Gorer and D. B. Amos, Passive immunity in mice against C578L leukosis E.L.4 by means of isoimmune serum. *Cancer Res.* **16,** 338 (1956).
9. G. Klein, H. O. Sjogren and E. Klein, Demonstration of host resistance against isotransplantation of lymphoma induced by the Gross agent. *Cancer Res.* **22,** 955 (1962).
10. B. Slettenmark and E. Klein, Cytotoxic and neutralization tests with serum and lymph node cells of isologous mice with induced resistance against Gross lymphomas. *Cancer Res.* **22,** 947 (1962).
11. J. C. Cerottini and K. T. Brunner, Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Advanc. Immunol.* **18,** 67 (1974).
12. R. B. Herberman, Cell-mediated immunity to tumor cells. *Advanc. Cancer Res.* **19,** 207 (1974).
13. K. T. Brunner, J. Mauel, J. C. Cerottini and B. Chapuis, Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology* **14,** 181 (1968).
14. J. C. Leclerc, E. Gomard, J. Pavie and J. P. Levy, Evolution de l'activité cytotoxique des lymphocytes immuns en réponse à une greffe de cellules leucémiques chez la souris. *C.R. Acad. Sc. (Paris)* **274,** 1233 (1972).
15. J. C. Leclerc, E. Gomard and J. P. Levy, Cell mediated reaction against tumors induced by Oncornaviruses. I. Kinetics and specificity of the immune response in murine sarcoma virus (MSV)-induced tumors and transplanted lymphomas. *Int. J. Cancer* **10,** 589 (1972).
16. J. Jami, C. Failly and E. Ritz, Lack of expression of differentiation in mouse teratoma-fibroblast somatic cell hybrids. *Expt. Cell Res.* **76,** 191 (1973).
17. J. Jami and E. Ritz, Nonmalignancy of hybrids derived from two mouse malignant cells. I. Hybrids between L1210 leukemia cells and malignant L cells. *J. nat. Cancer Inst.* **51,** 1647 (1973).
18. L. C. Stevens and J. A. Mackensen, Genetic and environmental influences on teratocarcinogenesis in mice. *J. nat. Cancer Inst.* **27,** 443 (1961).
19. K. T. Brunner, J. Mauel, H. Rudolf and B. Chapuis, Studies of allograft immunity in mice. I. Induction, development and *in vitro* assays of cellular immunity. *Immunology* **18,** 501 (1970).

20. J. L. Biesecker, Cellular and humoral immunity after allogeneic transplantation in the rat. I. Cellular and humoral immunity as measured by a ^{51}Cr cytotoxicity assay after allogeneic tumor and renal transplantation. *Transplantation* **15,** 298 (1973).
21. S. Denham, C. K. Grant, J. G. Hall and P. Alexander, The occurrence of two types of cytotoxic lymphoid cells in mice immunized with allogeneic tumour cells. *Transplantation* **9,** 366 (1970).
22. N. Rubio, Surface H2 antigen concentration requirement of somatic hybrid cells for IgM-mediated cytotoxicity. *Nature* (*Lond.*) **249,** 461 (1974).
23. P. Alexander, D. J. Connell and Z. B. Mikulska, Treatment of a murine leukemia with spleen cells or sera from allogeneic mice immunized against the tumor. *Cancer Res.* **26,** 1508 (1966).
24. P. Alexander, Immunotherapy of leukemia: the use of different classes of immune lymphocytes. *Cancer Res.* **27,** 2521 (1967).
25. R. Evans and P. Alexander, Cooperation of immune lymphoid cells with macrophages in tumour immunity. *Nature* (*Lond.*) 228, 620 (1970).
26. J. M. Zarling and S. S. Tevethia, Transplantation immunity to Simian Virus 40-transformed cells in tumor-bearing mice. I. Development of cellular immunity to Simian Virus 40 tumor-specific transplantation antigens during tumorigenesis by transplanted cells. *J. nat. Cancer Inst.* **50,** 137 (1973).

Europ. J. Cancer Vol. 12, pp. 19–23. Pergamon Press 1976. Printed in Great Britain

Distribution of ^{64}Cu-Bleomycin in Normal and Tumor-Bearing Rats*

K. H. TRAGL†, P. ANGELBERGER‡, P. PILS†, R. HRUBY‡, and E. DEUTSCH†

†*First Medical Department, University School of Medicine, Vienna, Austria*

‡*Research Center Seibersdorf, Österreichische Studiengesellschaft für Atomenergie, Vienna, Austria*

Abstract—*Accumulation of a radioactive label in tumor tissue is required for scintigraphic visualization of the tumor and has been achieved by application of bleomycin as a carrier. This chemotherapeutic polypeptide drug has been labeled with ^{64}Cu. Thin layer chromatography of ^{64}Cu-bleomycin which was incubated for 22 hr at 37°C showed no changes of the chelate. When urine of the ^{64}Cu-bleomycin treated animals was chromatographed considerable amounts (9·3%) of free ^{64}Cu appeared 2 hr after injection of the chelate and dissociation was found to be almost complete 4 hr after injection of labeled bleomycin.*

The distribution of ^{64}Cu-bleomycin is similar to that found with ^{99m}Tc bleomycin with long lasting accumulation in kidneys and liver. Highest concentration ratios of the activity in a mesenchymal tumor (Yoshida) and blood respectively were found 6 hr after the intravenous administration of ^{64}Cu-bleomycin.

INTRODUCTION

BLEOMYCIN is a group of water and methanol soluble basic glycopeptide antibiotics isolated from Streptomyces verticillus [1]. It has been used for the treatment of certain forms of cancer [2] and was also found to be a metal chelating agent. The latter property was used for radio-labeling of this antibiotic which was then applied for scintigraphic tumor visualization. While complexes of bleomycin formed with ^{67}Ga and ^{111}In were found not to clear very rapidly from the blood and liver, complexes with ^{57}Co are giving some contamination problems because of its slow radioactivity decay [3]. The scintigraphic imaging of tumors by accumulation of ^{99m}Tc-bleomycin was examined in previous experiments [4, 5]. The interpretation of the results obtained with ^{99m}Tc-bleomycin, however, were hampered by the temperature dependent, early dissociation of the chelate. The unstability of the ^{99m}Tc-bleomycin complex reduces the bleomycin directed distribution of the radioactive label and favors a distribution directed by technetium alone. Of the metal radionuclides available, ^{64}Cu was reported to form stable chelates with bleomycin [6] and was additionally attractive with regard to its radioactivity decay. ^{64}Cu decays with a 12·8 hr half-life by emission of beta particles and gamma rays whose energies and abundance are listed below:

beta	β^- 0·573 MeV	(38%)
,,	β^+ 0·656	(19%)
,,	EC	(43%)
gamma	0·0075 MeV	(characteristic Ni X-rays)
	0·511	(from β^+ annihilation, 38%)
	1·34	(0·5%)

The most prominent 0·511 MeV gamma rays can be utilized for scintigraphic imaging with the Anger Camera using a pinhole collimator.

MATERIAL AND METHODS

1. *Preparation of ^{64}Cu $(NO_3)_2$*

Approximately 1·0 mg of metallic Cu (99·999%) was irradiated to saturation (24–48 hr) in the reactor at a thermal neutron flux of 7×10^{13} n cm^{-2} s^{-2}. After decay to the desired

Accepted 25 September 1975.

*This work was supported by the Hochschuljubiläumsstiftung of the City of Vienna.

activity, Cu was dissolved in 0·1 ml of concentrated HNO_3, freed of NO and of NO_2 by heating, and finally adjusted to 10·0 ml by addition of distilled water. The activity of ^{64}Cu was measured in a calibrated ionisation chamber and the radionuclidid purity determined with Ge(Li)-gamma spectroscopy. Specific activities up to 56 mCi/mg Cu were obtained.

2. Five milligrams bleomycin (purchased from Lundbeck & Co. A/S, Copenhagen) were dissolved in 0·3 ml of 0·9% NaCl and an aliquot of the $^{64}Cu(NO_3)$ solution containing the desired activity and less than 120 μg Cu was added. The pH was adjusted to 1·6–1·8 and the volume brought to 2·0 ml with 0·9% NaCl. After stirring for one minute the solution was neutralized with 1·0 M $NaHCO_3$ (pH 5–7). The solution was finally sterilized by membrane ultrafiltration.

The radiochemical purity was determined by thin layer chromatography (Silica gel, methanol: 10% NH_4-acetate 1:1). Two peaks were found representing the bleomycin components A_2 (Rf—0·4) and B_2 (Rf—0·7), which contain more than 98% of the total ^{64}Cu activity. This ^{64}Cu-bleomycin chelate was absolutely stable for more than 24 hr at a temperature of 37°C. The specific activity of bleomycin for the consecutive experiments is kept at 0·25 mCi/mg.

3. *Animal experiments*

The experiments were performed on Wistar rats weighing between 230–280 mg. The animals were injected intravenously with ^{64}Cu-bleomycin (0·4 mCi/kg of body weight), killed and exsanguinated 1, 2, 4, 6, 9 or 22 hr respectively after the administration of ^{64}Cu-bleomycin. Urine of ^{64}Cu-bleomycin treated rats was obtained by puncture of the bladder. The radioactivity of the tissue samples (blood, skin, heart, lung, liver, spleen, pancreas, kidneys testicles and skeletal muscle) was determined in a well scintillation counter after they had been weighed in tared counting tubes. The counting rates were corrected for background and for decay of ^{64}Cu to the time of injection. They were finally expressed as per cent dose/g tissue of the injected ^{64}Cu.

In a second series of experiments an ascites form of Yoshida tumor (lymphosarcoma) was used to produce a solid tumor in the subcutaneous tissue of the left hind quarters of female rats. The tumor was allowed to develop for 10 days before the injection of the labeled bleomycin. The animals were treated as described before except that in this experiment tissue samples were also removed from the Yoshida tumor and from the ovaries instead of the testicles.

RESULTS

1. *Thin layer chromatography*

The radiochemical purity of ^{64}Cu-bleomycin was examined immediately after the reaction of the two components and after incubation of the chelate for 1, 2, 4, 9 and 22 hr at a temperature of 37°C. During this time no dissociation of ^{64}Cu from bleomycin could be found and the chromatographic pattern was unchanged except for the overall reduction of radioactivity according to the decay of the radioisotope (Fig. 1).

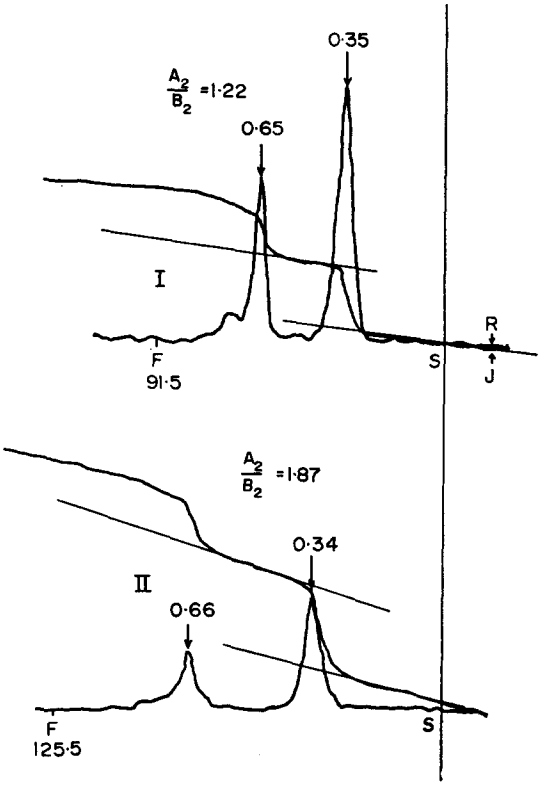


Fig. 1. Thin layer chromatography of ^{64}Cu-bleomycin immediately after its preparation (I) and after incubation at 37°C for 22 hr (II). Peak A_2 (0·35/0·34) and peak B_2 (0·65/0·66) correspond to fraction A_2 and B_2 of the bleomycin-glycopeptides. Twenty-two hours after preparation, no dissociation of the chelate could be detected.

2. *Excretion of radioactivity in the urine*

Measurement in a well scintillation counter revealed excretion of high activity in the first hour after injection of the chelate (Table 1) with a decrease in the following hour. When

Table 1. Urinary excretion of ^{99m}Tc and of ^{64}Cu following i.v. administration of either ^{99m}Tc-bleomycin or ^{64}Cu-bleomycin (% dose/g). The values were corrected for the half life of the radionuclides

Radiopharmaceutical	Time (hours) post injection 1 hr	2 hr	4 hr	6 hr	9 hr	22 hr
^{99}Tc-(bleo)	117·3	22·8	2·1	1·1	1·1	0·14
^{64}Cu-(bleo)	53·5	13·6	3·1	2·3	0·76	0·038

Table 2. Tissue distribution of ^{64}Cu, 1 2, 4, 6, 9, and 22 hr after i.v. administration of 0·4 mCi/kg body weight (%dose/g). The values were corrected for decay

Tissue	Time (hours) after i.v. injection 1 hr	2 hr	4 hr	6 hr	9 hr	22 hr
(1) Blood	0·148 ± 0·034	0·088 ± 0·062	0·064 ± 0·033	0·057 ± 0·003	0·065 ± 0·011	0·084 ± 0·058
(1) Skin	0·138 ± 0·031	0·083 ± 0·014	0·069 ± 0·022	0·070 ± 0·025	0·075 ± 0·017	0·062 ± 0·028
(1) Heart	0·062 ± 0·008	0·040 ± 0·010	0·040 ± 0·012	0·047 ± 0·014	0·057 ± 0·011	0·063 ± 0·017
(1) Lung	0·142 ± 0·025	0·085 ± 0·019	0·072 ± 0·024	0·075 ± 0·024	0·065 ± 0·006	0·074 ± 0·017
(1) Liver	0·229 ± 0·037	0·278 ± 0·044	0·294 ± 0·058	0·243 ± 0·031	0·291 ± 0·015	0·239 ± 0·029
(1) Spleen	0·064 ± 0·010	0·053 ± 0·024	0·061 ± 0·023	0·085 ± 0·043	0·083 ± 0·016	0·125 ± 0·053
(1) Kidney	1·286 ± 0·050	1·244 ± 0·184	1·448 ± 0·283	1·458 ± 0·376	1·336 ± 0·209	1·179 ± 0·472
(1) Pancreas	0·076 ± 0·017	0·069 ± 0·010	0·078 ± 0·013	0·087 ± 0·029	0·076 ± 0·020	0·053 ± 0·010
(1) Muscle	0·058 ± 0·011	0·023 ± 0·005	0·016 ± 0·006	0·016 ± 0·004	0·020 ± 0·009	0·018 ± 0·006
(2) Testicle	0·118 ± 0·034	0·105 ± 0·010	0·108 ± 0·015	0·117 ± 0·007	0·115 ± 0·003	0·089 ± 0·018
(3) Ovary	0·145	0·122	0·098	0·140	0·180	0·124
(3) Tumor*	0·163	0·088	0·153	0·184	0·172	0·171

(1) 5 rats.
(2) 4 rats.
(3) 1 rat.
*implanted lymphosarcoma (Yoshida).

Table 3. Tumor: tissue ratios for ^{64}Cu in rats bearing a subcutaneously implanted Yoshida tumor

Organ	Time (hr) post injection 1 hr	2 hr	4 hr	6 hr	9 hr	22 hr
Blood	1·09	1·00	2·39	3·22	2·64	2·04
Skin	1·18	1·06	2·21	2·62	2·29	2·77
Heart	2·62	2·20	3·82	3·91	3·01	2·73
Lung	1·14	1·03	2·12	2·45	2·64	2·32
Liver	0·71	0·31	0·52	0·75	0·59	0·71
Spleen	2·54	1·66	2·50	2·16	2·07	1·37
Kidney	0·12	0·07	0·10	0·12	0·12	0·14
Pancreas	2·14	1·27	1·96	2·11	2·26	3·24
Muscle	2·81	3·82	9·56	11·51	8·60	9·55
Testicle	1·38	0·83	1·41	1·57	1·49	1·93
Ovary	1·12	0·72	1·56	1·31	0·95	1·38

the excretion of ^{99m}Tc-bleomycin was examined in the same manner it was found to double the ^{64}Cu-bleomycin values in the first hour but to decrease to lower levels in the following hours. Since the radioactivity encountered in the urine was calculated per gram of urine it exceeded the injection dose in the ^{99m}Tc-bleomycin treated animals.

Thin layer chromatography of the urine indicated a rather stable ^{64}Cu-bleomycin complex for the first two hours of the animal experiment. Two hours after the injection of ^{64}Cu-bleomycin the fraction of free, unbound ^{64}Cu was 9·3% of the total activity in the urine. Four hours after the administration of the chelate, however, the ^{64}Cu-bleomycin complex in the urine was almost completely dissociated. At this time the ratio of free ^{64}Cu exceeded 70% of the total activity in the urine. Examination of ^{99m}Tc-bleomycin in a similar manner revealed accelerated dissociation: the fraction of free ^{99m}Tc in the urine exceeded 20% already one hour after the injection of ^{99m}Tc-bleomycin and was greater than 70% 2 hr after administration of the chelate [5].

3. *Distribution of ^{64}Cu animal tissue after i.v. injection of ^{64}Cu-bleomycin*

The results of the radioactivity distribution studies in rat tissue are summarized in Table 2. They indicate rapid reception of radioactivity

with consecutive slow delivery in skin, lung and muscle while the radioactivity in liver spleen, pancreas, testicles, ovaries, and in the implanted tumor is kept at a constant level during the observation period (22 hr). The very high radioactivity in kidney tissue is an expression of the excretory function rather an exceptional capability to accumulate radioactivity in this tissue. This suggestion is confirmed by the high radioactivity found in the urine of the rats. Table 3 shows the concentration ratio of radioactivity in samples of the mesenchymal tumor and in blood or in different tissue samples respectively. Four hours after the injection of ^{64}Cu-bleomycin the tumor/blood ratio was 2·4 and reaches a maximum of 3·2 6 hr after administration of the chelate. At this time concentration ratios exceeding 2·0 were found in spleen (2·16), in skin (2·6), in heart muscle (3·9), in lung (2·45), in pancreas (2·1), and in skeletal muscle (11·5). For liver tissue the tumor/tissue concentration ratio at no time reached 1·0.

DISCUSSION

Evaluation of bleomycin as a carrier of radioactive labels for scintigraphic imaging of malignant tumors has shown this drug to be a helpful tool in visualization of epidermoid carcinomas and malignant lymphomas [4, 5, 7]. The labels tagged to bleomycin have been ^{57}Co, ^{111}In, ^{67}Ga, and most recently ^{99m}Tc [4, 7, 8].

In fact, with ^{99m}Tc as a radioactive marker bleomycin fulfils most of the requirements expected in tumor visualization, i.e. short half life, sufficient contrast of the tumor or high tumor/neighbouring tissue concentration ratios respectively. The ^{99m}Tc-bleomycin chelate complex, however, has only a moderate thermal stability: after incubation of ^{99m}Tc-bleomycin for one hour at 37°C free pertechnetate will be encountered at concentrations up to 24% of the total radioactivity.

Since Cu^{2+} was reported to form very stable chelates with bleomycin [6, 8] this metal was chosen as a radioactive label of bleomycin and its distribution, excretion and its affinity to a mesenchymal tumor were examined in an animal experiment.

In the experiments presented above, ^{64}Cu-bleomycin was shown to be superior to ^{99m}Tc-bleomycin with regard to the stability of the chelate. This was found by incubation of ^{64}Cu-bleomycin at 37°C and succiding thin layer chromatography of the chelate. When the stability of the bleomycin chelate in the biological system was examined, thin layer chromatography of the urine of rats previously treated with either ^{99m}Tc-bleomycin or with ^{64}Cu-bleomycin revealed the ^{64}Cu-bleomycin chelate to be the more stable compound. However, in the biological system the degradation of ^{64}Cu-bleomycin was accelerated and 4 hr after the injection of ^{64}Cu-bleomycin to the rats free (unbound) ^{64}Cu exceeded 70% of the total radioactivity of the urine. With ^{99m}Tc-bleomycin this percentage of unbound label in the urine had already been found 2 hr after administration of the chelate [5].

With regard to the application of ^{64}Cu-bleomycin in scintigraphic tumor visualization its concentration ratios in tumor/blood and tumor/neighbouring tissue respectively were found to equal or even to surpass the results obtained with ^{99m}Tc-bleomycin [5]. Highest tumor/tissue and tumor/blood ratios were found 6 hr after the injection of ^{64}Cu-bleomycin. At this time we would expect optimum scanning conditions.

The disadvantage of any labeled bleomycin is the fact that strong accumulation of bleomycin is restricted to very specific and histologically defined tumors which confines positive imaging to a limited number of tumors. In addition, the accumulation of bleomycin in liver and kidneys gives a very high background activity and makes tumor visualization in this area almost impossible.

Acknowledgement—We thank Mrs. Margarethe Machanek for technical assistance.

REFERENCES

1. H. Umezawa, K. Maeda, T. Takeuchi and T. Okami, New antibiotics, bleomycin A and B. *J. Antibiot. (Tokyo), Ser. A* **19,** 210 (1966).
2. L. Wicart, Bleomycin in the treatment of cancer. *Proc. 6th Int. Cong. Chemotherapy*, p. 702, Tokyo 1969.
3. R. B. Grove, W. C. Eckelman and R. C. Reba, Distribution of labeled bleomycin in normal and tumor-bearing mice. *J. nucl. Med.* **14,** 917 (1973).
4. M. S. Lin, D. A. Goodwin and S. L. Kruse, Bleomycin as a ^{99m}Tc carrier in tumor visualization. *J. nucl. Med.* **15,** 338 (1974).

5. K. H. TRAGL, P. PILS, P. ANGELBERGER, R. HRUBY and H. IMHOF, Bleomycin als Trägersubstanz für ^{99m} Technetium in der Tumordiagnostik. *Wien. Klin. Wschr.* **9,** 308 (1975).
6. H. RENAULT, H. HENRY and J. RAPIN, Chélation de cations radioactifs par un polypeptide: la bléomycine. In: *New Developments in Radiopharmaceuticals and Labeled Compounds.* Vienna IAEA, 1974.
7. R. B. GROVE, W. C. ECKELMANN and R. C. REBA, Distribution of labeled bleomycin in normal and tumor bearing mice. *J. nucl. Med.* **14,** 917 (1973).
8. P. J. ROBBINS, E. B. SILBERSTEIN and D. L. FORTMAN, ^{111}In-bleomycin kinetics in mice bearing transplantable tumors of lung, skin, and bone. *J. nucl. Med.* **15,** 273 (1974).
9. U. Y. RYO, R. D. ICE, J. D. JONES and W. H. BEIERWALTES, Relative tissue distribution of radioactivity in rats with endocrine "autonomous" breast carcinomas after ^{3}H-, ^{99m}Tc-, and ^{64}Cu-bleomycin. *J. nucl. Med.* **16,** 127 (1975).

While this paper was being written U. Yun Ryo, *et al.* [9] reported on the distribution of ^{64}Cu-bleomycin in rats with endocrine "autonomous" breast carcinomas. They have also experienced a greater uptake of ^{64}Cu-bleomycin than ^{99m}Tc-bleomycin in the tumor as well as in liver and kidney. Though the data of both papers are hardly comparable for methodical reasons (different source of bleomycin, different tumors, different weight of the experimental animals, slightly different labeling procedure) the tumor/tissue rations seem to be lower for the experimental breast cancer than for the Yoshida tumor.

Europ. J. Cancer Vol. 12, pp. 25–31. Pergamon Press 1976. Printed in Great Britain

The Effect of Antilymphocytic and Normal Horse Serum on Growth of Precancerous Foci and Development of Tumours Induced by Diethylnitrosamine in Rat Liver

E. SCHERER, W. F. M. VAN DIJK and P. EMMELOT
Department of Biochemistry, Antoni van Leeuwenhoek-Laboratory, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Abstract—*Rats received either a single subcarcinogenic dose of 10 mg of diethylnitrosamine (DENA)/kg 24 hr after two-thirds hepatectomy followed by 2 ml of horse anti-rat antilymphocytic serum (ALS) once weekly for 6 weeks (procedure A), or a carcinogenic regimen of 8 mg DENA/kg·day together with 2 ml ALS once weekly for 6 weeks (procedure B). The number and size of the precancerous foci (ATPase-deficient islands) induced in the livers by procedures A and B were measured after fixed periods of time and found not to differ statistically from those occurring in the respective controls consisting of rats receiving normal pre-immune horse serum (HS) or no serum at all. Sera used were not toxic for rat liver.*

When rats treated according to procedure A were subjected after another 3 weeks to a daily dose of 2·4 mg DENA/kg until death from liver tumours resulted, no effect of the prior ALS treatment on the death rate, as compared with the non-ALS controls, was obtained. However, when rats subjected to procedure B were further left untreated, the rate of death from liver tumours in the ALS group was significantly enhanced as compared with the two control groups. The interpretation of this result was severely hampered because of the non-equivalence, both quantitatively and qualitatively, of the tumours in the ALS, HS and non-serum groups. The sera presumably contained a factor that furthered tumour development other than by immune suppression. However, if it is assumed that the effect of ALS on the neoplastic process was also mediated by immune suppression, then the esults indicated that cells participating in the process of liver carcinogenesis became immunogenic to the cellular immune system beyond the precancerous stage of the ATPase-deficient island cell proper.

INTRODUCTION

HEPATOCARCINOGENESIS by chemical agents is generally considered to be a multi-stage process in which successive alterations in liver and precancerous cells lead to liver tumours [1–3]. An early, morphologically detectable and proliferating cell stage—the ATPase-deficient island cell—that may act as precursor in the process of liver tumour formation, has recently been identified [4]. When induced by a subcarcinogenic dose of DENA, the island cells appear to serve as target for additional carcinogen leading to the formation of tumour cells [3], and, when induced by a carcinogenic dose, the island cells may by focal progression yield tumour cells in the course of time [5].

Accepted 18 September 1975.

Immune surveillance has been proposed as a protective mechanism for checking cells with aberrant, non-self surface expressions [6]. Since liver tumour cells may bear new antigens [7], the question arises at which stage of their development the antigenic change occurs.

Previously, Friedrich-Freksa and Hoffmann [8] have reported that islands induced in liver by DENA were considerably increased both in number and size by treatment of the rats with rabbit-ALS during the period of carcinogen administration. These authors concluded that the immune surveillance was already operating at the island cell stage. We have repeated and enlarged their experiments by studying islands induced by both subcarcinogenic and carcinogenic doses of DENA using horse-ALS after carefully assessing its properties —a precaution not taken by the former authors. Our results did not demonstrate a stimulating effect of ALS on the islands. In addition, we have studied the effect of ALS on liver tumour formation by two procedures in an attempt to find out at which stage, if any, of the process of liver tumour development participating cells become immunogenic to the cellular mechanism of host defence.

MATERIAL AND METHODS

Materials and methods used in the present experiments, except the sera, have been described in detail previously [3, 4].

Two batches of horse anti-rat antilymphocytic serum (ALS) of known protein content and immunosuppressive action, and the corresponding pre-immune sera were obtained from the Radiobiological Institute (TNO), Rijswijk, The Netherlands. The sera were non-sterile and inactivated for 30 min at 56°C. Two ml of the ALS preparation were administered by i.p. injection once weekly for 6 weeks into 2–3 months old Sprague–Dawley females used throughout the present experiments. During and after this period animals were observed for body weight, occurrence of wasting disease [9], hematocrite count, number of leukocytes and thrombocytes, liver weight, labelling indices of hepatic nuclei by ^{3}H-thymidine (4 injections of 200 μCi/kg each, with 6 hr intervals) and gross microscopic liver damage. Skin transplantation (two pieces of ventral skin of ♀ RxU rat, one cm^2 each) to the back of ♀ SD rats, which were treated with ALS for 6 weeks served as control for the immunosuppressive activity of the ALS preparations.

RESULTS AND DISCUSSION

Characterization of ALS

Two to three months old Sprague–Dawley females received 2 ml ALS once weekly for 6 weeks. During and after this period the two ALS batches did not differ much in their effects. Except for a transient drop of some 20% in the hematocrite count after 21 days (recovery to 90–95%, after 42 days), a persisting drop in leucocyte count to 40–50% of the controls within 2 weeks, and slight hepatoxicity (increased labelling index) of one ALS preparation they had no effect on any of the other parameters examined. The serum without signs of hepatotoxicity—thus behaving favourable in comparison with other preparations [9]—was selected for further use.

Skin transplantation performed after the 6 weeks ALS treatment resulted in 60% survival of the grafts after another 57 days, whereas controls similarly treated with HS or non-treated showed 100% rejection of grafts after 2 weeks. Thus the ALS preparation used in the present experiment was strongly immunosuppressive without being hepatotoxic.

Effect of ALS on number and size of ATPase-deficient islands induced by DENA

The effect of ALS was studied in two types of experiments, the design of which is illustrated in Fig. 1.

Procedure A. Three groups of 15 rats each were two-thirds hepatectomized followed after 24 hr by a single application of 10 mg DENA/kg. One group (A1) was left untreated, the second (A2) received 2 ml ALS once weekly for 6 weeks, and the third group (A3) received 2 ml HS once weekly also for 6 weeks. Number and size of the ATPase-deficient islands were measured at 9, 24 and 39 weeks after their induction. As shown in Table 1, the ALS treatment had not increased either number or size of the islands (or the fraction of liver occupied by island tissue). Rather ALS and HS tended to decrease the number of islands measured after 39 weeks.

Procedure B. To one group of 5 rats (B1) 8 mg DENA/kg·day was administered for 6 weeks. Two other groups were similarly treated and received in addition 2 ml ALS (group B2), respectively 2 ml HS (groups B3) once weekly during the same period. After another 4 weeks island number and size did not differ for the 3 groups (Table 2).

Table 1. Number and size-distribution of ATPase-deficient islands 63 resp. 273 days after a single dose of 10 mg DENA/kg and partial hepatectomy, with and without ALS resp. HS during the first 6 weeks after DENA application

Serum	Time after DENA application, days	Animals	Islands per cm^3 liver	Distribution (% of total) over groups of island diameter (µm)										Volumetric fraction occupied by island tissue (%)
				50-80	-115	-160	-225	-320	-450	-640	-900	-1280	-1800	
No serum	63	5	865	36	36	25	3.5	0.5	0.5	0	0	0	0	0.1
ALS	63	5	850	24	49	22	4.5	0	0.3	0	0	0	0	0.1
HS	63	5	810	12	55	29	4	0.3	0.3	0	0	0	0	0.1
No serum	273	4	1700	0	16	27	25	23	7	1.5	0.5	0.3	0.2	3.5
ALS	273	4	1250	0	14	37	29	12	5	2	0.1	0.05	0	1.2
HS	273	4	1280	0	9	33	28	18	9	3	1	0	0.1	2.2

Table 2. Number and size-distribution of ATPase-deficient islands after a 6 weeks treatment of intact rats with 8 mg DENA/kg·day, 70 days after the beginning of the experiment, with and without ALS or HS during the time of DENA application

Serum	Animals	Islands per cm^3 liver	Distribution (% of total) over groups of island diameter (µm)										Volumetric fraction occupied by island tissue (%)
			50-80	-115	-160	-225	-320	-450	-640	-900	-1280	-1800	
No serum	5	7080	0	25	36	25	11	2.5	0.7	0.1	0	0	4.0
ALS	5	5800	0	20	38	27	10	4.5	1	0.15	0	0	4.1
HS	5	5670	0	14	54	17	9	5	0.9	0.3	0	0	4.0

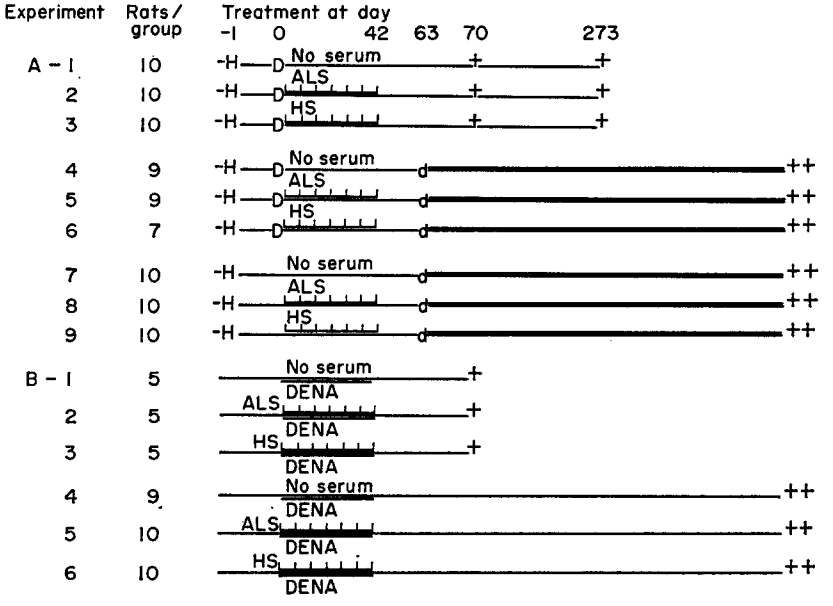


Fig. 1. Outline of DENA and serum applications. Experiment A: Induction of islands by a single, subcarcinogenic dose of DENA to partially hepatectomized rats. Island numbers and size distributions are illustrated in Table 1, death rates after continuous application of 2·4 mg DENA/kg·day in Fig. 2. Experiment B: Induction of islands by a carcinogenic treatment with DENA (8 mg/kg·day) for 6 weeks. Island numbers and size distributions are given in Table 2, the death rates in Fig. 3. Abbreviations: H, two-thirds hepatectomy; D, single dose of 10 mg DENA/kg; d, daily application of 2·4 mg DENA/kg until death; +, killed for microscopic evaluation of number and size of adenosine triphosphatase deficient islands in the liver; + +, observed until death.

It is concluded that in neither of the two experiments with rats receiving a non-carcinogenic treatment (A) or a carcinogenic one (B) did ALS stimulate or affect the rate of proliferation of the island cells at any time after their induction. This result stands in contrast to that obtained by Friedrich-Freksa and Hoffmann [8]. The differences between their and our experiments were the source and the quantity of ALS administered (0·5 ml 5 times/week used by the latter authors) and the enzymic marker for the island detection. Glucose-6-phosphatase deficiency was used by Friedrich-Freksa and Hoffmann, whereas we routinely used ATPase deficiency as marker. However, inspection of our samples for glucose-6-phosphatase deficiency and PAS staining after fasting, corroborated the present results obtained with ATPase deficiency as marker. Since the ALS used in the present experiments was strongly immunosuppressive, it does not seem likely that differences in this respect would have been of influence. The difference in results may be accounted for by the assumption that the rabbit anti-rat lymphocytic serum used by Friedrich-Freksa and Hoffmann had a hepatotoxic action and that the resulting regenerative response increased the proliferation of the island cells [5]. By the latter criterion ALS and HS used in the present experiments were not hepatotoxic.

Effect of ALS on liver tumour formation

Essentially the same experimental design as in the previous section was followed, except that in rats subjected to procedure A liver

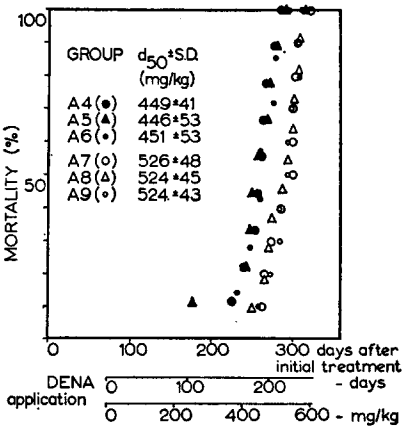


Fig. 2. Effect of ALS applied for 6 weeks after the induction of islands by a single subcarcinogenic dose of DENA on death rates from liver carcinoma following continuous application of DENA (see Fig. 1, exp. A).

tumours were now induced by application of a daily dose of 2·4 mg DENA/kg beginning at 9 weeks after the start of the experiment until death of the rats from lethal liver tumours followed (groups A4, A5 and A6—Fig. 2). Procedure B was carcinogenic by itself (groups B4, B5 and B6—Fig. 3). The present experiments were conducted concomitantly with those described in the previous section, so that the results obtained in the latter experiments are applicable to the present ones.

Procedure A, followed by 2·4 *mg DENA/kg·day.* No differences were observed between the times of death of the animals in the 3 groups—i.e., rats which had received either 2 ml ALS (A5) once weekly or 2 ml HS (A6) once weekly for 6 weeks, or no further treatment (A4, non-serum controls) following the initial partial hepatectomy and the dose of 10 mg DENA/kg. The number of days passed between the instalment

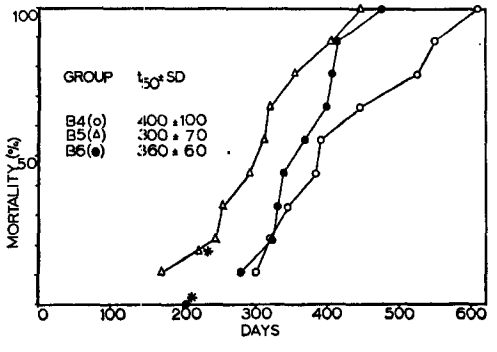


Fig. 3. *Effect of ALS applied for* 6 *weeks during a carcinogenic treatment with DENA on the death rates from liver carcinoma (see Fig.* 1, *exp. B).*

**Moribund from kidney tumour.*

of the daily 2·4 mg DENA/kg doses and death of 50% of the rats (t_{50}) in the 3 groups amounted to 189 ± 6·5 days (Fig 2, closed symbols).

A similar experiment was performed with partially hepatectomized rats which had not received the initial application of 10 mg DENA/kg (time scale identical to previous experiment). This experiment served as a control of the previous one, in order to examine whether ALS and HS had any effect in the absence of previously established islands (groups A7, A8 and A9). Again the death rates in the 3 groups were identical, the t_{50} now amounting to 213 ± 6·6 days (Fig. 2, open symbols). The slope of the dose-effect curves was similar in the two experiments. The difference of 24 days —corresponding to 57·6 mg DENA/kg— between the t_{50}'s in the two experiments was highly significant ($P < 0{\cdot}001$). It shows, in accord with previous results [3], that the initial 10 mg DENA/kg applied at day 0, and consequently, island tissue thus induced, contributed markedly to the carcinogenic process that was brought to completion by the daily low doses of carcinogen starting at day 63. The liver tumours at death consisted of those described previously [3], all rats in the various groups (A4–A9) dying from a qualitatively and quantitatively similar tumour burden.

It is concluded that the antilymphocytic treatment given in the initial phase of 6 weeks in which island cells induced by DENA proliferated before the carcinogenic regimen was installed, had no effect on the rate of liver tumour formation. Whereas it was shown in the previous section that ALS did not affect the growth of the island cells proper, the present result indicated that ALS, under the experimental conditions chosen, did not productively select clones from the island cell population that may have an advanced potential for conversion to malignancy by carcinogen supplied later.

Procedure B. The rats, having received a carcinogenic load of 8 mg DENA/kg·day for 6 weeks with concomitant ALS (group B5) or HS (group B6) or no further treatment (group B4) as indicated (Fig. 1), were kept and observed for lethal liver tumour formation In this experiment an effect of the ALS treatment given during the feeding of the carcinogen on the time of death from lethal liver tumours was observed, the t_{50} amounting to 300 ± 70 days, as against 360 ± 60 days ($P < 0{\cdot}1$) for the HS group, and 400 ± 100 days ($P < 0{\cdot}05$) for the rats in the non-serum control group. No significant difference ($t_{16} = 1$) was observed between the t_{50} of the HS and non-serum groups.

Several types of liver tumours are induced by DENA in rat liver, *viz* variously differentiated liver carcinoma and a more anaplastic hemorrhagic type of tumour [10–12] very probably also of parenchymal origin [3, 10, 13]. The former types of tumour were present in all animals of the various groups (B4, B5 and B6) at the time of death. About 50% of the rats treated with ALS or HS contained in addition the anaplastic type of tumour. The occurrence of these anaplastic tumours was connected with early death in the latter two groups, i.e.. rats dying at a time shorter than the t_{50} due to lethal bleeding from liver ruptures. However, the liver weight at the time of death varied significantly amounting to 15–38 g, average 21 g, for the ALS-treated animals (group B5) and to 15–95 g, average

47 g, for the HS-treated rats (group B6). The higher liver weights of the HS-treated rats, due to presence of the variously differentiated tumours, tended to accompany late death (beyond the t_{50}). Endpoints of tumour formation, i.e., causes of death were therefore not generally identical for the two groups. However, since the anaplastic tumours in the ALS- and HS-treated rats of the subgroups dying at a time earlier than the t_{50}, were qualitatively and quantitatively about similarly developed, it follows that ALS had accelerated the process of formation of these anaplastic tumours. This shortening of the process could have been due to the immunosuppressive action of ALS. If so, the finding that ALS did not affect number or size of the precancerous islands may lead one to conclude that ALS had facilitated, by immunosuppression, the outgrowth of either tumour cell precursors other than those represented by the island cells, or tumour cells arisen from the effect of the six-weeks DENA treatment. Since the island cells very probably serve as the first precancerous stage, it would then follow that cells participating in the process of liver carcinogenesis become immugenic beyond the stage of the island cell proper.

Interpretation of results beyond the t_{50} for the ALS- and HS-treated groups is impossible (compare also the difference in slopes of the time-effect curves, Fig. 3), and the concept that ALS merely acted through immunosuppression is further weakened by the finding that, in contrast to the ALS- and HS-groups, all rats of the non-serum control group died without anaplastic tumours but with variously differentiated liver tumours of relatively small weight and size (up to 2 cm dia), the liver weight at death amounting to 18–28 g, average 22 g. Thus if the latter rats are compared with the HS-treated ones, it appears that HS had qualitatively furthered the process of liver tumour formation, in respect of the non-serum controls. The enhancement of liver tumour formation by the sera (ALS *vs* HS *vs* the non-serum controls) cannot have been due to their toxic action on the liver, providing a regenerative stimulus that would increase tumour development, since the sera were non-toxic by various criteria. Neither could abrogation of the immune response have operated in the case of the non-immunosuppressive HS. However, a possibility to account for the effects is that horse serum may contain a (co) carcinogenic factor. This assumption is based on the following. First, it has very recently been shown in this laboratory [14] that normal horse serum may cause hepatomas to arise in a strain of mice (GRS/A) which has a very low incidence of spontaneous hepatomas. Secondly, in the presence of normal horse serum cultured embryonic cells are more prone to "spontaneous" neoplastic transformation than in the presence of serum from other species [15–17]; a dialysable principle appeared to be involved [18]. Thirdly, undialysed serum albumin (present sera were not dialysed before use) contains long-chain fatty acids and these chemicals may be promoters of skin carcinogenesis [19]. Finally, the hepatocarcinogenicity of Aflatoxin B_1 is markedly enhanced by cyclopropenoid fatty acids [20, 21].

Since the supposedly (co) carcinogenic factor may also be present in the ALS serum, it remains to be established whether the "extra" effect of ALS as compared with HS (below the t_{50}) is due to a different content of this factor or to an immunosuppressive effect.

REFERENCES

1. W. Nakahara, Critique of carcinogenic mechanism. In: *Progr. exp. Tumor Res.* (Edited by F. Homburger, Vol. 2, p. 158, Karger, Basel (1961).
2. E. Farber, Carcinogenesis—cellular evolution as a unifying thread: Presidential address. *Cancer Res.* **33,** 2537 (1973).
3. E. Scherer and P. Emmelot, Foci of altered liver cells induced by a single dose of diethylnitrosamine and partial hepatectomy: Their contribution to hepatocarcinogenesis in the rat. *Europ. J. Cancer* **11,** 145 (1975).
4. E. Scherer, M. Hoffmann, P. Emmelot and H. Friedrich-Freksa, Quantitative study on foci of altered liver cells induced in the rat by a single dose of diethylnitrosamine and partial hepatectomy. *J. nat. Cancer Inst.* **49,** 93 (1972).
5. E. Scherer and P. Emmelot, Kinetics of induction and growth of precancerous liver-cell foci, and liver tumour formation by diethylnitrosamine in the rat. *Europ. J. Cancer* **11,** 689 (1975).
6. M. L. Kripke and T. Borsos, Immune surveillance revisited. *J. nat. Cancer Inst.* **52,** 1393 (1974).
7. R. W. Baldwin, Immunological aspects of chemical carcinogenesis. *Adv. Cancer Res.* **18,** 1 (1973).

8. H. Friedrich-Freksa and M. Hoffmann, Immunological defense against preneoplastic stages of diethylnitrosamine induced carcinomas in rat liver. *Nature (Lond.)* **223,** 1162 (1969).
9. R. K. J. Koumans, J. B. Billote and J. F. Burke, Complications in prolonged administration of antilymphocytic sera. *Transplant. Proc.* **3,** 807 (1971).
10. C. Thomas, Zur Morphologie der durch Diäthylnitrosamin erzeugten Leberveränderungen und Tumoren bei der Ratte. *Z. Krebsforsch.* **64,** 224 (1961).
11. M. D. Reuber and C. W. Lee, Effect of age and sex on hepatic lesions in buffalo strain rats ingesting diethylnitrosamine. *J. nat. Cancer Inst.* **41,** 1133 (1968).
12. H. L. Stewart and K. C. Snell, The histopathology of experimental tumors of the liver of the rat. *Acta Un. intern. Cancr.* **13,** 770 (1957).
13. W. H. Butler, Pathology of liver cancer in experimental animals. *IARC scientific Publ.* **1,** 30 (1971).
14. L. Den Engelse, W. Misdorp and P. Emmelot, Hepatomas in GRS/A male mice treated with horse serum or horse anti-mouse antilymphocyte serum. *Europ. J. Cancer*, in press.
15. V. J. Evans and W. F. Andresen, Effect of serum on spontaneous neoplastic transformation *in vitro*. *J. nat. Cancer Inst.* **37,** 247 (1966).
16. J. T. Mitchell, W. F. Andresen and V. J. Evans, Comparative effects of horse, calf, and fetal serums on chromosomal characteristics and neoplastic conversion of mouse embryo cells *in vitro*. *J. nat. Cancer Inst.* **42,** 709 (1969).
17. V. J. Evans, F. M. Price and K. K. Sanford, Comparative effects of mare, stallion, gelding horse, and fetal bovine sera on neoplastic transformation *in vitro*. *J. nat. Cancer Inst.* **49,** 505 (1972).
18. F. M. Price, R. R. Gant and V. J. Evans, Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3H mouse cells in tissue culture. *In Vitro* **6,** 437 (1971).
19. K. Setälä, Neuere Cocarcinogene. In: *Berliner Symposion über Fragen der Carcinogenese* (Edited by H. Kraatz, A. Graffi and H. Gummel) p. 53, Akademie Verlag, Berlin (1960).
20. D. J. Lee, J. H. Wales, J. Layres and R. O. Sinnhuber, Synergism between cyclopropenoid fatty acids and chemical carcinogens in rainbow trout (Salmo gairdneri). *Cancer Res.* **28,** 2312 (1968).
21. R. O. Sinnhuber, D. J. Lee, J. H. Wales and J. L. Ayres, Dietary factors and hepatoma in rainbow trout (Salmo gairdneri). II. Cocarcinogenesis by cyclopropenoid fatty acids and the effect of gossipol and altered lipids on aflatoxin-induced liver cancer. *J. nat. Cancer Inst.* **41,** 1293 (1968).

Europ. J. Cancer Vol. 12, pp. 33–39. Pergamon Press 1976. Printed in Great Britain

Interspecies Mouse x Akodon Urichi Somatic Cell Hybrids: Comparative Immunogenicity of Parental and Hybrid Cells*

M. SATYA MURTHY†, JEAN BELEHRADEK, Jr. and GEORGES BARSKI††
Tissue Culture and Virology Laboratory (E.R. 38 of CNRS), Institut Gustave-Roussy 94–Villejuif, France

Abstract—*Immunizing capacity of an interspecies somatic hybrid cell line, HyRA4 (resulting from* in vitro *mating of R4/B malignant cells from the BALB/c mice, with normal embryonic cells of the South American rodent Akodon urichi venezuelensis), was compared with that of the parental R4/B cell line. Both R4/B and HyRA4 cells displayed cross-reacting tumour specific transplantation antigen(s) and were able to induce a relative transplantation resistance against challenge inoculation of R4/B cells in syngeneic BALB/c mice. However, immunization with non-pretreated HyRA4 hybrid cells carrying twice the number of R4/B chromosomes was not found to be more efficient than the immunization with X-irradiated R4/B parental cells in terms of the transplantation resistance induction. Furthermore, when mice in spite of pretreatment with either R4/B or HyRA4 cells failed to reject the challenge graft, the resulting tumours showed enhanced growth rates as compared to those growing in non pretreated control mice.*

Though the HyRA4 hybrid cells carried only a reduced number of chromosomes from the parental Akodon cell, they strongly displayed Akodon species specific antigen(s), thus rendering them unable to grow in normal BALB/c mice. In exceptional cases, in some mice in which tumours developed from the challenge inoculum of R4/B cells in spite of previous "immunization" with HyRA4 cells, a delayed tumour outgrowth at the site of HyRA4 inoculum was observed. These mouse tumour derivatives of HyRA4 cells were, however, devoid of any detectable Akodon antigenicity as well as of any identifiable complete Akodon chromosome. This phenomenon resulting in the outgrowth of an antigenically "curtailed" hybrid variant is described and discussed.

INTRODUCTION

SEVERAL methods have been developed in order to actively immunize experimental animals against neoplastic cells carrying tumour specific transplantation antigens (TSTA). These are generally considered weak antigens but their expression may be enhanced by "heterogenization" i.e., additional integration of foreign antigenic material into the neoplastic cells [1–4]. Since it is known that somatic cell hybrids share antigenic characters of both parental cells [5] heterogenization may conveniently be achieved by cell fusion. Further, by choosing two parental cells widely different in their antigenic properties, one may ensure rejection of hybrid graft when injected into hosts syngeneic for one of the parental cells. Watkins and Chen [6] were the first to successfully immunize mice against Ehrlich ascites

Accepted 17 September 1975.

*This work was supported by a grant from the New York Cancer Research Institute, U.S.A. Post-doctoral fellowship to one of us M.S. by NYCRI and partly by ICRO/UNESCO during these ivestigations is gratefully acknowledged.

†Present address: Institut du Cancer de Montréal, Laboratoires de Recherche, Hôpital Notre-Dame, Montréal 133, (Canada).

††To whom requests for reprints should be addressed.

tumour (EAT) cells by preinoculation of the hosts with cell hybrids formed by fusion between EAT cells and hamster cells. More recently, Jami & Ritz [7] Barski *et al.* [8] as well as Parkman [9], also demonstrated that somatic cell hybrids can immunize mice against the parental mouse malignant cells. It remains however not yet clear as to whether the somatic cell hybrids are in all instances better immunogens than the parental cells.

It is generally accepted that somatic cell hybrids are karyologically unstable [10–13], as most if not all cell hybrids gradually undergo less or more extensive chromosomal losses during their growth *in vitro*. In many interspecies hybrids, such losses may involve preferentially the chromosomes of one of the parents [8, 14]. As a consequence of such a progressive and frequently "asymmetrical" evolution of karyotype, many different types of chromosomal and antigenic variants may be generated. It is therefore desirable to know the extent to which these variants are able to retain and express their tumour specific transplantation antigen(s) as well as species specific antigens. Such a knowledge is important especially if one wishes to use cell hybrids for purposes of immunization.

In order to answer these questions, we utilized an interspecies cell hybrid system described elsewhere [15]. The hybrids were formed by fusion between mouse tumourigenic cells (R4/B) and normal embryonic cultured cells from the South American rodent Akodon urichi venezuelensis (AUV). The AUV cells contained 18 chromosomes all of them being biarmed excepting for the Y chromosome. Among the hybrid clones isolated and characterized, a tumourigenic clone designated as HyRA4, was chosen for the present investigations. Immunogenicity of the hybrid HyRA4 cells has been compared with that of X-irradiated parental neoplastic R4/B cells. Secondly, since the HyRA4 cells gradually lost AUV chromosomes, we were interested to know whether, at different stages of this loss, they were still able to, and if so, to what extent, express AUV specific membrane antigens responsible for evoking graft rejection reaction in BALB/c mice.

MATERIAL AND METHODS

Parental and hybrid cell lines

The malignant cell line R4 was kindly supplied by Dr. G. Todaro (National Cancer Institute, NIH, Bethesda, Md.). It was originally derived from a tumour obtained in a BALB/c mouse following inoculation of *in vitro* X-irradiated 3T3/BALB/c clone A31 cells. The R4 line was rendered resistant to 30 μg of 5-bromodeoxyuridine (BUdR) per ml of medium in our laboratory and designated R4/B. At the time of our experiments reported here, the parental R4/B cells were highly malignant, producing tumours in 100% of syngeneic mice with an inoculum of $1{\cdot}5 \times 10^6$ cells. On electron microscopic examination they showed abundant C-type particles. The Gross type cell surface antigen was demonstrated in the R4/B cells by means of indirect immunofluorescence tests with specific anti-Gross serum produced in rats.

The other parental cells were normal diploid cells obtained from secondary cultures developed from embryos of the rodent Akodon urichi venezuelensis (kindly supplied by Dr. C. Sonnenschein, Tufts University School of Medicine, Boston, Mass.).

Fusion was facilitated by u.v.-inactivated Sendai virus. Several hybrid clones were isolated in selective HAT medium. Details of the hybridization procedure and characteristics of the hybrid lines are described elsewhere [15].

All cultures were routinely maintained in the MEM medium supplemented with 10% fetal calf serum and usual antibiotics.

One of the hybrid clones isolated, designated as HyRA4, was employed for the present investigations. The hybrid nature of HyRA4 cells was confirmed by karyological analysis and the simultaneous presence of species-specific antigens from both parental cells. HyRA4 cells were malignant when tested in cortisone-treated Syrian hamster cheek pouches, whereas, they were systematically rejected when inoculated into normal BALB/c mice. The hybrid cells maintained these characteristics during long term cultivation approximating two years. At the time of present studies HyRA4 cells contained about 4–5 clearly identifiable AUV biarmed chromosomes and about twice (2S) the number of R4/B telocentric chromosomes. In spite of an extensive loss of AUV chromosomes, HyRA4 cells strongly expressed AUV species specific surface antigen(s). They also carried the Gross type surface antigen(s) as demonstrated by *in vitro* immunofluorescence tests with specific anti-Gross anti-serum (obtained from rats with regressing Gross lymphomas, kindly supplied by Dr. L. J. Old, Sloan-Kettering Cancer Center, New York).

Immunization and challenge procedures

Eight to ten weeks old male BALB/c mice,

supplied by the Central animal house of our Institute were used in all the experiments.

The immunization schedule was based on earlier experiments [15], which showed that a single subcutaneous inoculation of 4×10^6 HyRA4 cells rendered BALB/c mice resistant to subcutaneous challenge inoculation, 30 days later, with 5×10^5 R4/B cells; untreated parallel control mice showed a 100 per cent tumour takes with a similar inoculum of R4/B cells. On the other hand, the immunizing capacities of non-irradiated and X-irradiated HyRA4 cells were compared in another preliminary experiment. BALB/c mice pre-inoculated with HyRA4 cells either X-irradiated (10,000 rads) or not showed comparable degree of resistance to challenge with R4/B cells.

Therefore, the immunogenecities of non-irradiated HyRA4 and X-irradiated (10,000 rads) R4/B parental cells were studied by means of single subcutaneous or intradermal inoculation of 4×10^6 cells of the respective cell line into one flank, followed by subcutaneous challenge inoculation of $1{\cdot}5 \times 10^6$ R4/B cells into the opposite flank, 20 or 30 days later (see Table 1). All groups were challenged on the same day and with the same R4/B cell suspension. A group of non-pretreated mice was parallely inoculated as control.

The following three parameters were considered for analysing the effectiveness of the immunizing pretreatment in each experimental group: (1) proportion of mice developing tumours, (2) growth rate of tumours and (3) mean survival period of tumour bearing mice. Animals were examined twice a week and kept under observation till all the tumour bearing mice died (about 60 days). Growth rates of tumours were calculated following the method described by Attia and Weiss [16]. For each tumour 2 diameters perpendicular to each other were recorded and the tumour volumes calculated by the formula $v = 0{\cdot}4\ ab^2$ (where a is the longest diameter and b is the short diameter).

Demonstration of AUV specific antigens

Expression of AUV specific surface antigens was studied by the indirect immunofluorescence (IF) technique [17]. Anti-Akodon serum (kindly supplied by Dr. Sonnenschein, Tufts University School of Medicine, Boston, Mass.) was prepared in rabbits and absorbed extensively with R4/B cells to increase the specificity. Normal rabbit serum treated the same way served as control. The fluorescent serum was goat anti-rabbit IgG obtained from Hyland Laboratories, Los Angeles, Ca. Cells were examined under a 100 × oil immersion objective. IF index was calculated according to the method described by Möller and reaction considered as positive when IF > 0·30 [17].

Table 1. *Comparative immunizing abilities of the hybrid HyRA4 and irradiated parental neoplastic R4/B cells against a challenge of R4/B cells*

Immunizing cells	Route of inoculation	Delay between immunization and challenge in days	Number of mice with tumours / Number of inoculated mice*	Mean survival period of tumour bearing mice†
HyRA4	s.c.	20	6/10	39·3 ± 3·2
HyRA4	i.d.	20	8/10	38·4 ± 11·3
HyRA4	s.c.	30	8/10	34·5 ± 5·7
HyRA4	i.d.	30	7/10	46·1 ± 16·7
Irr. R4/B	s.c.	20	2/10	50·5 ± 6·5
Irr. R4/B	i.d.	20	2/10	40·5 ± 5·7
Irr. R4/B	s.c.	30	3/10	44·0 ± 5·7
Irr. R4/B	i.d.	30	5/10	44·6 ± 8·0
None	—	—	10/10	43·7 ± 8·9

*HyRA4 immunized mice show a significantly higher tumour take than Irr. R4/B immunized mice ($P < 0{\cdot}001$). Difference in tumour takes among HyRA4 immunized and control mice is statistically significant ($P < 0{\cdot}05$).

† ± standard deviation.

RESULTS

As shown in the Table 1, preimmunization with either non-irradiated HyRA4 or X-irradiated R4/B cells was able to reduce the

proportion of tumour takes following challenge inoculation with R4/B cells, but it did so to varying degrees. On the whole, the X-irradiated R4/B cells were significantly more efficient than the non-irradiated HyRA4 cells in terms of being able to protect a higher proportion of mice against the challenge inoculum with R4/B cells. From the total 40 mice immunized with HyRA4 hybrid cells only 11 were protected, whereas the corresponding figures for mice preinoculated with R4/B irradiated cells were 28 in 40. The P value as calculated by the Student's test was $< 0{\cdot}001$. Furthermore, irradiated R4/B cells were able to protect mice relatively better when the challenge delay was 20 rather than 30 days. No such time relationship seemed to exist in groups preimmunized with HyRA4 cells. The route of preimmunizing inoculation, whether subcutaneous or intradermal, did not seem to influence notably the proportion of takes after challenge inoculation.

Concerning the mean survival time of mice in which the tumour grew after challenge inoculation, no consistent and statistically significant differences were seen among the groups of mice preimmunized in different ways and the non-immunized control. Obviously, once tumour cells "took hold" in the inoculated animal, the malignant proliferation progressed fatally.

However, when growth rates of R4/B tumours were analyzed more thoroughly on the mean tumour volume curves, (Figs. 1 and 2) a striking pattern was noticed. In the mice which in spite of HyRA4 or R4/B pretreatment developed tumours from R4/B challenge inoculum, the tumours regularly showed faster growth rates as compared with those in non-pretreated control animals. There was no systematic correlation between the growth rates of tumours and either the route of immunizing inoculations or the delay of challenge (20 or 30 days).

Another interesting observation was concerning the influence of the challenge inoculum of parental R4/B cells on the earlier "immunizing" inoculum of HyRA4 cells. Inoculation of HyRA4 cells into normal, non pretreated BALB/c mice was invariably followed by a rejection of the inoculum. If occasionally, small, hardly palpable nodules appeared, they regressed rapidly. However, among the 40 mice given an immunizing inoculum of non-irradiated HyRA4 cells and later challenged with R4/B cells, four mice developed tumours not only at the site of R4/B inoculation but also on the other flank where HyRA4 cells had been previously injected.

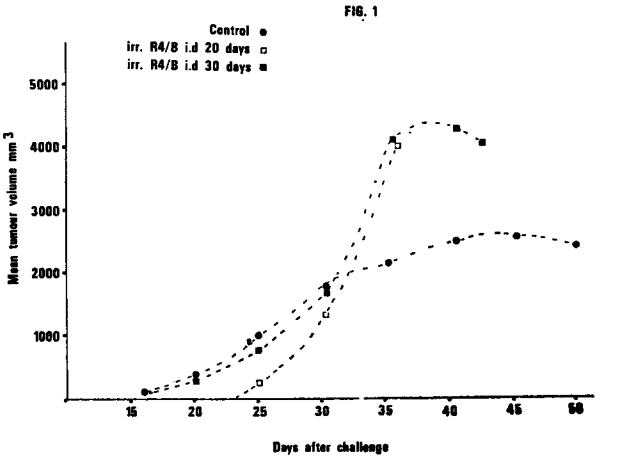


Fig. 1. Growth rate of R4/B (challenge) tumours in mice immunized once with irradiated R4/B dells abd in control non-immunized mice. Twenty days and 30 days refer to the intervals between immunization (i.d. route) and challenge.

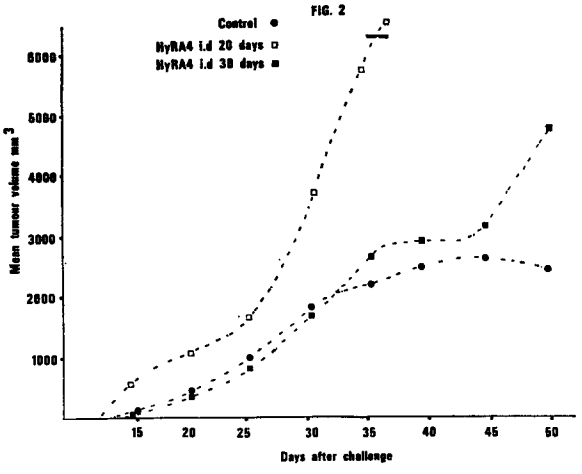


Fig. 2. Growth rate of R4/B (challenge) tumours in mice immunized once with HyRA4 cells and in control non-immunized mice.

These latter tumours grew progressively and two of them were successfully established as cell lines *in vitro*. They were designated as sublines HyRA4TM2 and HyRA4TM3. Karyologically, they were lacking any identifiable AUV chromosome, but they both contained a marker chromosome—an acrocentric one with secondary constriction—which was typical of HyRA4 hybrid cells and was never found either in R4/B or AUV cells [15]. Furthermore, the modal number of telocentric chromosomes approximating twice that of R4/B cells and characteristic for the original HyRA4 cells, was also found in both HyRA4-TM2 and HyRA4TM3 sublines. However, both these sublines, unlike the original HyRA4 cells, were devoid of AUV specific antigens as shown by immunofluorescence tests. Significantly, when HyRA4TM3 cells were inoculated

(10^6 cells subcutaneously) into BALB/c mice hyperimmunized against AUV cells, or into non-immunized control BALB/c mice, tumours appeared easily in both batches, indicating a clear loss of AUV antigeneicity in the HyRA4 TM3 cells.

DISCUSSION

The results reported here indicate that the HyRA4 somatic cell hybrids (resulting from a cross between mouse malignant and Akodon normal cells) carry and express tumour specific antigen(s) proper to the parental mouse neoplastic cells. The hybrid cells, when inoculated into mice, syngeneic to the parental neoplastic cells, induce a certain degree of immunity.

While the original findings of Watkins and Chen [6] showed that interspecies somatic cell hybrids immunize mice quite efficiently against parental neoplastic cells, our findings reveal a different situation. The hybrid HyRA4 cells carrying about twice the chromosome complement from the R4/B parent, were in fact no more immunogenic than the X-irradiated R4/B cells.

At least two possibilities may be envisioned to explain this difference in the immunogenicities. The first explanation rests on a suppression—at least partial—of cell surface antigens, in the hybrid cells, similar to that reported by Klein *et al.* [18] and Grundner *et al.* [19] in somatic cell hybrids involving Ehrlich ascites tumour (EAT) as one of the parental cells. This rather exceptional phenomenon seemed to be due to a particular nature of EAT cells which might have developed—during the very long history of their growth in allogeneic hosts—an active antigen-masking mechanism transmissible to their hybrids. Such a situation is highly improbable in our hybrid system, since the malignant parental cells did not undergo any selection by allotransplantation and were able to grow in syngeneic mice only. Furthermore, HyRA4 cells in earlier passages were able to induce an efficient protection of mice against challenge with R4/B parental malignant cells [15]. Moreover, they were repeatedly positive in immunofluorescence tests with specific antisera prepared against Gross-type antigen, characteristic of the R4/B cells.

Another explanation for the relatively low transplantation immunogenicity of HyRA4 hybrid cells could be based on the possibility of a partial loss and/or modified distribution of R4/B specific transplantation antigen(s) in the hybrid cells. Genetic rearrangements being common among somatic cell hybrids, HyRA4 cells might have lost some of the R4/B genetic material responsible for the expression of TSTA or some of its components. Such a genetic instability has been illustrated by some observations of different immunogenic ability of parallel hybrid clones formed by crossing the same parental cell lines. For instance, Parkman [9] reported that fusion between Cl 1D and EL-4 cells resulted in 5 hybrid clones more immunogenic than the irradiated parental EL-4 cells, while the other two clones were less so. Further, the quantitative differences in the expression of TSTA among different hybrid clones from the same parental cells [7, 9] or the same clone during its evolution allows one to admit that the expression of TSTA may be governed by more than one genetic locus. Therefore, one can assess that the somatic cell hybrids may be more, less or as immunogenic as the parental neoplastic cells, depending upon the extent of participation of the genetic material controlling expression of TSTA.

Immunization with either HyRA4 or R4/B cells was effective against R4/B challenge in some mice while not so in some others. However, once the immunological barrier was overcome, the tumour grafts progressed unabated and showed a different growth pattern when compared to those in the control mice. Further, the tumours which appeared in immunized animals showed even an enhanced growth rate compared to those in the controls. Differences in the tumour sizes between the control and the immunized groups were obvious, especially at the later stages of tumour growth. Similar situation was seen in another experiment (Satya Murthy and G. Barski, unpublished data) using another interspecies mouse/Chinese hamster somatic hybrid cells, the HyCS [8], as immunizing material against their malignant parental cells in mice.

Enhancement of growth of antigenic tumours was observed by several authors as being due to serum blocking factors impending the cell-mediated immune response [20–23]. An enhancement during the early stages of tumour growth can be due, as has been proposed recently [24–26], to a stimulatory effect of weak cell mediated immunity. This hypothesis that weak cell mediated immunity may stimulate the tumour growth, while a stronger one may be inhibitory, has been confirmed in other system also [27, 28]. Since in our experiments on the whole only moderate or weak immune response was observed after immunization with R4/B or HyRA4 cells, one can

admit that the situation here, at least in the limit cases, was similar to that described by the authors referred to above. Whatever is the mechanism involved in inducing increased growth rate of challenge graft tumours, the phenomenon itself is noteworthy.

In spite of an extensive loss of AUV chromosomes, HyRA4 cells, with as few as four AUV chromosomes, were able to evoke a graft rejecting reaction, when injected into normal BALB/c mice. However, when inoculated into BALB/c mice to immunize against a challenge of R4/B cells, a small proportion of mice developed progressively growing tumours not only from the R4/B challenge inoculum, but also at the site of previous "immunizing" HyRA4 inoculation. Although the karyological examination of the sublines obtained from these latter tumours did not show any identifiable complete AUV chromosomes, we consider them as originating from HyRA4 inoculum not only because the tumours were localized at the site of HyRA4 inoculation, but also because of the karyological identification of an abnormal, marker chromosome characteristic of HyRA4 cells. This conclusion is reinforced by the fact that the outgrowing tumours contained a number of mouse chromosomes close to the one characteristic of the HyRA4 hybrid cells, i.e., double that of the R4/B cells. When examined for the expression of AUV species specific surface antigens by IF test, the two sublines derived from these secondary tumours appeared to be negative. The absence of AUV species specific antigens on HyRA4 TM3 cells was further confirmed by *in vivo* transplantation test (growth in normal as well as anti-AUV hyperimmunized BALB/c mice). Thus, it became clear that the two tumour derivatives of HyRA4 were variants of HyRA4 cells with an apparent total loss of karyological and antigenic characters of the AUV parental cells.

These facts suggest that the presence of the R4/B tumour enhanced the growth of some—even if few—AUV antigen-free, but R4/B antigen(s) carrying "dormant" variant cells derived from the original HyRA4 inoculum. Such "dormant" variants might have been originally present in the inoculum, but due to their small number they might not have been able to grow normally in mice, unless enhanced by serum or other factor(s) supplied or induced by the presence of the R4/B tumour and protecting them against cell-mediated immune destruction. This production of variants from cell hybrids, intended for immunization, rings a warning signal. One redeeming feature, however, is that in case of human × rodent cell hybrids, most often the human genetic material is the one that is preferentially lost [14]. Thus, the evolution of the inoculated man × mouse hybrids towards malignant human parental cell—predominantly if not totally—seems to be a remote possibility. However, it calls for care when cell hybrids are considered as candidates for purposes of anti-tumour immunization.

Acknowledgement—We also appreciate the excellent technical assistance of Ms. B. Leon and Ms. M. Thonier.

REFERENCES

1. N. P. Czajkowski, M. Rosenblatt, F. R. Cushing, J. Vazquez and P. L. Wolf, Production of active immunity to malignant reoplastic tissue. Chemical coupling to an antigeneic protein carrier. *Cancer (Philad.)* **19,** 739 (1966).
2. G. Svet-Moldavsky and V. P. Hamburg, In: *Specific Tumour Antigens,* UICC Monograph Series 2 (Munksgrad, Copenhagen) (1967).
3. W. J. Martin, J. R. Wunderlich, F. Fletcher and J. K. Inman, Enhanced immunogenicity of chemically-coated syngeneic tumour cells. *Proc. nat. Acad. Sci. (Wash.)* **68,** 469 (1971).
4. C. W. Boone, M. Paranjpe, T. Orme and R. Gillette, Virus-augmented tumour transplantation antigens: evidence for a helper antigen mechanism. *Int. J. Cancer.* **13,** 543 (1974).
5. R. A. Spencer, T. S. Hauscha, D. B. Amos and B. Ephrussi, Co-dominance of isoantigens in somatic hybrids of murine cells grow *in vitro. J. nat. Cancer Inst.* **33,** 893 (1964).
6. J. F. Watkins and L. Chen, Immunization of mice against Ehrlich ascites tumours using a hamster/Ehrlich ascites tumour hybrid cell line. *Nature (Lond.)* **223,** 1018 (1969).
7. J. Jami and E. Ritz, Expression of tumour specific antigens in mouse somatic cell hybrids. *Cancer Res.* **33,** 2524 (1973).

8. G. Barski, M. G. Blanchard, J. K. Youn and B. Leon, Expression of malignancy in interspecies Chinese hamster × mouse cell hybrids. *J. nat. Cancer Inst.* **51,** 781 (1973).
9. R. Parkman, Tumour hybrid cells: an immunotherapeutic agent. *J. nat. Cancer Inst.* **52,** 1541 (1974).
10. G. Barski and F. Cornfert, Characteristics of "hybrid"-type clonal cell lines obtained from mixed cultures *in vitro*. *J. nat. Cancer Inst.* **28,** 801 (1962).
11. M. C. Weiss and B. Ephrussi, Studies on interspecific (rat × mouse) somatic hybrids. 1. isolation, growth and evolution of the karyotypes. *Genetics* **54,** 1095 (1966).
12. G. Barski, Cell association and somatic cell hybridization. *Int. Rev. exp. Pathol.* **9,** 151 (1970).
13. J. Belehradek, Jr. and G. Barski, Karyological patterns and expression of malignancy in some homologous mouse somatic hybrid cells. *Int. J. Cancer* **8,** 1 (1971).
14. M. C. Weiss and H. Green, Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. *Proc. nat. Acad. Sci. (Wash.)* **58,** 1104 (1967).
15. J. Belehradek, Jr., M. Satya Murthy, M. Thonier, B. Leon and G. Barski, Interspecies mouse × Akodon urichi somatic hybrid cells: Expression of malignancy. To be published 1975.
16. M. A. M. Attia and D. W. Weiss, Immunology of spontaneous mammary carcinomas in mice V. Acquired tumour resistance and enhancement in strain A mice infected with mammary tumour virus. *Cancer Res.* **26,** 1787 (1966).
17. G. Möller, Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. *J. exp. Med.* **114,** 415 (1961).
18. G. Klein, U. Gars and H. Harris, Isoantigen expression in hybrid mouse cells. *Expt. Cell Res.* **62,** 149 (1970).
19. G. Grundner, E. M. Fenyö, G. Klein, E. Klein, U. Bergula and H. Harris, Surface antigen expression in malignant sublines derived from hybrids of low malignancy. *Expt. Cell Res.* **68,** 315 (1971).
20. G. Möller, Studies on the mechanism of immunological enhancement of tumour homografts. 1. Specificity of immunological enhancement. *J. nat. Cancer Inst.* **30,** 1153 (1963).
21. K. E. Hellström, I. Hellström and R. J. Brawn, Abrogation of cellular immunity to antigenically foreign mouse embryonic cells by a serum factor. *Nature (Lond.)* **224,** 914 (1969).
22. G. H. Heppner, Studies on serum mediated inhibition of cellular immunity to spontaneous mouse mammary tumours. *Int. J. Cancer* **4,** 608 (1969).
23. N. Kaliss, Dynamics of immunologic enhancement. *Transplant. Proc.* **2,** 59 (1970).
24. R. T. Prehn and M. A. Lappé, An immunostimulation theory of tumour development. *Transplant Rev.* **7,** 26 (1971).
25. R. T. Prehn, Perspectives on oncogenesis: Does immunity stimulate or inhibit neoplasia? *J. Reticuloendothel. Soc.* **10,** 1 (1971).
26. R. T. Prehn, The immune reaction as a stimulator of tumour growth. *Science* **176,** 170 (1972).
27. I. J. Fidler, *In vitro* studies of cellular-mediated immunostimulation of tumour growth. *J. nat. Cancer Inst.* **50,** 1307 (1973).
28. I. J. Fidler, Immune stimulation-inhibition of experimental cancer metastasis. *Cancer Res.* **34,** 491 (1974).

Europ. J. Cancer Vol. 12, pp. 41–45. Pergamon Press 1976. Printed in Great Britain

Effect of CCNU on Survival, Rate of Objective Remission and Duration of Free Interval in Patients with Malignant Brain Glioma—First Evaluation*

EORTC BRAIN TUMOR GROUP†‡

Abstract—*The effect of CCNU on three clinical parameters was measured in patients with malignant brain gliomas, operated and treated by radiation therapy* (5500–6000 *rads*).

From 100 *selected patients included in the first trial* (*No.* 26741) 81 *were evaluable. Four questions were answered by this study:*

(*a*) *Free interval can be measured in a selected group of patients with malignant brain glioma.*

(*b*) *CCNU* (*mean dosage actually given was* 106 *mg/m*^{2} *once every* 6 *weeks*) *does not prolong the free interval.*

(*c*) *Objective remission was observed in* 4 *out of* 16 *patients where this parameter was evaluable.*

(*d*) *Total survival time was* 77 *weeks in patients treated with CCNU only after relapse and* 50 *weeks in those treated within* 3 *weeks after operation until death. This difference is significant at the* 5% *level.*

Only survival time was measured in the second trial (*No.* 26742), *it was* 33 *weeks in patients treated by CCNU and* 17 *in the control group. Although only* 17 *patients were evaluated, the difference is significant at* 5% *level.*

Combined results of both trials suggest so far that: CCNU prolongs the total survival time, produces objective remission in about 25% *of cases, and that it should be administered only after relapse, at least in patients with free interval.*

Accepted 15 September 1975.

*This research was supported by the "Fonds de la Recherche Scientifique Médicale (F.R.S.M., Belgium) and by a grant from The National Cancer Institute, Bethesda, MD 20014, U.S.A.

†The members of the Group who actively participated to this trial are: J. Brihaye, Service de Neurochirurgie, Université de Bruxelles, J. Brochi, Clinique Neuro-Chirurgicale, Université de Liège, L. Calliauw, Afdeling Neurochirurgie, St. Jans Hospital, Brugge, J. Hildebrand, Service de Médecine et d'Investigation Clinique, Institut Jules Bordet, Université Libre de Bruxelles, B. Le Mevel, Centre R. Gauducheau and F. Resche, Clinique Neuro-Chirurgicale, C. H. R. Nantes, G. Stroobant, Clinique de Neurologie et Nerochirurgie, Université de Louvain, and S. George and G. Flowerdew, EORTC Data Center—Institut J. Bordet, Bruxelles.

‡Requests for reprints should be sent to J. Hilldebrand —Secretary of the EORTC Brain Tumor Group, Institut Jules Bordet, 1000 Brussels, Belgium.

INTRODUCTION

THREE nitrosourea derivates BCNU, CCNU and methyl CCNU have been shown to be active against malignant brain glioma either in phase II-type studies measuring objective remissions [1–4] or in phase III studies where total survival time was determined [5].

The interest of the EORTC brain tumor group has been limited so far to CCNU [1-(2-chloroethyl-3-cyclohexyl-1-nitrosourea)]. The aim of our study is to investigate the effects of CCNU on three clinical parameters: total survival time, objective remission, and free interval between operation and relapse.

The purpose of the present communication is to report preliminary, yet statistically significant results of our first two trials.

MATERIAL AND METHODS

Criteria for patient selection and evaluation of therapy, the design of the two trials, and the administration of CCNU, dexamethazone and radiotherapy have been previously reported [6], and will be briefly summarized here.

Patients had to satisfy the following criteria to be admitted to the two trials (No. 26741 and 26742). Operation, consisting of maximum resection, had to be performed 3 weeks before the start of chemotherapy. The diagnosis had to be based on histology. Patients were expected to have a survival of 8 weeks or more. They had to have a normal hematopoietic, liver and renal function and no major medical disease. Two additional criteria were required for admission to trial 26741. Steroid therapy had to be stopped on the 10th day after surgery. The neurological examination had to be either normal or show incomplete deficits which could be measured quantitatively and serve as criteria for further evaluation.

STUDY DESIGN

(*a*) *Trial* 26741

Three weeks after neurosurgery and 10 days after complete discontinuation of steroids, the patients were randomized. Group I received CCNU 130 mg/m^2 once every 6 weeks, group II served as control. All patients received 5500–6500 rads radiotherapy and were followed with the same frequency once every 3 weeks until recurrence of neurological symptoms, to determine the effect of CCNU on the duration of the free internal. After recurrence, steroid administration was permitted and both groups received 130 mg of CCNU per m^2 once every 6 weeks until death. The rate and duration of objective remission after recurrence was noted in patients of group II. There were no remissions in group I since these patients had already been receiving CCNU prior to recurrence. The total survival time was reported for all patients.

(*b*) *Trial* 26742

Patients were randomized 20 days after surgery. Group I received CCNU 130 mg/m^2 once every 6 weeks until death, while group II served as control. Only total survival time was measured in this trial.

RESULTS

The results of trial 26741 and 26742 were evaluated separately.

Trial 26741

The purpose of this trial was to answer four questions.

(*a*) Can we measure the free interval, at least in selected patients with malignant glioma? The answer to this question appears to be yes. Out of 100 patients included to this trial 81 were evaluable. In all these selected patients with normal neurological examination or minimal deficit at outset, first *clinical* signs of tumor progression (suspected recurrence) were confirmed (confirmed recurrence) within two weeks by aggravation of neurological status and/or a worsening of the EEG, brian scan or both. Thus on basis of clinical examination, EEG and brain scan free interval could be determined with a two week accuracy.

(*b*) Does the early postoperative administration of CCNU prolong the free interval? The answer seems to be no. As shown on Fig. 1,

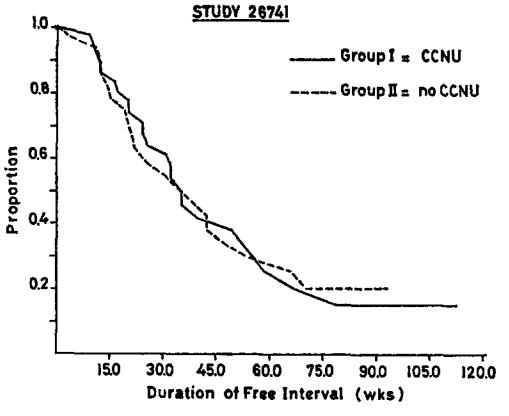


Fig. 1. *Trial* 26741. *Free interval length curves in* 45 *patients receiving CCNU* (——) *and* 36 *controls* (– – – –).

the curves of free interval for patients treated with CCNU and for controls are almost identical. The non-parametric maximum likelihood estimate of the median free interval is about 34 weeks in both groups. The mean dosage of CCNU actually given was 106 mg/m^2 per six weeks. This represents over 80% of maximal scheduled dosage.

(*c*) What is the rate of objective remission in patients receiving CCNU only after recurrence? So far there are only 16 patients for whom this

parameter is evaluable. Objective remission was observed in 4 of these 16 patients. The duration of remission ranged from 4 to 53 with a mean of more than 30 weeks.

(*d*) Finally, the survival time of patients treated with CCNU within 3 weeks after neurosurgery was compared to those who received CCNU only after relapse (Fig. 2).

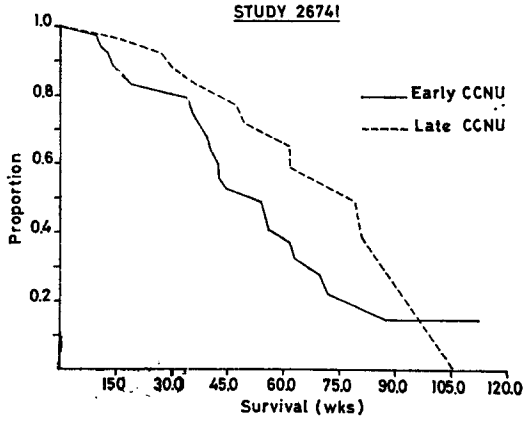


Fig. 2. *Trial* 26741. *Total survival time curves in* 45 *patients treated with CCNU* 3 *weeks after the neurosurgical procedure* (*early CCNU* ——) *and in* 36 *patients treated only after relapse* (*late CCNU* – – – –).

The estimated median survival in the group who received CCNU early was 50 weeks, as compared to 77 weeks in patients treated only after recurrence of neurological signs. This difference is significant at the 5% level. Since the free-interval curves (Fig. 1) suggest that the administration of CCNU does not influence the duration of the remission, the difference in overall survival must result from a longer survival after recurrence in the group receiving CCNU only after relapse. This is further demonstrated in Fig. 3, which indicates a significantly ($P < 0{\cdot}01$) shorter survival time after recurrence in patients treated early with CCNU (5 weeks) than in the group where CCNU was given after relapse (17 weeks).

Trial 26742

The aim of this trial was to compare the total survival times in patients receiving CCNU with control patients. As shown in Fig. 4, the estimated median survival of those receiving CCNU was 33 weeks, compared with 17 weeks in controls. This difference is significant at the 5% level.

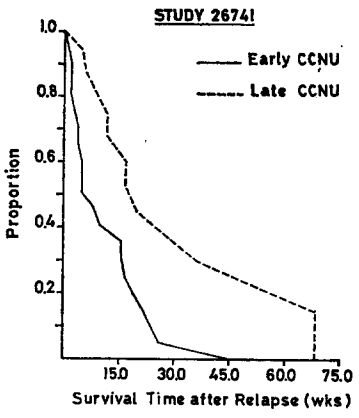


Fig. 3. *Trial* 26741. *Survival time curves after relapse in* 24 *patients treated with CCNU* 3 *weeks after the neurosurgical procedure* (*early CCNU* ——) *and in* 20 *patients treated only after relapse* (*late CCNU* – – – –).

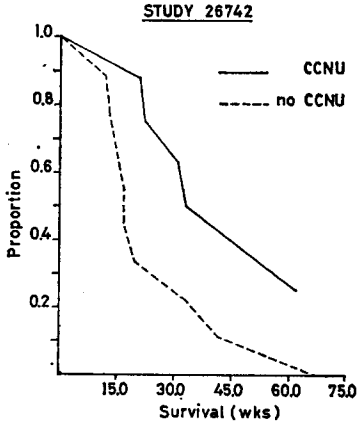


Fig. 4. *Trial* 26742. *Total survival time curves in* 8 *patients treated with CCNU* 3 *weeks after the neurosurgical procedure until death* (*CCNU* ——) *compared to* 9 *patients who received no chemotherapy* (*no CCNU* – – – –).

Toxicity

The toxicity was essentially hematological. It was cumumative in most patients and involved mainly a drop in platelets count. No patients died from drug toxicity.

DISCUSSION

The present study investigates the effect of CCNU on three clinical parameters: total survival time, rate of objective remission and free interval in patients with malignant brain glioma. All were treated by surgery and radiation therapy.

Because it is easy to measure with accuracy, survival time is generally considered as the best criterion for testing the activity of drugs in malignant brain tumors. Even though this parameter has been measured so far in only 17

patients, the results already indicate that total survival is significantly prolonged by administration of CCNU. The measure of total survival requires that treated patients and controls be kept under similar conditions since death may result from lack of supportive care. This condition was not fulfilled in the present study. For this reason, we feel it necessary to study two other parameters.

The occurrence of objective remission, defined here as a clear cut clinical improvement persisting 6 weeks or more after complete discontinuation of steroids, was only studied in patients treated by CCNU after relapse for a period of 12 weeks or more, usually corresponding to 3 courses of CCNU. An objective remission was observed in 4 patients out of 16. A similar rate was found in our previous study where CCNU was given in combination with methotrexate and vincristine [7], but a considerably higher percentage of favourable results has been reported by Rosenblum *et al.* [3]. In this study, however, stabilization of neurological signs was considered as a favourable result, whereas an objective improvement was required in our trial.

The main criticism to the study of the free interval between operation and relapse is that it is difficult to measure with accuracy. Our present experience shows that this difficulty can be overcome by selecting patients with minimal deficit or a normal neurological examination. It is fully realized by the authors that recurrence of neurological signs and symptoms does not necessary correspond to a change in the rate of tumor growth. The rationale for measuring this parameter is that it gives an idea about the quality of the survival. It should be also emphasized that during free interval no supportive therapy, in particular no steroids, which may interfere with the activity of CCNU, was ever given. The failure of CCNU administration to prolong the free interval is not inconsistent with its favourable effect on total survival time and production of objective remissions. One can speculate that patients with free interval are predominantly those with a slow postoperative tumor growth rate, possibly due to radiation therapy. Thus, early administration of CCNU will have little antitumor effect, but may select cells resistant to the drug. For the same reason, tumors of patients treated with CCNU only after recurrence may be more sensitive to the drug. In addition, because of cumulative toxicity of nitrosoureas, these patients can be treated more vigorously than those who received CCNU immediately after operation.

Patients who received CCNU 3 weeks after neurosurgery until death have an expected medium survival of 50 weeks in trial 26741 and only 33 weeks in trial 26742. The difference is significant ($P < 0{\cdot}05$) and was actually expected, since patients selected for trial 26741, who did not require the administration of DXM and had initially a minimal neurological deficit, had a better prognosis than those included into trial 26742. The median expected survival estimated by combining the patients of the two trials, who were treated by CCNU after neurosurgery until death was 46 weeks. This figure is quite close to the 42 week median survival time reported by Walker and Gehan [5] in patients with malignant glioma treated by radiotherapy and BCNU after surgery and selected according to general criteria similar to those used in this study.

To conclude, the combined results of the two trials suggest that CCNU adds something to the treatment of tumor malignant gliomas previously operated and treated by radiation therapy:

(*a*) it prolongs the survival time,

(*b*) it is able to produce objective remissions in about 25% of treated patients,

(*c*) in patients with free interval it seems that CCNU should be given only after relapse, and not before.

REFERENCES

1. M. D. Walker and B. S. Hurwitz, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea; NSC-409962) in treatment of malignant brain tumor. A preliminary report. *Cancer Chemother. Rep.* **54,** 263 (1970).
2. C. B. Wilson, E. B. Boldrey and K. Jean Enot, 1,3-bis(2-choroethyl)-1-nitrosourea (NSC-409962) in the treatment of brain tumors. *Cancer Chemother. Rep.* **54,** 273 (1970).
3. M. L. Rosenblum, A. F. Reynolds, K. A. Smith, B. H. Rumack and M. D. Walker, Chloroethyl-dicyclohexyl-nitrosourea (CCNU) in the treatment of malignant brain tumors. *J. Neurosurg.* **39,** 306 (1973).

4. T. H. WASSERMAN, R. L. COMIS, M. GOLDSMITH, H. HANDELSMAN, J. S. PENTA, M. SLAVIK, W. T. SOPER and S. K. CARTER, Tabular analysis of the clinical chemotherapy of solid tumors. *Cancer Chemother. Rep.* In press (1975).
5. M. D. WALKER and E. A. GEHAN, An evaluation of 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and irradiation alone and in combination for the treatment of malignant glioma. *Proc. 63rd Ann. Meeting Amer. Ass. Cancer Res.* (1972).
6. J. HILDEBRAND, J. BRIHAYE, J. C. GOFFIN and M. STAQUET, EORTC Protocol for the study of CCNU in the treatment of irradiated, operated, malignant glioma of the brain. *Europ. J. Cancer* **9,** 459 (1973).
7. J. HILDEBRAND, J. BRIHAYE, L. WAGENKNECHT, J. MICHEL and Y. KENIS, Combination chemotherapy with CCNU, vincristine and methotrexate in primary and metastatic brain tumors. *Europ. J. Cancer* **11,** 585 (1975).

Europ. J. Cancer Vol. 12, pp. 47–51. Pergamon Press 1976. Printed in Great Britain

In vitro Cellular Radiosensitivity of Human Malignant Tumors*

RALPH R. WEICHSELBAUM†, J. EPSTEIN‡ and JOHN B. LITTLE
Laboratory of Radiobiology, Department of Physiology, Harvard University School of Public Health, Boston, MA 02115, *U.S.A.* and

PAUL L. KORNBLITH
Department of Neurosurgery, Massachusetts General Hospital, Boston, MA 02114, *U.S.A.*

Abstract—*Cells from four human tumors considered to be either clinically curable by radiation therapy (medulloblastoma) or non-radiocurable (osteogenic sarcoma, glioblastoma multiforme) were grown in tissue culture and the* in vitro *X-ray survival curve parameters determined. These were compared with the survival parameters for normal human diploid skin fibroblasts grown and irradiated under identical conditions. There were no significant differences in response among the tumor cell lines: the D_0 values ranged from* 130 *to* 144 *rads, and $\bar{n}$ from* 1·4 *to* 1·9. *For normal lcells, D_0 = 132 rads, and the $\bar{n}$ = 0·95. These results suggest that the wide range of clinical radiocurabilities found among various human tumors cannot be explained on the basis of the results of the* in vitro *survival curve parameters.*

INTRODUCTION

HUMAN malignant tumors vary in their radiocurability. Some tumors, such as osteosarcoma and glioblastoma multiforme, can be locally controlled only infrequently using very high high doses of radiation [1, 2]. Others, such as medulloblastoma, may be radiocurable in up to 30% of all cases [3]. It has been postulated that a major determinant of radiocurability is the intrinsic cellular radiosensitivity of the various tumor cell types [4].

Accepted 15 September 1975.

*This work was supported by grants CA-05237, CA-07368, and CA-11751 from the National Cancer Institute and grant ES-00002 from the National Institute of Environmental Health Sciences.

†Clinical fellow, Joint Center for Radiation Therapy, Department of Radiation Therapy, Harvard Medical School, Boston, MA 02115, U.S.A.

‡Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, and The Clinical Genetics Unit, Department of Medicine, Children's Hospital Medical Center, Boston, MA 02115, U.S.A.

In an attempt to determine whether the clinical behavior of certain tumors has its basis in differing intrinsic sensitivities of the cells themselves to radiation, various human tumors considered to be either radiocurable (medulloblastoma) or essentially non-radiocurable (osteogenic sarcoma, glioblastoma multiforme) were grown in tissue culture and their X-ray survival parameters determined. In addition to these cells, normal human diploid skin fibroblasts derived from a normal adult were also grown in tissue culture to serve as a model for nonmalignant human cells.

MATERIAL AND METHODS

Origin and maintenance of cell lines

Two cell lines derived from different human medulloblastomas (designated TX-7 and TX-14), and a line derived from a human glioblastoma (TX-13) were established in culture by Dr. Paul Kornblith at the Massachusetts General Hospital as described in detail elsewhere [5, 6]. These uncloned tumor lines were grown in Ham's F-10 medium with Earle's

balanced salts supplemented with 15% fetal calf serum, and 50 mcg/ml of Aureomycin (Lederle Laboratories, Pearl River, New York).

A human osteogenic sarcoma line, designated TX-4, was isolated in this laboratory and cloned twice before use, once from monolayer colonies, and again from suspension culture. This procedure has been described in great detail elsewhere [7]. Stock and experimental cultures of TX-4 were maintained in a medium composed of 50% Eagle's minimal essential medium (MEM) and 50% Ham's F-10, both with Earle's balanced salts, and supplemented with 7·5% horse serum, 7·5% fetal calf serum, 450 mg/l glucose, and 3·3 mg/l sodium pyruvate.

EX-25 is a human diploid skin fibroblast strain which was derived from the volar surface of the forearm of a normal adult male; it has an *in vitro* lifespan of approximately 50 mean population doublings. This strain was maintained in MEM with Earle's balanced salts and 15% fetal calf serum. The details of biopsy and maintenance *in vitro* after explanting of EX-25 have been described in detail previously [8].

All stock cultures were grown in monolayer in unsealed plastic flasks (Falcon, Oxnard, California) in a water-saturated 5% CO_2–96% air environment at 37°C. Cultures were checked routinely and found to be free of mycoplasma contamination during the course of these experiments.

Survival assay methods

For survival experiments, cells were trypsinized from exponentially growing monolayers of stock cultures and cell counts determined in a hemacytometer. By microscopic examination greater than 98% of trypsinized cells appeared to be present as single cells. The single cell suspensions were then distributed to 4 replicate 100 mm dia plastic petri dishes, containing 10 ml of medium, for each X-ray dose. In different experiments, enough cells were plated to allow for either 15–20 or 30–40 surviving clones per dish. That is, in different experiments, the initial cell innoculum was varied by a factor of two to insure that the number of survivors was dependent only on X-ray dose and not on the initial number of cells plated.

Twenty-four hours after plating into petri dishes, the cells in monolayer were irradiated at room temperature with a 220 kVp General Electric Maximar generator operating at 15mA (HVL = 0·3 mm Cu) with a dose rate of 80 rads/min. After irradiation, the cells were returned to the same incubator as the stock cultures where they were grown for 21–28 days. At weekly intervals, media changes were performed using the same media in which the stock cultures were maintained.

Data evaluations

Dishes were fixed and stained, and only those clones with a minimum of 50 cells each were counted as survivors. Since greater than 98% of the cells were originally plated as single cells, it is unlikely that a significant number of survivors arose from pairs or clumps of cells. Care was also taken to insure that surviving clones were of uniform size distribution, and in no case were surviving clones found to consist of more than 75 cells which might have suggested a multiple cell origin.

Each survival point represents the mean of four dishes, each dish containing a minimum of 15 clones. The curves shown for each cell line were fitted by a least squares regression analysis of data gathered from 2 to 4 separate experiments performed on each line.

The population doubling times of all cell lines during exponential growth were determined by plating 5×10^4 cells in replicate 60 mm petri dishes containing 5 ml of the medium in which the stock culture was maintained; no media changes were made after the initial plating. At 24 hr intervals after plating, cell counts were made in a hemacytometer and the doubling times determined graphically for each line.

RESULTS

Radiation survival curves for the four tumor lines are presented in Figures 1–4, and that for the normal diploid strain in Figure 5. The survival parameters derived from these curves are tabulated in Table 1. For the tumor lines, it can be seen that the D_0 values ranged from 130 to 144 rads, while the extrapolation number ($\bar{n}$) values ranged from 1·4 to 1·9. The D_0 value for the normal human diploid skin fibroblast strain (EX-25) was 132 rads. This is well within the range for all 4 tumor lines. The $\bar{n}$ for EX-25, however, was 0·95, which is smaller than that of any of the tumor lines. In similar experiments with several other normal diploid skin strains we have consistently found $\bar{n}$ to be less than 1·2, and to average about 1·0.

The population doubling times for each of the tumor lines varied greatly from experiment to experiment, but ranged from 39 to 72 hr. This is in contrast to the normal human skin

strain which consistently gave a mass culture doubling time of 22–24 hr when examined between the 20th and 30th *in vitro* passage, the age at which the survival curves were performed for this report.

It should be noted that the cloning efficiencies for the tumor lines ranged from 3 to 12%, and those for the normal cells from 8 to 22%. These experiments were performed by 3 investigators independently over the course of 8 months, and the cloning efficiencies were consistently within these ranges.

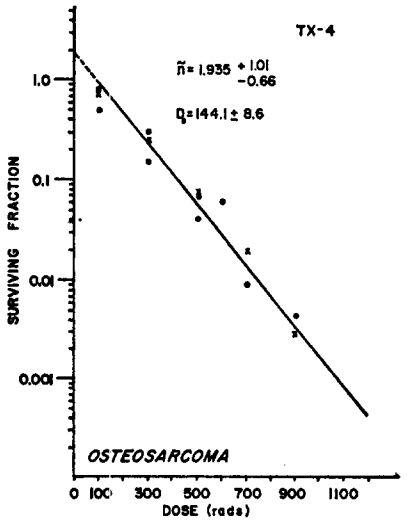


Fig. 1. *X-ray survival curve for cells from a human osteogenic sarcoma (TX-4). Different symbols represent data points from separate experiments. In these experiments, single cell suspensions were plated, irradiated 24 hr later, and after 21–28 days of incubation for colony growth, the survival parameters determined. The curve shown was fitted by a least squares regression analysis of the points shown; the* $\bar{n}$ = 1·9, *and the* D_0 = 144 *rads.*

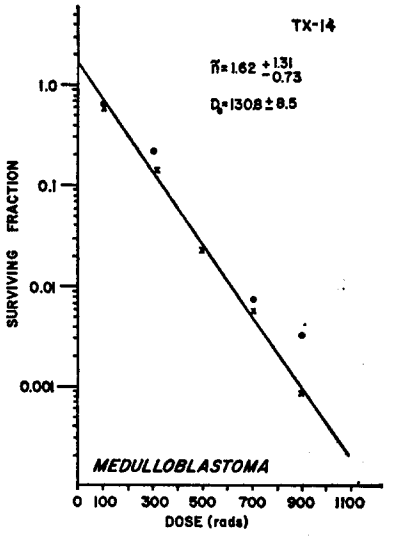


Fig. 3. *X-ray survival curve for cells from a second human medulloblastoma (TX-14). Different symbols represent data points from separate experiments. In these experiments, single cell suspensions were plated, irradiated 24 hr later, and after 21–28 days of incubation for colony growth, the survival parameters determined. The curve shown was fitted by a least squares regression analysis of the points shown; the* $\bar{n}$ = 1·6, *and the* D_0 = 130 *rads.*

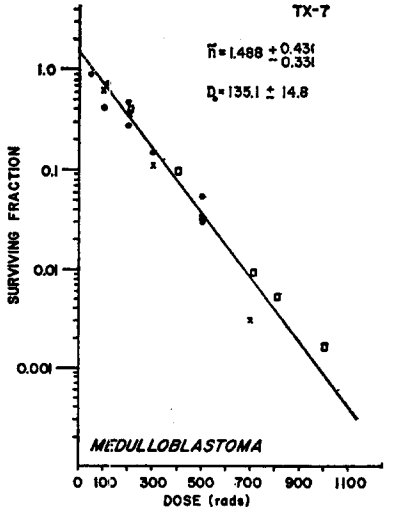


Fig. 2. *X-ray survival curve for cells from a human medulloblastoma (TX-7). Different symbols represent data points from separate experiments. In these experiments, single cell suspensions were plated, irradiated 24 hr later, and after 21–28 days of incubation for colony growth, the survival parameters determined. The curve shown was fitted by a least squares regression analysis of the points shown; the* $\bar{n}$ = 1·5 *and the* D_0 = 135 *rads.*

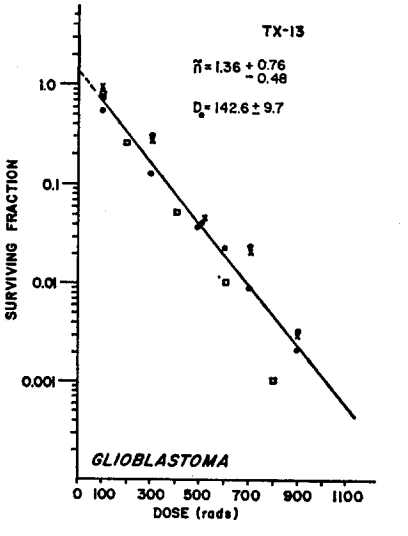


Fig. 4. *X-ray survival curve for cells from a human glioblastoma multiforme (TX-13). Different symbols represent data points from separate experiments. In these experiments, single cell suspensions were plated, irradiated 24 hr later, and after 21–28 days of incubation for colony growth, the survival parameters determined. The curve shown was fitted by a least squares regression analysis of the points shown;* $\bar{n}$ = 1·4, *and the* D_0 = 143 *rads.*

Table 1. In vitro *growth and X-ray survival parameters for four human malignant tumors and normal human diploid skin fibroblasts*

Cell line*	Type	Doubling time (hr)	No. of experiments	Cloning efficiency	D_0	$\bar{n}$
TX-7	Medulloblastoma	= 42	4	5–10	135	1·5
TX-14	Medulloblastoma	= 40	2	9–11	130	1·5
TX-13	Glioblastoma	= 39–59	4	5–12	143	1·4
TX-4	Osteogenic sarcoma	= 44–72	4	3–12	144	1·9
EX-25	Normal skin	= 22–24	3	8–22	132	0·95

*Some of these cells have also been designated as follows: TX-7, TC898; TX-14, TC971; TX-13, TC656.

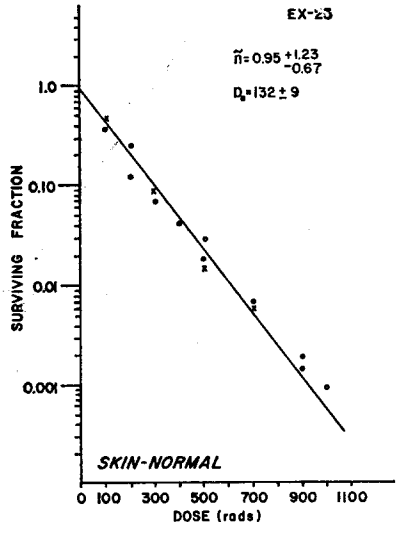


Fig. 5. *X-ray survival curve for fibroblasts isolated from human skin (EX-25). Different symbols represent data points from separate experiments. In these experiments, single cell suspensions were plated, irradiated 24 hr later, and after 21–28 days of incubation for colony growth, the survival parameters determined. The curve shown was fitted at least squares regression analysis of the points shown;* $\bar{n}$ = 0·95, *and the* D_0 = 132 *rads.*

DISCUSSION

The tumor lines chosen for this study were known to have greatly different radiocurabilities. Medulloblastoma has been reported to be controlled locally (cure of the primary tumor) in 30–40% of affected patients after a total dose of 5000–5500 rads [3]. Glioblastoma multiforme, on the other hand, has less than a 5% incidence of local control with cumulative doses as high as 6500 rads [2]. Similarly, osteogenic sarcomas are notoriously difficult to cure even with very high doses of fractionated radiation. Thus, in terms of clinical curability, these three tumors offer examples of good and poor results with radiation. When these tumors were cultured and irradiated *in vitro* under identical conditions of exponential growth, however, there was no significant difference among the survival parameters (D_0, $\bar{n}$); thus the intrinsic cellular radiosensitivity of the four tumor lines examined cannot be said to vary greatly under the conditions of exponential cell growth *in vitro*. It could be argued that because of the low cloning efficiencies in these experiments (4–22%), only a subpopulation of each cell line was actually being examined. However, the survival characteristics were not influenced by cloning efficiences which varied over this six-fold range. Furthermore, there was no evidence of a biphasic nature to the survival curves in any of these experiments. We thus feel that the low cloning efficiencies have not greatly influenced the results, and the survival characteristics are most likely representative of the entire population of tumor cells. It could also be argued that the conditions of the *in vitro* growth are quite different from those in which the tumors grow *in vivo*. Since normal skin cells were cultured under similar conditions and found not to differ greatly in their response from any of the tumor lines, this can be taken as additional evidence that no intrinsic difference in radiosensitivity as measured by D_0 (and also $\bar{n}$) values could be found among the various human tumor cells we have examined.

The shoulder size has great influence in the ultimate results of clinically fractionated radiotherapy. Split dose experiments might give a clearer picture of shoulder size as well as the ability of these tumors to repair sublethal radiation damage as compared to normal cells. These experiments are particularly important since there was one slight but consistent difference between the response of the tumor cells and that of the normal skin fibroblasts, namely, there was a small shoulder on all of the tumor cell survival curves which was not seen for the normal fibroblast cells.

Borranco *er al.* [9] have reported an $\bar{n}$ of 12 for a human melanoma culture *in vitro*. This value is considered larger than that found in either of the poorly radiocurable

tumors we have examined. However, as these authors did not examine normal human cells in a parallel study, their result is difficult to interpret. In addition, since small variations in technique can account for large variations in survival parameters, we hesitate to compare our results to theirs, particularly since we have not examined melanoma cells.

The *in vitro* survival characteristics of the human osteosarcoma are similar to those described by Nelson for the 23022 subline of the $C_{22}RL$ mouse osteogenic sarcoma [10]. The lack of clinical curability for this mouse tumor *in vivo* similarly could not be accounted for by its *in vitro* survival characteristics.

From data presented here in which identical conditions of exponential growth and irradiation were employed, we were unable to find any significant differences in single dose survival curve parameters among cells derived from a human osteogenic sarcoma, a human glioblastoma, and two human medulloblastomas. This finding would suggest that other factors account for the differing clinical responses to fractionated radiotherapy protocols exhibited by these tumors. Among these other factors might be the fraction of hypoxic cells and their ability to re-oxygenate during fractionated radiotherapy, growth kinetics including the fraction and response of non-cycling cells, and tumor cell repopulation kinetics. We plan further investigations into the ability of these various tumor lines and normal strains to repair sublethal damage following multiple doses of X-rays comparable to those used in clinical radiotherapy schemes.

REFERENCES

1. M. Copeland, *Tumors of Bone and Soft Tissues* pp. 299–311. Yearbook Medical Publishers, Chicago (1963).
2. J. Bouchard, *Radiation Therapy of Tumors and Diseases of the Central Nervous System* pp. 69–90. Lea & Febiger, Philadelphia (1966).
3. H. J. G. Bloom, E. N. K. Wallace and J. M. Henk, Treatment and prognosis of medulloblastoma in children. *Am. J. Roentgenol.* **105,** 43 (1969).
4. M. Friedman, Aspects of radiation biology and radiation therapy observed during treatment of cancer in man. *Brit. J. Radiol.* **48,** 81 (1975).
5. M. C. Trachtenberg, P. L. Kornblith and J. Häuptli, Biophysical properties of cultured human glial cells. *Brain Res.* **38,** 279 (1972).
6. P. L. Kornblith, F. C. Bohan, Jr., William C. Wood and B. O. Whitman, Human astrocytomas: serum mediated immunological reactivity. *Cancer (Philad.)* **33,** 1512 (1974).
7. R. Weichselbaum, J. Epstein and J. B. Little. Submitted for publication.
8. J. Epstein, J. R. Williams and J. B. Little, Rate of DNA repair in progeric and normal human fibroblasts. *Biochem. biophys. Res. Comm.* **59,** 850 (1974).
9. S. C. Borranco, M. D. Romsdahl and R. M. Humphrey, The radiation response of human malignant melanoma cells grown *in vitro*. *Cancer Res.* **31,** 830 (1971).
10. J. M. Nelson, Cellular radiosensitivity of a cultured mouse osteosarcoma subline derived from a transplantable tumor to different ionizing radiations. *Radiat. Res.* **58,** 209 (1974).

Europ. J. Cancer Vol. 12. pp. 53–59. Pergamon Press 1976. Printed in Great Britain

The Interaction of Bifunctional Alkylating Agents with the DNA of Tumour Cells

K. R. HARRAP and E. W. GASCOIGNE*

Department of Applied Biochemistry, Institute of Cancer Research, Sutton, Surrey, England

Abstract—*Differences are described in the extent and rate of removal of inter-strand DNA cross-links in drug-sensitive and resistant strains of the Yoshida ascites sarcoma following their exposure to various bifunctional alkylating agents.*

INTRODUCTION

THE CYTOTOXIC effects of bifunctional alkylating agents have been attributed to their interaction with DNA [1]. Exposure of cells to these compounds results in the insertion of inter- and intra-strand cross-links into their DNA [2]. When sensitive and resistant strains of *E. coli* were exposed to ^{35}S-mustard gas, the DNA of both cell types was alkylated to a comparable extent; however, alkyl groups were removed from the DNA of resistant cells, implicating the existence of excision and repair processes [3, 4]. Similar studies with sub-lines of L cells exhibiting acquired resistance to sulphur mustard showed that alkylation products were removed from their DNA approximately three times faster than from the DNA of cells of the parent strain [5]. However, attempts to quantitate the relative excision and repair capacities of alkylating agent sensitive and resistant strains of the Yoshida sarcoma have led to equivocal results [6–8]. Previous studies in this laboratory have been concerned with the subcellular distribution of chlorambucil, its binding to macromolecules, and in particular its interaction with nuclear proteins [9–11] of the Yoshida sarcoma. In the present paper we have compared the rate of insertion of inter-strand cross-links into the DNA of sensitive and resistant Yoshida cells following their exposure *in vivo* and *in vitro* to a range of alkylating agents including chlorambucil [$(ClCH_2 \cdot CH_2)_2N \cdot C_6H_4 \cdot (CH_2)_3 \cdot COOH$], nitrogen mustard [$(ClCH_2 \cdot CH_2)_2 \cdot N \cdot CH_3$] and CB 3034 [$(ClCH_2 \cdot CH_2)_2 \cdot N \cdot C_6H_4 \cdot CH_2CH_2NH_2$]. The doses of these compounds administered to tumour-bearing animals were sufficient to produce cures in animals carrying the sensitive tumour, but were without effect on the growth of the resistant tumour. In tissue culture experiments, chlorambucil was used at a concentration which inhibited the growth of sensitive cells, but was without effect on the growth of resistant cells.

Inter-strand DNA cross-linking was measured by two techniques: (i) by liquid-liquid partition between polyethylene glycol and dextran, broadly according to the procedure of McCann *et al.* [12]; (ii) by alkaline sucrose density gradient centrifugation of DNA using a modification of the technique of Peterson *et al.* [13].

Accepted 8 September 1975.

*Present address: Department of Biochemistry, Marischal College, University of Aberdeen, Aberdeen, Scotland.

MATERIAL AND METHODS

Chemicals

Chlorambucil and CB 3034 were synthesised at the Institute of Cancer Research, London. Nitrogen mustard (as Mustine Hydrochloride) was obtained from The Boots Pure Drug Co. Ltd.; [6-^{3}H] thymidine (25Ci/mmol) and [2-^{14}C] thymidine (62 mCi/mmol) were purchased from the Radiochemical Centre, Amer-

sham. Dextran T-500 was obtained from Pharmacia Fine Chemicals Ltd., and polyethylene glycol (as Carbowax 6000) from Union Carbide Ltd. Foetal bovine serum was obtained from Flow Laboratories Ltd. All other chemicals were obtained from British Drug Houses Ltd.; Hopkin and Williams Ltd.; or May and Baker Ltd., analytical grades being used where available. Drug solutions were dispensed immediately before use and were sterilised by filtration (Millipore, 0·22 μm). Nitrogen mustard and CB 3034 were dissolved in physiological saline. Chlorambucil was dissolved in 0·5 ml of 2% (w/v) ethanolic HCl and diluted to 10 ml with a solution of 2% (w/v) K_2HPO_4 in 50% v/v aqueous propylene glycol. Subsequent dilutions were made using isotonic saline.

Yoshida ascites sarcoma

(a) Growth in Wistar rats: full details of animal experimentation and tumour transplantation techniques have been provided previously [14, 15]. Drugs were administered sub-cutaneously to tumour-bearing animals 3 days after transplant at the following doses: chlorambucil 8 mg/K; nitrogen mustard 0·7 mg/K; CB 3034 12·5 mg/K.

Animals were killed at various times after treatment and the ascitic fluid aspirated with isotonic saline.

(b) Growth in suspension culture: an aliquot of ascites tumour, obtained from an animal under aseptic conditions, was diluted to a concentration of 5×10^4 cells/ml in Fischer's medium containing 20% foetal bovine serum, benzyl penicillin (60 μg/ml) and streptomycin (100 μg/ml) and incubated in closed tubes at 37°. Chlorambucil was added to logarithmically growing cultures to a final concentration of $3{\cdot}3 \times 10^{-6}$M, which represented an LD_{90} dose for the sensitive cells, but was without effect on the viability of resistant cells [16].

Labelling of DNA

The DNA of cells growing in host animals was labelled by intraperitoneal injection of 100 μCi [6-^{3}H] thymidine (25 Ci/mmol) 16 hr prior to administration of drug.

The DNA of cultured cells was pre-labelled by growing the cells for 16 hr in the presence of [2-^{14}C] thymidine (0·2 μCi/ml; 62 mCi/mmol), followed by 6 hours' incubation in unlabelled medium. Cells were incubated in the presence of [6-^{3}H] thymidine (10 μCi/ml; 25 Ci/mmol) for 30 min immediately prior to harvesting: where cells had been exposed to chlorambucil the incubation time in the presence of [6-^{3}H] thymidine was increased proportionately in order that all cells under study contained comparable amounts of [^{3}H] thymidine in their DNA.

Estimation of inter-strand DNA cross linking

(a) Liquid-liquid partition of DNA: cross-linked DNA, when denatured, will spontaneously "zipper up" to reform double-stranded DNA [17, 18]. DNA was isolated from ascites cells by the method of Diamond *et al.* [19], and dissolved in 10 mM sodium phosphate, pH 6·8, at a concentration of 400 μg/ml. The DNA was denatured by carefully mixing with an equal volume of 0·1 N NaOH, followed by incubation at 37°C for 10 min. The solutions were then reneutralised by addition of an equal volume of ice-cold 0·1 M sodium dihydrogen phosphate. After extensive dialysis at 4°C against 10 mM sodium phosphate, pH 6·8, the DNA samples were sonicated at 20 kHz for one minute at 0°C, prior to their partition in the 2-phase system. Single-stranded and double-stranded DNA were separated by a modification of the method of Alberts [20]. A stock solution containing 9·2% (w/w) polyethylene glycol and 16·8% (w/w) Dextran T500 was prepared. The proportion of DNA solution and phase stock which resulted in optimal separation between single- and double-stranded DNA was determined empirically. The mixture of DNA and phase stock was allowed to stand at 4°C for 30 min, with occasional mixing, prior to centrifugation at 600 *g* for 10 min at 4°C. Upper phase was removed with a Pasteur pipette, and a known volume mixed with an equal volume of chloroform to precipitate the phase polymers. After centrifugation, an aliquot of the aqueous upper phase was mixed with an equal volume of 1 N perchloric acid and then hydrolysed at 70°C for 20 min. The DNA content was then estimated colorimetrically by the method of Burton [21]. Appropriate controls were run simultaneously in the 2-phase system, including native DNA, alkali-denatured DNA and phosphate buffer alone.

(b) Alkaline sucrose density gradient centrifugation of DNA: the technique used was a modification of the procedure described by Peterson *et al.* [13]. At various times after drug administration, cells were harvested and then stored in ice-cold saline for 45 min at a concentration of 8×10^5 cells/ml, when a limited fragmentation of DNA occurred due to the presence of previously incorporated [^{3}H]

thymidine [22]. The cells were then used for sedimentation analysis.

Sucrose solutions were prepared immediately prior to gradient formation. Five to twenty-five percent (w/v) linear sucrose gradients (11·5 ml) were used, containing 0·001 M EDTA; 0·7 M NaCl; 0·3 M NaOH; 0·01 M Tris and 0·06 M sodium *p*-aminosalicylate, and buffered to pH 12·5 with 10 N NaOH. Sodium hydroxide (0·3 ml, 1 N) was layered on top of the gradients and 0·3 ml of cell suspension carefully added to this layer. The tubes were allowed to stand in the centrifuge rotor (MSE Superspeed 65; 6 × 10 ml swing-out rotor), maintained at 20°C in the centrifuge (without spinning), for 30 min. The gradients were then centrifuged at 30,000 rev/min (110,000 *g*) for 120 min at 20°C. After centrifugation, 30 fractions (each of 20 drops) were collected from the top of the gradient by pumping 60% (w/v) sucrose into the bottom of the tube.

The fractions were collected in small glass tubes, to each of which was added 0·1 ml of calf thymus DNA solution (250 μg/ml), followed by approximately 5 ml of ice-cold 10% TCA. The samples were allowed to stand overnight at 4°C and then filtered through glass-fibre discs (2·4 cm, Whatman GF/C). The discs were washed twice with ice-cold 10% TCA (5 ml), twice with ice-cold ethanol (5 ml) and then dried at 50°C. The discs were then counted in 10 ml of scintillation fluid (butyl-PBD, 7·0 g; napthalene, 80·0 g; toluene, 385 ml; dioxan, 385 ml; methanol, 230 ml) using an Intertechnique Model SL40 scintillation counter. Results were plotted as a percentage of the radioactivity in a particular fraction relative to the total contained in 30 fractions. Of the total radioactivity put onto each gradient, the proportion recovered varied between 80 and 100%.

All profiles shown in this communication refer to pre-labelled DNA, and not to DNA labelled immediately prior to harvesting of cells.

RESULTS

(*i*) *Liquid–liquid partition*

Table 1 shows the effect of sonication on the partition of native and denatured DNA. As a routine we sonicated all our DNA preparations and used 0·9 gm. of phase stock per 1 ml DNA solution, which provided optimal separations. Figure 1 shows the differences in rate of insertion of cross-links into the DNA of sensitive and resistant Yoshida cells, as measured by

Table 1. *The effect of sonication on the partition of native and alkali denatured Yoshida DNA* (100μg/ml)

Grams of phase stock added to 1ml DNA solution	Percent DNA in upper phase			
	Native DNA		Denatured DNA	
	Unsonicated	Sonicated	Unsonicated	Sonicated
0.7	61.2	89.1	32.9	35.2
0.8	27.4	90.3	6.6	7.5
0.9	21.2	88.1	5.9	5.6
1.0	12.8	84.2	3.8	4.0

Each value represents the mean of 4 determinations
Overall scatter at each point ⩾ 10%.

partition in the two-phase system, following the administration of chlorambucil to tumour-bearing animals. Crosslinks appeared rapidly in the DNA from resistant cells, maximum cross-linking occurring 6 hr after treatment. Thereafter the cross-links were removed, the level returning almost to control proportions by 10 hr after treatment. In the DNA of sensitive cells, however, cross-links appeared much more slowly: significantly there was scarcely any increase in cross-linking during the first 6 hr of exposure to the drug. Thereafter the level of cross-linking increased progressively, reaching a plateau at about 24 hr after treatment. At no time did the level of cross-linking in the DNA of sensitive cells attain that observed in the DNA of resistant cells. Nitrogen mustard produced a similar pattern of events,

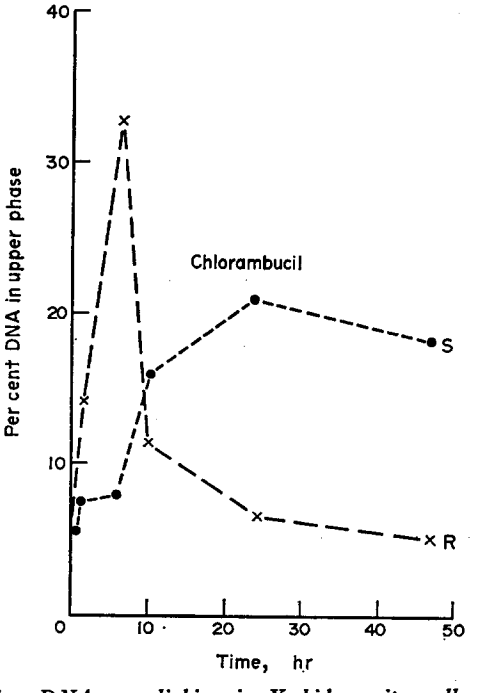


Fig. 1. *DNA cross-linking in Yoshida ascites cells after administration of chlorambucil to tumour-bearing animals. Each point represents the mean of six determinations. Overall scatter at each point* ⩾ 10%.

though the peak of cross-linking in the DNA of resistant cells occurred earlier (see Fig. 2). The basic mustard CB 3034 produced maximum DNA cross-linking in the resistant cells 1 hr after treatment, though the peak height was much reduced compared with those produced in response to the other agents: cross-linking of sensitive cell DNA by CB 3034 occurred at a much slower rate than with chlorambucil or nitrogen mustard (Fig. 3).

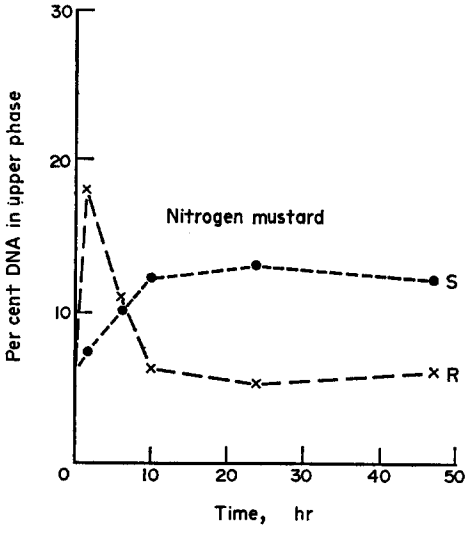


Fig. 2. DNA cross-linking in Yoshida ascites cells after administration of nitrogen mustard to tumour-bearing animals. Each point represents the mean of six determinations. Overall scatter at each point ≯ 10%.

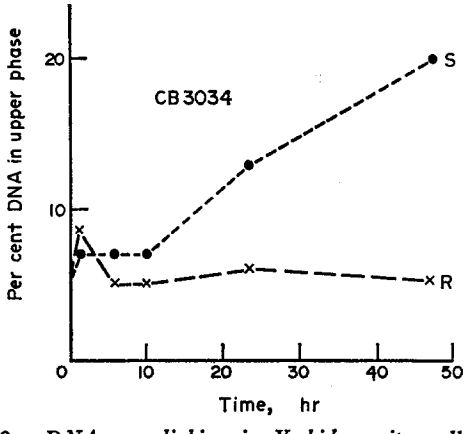


Fig. 3. DNA cross-linking in Yoshida ascites cells after administration of CB 3034 to tumour-bearing animals. Each point represents the mean of six determinations. Overall scatter at each point ≯ 100%.

(ii) Alkaline sucrose density gradient centrifugation

In the case of chlorambucil, the patterns just described were essentially confirmed by alkaline sucrose density gradient centrifugation of DNA previously labelled by injection of ^{3}H-thymidine into tumour-bearing animals. Fig. 4 shows the sedimentation profiles of DNA from resistant cells at various times after treatment. DNA from untreated cells sedimented as a clearly-defined peak around fraction 16; during the first 6 hr of treatment the sedimentation rate increased, and at 24 hr after treatment there was evidence of a return to the control profile, which appeared complete at 48 hours. Figure 5 shows the sedimentation profiles of DNA from sensitive cells (the control profile was similar to that observed in resistant cells);

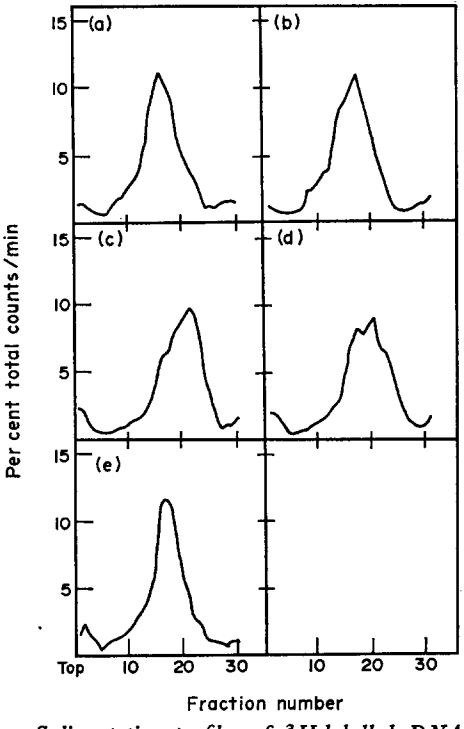


Fig. 4. Sedimentation profiles of ^{3}H-labelled DNA from resistant Yoshida ascites cells after administration of chlorambucil to tumour-bearing animals.
(a) *control;* (b) 1 *hour after treatment;* (c) 6 *hours after treatment;* (d) 24 *hours after treatment;* (e) 48 *hours after treatment.*

at 1 hr after treatment the DNA sedimented more slowly, with a peak occurring at fraction 14; by 6 hours the profile exhibited two discrete peaks, one at fraction 14 and a second at fraction 17; by 24 hr the DNA was again sedimenting as a single homogeneous band with a peak at fraction 19; at 48 hr the DNA was still sedimenting faster than control material, with a peak at fraction 18.

These observations were extended to include an investigation of the effects of chlorambucil on Yoshida cells growing in suspension culture. The results are summarised in Table 2. In resistant cells the sedimentation rate of the

DNA increased during the first 4 hr of exposure to the drug indicating the presence of cross-linked material, though the sedimentation rate reverted to that of the control by 24 hr. In sensitive cells the sedimentation rate of the DNA decreased during the first 4 hr probably due to single-strand breaks, but by 24 hr the DNA was sedimenting more rapidly than that of control cells, indicating the presence of cross-linked material.

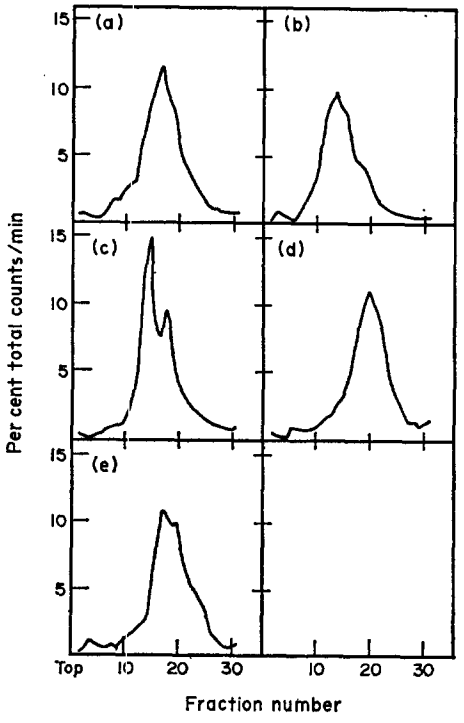


Fig. 5. *Sedimentation profiles of* ^{3}H*-labelled DNA from sensitive Yoshida ascites cells after administration of chlorambucil to tumour-bearing animals.*
(a) *Control;* (b) 1 *hr after treatment;* (c) 6 *hr after treatment;* (d) 24 *hr after treatment;* (e) 38 *hr after treatment.*

Table 2. *Mean fraction numbers in sedimentation profiles of* $[^{14}C]$*-labelled DNA from Yoshida ascites cells treated with* $3{\cdot}3 \times 10^{-6} M$ *chlorambucil*

Cell type	Time after treatment (hours)	Mean fraction number (fractions from top of gradient)	Range	Number of expts.
Resistant	Control	16.3	16.0-16.6	16
	1	17.6	17.3-17.8	4
	4	17.9	17.7-18.2	4
	24	16.2	16.0-16.5	4
Sensitive	Control	16.2	15.9-16.4	14
	1	15.1	15.0-15.3	4
	4	15.2	15.0-15.4	4
	24	17.1	16.9-17.5	4

DISCUSSION

These data indicate the existence of discrete differences in the mechanism by which alkylating agents promote the formation of inter-strand cross-links in the DNA of the two cell types. In resistant cells there was a rapid appearance and removal of inter-strand DNA cross-links: maximum cross-linking occurred between 1 and 6 hr after the initial exposure of the cells to the agents, but had effectively returned to control values by 24 hr. In contrast, cross-linking of DNA in sensitive cells occurred at a much slower rate and was an irreversible process. With the two aromatic mustards, essentially no cross-linking of sensitive cell DNA occurred during the first six hours of exposure, as though the DNA were in some way being "spared" from the effects of these agents: in the case of nitrogen mustard, however, no such early delay was seen. There appears to be no intuitively apparent chemical explanation for this delayed appearance of cross-links in the DNA of sensitive cells following their exposure to either of the two aromatic mustards. It may be that these compounds associate initially with components of the chromatin other than DNA. Chlorambucil has been shown to induce rapid conformational changes in the chromatin of sensitive Yoshida cells, to bind non-covalently to non-histone nuclear proteins and to induce changes in nuclear protein structure [11, 23]. It may be that CB 3034, which exhibits certain structural similarities to chlorambucil, behaves analogously. The early association of chlorambucil with nuclear proteins and the subsequent reorganisation of the chromatin may delay the formation of DNA cross-links.

While a correlation has been demonstrated between cellular sensitivity and the amount of repair synthesis after exposure of Yoshida cells to nitrogen mustard or methylene dimethanesulphonate [7], no such correlation can be observed after exposure of cells to sulphur mustard [6, 8]. We have seen no evidence for the removal of cross-links from the DNA of sensitive Yoshida cells previously exposed to bifunctional alkylating agents. It may be that the function of the relevant enzymes is inhibited by the presence of the alkylating agent in the chromatin.

Density gradient centrifugation of DNA from cells exposed both *in vivo* and *in vitro* to chlorambucil confirmed the findings of the liquid–liquid partition technique. In addition, however, the sedimentation studies revealed the presence of single-strand breaks in the DNA of

sensitive cells shortly after their exposure to the drug. A similar study by Yin *et al.* [25] has revealed the presence of strand breaks in the DNA of sensitive Ehrlich cells following their exposure to nitrogen mustard.

Acquired resistance in Yoshida cells may be associated with failure of the alkylating agent to elicit the conformational and structural changes in the chromatin which are observed (in sensitive cells). We are currently comparing the nuclearprotein contents of the two cell types and studying how some of their properties are modified by alkylating agents, in an attempt to define the basis of the resistance mechanism.

Acknowledgements—We wish to thank Dr. J. A. Stock for the synthesis of CB 3034, also Dr. P. G. Riches for much helpful advice and discussion. One of us (EWG) acknowledges receipt of an MRC studentship.

REFERENCES

1. P. D. Lawley, Effects of some chemical mutagens and carcinogens on nucleic acids. *Prog. nucleic acid Res.* **5,** 89 (1966).
2. I. G. Walker, Intrastrand bifunctional alkylation of DNA in mammalian cells treated with mustard gas. *Canad. J. Biochem.* **49,** 332 (1971).
3. K. W. Kohn, N. H. Steigbigel and C. L. Spears, Cross-linking and repair of DNA in sensitive and resistant strains of *E. coli* treated with nitrogen mustard. *Proc. nat. Acad. Sci. (Wash.)* **53,** 1154 (1965).
4. P. D. Lawley and P. Brookes, Cytotoxicity of alkylating agents towards sensitive and resistant strains of *Escherichia coli* in relation to extent and mode of alkylation of cellular macromolecules and repair of alkylation lesions in deoxyribonucleic acid. *Biochem. J.* **109,** 433 (1968).
5. I. G. Walker and B. D. Reid, Some properties of substrains of L-cells with a decreased sensitivity to bis(2-chloroethyl)sulfide. *Cancer Res.* **31,** 510 (1971).
6. C. R. Ball and J. J. Roberts, DNA repair after mustard gas alkylation by sensitive and resistant Yoshida sarcoma cells *in vitro*. *Chem. biol. Intera.* **2,** 321 (1970).
7. M. Fox and B. W. Fox, Repair replication and unscheduled DNA synthesis in mammalian cell lines showing differential sensitivity to X-rays and sulphur mustard. *Mutation Res.* **19,** 119 (1973).
8. D. Scott, M. Fox and B. W. Fox, The relationship between chromosomal aberrations and DNA repair in tumour cell lines of differential sensitivity to X-rays and sulphur mustard. *Mutation Res.* **22,** 207 (1974).
9. B. T. Hill, Studies on the transport and cellular distribution of chlorambucil in the Yoshida ascites sarcoma. *Biochem. Pharmacol.* **21,** 495 (1972).
10. B. T. Hill and P. G. Riches, The absorption, distribution and excretion of ^{3}H-chlorambucil in rats bearing the Yoshida ascites sarcoma. *Brit. J. Cancer* **25,** 831 (1971).
11. P. G. Riches and K. R. Harrap, Some effects of chlorambucil on the chromatin of Yoshida ascites sarcoma cells. *Cancer Res.* **33,** 389 (1973).
12. J. J. McCann, T. M. Lo and D. A. Webster, Cross-linking of DNA by alkylating agents and effects on DNA function in the chick embryo. *Cancer Res.* **31,** 1573 (1971).
13. A. R. Peterson, B. W. Fox and M. Fox, Alkaline sucrose sedimentation studies of DNA from P388F lymphoma cells treated with difunctional alkylating agents. *Biochim biophys. Acta.* **299,** 385 (1973).
14. K. R. Harrap and B. T. Hill, The selectivity of action of alkylating agents and drug resistance. Part I: Biochemical changes occurring in sensitive and resistant strains of the Yoshida ascites sarcoma following chemotherapy. *Brit. J. Cancer* **23,** 210 (1969).
15. K. R. Harrap and B. T. Hill, The selectivity of action of alkylating agents and drug resistance. Part II: A comparison of the effects of alkylating drugs on growth inhibition and cell size in sensitive and resistant strains of the Yoshida ascites sarcoma. *Brit. J. Cancer* **23,** 227 (1969).
16. K. R. Harrap and M. E. Furness, The cytotoxicity of chlorambucil and its associated effects on NAD metabolism. *Europ. J. Cancer* **9,** 343 (1973).
17. E. P. Geiduschek, "Reversible" DNA. *Proc. natl. Acad. Sci. (Wash.)* **47,** 950 (1961).
18. K. W. Kohn, C. L. Spears and P. Doty, Interstrand cross-linking of DNA by nitrogen mustard. *J. mol. Biol.* **19,** 266 (1966).

19. L. DIAMOND, V. DEFENDI and P. BROOKES, The interaction of 7,12-dimethylbenz(a)anthracene with cells sensitive and resistant to toxicity induced by this carcinogen. *Cancer Res.* **27,** 890 (1967).
20. B. M. ALBERTS, Efficient separation of single-stranded and double-stranded deoxyribonucleic acid in a dextran-polyethylene glycol two-phase system. *Biochemistry* **6,** 2527 (1967).
21. K. BURTON, A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemistry J.* **62,** 315 (1956).
22. A. R. PETERSON and B. W. FOX, Toxicity of tritiated thymidine in P388F lymphoma cells. II—Effects on DNA molecular weight. *Int. J. Radiat. Biol.* **19,** 237 (1971).
23. P. G. RICHES and K. R. HARRAP, The binding of chlorambucil to nuclear proteins of the Yoshida ascites sarcoma. *Chem. biol. Inter.* **11,** 291 (1975).
24. P. G. RICHES, S. M. SELLWOOD and K. R. HARRAP, The effects of chlorambucil on nuclear protein phosphorylation in the Yoshida ascites sarcoma. Submitted to *Cancer Res.*
25. L. YIN, E. H. L. CHUN and R. J. RUTMAN, A comparison of the effects of alkylation on the DNA of sensitive and resistant Lettre–Ehrlich cells following *in vivo* exposure to nitrogen mustard. *Biochim. biophys. Acta.* **324,** 472 (1973).

Europ. J. Cancer Vol. 12, pp. 61–75. Pergamon Press 1976. Printed in Great Britain

An Immunohistological Study of Follicular Lymphoma, Reticulum Cell Sarcoma and Hodgkin's Disease*

CLIVE R. TAYLOR

Department of Pathology, Gibson Laboratories, Radcliffe Infirmary, Oxford OX2 6HE, Great Britain

Abstract—*Immunoperoxidase techniques were applied to the study of intracellular immunoglobulin and muramidase (lysozyme) in formalin paraffin sections of more than 150 lymph node biopsies, including reactive lymphadenopathies, follicular lymphoma, reticulum cell sarcoma and Hodgkin's disease. Immunoglobulin components were demonstrated in the neoplastic cells of cases of follicular lymphoma, and in the malignant reticulum cells of some cases of reticulum cell sarcoma and Hodgkin's disease. A tentative scheme is proposed relating some of the lymphomas to the different morphological expressions of the B lymphocyte during its transformation and maturation to the plasma cell.*

INTRODUCTION

THE PATHOLOGY of the lymphomas poses two major problems, quite apart from the clinical aspects of therapy.

The first problem concerns the nature of the pathological process and its causation. The aetiological factors which have been considered in relation to the lymphoid neoplasms are those of neoplasia in general. In addition, there are a number of reports in human and animal pathology where a lymph node subjected to prolonged immunological stimulation develops a progressive hyperplasia, which terminates as a frank malignant neoplasm (usually reticulum cell sarcoma) of one of the component cells of the lympho-reticular system [1–6].

The second problem relates to diagnosis and classification, and stems from a continuing lack of clear understanding of the basic cell types from which these neoplasms take origin.

In particular there is the problem of those neoplasms which are traditionally believed to be derived from the reticulum cell—principally Hodgkin's disease and the reticulum cell sarcoma group (the histiocytic and stem cell lymphomas of the American literature [7].

The term "reticulum cell" lacks any clear definition, but has been used extensively to represent a pluripotential lymphoreticular and haematopoietic stem cell. This concept of the reticulum cell (mesenchymal stem cell) provides the theoretical basis for many of the existing ideas of the nature of the lymphomas (or reticuloses and reticulo-sarcomas [8, 9]), and has been evoked to explain the mixed cell population characteristic of Hodgkin's disease [10], as well as the inter-relations of the different histological lymphomas [11].

The application of experimental techniques for distinguishing T and B lymphocytes to the study of the malignant lymphomas has provided new information as to the nature of some of these malignant cell types [12–14].

In particular immunoglobulin components have been identified within Reed–Sternberg cells [15–18] and within the cells of some cases of reticulum cell sarcoma [17–19], by the application of immunoperoxidase methods to formalin fixed paraffin embedded tissue. These initial observations have been confirmed and

Accepted 25 June 1975.

*This work was partly supported by a grant from the Leukaemia Research Fund.

consolidated by the present more extensive study of follicular lymphoma, reticulum cell sarcoma, and Hodgkin's disease. The finding of intracellular immunoglobulin within some so-called "malignant reticulum cells" supports the suggestion [17] that these cells are in fact neoplastic derivatives of the transformed lymphocyte, or the immediate precursors and progeny thereof.

Immunoperoxidase methods have also been used for the demonstration of cytoplasmic muramidase (lysozyme) within some cells of the histiocytic series [20]. Under controlled conditions the presence of muramidase within a cell may be regarded as a positive indication of the histiocytic nature of that cell. In the present study this method for demonstrating cytoplasmic muramidase has been applied in parallel with the study of intracellular immunoglobulin in the malignant lymphomas.

MATERIAL AND METHODS

All the investigations were carried out on formalin-paraffin sections of diagnosed cases of the lympho-reticular neoplasia (Hodgkin's disease, classification 201, International Classification Disease, 8th Revision; Follicular Lymphoma, classification 202; Reticulo-sarcoma, classification 200) from the surgical histology and autopsy files of the Radcliffe Infirmary, Oxford.* A number of cases diagnosed as "reactive lymphadenopathy" were also taken for comparison with the neoplastic tissues.

All tissues had been routinely processed on the laboratory histokinettes by the recommended schedule using industrial methylated spirits and chloroform, following adequate fixation in 10% buffered formalin. Paraffin blocks were stored at room temperature.

Preliminary examination was made of an H & E section of each of the selected cases. A small number of cases were rejected for subsequent study because of unsatisfactory fixation, inadequate tissue, or uncertain diagnosis.

Table 1. *Immunoglobulin content of lympho-reticular neoplasms classified according to Robb-Smith* [8, 9]

Group*	No.	*Tumour cells* Positive for immunoglobulin	*Tumour cells* Negative for immunoglobulin
1. Lymphoid follicular reticulosis and lymphoblastic reticulosarcoma (follicular)†	20	14	6
2. Lymphosarcoma	5	—	5
3. Lymphoblastic reticulosarcoma (diffuse)	11	4	7
4. Syncytial reticulosarcoma	16	12	4
5. Dictyosyncytial reticulosarcoma	5	—	5
6. Dictyocytic/histocytic reticulosarcoma	7	6	1
7. Polymorphic reticulosarcoma	11	9	2
8. Plasma Cell reticulosarcoma	9	9	—
Unclassified	7	4	3
Total	91		

*Crude matching of alternative terminology: (1) lymphocytic lymphoma, nodular, well and poorly differentiated, (2) and (3) lymphocytic lymphoma, diffuse, well and poorly differentiated, (4)–(7) reticulum-cell sarcoma (unqualified) or stem-cell lymphoma and histiocytic lymphoma.
†This group includes cases which have undergone sarcomatous transformation with some loss of follicular pattern.

The diagnosis in the remaining 91 cases of non-Hodgkin lymphoma is recorded in Table 1. In addition 45 cases of Hodgkin's disease were examined together with nineteen examples of reactive lymphadenopathy, mostly of non-specific type, but including some cases of infectious mononucleosis, toxoplasmosis, tuberculosis and sarcoidosis. Where possible the diagnosis recorded is that made by the pathologist at initial presentation. In a small number of cases some revision of terminology was necessary, and other cases were sub-classified more precisely. A few cases with atypical features remained unclassifiable.

*Cases referred from other hospitals were included when sufficient numbers of sections were available. Details of tissue processing to paraffin sections were not known for this group.

IMMUNOPEROXIDASE PROCEDURE FOR INTRACELLULAR IMMUNOGLOBULIN OR CYTOPLASMIC MURAMIDASE

Thin sections were taken from the paraffin blocks of each case and serial sections were examined with the following panel of antisera (raised in rabbit, *vide infra*) applied to the dewaxed section at the dilutions shown:

	PAP	SWAR/P
anti-Kappa (light chain specific)	1/100	1/40
anti-Lambda (light chain specific)	1/100	1/40
anti-gamma (G, heavy chain specific)	1/40	1/20
anti-alpha (A, heavy chain specific)	1/40	1/20
anti-mμ (M. heavy chain specific)	1/40	1/20
anti-muramidase	1/40	1/20

Table 2. Procedure for the immunoperoxidase technique: the peroxidase conjugate (SWAR/P) method

1. Paraffin sections—xylol–alcohol.
2. Block endogenous peroxidase with methanol containing 0·3% hydrogen peroxide 30 min.
3. Normal swine serum 1/20 10 min.
4. Rabbit antiserum to human immunoglobulin components or to muramidase* 30 min.
5. Peroxidase-conjugated swine anti-rabbit serum IgG 1/20 30 min.
6. Diaminobenzidine reaction, couterstain with haematoxylin, dehydrate, and mount in D.P.X.

The reactions are carried out in tris buffer (pH 7·6), with washes after Stages 3, 4 and 5 in tris saline (a dilution of tris buffer 1/10 in normal saline).

*Dilutions given in text.

Table 3. Procedure for the immunoperoxidase technique: the peroxidase–anti-peroxidase immune complex (PAP) method

1. Paraffin sections—xylol-alcohol.
2. Block endogenous peroxidase with methanol containing 0·3% hydrogen peroxide 30 min.
3. Normal swine serum 1/20 10 min.
4. Rabbit antiserum to human immunoglobulin components or to muramidase* 30 min.
5. Swine anti-rabbit serum protein 1/20 30 min.
6. PAP 1/20 30 min.
7. Diaminobenzidine reaction, counterstain with haematoxylin, dehydrate, and mount in D.X.P.

The reactions are carried out in tris buffer (pH 7·6, with washes after Stages 3, 4, 5 and 6 in tris saline (a dilution of tris buffer 1/10 in normal saline).

*Dilutions given in text.

The subsequent procedure is summarised in the accompanying Tables 2 and 3 using either the peroxidase conjugate method (SWAR/P) or the peroxidase–anti-peroxidase immune complex (PAP) method. The methods have been described in more detail elsewhere [15–20].

In the peroxidase conjugate method (Table 2) the sites of specific binding of the rabbit anti-human immunoglobulin (or anti-muramidase) antiserum are revealed by the addition of a swine anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase. The specific localization of peroxidase-antibody conjugate is then demonstrated by use of the diaminobenzidine (DAB) reaction using 6 mg of diaminobenzidine with 0·01% hydrogen peroxide in 10 ml of Tris buffer. This substrate produces a clear brown colouration, which is visible on light microscopy after dehydration through the alcohols and mounting in DPX.

In later studies a more sensitive variation of the method was adopted. This involves the use of peroxidase–anti-peroxidase (PAP) immune complexes (horseradish peroxidase antigen complexed with rabbit antiserum to horseradish peroxidase) to identify the sites of specific antibody binding. In Table 3 the specifically bound rabbit anti-human immunoglobulin or anti-muramidase antiserum (Stage 4) is linked to the PAP immune complexes (Stage 6) by the intermediate addition (Stage 5) of swine antiserum to rabbit serum protein. The anti-rabbit immunoglobulin activity in the swine antiserum (Stage 5) specifically attaches both to the tissue bound rabbit immunoglobulin (Stage 4) and to the rabbit immunoglobulin in the PAP complexes (Stage 6).

In Stage 2 of Tables 2 and 3 reference is made to the blocking of endogenous peroxidase activity by methanol/hydrogen peroxide. This is necessary in tissues containing significant numbers of neutrophils or eosinophils, both of which contain large amounts of endogenous peroxidase which stains brown on the addition of diaminobenzidine and thus interferes with the interpretation of immunoglobulin positive cells (also stained brown by immunoperoxidase technique). The method described for blocking endogenous peroxidase does not interfere with the subsequent reactions of antisera either for immunoglobulin or muramidase [16, 20].

Non-specific staining of collagenous elements in tissue sections can be usefully reduced by prior treatment of the dewaxed sections with normal swine serum (Stage 3 in Tables 2 and 3). This reduces non-specific binding of the rabbit anti-human immunoglobulin antisera (Stage 4) and also does not cross react with the subsequent

swine antiserum to rabbit serum protein (Stage 5).

With the exception of a rabbit anti-muramidase serum prepared in these laboratories by Dr. D. Y. Mason [20], all of the antisera used in this study were obtained from Dakopatts A/S, through Mercia Diagnostics, Sandown Road, Watford, Herts. The specificity and sensitivity of the anti-immunoglobulin antisera used in this study were assessed in a previously reported study of tissues from multiple myeloma [19], and independently by haemagglutination methods [21].

Extensive control preparations were used, omitting each of Stages 4, 5 and 6 in turn, and substituting normal rabbit serum for Stage 4. In addition the different antisera (i.e. anti-K, anti-L, anti-G, anti-A, anti-M, anti-muramidase, all derived from rabbit) applied to serial sections of each case served as inbuilt controls.

RESULTS

Immunoperoxidase methods applied to formalin fixed paraffin embedded material result in excellent morphological detail equivalent to any orthodox haematoxylin and eosin paraffin section. There was the added advantage that cells of certain functional groups were independently identified according to their intracellular content of immunoglobulin or muramidase, staining brown by the reaction used.

By treating serial sections of each case with anti-K, anti-L, anti-G, anti-A and anti-M, it was possible to classify individual cells for type of light and heavy chain production. In each case the reaction of the individual cytological cell types for immunoglobulin was compared with the cytoplasmic reaction for muramidase. In the results described below reference is principally made to the demonstration of intracellular immunoglobulin. Results of studies of cytoplasmic muramidase are included where clearly positive.

Reactive hyperplasias

The majority of reactive tissues examined carried the diagnosis of non-specific reactive hyperplasia. In these cases the morphology of the plasma cell and its immediate precursors (including the transformed B lymphocyte) was clearly established (Fig. 1). These cells were mainly distributed deep to the reactive centres or around the periphery of reactive centres.

Within the reactive centres the large pale cells were predominantly immunoglobulin negative. However a small proportion of large folded cells (the "cleaved" cells of Lukes and Collins [13, 14]) showed definite staining for kappa or lambda chain and a somewhat larger proportion of rounded cells ("non-cleaved" cells of Lukes) were also positive. These latter cells resembled the transformed lymphocytes observed outside the germinal centre areas (Fig. 1).

Non-Hodgkin lymphomas

An attempt was made to identify light chain staining in every case, and to assess the population of positive cells for evidence of a monoclonal distribution (exclusively K positive or exclusively L positive). The results of studies with anti-heavy chain antisera (anti-G, anti-A and anti-M) were in agreement with light chain studies, but as anti-heavy chain antisera were not employed in every case, these observations are only reported where they contribute specially to the understanding of a particular case.

In a number of cases stained by the peroxidase conjugate method clear cut results were obtained, and the tumour cells were either strongly positive or entirely negative.

There were some cases, however, (twenty-eight in all) in which staining was equivocal. Often the appearance was of very light brown staining in a small proportion of tumour cells, either with anti-kappa or anti-lambda, or both. In other cases interpretation was confused by the presence of mature plasma cells at the periphery of the tumour, or distributed throughout. A proportion of these plasma cells stained with anti-K and a proportion with anti-L, indicating their polyclonal (reactive) nature.

All negative cases, and those cases in which the results were equivocal by the peroxidase conjugate method, were re-examined by the more sensitive PAP method. In the majority of cases difficulties in interpretation were resolved by this second examination, but there remained a small number of neoplasms in which the final decision was uncertain.

Multiple myeloma and plasma cell reticulosarcoma

In a previous study [19] a detailed examination was made of tissues from thirty-three cases of diagnosed plasma cell neoplasms. That study was particularly valuable in confirming the specificity and sensitivity of the peroxidase conjugate method and served to establish the specificity of the rabbit anti-human immunoglobulin antisera. The PAP method gave identical results with selected myeloma tissues

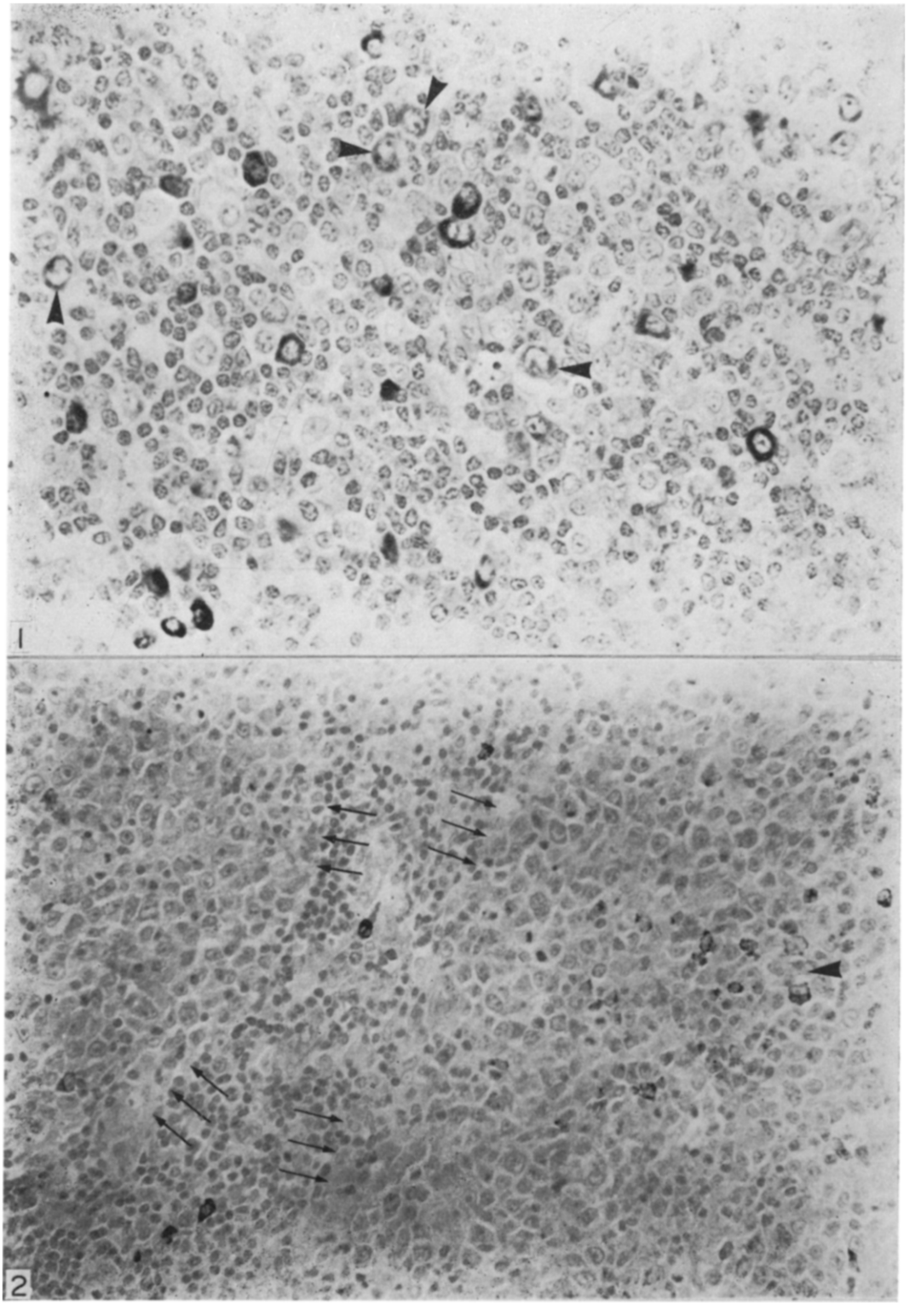


Fig. 1. *Reactive lymphadenitis. Paraffin section with anti-K* 1/100 *by the PAP method. Area of lymph node deep to reactive centre. Large cells containing traces of immunoglobulin (appearing as black granules—arrowed) are identifiable as transformed lymphocytes and are characterised by a large vesicular nucleus, with distinct nuclear membrane and prominent nucleolus. Plasma cells and large rounded plasma cell precursors show uniform dense staining for immunoglobulin. Paraffin Section. Cyan blue filter** × 750.

Fig. 2. *Follicular lymphoma. Paraffin section, anti-L* 1/100 *by the PAP method. Part of perimeter of two neoplastic follicles (arrows). The cells comprising the neoplastic follicles are large transformed type cells, resembling those illustrated in Fig.* 1. *A small proportion are positive for immunoglobulin (large arrow). Cyan blue filter** × 750.

(to face p. 64)

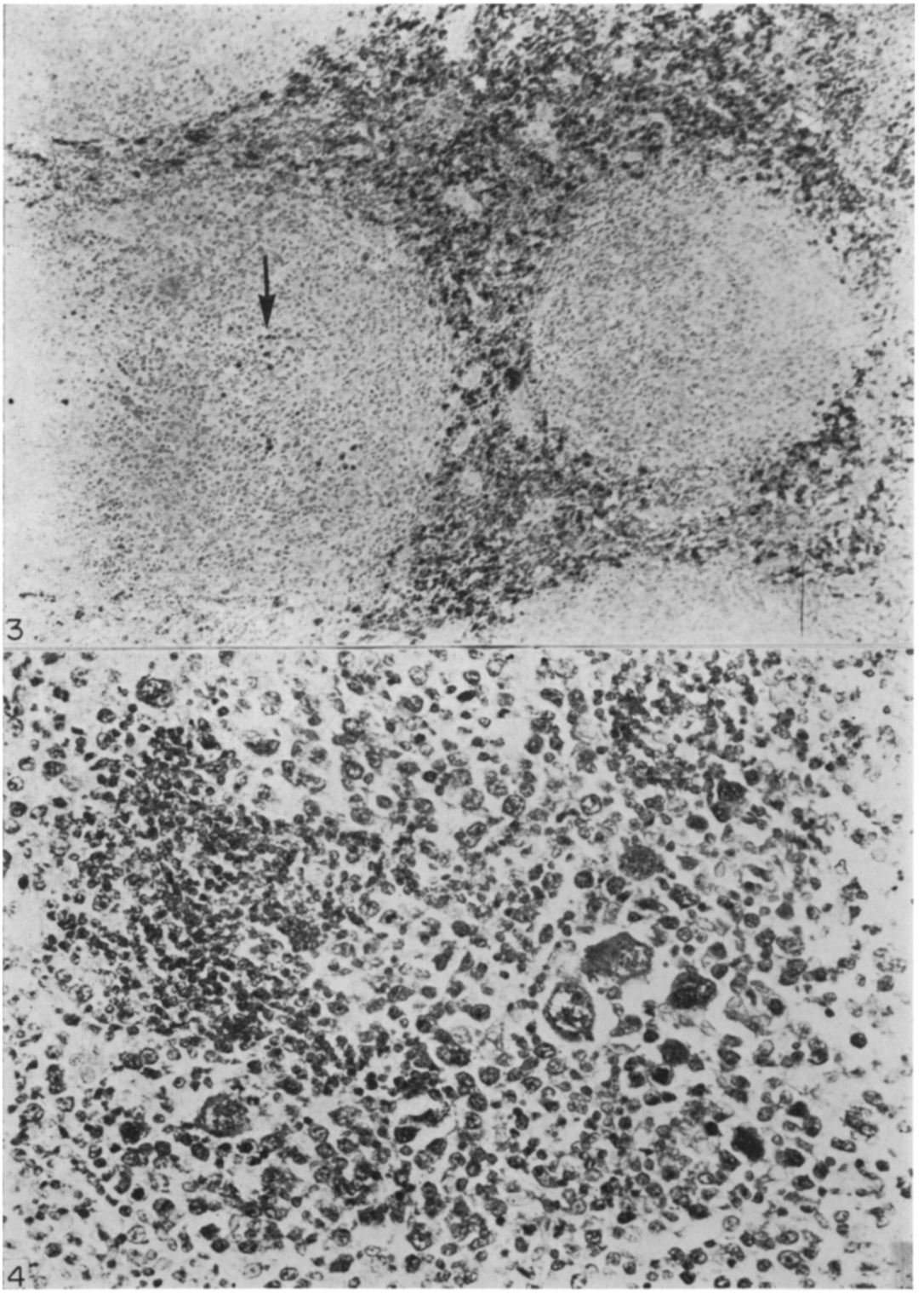


Fig. 3. *Follicular lymphoma. Paraffin section with anti-L* 1/100 *by the PAP method. Immunoglobulin containing cells appear black, and are present in a perifollicular distribution. Within the neoplastic follicles only a minority of cells are immunoglobulin positive (arrow). Monoclonal staining pattern. Anti-L positive, anti-K negative. Cyan blue filter** × 300.

Fig. 4. *Polymorphic—Reticulum cell sarcoma (histiocytic lymphoma) Paraffin section, anti-L,* 1/100 *by the PAP method. Same case as shown in Figure* 2, *tissue taken following death nine months later. The neoplastic cells are variable in morphology. Some of the giant cell forms resemble Reed–Sternberg cells. The majority of cells are positive for immunoglobulin, both kappa and lambda chain. Cyan blue filter** × 750.

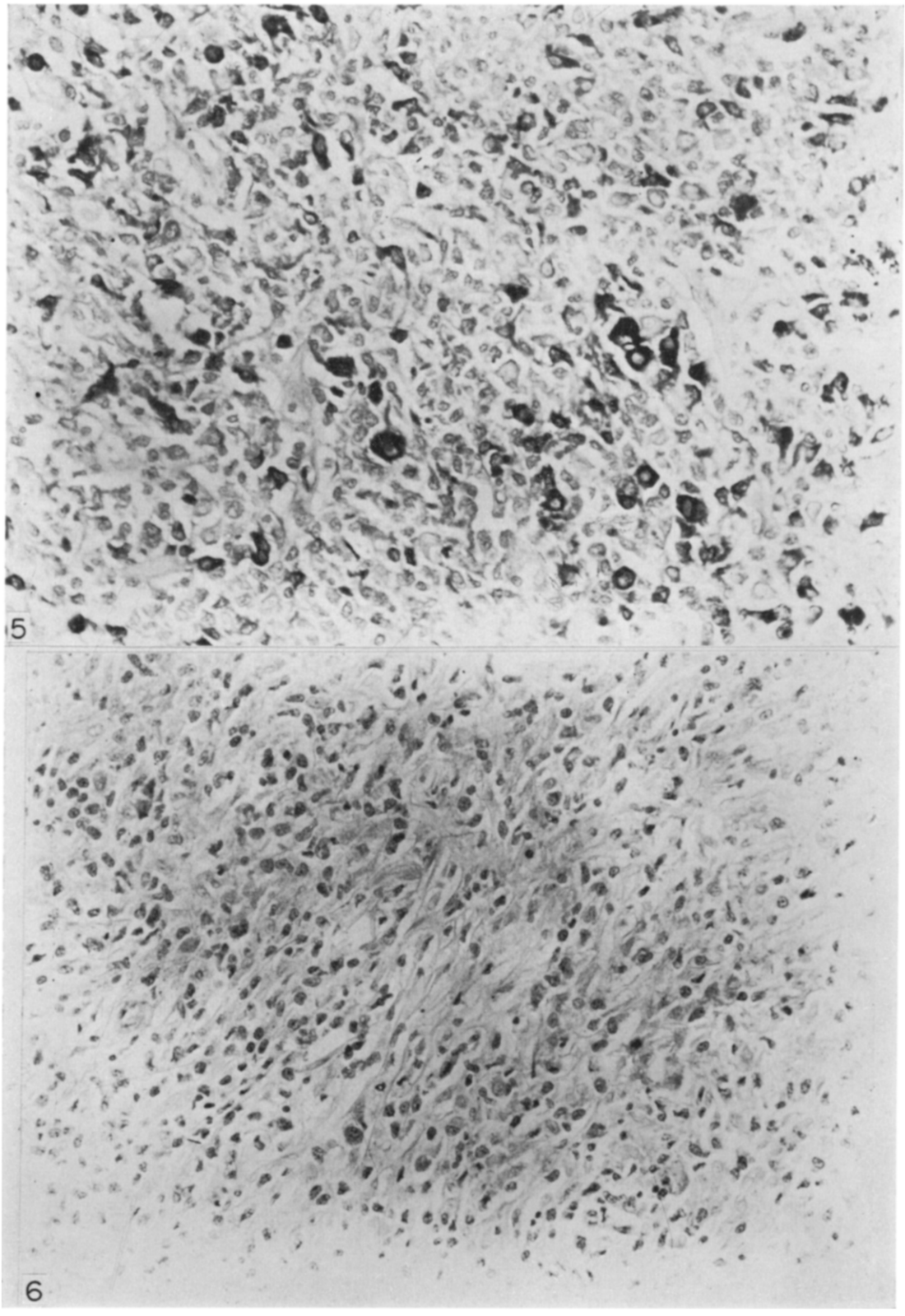


Fig. 5. *Reticulum cell sarcoma (histiocytic lymphoma) Paraffin section, anti-K* 1/100 *by the PAP method. Parallel section to Figs.* 6 *and* 7. *A proportion of the irregularly shaped neoplastic cells show an intense positive reaction (black). Cyan blue filter** × 750.

Fig. 6. *Reticulum cell sarcoma (histiocytic lymphoma). Paraffin section, anti-L,* 1/100 *by the PAP method. Parallel section to Figs.* 5 *and* 7. *There is no positive reaction with anti-L antiserum. The neoplastic cells, therefore, appear to be monoclonal for kappa chain. Cyan blue filter** × 750.

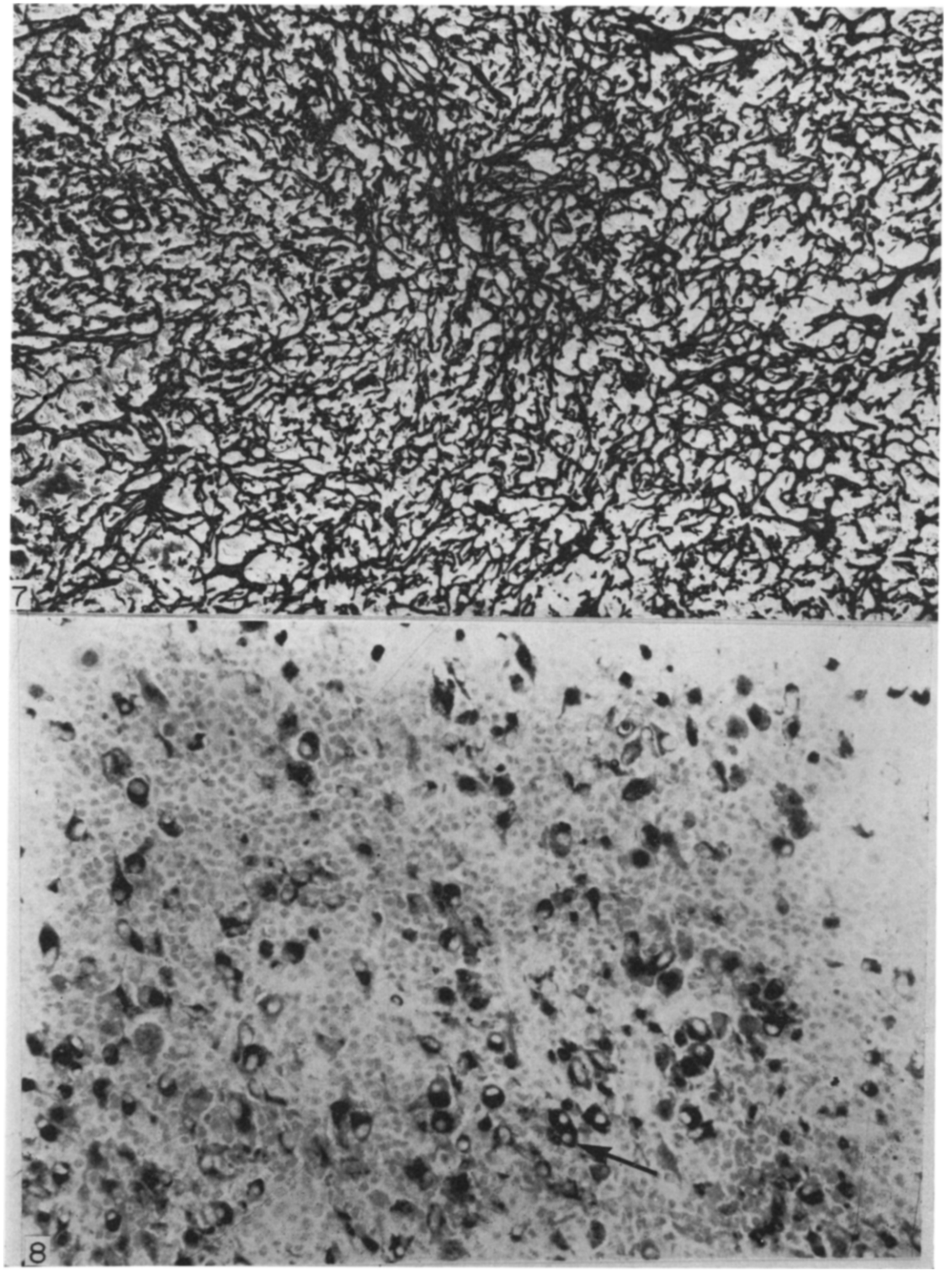


Fig. 7. *Reticulum cell sarcoma (histiocytic lymphoma). Paraffin section, Sweet's method for reticulin. Parallel section to Figs.* 5 *and* 6. *Dense reticulin formation throughout tumour.* × 400.

Fig. 8. *Epithelioid Hodgkin's disease (lymphocyte/histiocyte predominant). Paraffin section, anti-muramidase* 1/40 *by the PAP method. Many muramidase positive cells (black) including occasional binucleate forms (arrow). In parallel H & E section the muramidase positive cells correspond to eosinophilic epithelioid histiocytes. Cyan blue filter** × 400.

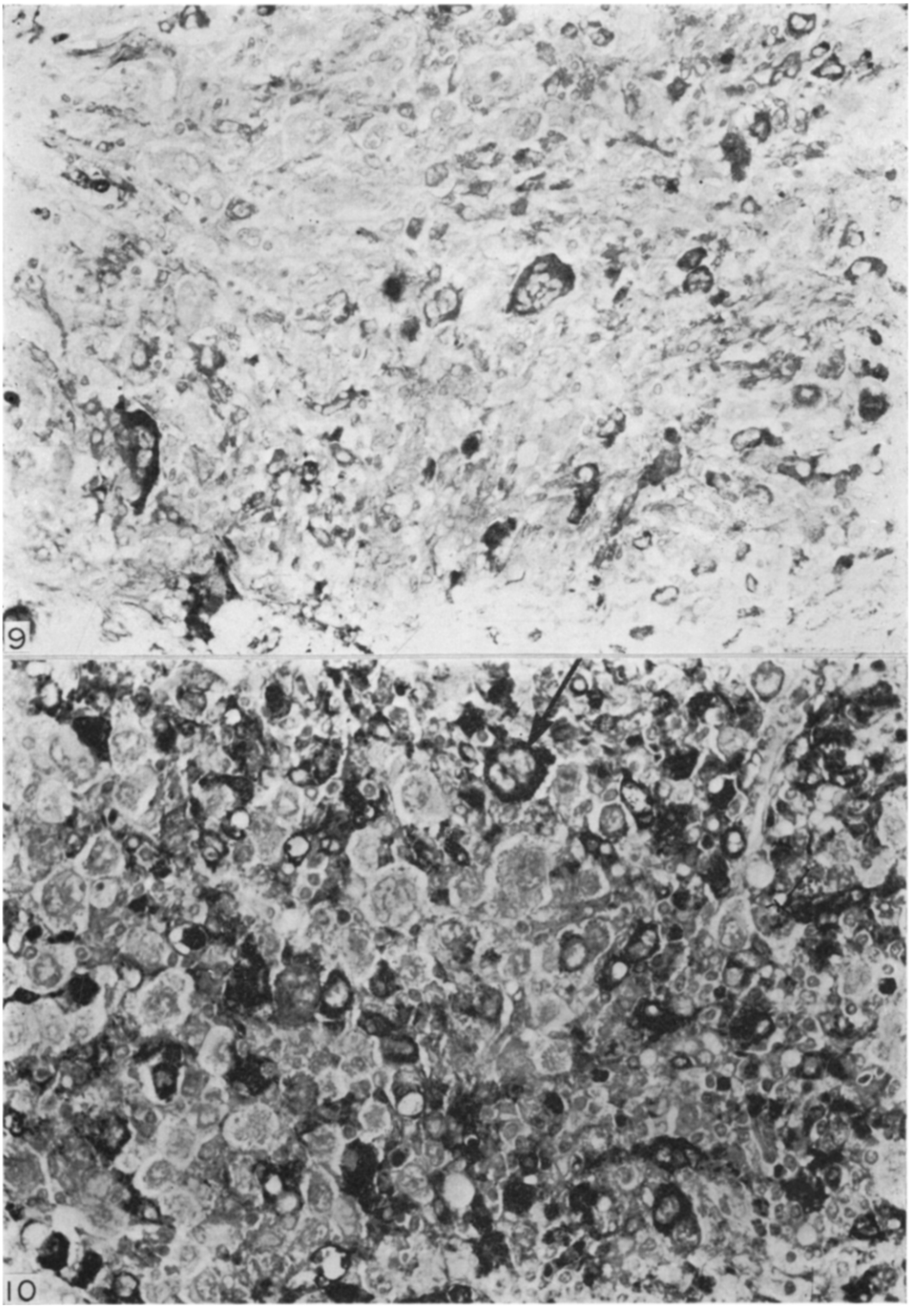


Fig. 9. *Hodgkin's disease (Mixed cellularity). Paraffin section, anti-K* 1/100 *by the PAP method. A proportion of mononuclear Hodgkin cells and giant Reed–Sternberg cell variants are positive for immunoglobulin. In parallel sections a proportion of these cells can be shown to contain both kappa and lambda light chains. Cyan blue filter** × 750.

Fig. 10. *Hodgkin's (disease mixed cellularity → lymphocyte depleted) Paraffin section, anti-K* 1/100 *by the PAP method. Many mononuclear Hodgkin cells and Reed–Sternberg type cells are present, and a proportion are positive for immunoglobulin (Classical Reed–Sternberg cell arrowed). Cyan blue filter** × 750.

*Cyan blue filter used to enhance contrast in mono chrome plates results in loss of nuclear detail. For better appreciation of results obtainable see colour Plate I [15].

but was clearly more sensitive in that positive results were obtained with dilutions of anti-kappa or anti-lambda up to 1/500.

Also in the myeloma study the presence of monoclonal immunoglobulin components within poorly differentiated sarcoma cells was noted in sections from four neoplasms, which on purely morphological grounds were indistinguishable from other lesions independently classified as "reticulum cell sarcomata". These four cases are of particular relevance to the observations made of immunoglobulin components within other lymphomata. Five similar poorly differentiated plasma cell neoplasms were discovered among the reticulosarcoma cases making a total of nine in all (Table 1).

Follicular lymphoma

Twenty cases of lymphoma were examined in which a nodular pattern was apparent. Six cases were classified as negative for intracellular immunoglobulin. In five of these cases the cells comprising the neoplastic follicles were of relatively uniform size and resembled medium sized lymphocytes. In the sixth case there was partial loss of the follicular pattern and the cells were large with vesicular nuclei and frequent mitoses. This case was interpreted as sarcomatous change of a pre-existing follicular lymphoma (diagnosed 10 years earlier), and was classified as "reticulum cell sarcoma".

Definite staining for intracellular immunoglobulin was observed in fourteen cases of follicular lymphoma. In these cases the cells comprising the neoplastic follicles were variable in size and included larger transformed type cells with prominent nucleoli and basophilic cytoplasms. In some cases a number of folded (cleaved) type cells were also present. A small proportion of these neoplastic follicle cells showed definite staining for kappa chain or for lambda chain in a monoclonal pattern (Fig. 2).

More remarkable however was the great number of positive immunoglobulin containing cells in the perifollicular area of a minority of cases (Fig. 3). Many of these perifollicular cells were lymphocytoid, but some showed features of plasma cell differentiation. These perifollicular cells showed a monoclonal pattern of immunoglobulin staining identical to that observed within the neoplastic follicles. It therefore appeared that the perifollicular cells were part of the same neoplastic process.

Other cases contained fewer perifollicular immunoglobulin positive cells but showed a larger proportion of immunoglobulin containing cells actually within the neoplastic follicles.

Lymphosarcoma (diffuse lymphocytic lymphoma, well differentiated).

These cases consisting of well differentiated small lymphocytes in a diffuse pattern showed no evidence of staining for intracellular immunoglobulin.

Diffuse lymphoblastic reticulosarcoma (Lymphocytic lymphoma, diffuse, poorly differentiated)

These neoplasms consisted of larger lymphoid cells with prominent nucleoli and frequent mitoses. Of the eleven cases in this group seven showed no trace of immunoglobulin staining while four were classified as immunoglobulin positive. On closer examination all four immunoglobulin positive cases showed atypical features. In two a poorly defined nodular pattern was apparent and the cell morphology and immunoglobulin staining closely resembled that of some cases of follicular lymphoma. Another case containing a large proportion of "blast-like" cells was reclassified as Waldenstrom's disease.* The fourth case was reclassified as a plasma cell reticulosarcoma. All four cases showed monoclonal immunoglobulin staining.

Reticulum cell sarcoma group (4 to 8 in Table 1)

The histological sub-divisions of this group presented in Table 1 are based upon the classification of Robb-Smith [8 and 9] and depend upon overall morphological features together with evidence of reticulin fibre production. The majority of those neoplasms consisting of large cells with vesicular nuclei but poorly defined cytoplasm (termed syncytial—Table 1) were immunoglobulin positive, with the notable exception of five cases having marked reticulin formation (dictyosyncytial—Table 1). In some of the immunoglobulin positive cases the neoplastic cells showed deep folding of the nuclear membrane (cleaved cells), while other cases consisted predominantly of nuclei of the "non-cleaved" type, with a central prominent nucleolus. The latter neoplasms show morphological correspondence to the immunoblastic sarcoma group as described by Lukes [23].

Of the twelve immunoglobulin positive cases, nine were clearly kappa chain and one lambda chain. The other two cases showed evidence of both kappa and lambda chains within the malignant cell population.

*Monoclonal staining pattern KM [22].

Others of the reticulum cell sarcoma group showed considerable variation in cell morphology but were characterised by distinct cytoplasmic boundaries. The majority of these cases showed definite staining for intracellular immunoglobulin usually of kappa type. However, in five cases both kappa and lambda chains were apparently present within individual neoplastic cells. Six of the immunoglobulin positive cases showed marked reticulin formation, a phenomenon also observed in three of the plasma cell reticulosarcomas. Thus reticulin formation was observed in both immunoglobulin positive and negative cases (*vide supra*).

The staining pattern in the polymorphic reticulosarcoma group is exemplified by one case (Fig. 4) diagnosed at autopsy and shown by immunoperoxidase methods to contain both kappa and lambda light chains within the malignant cell population. A previous biopsy (some six months earlier) of this case had been diagnosed as follicular lymphoma. On subsequent examination of this biopsy it was possible to demonstrate lambda light chain within the cells of the neoplastic follicle (Fig. 2).

The case illustrated by Figures 5, 6 and 7 was morphologically of the histiocytic type in that the cells were clearly defined, angular, and showed cytoplasmic eosinophilia. However these cells were clearly positive for immunoglobulin (kappa chain positive, Fig. 5, lambda chain negative, Fig. 6) and the angular form of the cells was related to the dense reticulin pattern (Fig. 7).

Finally some of the cases included in this reticulum cell sarcoma group showed marked plasmacytoid features and these were additional to those nine cases in which a diagnosis of plasma cell reticulosarcoma had been made on purely morphological grounds.

Cytoplasmic muramidase—Non-Hodgkin's lymphomas

In all of these cases serial sections were examined for the presence of cytoplasmic muramidase by the immunoperoxidase method. In the great majority of cases the neoplastic cells showed no trace of positive reaction with the anti-muramidase antisera, although varying numbers of reactive histiocytes and polymorphs were seen in most sections. However two cases were discovered in which cells, which were interpreted as part of the neoplastic population, clearly contained muramidase. One of these cases had been classified as syncytial reticulosarcoma and the other remained unclassified (but would fit most closely with the reticulum cell medullary reticulosis group of Robb-Smith [8]).

The findings in Hodgkin's disease—Immunoglobulins

Here the results are reported with the minimum of interpretation, the possible significance is considered in more detail in the discussion.

Interpretation of a positive staining reaction was more difficult in cases of Hodgkin's disease than in other lymphomas. Some of the reasons for these difficulties of interpretation were as follows: uncertain histological sub-grouping, specific and non-specific background staining, the dispersed nature of the Hodgkin and Reed–Sternberg cell population, the presence of kappa and lambda chain components within the same cell.

A positive brown reaction visible at high magnification was considered significant in the absence of specific or non-specific background staining. An assessment was made of the intensity and number of positive mononuclear cells (Hodgkin cells) and Reed–Sternberg cells relative to the total number of these cell types in the section. Plasma cells were present in every case and served as a valuable positive control. In no case did the staining of Reed–Sternberg cells equal in intensity that of plasma cells which appeared dark brown or black.

Results of cytoplasmic muramidase study in Hodgkin's disease

Sections treated with anti-muramidase antisera were examined for three principal findings: firstly the number of muramidase positive polymorphonuclear cells, mainly eosinophils; secondly an assessment of the number of muramidase positive mononuclear cells (histiocytes); and thirdly an examination of Hodgkin and Reed–Sternberg cells for evidence of cytoplasmic muramidase, as a possible indication of histiocytic origin for these cell types.

The results of these studies were complex but some general patterns emerged. These are summarised below and illustrated by Figures 8, 9 and 10.

Lymphocyte histiocyte predominant Hodgkin's disease (4 cases)

In these cases many epithelioid histiocytes were present, scattered diffusely throughout the lymph node. Some of these cells contained prominent nucleoli and binucleate cells were present showing some resemblance to classical

Reed–Sternberg cells, from which they were distinguishable by their markedly eosinophilic cytoplasmic reaction. These epithelioid cells were strongly positive for muramidase (Fig. 8) but showed no evidence of staining with anti-kappa or anti-lambda antisera.

Mixed cellularity Hodgkin's disease (9 cases)

In this group classical Reed–Sternberg cells were more prominent and more numerous than in the lymphocyte/histiocyte predominant group. However a number of histiocytes were present in some cases and showed a strong cytoplasmic reaction for muramidase. Typical Reed–Sternberg cells and mononuclear Hodgkin cells were negative for muramidase but in all cases there was evidence of kappa and lambda light chain within the cytoplasm of a proportion of these cell types (Figs. 9 and 10). The proportion of immunoglobulin containing cells was generally small (less than 30%) but in some cases many Hodgkin-type cells were positive (Fig. 10). In the majority of cases both kappa and lambda light chains appeared to be present within individual Reed–Sternberg cells but in two cases a monoclonal pattern for kappa chain was observed. The immunoglobulin positive Hodgkin and Reed–Sternberg cells were characterised by a prominent central nucleolus and vesicular type of nucleus. Cytoplasm was generally extensive and in some cases showed basophilia on H & E preparations. Many of these cells were clearly pyroninophilic (Unna Pappenheim stain).

Nodular sclerosis group (30 cases)

The findings in this group were most complex and generally difficult to interpret.

In a number of cases many histiocytes were present and some of these showed extensive pink cytoplasm with peripheral cytoplasmic collapse giving some resemblance to classical lacunar cells. Resemblance was enhanced by the presence of occasional binucleate forms, but forms with complex multilobulate nuclei were not observed. These cells with eosinophilic cytoplasm generally contained cytoplasmic muramidase, whereas classical lacunar cells were negative for muramidase.

Diagnostic Reed–Sternberg cells with prominent nucleoli and vesicular nuclei were frequently observed to contain immunoglobulin which was judged to have a monoclonal pattern in seven cases (four kappa and three lambda). In eighteen cases both kappa and lambda chains were present in a small proportion of the cells and in the remaining five cases there was no trace of immunoglobulin staining within mononuclear Hodgkin cells or Reed–Sternberg cells. Immunoglobulin negative Hodgkin cells were observed in all cases and generally lacked the prominent nucleolus and distinct nuclear membrane of the immunoglobulin positive cells. In addition the negative cells often showed complex nuclear folding. Cytoplasmic muramidase was not detected in mononuclear Hodgkin cells or in Reed–Sternberg cells. In the nodular sclerosis group staining for intracellular immunoglobulin was most prominent near areas of tissue necrosis, and a proportion of these cells showed degenerative features.

Lymphocyte depleted Hodgkin's disease

There were two cases of lymphocyte depleted Hodgkin's disease in this group and in addition three of the polymorphic reticulosarcoma group might, by others, be termed Hodgkin's Sarcoma (equivalent to lymphocyte depleted Hodgkin's disease). Four of these five cases were negative both for immunoglobulin and muramidase, and the fifth showed a weak reaction for both kappa and lambda.

DISCUSSION

The specificity and sensitivity of the method has been compared favourably with that of established immunofluorescence techniques [16]. The principal advantages of the immunoperoxidase method over immunofluorescence methods are three: firstly the excellent tissue morphology, secondly the production of permanent preparations for examination by light microscopy, thirdly the facility for conducting retrospective studies of formalin fixed material stored in paraffin blocks.

The significance of a positive reaction for intracellular immunoglobulin by immunoperoxidase methods

The specificity of the rabbit anti-human immunoglobulin antisera, used in the first stage of the immunoperoxidase methods was confirmed in the study of tissues from multiple myeloma [19].

Known positive controls (tissues from typed cases of myeloma) were used throughout the study, together with normal rabbit serum controls and negative controls omitting each stage of the immunoperoxidase sequence in turn. Finally for every case serial sections were treated with a standard panel of rabbit anti-

human antisera. The different proportions of immunoglobulin containing cells observed in these preparations provided a valuable inherent control, and in polyclonal plasma cell populations positive cells could be seen adjacent to clearly negative cells, indicating a high degree of specificity. At the dilutions used in the PAP method (anti-K 1/100) the specificity of the antisera would be expected to be extremely high, and the probability of cross reactions minimal.

Non-specific background staining

This form of background staining is generally attributed to the non-immune binding of immunoglobulin onto basically charged tissue components, particularly collagen. In multiple layer methods non-immune binding is maximal for the first antiserum applied. Thus the application of a neutral antibody (i.e., one not participating in the immunoperoxidase reaction under study) as the first layer may be expected to reduce non-specific binding of the test antisera applied subsequently. In this study pretreatment with normal swine serum was of particular value in those cases of Hodgkin's disease where there was extensive fibrosis.

Specific background staining

In addition to non-specific background staining the demonstration of intracellular immunoglobulin may be obscured by the presence of free tissue immunoglobulin or by the presence of immunoglobulin bound in germinal centre areas [24–26]. Both these forms of "specific background staining" were more obvious in sections examined by the PAP method, as a direct result of the increase in sensitivity achieved by the method.

Within these limits, and in the presence of a suitable control preparation, the presence of positive reaction of any cell can be confidently equated with the presence of a particular immunoglobulin component within that cell.

Immunoglobulin containing cells

The demonstration of immunoglobulin in a plasma cell can reasonably be equated with the production of that immunoglobulin by that cell. However, the demonstration of immunoglobulin within the malignant reticulum cells of reticulum cell sarcoma and within Reed–Sternberg cells may be open to several interpretations.

1. The immunoglobulin may have been produced by the cell containing it.

At present immunoglobulin production is considered to be confined to members of the B lymphocyte series, including plasma cells. Transforming B lymphocytes and their differentiating progeny (plasma cell precursors) are engaged in active immunoglobulin synthesis [27] and may therefore be distinguished from other cells by their content of immunoglobulin [25]. According to Takahashi and colleagues [28], in studies of synchronised cells in culture, immunoglobulin synthesis is maximal in G1 and early S phase of the cell cycle, corresponding to the period of morphological transformation.

At this stage of the lymphocyte cell cycle those cells destined to become mature plasma cells

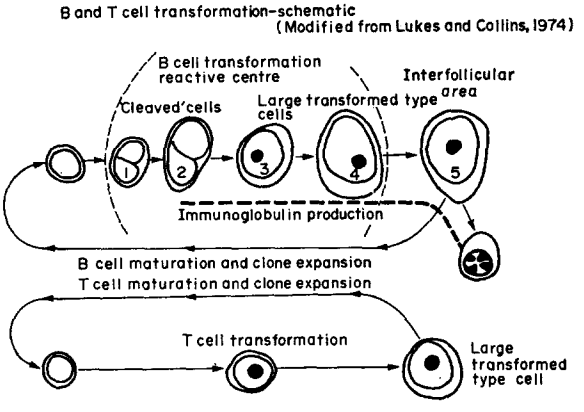


Fig. 11. *B and T cell transformation—schematic (Modified from Lukes and Collins,* [14].
Scheme shows changes in B lymphocyte morphology during the physiological process of B cell transformation in the reactive centre.
Cells 1 *and* 2*—small and large cleaved cells.*
Cells 3, 4 *and* 5*—non-cleaved cells—various expressions of the transformed lymphocyte.*
Types of cells in which immunoglobulin may be observed are indicated by the heavy interrupted line.

may be morphologically indistinguishable from other lymphocytes of different origin and destiny (e.g. transforming T cells) but may be recognised as B cells by their content of immunoglobulin.

Thus it is possible that all immunoglobulin producing cells, whatever their morphological appearance, are part of the B lymphocyte series, and that their morphological features vary according to the phase of the cell cycle (Fig. 11).

However, neoplastic cells may on occasion display potentialities, such as hormone production, which are not related to the normal functional role of the progenitor cell. In this context a number of non-lymphoreticular neoplasms have been examined for intracellular immunoglobulin, and have been negative,

except for inconsistent staining of obviously degenerating cells in areas of necrosis.

In this respect the example of plasma cell reticulosarcoma is of particular significance for given the natural history of the process [19] the intracellular monoclonal immunoglobulin clearly has been produced by the tumour cells themselves. In addition the similarity of such cells to the large immunoglobulin containing cells (malignant "reticulum cells") demonstrated in cases of Hodgkin's disease and reticulosarcoma, supports the hypothesis that the latter cell types are also capable of immunoglobulin production.

One further observation supports the view that cells containing monoclonal immunoglobulin have also produced it. Thus it would be difficult to account for the presence of monoclonal immunoglobulin components within a cell by passive absorption, or by phagocytosis, whereas it is generally accepted that individual immunoglobulin producing cells are usually restricted to a single light and heavy chain type (*vide infra*).

2. The intracellular immunoglobulin may have been passively absorbed onto the neoplastic cell, as a manifestation of a host anti-tumour response.

This presupposes that the neoplastic cells display some tumour specific antigen to which the host can respond. There is some evidence that such antigens do exist in Hodgkin's disease [29–31] and other lymphomas [32].

However, in a host anti-tumour response one might expect to find the tumour specific antibody concentrated at the cell surface, and the penetration of antibody into the cell cytoplasm would signify cell death. Thus cells showing cytoplasmic staining should also show evidence of cellular degeneration. In nodular sclerosis a proportion of immunoglobulin positive cells showed nuclear pyknosis and cytoplasmic degeneration, but these features were observed rarely in other cases.

3. The intracellular immunoglobulin may have been actively phagocytosed by the neoplastic cells.

It is as difficult to account for the presence of intracellular monoclonal immunoglobulin by active phagocytosis as it is by passive absorption.

In some cases of Hodgkin's disease and reticulum cell sarcoma, both K and L chains were demonstrated within the same cell, as might occur following indiscriminant phagocytosis. In other cases only a single light or heavy chain type was present, favouring the concept of synthesis within the cell. Thus, while cases of myeloma and the majority of the reticulosarcoma group were clearly monoclonal, this was not the case for Hodgkin's disease. Examples where all of the positive Hodgkin cells or reticulum cell sarcoma cells contain both kappa and lambda chain may represent the occurrence of a clone with the capacity for production of more than one type of immunoglobulin, such as occurs rarely in myelomatosis (bi-clonal gammapathies [33, 34], or in reactive plasma cells [25, 28, 35].

Also the immunoglobulin containing neoplastic cells in Hodgkin's disease and in reticulum cell sarcoma showed no other evidence of phagocytic activity. The extensive histochemical studies of other authors have produced conflicting results and the demonstration of histiocyte associated enzymes within Reed–Sternberg cells has not always been confirmed [36–38]. The observations made in this study using anti-muramidase antiserum are pertinent to this question, and are reported below.

Of these three possible interpretations of the significance of intracellular immunoglobulin in "malignant reticulum cells" the first is preferred, relating the presence of intracellular immunoglobulin in a tumour cell to *in situ* production by the cell.

The significance of cytoplasmic muramidase

Two anti-muramidase antisera, obtained from separate sources, were used in this study and produced identical staining patterns. The distribution of the muramidase containing cells has been reported [20] and evidence was given of muramidase production in activated or reacting histiocytes. Epithelioid histiocytes and their related giant cells commonly gave a positive reaction with anti-muramidase antiserum.

Similar observations were made in the study of the lymphomas in which it was observed that epithelioid histiocytes occurring singly or in small congeries were frequently muramidase positive. In some cases of Hodgkin's disease epithelioid histiocytes were numerous and included larger cell types with extensive cytoplasm and one or two nuclei with distinct nucleoli (Fig. 8). These large cells, (positive for muramidase, negative for immunoglobulin components) were believed to be distinct from true Reed–Sternberg cells (negative for muramidase, positive for immunoglobulin). Morphologically the large muramidase containing cells could be differentiated on H & E section by the occurrence of distinct cytoplasmic eosinophilia, by the smaller nucleolus and the

more delicate nuclear membrane in comparison with classical Reed–Sternberg cells. However, it must be admitted that in some cases distinction was impossible on H & E section, and difficult in immunoperoxidase preparations for some of these cells were negative both for muramidase and intracellular immunoglobulin.

In addition two of the immunoglobulin negative reticulum cell sarcoma cases were found to contain a small proportion of neoplastic cells which were muramidase positive. This suggested that "histiocytic" sarcomas may occur, somewhat less frequently than the corresponding neoplasms of lymphoid cells.

Morphology of immunoglobulin containing cells

In most sections the majority of immunoglobulin containing cells possessed a classical plasma cell morphology. In other cases a proportion of larger, more rounded cells were present. These were identified as plasma cell precursors and some showed evidence of plasmacytoid differentiation (Fig. 1). Other large immunoglobulin containing cells showed no morphological evidence of plasma cell differentiation and were characterised by a vesicular nucleus with distinct nuclear membrane and prominent central nucleolus (Fig. 1). These cells are the earliest recognisable precursor of the plasma cell and are thought to be equivalent to the transformed B lymphocyte. Immunoglobulin containing transformed lymphocytes were identified in reactive lymphoreticular tissues and also in some of the lymphoreticular neoplasms. The morphology of these cells is shown in Figure 1. Within reactive centres a minority of the transformed type lymphocytes (non-cleaved) were also immunoglobulin positive, together with a somewhat smaller proportion of positive cells of the large "cleaved" variety. The morphology of these immunoglobulin containing cells may be compared with the suggested sequence [14] of morphological changes occurring during transformation of the B lymphocyte in germinal centre areas (Fig. 11).

From their purely morphological studies Lukes and Collins suggested that a significant proportion of the lymphomas might be derived from follicular centre cells (B lymphocytes). This suggestion was reinforced by a developing understanding of functional aspects of the lymphomas [14].

The observations reported here, of intracellular immunoglobulin within follicular and diffuse lymphomas (including those classifiable as reticulum cell sarcoma), extend and support this concept and make it possible to interrelate the diverse morphological forms of lymphoma through their common origin from the B lymphocyte (see Fig. 12).

Postulated relationship of lymphomas to the transformed lymphocyte

A tentative scheme may then be suggested linking these various histological types of lymphoreticular neoplasms to the different morphological forms of the lymphocyte during its cell cycle. It is postulated that the neoplastic cells retain some of the functional and morphological attributes of the lymphocyte at any particular phase of the cell cycle.

Clearly the B lymphocyte undergoes remarkable morphological changes during the physiological process of transformation (see Figs. 11 and 12). Recognition of this radical metamorphosis of the B lymphocyte establishes the developmental relationships of five morphological cell types, previously inter-related by pathologists with varying degrees of uncertainty, (a) the small lymphocyte, (b) the folded reactive centre cell (cleaved cell of Lukes), (c) the early transformed type lymphocyte (small non-cleaved cell), (d) the fully transformed lymphocyte (large non-cleaved cell, plasmablast) and (e) the plasma cell. Cells (b), (c) and (d) have commonly been regarded as varieties of "reticulum cell" [18].

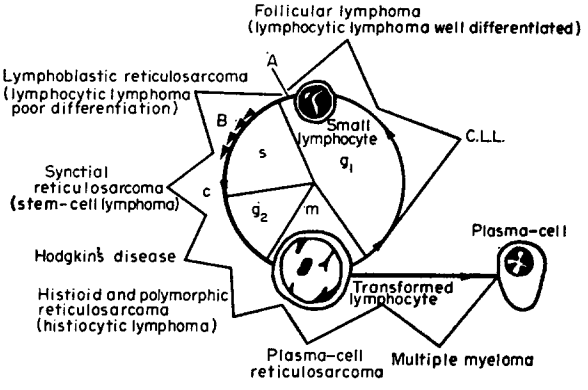


Fig. 12. Postulated relation between lympho-reticular neoplasms and the various morphological forms assumed by the lymphocyte during its cell cycle.

Just as these separate morphological cell types may be interrelated by recognition of the morphological changes of B lymphocyte transformation, so also the corresponding neoplasms (Fig. 12). Thus the behaviour and appearance of the lymphoid neoplasms depends upon the mode of cycle of the majority of the neoplastic cells. For example a follicular pattern would only be expected in those neoplasms corres-

ponding to the forms of the B lymphocyte normally retained within the follicular areas, and similarly significant immunoglobulin production would tend to occur in those neoplasms corresponding to the phases of the cycle in which immunoglobulin secretion normally occurs. In this respect it is notable that those transformed B lymphocytes destined to become plasma cells normally leave the reactive centre and pass towards the medulla of the lymph node where they assume characteristic plasma cell morphology [39].

Those lymphoreticular neoplasms in which it was not possible to demonstrate intracellular immunoglobulin may, on this basis, be tumours of B cells in a non-productive phase, of B cells containing traces of immunoglobulin below the threshold of sensitivity of the techniques used, T cells, or indeed of any other cell type. In addition B cell neoplasms, which during the course of the neoplastic process have evolved far from the original lymphoid progenitor cell, may have lost any of the functional characteristics of the lymphocyte and therefore remain unclassifiable by the present method.

This scheme is in accordance with the concepts of follicular centre cell lymphomas recently advanced by Lukes and Collins (see above). The suggested sequence of small lymphocyte to cleaved cell to non-cleaved cell to plasma cell appears to be confirmed for the B cell (Fig. 11) and is similar in concept to the more general scheme presented here (Fig. 12).

In addition the tritiated thymidine and Feulgen microspectrophotometry studies of Peckham and Cooper support to some extent the relative positions allocated to various lymphoid tumours.

Thus in Hodgkin's disease [40, 41] mononuclear Hodgkin cells and Reed–Sternberg cells show some evidence of DNA synthesis (S phase) and possess a DNA content varying from diploid to near tetraploid (late S phase or G2 phase).

The phenomenon of giant cell formation in Hodgkin's disease (and other lymphomas) remains unexplained. However it has been reported that interference with the synthetic processes of the cell cycle can result in radical changes in morphology, with accumulation of the affected cell type. The possible consequences of interference with the cell cycle have been summarised by Harris [42] who recorded the occurrence of giant cells following specific inhibition of DNA synthesis in S phase or inhibition of mitosis itself.

A study of non-Hodgkin lymphomas by similar methods of DNA analysis and thymidine incorporation [41, 43] has also produced results compatible with the positioning of these lymphomas in the accompanying scheme (Fig. 12). Thus well differentiated lymphocytic lymphomas possess a diploid content of DNA in accordance with a G1 mode, while poorly differentiated lymphocytic lymphomas (lymphoblastic) possess a modal DNA content varying from diploid to hyperdiploid (early S phase). The results in those neoplasms classified as reticulum cell sarcoma were more varied in keeping with the diverse nature of neoplasms included in this group, and diploid, hyperdiploid and tetraploid forms were recorded.

The interrelation of the various lymphomas through the transformation sequence of the B lymphocyte offers a ready explanation of the numerous observations of transmutation or transition of histological forms of lymphoma from one to another (see Figs. 2 and 4). However while neoplastic lymphocytes may resemble the phases of transformation of the normal B lymphocyte in morphology, behaviour, DNA content and synthetic activity, they have departed significantly from the normal mode of existence of the B lymphocyte by the very nature of the neoplastic process itself.

B cell neoplasms

From the observations of intracellular immunoglobulin reported here it may be concluded that multiple myeloma, follicular lymphoma, and some cases of "reticulum cell sarcoma" and Hodgkin's disease are B lymphocyte derived.

There is some independent evidence of the B cell nature of follicular lymphoma [44] and in addition there have been reports of immunoglobulin production by various of the diffuse lymphomas [45, 46].

Hodgkin's disease has traditionally been regarded as a T cell disease on the basis of the clinically observed deficit of T cell function and the occurrence of early Hodgkin lesions in the paracortical area of the lymph nodes. However, the observations reported in this study would favour a B cell origin for Hodgkin's disease and there have been independent reports of possible immunoglobulin production by Reed–Sternberg cells [30, 47, 48].

The conclusion that Hodgkin's disease may be a neoplasm of transformed lymphocytes has certain other implications for the understanding of its histological features. Thus uncontrolled lymphocyte transformation may result in local immunoglobulin production with complement mediated chemotaxis for eosinophils and poly-

morphs [49], or the generation of immune complexes leading to granuloma formation [50]. In addition the release of lymphokines may include factors chemotactic for eosinophils [51, 52], as well as other factors effecting macrophage morphology and function [53].

The concept of progressive hyperplasia

In addition the relation of the lymphoreticular neoplasm to the various morphological forms of the lymphocyte allows a clearer understanding of Robb-Smith's original concept of progressive hyperplasia [8, 9, 12] with subsequent transition to true sarcoma.

This sequence of events is well illustrated by the observation in mice of different phases of reaction of lymphoid tissues prior to the appearance of "reticulum cell sarcomas". In chronic allogeneic disease (low grade graft versus host reaction) there is an initial phase of plasma cell hyperplasia followed by the progressive appearance of large numbers of "reticulum cells" and the development of morphological reticulum cell sarcoma [6, 18, 54]. A similar series of changes occurs spontaneously in certain inbred strains of mice with a high incidence of reticulum cell sarcoma [18, 54].

The development of reticulum cell sarcoma after a prolonged plasma cell hyperplasia assumes a logical aspect when related to a period of sustained lymphocyte transformation. The development of progressive maturation arrest in the plasma cell series results in an accumulation of abnormal transformed lymphocytes (previously termed reticulum cells) and frank malignant neoplasia. The reported production of immunoglobulin by such tumours of mice [18, 54, 55] evokes no surprise and the corresponding observation of immunoglobulin production by reticulum cell sarcomata of man [45] becomes a matter of course.

It is suggested that many of the conceptual problems of the nature and relations of the lymphomas may be resolved by recognising that the reticulum cell has been used to represent two principal groups of cells:

1. The histiocyte group.
2. The transformed lymphocyte.

There would be little argument to the first proportion that the term reticulum cell has been applied to members of the histiocytic series [56–58]. Two possible sarcomas of this cell type have been recognised by their content of cytoplasmic muramidase.

Secondly, in the literature the term reticulum cell has been used to represent a stem cell function, but the form and nature of the stem cell remained ill-defined. Many different terminologies have been applied to cells of this group as illustrated by the example of nomenclature applied to the large atypical cells observed in histological sections of infectious mononucleosis [59].

The demonstration of immunoglobulin within some of this reticulum cell group has allowed the identification of the transformed B lymphocyte and possible neoplasms of this cell type have been identified by their content of intracellular immunoglobulin.

Acknowledgements—I would like to thank Maria Hambridge for preparation of the sections used in this study, and Dr. T. Parry for the photomicrographs. I am similarly indebted to Mrs. J. Braidwood who deciphered the manuscript.

This work was carried out in the department of Dr. A. H. T. Robb-Smith, and I am grateful to him for critical encouragement throughout.

REFERENCES

1. A. D. Rubin, L. I. Johnson, and S. M. Brown, Lymphocyte-lymphoproliferative disorders. *Prog. exp. Tumour Res.* **13,** 135 (1970).
2. L. G. Anderson and N. Talal. Sjogren's syndrome and reticulum cell sarcoma. The spectrum of benign to malignant lymphoproliferation in Sjogren's syndrome. *Clin. exp. Immunol.* **10,** 199 (1972).
3. A. A. Liebow, C. R. Carrington and P. G. Friedman. Lymphomatoid granulomatosis. *Hum. Pathol.* **3,** 457 (1972).
4. R. S. Schwartz and L. Beldotti, Malignant lymphomas following allogeneic disease: Transition from an immunological to a neoplastic disorder. *Science* **149,** 1511 (1965).
5. M. Y. K. Armstrong, R. S. Schwartz, L. Beldotti, Neoplastic sequelae of allogeneic disease. 3. Histological events following transplantation of allogeneic spleen cells. *Transplantation* **5,** 1380 (1967).
6. M. Y. K. Armstrong, E. Gleichman, H. Gleichman, L. Beldotti, J. Andre-Schwartz and R. S. Schwartz, Chronic allogeneic disease. II. The development of lymphomas. *J. exp. Med.* **132,** 417 (1970).

7. H. RAPPAPORT, Tumours of the Hematopoietic System. *Atlas of Tumour Pathology*. Section III—Fascicle 8. Armed Forces Institute of Pathology, Washington (1966).
8. A. H. T. ROBB-SMITH, Reticulosis and reticulosarcoma: A histological classification. *J. Path. Bact.* **47,** 457 (1938).
9. A. H. T. ROBB-SMITH, The lymph node biopsy. *Recent Adv. clin. Path.* 350, Churchill Livingstone, London (1947).
10. B. D. PULLINGER, Histology and histogenesis, *Rose Research on Lymphadenoma*, p. 117, John Wright, Bristol (1932).
11. R. A. WILLIS, *Pathology of Tumours* 4th Ed. p. 772. Butterworths, London (1967).
12. A. H. T. ROBB-SMITH, Immunotopography of lymphoreticular disorders. *Lancet* **i,** 513 (1974).
13. R. J. LUKES and R. D. COLLINS, New observations on follicular lymphoma. *Gann. Monogr. Cancer Res.* **15,** 209 (1973).
14. R. J. LUKES and R. D. COLLINS, A functional approach to the classification of malignant lymphoma. *Recent Results in Cancer Res.* **46,** 18. (Edited by K. MUSSHOFF) (1974).
15. C. R. TAYLOR and J. BURNS, The demonstration of plasma cells and other immunoglobulin-containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase-labelled antibody. *J. clin. Path.* **27,** 14 (1974).
16. J. BURNS, MARIA HAMBRIDGE and C. R. TAYLOR, Intracellular immunoglobulins. A comparative study on three standard tissue processing methods using horseradish peroxidase and fluorochrome conjugates. *J. clin. Path.* **27,** 548 (1974).
17. C. R. TAYLOR, The nature of Reed–Sternberg cells and other malignant "reticulum" cells. *Lancet* **ii,** 802 (1974).
18. C. R. TAYLOR, An immunohistological study of human and murine lymphomata. D.Phil. Thesis, Oxford, 1974.
19. C. R. TAYLOR and D. Y. MASON, The immunohistological detection of intracellular immunoglobulin in formalin-paraffin sections from multiple myeloma and related conditions using the immunoperoxidase technique. *Clin. exp. Immunol.* **18,** 417 (1974).
20. D. Y. MASON and C. R. TAYLOR, The distribution of muramidase (lysozyme) in human tissues. *J. clin. Path.* **28,** 124 (1975).
21. D. Y. MASON, Measurement of kappa/lambda light chain ratios by haemagglutination techniques. *J. immunol. Methods*, In press (1975).
22. J. B. HEENAN, D. Y. MASON, J. SKINNER and C. R. TAYLOR, Waldenstrom's disease of the jejunum. *Beitr. zur. Path.* In press (1975).
23. R. J. LUKES and B. H. TINDLE, Immunoblastic lymphadenopathy. A hyperimmune entity resembling Hodgkin's disease. *New Engl. J. Med.* **292,** 1 (1975).
24. G. SAINTE-MARIE, A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10,** 250 (1962).
25. R. C. MELLORS and L. KORNGOLD, The cellular origin of human immunoglobulin. *J. exp. Med.* **118,** 387 (1963).
26. W. STRAUSS, Cytochemical detection of site of antibody to horseradish peroxidase in spleen and lymph nodes. *J. Histochem. Cytochem.* **16,** 237 (1968).
27. J. B. HAY, M. J. MURPHY, B. MORRIS, and M. C. BESSIS. Quantitative studies on the proliferation and differentiation of antibody forming cells in lymph. *Amer. J. Path.* **66,** 1 (1972).
28. M. TAKAHASHI, Y. YAGI, G. E. MOOR and D. PRESSMAN. Immunoglobulin production in synchronized cultures of human haematopoietic cell lines. 1. Variation of cellular immunoglobulin level with the generation cycle. *J. Immunol.* **103,** 834 (1969).
29. S. E. ORDER, S. E. CHISM and S. HELLMAN, Studies of antigens associated with Hodgkin's disease. *Blood* **40,** 621 (1972).
30. P. M. DENTON, Immune responsiveness in Hodgkin's disease. *Brit. J. Cancer* **28,** Suppl. 119 (1973).
31. K. B. SHARMA and M. H. CHO, Demonstration of cell bound antigen in Hodgkin's disease. A study of the cells in the blood by indirect immunofluorescence. *Lab. Invest.* **28,** 397 (1973).

32. M. Braun, L. Sen, A. E. Bachmann and A. Ravlovsky, Cell migration inhibition in human lymphomas using lymph node and cell line antigens. *Blood* **39,** 368 (1972).
33. R. Van Furth, H. R. E. Schuit and W. Hijmans. In *Gamma Globulins* p. 443, Interscience, New York (1967).
34. J. H. Sanders, J. L. Fahey, I. Finegold, D. Ein, R. Reisfeld and C. Berard, Multiple anomalous immunoglobulins. Clinical, structural and cellular studies in three patients. *Amer. J. Med.* **47,** 43 (1969).
35. G. Attardi, M. Cohen, K. Horibata and E. S. Lennox, Symposium on the biology of cells modified by viruses or antigens. II. On the analysis of antibody synthesis at the cellular level. *Bact. Rev.* **23,** 213 (1959).
36. G. A. Ackerman, R. A. Knouff and M. A. Hoster, Cytochemistry and morphology of neoplastic and non-neoplastic human lymph node cells with special reference to Hodgkin's disease. *J. nat. Cancer Inst.* **12,** 465 (1951).
37. H. Braunstein, D. G. Freiman, W. Thomas and E. A. Gall, A histochemical study of the enzymatic activity of lymph nodes. III. Granulomatous and primary neoplastic conditions of lymphoid tissue. *Cancer (Phil.)* **15,** 139 (1962).
38. R. F. Dorfman, Enzyme histochemistry of normal, hyperplastic and neoplastic lymphoreticular tissues. In *Symposium on Lymphoreticular Tumours in Africa* (Edited by F. C. Roulet) p. 304, S. Karger, Basel, New York (1963).
39. G. J. V. Nossal and G. L. Ada, *Antigens, Lymphoid Cells and the Immune Response.* Academic Press, London (1971).
40. M. J. Peckham and E. H. Cooper, Proliferation characteristics of the various classes of cells in Hodgkin's disease. *Cancer (Phil.)* **24,** 135 (1969).
41. M. J. Peckham and E. H. Cooper, Cell biology. In *Hodgkin's Disease* (Edited by D. Smithers) p. 64 Churchill Livingstone, London (1973).
42. H. Harris, Cell growth and multiplication. In *General Pathology* (Edited by H. W. Florey) p. 630, Loyd-Lukes, London (1970).
43. E. M. Cooper, M. J. Peckham, R. E. Millard, I. M. E. Hamlin and R. Gerard-Marchant, Cell proliferation in human malignant lymphomas. Analysis of labelling index and DNA content in cell populations obtained by biopsy. *Europ. J. Cancer* **4,** 287 (1968).
44. E. S. Jaffe, E. M. Shevach, M. M. Frank, C. W. M. Berard and I. Green, Nodular lymphoma—Evidence for origin from follicular B lymphocytes. *New Engl. J. Med.* **290,** 813 (1974).
45. H. Stein, K. Lennert and M. R. Parwaresch, Malignant lymphomas of B cell type. *Lancet* **ii,** 855 (1972).
46. C. R. Peter, K. M. Mackenzie and F. J. Glassy, T or B cell origin of some non-Hodgkin lymphomas. *Lancet* **ii,** 686 (1974).
47. J. Leech, Immunoglobulin positive Reed–Sternberg cells in Hodgkin's disease. *Lancet* **ii,** 265 (1973).
48. A. J. Garvin, S. S. Spicer, R. T. Parmley and A. M. Munster, Immunohistochemical demonstration of IgG in Reed–Sternberg cells and other cells in Hodgkin's disease. *J. exp. Med.* **139,** 1077 (1974).
49. P. A. Ward, Chemotaxis of human eosinophils. *Amer. J. Path.* **54,** 121 (1969).
50. W. G. Spector and N. Heesom, The production of granulomata by antigen-antibody complexes. *J. Path.* **98,** 31 (1969).
51. A. Basten and P. B. Beeson, Mechanism of eosinophilia. II. Role of the lymphocyte. *J. exp. Med.* **131,** 1288 (1970).
52. S. Cohen and P. A. Ward, *In vitro* and *in vivo* activity of a lymphocyte and immune complex dependent chemotactic factor for eosinophils. *J. exp. Med.* **133,** 133 (1971).
53. G. A. Granger, Lymphokines—the mediators of cellular immunity. *Ser. Haemat.* **5.4,** 8 (1972).
54. C. R. Taylor, The development of reticulum cell sarcoma in mice. An immunohistological study of chronic allogeneic disease and of murine strains SJL/J, NZB and PBA. *J. Path.* In press (1975).
55. K. R. McIntire and L. W. Law, Abnormal serum immunoglobulins occurring with reticular neoplasm in an inbred strain of mice. *J. nat. Cancer Inst.* **39,** 1197 (1967).

56. E. A. GALL, Cytological identity and inter-relation of mesenchymal cells of lymphoid tissue. *Ann. N.Y. Acad. Sci.* **73,** 120 (1958).
57. K. LENNERT, Classification of malignant lymphomas (European concept). In *Progress in Lymphology* (Edited by A. RUTTIMAN), p. 103, Georg Thieme Verlag, Stuttgart (1967).
58. R. J. LUKES, A review of the American concept of malignant lymphoma. In *Progress in Lymphology.* (Edited by A. RUTTIMAN), p. 109, Georg Thieme Verlag, Stuttgart (1967).
59. B. H. TINDLE, J. W. PARKER, R. J. LUKES, "Reed–Sternberg cells" in infectious mononucleosis? *Amer. J. clin. Path.* **58,** 607 (1972).

Europ. J. Cancer Vol. 12, pp. 77–78. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *January, 1976*

Human Cancer

P. Gerber, F. K. Nkrumah, R. Pritchett and E. Kieff: Comparative studies of Epstein–Barr virus strains from Ghana and the United States.

L. Zech, U. Haglund, K. Nilsson and G. Klein: Characteristic chromosomal abnormalities in biopsies and lymphoid cell lines from patients with Burkitt and non-Burkitt lymphomas.

G. Klein, E. Svedmyr, M. Jondal and P. O. Persson: EBV-determined nuclear antigen (EBNA) positive cells in the peripheral blood of infectious mononucleosis patients.

G. Henle and W. Henle: Epstein–Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma.

A. de Schryver, A Rosén, P. Gunvén and G. Klein: Comparison between two antibody populations in the EBV system: Anti-MA versus neutralizing antibody activity.

G. Steele, Jr., H. O. Sjögren and I. Stadenberg: *In vitro* cell-mediated immune reactions of melanoma and colorectal carcinoma patients demonstrated by long-term ^{51}chromium assays.

R. Mažuran, H. Mujagič, B. Malenica and V. Silobrčić: *In vitro* detection of cellular immunity to melanoma antigens in man by the monocyte spreading inhibition test.

L. C. Andersson, C. Wasastjerna and C. G. Gahmberg: Different surface glycoprotein patterns on human T, B and leukemic lymphocytes.

J. M. Friedman and P. J. Fialkow: Viral 'tumorigenesis' in man: Cell markers in condylomata acuminata.

M. Lieber, B. Smith, Al Szakal, W. Nelson-Rees and G. Todaro: A continuous tumor cell line from a human lung carcinoma with properties of type II alveolar epithelial cells.

Experimental Cancer

M. Ran, G. Klein and I. P. Witz: Tumor bound immunoglobulins. Evidence for the *in vivo* coating of tumor cells by potentially cytotoxic anti-tumor antibodies.

G. Dorval, I. P. Witz, E. Klein and H. Wigzell: Tumor-bound immunoglobulins: I. Further analysis of the characteristics of binding of immunoglobulins to *in vivo* grown tumor cells.

P. S. Morahan and A. M. Kaplan: Macrophage activation and antitumor activity of biologic and synthetic agents.

M. Zöller, M. R. Price and R. W. Baldwin: Inhibition of cell-mediated cytotoxicity to chemically-induced rat tumours by soluble tumour and embryo cell extracts.

D. Fritze, D. H. Kern, J. A. Humme, C. R. Drogemuller and Y. H. Pilch: Detection of private and common tumor-associated antigens in murine sarcomas induced by different chemical carcinogens.

H. P. Charman, M. B. Gardner, R. M. McAllister, N. Kim and R. V. Gilden: Humoral immune responses of cats to mammalian type-C virus p30s.

M. Schülein, J. L. Daehnfeldt and P. Briand: Biochemical changes during regression and regrowth of hormone-dependent GR mouse mammary tumors.

British Journal of Cancer *January, 1976*

P. G. Smith, M. C. Pike, M. M. Till and R. M. Hardisty: Epidemiology of childhood leukaemia in Greater London: A search for evidence of transmission assuming a possibly long latent period.

G. C. Davey, G. A. Currie and P. Alexander: Spontaneous shedding and antibody-induced modulation of histocompatibility antigens on murine lymphomata: Correlation with metastatic capacity.

S. K. Chatterjee, U. Kim and K. Bielat: Plasma membrane-associated enzymes of mammary tumours as the biochemical indicators of metastasizing capacity, analyses of enriched plasma membrane preparations.

K. D. Chandradasa and J. Bradley: Studies on the stimulation and suppression of deoxyribonucleic acid (DNA) synthesis in lymph nodes of mice bearing progressively growing tumours.

S. Szmaniec and K. James: Studies on the Fe receptor bearing cells in a transplanted methylcholanthrene induced mouse fibrosarcoma.

G. A. Currie and S. A. Eccles: Serum lysozyme as a marker of host resistance I. Production by Macrophages resident in rat sarcomata.

H. A. S. Van den Brenk, M. G. Stone, H. Kelly and C. S. Sharpington: Lowering of innate resistance of the lungs to the growth of blood-borne cancer cells in states of topical and systemic stress.

K. Kudd, Y. Hanaoka and H. Hayashi: Characterisation of tumour cell aggregation promoting factor from rat ascites hepatoma cells: Separation of two factors with different antigenic property.

H. J. Brenner and A. Yerushalmi: Combined local hyperthermia and X-irradiation in the treatment of metastatic tumours.

A. C. Horsfall and B. Ketterer: The fractionation of isolated liver cells from normal and carcinogen treated rats.

P. Dyson and A. G. Heppleston: Cell kinetics of urethane-induced murine pulmonary adenomata. II. The growth fraction and cell loss factor.

Short Communications

C. R. Franks, D. Bishop and D. Reeson: The growth of tumour xenografts in thymectomised high dose irradiated mice reconstituted with syngeneic bone marrow cells incubated with anti-thymocyte serum.

W. R. Miller and A. P. M. Forrest: Oestadiol synthesis from C19 steroids by human breast cancers.

T. K. Basu, D. Donaldson, M. Jenner and A. Sakula: Plasma vitamin A in patients with bronchial carcinoma.

Book Reviews

J. Koweldon: Cancer epidemiology and prevention.

R. Saffhill: Ozone chemistry and technology: A review of the literature 1961/1974.

Europ. J. Cancer Vol. 12, pp. 79–85. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Synchronization in Tumour Chemotherapy

L. M. VAN PUTTEN,* H. J. KEIZER* and J. H. MULDER†
**Radiobiological Institute TNO, Rijswijk and*
†Rotterdams Radiotherapeutisch Instituut, Rotterdam, The Netherlands

IN MOST cell populations—in tissue culture and *in vivo*, both in many normal tissues and in tumours—cell divisions are distributed at random in time. Various types of treatment may disturb this random distribution to some extent for a limited period of time. There is a larger fraction of cells passing simultaneously through the phases of the cell cycle, until the variation between cells in the speed of progress through the cycle once more evens out the inequality in distribution. Synchronization is the name given to the phenomenon where larger than average fractions pass simultaneously through the various recognizable phases of the cycle. It can be identified by a temporary peak in the mitotic index or in the thymidine labelling index.

Synchronized cells have been used in tissue culture on a large scale for experimental studies of the biochemical processes associated with the various phases of the cell division cycle and for this purpose highly effective methods of *in vitro* synchronization have been developed such as treatment with cold shock [1], excess thymidine [2, 3], detachment of mitotic cells [4, 5] or isoleucine deprivation [6, 7]. Study of synchronized cells has shown that cells vary over the division cycle in their sensitivity to radiation and to a large number of cytostatic drugs. One group of drugs kills cells especially if applied during DNA synthesis, other agents kill cells preferentially after exposure in G_2 or during mitosis (for a systematic review see Madoc-Jones and Mauro [8]).

Conversely it has become evident that many types of drug treatment or irradiation may also lead to partial synchronization and cytostatic drugs have also been used in tissue culture to obtain synchronized cell populations. This synchronization is reached *in vivo* by at least two mechanisms (see Fig. 1); on the one hand selective killing of cells in a sensitive phase of the cell cycle leads to a predominance of cells in the insensitive phase; on the other hand many agents will block or slow down the progress of cells through certain phases of the cell cycle and this may well be the more effective method of synchronization. In the third place synchronization may be an indirect consequence

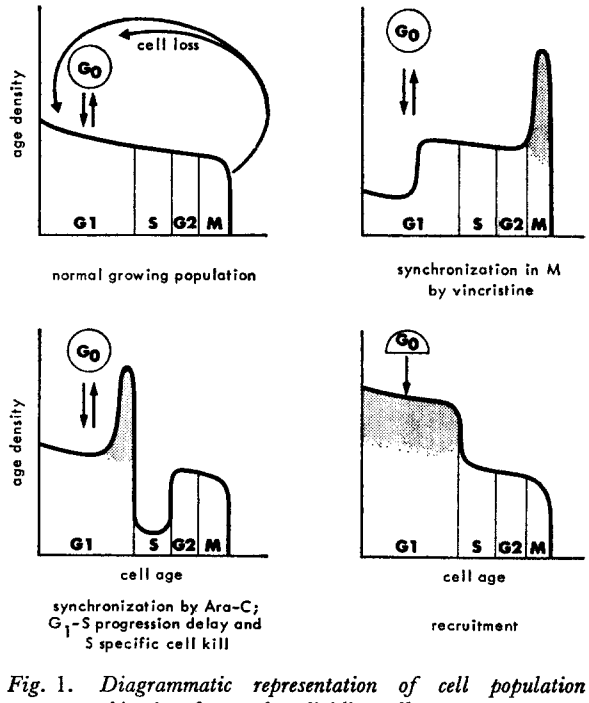


Fig. 1. Diagrammatic representation of cell population kinetics of at random dividing cell system. Synchronization (by vincristine and Ara-C) and recruitment are also represented. The areas of the age density diagrams are intended to roughly indicate the relative sizes of the populations in the different cell cycle phases.

J.H.M. gratefully acknowledges financial support from Promeso.

of cell killing when the cell depletion leads to a simultaneous recruitment of resting cells into the mitotic cycle.

Synchronization of cells *in vivo* has been attempted by phase specific drugs mainly by accumulation of cells in early S-phase or in mitosis. Synchronization in early S-phase has been achieved with hydroxyurea and cytosine arabinoside; synchronization in mitosis especially with vincristine. After Bleomycin progressive delay in G_2 phase has been noted. When drug levels are reduced by excretion or metabolism of the drug, the block is released after which the cells pass more or less synchronously through the mitotic cycle. Many cytostatic drugs and also radiation may cause partial synchronization *in vivo* in the sense of a disturbance of the random distribution over the cell cycle.

The consequent variation in sensitivity of the cells may be important for the effectiveness of combination chemotherapy or fractionated radiotherapy and attempts have been made to synchronize cells to exploit the temporarily increased drug sensitivity which may occur in synchronized populations. Model studies in tissue culture and in transplantable tumours have shown the feasibility of exploiting this principle.

THEORETICAL ASPECTS

A number of factors are of theoretical importance in determining the efficacy of cell synchronization procedures *in vivo*. They have been listed in Table 1 but will not be discussed in detail. It should be noted that the synchronizing agent must meet certain criteria: the cell cycle progression delay must be phase specific, in order to get an accumulation in one phase. The block must be released over a short period, otherwise the population will become asynchronous during the release; so the half life of the synchronizing agent must be short.

A major problem is associated with the differences between the model tumour systems and the clinical tumours at which the treatment is aimed: in most models all cells are in cycle and proliferation is rather uniform. This is in marked contrast to the tumours observed in patients. Usually a small fraction of cells is in cycle and the kinetic parameters of the dividing cells may show a wide variation. Finally it should be remembered that therapeutic gain is revealed only if the treatment enhances tumour cell killing without simultaneously increasing the killing of critical normal tissue cells. This is usually aimed at by a procedure which permits differential synchronization of normal and malignant cells. Such differences may be found in their cycle time. As shown in an example from Klein and Lennartz [10] this can be exploited in the following way. Cells are blocked in mitosis by a non-killing dose of vincristine. After release from the block the accumulated cells start their progress through the cycle; normal bone marrow cells, having a shorter cell cycle time first reach S-phase and most of these cells have completed DNA-synthesis when the tumour cells are entering this phase. At that time an S-phase specific drug (effector agent) will cause increased tumour cell killing but decreased normal bone marrow cell killing. This example will make it clear why it is necessary for the tumour cells

Table 1. *Conditions for optimal synchronization* in vivo

(a) *The synchronizing agent*
- should cause a phase specific cell cycle progression delay.
- should permit rapid simultaneous release of cells from the block.
- should be tolerated well so as to permit accumulation of a larger fraction of cycling cells.

(b) *The tumour cell population*
- should contain a large fraction of cells in cycle.
- should show a minimal variability in cell cycle parameters.
- should have cell cycle parameters different from the critical normal cell population.

(c) *Practical aspects to be fulfilled in order to obtain better results*
- the tumour cells should be available for monitoring synchronization since preferably multiple synchronization sequences should be tolerated.
- normal tissue cell kill by the synchronizing agent should be limited. In this respect VCR [11] may have some preference over HU [9].
- if the tumour contains many resting cells, synchronization by recruitment will be achieved only by agents which kill cells rapidly (e.g. vincristine is more effective than radiation since the latter permits longer survival of doomed cells and consequently a slower recruitment).

and normal tissue cells to have different cycle times and why a large variation in tumour cell cycles is decreasing the effectiveness of synchronization treatment: such large variations will cause a significant fraction of the tumour cells to be in a non-sensitive phase at the time of treatment with the effector agent.

RODENT MODEL STUDIES *IN VIVO*

In Table 2 a list is presented of the procedures proposed and tested in various tumour systems. Some of the systems listed have been tested only for their capacity to cause synchronization as measured by mitotic index peaks or thymidine labelling index peaks. Others have been tested by applying a second drug after synchronization and noting the variation in response depending on the time interval between the administration of the first (synchronizing) agent and the second (effector) drug. This makes it very hard to make meaningful comparisons between the different drug schedules. Note that synchronization has not only been described in tumour cells but also in normal tissue cells, both in the bone marrow and the intestinal tract.

A direct comparison of the degree of synchronization in normal and malignant tissues has only rarely been performed in the same experimental model. Some authors [23, 24] have compared the synchronization reached after hydroxyurea in different systems and from such a comparison (Fig. 2) it may be noted that the peak of thymidine labelling in the rat mammary carcinoma of Rajewsky *et al.* [14, 15] (although shown to differ from rat spleen cells) does overlap with the peak of labelling period of mouse bone marrow stem cells as determined by Vassort *et al.* [16]. The peak of thymidine incorporation in the mouse lymphoma of Madoc-Jones and Mauro [9] does not coincide with these peaks but it does overlap with the peak of thymidine incorporation in

Table 2. Drug treatment used in experimental models in vivo *in which synchronization may have played a role*

Author(s)	Cell type	Synchronizing agent(s)	Evidence for synchronization was obtained from: LI*	MI*	increased cell kill after second drug:	Tumour cells were compared to normal cell population?
Madoc-Jones and Mauro (1970) [9]	mouse lymphoma	HU			HU, VLB, VCR	
Klein *et al.* (1970) [10–13]	mouse Ehrlich ascites	VCR	+		CY	bone marrow
Gillette *et al.* (1970) [25]	mouse intestinal crypt cells	HU	+			no tumour
Rajewski *et al.* (1971) [14, 15]	rat mammary carcinoma	HU	+			spleen
Vassort *et al.* (1971) [16]	mouse bone marrow	HU			HU	no tumour
Gibson and Bertalanffy (1972) [17]	mouse B16-melanoma	Ara-C	+	+		
Verbin *et al.* (1972) [18]	rat intestinal crypt cells	Ara-C + CCM		+		no tumour
Neil and Homan (1973) [19]	mouse L1210	Ara-C, HU, 5-HP	+		Ara-C, HU 5-HP	
Edelstein *et al.* (1973) [20]	mouse L1210	Ara-C			daunomycin	bone marrow
Razek *et al.* (1974) [21]	mouse L1210 and AKR lymphoma	VCR			CY	bone marrow
Pouillart *et al.* (1974) [22]	mouse L1210	VCR, VM26	+		MTX, CY, 5-FU, MTC, nitrosoureas	

VCR: vincristine; HU: hydroxyurea; Ara-C: arabinosyl cytosine; CCM: colcemid; VM26: 4-dimethyl-epipodophyllotoxin thenylidene glucoside; CY: cyclophosphamide; 5-HP: 5-hydro-2-formylpyridine thiosemicarbazon; MTC: mitomycin; MTX: methotrexate; 5-FU: 5-Fluorouracil; nitrosoureas: BCNU: bis-chloroethyl nitrosourea; CCNU: chloroethyl cyclohexyl nitrosourea; *LI: thymidine labelling index; MI: mitotic index.

intestinal tract epithelial cells [25]. It should be noted however that the selection of an effector drug which has low toxicity for the intestinal epithelium, could partly overcome this problem. Klein and his coworkers [10, 11] could demonstrate in model studies a clear separation of the mitotic peaks in ascites tumour cells and bone marrow after synchronization with vincristine, without much cell killing.

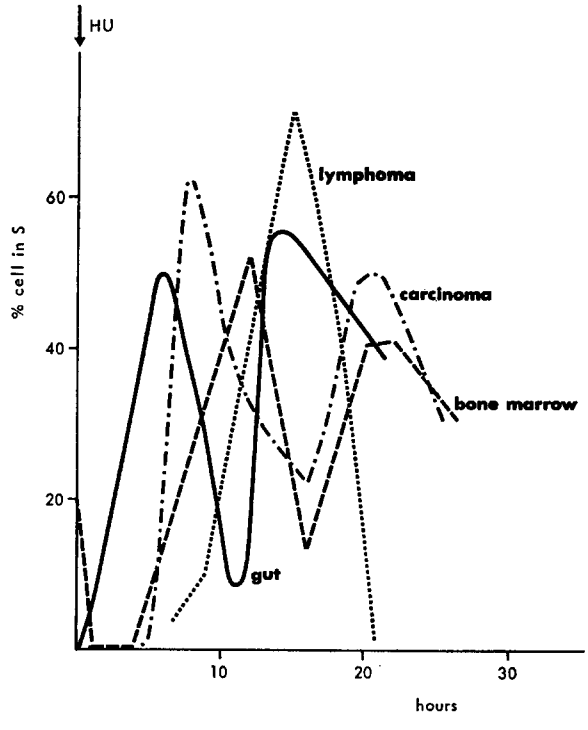


Fig. 2. Time course changes of number of cells in S-phase after Hydroxyurea administration (modified after Tubiana [23]). *Data for bone marrow stem cells from Vassort* et al. [16], *for intestinal cells from Gilette* et al. [25], *for mammary carcinoma from Rajewski* et al. [14] *and for lymphoma cells from Madoc-Jones et al.* [9].

These examples show increased tumour cell killing after synchronization. On the other hand a therapeutic gain after sequential drug treatment is not necessarily based on effective cell synchronization. The group at the Mallinckrodt Institute in St. Louis demonstrated a clear therapeutic gain by two types of sequential drug administration. One of the sequences is vincristine (in doses 40–50 times higher than used by Klein *et al.* [10]) followed by Cyclophosphamide [21]. A therapeutic gain was observed when the time interval between drugs was 5 to 15 hr. This ten hour period of constant sensitization of cells having a cycle time also of the order of ten hours cannot be explained by synchronization. This is also true for the combination of Cytosine Arabinoside followed by Daunomycin in which maximum therapeutic benefit over additive treatment (30 to 100 fold gain) was observed after drug intervals between 8 and 25 hr [20]. The authors suggest that their results may not be based on cell kinetic mechanisms alone. The studies of Pouillart *et al.* [22] point in a similar direction. They specifically studied the correlation between synchronization and potentiation seen after two-drug treatment of L1210 leukaemia in mice. A most striking finding is that potentiation in the sequence vincristine-methotrexate is near maximal at a time interval of 8 hours between drugs, showing a paradoxically increased effect of MTX at a time interval after vincristine at which thymidine labelling is maximally depressed. The sequences vincristine-cyclophosphamide and vincristine-MTX were found to have an effectiveness that was markedly increased over simple additivity when time intervals between drugs ranged from 8 to 48 hr. Since the cycle time of L1210 leukaemia in the phase of growth used for these studies was 11·5 hr, it is impossible to explain these results by synchronization. The authors suggest that a pharmacological interaction between drugs is at the basis of this effect rather than cell kinetic synchronization effects. Such mechanisms have indeed been identified [26–28] mainly through enhanced penetration of one drug after pretreatment with another agent. Does this mean that synchronization is not involved and that it cannot be used practically? We do not know but it seems that we must look very critically at the various clinical studies in order to make sure that the good results claimed are indeed caused by modification of cell kinetics.

CLINICAL STUDIES

Lampkin, Nagao and Mauer [29] showed in man a marked increase in labelling index of leukaemic cells after cytosine arabinoside and proposed to follow this by treatment with vincristine "in an effort to increase the number of susceptible cells by synchronizing the mitotic cycle".

In further studies [30] they discussed the mechanisms of synchronization and observed that more cells were killed after sequential treatment than with a single drug. The benefit was only partly ascribed to synchronization and the importance of recruitment of resting cells into a sensitive phase of the mitotic cycle was stressed. Pouillart *et al.*, saw some good results in leukaemias [31] and solid tumours [32] of the following sequence: days 1 and 2, vincristine or VM26; days 3 through 6, cyclophospha-

mide, adriamycin or cytosine arabinoside. The data suggest that recruitment did occur under these treatment schedules.

As mentioned earlier Klein and coworkers [10, 11] have reported an analysis of cell kinetics and optimal treatment times for the combination of vincristine (in very low doses) with cyclophosphamide. After a detailed analysis of conditions prevailing in two types of mouse ascites tumour they have also reported on the cell kinetic parameters of a number of tumours in patients [11, 33]. In a cooperative trial a total number of 87 patients mostly treated previously for Hodgkin's disease stage IIIB and IV and with advanced non-Hodgkin's lymphoma were treated with two spaced low doses of vincristine followed by a high dose of cyclophosphamide at an interval of 30–48 hr after the first vincristine injection.

After three to four courses the complete remission rates were 74, 39 and 49% respectively. Severe side effects were not observed. Also in this study there are indications that synchronization may not be the only explanation of the clinical results. In the experimental studies the ratio of increased cell killing was between two and three in terms of decreased cell survival and it is unlikely that this could be much better in a solid tumour in which at most 25% of the cells are in cycle. Since a complete remission requires a reduction of cell survival by a factor of at least 100, it is likely that part of the effectiveness of this schedule is due to killing of non-cycling cells by cyclophosphamide. This is apparently facilitated by pretreatment with vincristine through a pharmaco-kinetic rather than through a cell kinetic mechanism. Since partial synchronization has undoubtedly occurred in these studies it is very hard to conclude with certainty what part of the effect is due to synchronization and what part due to other effects.

Thus it seems that the efforts aimed at cell kinetic manipulations have had a good effect in introducing treatment schedules which promise an improvement over traditional schedules, although not necessarily by the mechanism which was the conceptional starting point for its development. It should be noted that not all schedules based on this concept have met with equal success. Clavel *et al.* [34] reported disappointing results and unexpected toxicity when vincristine was followed by three days of methotrexate treatment for squamous cell carcinoma. Obviously further studies are required to elucidate the mechanisms of the differential effects of combination treatments of this type on normal and malignant cell populations.

These results do not imply that synchronization attempts have no place in chemotherapy; they indicate that the theoretically predictable gain from such attempts may be small in comparison to other modifying factors encountered in sequential chemotherapy. As was pointed out elsewhere [35], the small gain to be obtained by synchronization in a tumour having a small growth factor can only be made useful if the treatment schedule is repeated.

This was attempted by Wannenmacher *et al.* [36, 37] in two systems using combination of chemotherapy and irradiation. In these studies the degree of synchronization was systematically analysed. In the first study 5-FU infusion for 12 hours was followed by irradiation after an 8 hour interval. This sequence was shown to synchronize cells in G_2, but its effect was ambiguous [36]. In experimental models tested by others, this sequence had not been found effective in contrast to the reverse order of administration [38]. A second series of clinical studies involved repeated use of Bleomycin followed by radiation. In this case synchronization in G_2 was demonstrated. Treatment effectiveness is hard to evaluate but some striking benefits were noted as well as clearly increased normal tissue responses [37]. These studies deserve mention especially as they indicate the possibility of a systematic monitoring of the degree of synchrony obtained in individual solid tumours. The technique of cytofluorometry seems to be useful, also for the kinetic analysis of cells in solid tumours. However, the use of Bleomycin at a short interval before radiotherapy does not seem rational if there is no reason to assume a differential effect on tumours and normal tissue cells. A similar effect may then be obtained by increasing the radiation dose at each session, unless it is shown that Bleomycin is far less effective in synchronizing the critical normal tissue cells.

In conclusion, it seems that so far there are only a few indications that it is possible to reach a satisfactory degree of differential synchronization between tumour cells and normal cells and that the majority of the beneficial effects observed are not due to cell synchronization. Although the concept has no doubt had a favourable effect upon the development of better chemotherapy sequences, its hazards should not be forgotten.

Note added in proof: At this time two important new sets of data which are relevant for the problem of synchronization have become available (Presented at the meeting on Cell Kinetics and Cancer Chemotherapy; Annapolis, November 4–6, 1975). In the first place Rao *et al.* (*Cancer Research* November, 1975) have collected evidence that the block in G2 observed after a number of cytostatic drugs may largely be due to an accumulation of doomed cells and thus should not be used as an argument for synchronization of the surviving cells. In the second place Valleron presented data (to be published by Rockwell *et al.*) indicating that analysis of the best time for periodical exposure of tissue culture cells to hydroxyurea gave different results if derived from total cell labelling data—which includes non-clonogenic cells—and if derived from thymidine suicide data. Differences were not only shown to affect cell kinetic parameters but also the optimal treatment interval predicted by the latter method was shown to be markedly more effective in eliminating clonogenic cells. Evidently our cell kinetic techniques may be inadequate to follow the degree of synchronization of the surviving cells if the initial treatment caused significant cell killing.

REFERENCES

1. A. A. Newton and P. Wildy, Parasynchronous division of HeLa cells. *Exp. Cell Res.* **16,** 24 (1959).
2. N. Xeros, Deoxyriboside control and synchronization of mitosis. *Nature* (*Lond.*) **194,** 682 (1962).
3. D. Bootsma, L. Budke and O. Vos, Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res.* **33,** 301 (1964).
4. T. Terasima and L. J. Tolmach, Changes in X-ray sensitivity of HeLa cells during the division cycle. *Nature* (*Lond.*) **190,** 1210 (1961).
5. W. K. Sinclair and R. A. Morton, Variations in X-ray response during the division cycle of partially synchronized Chinese hamster cells in culture. *Nature* (*Lond.*) **199,** 1158 (1963).
6. M. D. Enger and R. A. Tobey, Effects of isoleucine deficiency on nucleic acid and protein metabolism in cultured Chinese hamster cells. Continued Ribonucleic acid and protein synthesis in the absence of deoxyribonucleic acid synthesis. *Biochemistry* **11,** 269 (1972).
7. R. A. Tobey, Production and characterization of mammalian cells reversibly arrested in G_1 by growth in isoleucinedeficient medium. In *Methods in Cell Biology*. Edited by D. M. Prescott. Vol. 6, chap. 3, pp. 67–112. Academic Press, Inc., New York (1973).
8. H. Madoc-Jones and F. Mauro, Site of action of cytotoxic agents in the cell life cycle. In *Handb. Exp. Pharm.* XXXVIII/1. Antineoplastic and immunosuppressive agents. pp. 205–219. Springer Verlag (1974).
9. H. Madoc-Jones and F. Mauro, Age responses to X-rays, Vinca alkeloids and hydroxyurea of murine lymphoma cells synchronized *in vivo*. *J. nat. Cancer Inst.* **45,** 1131 (1970).
10. H. O. Klein and K. J. Lennartz, Proliferationskinetische Grundlagen der Behandlung von akuten Leukosen. In *Leukämie*. Edited by R. Gross and J. van der Loo. pp. 479–500. Springer Verlag (1972).
11. H. O. Klein, Relevance of chemically induced synchronization of cell cycle to chemotherapy of human solid tumours. In *Proc. XI Int. Cancer Cong. Florence.* pp. 208–216 (1974).
12. H. O. Klein and K. J. Lennartz, Chemotherapy after synchronization of tumor cells. *Seminars in Hematology* **11,** 203 (1974).
13. H. O. Klein, K. J. Lennartz, W. Habicht, M. Eder and R. Gross, Synchronisation von Ehrlich-Ascites-Tumorzellen und ihre Bedeutung bei der Anwendung eines alkylierenden Cytostaticum. *Klin. Wschr.* **16,** 1001 (1970).
14. M. F. Rajewski, D. F. Hülser and E. Fabricius, Untersuchungen zur Synchronisation *in vivo*: Inhibition der DNA-synthese durch Hydroxyharnstoff in normaler und maligner Saügerzellsystemen. *Z. Krebsforsch.* **76,** 266 (1971).
15. M. F. Rajewski, Synchronization *in vivo*: kinetics of a malignant cell system following temporary inhibition of DNA synthesis with hydroxyurea. *Exp. Cell. Res.* **60,** 269 (1970).
16. F. Vassort, E. Frindel and M. Tubiana, Effects of hydroxyurea on the kinetics of colony forming units of bone marrow in the mouse. *Cell Tiss. Kinet.* **4,** 423–431 (1971).
17. M. H. Lindsay Gibson and Felix D. Bertalanffy, *In vivo* synchrony of solid B16 Melanoma by cytosine arabinoside, an inhibitor of DNA synthesis. *J. nat. Cancer Inst.* **49,** 1007 (1972).

18. Robert S. Verbin, Gloria Diluiso, Hilde Liang and Emmanuel Farber, Synchronization of cell division *in vivo* through the combined use of cytosine arabinoside and colcemid. *Cancer Res.* **32,** 1489 (1972).
19. G. L. Neil and E. R. Homan, The effect of dose interval on the survival of L1210 leukemic mice treated with DNA synthesis inhibitors. *Cancer Res.* **33,** 895 (1973).
20. Mark Edelstein, Teresa Vietti and Fred Valeriote, Schedule dependent synergism for the combination of 1-β-D-Arabinofuranosylcytosine and Daunorubicin. *Cancer Res.* **34,** 293 (1974).
21. Aly Razek, Teresa Vietti and Fred Valeriote, Optimum time sequence for the administration of vincristine and cyclophosphamide *in vivo*. *Cancer Res.* **34,** 1857 (1974).
22. P. Pouillart, T. Hoang Thy Huong, E. Brugerie and J. Lheritier, Sequential administration of two oncostatic drugs: study of modalities for pharmacodynamic potentiation. *Biomedicine* **21,** 471 (1974).
23. M. Tubiana, Kinetics of cell proliferation of solid tumours and chemotherapy. In: *Proc. 8th Int. Cong. of Chemotherapy, Athens*, vol. III, p. 208. Edited by George K. Daikos (1974).
24. E. Frindel, Pertubations of cellular kinetics by physical and chemical agents. In: The cell cycle in malignancy and immunity (1975). p. 156–181. *Proc. Thirteenth Ann. Hanford Biology Symp., Richland,* (1973). Technical information center. Office of Public Affairs. U.S. Energy Research and Development Administration.
25. Edward L. Gilette, Rodney Withers and Ian F. Tannock, The age sensitivity of epithelial cells of mouse small intestine. *Radiology* **96,** 639 (1970).
26. I. D. Goldman and M. J. Fyfe, Free intracellular Methotrexate (Mtx) is required for maximum inhibition of DNA synthesis—augmentation by vincristine. *Proc. Amer. Ass. Cancer Res.* **14,** 50 (abstract 190) (1973).
27. V. T. Oliveiro and D. S. Zaharko, Tissue distribution of folate antagonists. *Ann. N.Y. Acad. Sci.* **186,** 387 (1971).
28. F. M. Sirotirak and R. C. Donsbach, Differential cell permeability and the basis for selective activity of methotrexate during therapy of the L1210 leukemia. *Cancer Res.* **33,** 1290 (1973).
29. B. C. Lampkin, T. Nagao and A. M. Mauer, Synchronization and recruitment in acute leukemia. *J. clin. Invest.* **50,** 2204 (1971).
30. B. C. Lampkin, T. Nagao and A. M. Mauer, Synchronization of the mitotic cycle in acute leukemia. *Nature (Lond.)* **22,** 1274 (1969).
31. P. Pouillart, L. Schwarzenberg and G. Mathé, Une nouvelle approche pour les combinaisons chimiothérapiques dans le treatment des tumeurs solides: essai de synchronisation, recrutement. *Ann. Méd. int.* **124,** 437 (1973).
32. P. Pouillart, Roy Weiner, Jean-Louis Misset and L. Schwarzenberg, Combination chemotherapy based on model of recruitment by partial synchronization. *Proc. Amer. Assoc. Cancer Res.* **14,** 115 (1973).
33. H. O. Klein, D. Gerecke, H. Borberg, R. Gross *et al.*, Ergebnisse der Synchronisationstherapie mit Vincristin und Endoxan bei Lymphogranulomatose, Retikulumzell- und Lymphosarkom-eine Phase II—Studie. *Dtsch. med. Wschr.* **100,** 1719 (1975).
34. J. Clavel, J. Gary-Bobo, J. Guerrin, T. Klein, E. Pommatau, G. Brule and J. Chauvergne, Essais de chimiothérapie des cancers épidermoides en phase évolutive avancée par un protocole basé sur la notion de synchronisation cellulaire. *Sem. Hôp. Paris* **50,** 3107 (1974).
35. L. M. Van Putten, Problems in the treatment of slow-growing tumours. In *Recent Results in Cancer Research.* Edited by E. Grundmann and R. Gross. Vol. 52, pp. 225–233. Springer Verlag (1975).
36. M. Wannenmacher, E. Esser, J. Glupe and J. Schumann, Klinische und experimentelle Untersuchungen zur Strahlenbehandlung in operabelen Tumoren nach Teilsynchronisation. *Strahlentherapie* **147,** 1 (1974).
37. M. Wannenmacher, E. Esser and J. Schumann, Erste klinische Ergebnisse der Strahlenbehandlung nach Teilsynchronisation mit Bleomycin. *Strahlentherapie* **149,** 131 (1975).
38. T. Vietti, E. Eggerding and F. Valeriote, Combined effect of X-irradiation and 5-Fluorouracil on survival of transplanted leukemic cells. *J. nat. Cancer Inst.* **47,** 865 (1971).

Europ. J. Cancer Vol. 12, pp. 87–94. Pergamon Press 1976. Printed in Great Britain

Rheumatoid Factor as an Indicator of Serum Blocking Activity and Tumour Recurrences in Bladder Tumours*

S. PYRHÖNEN,† T. TIMONEN,‡ A. HEIKKINEN,§ K. PENTTINEN†,
O. ALFTAN,§ E. SAKSELA,‡ and O. WAGER¶

Abstract—*Serum rheumatoid factor (RF) was measured with four methods (latex baboon, Ripley, Waaler-Rose) and the blocking of lymphocyte-mediated cytotoxicity against bladder tumour cells was tested for* 33 *patients with bladder tumours.*

The results showed that latex titre correlated significantly with blocking activity: 10 *out of* 11 *sera with a latex titre* ≧ 16 *were capable of blocking, whereas in none of the* 12 *sera that lacked latex activity could this capacity be demonstrated. Of the other RF assays performed the results achieved with the Ripley test correlated well with blocking activity whereas the baboon and Wa-Ro did not.*

The presence of RF activity was also correlated with the clinical stage of the bladder tumours of 195 *patients. In seven out of nine* (78%) *clinically tumour-free patients whose latex titre was* ≧ 16 *a malignant tumour recurred within the following two years. In contrast, in only two out of the* 39 *patients* (5%) *whose latex activity was low (titre* 8) *or non-detectable did the malignancy recur during this same period.*

INTRODUCTION

THE PRESENCE of rheumatoid factor (RF) activity in the sera of cancer patients has been reported [1–4].

While screening the sera of patients with urinary bladder cancer for RF activity, we found an unexpected correlation of such activity with the capacity of these same sera to block lymphocyte-mediated cytotoxicity as measured by *in vitro* microcytotoxicity assay. The results of both tests were apparently related to the course of the disease.

Accepted 25 September 1975.

*This survey was supported in part by grants from the Finnish Cancer Foundation, the Finnish Cultural Foundation and the Sigrid Jusélius Foundation.

†Reprint request and correspondence to this author at Laboratory of Viral Immunopathology, Dept. of Virology, University of Helsinki, Haartmaninkatu 3, Helsinki 29, SF–00290, Finland.

‡Third Department of Pathology, University of Helsinki.

§Urological Department of the Second Surgical Clinic, Helsinki, University Central Hospital.

¶Municipal Bacteriological Laboratory, Aurora Hospital, Helsinki, Finland.

MATERIAL AND METHODS

Patients

One hundred and ninety-five patients in the Urological Department of the Second Surgical Clinic, Helsinki University Central Hospital, who had a histologically-verified transitional-cell papilloma or carcinoma of the bladder were studied. The first clinical evaluation was based on cystoscopic examination, histologic examination of biopsy specimens, palpation under anaesthesia and cytological analysis of urine and bladder washings. Follow-up consisted of cystoscopic examination and cytological analyses every third month and, after two recurrence-free years twice yearly. The tumours were grouped according to the clinical stage and the grade of malignancy.

The clinical grouping represents the TNM

classification proposed by the UICC Committee 1963 [5].

T_x —histologically benign papilloma

T_1 —all tumours not infiltrating deeper than the subepithetial connective tissue

T_2 —tumour with infiltration of superficial muscle

T_3 —tumour with infiltration of deep muscle

T_4 —tumour fixed or invading adjoining organs

The grade of malignancy was estimated according to the classification, based on cellular deviations, of Bergkvist *et al.* [6]. The borderline between benign and malignant tumours was between grades 1 and 2.

Sera

Sera were obtained from all 195 patients studied, serial samples were taken from some patients when possible. All sera were stored at −20°C for RF tests and blocking experiments.

Test for Rheumatoid Factor(s) (RF)

For the sake of convenience, the designation of rheumatoid factor (RF) in the present paper is applied collectively for all anti-globulin (anti-IgG) factors irrespective of their species specificity and clinical association.

Human ORh positive cells were coated with rabbit anti-human erythrocyte serum ("Waaler-Rose"), with baboon anti-human fibroblast serum ("baboon") or with human anti-D ("Ripley"). The latex titration was carried out with Hyland's RA test® latex-globulin reagent.

For all titrations U-microplates (Daniel Co.) and microtiter^R equipments were used as described earlier [7]. In the Waaler-Rose test, PBS containing 0·2% pooled normal human serum (NHS) was used as diluent. In the Ripley and baboon tests 0·2% bovine serum albumin (BSA) was used instead on NHS. In the latex test PBS alone was used as diluent. The incubation for the three haemagglutination tests was over night at 4–8°C, for the latex test 2–3 hr at room temperature.

DETERMINATION OF BLOCKING ACTIVITY

Target cells

For the microcytotoxicity assays, the established TCC cell line RT4 [8] obtained by the courtesy of professor Perlmann (Stockholm, Sweden) was used. The control target cells were human adult skin fibroblasts in the fifth to the fifteenth passage.

Effector lymphocytes

Heparinized blood was drawn (into 20 ml plastic syringes, Jintan, Tokyo, Japan) from the patients and control subjects (healthy students). Lymphocytes were separated by gradient centrifugation on Ficoll–Isopaque [9], washed three times in PBS, suspended in Hank's solution supplemented with 5% foetal bovine serum (Flow Laboratories, Irvine, Scotland), run through nylon wool columns [10] and suspended in Ham's F10 growth medium (Flow) supplemented with 10% foetal bovine serum (Flow), 10 μg of streptomycin and 100 i.u. of penicillin per ml.

Cytotoxicity tests

Microcytotoxicity was determined as described by Takasugi and Klein [11]. Briefly, 200 target cells suspended in the growth medium described above were plated in each well of plastic microcytotoxicity plates (Falcon 3034, Falcon Plastics, Los Angeles, Calif., USA). After incubation overnight at 37°C in humidified air +5% CO_2 50–100 cells had adhered to the bottom of the well. The addition of purified lymphocytes to wells in aliquots of 20 μl (5×10^4 per well) resulted in an effector target cell ratio of about 500/1. After incubation for 48 hr the plates were washed in two changes of PBS to remove non-adherent cells, fixed in methanol, air dried and stained with Giesma. Six to eight wells of each effector-target cell combination were prepared. Control effector lymphocytes were added to the same plates as effector lymphocytes. The remaining cells were counted and the mean ± standard error was determined. Cytotoxicity was calculated from the formula:

$$Cx\% = \frac{C-P}{C} \times 100$$

where C = attached cells remaining in the wells with control effector lymphocytes P = attached cells remaining in the wells with patient effector lymphocytes.

Blocking experiments

Sera previously collected from patients with bladder tumours and stored at −20°C were used in the blocking experiments. All sera were inactivated at 56°C for 30 min before use. Effector lymphocytes, previously shown to be cytotoxic against RT4 cells, but not against control target cells, were suspended in serum-free growth medium (Ham's F10 plus antibiotics). Five to seven sera were tested in each

Table 1. *The blocking of cell-mediated anti-RT4 cytotoxicity with sera from tumour patients*

		RT4			Control fibroblasts	
Effector cell donor	Serum donor*	Cells/well ± S.E.	Cx %†	Bl %‡	Cells/well ± S.E.	Cx %†
Patient LU	None	40 ± 2	43‡		54 ± 4	5
	2	74 ± 4		100‡	44 ± 5	
	6	72 ± 5		94†	58 ± 4	
	9	62 ± 8		65*	62 ± 5	
	13	41 ± 8		3	41 ± 8	
	27	42 ± 6		6	55 ± 4	
Control	None	74 ± 5			57 ± 4	
None (Medium control)		106 ± 7			62 ± 4	

*The numbers refer to the patients in Table 2.
†Cx % = Cytotoxicity in percent exerted by bladder tumour patient effector cells as compared to the effector cells of a normal healthy control. The significance level: $*P < 0{\cdot}05$, $\dagger P < 0{\cdot}005$, $\ddagger P < 0{\cdot}0005$.
‡Bl % = The percentual blocking of the cytotoxicity by the presence of bladder tumour patient sera in the nutrient medium incubation.

experiment and altogether 8 different bladder carcinoma patients' lymphocytes were used. All calculations were made from results obtained on a single plate as exemplified in Table 1. One hundred microlitres of the serum to be tested for blocking activity were added to 500 µl of effector cell suspension, yielding a serum of 1/6 dilution. This suspension was added in aliquots of 20 µl to the wells in the microcytotoxicity plates. (Hence the sera were present in the microwells for the entire 48-hr incubation period). Cytotoxicity in the absence of the patient serum was measured with effector and control-effector lymphocytes suspended in growth medium supplemented with 10% foetal bovine serum as described above.

Blocking activity was calculated from the formula:

$$Bl\% = \frac{(C-P)-(C-P_b)}{C-P} = 100$$

where C and P are as described above and P_b = attached cells remaining in the wells with effector lymphocytes and patient serum.

To exclude the possibility that blocking sera were cytotoxic against effector lymphocytes the direct effect of various sera on lymphocytes was also studied. Trypan-blue staining of lymphocytes incubated with the sera under conditions maintained in the blocking experiments showed that blocking sera did not destroy effector lymphocytes.

Statistical analysis

Differences between course of curves were determined by testing simultaneous χ^2-tests.

Table 2. *Blocking activities and latex titres of sera from patients with bladder tumours*

Patients	Blocking %	Latex titre lab. 1	Latex titre lab. 2
1.	100‡	16	32
2.	100‡	64	32
3.	174†	32	32
4.	200†	16	16
5.	74†	32	32
6.	94†	16	16
7.	53†	32	64
8.	72†	8	16
9.	65*	16	16
10.	19*	16	8
11.	19*	8	8
12.	209*	8	< 8
13.	3	64	≧ 64
14.	−19	8	8
15.	−31	8	< 8
16.	−23	8	< 8
17.	−131	8	< 8
18.	−96	8	< 8
19.	0	8	< 8
20.	10	8	< 8
21.	29	8	< 8
22.	−8	< 8	< 8
23.	−24	< 8	< 8
24.	−47	< 8	< 8
25.	−40	< 8	< 8
26.	19	< 8	< 8
27.	6	< 8	< 8
28.	9	< 8	< 8
29.	−4	< 8	< 8
30.	−19	< 8	< 8
31.	−42	< 8	< 8
32.	−5	< 8	< 8
33.	−38	< 8	< 8

Level at which blocking activity was significant $*P < 0{\cdot}05$ $\dagger P < 0{\cdot}005$ $\ddagger P < 0{\cdot}0005$.

Table 3. Correlation of results of latex, Ripley, baboon and Wa-Ro tests with demonstration of the blocking of lymphocyte-mediated cytotoxicity in sera of 33 patients with urinary bladder tumours

Technique	Titre	No. of sera	No. of blocking sera	% of blocking sera
Latex	≧ 16	11	10	91
	8	10	2	20
	< 8	12	0	0
Ripley	≧ 32	19	11	58
	< 32	14	1	7
Baboon	≧ 32	10	5	50
	< 32	23	7	30
Wa-Ro	≧ 32	4	1	25
	< 32	29	11	38

RESULTS

Rheumatoid factor as an indicator of blocking activity

In the sera of 33 patients with bladder tumours (Tables 2 and 3) RF titres correlated significantly with the capacity to block lymphocyte-mediated cytotoxicity. All RF titres (Table 2) were measured by the latex test in two laboratories, first as a control of the results of one laboratory, and second, to demonstrate the variations in the limits for positive and negative results. Fewer of the sera were latex positive in laboratory 2 than in laboratory 1. In laboratory 2 all but two of 21 non-blocking sera were latex negative, whereas in laboratory 1 an additional seven sera gave a weakly positive reaction (titre 8).

As shown in Table 3, 10 out of the 11 sera that had a latex titre of ≧ 16 in either laboratory also displayed blocking activity when tested in microcytotoxicity assays, whereas in none of the 12 latex negative sera could such activity be demonstrated. When tested with the chi-squared test the difference in blocking activity between these two groups of sera was significant at a level of $P < 0{\cdot}001$. Two of the 10 sera with a latex titre 8 showed blocking activity; the remaining eight did not. However these two blocking sera that had low latex titres, had high titres in the Ripley test (≧ 1000), whereas only one (patients No. 13) of the 21 non-blocking sera had such a high Ripley titre.

As shown in Table 3, the results of the two tests that measured RF against human IgG, i.e., the Ripley and especially the latex test, were good indicators of blocking; the results of the baboon and Wa-Ro test did not correlate as well with blocking activity.

Rheumatoid factor correlated with clinical stages and as an indicator of recurrences

Changes in the level of RF activity during the observation period were few. Of those seven patients who had latex-positive sera in their first samples and from whom at least two additional samples could be collected during the next two years, the later samples of one had higher titres, of two had lower titres or no latex activity and of four had unchanged titres. Of those whose first serum sample was latex negative, one had weak latex activity in his later samples and 28 remained latex negative during the 2-years' observation period.

The correlation of latex activity (measured in lab. 2) with the clinical stage of tumours in all 195 patients is presented in Tables 4 and 5. Of those patients who were clinically tumour-free at the time their serum was collected but in whom recurrence occurred within a year (Table 4), 57% were latex positive. In contrast, latex positivity was present in significantly fewer ($P < 0{\cdot}01$), i.e., 15%, of those patients who were tumour-free and remained free of a recurrence for at least a year. When latex activity was studied in patients with the presence of bladder tumours (Table 5) latex titres had some correlation with the stage of tumours. About one-third of the patients with malignant bladder tumours in stages 1, 2 or 3

Table 4. Correlation of latex titres with recurrence during first follow-up year in post-operatively tumour-free patients

One year follow-up	No. of patients	No. of latex positive patients titre 8	titre ≧ 16	% of latex positive patients
Without recurrence	41	3	3	15
Recurrence	14	1	7	57
Total	55			

Table 5. *Correlation of latex titres with clinical stage of tumour*

Clinical stage	No. of patients	No. of latex-positive patients titre 8	titre 16	% of latex-positive patients
Without metastases				
T_x	43	2	4	14
T_1Gr 2–3*	41	11	3	35
T_2Gr 2–4	30	3	5	27
T_3Gr 2–4	26	3	5	31
T_4Gr 2–4 or Tumours with metastases	24	—	4	17
Total	164			

*No Gr 4 tumours were found in this group.
Infiltration stage T_x–T_4 was based on UICC classification 1963
Grade of malignancy was estimated according Bergkvist *et al.* 1965.

had latex activity in their sera, whereas about one-sixth or one-seventh of the patients with benign papillomas (T_x) and with the most widespread tumours (stage T_4 or other stages with metastases) had latex-positive sera.

The results showed that latex positivity of serum correlated well with tumour growth *in vivo*. In Fig. 1 the recurrence frequency among clinically tumour-free patients during the observation period of two years is correlated with latex titres. During this 2-year period the risk of recurrence was significantly ($P < 0{\cdot}001$) higher in patients who had elevated latex positivity (titre ≧ 16) than in patients with low (titre 8) or non-detectable latex activity.

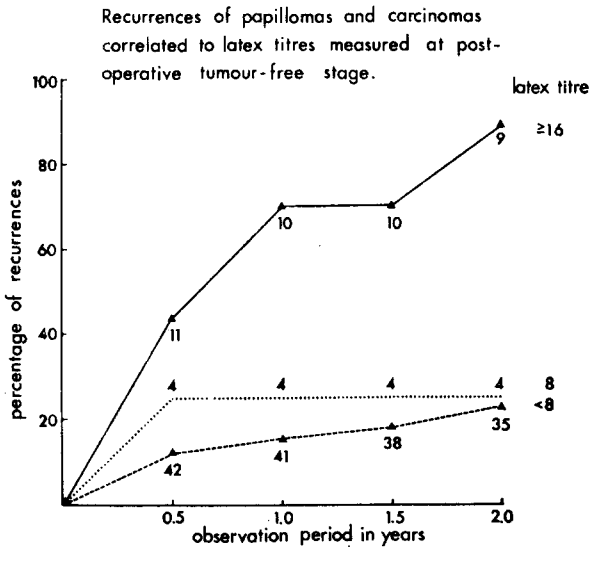


Fig. 1. *Figures on the curves represent the number of patients followed up. The difference of the proportion of patients with recurrences between the groups of patients with latex titres* ≧ 16 *and* < 8 *is significant from a* 6 *months' observation period onwards and between the groups with latex titres* ≧ 16 *and* 8 *at* 2 *years' observation.*

In 89% (8/9) of these patients, a recurrent tumour appeared within the observation period of two years. The only patient who remained tumour-free had cytologically malignant cells (Papa R_{IV-V}) in repeated urine specimens, when his serum latex titres was 16. In later serial serum samples RF activity disappeared and cytological findings returned to normal. The other, till now up to 18th month tumour-free patient who had a high latex titre has had a prolonged haemorrhagic cystitis. His serum induced, in the presence of lymphocytes, a cytotoxic reaction against the normal skin fibroblasts used as control target cells in cytotoxicity tests. In patients with a low or non-measurable latex titre (≦ 8) the percentage of recurrence was 23% (9/39).

In the latter cases 2 out of 9 recurrent tumours were histologically malignant, while 7 were benign papillomas; in contrast, in cases with latex titre ≧ 16 7 out of 8 recurrent tumours were malignant and two were benign papillomas. In summary as presented in Fig. 2, the frequency of recurrent malignant bladder tumours was 78% (7/9) in patients with high latex activity and 5% (2/39) in patients with low or non-detectable latex activity.

DISCUSSION

In the present study lymphocytes from bladder carcinoma patients that killed only RT4 cells but not control fibroblasts were tested in serum blocking experiments. Human fibroblasts are, in our experience [12], sensitive targets to non-specific cellular cytotoxic reactions *in vitro* [13] and therefore we interpret the cytotoxicity against RT4 cells only to represent a tumour specific reaction in confirmation of O'Toole *et al.*'s [17] data.

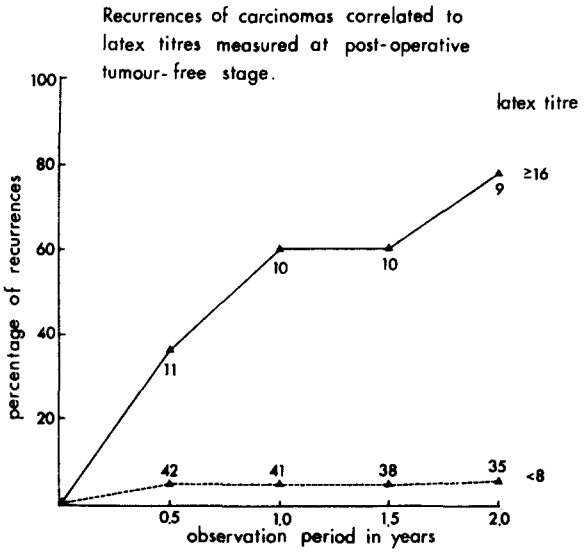


Fig. 2. Figures on the curves represent the number of patients followed up. None of the 4 patients with latex titre 8 had recurrent carcinoma. The difference of the proportion of patients with recurrent carcinomas between the groups of patients with latex titres ≧ 16 and < 8 is significant from a half year's observation period onwards.

An association was revealed between RF and blocking activity in the serum of patients with bladder tumours. The blocking activity detected with microcytotoxicity tests was tumour specific. Lymphocytes cytotoxic to ovarian carcinoma cells were not blocked by even the most potent blocking sera used in these experiments [14].

Sjögren *et al.* [15] and Baldwin *et al.* [16] have shown that the blocking activity present in the sera of tumour patients is in most instances due to circulating complexes of tumour antigen and IgG antibody. Rheumatoid factors are considered a response to a continuous production of IgG complexes, as observed, for example, in patients with rheumatoid arthritis. It is possible that the observed association between RF and blocking activity in the present series of patients might be related to a RF response against tumour antigen antibody complexes.

However, RF might be not only a consequence of but possibly also an active participant in the blocking phenomenon. Previous studies reveal that antibody-mediated lymphocytotoxic reaction can be effective in bladder tumours [17, 18]. Hallberg *et al.* [19], who used a red-cell model system, reported blocking of antibody-mediated lymphocytotoxicity by RF-positive rheumatoid sera. Purified RF is known to interfere with the reactivity of the Fc-sites of the IgG molecules in other systems. It inhibits, for example, the ability of IgG complexes to aggregate human platelets [7] and to fix complement [20], and it amplifies antibody-mediated virus neutralization [21, 22]. Of special interest in this context is the finding that the addition of RF to non-blocking sera of patients with ovarian carcinoma can induce them to block [14]. Previous studies [23, 24] have shown that the RF may alter the physicochemical state (precipitability, solubility, size, stability, etc.) of the IgG complexes. Available data do not permit conclusions to be drawn as to the mechanism of the blocking induced by RF. Either an inhibition of the contact between the Fc-site and the lymphocyte, some alteration of the tumour antigen-antibody complexes into more potent blockers, or both are, however, possible mechanisms. Meticulous studies in model systems will be needed to define the mechanism.

The presence of RF in the serum of bladder tumour patients at the start of follow-up correlated well with tumour recurrences. These findings suggest that the presence of RF, perhaps as evidence of blocking activity, may be of prognostic value. The presence of serum blocking activity has been shown to be a useful indicator of recurrent tumour growth in experimental tumour systems [25, 26] and possibly also in human malignant melanoma [27]. It would be of considerable practical value if determinations of serum RF activity could be carried out to screen those patients in whom the risk of tumour recurrence is high. Large-scale clinical studies are needed before a final evaluation of the usefulness of the latex test as a tool in the follow-up of bladder tumours can be made. Further RF studies dealing with other human tumours as well, especially those in which early diagnosis of recurrence is difficult, should prove interesting.

Acknowledgements—The expert technical assistance of Miss Ann-Maj Hasselström, Mrs. Anja Kallionpää and Miss Helena Kainulainen is gratefully acknowledged.

REFERENCES

1. O. Wager, On the factor producing agglutination of sensitized red cells and its relation to the agglutination of hemolytic streptococci in rheumatoid arthritis sera. *Ann. Med. exp. Fenn. suppl.* 8, **28,** (1950).

2. K. SIEVERS, The rheumatoid factor in definite rheumatoid arthritis. *Acta rheum. Scand.* suppl. 9 (1965).
3. L. HURRI and Y. PERTTALA, Observations on non-specific Waaler–Rose and latex reactions in cancer patients. *Ann. Med. intern. Fenn.* **54,** 181 (1965).
4. H. BARTFELT, Distribution of rheumatoid factor activity in nonrheumatoid states. *Ann. N.Y. Acad. Sci.* **168,** 30 (1969).
5. UICC, TNM classification of malignant tumours. Research Commission. Committee of clinical stage classification and applied statistics 1963–1967. UICC 17. Laurain.
6. A. BERGKVIST, A. LJUNGQVIST and G. MOBERGER, Classification of bladder tumours based on the cellular pattern. *Acta. chir. scand.* **130,** 371 (1965).
7. O. WAGER, K. PENTTINEN, J. A. RÄSÄNEN and G. MYLLYLÄ, Inhibition of IgG complex-induced platelet aggregation by antiglobulin-active cryoglobulin IgM components. *Clin. exp. Immunol.* **15,** 393 (1973).
8. C. C. RIGBY, L. M. FRANKS, A human tissue culture cell line from a transitional cell tumour of the urinary bladder: growth, chromosome pattern and ultrastructure. *Brit. J. Cancer* **24,** 746 (1970).
9. A. BÖYUM, Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest.* suppl. 97 **21,** 77 (1968).
10. M. H. JULIUS, E. SIMPSON and L. A. HERZENBERG, A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Europ. J. Immunol.* **3,** 645 (1973).
11. M. TAKASUGI and E. KLEIN, A microassay for cell-mediated immunity. *Transplantation* **9,** 219 (1970).
12. T. TIMONEN and E. SAKSELA, Cell-mediated anti-embryo cytotoxicity in human pregnancy. *Clin. exp. Immunol.* In press.
13. M. TAKASUGI, M. R. MICKEY and P. I. TERASAKI, Studies on specificity of cell-mediated immunity to human tumours. *J. nat. Cancer Inst.* **53,** 1527 (1974).
14. E. SAKSELA, *et al.*, manuscript in preparation.
15. H. O. SJÖGREN, I. HELLSTRÖM, S. C. BANSAL and K. E. HELLSTRÖM, Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen-antibody complexes. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1372 (1971).
16. R. W. BALDWIN, M. R. PRICE and R. A. ROBINS, Blocking of lymphocyte-mediated cytotoxicity for rat hepatoma cells by tumour-specific antigen-antibody complexes. *Nature New Biol.* **238,** 185 (1972).
17. C. O'TOOLE, P. PERLMANN, H. WIGZELL, B. UNSGAARD and C. G. ZETTERLUND, Lymphocyte cytotoxicity in bladder cancer: No requirement for thymus-derived effector cells? *Lancet* **i,** 1085 (1973).
18. T. R. HAKALA, P. H. LANGE, A. E. CASTRO, A. Y. ELLIOT and E. E. FRALEY, Antibody induction of lymphocyte-mediated cytotoxicity against human transitional-cell carcinomas of the urinary tract. *New Engl. J. Med.* **291,** 637 (1974).
19. T. HALLBERG, *In vitro* cytotoxicity of human lymphocytes for sensitized chicken erythrocytes is inhibited by sera from rheumatoid arthritis patients. *Scand. J. Immunol.* **1,** 329 (1972).
20. K. PENTTINEN, O. WAGER, S. PYRHÖNEN, P. LEINIKKI and A. M. TEPPO, Negative complement-fixation tests as an immunopathological finding. *Lancet* **i,** 367 (1974).
21. R. VON ESSEN, Amplification of virus neutralization by RF. II International Congress of Immunology, Brighton 1974. Workshop 154: Antiglobulins including rheumatoid factors. *Progress in Immunology* II, Vol. 5, p. 307 (Edited by L. BRENT and J. HOLBOROW) North-Holland, Amsterdam (1974).
22. T. G. GIPSON, C. A. DANIELS and A. L. NOTKINS, Interaction of rheumatoid factor with infectious vaccinia virus-antibody complexes. *J. Immunol.* **112,** 2087 (1974).
23. K. AHO, N. RIPATTI, N. E. SARIS and O. WAGER, Reaction of the rheumatoid factor with human specific precipitates. II Quantitative studies of the protein adsorbable from rheumatoid serum onto diphtheria toxoid-human antitoxin precipitates. *Ann. Med. exp. Fenn.* **40,** 481 (1962).
24. J. T. TESAR and F. R. SCHMID, Conversion of soluble immune complexes into complement-fixing aggregates by IgM-rheumatoid factor. *J. Immunol.* **105,** 1206 (1970).

25. I. Hellström, K. E. Hellström, C. A. Evans, G. Heppner, G. E. Pierce and J. P. S. Yang, Serum mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor specific antigens. *Proc. nat. Acad. Sci. (Wash.)* **62,** 362 (1969).
26. S. C. Bansal, R. Hargreaves and H. O. Sjögren, Facilitation of polyoma tumor growth in rats by blocking sera and tumor eluate. *Int. J. Cancer* **9,** 97 (1972).
27. I. Hellström, G. A. Warner, K. E. Hellström and H. O. Sjögren, Sequential studies on cell-mediated tumor immunity and blocking serum activity in ten patients with malignant melanoma. *Int. J. Cancer* **11,** 280 (1973).

Europ. J. Cancer Vol. 12, pp. 95–100. Pergamon Press 1976. Printed in Great Britain

Binding of Chlorambucil with Antitumor Globulins and its Effect on Drug and Antibody Activities*

A. GUCLU, T. GHOSE, J. TAI and MOLLY MAMMEN
Departments of Pathology and Microbiology, Dalhousie University, Halifax, Nova Scotia, Canada

Abstract—*A method for noncovalently binding chlorambucil to antitumor globulins (ATG) at a pH of about 2·0 is described. Binding of chlorambucil to ATG by this method did not impair either the alkylating activity of chlorambucil or the immunological reactivity of ATG and led to more consistent binding and better retention of both drug and antibody activities than binding at alkaline pH. Variations of temperature between 0°–37°C had no effect on the binding. Gammaglobulins from different species showed different patterns of binding with chlorambucil. Chlorambucil to y-globulin ratio and the concentration of chlorambucil in the reaction mixture were important factors in determining the amount of drug which bound to ATG. Up to 3000 moles of chlorambucil could be bound to each mole of a goat anti-EL4 IgG, however, there was substantial reduction of antibody activity when 20 moles of chlorambucil bound to a mole of the ATG. Binding of chlorambucil also changed the immunoelectrophoretic mobility of the ATG. Chlorambucil bound anti-EL4 globulin carried more chlorambucil to target EL4 cells in vitro than equivalent amounts of chlorambucil bound to normal goat globulin.*

INTRODUCTION

WE HAVE demonstrated that chlorambucil could be non-covalently bound to antitumor globulins (ATG) without impairing the alkylating activity of chlorambucil or the immunological reactivity of antitumor antibodies and also that chlorambucil bound ATG is a more effective tumor inhibitor (both *in vivo* and *in vitro*) than chlorambucil or ATG alone or comparable amounts of chlorambucil bound to "normal" globulins [1, 2]. Our observation on the increased tumor inhibition by chlorambucil bound ATG has been confirmed [3–6] and extended to demonstrate improved selective tumor cell destruction after exposure to antitumor antibodies chemically linked to potent toxins [7–11]. The mechanism of increased tumor inhibition by chlorambucil bound antitumor antibodies might be any combination of the following (a) synergism between cytotoxic drugs and antitumor antibodies [3, 4, 12] (b) antibody mediated selective concentration of cell damaging agents in tumor tissue [13, 14] (c) facilitation of the transport of cytotoxic drugs across cell membrane following "capping" [15]. Though the possibility of synergism between cytotoxic drugs and antitumor antibodies is supported by observations in some models showing that the exposure of target cells to cytotoxic drugs render them more susceptible to damage by antibody [16], in other models chlorambucil had to be linked to antibody to exert its maximal tumor inhibitory effect [3, 17]. Since the conditions under which chlorambucil is bound to y-globulins is likely to affect the alkylating activity of chlorambucil as well as the immunological reactivity of ATG [18], we report here a reproducible method of

Accepted 11 August 1975.

*This work was supported by grants from the National Cancer Institute of Canada and Cancer Research Fund, Faculty of Medicine, Dalhousie University.

non-covalently binding chlorambucil with y-globulins including ATG, and the effects of variables like pH, temperature, nature of the y-globulin (i.e., whether of rabbit, goat or human origin), chlorambucil to ATG ratio and also chlorambucil and ATG concentrations on the extent of binding of chlorambucil to y-globulins and on the retention of antitumor activity in ATG.

MATERIAL AND METHODS

Binding of globulins to chlorambucil

Aqueous solutions of chlorambucil (Burroughs Wellcome & Co., Canada Ltd., La Salle, P.Q.) were prepared by dissolving the drug in 12 N HCl (1 drop/mg of chlorambucil) and then adding the necessary amount of 0·01 M, phosphate buffered saline (PBS, pH 7·1) to obtain desired chlorambucil concentration.

Chlorambucil solution was then added slowly, drop by drop into the desired amount of gamma globulin solution (5 mg/ml PBS at 4°C) with gentle stirring. The pH of the mixture was determined and adjusted to pH 2 by adding as necessary 0·1 N NaOH or 0·1 N HCl. The mixture was gently shaken at the desired temperature for 30 minutes after which protein bound chlorambucil (PBC) was separated from free chlorambucil by Amicon Centriflo C F50A ultrafiltration (Amicon Corp., Lexington, Mass.). Immunologic reactivity of chlorambucil bound ATG was tested by cytoplasmic and membrane immunofluorescence staining of appropriate tumor cells [1, 2].

Estimation of the alkylating activity of protein bound chlorambucil

To determine the % alkylating activity of PBC (i.e., 100 × alkylating activity PBC/alkylating activity of chlorambucil used for binding) the Epstein method [19] was used. The lack of development of the color reaction in parallel aliquots of chlorambucil pre-incubated for 30 min at 4°C with sodium thiosulfate established the specificity of the Epstein color reaction [20, 21].

Every experiment involving determination of the percent alkylating activity of PBC was repeated at least three times.

Production of antitumor globulins and assessment of their reactivity

EL4 lymphoma was maintained by serial intraperitoneal passage in C57BL/6J mice purchased from the Jackson Memorial Laboratories (Bar Harbour, Maine). The methods of production and purification of goat and rabbit anti-mouse EL4 lymphoma sera, the tests and criteria establishing their specificity have been recently described [15, 22]. The goat and rabbit anti-EL4 globulins (GAEG and RAEG) were obtained by fractionation with 33·3% saturated ammonium sulfate solution and immunoelectrophoresis revealed these to be IgG. Human lyophilized IgG was obtained commercially (Mann Research Laboratories, New York) and was also separated from normal human serum following a batch method [23]. Protein was estimated by the method described by Lowry [24] and immunoelectrophoresis was performed with barbital buffer (pH 8·6) following the method of Scheidegger [25]. Cytoplasmic and membrane immunofluorescence of EL4 lymphoma cells with chlorambucil bound antitumor globulins were performed by the sandwich method with appropriate controls [2, 22]. Fluorescein isothiocyanate conjugated antibody globulins against rabbit, goat and human whole sera and their IgG fractions were obtained commercially (Hyland Laboratories, California).

RESULTS

Effect of variations in pH on the binding of goat anti-EL4 lymphoma IgG with chlorambucil

At a chlorambucil to GAEG ratio of 1 : 80 (w/w), at 4°C, there was considerable amount of precipitate formation when chlorambucil and GAEG were mixed at the pH range between 5 to 9·5. At pH 2, the % alkylating activity of PBC varied between 60 and 65 whereas after reactions at pH 9·5 and 10, PBC alkylating activity varied between 10 to 20%. When the pH was readjusted to 7, the chlorambucil bound GAEG obtained after both acid and alkaline binding was found to have retained its reactivity with EL4 cells as well as with rabbit anti-goat IgG.

Effect of variation in temperature

When aliquots of chlorambucil and GAEG (ratio 1/80, w/w) were incubated at 0°, 4°, 15°, 20°, 30° and 37°, PBC alkylating activity varied between 62 to 71% irrespective of the temperature of the reaction mixture.

Binding of chlorambucil with different serum proteins and IgG from different species

The alkylating activity of PBC obtained after reaction at 4°C, pH 2·0 and at chlorambucil protein ratios of 1/80 (w/w) were between 91–97% with bovine serum albumin (crystalline, Sigma, St. Louis, Missouri) as well as with fractions of human, goat and rabbit sera obtained after a single precipitation with 40% saturated ammonium sulfate (containing

mainly y-globulins with a trace of albumin); between 60 to 65% with GAEG, normal human IgG, normal human and goat globulins precipitated 3 times with 33·3% saturated ammonium sulfate to eliminate albumin contamination; and between 44 and 50% with albumin free rabbit anti-EL4 IgG or rabbit whole y-globulin (Fig. 1). Prolonged storage and

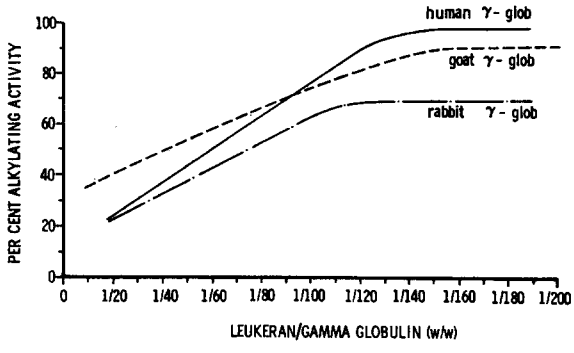


Fig. 1. *Percentage of the original alkylating activity of chlorambucil in chlorambucil-y-globulin complexes after reaction with human* (—), *goat* (— — —) *and rabbit* (—·—) *y-globulins with chlorambucil at different chlorambucil* (*Leukeran*)*/y-globulin ratios* (*w/w*). (*pH* 2·0. 4°*C, chlorambucil concentration* 0·1 *mg/ml*).

repeated freezing and thawing of albumin free IgG or whole y-golbulin solutions usually led to the formation of fine precipitates containing polymerized immunoglobulins. Similar precipitates were also found after dissolving the commercial lyophilized human IgG preparation. Aliquots containing precipitates of polymerized immunoglobulins were found to bind poorly with chlorambucil.

Chlorambucil/y-globulin ratio

When varying ratios of chlorambucil to human IgG, GAEG and RAEG were reacted at 4°C, pH 2, the percent of binding increased with the lowering of chlorambucil/protein ratio from 1/10 to 1/140 (w/w) (Fig. 2). At the ratio of 1/140 the maximal percent binding of chlorambucil under the test conditions was obtained, i.e., between 90 to 97% PBC alkylating activity was obtained with human IgG and GAEG. However, with RAEG, the maximal binding was 70% and was obtained at 1/120 chlorambucil to RAEG ratio (w/w) (Fig. 1). When GAEG was reacted to chlorambucil at chlorambucil to GAEG ratios (w/w) starting from 1/200 to 250/1 at 4°C and at a pH of 2·0, with increasing chlorambucil GAEG ratio, the ratio of moles of chlorambucil bound to mole of GAEG gradually increased though the percentage of chlorambucil which bound to GAEG gradually decreased. However, at a chlorambucil to GAEG ratio of 1/5 (w/w), (i.e., when 20 moles of chlorambucil bound to 1 mole of GAEG) the titre of reactivity of GAEG with EL4 cells as detected by immunofluorescence decreased from 1/128 to 1/16 and at a chlorambucil to GAEG ratio of 25/1 (w/w), GAEG did not react with EL4 cells (Fig. 2).

Changes in the immunoelectrophoretic mobility of the chlorambucil bound GAEG could be seen even when chlorambucil was bound to GAEG at 1 : 80 ratio (w/w) with the formation of a slow moving tail, at 1/10 (w/w) ratio the entire migration pattern changed. At a chlorambucil to GAEG ratio of 10/1 and above, immunoelectophoresis did not reveal any reaction between chlorambucil bound GAEG and rabbit anti-goat globulin (Fig. 3).

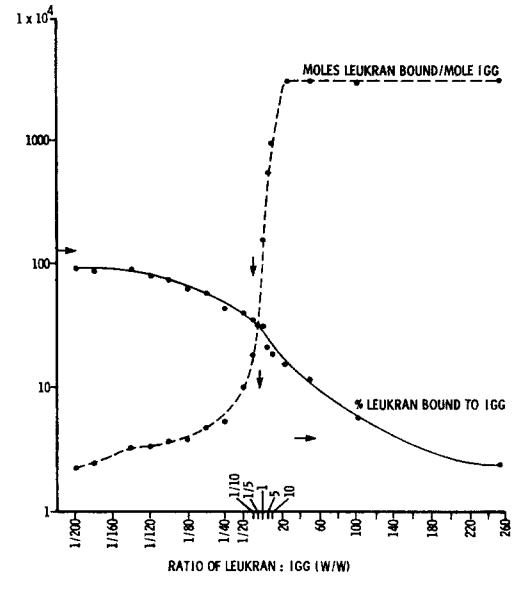


Fig. 2. *Percentage of the original alkylating activity of chlorambucil* (—) *and the ratio of the moles of alkylating form of chlorambucil bound to a mole of a goat anti-EL4 lymphoma IgG* (— — —) *after reacting the antitumor IgG with chlorambucil at varying chlorambucil antitumor IgG ratios* (*pH* 2·0, 4°*C, chlorambucil concentration* 0·1 *mg/ml*). *Each point represents the mean of at least three separate determinations. The vertical arrows* (↓) *represent the partial loss and the horizontal arrow* (→) *complete loss of the reactivity of the chlorambucil-anti-EL4 globulin complexes with EL4 cells as assessed by immunofluorescence.*

LEUKRAN = Leukeran brand of chlorambucil.

Effect of varying chlorambucil and y-globulin concentrations on PBC alkylating activity

When chlorambucil was bound to GAEG at a pH of 2·0, at 4°C at a chlorambucil to globulin ratio of 1/140 variations in the concentration of chlorambucil revealed maximum binding in aliquots containing 0·05 and 0·1 mg of chlorambucil/ml (Table 1).

Table 1. *Effect of varying the concentration of chlorambucil on the percent binding of chlorambucil to goat anti-EL4 globulin, at* 4°C, *pH* 2·0 *and chlorambucil/antitumor globulin ratio* 1/140 (*w*/*w*). *The range of percent chlorambucil binding shown represents results of five experiments for each chlorambucil concentration.*

Chlorambucil/ml reaction mixture	Percent of chlorambucil bound to antitumor globulin
1 mg	62 - 70
0.25 mg	70 - 76
0.1 mg	90 - 96
0.05 mg	88 - 97
0.025 mg	36 - 48

Reactivity of chlorambucil bound GAEG to EL4 cells in vitro

Aliquots of 10^9 EL4 cells after incubation in 4 ml of either PBS or inactivated normal C57BL serum (i.e., approximate blood volume of adult C57BL mice) at 37°C for 20 min at pH 7 with either 50 μg of chlorambucil alone, 50μg chlorambucil bound to 5000 μg of GAEG, or 50 μg of chlorambucil bound to 5000 μg of NGG were washed in PBS prior to the determination of the alkylating activity in the cells. No chlorambucil activity could be detected in EL4 cells incubated with chlorambucil alone. In every experiment the % alkylating activty of chlorambucil associated with EL4 cells incubated in chlorambucil bound GAEG (range 3·4–15·6%) was at least twice the amount of % alkylating activity found in EL4 cells incubated with chlorambucil bound NGG (range 1·2–1·74%).

At least 90% of EL4 cells incubated for 30 min with chlorambucil bound GAEG showed membrane staining and "cap formation" could be seen in about 50% of the EL4 cells after incubation for two hours (Fig. 4). No membrane staining could be seen on the EL4 cells incubated with chlorambucil bound NGG.

DISCUSSION

Though chlorambucil is only sparingly soluble in water at a pH 7·0, it readily dissolves in a number of organic solvents. However, most of these organic solvents are water immiscible toxic to animals (e.g. dioxane) and denature proteins. When attempts were made to dissolve chlorambucil in a water miscible and a relatively nontoxic solvent like ethanol, chlorambucil tended to precipitate out at more than × 2 dilutions with PBS. Chlorambucil can, however, be completely dissolved in water either at any pH in the alkaline range when it forms anionic molecular species (Form 1—Fig. 5); or below pH 2 forming cationic molecular species; between pH 2 and 7 only nonionized chlorambucil molecules of limited solubility are formed [26]. In both hydrolysis (i.e. the replacement of the chlorine atoms of chlorambucil by hydroxyl groups) and alkylation reactions (i.e. the substitution of a hydrogen atom in a receptor molecule by chlorambucil), chlorambucil is first activated relatively slowly to form carbonium ions which then rapidly reacts with hydroxyl or nucleophilic groups (Fig. 5). However, only the anionic form of chlorambucil molecules (i.e. in aqueous solution of chlorambucil in the alkaline pH) can be activated to give rise to carbonium ions and as a result, the rate of both hydrolysis and alkylation reactions of chlorambucil in aqueous solution decreases below pH 7·0 [26].

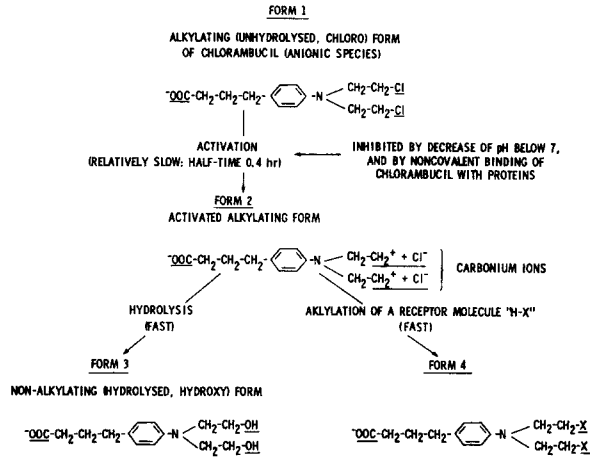


Fig. 5. *Different forms of the chloranbucil molecule involved in alkylation and hydrolysis.*

Our own results and those of Linford [27] further show that at 0°–4°C the alkylating activity of chlorambucil remains unimpaired for a period of at least 70 days irrespective of the pH of the chlorambucil solution. At 37°C the half life of the alkylating activity of chlorambucil is 3·5 days at a pH ≦ 2, 1·4 hr at pH 7 and 0·3 hr at the alkaline ranges.

As the alkylating activity of chlorambucil appears to be the chemical basis of its anticancer action [1, 2], binding antitumor globulins with chlorambucil at 4°C and at a pH ≦ 2 appeared to be ideal because (i) chlorambucil is soluble under these conditions and does not form precipitates when mixed with proteins (ii) the cationic form of chlorambucil formed at this pH can neither alkylate the antitumor globulin nor undergo hydrolysis and (iii) at this temperature chlorambucil retains its alkylating activity unimpaired. As can be seen from our

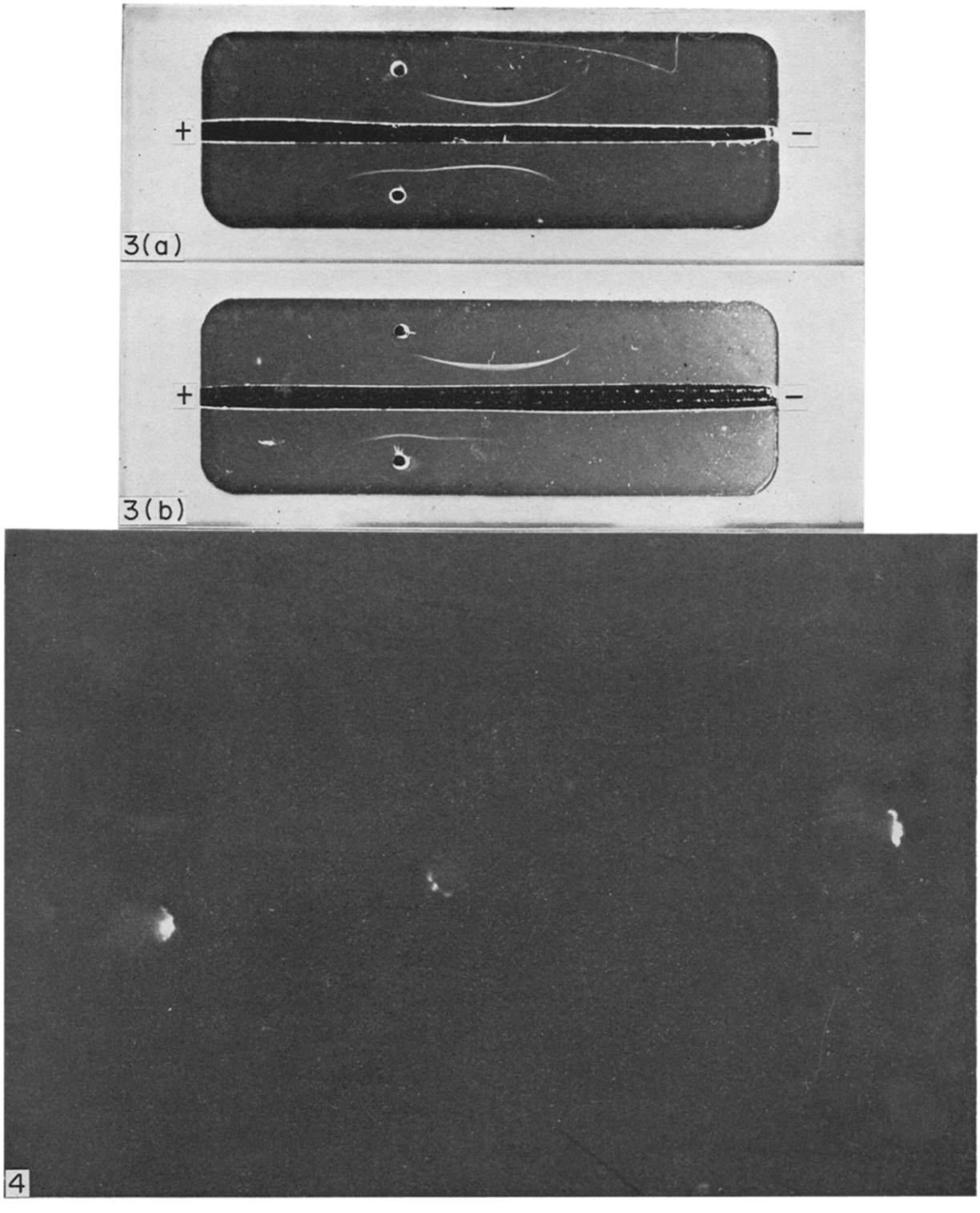


Fig. 3. *Agar gel immunoelectrophoresis patterns of goat anti-EL4 globulin and goat anti-EL4 globulin bound to chlorambucil at varying ratios.*

Centre troughs: Rabbit antigoat γ-globulins.
Upper wells: Goat anti-EL4 globulin.
Lower wells: Goat anti-EL4 globulin after reaction with chlorambucil at pH 2·0. 4°*C, chlorambucil concentration* 0·1 *mg/ml, chlorambucil: goat anti-EL4 globulin* (*A*) 1/80 *and* (*B*) 1/10.

Fig. 4. *Fluorescence photomicrograph of EL4 cells from a suspension of* 10^9 *viable cells/ml Hank's balanced salt solution incubated for* 2 *hours with* 50 *μgm of chlorambucil bound to* 5 *mg of goat anti-EL4 globulin, washed and then treated with fluoresceinated rabbit antigoat globulin. Granula membrane immunofluorescence and "capping" of EL4 cells show that the chlorambucil bound antitumor globulin had retained its reactivity with a surface antigen on EL4 cells.*

(to face p. 98)

results on the effect of variation of temperature on the binding of chlorambucil with GAEG, all these conditions are fulfilled by adjusting the pH of the chlorambucil solution to ≦ 2 only and variation in temperature in the range between 0° to 37°C had no effect on the non-covalent binding of chlorambucil with immunoglobulins at this pH.

Subsequent to our demonstration of increased antitumor activity of chlorambucil bound ATG, binding of chlorambucil to IgG has been investigated at an alkaline pH by Blakeslee and Kennedy [28] and Ross [29] and to both alkaline and acid pH by O'Neil *et al.* [30]. In keeping with our observation, O'Neil *et al.* found about 9% PBC alkylating activity at pH 3·5 at a chlorambucil to protein ratio of 1/10 (w/w). At alkaline pH (8·0) the PBC alkylating activity was only 2%.

Using a Na salt of chlorambucil and a lyophilized preparation of human IgG, Ross [29] observed that only about 3·3% of chlorambucil bound to the IgG after reaction for 5 min at 20°C, pH 8·0 and chlorambucil to IgG molar ratio of 60 : 1 (i.e. about 1/8 w/w). This observation on chlorambucil IgG interaction at an alkaline pH is consistent with the results of Blakeslee and Kennedy [28] who studied chlorambucil/rabbit IgG interaction under comparable circumstances and also demonstrated that only a maximum of about 12 moles of chlorambucil could be bound to a mole of rabbit IgG at a molar reaction ratio of 750: 1 in contrast to the maximum binding of 3000 moles of chlorambucil to a mole of IgG at pH ≦ 2 reported here. No attempt was made previous to this report to find out whether antibody activity is retained by chlorambucil-immunoglobulin complexes.

Our own investigation on the binding of chlorambucil with antitumor globulins at alkaline pH following the method described by Israels and Linford [17] gave rise to variable amounts of PBC alkylating activity which was always less than the binding at pH ≦ 2. Tumor inhibition by antitumor globulins bound with chlorambucil at alkaline pH was also very variable and inferior to that of chlorambucil bound at pH ≦ 2.

The results of our *in vitro* study demonstrating that ATG non-covalently bound to chlorambucil carried more chlorambucil to EL4 cells than NGG similarly bound to chlorambucil and our previous report on the endocytosis of chlorambucil bound ATG into EL4 cells subsequent to capping [15] might explain, at least in part, the increased tumor inhibition by chlorambucil bound ATG in experimental models as well as in the treatment of clinical cancer [2, 31].

REFERENCES

1. T. Ghose and S. P. Nigam, Antibody as carrier of chlorambucil. *Cancer (Philad.)* **29,** 1398 (1972).
2. T. Ghose, S. T. Norvell, A. Guclu, D. Cameron, A. Bodutha and A. S. Macdonald, Immunochemotherapy of cancer with chlorambucil-carrying antibody. *Brit. med. J.* **3,** 495 (1972).
3. I. Flechner, The cure and concomitant immunization of mice bearing Ehrlich ascites tumors by treatment with an antibody-alkylating agent complex. *Europ. J. Cancer* **9,** 741 (1973).
4. D. A. L. Davies and G. J. O'Neil, *In vivo* and *in vitro* effects of tumor specific antibodies with chlorambucil. *Brit. J. Cancer (Suppl. I)* **28,** 285 (1973).
5. R. D. Rubens and R. Dulbecco, Augmentation of cyto-toxic drug action by antibodies directed at cell surface. *Nature (Lond.)* **248,** 81 (1974).
6. G. V. Smith, J. B. Grogan, J. Stribling and J. Lockard, Immunochemotherapy of hepatoma in rats. *Amer. J. Surg.* **129,** 137 (1975).
7. F. L. Moolten, N. J. Capparell and S. R. Cooperband, Antitumor effects of antibody-diphtheria toxin conjugates: Use of hapten-coated tumor cells as an antigenic target. *J. nat. Cancer Inst.* **49,** 1057 (1972).
8. G. W. Philpott, R. J. Bower and C. W. Parker, Selective iodination and cytotoxicity of tumor cells with an antibody–enzyme conjugate. *Surgery* **74,** 51 (1973).
9. G. W. Philpott, R. J. Bower, K. L. Parker, W. Shearer and C. W. Parker, Affinity cytotoxicity of tumor cells with an antibody-glucose oxidase conjugate, peroxidase and arsphenamine. *Cancer Res.* **34,** 2159 (1974).
10. J. H. Linford, G. Froese, J. Berezi and L. G. Israel, An alkylating agent-globulin conjugate with both alkylating and antibody activity. *J. nat. Cancer Inst.* **52,** 1665 (1974).

11. R. Levy, E. Hurwitz, R. Maron, R. Arnon and M. Sela, The specific cytotoxic effects of Daunomycin conjugated to antitumor antibodies. *Cancer Res.* **35,** 1182 (1975).
12. E. Mihic, Combined effects of chemotherapy and immunity against leukemia L1210 on DBA/2 mice. *Cancer Res.* **29,** 848 (1969).
13. T. Ghose, A. Guclu, J. Tai, A. S. Macdonald, S. T. Norvell and J. Aquino, Antibody as carrier of ^{131}I in cancer diagnosis and treatment. *Cancer* **36,** (1975).
14. T. Ghose, J. Tai, J. Aquino, A. Guclu, S. T. Norvell and A. S. Macdonald, Tumor localization of ^{131}I-labelled antibodies by radionuclide imaging. *Radiology* **116** (1975).
15. A. Guclu, J. Tai and T. Ghose, Endocytosis of chlorambucil-bound antitumor globulin following "capping" in EL4 lymphoma cells. *Immunol. Commun.* **5** (1975).
16. M. Segerling, S. H. Ohanian and T. Borsos, Metabolic inhibitors increase killing of tumor cells by antibody and complement. *J. nat. Cancer Inst.* **53,** 1411 (1974).
17. T. Ghose, A. Guclu, and J. Tai, Suppression of an AKR lymphoma by antibody and chlorambucil. *J. nat. Cancer Inst.* **55,** (1975).
18. L. G. Israel and J. H. Linford, Some observations on the reaction of chlorambucil, azo-mustard (CB 1414) and cyclophosphamide. *Proc. of the 5th Canadian Cancer Conference,* p. 399, Academic Press, New York.
19. J. Epstein, R. W. Rosenthal and R. J. Ess, Use of y-(4-nitrobenzyl) pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.* **27,** 1435 (1955).
20. C. A. Ross, D. M. Carberry and G. E. Kraus, Protection against systemic toxicity due to nitrogen mustard. *Surg. Forum* **11,** 43 (1960).
21. R. K. Ausman, G. E. Crevar, H. Hagedom, T. J. Bardos and J. L. Ambrus, Studies in the pharmacodynamics of mechlorethamine and AB 100. *J. Amer. med. Ass.* **178,** 735 (1961).
22. T. Ghose and A. Guclu, Cure of a mouse lymphoma with radio-iodinated antibody. *Europ. J. Cancer* **10,** 787 (1974).
23. J. S. Baumstark, R. J. Laffin and W. A. Bardawit, A preparative method for the separation of 7S gamma globulin from human serum. *Arch. Biochem. Biophys.* **108,** 514 (1964).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
25. J. J. Scheidegger, Une micro-méthode de l'immuno-électrophorèse. *Int. Arch. Allergy* **7,** 103 (1955).
26. J. H. Linford, The influence of pH on the reactivity of chlorambucil. *Biochem. Pharmacol.* **12,** 317 (1963).
27. J. H. Linford, The role of adsorption in controlling the rate of reaction of chlorambucil with protein. *Canad. J. Biochem. Physiol.* **41,** 931 (1963).
28. D. Blakeslee and J. C. Kennedy, Factors affecting the noncovalent binding of chlorambucil to rabbit immunoglobulin G. *Cancer Res.* **34,** 882 (1974).
29. W. C. J. Ross, The interaction of chlorambucil with human γ-globulin. *Chem.-Biol. Interations* **8,** 261 (1974).
30. G. J. O'Neil, B. A. Pearson and D. A. L. Davies, *In vitro* cytotoxicity of anti-θ (Thy-1) antibodies combined with chlorambucil. *Immunology* **28,** 323 (1975).
31. T. Ghose, S. T. Norvell, A. Guclu and A. S. Macdonald, Immunochemotherapy of human malignant melanoma with chlorambucil-carrying antibody. *Europ. J. Cancer* **11,** 321 (1975).

Europ. J. Cancer Vol. 12, pp. 101–106. Pergamon Press 1976. Printed in Great Britain

Hepatomas in GRS/A Male Mice Treated with Horse Serum or Horse Anti-Mouse Antilymphocyte Serum

L. DEN ENGELSE,* W. MISDORP† and P. EMMELOT*

Departments of Biochemistry and Pathology,† The Netherlands Cancer Institute, Amsterdam, The Netherlands*

Abstract—*Repeated* (11–16) *subcutaneous injections of horse anti-mouse antilymphocyte serum (HALS), normal horse serum (NHS) or physiological saline (PS) were given to GRS/A male mice starting at the ages of* 10 *days or* 8 *weeks. All animals received two injections weekly. At the age of* 49–58 *weeks all surviving animals were killed and autopsied. Hepatomas were found predominantly in groups started at the age of* 10 *days. Highest hepatoma incidence was found in a group of animals treated from the age of* 10 *days with* 13 *injections of* 0·2 *ml NHS* (16 *out of* 29 *animals surviving at two weeks after last injection*; 55%). *Small numbers of hepatomas were found in mice injected with either HALS or PS. No spontaneous hepatomas were detected in a control group of* 53 *untreated GRS/A male mice. None of the treatments resulted in significantly increased lung tumour incidences. The results are discussed with regard to possible mechanisms of hepatoma formation in GRS/A male mice.*

INTRODUCTION

THE ABSENCE of an effect of ALS or ATS on the incidence of "spontaneous" tumours in mice has been observed by several authors [1–3] suggesting a small or even negligible role of cellular lymphocyte functions in the process of "spontaneous" tumour formation. Only a few cases were reported in which the formation of lymphoreticular neoplasms was inhibited [4] or stimulated [5–7] by ALS or ATS treatment.

In preliminary experiments started several years ago we investigated immunological factors in lung adenoma formation in GRS/A mice. Untreated GRS/A male mice, as reported previously [8], were very sensitive towards "spontaneous" lung tumour formation. In a small series of experiments we treated GRS/A male mice with HALS; control groups were injected with NHS or PS. In agreement with other authors [1–7], no significantly increased lung tumour incidences were observed, but, unexpectedly a number of hepatomas were found in surviving HALS- and NHS-treated animals. Since in our laboratory no hepatomas had been observed in untreated GRS/A males of corresponding age, we decided to repeat the experiment with larger groups and under slightly different experimental conditions. The results of this second series of experiments—confirming and extending our earlier results—are reported in this paper.

Accepted 15 October 1975.

Abbreviations: ALS, antilymphocyte serum; ATS, antithymocyte serum; PS, physiological saline; HALS, horse anti-mouse antilymphocyte serum; NHS, normal horse serum.

MATERIALS AND METHODS

Animals

Male mice of the inbred strain GRS/A (formerly designated GR or GR/A) received drinking water and standard laboratory diet (Hope Farms, Woerden, The Netherlands) *ad libitum*. The animals were subjected to a strict light-dark regimen and injected (with PS or serum) between 6 and 8 hr after the start of the 11-hr light period (8.00 a.m.).

Sera

Horse sera were obtained from the Radiobiological Institute TNO (Rijswijk-Z.H., The Netherlands). HALS was prepared by injecting a $2\frac{1}{2}$ years old virgin mare with a suspension of thymic cells and mesenterial lymph node cells from 10 weeks old mice of various strains according to the method of Monaco [9]. Graft survival was assayed at the Radiobiological Institute TNO in 10–12 weeks old animals. At day-1 recipient (CBA/Rij × C57BL/Rij)F_1 animals received an intraperitoneal injection of 1 ml HALS followed by a skin graft from RFM mice at day 0. Graft survival time in HALS treated animals was 14 ± 1 days; graft survival time in control (untreated) animals was 8 ± 1 days. Normal horse serum was taken from another $2\frac{1}{2}$ years old virgin mare. Both HALS and NHS were inactivated for 30 min at 56°C and not absorbed against red blood cells.

Experiments

GRS/A male mice were divided into 12 groups as shown in Tables 1 and 2 and received 11–16 (each week 2) subcutaneous injections of 0·2 or 0·5 ml HALS, NHS or PS respectively. In some groups (see Tables 1 and 2) a number of animals were found dead or had to be killed when moribund before the end of the experimental period. Autopsies were performed on all animals dying or sacrificed between the

Table 1. Hepatomas and lung adenomas in GRS/A male mice. First injection at age 9–11 days

Group	1	2	3	4	5	6
Treatment	HALS	HALS	NHS	NHS	PS	PS
Volume injected (ml)	0.2	0.5	0.2	0.5	0.2	0.5
Number of injections	16	12	13	11	16	16
Number of animals at the start of the experiment	28	26	54	39	27	25
End of the experiment (age in weeks)	57	56	56	58	49	49
Number of animals alive at two weeks after last injection (%) *	14(50%)	4(15%)	29(54%)†	37(95%)	25(93%)	24(96%)
Number of hepatoma-bearing animals (%)‡	2(14%)	3(75%)	16(55%)	7(19%)	2(8%)	1(4%)
Number of lung-adenoma-bearing animals (%)‡	3(21%)	2(50%)	13(45%)	18(49%)	4(16%)	6(25%)
Mean number of lung adenomas per animal‡	0.29	0.50	0.66	0.65	0.20	0.29

* Based on the number of animals at the start of the experiment.

† 20 animals died before the age of 16 days.

‡ Based on the number of animals alive at 2 weeks after last injection.

Table 2. Hepatomas and lung adenomas in GRS/A male mice. First injection at age 8 weeks

Group	7	8	9	10	11	12
Treatment	HALS	HALS	NHS	NHS	PS	PS
Volume injected (ml)	0.2	0.5	0.2	0.5	0.2	0.5
Number of injections	16	12	13	12	16	16
Number of animals at the start of the experiment	25	25	53	49	25	20
End of the experiment (age in weeks)	63	49	57	58	56	56
Number of animals alive at two weeks after last injection (%)*	25(100%)	24(96%)	34(64%)†	49(100%)	24(96%)	20(100%)
Number of hepatoma-bearing animals (%)‡	0(0%)	0(0%)	1(3%)	1(2%)	0(0%)	0(0%)
Number of lung-adenoma-bearing animals (%)‡	5(20%)	5(21%)	6(18%)	8(16%)	1(4%)	2(10%)
Mean number of lung adenomas per animal‡	0.24	0.21	0.21	0.20	0.04	0.15

* Based on the number of animals at the start of the experiment.

† 17 animals died within 3 days after the 13th injection.

‡ Based on the number of animals alive at 2 weeks after last injection.

14th day after the last injection and the end of the experiment (at the age of 49–58 weeks; see Tables 1 and 2). Only a limited number of animals dying before that period were autopsied. All animals surviving the experimental period were sacrificed and autopsied. At autopsy all animals were inspected for tumours and other lesions. Liver, kidneys, and (when grossly abnormal) spleen and lung, as well as all macroscopic lesions (except most lung tumours) were routinely stained with hematoxylin-azophloxin and examined microscopically. Tumours of the lung were counted with the aid of a dissecting microscope (magnification × 10); only nodules with a diameter of 0·1 mm or larger were counted. A control group of 53 untreated, GRS/A male mice was sacrificed and autopsied at the age of 56 weeks.

RESULTS

Survival

Survival data are listed in Tables 1 and 2. In most groups (4–8, 10–12) the percentage of animals dying either in the injection period or within two weeks following that period was less than 7%. Survival was low in both groups receiving HALS starting at the age of 10 days (groups 1 and 2). In group 2 further treatment had to be stopped after 12 injections because of poor condition of the animals. To facilitate comparison group 8 then also received 12 injections. Survival after NHS injections seemed to be dose rather than age dependent. Whereas 0·5 ml NHS injections caused some occasional death only, injections of 0·2 ml NHS proved to be toxic to both baby mice (group 3) and adult ones (group 9). A possible explanation might be that the higher dose induces a state of immunological unresponsiveness (see "Discussion").

Liver tumours

On microscopic examination, all tumours in the liver of GRS/A male mice proved to be hepatomas. The hepatomas did not structurally differ from those described by other authors [10] and should be regarded as low grade malignant tumours [11, 12]. Hepatoma incidences are recorded in Tables 1 and 2. In mice treated from the age of 8 weeks (groups 7–12, see Table 2) only 2 hepatoma-bearing animals were found. Both tumours occurred in NHS-treated animals (group 9: 1/34, (3%); group 10: 1/49, (2%)). Although the percentage of hepatoma-bearing animals was low in PS-treated animals (group 5: 2/25, (8%); group 6: 1/24, (4%)), hepatomas were observed in all groups of mice treated from the age of 10 days (groups 1–6). The quantitative interpretation of tumour data in group 2 and to a lesser extent also in groups 1 and 3 is hampered by considerable death rates in these groups during the injection period. The control group of 53 untreated GRS/A male mice, sacrificed at the age of 56 weeks, showed neither macroscopic nor microscopic signs of hepatomas.

Tumours in other organs

In 22 out of 53 (42%) untreated GRS/A male mice one or more lung tumours were observed at the age of 56 weeks (mean number of lung tumours per animal 0·49). None of the treated groups showed a statistically significant increased percentage of animals with lung tumours; in contrast, most groups even showed appreciably lower lung tumour incidences when compared with untreated controls. Lymphoreticular tumours were not observed in groups 1, 2, 5 and 12; in the remaining groups incidences of 3–8% were found. In untreated control animals the incidence of lymphoreticular tumours was 6% (3 leukemic animals out of 53). Other tumours found were lipomas (1 animal in groups 2, 6, 7, 9 and 10; 2 animals in group 3), skin tumours (1 highly differentiated squamous cell carcinoma in group 4; 1 trichoepithelioma in group 6; 1 papilloma in group 11). An adenocarcinoma of the mammary gland was observed in group 5.

Non-neoplastic lesions

Two types of non-neoplastic lesions were observed in lung tissue. In group 1 (16 × 0·2 ml HALS, first injection on the 10th day) and group 9 (13 × 0·2 ml NHS, first injection at 8 weeks) most autopsied animals dying within or in two weeks after the injection period showed extensive bleeding throughout the lung. In group 2 (16 × 0·5 ml HALS, first injection on the 10th day) most animals died within the injection period or in two weeks following this period and showed severe (interstitial) pneumonia at autopsy.

Liver damage in animals dying within or in the two weeks following the injection period was observed in some animals in group 9 (see Table 2) and included dilated veins, perivascular inflammatory infiltrate, local necrosis, fatty degeneration and, in one liver, peliosis (see Ref. 13). In untreated GRS/A male mice at the age of 56 weeks non-neoplastic liver lesions were found to be absent or very weak in the majority of animals; only an occasional liver showed moderate or pronounced centrilobular fatty degeneration. In none of the

groups in which treatment was started at the age of 8 weeks (groups 7–12), increased centrilobular fatty degeneration was observed at the end of the experimental period when compared with untreated controls. Moderate to pronounced centrilobular fatty degeneration was observed in the livers of most animals in groups 1–6 (first injection at the age of 10 days); the stage of lesion proved to be independent of the material or volume injected.

DISCUSSION

In this paper we describe the formation of substantial numbers of hepatomas in GRS/A male mice treated with NHS or HALS (see Tables 1 and 2). Apart from group 2 in which many animals died within two weeks after the last injection, highest hepatoma incidence (55%) was observed in 57 weeks old animals, treated from the age of 10 days with control horse serum. Effects of antilymphocyte and control sera on the development of tumours in otherwise untreated mice have been investigated by a number of authors [1–7]. Although incidences of "spontaneous" lymphoreticular tumours ("leukemia") were influenced [4–7], changes in the incidences of epithelial tumours have not been reported previously. It is important to note that our experimental conditions differed from those used by other authors with regard to age at treatment, type of serum, duration of the experiment, or strain of mice. Adult animals were used by many authors [1, 2, 4, 6, 7]; our experiments show a dramatic decrease in hepatoma incidence with increasing age at the first injection (compare groups 1–6 with groups 7–12). Some investigators [4–6] used rabbit sera. Preliminary experiments in our laboratory indicated that, although 16 injections of pooled, heat-inactivated control rabbit sera to GRS/A male mice (each week 2 injections of 0·2 ml; first injection at the age of 10 days) resulted in a number of hepatoma-bearing animals, the incidence (7%) was appreciably lower than after comparable NHS treatment but not significantly different from that in group 1 (14%) or group 5 (8%). In a number of reported experiments [14–16] in which control serum was given to carcinogen-treated animals, most or all animals were sacrificed before the age of 8 months. It is known that the majority of both spontaneous and induced liver tumours in mice appears after this age. It is possible that sensitivity towards hepatoma formation after horse serum injections is restricted to the GRS/A strain. This inbred mouse strain has been developed in Europe and is not related to the American gene pool of *Mus musculus*.

It is a well-known phenomenon that hepatoma incidence in mouse strains liable to spontaneous hepatoma formation is dependent on in- and external factors like hormonal status, diet and number of animals per cage. In GRS/A male mice however, no "spontaneous" hepatomas were observed in 53 animals sacrificed at the age of 56 weeks. Hepatomas in GRS/A male mice could be induced in two out of twenty animals by treating adult animals with the carcinogen dimethylnitrosamine in drinking water [8]. Our attempts to induce higher incidences of hepatomas by dimethylnitrosamine administration to baby GRS/A male mice failed because all animals developed high numbers of lung adenomas and therefore had to be killed at the age of 3–4 months. So it cannot be decided at this moment whether or not formation of hepatomas in groups 1–6 (first injection at the age of ten days) and not or to an appreciably lesser degree in groups 7–12 (first injection at the age of 8 weeks), is primarily an age-dependent or dose-(ml/g body weight) dependent phenomenon. However, the relatively high sensitivity of newborn or baby mice to tumour induction is well documented [17, 18] and seems to offer a plausible explanation for the widely diverging hepatoma incidences in groups 1–6 vs groups 7–12.

The finding that hepatomas occurred in all groups treated from the age of 10 days (groups 1–6) suggest that handling and injecting young animals *per se* might lead to hepatoma formation. Since highest hepatoma incidences were observed in HALS and NHS treated animals (groups 1–4) whereas PS treatment (groups 5 and 6) resulted in an occasional hepatoma only, it is concluded that horse serum factors are of prime importance in hepatoma formation in treated GRS/A male mice. Results of our experiment with pooled sera of untreated rabbits (as mentioned before) indicate that these factors (or factor) are not or to a much lesser degree connected with the latter type of serum.

Besides hepatomas a number of other lesions were observed. Extensive bleeding throughout the lung was observed in animals from group 1 ($16 \times 0{\cdot}2$ ml HALS, first injection at 8 weeks). This lesion was restricted to animals dying within the injection period or in two weeks following this period and might be similar to the allergic shock described by other authors [19] for mice receiving horse anti-mouse lymphocyte globulin. In most experimental groups lung tumour incidence was

lower than in untreated controls, the latter incidence (42%) being equal to the incidence of 40% reported [8] several years ago for male mice of the same strain. At this moment no explanation can be given for the decrease in lung tumour incidences as observed after most treatments. Evidence for an effect of hormonal or environmental factors on lung adenoma formation has been reported only incidentally [20, 21]; in both reports effects related to the formation of lung tumours after administration of chemical compounds. Leukemia was observed in small numbers of untreated as well as treated GRS/A male mice. We conclude that leukemia incidences are not influenced appreciably by any of the treatments [7].

Our results indicate that, at least in GRS/A male mice, neither the development of hepatomas nor the spontaneous growth of lung tumours, is controlled by *cellular* lymphocyte functions. The immunosuppressive effect of HALS does not seem to play a significant role in the process of hepatoma formation as described in this paper. Comparison of survival data in groups 3 and 4 suggests that mice injected with the higher dose of NHS might have developed immunological tolerance [22] towards injected horse antigens, whereas the lower dose in group 3 probably did not induce immunological tolerance towards horse antigens.

With regard to the phenomenon reported in this paper it is of interest that different types of sera showed strongly diverging effects on the transformation-rate of cultured fibroblasts of some mouse strains (and not of others) when added to the growth medium [23–25]. Male horse serum was found to be more active in this respect when compared with fetal bovine serum or mare horse serum [23]. It has been suggested [26] that the active substance(s) in gelding horse serum is (are) of low molecular weight. It is known that a number of serum factors control growth of cultured cells [27, 28]; as yet no information is available on the nature of serum factors influencing processes of cellular transformation. In this connection it would be of interest to see whether purified horse serum fractions (*e.g.*, immunoglobulin-G) will give rise to hepatoma formation in GRS/A male mice or not.

Direct effects of HALS on mouse liver cells have been reported [29]; theoretically, a process of differential toxicity on GRS/A mouse liver cells might act to stimulate outgrowth of dormant tumour cells *in situ*. Although moderate liver damage in some animals dying within or in the two weeks following the injection period was observed in group 9, the occurrence of hepatomas in this and other groups was not related to the extent of liver damage at death. Hence, in our experiments differential toxicity on liver cells is thought to be of minor importance in hepatoma formation.

Evidence from our experiments that NHS and probably also HALS contain factors influencing tumour formation, might have consequences for the interpretation of those experiments in which the influence of ALS on carcinogenesis has been studied without inclusion of the appropriate control groups (untreated or treated with either control serum or PS).

Acknowledgements—We acknowledge the skilful assistance of Mr. B. De Graaf, Mr. R. Bloemraad (animal care), Ms. L. Kuiper and Ms. F. Nilant (histologic preparations).

REFERENCES

1. H. Balner, Reduced incidence of X-ray induced leukemia after treatment with anti-lymphocyte serum. *Rev. Europ. Etud. clin. et biol.* **16,** 981 (1971).
2. R. H. Denlinger, J. A. Swenberg, A. Koestner and W. Wechsler, Differential effect of immunosuppression on the induction of nervous system and bladder tumors by N-methyl N-nitrosourea. *J. nat. Cancer Inst.* **50,** 87 (1973).
3. E. M. Lance, P. B. Medawar and R. N. Taub, Antilymphocyte serum, In *Advances in Immunology* (Edited by F. J. Dixon and H. G. Kunkel) Vol. 17, p. 1(1973).
4. D. L. Vredevoe and E. F. Hays, Effect of antilymphocytic and antithymocytic sera on the development of mouse lymphoma. *Cancer Res.* **29,** 1685 (1969).
5. S. L. Nehlsen, Immunosuppression, virus and oncogenesis in mice. *Transpl. Proc.* **3,** 811 (1971).
6. N. A. Burstein and A. C. Allison, Effect of antilymphocytic serum on the appearance of reticular neoplasms in SJL/J mice. *Nature (Lond.)* **225,** 1139 (1970).
7. L. W. Law, Effects of antilymphocyte serum on the induction of neoplasms in lymphoreticular tissues. Discussion. *Fed. Proc.* **29,** 171 (1970).

8. L. Den Engelse, P. A. J. Bentvelzen and P. Emmelot, Studies on lung tumours. I. Methylation of deoxyribonucleic acid and tumour formation following administration of dimethylnitrosamine to mice. *Chem.-biol. Inter.* **1,** 395 (1969).
9. A. P. Monaco, M. L. Wood, J. G. Gray and P. S. Russell, Studies on heterologous anti-lymphocyte serum in mice, II. Effect on the immune response. *J. Immunol.* **96,** 229 (1966).
10. H. B. Andervont and T. B. Dunn, Transplantation of spontaneous and induced hepatomas in inbred mice. *J. nat. Cancer Inst.* **13,** 455 (1952).
11. P. Emmelot and C. J. Bos, Studies on plasma membranes, X. A survey of enzyme activities displayed by plasma membranes isolated from mouse liver and three mouse hepatoma strains. *Int. J. Cancer* **4,** 723 (1969).
12. C. F. Hollander, G. A. Boorman and C. Zurcher, Classification of malignant tumours in rodents. *Proc. 5th Perugia Quadrennial Int. Conf. on Cancer,* 1973 (In press).
13. L. Den Engelse, C. F. Hollander and W. Misdorp, A sex-dependent difference in the type of tumours induced by dimethylnitrosamine in the livers of C3Hf mice. *Europ. J. Cancer* **10,** 129 (1974).
14. P. M. Bolton, The effects of immune depression on chemical carcinogenesis in the rat, II. Antilymphocyte serum. *Oncology* **27,** 520 (1973).
15. A. G. Rabbat and H. F. Jeejeebhoy, Heterologous antilymphocyte serum (ALS) hastens the appearance of methylcholanthrene-induced tumours in mice. *Transplantation* **9,** 164 (1970).
16. C. D. Baroni, R. Scelsi, L. Peronace, S. Uccini and A. Cavallero, Effects of repeated injections of rabbit anti-mouse lymphocyte serum on tumors induced in mice by 7,12-dimethylbenz(a)anthracene given at birth. *Tumori* **59,** 1 (1973).
17. B. Toth, A critical review of experiments in chemical carcinogenesis using newborn animals. *Cancer Res.* **28,** 727 (1968).
18. G. Della Porta and B. Terracini, Chemical carcinogenesis in infant animals. *Progr. exp. Tumor Res.* **11,** 334 (1969).
19. H. Balner and H. Dersjant, Increased oncogenic effect of methylcholanthrene after treatment with anti-lymphocyte serum. *Nature (Lond.)* **224,** 376 (1969).
20. R. Truhaut and R. P. Dechambre, Modalités de l'induction chez la souris de tumeurs pulmonaires par le benzo-(a)-pyrène: influence de la dose d'hydrocarbure aromatique et de facteurs écologiques. *C.R. Acad. Sci. (D) (Paris)* **274,** 2263 (1972).
21. C. Biancifiori, Existence of a hormonal factor in pulmonary carcinogenesis in response to hydrazine. *Lav. Ist. Anat. Istol. Univ. Perugia* **29,** 29 (1969).
22. W. O. Weigle, Immunological unresponsiveness, In *Advances in Immunology,* Vol. 16, p. 61 (Edited by F. J. Dixon and H. G. Kunkel) (1973).
23. V. J. Evans, F. M. Price, K. K. Sanford, H. A. Kerr and S. L. Handleman, Comparative effects of mare, stallion, gelding horse, and fetal bovine sera on neoplastic transformation *in vitro. J. nat. Cancer Inst.* **49,** 505 (1972).
24. K. K. Sanford, J. L. Jackson, R. Parshad and R. R. Gantt, Evidence for an inhibiting influence of fetal bovine serum on "spontaneous" neoplastic transformation *in vitro. J. nat. Cancer Inst.* **49,** 513 (1972).
25. R. Parshad and K. K. Sanford, Search for genetic influences on neoplastic transformation and chromosomal stability of mouse cells cultured in medium supplemented with horse or fetal bovine serum. *J. nat. Cancer Inst.* **49,** 1155 (1972).
26. F. M. Price, R. R. Gantt and V. J. Evans, Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3Hf mouse cells in tissue culture. *In Vitro* **6,** 437 (1971).
27. R. W. Holley and J. A. Kiernan, Studies of serum factors required by 3T3 and SV3T3 cells. In *Growth Control in Cell Cultures* p. 3 (Edited by G. E. W. Wolstenholme and J. Knight). Churchill Livingstone, London (1971).
28. H. L. Leffert, Growth control of differentiated fetal rat hepatocytes in primary monolayer culture. V. Occurrence in dialyzed fetal bovine serum of macromolecules having both positive and negative growth regulatory functions. *J. Cell Biol.* **62,** 767 (1974).
29. J. C. Pisano, J. T. Patterson, R. Trejo, E. Hoffman, P. E. Lentz and N. R. Di Luzio, Hepatotoxic effects of horse anti-mouse lymphocyte serum. *Exp. mol. Pathol.* **16,** 302 (1972).

Europ. J. Cancer Vol. 12, pp. 107–111. Pergamon Press 1976. Printed in Great Britain

Time Dependent Cytotoxicity of Adriamycin and Daunomycin in Primary Cultures of Normal and Neoplastic Mammary Glands*

L. MORASCA, E. G. FOGAR-OTTAVIANO and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea 62—20157 *Milan, Italy*

Abstract—*Adriamycin and daunomycin exert a cytotoxic effect on primary cultures of mammary carcinoma in* $(C_3H \times O_{20})F_1$ *mice.*

This effect increases with the time of contact with cells and with the time elapsed between the end of the treatment and the evaluation of the effect.

Daunomycin appears to be more cytotoxic than adriamycin while both antibiotics are more active on the cancer cells than on cultures of lactating mammary glands.

Some known metabolites of daunomycin (the aglicones of daunorubicinol, daunomycin and 7-deoxydaunorubicinol) and the aglicone of adriamycin appear to be inactive on the mammary carcinoma in vitro. *Daunorubicinol shows instead an activity which is* 10–40 *times lower than daunomycin.*

INTRODUCTION

ADRIAMYCIN and daunomycin are two antibiotics of the antracycline group, with a relatively minor structural difference, i.e., the substitution of a hydrogen atom by a hydroxyl group [1]. Adriamycin has been found to be more active than daunomycin in several experimental tumors [2] and in human solid tumors [3]. The reason for this difference in activity is not known and therefore it is interesting to try and establish which experimental parameter may be relevant in producing this difference.

Previous studies have indicated that, if anything, daunomycin is more cytotoxic *in vitro* than adriamycin [4] but this finding may depend on the experimental conditions utilized.

Pharmacokinetic studies have shown that two antibiotics follow a biphasic pattern of disappearance from the plasma. A first phase is very rapid and corresponds to the entry into the various tissues including the tumor while the second phase is relatively slow and it probably represents the processes of metabolism and excretion [5].

These data suggest that the time of exposure of cancer cells to adriamycin and daunomycin may be extremely important in determining the extent of their antitumoral activity. Since no information is available on the effect of the length of exposure on cytotoxicity, nor on the interval between the end of the exposure and the onset of cytotoxicity, it was decided to investigate these aspects in an *in vitro* model. A mammary tumor not hormone dependent which develops spontaneously in force bred females or in castrated estrogen treated males of the $(C_3H \times O_{20})F_1$ mouse strain [6, 7] was utilized because this tumor is sensitive to both adriamycin and daunomycin (unpublished results).

The effect of the two antibiotics was studied not only on the tumor but also on the normal lactating mammary gland obtained from the same strain of mice and cultured *in vitro*.

Accepted 15 October 1975.

*This work was supported by NCI contract No. NIH-NCI-C-72-3242.

Furthermore, we investigated the possibility that some metabolites of adriamycin or daunomycin could contribute to the cytotoxic activity exerted by the two antibiotics *in vivo*.

MATERIAL AND METHODS

Tissues

Normal mammary glands were obtained from lactating $(C_3H \times O_{20})F_1$ mice. Tumor bearing animals were 9 month old females of the same strain [6, 7]. Both groups of animals were supplied by the International Center for the Provision and Study of Tumor Bearing Animals (Prof. O. Muhlbock, Amsterdam).

In vitro *procedure*

Each tumor was excised under ether anesthesia by aseptic techniques. Fragments, approximately 1 mm in size were dissected, washed in phosphate buffered saline and placed in Rose chambers under perforated cellpohane [8]. Incubation was performed at $36{\cdot}5 \pm 0{\cdot}5$°C in air. Culture medium was 199 buffered with Hepes and fortified with 20% calf serum.

In this experimental situation outgrowth started 48 hr after implantation. At this time representative microscopic fields were photographed and identified by a marker objective on the outside of the Rose chamber coverslip. Each chamber was then treated with adriamycin or daunomycin at concentrations ranging from 0·1 to 100 μg/ml. For each sample three microscopic fields were evaluated. The same fields were photographed again at the end of treatment and at the end of the recovery period.

By this method we were able to obtain four microscopic records of the same field: (a) before treatment; (b) at the end of treatment; (c) at 24 hr and (d) at 120 hr, following the end of the treatment. Then microscopic records were used to detect the destruction of the cell population in the recorded area (Lethal Effect—LE). The ratios between number of LEs reached and total experiments (LE/TE) performed were used to establish the LD_{50} concentration by the method of Spearman-Karber [9]. The cultures used for the statistical analysis were obtained from 18 separate experiments performed on tumors taken from different animals.

In other experiments the effect of adriamycin and daunomycin were studied in more detail. The antibiotics were added at various concentrations for one hour. The chambers were then washed and the behavior of the cultures was followed up to 144 hr. The score of the activity of the two antibiotics was made on the surface of cultures having 2 mm of diameter, according to the following scale:

0 = normal epithelium
1 = alterations of cellular shape
2 = reduction of outgrowth layer
3 = fragmentation of outgrowth layer with necrotic elements
4 = few cells surviving (< 10 cells)
5 = no surviving cells.

Adriamycin, daunomycin and their possible metabolites daunorubicinol, and aglicones of daunorubicinol, 7-deoxydaunorubicinol, daunomycin and adriamycin, were kindly donated by Farmitalia, Milan, Italy.

RESULTS

Table 1 reports the LD_{50} and the 0·05 confidence limits of adriamycin and dauno-

Table 1. *C_3H mammary cancer LD_{50} μg/ml after various times of treatment and recovery*

Treatment	Time (hr)	Recovery at end of treatment 0 hr	24 hr	120 hr
Adriamycin	1	> 100	88 (18–437)	2·8 (1·5–5·5)
	8	24 (17–35)	6·8 (4·0–11·5)	3·16 (1–10)
	16	21 (14–33)	3·6 (2·8–4·7)	2·3 (1·5–3·6)
	24	2·9 (1·6–5·3)	2·2 (1·3–3·8)	0·6 (0·3–1·3)
Daunomycin	1	> 100	21 (14–33)	1·3 (0·7–2·2)
	8	15 (7–25)	2·4 (1·7–3·5)	1·1 (0·6–2·0)
	16	3·6 (2·8–4·6)	3·16 (1–10)	0·8 (0·4–1·3)
	24	1·3 (0·7–2·2)	0·8 (0·4–1·3)	0·1 (0·06–0·17)

Means and 0·05 confidence limits.

mycin on the spontaneous mammary tumor cultivated *in vitro*. Both drugs became more cytotoxic (decrease of LD_{50} concentrations) as a function of the time of contact with cells (treatment) and as a function of the time elapsed between the end of the treatment and the evaluation of the effect (recovery period).

In fact for adriamycin the LD_{50} decreases from > 100 μg/ml to 2·9 μg/ml when the treatments were prolonged from 1 to 24 hr. The same level of cytotoxicity recorded after 24 hr of treatment was present also when the treatment was limited to 1 hr but the recovery period was 120 hr.

The LD_{50} values recorded after 120 hr recovery appear to be similar when the treatments ranged from 1 to 16 hr but the 24-hr treatment permitted us to obtain a sharp decrease of the LD_{50} concentration.

Daunomycin shows the same pattern of activity but this antibiotic appears to be consistently more cytotoxic than adriamycin in each tested experimental condition. The results were not different if the total time of treatment plus recovery was kept constant at 144 hr. Other experiments not reported in detail showed that the cytotoxic effect of both antibiotics was present even when the time of contact was limited to 5 min followed by a period of 120 hr of recovery. No differences in the level of cytotoxicity were recorded in absence of serum nor in Leighton tube cultures.

Table 2 reports in the same experimental conditions the cytotoxic effect of a number of possible metabolites of adriamycin and daunomycin. It is evident that with the possible exception of daunorubicinol, the other derivatives if they are found *in vivo* are probably not important in sustaining the cytotoxic effect of the two antibiotics. The analysis of the morphological effects of the two drugs carried out in more detail is reported in Table 3,

Table 2. LD_{50} on primary cultures of the mammary cancer $(C_3H \times O_{20})F_1$ mice*

Treatment	Length of treatment (hr)			
	1	8	16	24
Daunorubicinol	32 (10–100)†	14·7 (6·8–31·6)†	14·7 (7·6–28·2)†	4·3 (2·8–6·5)†
Daunorubicinol aglicone	> 100	100	100	> 100
Daunomycin aglicone	> 10	—	—	> 10
7-Deoxydaunorubicinol aglicone	> 10	—	—	> 10
Adriamycin aglicone	> 10	—	—	> 10

*The lethal effects have been measured 120 hr after the end of treatment.
†0·05 confidence limits.

Table 3. Increase of the toxicity of adriamycin and daunomycin as function of recovery time of spontaneous mammary tumor cultivated in vitro

Treatment	Recovery hours			
	12 (hr)	96 (hr)	120 (hr)	144 (hr)
Controls	0	0	0·06	0·12
Adriamycin				
0·2 μg/ml	0	2	2·5	3·5
0·4	1	2	3	3·5
0·8	1	2·5	3·5	4·5
1·6	3	4·5	4·5	4·5
3·2	3	4·5	4·5	5
6·4	5	5	5	5
Daunomycin				
0·2 μg/ml	0	0·5	1·5	2
0·4	0	1	1·5	2
0·8	1	1·5	3	3
1·6	1	3	4	4
3·2	3	4·5	4·5	5
6·4	5	5	5	5

For the meaning of this score see Material and Methods.

Table 4. ($C_3H \times O_{20}$) Mice lactating mammary gland LD_{50} μg/ml after various lengths of treatment and recovery

Treatment	Time (hr)	Recovery at end of treatment 0 (hr)	24 (hr)	120 (hr)
Adriamycin	1	> 100	> 10	> 10
	8	> 10	> 10	> 10
	16	> 10	> 10	0·7 (0·15–3·1)
	24	6·8 (3·2–14·7)	5·3 (2·1–13·3)	0·5 (0·09–3·2)
Daunomycin	1	> 100	21 (10–46)	5·4 (1·4–21·2)
	8	> 10	> 10	0·3 (0·04–2·8)
	16	> 10	> 10	0·3 (0·04–2·8)
	24	4·1 (1·5–11·6)	2·4 (0·9–6·8)	1·1 (0·5–2·5)

Means and 0·05 confidence limits.

which gives the sequence of events underlying the cytotoxic effect of the two antibiotics.

Table 4 reports the LD_{50} evaluated in cultures of normal lactating mammary glands of the same strain of animals. It is evident that both antibiotics are less active as inhibitors of the growth of the normal mammary gland cultured *in vitro* in respect to the cancerous tissue. In fact daunomycin after 24 hr of treatment shows a particularly significant difference. After 120 hr recovery this treatment gave an LD_{50} ranging from 0·5 to 2·5 μg/ml for the normal tissue in comparison to the range of 0·06 to 0·17 obtained for the cancer tissue.

DISCUSSION

The experiments performed on a mammary tumor cultured *in vitro* indicate that daunomycin is more cytotoxic than adriamycin. This finding is in contrast with the fact that adriamycin is *in vivo* more active on this experimental tumor (unpublished data), but it is in agreement with data obtained *in vitro* on HeLa cells showing that daunomycin is more active than adriamycin in depressing cellular growth and in decreasing the uptake of tritiated thymidine and uridine [4]. The effect on the mammary tumor is higher for both antibiotics in respect to their effect on the normal lactating mammary gland. An interesting observation is represented by the fact that the cytotoxicity depends not only on the time of contact but also on the time elapsing between the washing of the cells from the medium containing the antibiotics to the time of the morphological evaluation. That the action of adriamycin depends on the length of the treatment has been previously shown with fast growing cells [10] while in our case the cancer cells were kept under experimental conditions not conducive to their growth.

These findings indicate that the evaluation *in vitro* of the cytotoxic effect of drugs such as adriamycin and daunomycin may be misleading unless different times of incubation with the drug and different times of recovery are considered. Furthermore, they point out the difficulty in correlating the results *in vitro* with the results *in vivo* where kinetic aspects of drug distribution and metabolism expose the tumor to concentrations of drugs which are different in relation to the time of administration. It should be pointed out in this respect that the available metabolites of daunomycin as well as the aglicone of adriamycin show very little cytotoxic activity in respect to the parent compounds and therefore they are not likely to play any role in the antitumoral activity of both antibiotics. The fact that only a short contact of the cells with adriamycin and daunomycin is sufficient to elicit a marked antitumoral activity indicates that there must be a rapid uptake of these antibiotics into the cancer cells. Furthermore the uptake of adriamycin and daunomycin must occur also in cells which are not in the phase of mitosis. The drugs must be stored in these cells long enough so as to become active when the cells enter mitosis. This may explain why the activity of adriamycin and daunomycin increases with the time after substituting the medium containing the antibiotics with a normal one.

The rapid entry into the cells of adriamycin and daunomycin occurs also *in vivo* considering that plasma levels of both compounds decrease rapidly during the first 15 min after the treatment [5]. Obviously this rapid uptake concerns

not only the tumor but also the normal tissues (DONELLI, in press).

The observations made in this study may be of importance in trying to define a threshold for the concentration of adriamycin and daunomycin showing cytotoxic activity to be confronted with concentrations actually present in the tumor *in vivo*.

Acknowledgements—The technical assistance of Mrs. Anna Lisa Cambini is gratefully appreciated.

REFERENCES

1. F. ARCAMONE, G. FRANCESCHI, S. PENCO and A. SELVA, Adriamycin (14-hydroxydaunomycin) a novel antitumor antibiotic. *Tetrahedron Lett.* **13,** 1007 (1969).
2. A. DI MARCO, Adriamycin: the therapeutic activity on experimental tumors. In *International Symposium on Adriamycin.* (Edited by S. K. CARTER, A. DI MARCO, M. GHIONE, I. N. KRAKOFF and G. MATHÉ) p. 53. Springer-Verlag, Berlin (1972).
3. N. R. BACHUR, Interdisciplinary session report: current status of studies with adriamycin (NSC-123127). *Cancer Chemother. Rep.* **4,** 47 (1973).
4. R. SILVESTRINI, C. GAMBARUCCI and T. DASDIA, Attività biologica dell'adriamicina *in vitro*. *Tumori* **56,** 137 (1970).
5. D. W. YESAIR, E. SCHWARTZBACH, D. SHUCK, E. P. DENNIE and M. A. ASHELL, Comparative pharmacokinetics of daunomycin and adriamycin in several animal species. *Cancer Res.* **32,** 1177 (1972).
6. O. MÜHLBOCK, Biological studies on the mammary tumor agent in different strains of mice. *Acta Un. int. Cancr.* **12,** 665 (1956).
7. W. G. HOAG, Spontaneous cancer in mice. *Ann. N.Y. Acad. Sci.* **108,** 805 (1963).
8. G. G. ROSE, C. M. POMERAT, T. O. SHINDLER and J. B. TRUNNEL, A cellophan-strip technique for culturing tissue in multipurpose culture chambers. *J. biophys. biochem. Cytol.* **4,** 761 (1958).
9. D. J. FINNEY, *Probit Analysis.* Cambridge University Press, Cambridge (1952).
10. B. DREWINKO and J. A. GOTTLIEB, Survival kinetics of cultured human lymphomas cells exposed to adriamycin. *Cancer Res.* **33,** 1141 (1973).

Europ. J. Cancer Vol. 12, pp. 113–123. Pergamon Press 1976. Printed in Great Britain

Effect of *Corynebacterium parvum* on Cellular and Humoral Antitumoral Immune Effector Mechanisms*

ALBERTO MANTOVANI,† ALDO TAGLIABUE, ANNUNCIATA VECCHI and FEDERICO SPREAFICO‡

Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea 62—20157 *Milan, Italy*

Abstract—*In an attempt to characterize the mechanism of action of* Corynebacterium parvum (C. parvum) *an analysis was performed of the changes in specific antitumoral immune effector mechanisms in C_3H mice showing increased protection against challenge with high inocula of the allogeneic L*1210 *leukemia after treatment with this immunoadjuvant.* C. parvum*-treated spleens displayed an increase in DNA synthesis, total cell number and in the percentage of macrophages; the percent of theta bearing lymphocytes was concomitantly decreased whereas no modification in the percent of EA-rosette forming cells was observed. Although the efficiency of target cell lysis by immune splenocytes was unchanged, the raise in spleen cellularity of* C. parvum*-treated mice resulted in a marked increase in this organ's capacity to express cell-mediated cytotoxicity. Treatment with this adjuvant produced also a sizable increase in the spleen capacity to mediate antibody-dependent cellular cytotoxicity whereas arming and potentiating serum activities and the levels of complement-dependent cytolytic antibody were not modified.*

INTRODUCTION

THE POTENTIAL of *Corynebacterium parvum* (*C. parvum*) in the immunotherapy of experimental tumors has been demonstrated in various studies [1–8] and this agent has also proven of value in the treatment of human neoplastic conditions [9]. The exact mechanisms through which *C. parvum* can induce tumor growth retardation are still not completely clarified. The powerful stimulation of the reticuloendothelial system by *C. parvum* has been repeatedly confirmed [10–12] and evidence has been presented supporting the contention that in the antitumoral effect of this compound a primary role is played by increases in non specific resistance through an activation and multiplication of macrophages [7, 13–15]. However, the possibility that this agent also induces a stimulation of specific immune responses could not be ruled out especially since a detailed analysis of the changes in humoral and cellular antitumoral immune effector mechanisms induced by *C. parvum* is still lacking.

In this study, an experimental system in which treatment with *C. parvum* conferred increased protection against challenge with high inocula of an allogeneic tumor has been employed for evaluating the effects of this agent on cell-mediated cytotoxicity (CMC) and the capacity to mediate antibody-dependent cellular cytotoxicity (ADCC) by spleen cells as well as on complement-dependent, arming and potentiating serum activities.

MATERIAL AND METHODS

Animals and tumor

Female C_3H mice (Charles River, Italy; 22 ± 2 g of body weight) were used throughout. The mice were injected intraperitoneally on

Accepted 14 October 1975

*This work was supported by CNR Contract 74-00648-04 and Euratom Contract 088-72-1-BIA C.

†Alberto Mantovani was supported by a fellowship from the Italian Association Against Leukemias.

‡To whom reprint requests should be addressed.

day 0 with 15×10^6 cells of the allogeneic L1210 leukemia which had been maintained by weekly i.p. passages in compatible DBA/2 hosts. Leukemia cell viability was assessed microscopically by dye exclusion and only preparations showing over 95% viability were used for transplantation.

Drug

C. parvum (Lot No. PX 374) was obtained from Wellcome Research Laboratories, Beckenham, England, and a dose of 0·7 mg was injected intravenously at different times before tumor allografting.

Sera

Blood was collected from the retroorbital plexus of 4–6 mice per group on various days after tumor transplantation; the sera (henceforth referred to as S6, S7, etc. according to the day of collection) were heat inactivated (56°C for 30 min) and stored at −20°C until used.

Spleen cell populations

An anti-theta C_3H serum prepared in AKR/J mice [16] was used to determine the percentage of T cells on preparations from individual spleens resuspended after twice washing in RPMI 1640 medium (Biocult, Scotland) supplemented with 10% fetal calf serum (FCS). Specifically, 2×10^5 spleen cells in 50 μl RPMI-FCS were incubated for 45 min at 37°C with 50 μl of 1:8 anti-theta serum and 50 μl of 1:4 diluted guinea pig serum previously absorbed with agarose [17] as a source of complement. The number of viable cells was counted by Trypan blue exclusion on 5 replicates per test; in the conditions described the anti-theta serum killed > 95% of C_3H thymocytes and < 5% of bone marrow cells. Identification of leukemic cells in spleen cell suspensions was routinely performed using morphologic criteria after preliminary experiments in which the same suspensions were also treated with cytotoxic C_3H anti-L1210 serum had confirmed the reliability of this approach.

The percentage of EA-rosette forming cells (EA-RFC) was determined as described by Kedar *et al.* [18] employing sheep erythrocytes (E) sensitized with rabbit anti-E serum. At least 200 cells per sample were scored with 5 replicates per test; those elements having 3 or more erythrocytes bound at their periphery were considered EA-RFC.

The percentage of spleen macrophages was evaluated by a modification of the procedure of Evans [19]. Spleen cells (2×10^6/ml RPMI-FCS) were incubated in a hemocytometer for 60 min at 37°C in a humidified 95% air–5% CO_2 atmosphere in the presence of carbon particles [20]; the hemocytometer was then immersed in Hank's and the floating coverslips lifted off, thus removing non-ingested carbon and non-adhering cells. The number of adherent cells which had ingested carbon particles was then determined microscopically. Depletion of macrophages from spleen cell preparations was performed by the carbonyl iron method [21].

DNA synthesis in vivo

Mice were injected i.p. with 50 μCi of thymidine-methyl-^{3}H(^{3}H-TdR; The Radiochemical Center; specific activity 18·4Ci/mmol) 1 hr before sacrifice. After twice washing, 10^7 spleen cells in 1 ml PBS were layered on 0·8 μ filters (Sartorius GMBH) in a Millipore multimanifold sample collector. Filters were then submitted to 3 washings with 5 ml PBS and 1 with 5% cold trichloroacetic acid, dried overnight and radioactivity then determined in a liquid scintillation counter. At least 3 replicates were counted per spleen with 5 animals per group per time.

Cell-mediated cytotoxicity

A modification of the ^{51}Cr release assay described by Brunner *et al.* [22] was used and experimental conditions were as previously described [23, 24]; 3 replicates per individual spleen were used with 5 spleens per experimental group. Specific cytotoxicity was calculated by subtracting the percent isotope release in the presence of normal splenocytes which averaged 12·5 ± 1·5% in different experiments.

Antibody-dependent cellular cytotoxicity

In this study a separate analysis was performed of *C. parvum* effects on serum potentiating and arming activity and on the capacity of the spleen cells to mediate ADCC. The effects of *C. parvum* treatments on ADCC-effector cells were investigated by assessing the cytotoxic capacity of control and test splenocytes in the presence of reference C_3H anti-L1210 ADCC-positive sera. To tubes containing labelled target cells (5×10^4 in 1 ml RPMI-FCS), 0·1 ml of 1:10 diluted normal or reference serum were added followed by 5×10^6 splenocytes in 0·2 ml RPMI-FCS from control or *C. parvum*-treated mice. Results presented are means of 3 replicates per individual spleen employing 5 animals per experimental group. Reference ADCC-positive sera (S15, S29, etc.) were obtained on various days after the i.p. transplantation of 5×10^6 L1210 cells into C_3H mice

and their arming and potentiating activities characterized as described below. An index expressing the total spleen capacity to mediate ADCC was calculated from the formula:

(% specific cytotoxicity × No. × 10^6 of spleen cells)/100, where per cent specific cytotoxicity in this case is the difference between the isotope released by spleen cells in the presence of ADDC-positive and normal C_3H serum.

For the evaluation of serum arming activity, 0·1 ml of the test serum was mixed with 5×10^4 L1210 cells in 1 ml RPMI-FCS and splenocytes from normal C_3H donors added at an attacker to target cell (A:T) ratio of 100:1. For serum potentiating activity, immune splenocytes were employed, i.e. cells harvested from C_3H mice 7 days after the i.p. inoculation of 5×10^6 L1210 cells, a challenge which is resisted by 100% of the C_3H hosts. Since the same serum may result in a potentiation or in a blocking of CMC depending on the A:T ratio employed in the assay [25], for evaluating serum potentiating activity a range of A:T ratios (20:1 to 100:1) was used.

In the absence of lymphoid cells all sera employed in these studies did not modify the isotope release from target cells as compared to normal C_3H serum, thus excluding the presence of feeder or toxic effects.

Complement-dependent cytolytic antibody

Conditions of testing were as described in reference 24. The mean percentage of isotope release in the presence of normal serum and complement was subtracted and the highest serum dilution still giving at least 20% specific cytotoxicity taken as cytotoxic antibody titer.

Statistical analysis

Statistical significance of *in vitro* data was assessed by analysis of variance, multiple tests, and *in vivo* results were analyzed by Fisher's exact test.

RESULTS

Effect of C. parvum *on allograft rejection*

When an inoculum of 15×10^6 cells of the allogeneic L1210 leukemia was given i.p. to C_3H mice, from 50 to 65% of the recipients succumbed to progressive tumor growth with a mean survival time of 9·2 ± 2·0 days (Table 1). If the C_3H recipients were pretreated with single i.v. injections of *C. parvum*, either increased or decreased protection against this leukemia challenge could be observed depending on the day of immunoadjuvant injection.

Table 1. *Effect of* C. parvum *on tumor allograft rejection*

C. parvum	Day of treatment	MST*	D/T†
−	—	9·2 ± 2·0	12/20
+	−2	10·4 ± 2·5	16/20
+	−4	—	2/20§
+	−5	10·5 ± 1·3	4/20‡
+	−9	10·4 ± 3·1	20/20§

*Mean survival time in days ± S.E.
†Dead with leukemia over total number of mice injected.
‡$P < 0·05$.
§$P < 0·01$.

In fact when *C. parvum* was given 4 or 5 days before transplantation, from 90 to 100% of the hosts were capable of rejecting the tumor whereas when the treatment preceded leukemia inoculation by 9 days an enhancement of tumor takes was observed since 100% of the animals died of leukemia versus 60% of the controls. Administration of *C. parvum* 2 days before tumor transplantation either had no effect or was followed by a slightly increased incidence of progressive tumor takes.

In view of the fact that the highest antitumoral resistance in these conditions was seen after injecting *C. parvum* 4 days before leukemia allografting, this schedule of treatment was selected for subsequent analysis of the immunological modifications accompanying protective treatments with *C. parvum.*

Effect on spleen cell populations

Injection of *C. parvum* induced marked changes in the spleen cellularity of both normal and tumor-transplanted mice (Fig. 1). In the former group the number of mononuclear spleen cells was significantly increased already 3 days after treatment with a peak reached on day 5 at values which were approximately

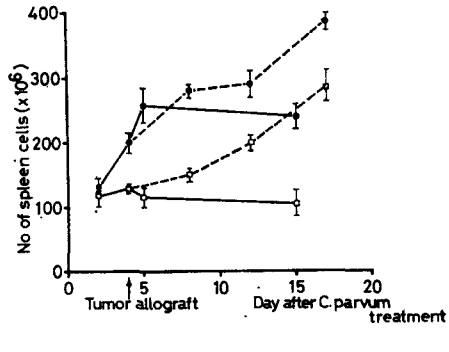


Fig. 1. *Spleen cell numbers in control* (□) *and* C. parvum-*treated* (●) *mice. Continuous and dotted line represent normal and tumor allografted mice respectively.*

130% higher than controls, and no significant decreases in spleen cellularity were observed up to day 15 when observation was discontinued. In tumor-allografted control mice, a progressive increase in spleen cell number was observed from day 4 after leukemia inoculation and on day 15 the values found were still 140% above control level; a similar pattern was seen in those mice which had been pretreated with *C. parvum*, the difference between control and stimulated animals averaging approximately 100 million cells throughout the period of observation. Values presented in Fig. 1 have been subtracted of the leukemia cells present in the organ, which never exceeded a value of 5% in these conditions.

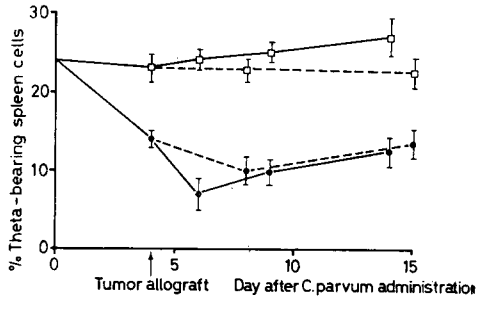


Fig. 2. Percent theta-bearing cells in control (□) and C. parvum*-treated (●) spleens. Continuous and dotted line represent normal and tumor allograft mice respectively.*

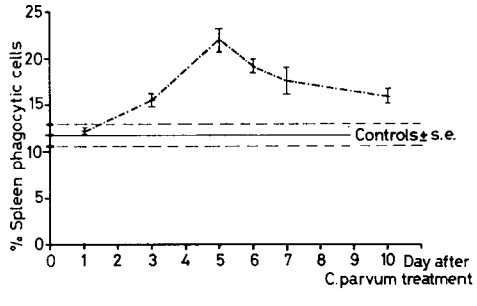


Fig. 3. Percent macrophages in non-tumor allografted mice, treated with C. parvum.

The percentage of theta-bearing spleen cells was also evaluated in these animals (Fig. 2). It was observed that treatment with *C. parvum* was followed in both normal and tumor-allografted mice by a consistent and marked decrease in the percentage of T cells which reached its nadir (7%) 6 days after adjuvant injection. Although a tendency to an increase in this value was present at later times, on day 15 after *C. parvum* the percent of theta-positive cells in the spleen was still about one half (12·5%) of control values. As can be seen in Fig. 2, the changes observed in normal mice were practically indistinguishable from those seen in allografted hosts. Taking into account the increase in organ cellularity present in *C. parvum*-treated mice, the total number of theta-bearing splenocytes was not however significantly changed in respect to normal values.

In normal, untreated mice the percentage of spleen macrophages was found to be $11{\cdot}8 \pm 1{\cdot}8\%$; treatment with *C. parvum* resulted in an increase in the percentage of phagocytic-adherent cells which was detectable already 3 days after drug injection (Fig. 3), reached a peak on day 5 ($22 \pm 1{\cdot}4\%$) and slowly declined thereafter. Results obtained in tumor-transplanted, adjuvant-pretreated mice were qualitatively and quantitatively similar. Considering the increase in organ cellularity following *C. parvum* injection, the total number of phagocytic elements in treated spleens was greater than is apparent from Fig. 3. Indeed, on day 5 the number of macrophages in adjuvant-treated organs was calculated to be $31{\cdot}5 \pm 3{\cdot}3 \times 10^6$ as compared to $12{\cdot}4 \pm 2{\cdot}1 \times 10^6$ of the controls.

Table 2. EA-rosette forming cells in the spleen of control and C. parvum*-treated mice*

Day after tumor allograft*	Experimental group	% EA-RFC
0	Control	$42 \pm 1{\cdot}2$
	C. parvum	$43 \pm 1{\cdot}3$
3	Control	$49 \pm 1{\cdot}5$
	C. parvum	$48 \pm 1{\cdot}9$
7	Control	$50 \pm 1{\cdot}1$
	C. parvum	$52 \pm 2{\cdot}1$

**C. parvum* was injected 4 days before challenge with allogeneic tumor cells.

As shown in Table 2, no differences were observed in the percentage of EA-RFCs between control and *C. parvum*-treated animals, whether tumor allografted or not. Therefore, taking as average a value of 100% for the increase in spleen cellularity given by the *C. parvum* treatment employed, the total number of EA-RFCs in this organ was in effect approximately doubled during the 5–15 day period after agent injection.

In order to evaluate the role played by cell proliferation in the observed rise in spleen cell numbers in adjuvant-treated mice, *in vivo* DNA synthesis was investigated in these animals. Figure 4 shows that a slight (22%) though significant ($P < 0{\cdot}05$) increase in ^{3}H-TdR uptake was present already one day after *C. parvum*; the maximum was found on day 2 with levels which were approximately 90% higher than baseline values, thereafter a tendency

towards a gradual decline in ^{3}H-TdR uptake was recognizable.

Effect on immune effector mechanisms

The first immune parameter examined in these mice showing increased protection against allogeneic tumor transplants after *C. parvum*, was spleen CMC. Since it had been reported [7, 14, 15] that cellular cytotoxicity could be non specifically stimulated by *C. parvum* also in non-tumor transplanted animals, a group of these animals was also included in the study. Splenocytes harvested 4 days after treatment with *C. parvum* of normal C_3H mice did not show any significant cytotoxic capacity towards L1210 cells, the values observed being indistinguishable from background values (Table 3). Similar results were obtained with cells collected up to 12 days after *C. parvum* administration.

Spleen anti-L1210 CMC was than evaluated

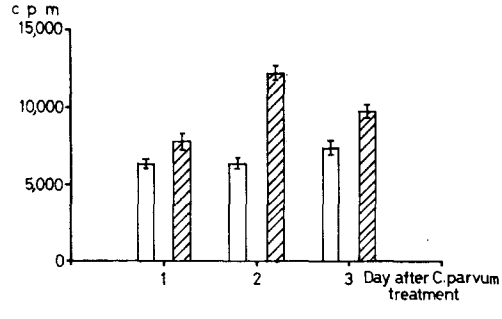


Fig. 4. ^{3}H-TdR incorporation in control (white column) and C. parvum*-treated (hatched column) spleens.*

in tumor allografted mice pretreated or not with *C. parvum*; no significant differences in cytotoxic capacity between the 2 groups of animals could be observed (Table 3) at any of the times examined over the day 5–30 range investigated and employing A:T ratios of 100:1 to 500:1. When splenocytes from both groups were tested against the EL_4 leukemia employed as a specificity control, ^{51}Cr release did not rise above background values. Since the total number of spleen mononuclear cells was markedly increased in *C. parvum*-treated hosts, the total spleen CMC capacity was in effect significantly higher in these animals showing an increased tumor resistance after *C. parvum* treatment. Prior removal of phagocytic cells from these splenocyte suspensions did not reduce their cytotoxic activity (Table 4); it may be noted on the contrary that, in some experiments, a significantly increased cytotoxic capacity could be seen in phagocyte-deprived cellular preparations.

The effects of *C. parvum* on the cellular and

Table 3. Spleen CMC in control and C. parvum*-treated mice*

Day after tumor allograft	Experimental group	A:T Ratio	% Specific cytotoxicity (± S.E.)
0	Control	100:1	—
		500:1	—
	C. parvum	100:1	—
		500:1	—
5	Control	100:1	4·3 ± 1·2
		500:1	5·9 ± 0·9
	C. *parvum**	100:1	2·0 ± 0·6
		500:1	3·9 ± 1·1
7	Control	100:1	9·9 ± 0·5
		500:1	18·3 ± 2·1
	*C. parvum**	100:1	7·3 ± 1·4
		500:1	18·6 ± 0·8
13	Control	100:1	10·4 ± 2·5
		500:1	27·9 ± 3·0
	*C. parvum**	100:1	14·2 ± 3·1
		500:1	31·9 ± 2·8
15	Control	100:1	14·4 ± 1·8
	*C. parvum**	100:1	10·3 ± 2·5
30	Control	100:1	13·6 ± 0·8
	*C. parvum**	100:1	10·9 ± 0·9

**C. parvum* was injected 4 days before challenge with allogeneic tumor cells.

humoral arms of ADCC were then examined. When the capacity of spleen cells from adjuvant-injected, non-tumor-allografted hosts to mediate ADCC in the presence of a reference arming serum (S29) was investigated, significantly higher cytotoxicity values were observed than seen with untreated controls (Table 5). Moreover, significant cytotoxicity was detected also when splenocytes from *C. parvum*-treated mice were tested in the presence of a late serum (S64) unable in the conditions employed to render cytotoxic normal C_3H spleen cells.

Table 4. Effect of removal of phagocytic cells on spleen cell-mediated cytotoxicity

Experimental group*		Treatment with carbonyl iron	% Specific cytotoxicity
Exp. 1	Control	−	14·4 ± 0·9
	Control	+	34·4 ± 3·0†
	C. parvum	−	10·3 ± 0·4
	C. parvum	+	12·7 ± 0·7
Exp. 2	Control	−	13·6 ± 1·1
	Control	+	13·4 ± 1·8
	C. parvum	−	10·9 ± 1·5
	C. parvum	+	14·8 ± 1·3

**C. parvum* was injected 4 days before tumor allograft. Spleen cells were collected 15 days (exp. 1) or 18 days (exp. 2) after tumor transplantation.

†$P < 0{\cdot}01$ versus untreated control spleen cell preparations.

Table 5. *Effect of* C. parvum *on spleen effector cells involved in antibody-dependent cellular cytotoxicity*

Splenocytes	Arming serum	% Specific cytotoxicity	No. cells/ spleen ($\times 10^6$)	Spleen ADCC activity index
Control*	S29	4·6 ± 0·4†	128·6 ± 3·8	5·9
*C. parvum**	S29	9·2 ± 0·4†‡	198·6 ± 13·1	18·3
Control*§	S29	4·2 ± 0·4†	103·0 ± 10·1	4·3
*C. parvum**§	S29	8·3 ± 0·3†‡	160·5 ± 19·3	13·4
Control*	S64	1·2 ± 0·8	114·8 ± 19·5	2·4
*C. parvum**	S64	4·8 ± 0·6†‡	258·0 ± 25·6	12·4
Control¶	S15	10·2 ± 0·9†	130·5 ± 15·2	13·2
C. parvum¶	S15	12·3 ± 1·4†	205·2 ± 18·8	25·2
Control	S15	11·7 ± 1·2†	94·6 ± 6·9	11·1
Sheep erythrocytes‖	S15	4·4 ± 0·9†	178·4 ± 22·7	7·8

*Spleen cells were collected from normal mice 6 days after treatment with *C. parvum*.
†$P < 0{\cdot}05$ versus samples containing normal C_3H serum.
‡$P < 0{\cdot}05$ versus control splenocytes.
§Phagocytic cells were removed by carbonyl iron.
¶Spleen cells were collected 6 days after tumor allograft, i.e., 10 days after *C. parvum*.
‖4×10^8 sheep erythrocytes were injected i.p. 5 days before testing.

Results presented in Table 5 were obtained using spleen cells collected 6 days after *C. parvum* injection, but no qualitative differences were seen when cells harvested from 5 to 10 days after adjuvant injection were tested or other arming sera employed. Considering the increase in spleen cell numbers present in *C. parvum*-treated mice, the total capacity of this organ to mediate ADCC was increased to a greater extent than it would appear from the *in vitro* data. The spleen ADCC index was in fact more than tripled in respect to control on day 6 and for days 5, 8 and 10 the calculated values were 17, 19 and 13 respectively. Table 5 also shows that increases in spleen cell numbers of a degree comparable to that given by *C. parvum* could be seen after the i.p. injection of 4×10^8 sheep erythrocytes; however, the spleen ADCC index computed for this group of animals was not significantly different from normal control levels. From the same table it can also be observed that previous removal of phagocytic cells did not modify the ADCC-mediating capacity of spleen cell preparations from control and *C. parvum*-treated mice.

At variance with non-tumor-transplanted mice, in leukemia allografted mice pretreated with *C. parvum* the capacity of splenocytes to mediate ADCC in the presence of a potentiating serum was not significantly higher than found in the appropriate controls (Table 5). Again, however, the total spleen ADCC indexes were markedly higher taking into consideration the raised cell number present in this organ.

Finally, the effects of *C. parvum* treatment on the serum cytolytic, arming and potentiating activities were investigated. As shown in Table 6, where the results of 2 representative experiments are pooled, no significant differences were observed between adjuvant-treated and untreated mice in the levels of complement-dependent cytolytic antibody in sera taken from 6 to 13 days after tumor transplantation. In these experimental conditions a significant serum arming activity was first detected in

Table 6. *Anti-L*1210 *complement-dependent cytolytic antibody levels in control and* C. parvum*-treated mice*

Day after tumor allograft	Experimental group	Antibody titer
6	Control	1:2
	*C. parvum**	1:2
7	Control	1:4
	*C. parvum**	1:4
11	Control	1:32
	*C. parvum**	1:32
13	Control	1:128
	*C. parvum**	1:128

**C. parvum* injected 4 days before tumor transplantation.

control mice 8 days after tumor inoculation, increasing progressively thereafter until day 13 when observation was discontinued (Fig. 5); no differences in the kinetics of the response nor in the levels of this serum activity were observed in adjuvant-injected hosts. Results presented in Fig. 5 were obtained employing final serum dilutions of 1:15, but no differences between control and *C. parvum* sera were detected with lower (1:7) or higher (up to

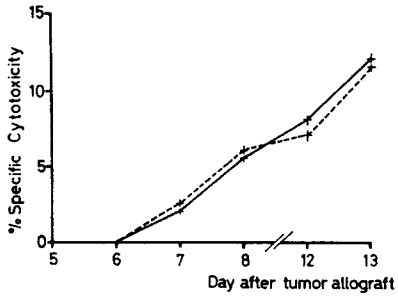


Fig. 5. *Serum arming activity in control (continuous line) and* C. parvum*-treated (dotted line), tumor-allografted.*

1:60) serum dilutions. Potentiating activity was also evaluated in the same sera, employing for these tests A:T ratios ranging from 20:1 to 100:1; representative results employing S8 are given in Table 7. It can be seen that when a low A:T ratio was used, serum from adjuvant-treated and untreated donors potentiated CMC to a similar extent, showing 5–6% increases in specific cytotoxicity. At a higher (100:1) A:T ratio neither *C. parvum* nor control sera significantly modified the cytotoxic capacity displayed by immune splenocytes. Results qualitatively similar to those just described were obtained employing sera taken 7, 9 and 11 days after tumor transplantation.

DISCUSSION

The results obtained in this study confirm previous observations that treatment with *C. parvum* can increase host's antitumor resistance. As described by others also in various syngeneic tumor models, in this system the conditions employed for adjuvant treatment were critical, since either no effect, increased protection or enhanced tumor takes could be seen depending on the relative timing between *C. parvum* injection and tumor transplantation. In this regard *C. parvum* does not therefore appear to be different from other immunomodulators such as BCG or Levamisole, agents for which a careful choice of treatment conditions must also be made in order to obtain an antitumoral effect [24, 26–29].

Table 7. *Serum potentiating activity in control and* C. parvum*-treated mice*

	% Specific cytotoxicity (± S.E.)	
Serum*	20:1†	100:1†
Normal	1·1 ± 0·9	58·9 ± 1·1
Control	6·7 ± 1‡	55·8 ± 1·4
C. parvum§	7·1 ± 1·2*	56·3 ± 1·3

*Serum was collected 8 days after tumor transplantation.
†A:T ratio.
‡$P < 0{\cdot}05$ versus normal serum.
§*C. parvum* was administered 4 days before tumor allograft.

The main purpose of this study was to investigate the modifications in immune parameters following a protective treatment with *C. parvum* in the effort to obtain a better knowledge of the mechanism(s) of action of this adjuvant. An increase in the number and functional capacity of macrophages has been proposed as playing the major role in the antitumoral activity of systemically injected *C. parvum* [6, 7, 11, 13], a well known reticulo-endothelial stimulant [10, 22]. In addition, peritoneal macrophages from normal animals treated with *C. parvum* have been reported to be cytostatic against a variety of tumor cells *in vitro* [7, 14, 15] as seen also with other stimulants such as BCG or endotoxin [30–32]. In support of the view that non specific mechanisms underly the antitumor effect of *C. parvum*, were also the facts that a depression of T-cell function can be seen in animals given this adjuvant systemically [33] and that the antitumor effect of i.v. *C. parvum* could still be observed in T-cell deprived mice [34]. The finding, however, of a direct relationship between tumor immunogenicity and the protective effect of *C. parvum* and *C. granulosum* [3, 35] suggested that the antineoplastic activity of these agents involves other mechanisms in addition to non-specific stimulation of macrophages. More directly, the development of a systemic antitumor immunity has been reported to occur after injections of *C. parvum* [4, 8, 36]. The possibility that a stimulation of specific immune mechanisms plays a role also in the antitumoral effect of i.v. *C. parvum*, at least in allogeneic conditions, appears to be supported by the results presented here.

Considering first CMC, although the cytocydal efficiency of splenocytes from adjuvant-treated mice was not significantly higher than that of control cells, because of the increase in spleen cellularity the total capacity of this organ to attack tumor cells was in effect substantially raised and could thus have significantly contributed to the increased tumor resistance displayed by *C. parvum* injected animals.

In allogeneic tumor systems, both T and non-T cells appear to mediate CMC [37]; in view of the fact that splenocyte preparations from adjuvant-treated mice displayed the same *in vitro* cytocydal capacity of control cells in spite of a lower percentage of T cells, non-T elements would thus seem to contribute to

cytotoxicity in a larger measure than in controls. This observation is in keeping with previous evidence that B-cell responses are potentiated by *C. parvum* [38], whereas spleen T-cell responses such as PHA and mixed lymphocyte reactivity or GVH-inducing capacity [33] are depressed after i.v. injections of this agent. The lower percentage of T cells observable in lymphoid organs of *C. parvum*-treated mice could provide an explanation of this depressed reactivity, for which an interaction requiring cell contact between activated macrophages and T lymphocytes has also been proposed [39] as an additional mechanism. The latter mechanism could also explain our observations of a higher CMC in macrophage-depleted splenocyte preparations.

The increase observed in spleen ADCC-mediating capacity can also be regarded as an important factor in the higher antitumoral resistance given by *C. parvum* in our conditions, as this effector mechanism can indeed play a crucial role in tumor resistance [40, 41]. A similar increase in ADCC-mediating activity has been reported by Allison [42] following treatment with Freund's complete adjuvant. Consensus exists that T cells are not involved in mediating ADCC [43] but the precise nature of the relevant population(s) is still unclear [43, 44]. Our data showing a higher number of EA-RFCs in the spleens of *C. parvum*-treated mice displaying an increased ADCC effector activity, cannot resolve this point since this type of rosette can be formed by B and other types of cells, such as macrophages or granulocytes [18].

The possible role of macrophages in mediating the increased tumor resistance found in our conditions cannot be assessed with certainty. We could not confirm that the administration of *C. parvum* to normal animals is followed by the appearance of unspecific tumorocydal activity in the spleen [7, 15]. As previously mentioned, the removal of phagocytes from splenocyte preparations from allografted, adjuvant-treated mice did not impair their CMC and ADCC capacities. These results would apparently seem to argue against the possibility that macrophages acted as effector cells in these conditions. A definite conclusion on this point cannot, however, be reached; not only the short term cytotoxicity assay employed might not have provided the best conditions for revealing a cytocydal macrophage activity, but results obtained in the spleen may not be representative of the conditions operative in the peritoneal cavity where tumor rejection took place.

Whether on the other hand, the marked and prolonged rise in macrophage numbers found in the spleen after *C. parvum* plays a role in the inductive if not effector phases of antitumoral response remains also to be resolved.

Increased lymphocyte trapping has been reported to play a major role in the higher spleen cellularity given by *C. parvum* [45] and activated macrophages considered the principal mediators in this process [46]. Whether this occurred also in our conditions was not tested; if so it may be reasoned that non-T cells possibly constituted the majority of the lymphoid cells trapped in the spleen. From our data it is clear however that this immunoadjuvant also induced a marked degree of cell multiplication in this organ, whose kinetics paralleled the increase in cellularity. In this connection, the fact that the total number of T cells in the spleen did not change and that only a dilution occurred, may be taken as an indication that non-T elements and macrophages were the populations mostly undergoing multiplication.

Evidence has been presented that *C. parvum* can stimulate the antibody response to various types of antigens in normal and tumor-bearing animals [12, 47]. In our conditions, however, an increase in the humoral response to this tumor was not detectable and such a mechanism did not appear to significantly contribute to the higher capacity to reject an allogeneic leukemia induced by this adjuvant.

In conclusion, our results indicate that the enhanced resistance towards allogeneic neoplasms caused by *C. parvum* is accompanied by an increase in the total spleen CMC activity and ADCC-mediating capacity. Both these findings can be explained by an increase in the total number of non-T cells observable in these animals, which is, at least partially, the consequence of a higher cell multiplication occurring in the organ. These results thus indicate that after systemic injections of this adjuvant an augmentation of specific immune responses occurs and not only an activation of non specific mechanisms as proposed by others [7]. Whether these conclusions also hold true for syngeneic tumor systems is currently under investigation.

For its possible therapeutic implications, one point deserves comment, namely the possible interest in associating *C. parvum* treatments leading to a potentiation of ADCC-mediating cells with passive immunotherapy with ADCC-positive sera. Preliminary data have in fact given clear indications of therapeutic synergism employing this combined approach.

REFERENCES

1. B. N. Halpern, G. Biozzi, C. Stiffel and D. Mouton, Inhibition of tumour growth by administration of killed Corynebacterium parvum. *Nature (Lond.)* **212,** 853 (1966).
2. M. F. A. Woodruff and J. L. Boak, Inhibitory effect of injection of Corynebacterium parvum on the growth of tumour transplants in isogeneic hosts. *Brit. J. Cancer* **20,** 345 (1966).
3. S. E. Smith and M. T. Scott, Biological effects of Corynebacterium parvum: III. Amplification of resistance and impairment of active immunity to murine tumours. *Brit. J. Cancer* **26,** 361 (1972).
4. V. V. Likhite and B. N. Halpern, Lasting rejection of mammary adenocarcinoma cell tumors in DBA/2 mice with intratumor injection of killed Corynebacterium parvum. *Cancer Res.* **34,** 341 (1974).
5. V. V. Likhite and B. N. Halpern, The delayed rejection of tumors formed from the administration of tumor cells mixed with killed Corynebacterium parvum. *Int. J. Cancer* **12,** 699 (1973).
6. J. E. Castro, Antitumor effects of "Corynebacterium parvum" in mice. *Europ. J. Cancer* **10,** 121 (1974).
7. M. T. Scott, Corynebacterium parvum as a therapeutic antitumor agent in mice. I. Systemic effects from intravenous injection. *J. nat. Cancer Inst.* **53,** 855 (1974).
8. M. T. Scott, Corynebacterium parvum as a therapeutic antitumor agent in mice. II. Local injection. *J. nat. Cancer Inst.* **53,** 861 (1974).
9. L. Israël and B. N. Halpern, Le Corynebacterium parvum dans les cancers avancés. Première évaluation de l'activité thérapeutique de cette immunostimuline. *Nouv. Presse méd.* **1,** 19 (1972).
10. B. N. Halpern, A. R. Prévot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard and Y. Bouthillier, Stimulation de l'activité phagocytaire du système réticuloendothélial provoquée par Corynebacterium parvum. *J. reticuloendoth. Soc.* **1,** 77 (1964).
11. J. E. Castro, The effect of "Corynebacterium parvum" on the structure and function of the lymphoid system in mice. *Europ. J. Cancer* **10,** 115 (1974).
12. G. J. O'Neill, D. C. Henderson and R. G. White, The role of anaerobic coryneforms on specific and non-specific immunological reactions. I. Effect on particle clearance and humoral and cell-mediated immunological responses. *Immunology* **24,** 977 (1973).
13. R. Bomford and M. Olivotto, The mechanism of inhibition by "Corynebacterium parvum" of the growth of lung nodules from intravenously injected tumour cells. *Int. J. Cancer* **14,** 226 (1974).
14. M. Olivotto and R. Bomford, *In vitro* inhibition of tumor cell growth and DNA synthesis by peritoneal and lung macrophages from mice injected with Corynebacterium parvum. *Int. J. Cancer* **13,** 478 (1974).
15. A. Ghaffar, R. T. Cullen, N. Dunbar and M. A. F. Woodruff, Antitumour effect *in vitro* of lymphocytes and macrophages from mice treated with Corynebacterium parvum. *Brit. J. Cancer* **29,** 199 (1974).
16. A. E. Reif and J. M. V. Allen, The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. exp. Med.* **120,** 413 (1964).
17. A. Cohen and M. Schlesinger, Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation* **10,** 130 (1970).
18. E. Kedar, M. Ortiz de Landazuri and B. Bonavida, Cellular immunoadsorbents: a simplified technique for separation of lymphoid cell populations. *J. Immunol.* **112,** 1231 (1974).
19. R. Evans, Macrophages in syngeneic animal tumours. *Transplantation* **14,** 468 (1972).
20. F. de Halleux, H. S. Taper and C. Deckers, A simple procedure for identification of macrophages in peritoneal exudates. *Brit. J. exp. Path.* **54,** 352 (1973).
21. O. Sjöberg, J. Andersson and G. Möller, Requirement for adherent cells in the primary and secondary immune response *in vitro*. *Europ. J. Immunol.* **2,** 123 (1972).

22. K. T. Brunner, J. Mauel, J. C. Cerottini and B. Chapius, Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells *in vitro*: inhibition by isoantibody and by drugs. *Immunology* **14,** 181 (1968).
23. A. Mantovani and F. Spreafico, On the nature of blocking factors and their lymphoid target cells in an allogeneic tumor system. *Europ. J. Cancer* **11,** 451 (1975).
24. A. Mantovani and F. Spreafico, Allogeneic tumor enhancement by levamisole, a new immunostimulatory compound: studies on cell-mediated immunity and humoral antibody response. *Europ. J. Cancer* **11,** 537 (1975).
25. A. Mantovani, A. Vecchi and F. Spreafico, The attacker: target cell ratio and serum effects on "in vitro" cell-mediated immunity. *J. nat. Cancer Inst.* In press.
26. D. W. Weiss, R. S. Bonhag and P. Leslie, Studies on the heterologous immunogenicity of a methanol-insoluble fraction of attenuated tubercle bacilli (BCG). II. Protection against tumor isografts. *J. exp. Med.* **124,** 1039 (1966).
27. W. F. Piessens, F. L. Lachapelle, N. Legros and J.-C. Heuson, Facilitation of rat mammary tumour growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).
28. D. O. Chee and A. J. Bodurtha, Facilitation and inhibition of B16 melanoma by BCG "in vivo" and by lymphoid cells from BCG-treated mice "in vitro". *Int. J. Cancer* **14,** 137 (1974).
29. F. Spreafico, A. Vecchi, A. Mantovani, A. Poggi, A. Anaclerio and S. Garattini, Characterization of the immunostimulants levamisole and tetramisole. *Europ. J. Cancer* **11,** 555 (1975).
30. P. Alexander and R. Evans, Endotoxin and double stranded RNA render macrophages cytotoxic. *Nature New Biol.* **232,** 76 (1971).
31. B. S. Zwilling, E. J. Leonard, R. C. Jr. Bast and B. Zbar, Destruction of syngeneic tumors by tuberculin-stimulated peritoneal exudate cells from guinea pigs immunized to Mycobacterium bovis (Strain BCG). *J. nat. Cancer Inst.* **53,** 541 (1974).
32. J. B. Jr. Hibbs, Macrophage nonimmunologic recognition: target cell factors related to contact inhibition. *Science* **180,** 868 (1973).
33. M. T. Scott, Biological effects of the adjuvant Corynebacterium parvum. I. Inhibition of PHA, mixed lymphocyte and GVH reactivity. *Cell. Immunol.* **5,** 459 (1972).
34. M. F. A. Woodruff, N. Dunbar and A. Ghaffar, The growth of tumours in T-cell deprived mice and their response to treatment with Corynebacterium parvum. *Proc. roy. Soc. B.* **184,** 97 (1973).
35. L. Milas, N. Hunter and R. Withers, Corynebacterium granulosum-induced protection against artificial pulmonary metastases of a syngeneic fibrosarcoma in mice. *Cancer Res.* **34,** 613 (1974).
36. B. Fisher, N. Wolmark and J. Coyle, Effect of "Corynebacterium parvum" on cytotoxicity of regional and nonregional lymph node cells from animals with tumors present or removed. *J. nat. Cancer Inst.* **53,** 1793 (1974).
37. J. Forman and S. Britton, Heterogenicity of the effector cells in the cytotoxic reaction against allogeneic lymphoma cells. *J. exp. Med.* **137,** 369 (1973).
38. J. G. Howard, M. T. Scott and G. H. Christie, Cellular mechanisms underlying the adjuvant activity of "Corynebacterium parvum": interactions of activated macrophages with T and B lymphocytes. In: *Immunopotentiation*, CIBA Foundation Symposium No. 18, p. 101, Elsevier, Amsterdam (1973).
39. M. T. Scott, Biological effects of the adjuvant "Corynebacterium parvum". II. Evidence for macrophage-T-cell interaction. *Cell. Immunol.* **5,** 469 (1972).
40. P. Hersey, New look at antiserum therapy of leukaemia. *Nature New Biol.* **244,** 22 (1973).
41. J. Zighelboim, B. Bonavida and J. L. Fahey, Antibody-mediated *in vivo* suppression of EL4 leukaemia in a syngeneic host. *J. nat. Cancer Inst.* **52,** 879 (1974).
42. A. C. Allison, Effects of adjuvants on different cell types and their interactions in immune responses. In: *Immunopotentiation*, CIBA Foundation Symposium No. 18, p. 73, Elsevier, Amsterdam (1973).
43. P. Perlmann, H. Perlmann and H. Wigzell, Lymphocyte-mediated cytotoxicity *in vitro*. Induction and inhibition by humoral antibody and nature of effector cells. *Transpl. Rev.* **13,** 91 (1972).

44. A. H. GREENBERG, L. HUDSON, L. SHEN and I. M. ROITT, Antibody-dependent cell-mediated cytotoxicity due to a "null" lymphoid cell. *Nature New Biol.* **242,** 111 (1973).
45. M. T. SCOTT, Depression of delayed-type hypersensitivity by "Corynebacterium parvum": mandatory role of the spleen. *Cell. Immunol.* **13,** 251 (1974).
46. P. FROST and E. M. LANCE, The relation of lymphocyte trapping to the mode of action of adjuvants. In *Immunopotentiation*, CIBA Foundation Symposium No. 18, p. 29, Elsevier, Amsterdam (1973).
47. K. JAMES, A. GHAFFAR and I. MILNE, The effect of transplanted methylcholanthrene induced fibrosarcomata and "Corynebacterium parvum" on the immune response of CBA and A/Hej mice to thymus dependent and independent antigens. *Brit. J. Cancer* **29,** 11 (1974).

Europ. J. Cancer Vol. 12, pp. 125–129. Pergamon Press 1976. Printed in Great Britain

Modification of Interstitial Fluid Close to Chemically-Induced Tumours and Correlation with a Growth Stimulation of Target Cells*

D. VAILLIER, J. VAILLIER and M. DONNER
Research Unit of Experimental Cancerology and Radiobiology, U 95-INSERM, 54500 Vandoeuvre-lez-Nancy, France

Abstract—*Subcutaneous implantation of diffusion chambers in mice close to some growing tumours resulted in a growth stimulation of target cells in chambers.*

Proteins of interstitial subcutaneous fluid were analyzed by electrophoresis in 7·5% polyacrylamide gel. Total protein content was determined according to Lowry's method.

A significant decrease of some proteic fractions was found in the s.c. interstitial fluid of stimulated tumour-bearing mice.

Serum and peritoneal fluid failed to demonstrate these differences.

The hypothesis of loss of growth inhibitory substances was proposed to explain the decrease of protein fraction in connection with the stimulation of target cells growth.

INTRODUCTION

IN A PREVIOUS work [1] the growth of tumour target cells in diffusion chambers has been studied when the chambers were s.c. implanted close to solid tumours of the same origin than the target cells.

For some tumours a high increase of multiplication of target cells in diffusion chambers was found. From these data, it has been hypothesized that the local s.c. interstitial fluid might be responsible for this phenomenon, Indeed, the stimulation of target cells growth decreased with the distance between the tumour and the diffusion chambers and is going out when the diffusion chambers were implanted in the peritoneum.

This stimulating effect might be explained by the release of growth stimulating substances by the tumour cells. An alternative hypothesis is that some tumours possess the capacity to destroy growth inhibitory substances.

Accepted 14 October 1975.
*This work was supported by a grant (ATP 11-74-32) from I.N.S.E.R.M.

The aim of the present work was to verify these different hypotheses. For this purpose, we used the acrylamide gel electrophoretic analysis to investigate the composition of interstitial s.c. fluid in mice bearing tumours having or not these stimulating properties.

The data presented here support the second hypothesis. Indeed the results present evidence for a decrease of proteins in interstitial s.c. fluid of stimulated tumour-bearing mice.

MATERIAL AND METHODS

Animals and tumours

The experiments were carried out with inbred mice aged 2–3 months of C3H/He, Swiss/B and CBA strains.

The tumours were rhabdomyosarcoma induced by i.m. injection of methylcholanthrene or benzpyrene and maintained by serial transplantation in syngeneic recipients. The RV2 tumour was induced by methylcholanthrene in a male C3H and the experiments performed from passage 100 to passage 110. The TCH1 tumour was induced by benzpyrene

in a female C3H and used from passage 35 to passage 45. The MC2 and MC3 tumours induced by methylcholanthrene were used from passage 16 to passage 22 in CBA strain.

Diffusion chambers

The diffusion chambers were derived from Algire's chambers [2]. Millipore filters effective pore size 0·1 μm were cemented onto plastic rings. The 0·1 μm porosity selected the chambers impermeable to cellular penetration [3].

Operative procedure

The target cells consisted of a single tumour cell suspension prepared as described previously [4]. Target cells of the same or different origin of the growing tumour were put in diffusion chambers. The diffusion chambers were s.c. inserted into the back of 5 mice bearing tumours ranging from 10 to 20 mm (experimental group) and in 5 normal mice (control group). After 5 days, the chambers were withdrawn and the target cells were counted. The mean of target cells was calculated in each group. Statistical significance of the results was evaluated by Student's *t* test. The percentage of increase of tumour cells in diffusion chambers was expressed as follows:

$$100 \times \left[1 - \frac{\text{Mean number of target cells in experimental group}}{\text{Mean number of target cells in control group}} \right]$$

For each tumour 4 experiments were carried out, and the stimulation index corresponded to the mean of the percentage of increase of each series of experiments.

Collection of interstitial s.c. and peritoneal fluid and serum

The diffusion chambers containing culture medium were s.c. inserted either into the back close to the tumour or in the peritoneum. Five days later, the chambers were removed and the fluids collected.

Blood was collected from retro-orbital sinus of normal mice and tumour-bearing mice. The blood was allowed to clot overnight at 4°C after which the serum was separated and centrifugated.

Proteins determination

Total protein content of interstitial fluid and serum was determined according to Lowry's method [5] using crystalline bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

The electrophoresis run was carried out employing a 2·5% upper polyacrylamide gel and 7·5% lower gel (pH 8·3). During each run, a constant current of 2·5 mA/tube was maintained for two hr. The gels were stained with Amidoblack, destained in 7% acetic acid and scanned in a densitometer Gelman. Only the gels electrophoresed at the same time were compared.

RESULTS

Comparison of tumour cells growth in diffusion chambers implanted in subcutaneous in normal and tumour-bearing mice

These experiments confirm previous data [1] enlarging at other tumours. Diffusion chambers containing cells from MC2 or MC3 tumours were s.c. implanted in normal CBA mice or respectively in MC2 and MC3 tumour-bearing CBA mice. The tumour diameter is ranging between 10 and 20 mm.

For each experiment, 5 normal mice and 5 tumour-bearing mice were used. The results of 4 experiments are pooled in Table 1. The growth of MC3 target cells is significantly higher when the diffusion chamber is implanted in MC3 tumour-bearing mice than in normal mice.

In contrast, the growth of MC2 cells is not different when the diffusion chamber is implanted in normal or MC2 tumour-bearing mice.

To study if the multiplication of tumour cells in diffusion chambers corresponded to a specific phenomenon, diffusion chambers containing cells from MC2 tumour were implanted in normal CBA mice and MC3 tumour-bearing mice. Similarly, diffusion chambers containing cells from MC3 tumour were implanted in normal CBA mice and MC2 tumour-bearing mice.

The results of 4 experiments are pooled in Table 2. In both cases, there is no stimulation for the growth of tumour cells in diffusion chambers implanted in tumour-bearing mice.

The results confirm previous data [1]. It seems that some tumours draw the growing stimulation of target cells—of the same origin—through the s.c. interstitial fluid.

Polyacrylamide gel electrophoresis of interstitial s.c. fluid of normal and tumour-bearing mice

Polyacrylamide gel electrophoresis of s.c. interstitial fluid of 6 normal mice and 6 mice bearing tumours of median diameter 15 mm

Table 1. *Number of tumour cells harvested in diffusion chambers implanted s.c. in normal and tumour-bearing mice*

Strain and tumour	Exp. No.	Normal mice	Tumour-bearing mice	Dia. (mm)	% increase	Student's *t* test
CBA ♀	1	2·16 ± 0·33[1]	5·82 ± 0·91	(22·3 ± 3·4)[2]	169·4	$P < 0·01$
MC3 tumour	2	2·08 ± 0·41	7·24 ± 0·70	(18·2 ± 2·1)	248·0	$P < 0·001$
	3	2·25 ± 0·27	4·09 ± 0·78	(16·9 ± 1·7)	81·8	$P < 0·05$
	4	1·75 ± 0·36	4·82 ± 0·63	(20·0 ± 1·5)	175·4	$P < 0·005$
				Stimulation index[4]	168·6 ± 34·0	
CBA ♂	1	6·31 ± 1·10	5·04 ± 0·53	(17·1 ± 0·8)	−20·1	NS[3]
MC2 tumour	2	3·66 ± 0·49	1·75 ± 0·22	(12·5 ± 1·1)	−52·2	NS
	3	3·80 ± 0·21	4·02 ± 0·43	(11·1 ± 1·8)	5·8	NS
	4	1·81 ± 0·58	1·89 ± 0·69	(13·0 ± 0·7)	4·2	NS
				Stimulation index	−15·5 ± 13·5	NS

[1]Mean number of tumour cells ($\times 10^6$/ml) ± S.E. harvested in diffusion chambers.
[2]Mean diameter ± S.E. of 5 tumours.
[3]NS: not significant.
[4]Stimulation index refers to the mean ± S.E. of % increases.

Table 2. *Number of tumour cells harvested in diffusion chambers implanted s.c. in normal mice and mice bearing tumours of a different origin*

Strain and tumour	Exp. No.	Normal mice	Tumour bearing mice	Diameter (mm)	% increase	Student's test
		MC2 tumour cells in diffusion chambers				
CBA ♀	1	1·30 ± 0·19[1]	0·86 ± 0·14	(20·2 ± 1·5)[2]	−33·8	NS[3]
MC3 tumour	2	4·14 ± 0·82	3·50 ± 0·34	(12·5 ± 2·8)	−15·4	NS
	3	4·46 ± 0·49	4·06 ± 0·74	(14·2 ± 3·1)	− 9·0	NS
	4	3·51 ± 0·41	3·23 ± 0·41	(15·7 ± 1·2)	− 8·0	NS
				Stimulation index[4]	−16·5 ± 6·0	
		MC3 tumour cells in diffusion chambers				
CBA ♂	1	2·88 ± 0·82	2·61 ± 0·70	(14·0 ± 0·9)	− 9·4	NS
MC2 tumour	2	1·57 ± 0·35	2·06 ± 0·40	(12·5 ± 2·1)	31·2	NS
	3	3·34 ± 0·41	3·01 ± 0·83	(17·2 ± 1·5)	− 9·9	NS
	4	3·98 ± 0·31	4·21 ± 0·55	(15·4 ± 1·8)	5·8	NS
				Stimulation index	4·4 ± 9·6	

[1]Mean number of tumour cells ($\times 10^6$/ml) ± S.E. harvested in diffusion chambers.
[2]Mean diameter ± S.E. of 5 tumours.
[3]NS: not significant.
[4]Stimulation index refers to the mean ± S.E. of % increases

have been carried out. Four host-tumour systems have been tested, the C3H male mice normal and bearing the RV2 tumour, C3H female mice normal and bearing the TCH1 tumour, the CBA male mice normal and bearing the MC2 tumour, and the CBA female mice normal and bearing the MC3 tumour. The gels obtained for each group of 6 mice are comparable.

Figure 1 shows the gels obtained after the electrophoresis of interstitial fluid of 3 normal C3H and 5 RV2 tumour-bearing C3H.

Figure 2 reproduces the curves obtained after scanning of the gels on the densitometer of interstitial fluid of a normal and tumour-bearing mouse for the 4 tested systems. The gel scan was divided into 7 fractions. The albumin (fraction 7) and the transferrin

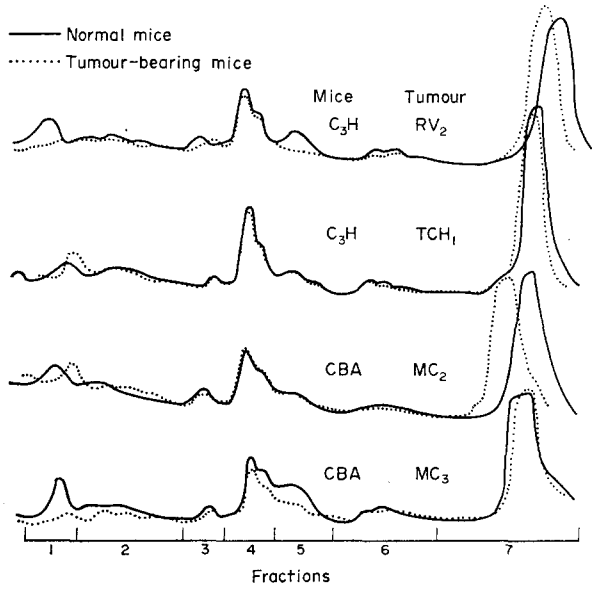


Fig. 2. Superimposed densitometric tracings of s.c. interstitial fluid of a normal mouse and a mouse bearing tumour of diameter 14 mm (the 4 groups of curves resulted from 4 different electrophoreses).

(fraction 4) have been localized with regard to the position of human albumin and transferrin.

The results show that the deviations of different proteic fractions of interstitial fluid of tumour-bearing mice with regard to normal mice vary following the tumour. For the RV2 and MC3 tumours, there is a decrease for each fraction, but most important for the 1st, 5th and 6th fractions. But, for MC2 and TCH1 tumours, there are not notorious differences.

The data are consistant with the content of total proteins determined by Lowry's method [5]. The content of total proteins of interstitial fluid of 6 normal mice and 6 mice bearing tumours of median diameter 15 mm has been measured.

For the interstitial fluid of RV2 and MC3 tumour-bearing mice, the mean of values obtained for 6 mice equals 2·6 g/100 ml (S.D. 0·35 range 2·3–3·0 g/100 ml) and 2·2 g/100 ml (S.D. 0·41 range 2·0–2·8 g/100 ml) compared respectively to values obtained for normal mice 3·1 g/100 ml (S.D. 0·52 range 2·8–3·5 g/100 ml) and 2·9 g/100 ml (S.D. 0·40 range 2·5–3·1 g/100 ml).

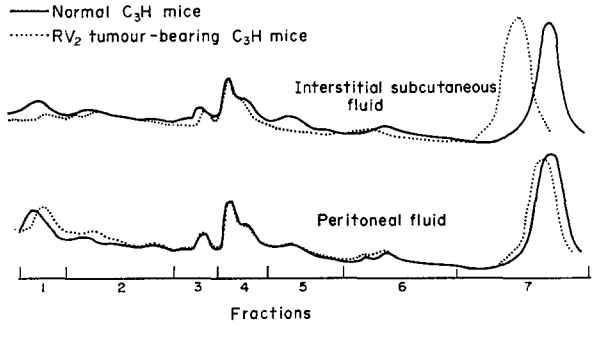


Fig. 3. Superimposed densitometer tracing of interstitial s.c. fluid and peritoneal fluid of a normal C3H mouse and a C3H mouse bearing RV2 of diameter 12 mm.

For the interstitial fluid of TCH1 and MC2 tumour-bearing mice, the values are 2·9 g/100 ml (S.D. 0·24 range 2·7–3·2 g/100 ml) and 2·8 g/100 ml (S.D. 0·34 range 2·5–3·0 g/100 ml) compared respectively to values obtained for normal mice 3·0 g/100 ml (S.D. 0·49 range 2·7–3·6 g/100 ml) and 2·8 g/100 ml (S.D. 0·24 range 2·7–3·4 g/100 ml).

Comparison of interstitial s.c. fluid and peritoneal fluid of normal or RV2 tumour-bearing C3H mice

In a previous work [1] Vaillier *et al.* have shown that when the diffusion chambers were implanted in the peritoneum, the tumour being implanted s.c., there is no difference of growth of target cells.

Therefore, the composition of the interstitial s.c. fluid and peritoneal fluid of 3 normal mice and 3 RV2 tumour-bearing mice were compared after gel electrophoresis. The gels obtained for each group are similar.

Figure 3 shows that, for the peritoneal fluid, there are no notorious variations, whether the mice bear or do not bear a s.c. tumour.

Comparison of variations of proteins fractions obtained after polyacrylamide electrophoresis of s.c. interstitial fluid and serum of normal or RV2 tumour-bearing C3H

As important decreases were found in the interstitial s.c. fluid of RV2 tumour-bearing mice, it seemed of interest to investigate serum proteins.

For this purpose, electrophoresis of serum and s.c. interstitial fluid of 3 normal C3H and 3 C3H mice bearing RV2 tumour of median diameter 15 mm has been made within the same experiment. Similar results are found for both groups. Figure 4 shows the gels obtained after the electrophoresis of interstitial fluid and serum of one normal and tumour-bearing mouse. Important decreases are observed for interstitial s.c. fluid of tumour-bearing mice, for the serum, no notable difference was detected between normal and tumour-bearing mice.

DISCUSSION

The present work confirms and extends previous results presenting evidence that the

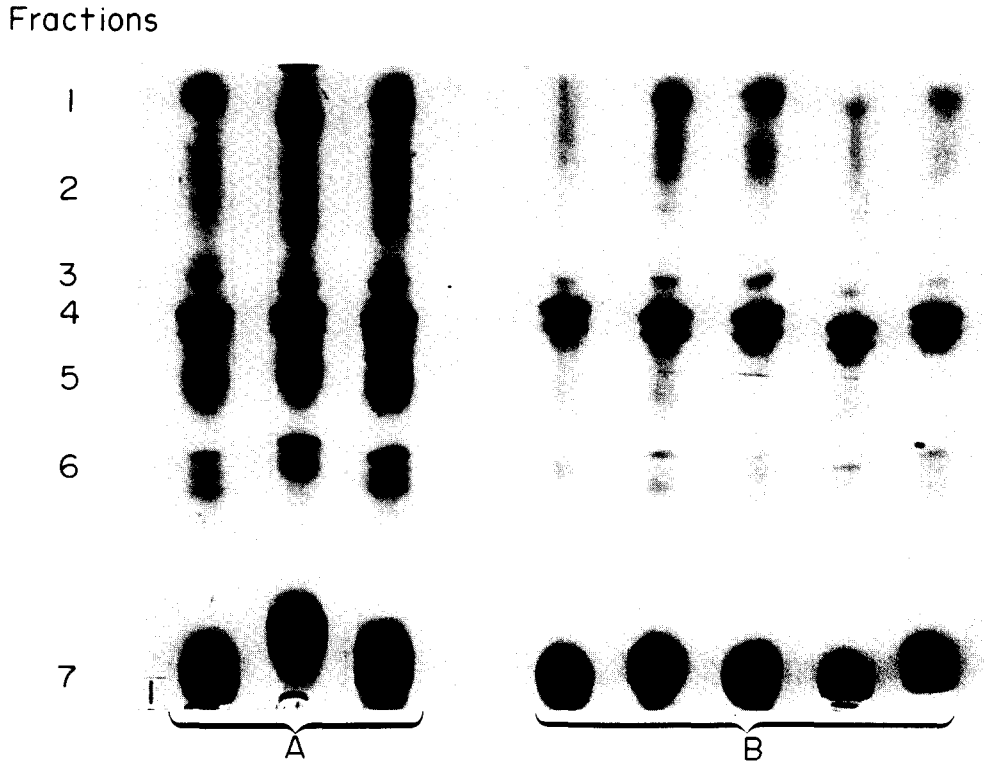


Fig. 1. *Polyacrylamide gel electrophoresis of s.c. interstitial fluid. A = from normal C3H mice, B = from C3H mice bearing RV2 tumour of median diameter* 15 *mm.*

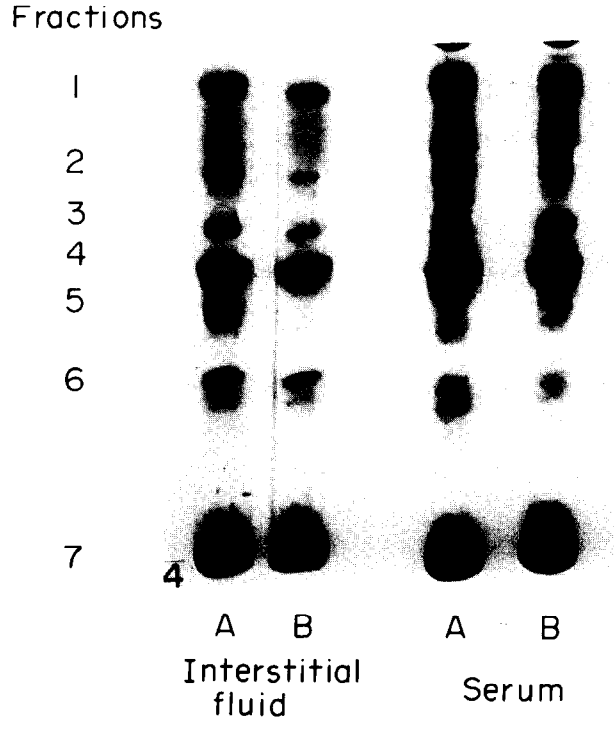


Fig. 4. *Polyacrylamide gel electrophoreis of subcutaneous interstitial fluid and serum of normal C3H (A) and C3H bearing a RV2 tumour of diameter* 15 *mm (B).*

(to face p, 128)

growth of target cells in diffusion chambers was stimulated close to some chemically-induced tumours.

Number of tumour transplantations does not seem to interfere since this phenomenon has been found for tumour transplanted many times and recent induced tumour.

The analysis of s.c. interstitial fluid of stimulated tumour-bearing mice compared to the interstitial fluid of normal or not stimulated tumour-bearing mice have shown a significant decrease of proteins.

This important diminution of different proteic fractions in the s.c. fluid of stimulated tumour-bearing mice is not associated with similar variation in the serum.

The absence of variation in serum proteins is somewhat surprising. Numerous works have presented evidence that modifications occurred in serum of tumour-bearing mice characterized either by a decrease of some proteins such as albumin [6] either an increase of others proteins [6, 7] or the apparition of a new protein [8].

Now in the case of our experiments, the important diminution of different proteic fractions in the s.c. interstitial of stimulated tumour-bearing mice is not associated with similar variations in the serum. Indeed the stimulation that we observed, implicated the interstitial fluid of tumour-bearing mice close to the tumour site.

Our results can be compared to these of Nowotny *et al.* [9] that showed a growth stimulation *in vivo* of an ascitic tumour if the animal is injected by ascitic fluid, the serum having no effect. Yet this phenomenon has been shown to implicate some compounds of ascitic fluid in origin of tumour cells membrane.

Concerning the growth stimulation of target cells close to some tumours, the hypothesis that tumours released factors responsible for stimulation growth of target cells is failed. Indeed both the growth stimulation phenomenon and the decrease of some proteic fractions were simultaneously observed.

The local decrease of some proteins can be explained by an action of tumour cells themselves. Indeed, they are susceptible to use substrates that the normal tissues do not use as a nutritional source [10–11].

If this is correct, it may be suggested that some tumours—but not all—use growth inhibitory substances naturally occurring in s.c. interstitial fluid. This hypothesis could account for both decrease of proteins and simultaneous growth stimulation of target cells.

Acknowledgements—We wish to thank Mrs Maryse Girr for her technical assistance.

REFERENCES

1. D. Vaillier, M. Donner, J. Vaillier and C. Burg, Stimulation *in vivo* de la croissance de cellules tumorales en chambres à diffusion implantées chez des souris portant des tumeurs chimio-induites. *Int. J. Cancer* **15,** 457 (1975).
2. G. H. Algire and F. Y. Legallais, Recent developments in the transparent chambers technique as adapted to the mouse. *J. nat. Cancer Inst.* **10,** 225 (1949).
3. E. Capalbo, J. F. Albright and W. E. Bennet, Evaluation of the diffusion chamber culture technique for study of the morphological and functional characteristics of lymphoid cells during antibody production. *J. Immunol.* **12,** 243 (1964).
4. D. Vaillier, M. Donner, J. Vaillier and C. Burg, Release of lymphotoxins by spleen cells sensitized against mouse tumour associated antigens. *Cell. Immunol.* **6,** 466 (1973).
5. O. H. Lowry, H. J. Roselrough, A. L. Farr and R. J. Randall, Proteins measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
6. I. Zeidman, J. W. Dempsey and P. B. Shelley, Serum protein changes in neoplasia. *Arch. Path.* **85,** 481 (1968).
7. D. P. Lilley, D. R. Burger and R. M. Vetto, Tumor growth in the guinea pig: Alpha globulin changes associated with lymphocyte suppression. *J.N.C.I.* **53,** 701 (1974).
8. W. G. Palmer, T. W. Orme and C. W. Boone, A unique serum protein in mice with various tumours. *J.N.C.I.* **52,** 279 (1974).
9. A. Nowotny, J. Groshman, A. Abdelnoor, N. Note, C. Yang and R. Waltersdorff, Escape of TA 3 tumors from allogeneic immune rejection: Theory and experiments. *Europ. J. Immunol.* **4,** 73 (1974).
10. H. Busch, J. R. Davies, G. R. Honig, D. C. Anderson, P. V. Nair and W. L. Nyhan, The uptake of a variety of amino-acides into nuclear proteins of tumor and other tissues. *Cancer Res.* **19,** 1030 (1959).
11. J. F. Henderson and G. A. Lepage, Utilization of host protein by 6 C3H ED ascites lymphosarcoma in C3H and Swiss mice. *Cancer Res.* **19,** 749 (1959).

Europ. J. Cancer Vol. 12, pp. 131–135. Pergamon Press 1976. Printed in Great Britain

Increased Erythrocytic and Neutrophilic Progenitors in Myelofibrosis with Myeloid Metaplasia*†‡

S. ÖHL§, A. L. CARSTEN, A. D. CHANANA, G. CHIKKAPPA and E. P. CRONKITE
Medical Research Center, Brookhaven National Laboratory, Upton, L.I., New York 11973, *U.S.A*

Abstract—*Nucleated blood or bone marrow cells obtained from patients suffering from myelofibrosis with myeloid metaplasia (MMM) were cultured in diffusion chambers implanted in the peritoneal cavities of X-irradiated mice. The data obtained from MMM cell culture studies were compared with those obtained from normal or chronic myelocytic leukemia marrow cell cultures. The results indicate that MMM patients have increased numbers of erythrocytic and neutrophilic progenitors in their blood and marrow.*

INTRODUCTION

COLONY-FORMING cells have been reported to increase in the blood of patients with myelofibrosis with myeloid metaplasia [1]. The *in vitro* culture technique used in that study permits the proliferation solely of progenitors committed to form granulocytes and/or macrophages [2, 3]. In contrast, the diffusion chamber culture system (DC) closely approximates the *in vivo* conditions for the proliferation of committed as well as pluripotent hemopoietic progenitors [4]. Accordingly, the present studies were performed to study the proliferative patterns of blood and marrow cells of patients with myelofibrosis with myeloid metaplasia (MMM).

Accepted 30 September 1975.

*Research supported by the U.S. Energy Research and Development Administration and in parts by NIH Grant # 5 R01 CA 14742 and American Cancer Society Grant # DT-5.

†Written informed consent was obtained from the human subjects after the nature of the procedure had been fully explained.

‡Presented in part at the American Federation for Clinical Research Meeting, May 4–7, 1974, Atlantic City, New Jersey.

§Present address: Innere Klinik Tumorforschung, der Ruhruniversität, Hufelandstrasse, Essen, West Germany.

Address communications to Dr. G. Chikkappa, Medical Research Center, Brookhaven National Laboratory, Upton, L.I., New York 11973, U.S.A.

MATERIAL AND METHODS

Patients

Clinical data of the two males with MMM are given in Table 1. Patient FG, had patchy fibrosis in his marrow biopsy sections. His marrow could still be aspirated though with some difficulty. He was not on any cytotoxic drugs prior to or at the time of the study. Patient JC had classical features of the disease. Four years prior to the first study (JC I), a hemoglobin (Hgb) value of 24 g% was recognized elsewhere. Elevation of Hgb was neither investigated nor treated. Two years prior to the study a bleeding peptic ulcer requiring transfusions was documented. Marked fibrosis was noted in his marrow biopsy sections at the time of the present studies. His marrow could not be aspirated and his blood cells were cultured on two occasions 9 months apart. He did not receive any cytotoxic drugs before or during the first study and was on Melphalan (L-sarcolysin) 2 mg/day for 90 days prior to and during the second study.

Diffusion chamber cultures

Cultures were performed as previously described [4–7]. Briefly, blood or marrow samples were obtained in syringes containing 1·0% EDTA. The nucleated cells were separated by density gradient sedimentation technique of Bøyum [8]. The nucleated cells were washed

Table 1. *Clinical data*

Patients	Age (yr)	Spleen cms (LCM)*	Liver cms (RCM)†	Bone marrow fibrosis	BLOOD Hct (%)	WBC X10³/mm³	Myeloblasts, promyelocytes and myelocytes (%)	Nucleated RBC's/100 WBC
FG	38	8	3	patchy	33	7·0	3	3
JC I‡	60	25	7	diffuse	35	89·0	14	0·5
JC II‡	60	24	10	diffuse	36	52·3	11	0·0§

*Under left costal margin.
†Under right costal margin.
‡Two separate studies on patient JC (for details see text).
§Occasional nucleated erythrocytic cell seen on scanning, blood smears.

twice and suspended in Hanks' BSS (10X) medium*. One-tenth millilitre of the suspension containing $4{\cdot}4\times10^5$, $7{\cdot}5\times10^5$, and $9{\cdot}9\times10^5$ cells from FG, JC I, and JC II

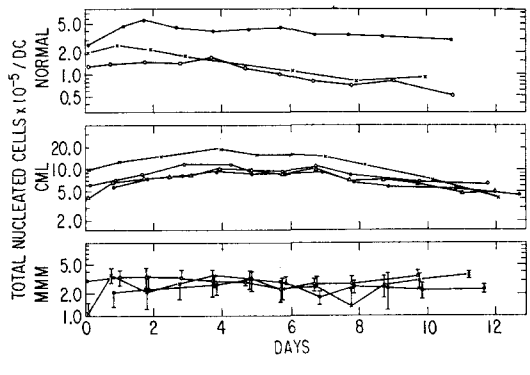


Fig. 1. *Total nucleated cells recovered from the cultures at various times following chamber implantation are shown. Top section represents three separate normal marrow cultures; middle section represents three separate studies* (−○−, −△−, −●−) *of marrow cells of one patient and one study* (−×−) *of marrow cells of another patient with chronic myelocytic leukemia* (*CML*)*; the bottom section represents two studies* (−○−, −●−) *of blood cells of one* (*JC*) *and one study* (−×−) *of marrow cells of another* (*FG*) *patient with myelofibrosis with myeloid metaplasia* (*MMM*). *The vertical bars in the bottom section represent the standard error of the mean* (*SEM*). *SEM's are not given in the top two sections since they tend to confuse the picture and are very small* (*average* 7·55% *of the mean for CML and* 7·50% *for normal*).

respectively was placed in each of the diffusion chambers. The chambers (2/mouse) were placed in the peritoneal cavities of 6–8 week old, male mice of the Hale–Stoner–Brookhaven strain. Eighteen to 24 hr previously the mice had received a total body X-ray dose of 700 rads. The chambers from original hosts were reimplanted into newly irradiated hosts on day 7 or 8. Commencing at 2 hr in JC I, 10 hr in JC II and 20 hr in FG after the implantation of the chambers and at various intervals

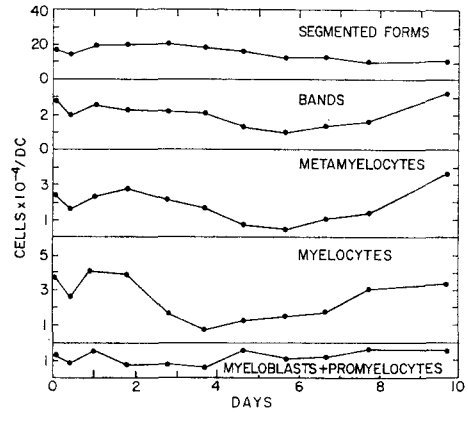


Fig. 2. *Recovery of neutrophilic cells from cultures of JC II blood* (*MMM*) *cells at various times following chamber implantation.*

thereafter, groups of 6–8 chambers were removed and total and differential cell counts made on their contents.

RESULTS

The total nucleated cell recovery at different times following chamber implantation is shown in Fig. 1. For comparison, the data from previously published studies on normal individuals [4] and chronic myelocytic leukemia (CML) patients [7] are also shown. Total cell recovery from MMM blood (JC I, JC II) and marrow (FG) cell cultures was similar and exhibited minor fluctuations during the observation period.

*Obtained from Microbiological Associates, Inc., Bethesda, MD, USA.

Table 2. Proliferative (M_{1-4}) and non-proliferative (M_{5-7}) granulocytic cells in diffusion chamber cultures

	M_{1-4}:M_{5-7}		M_{1-4} of Wave 1: Wave 2	M_{1-4} on day 10: Cell inoculum
	First wave*	Second wave*		
MMM†	1·0:4·7	1·0:2·9	1·0:0·8	1:14,342
CML‡	1·0:2·7	Not observed	§	1:19,670
Normal	1·0:5·0	1·0:1·0	1·0:0·4	1:34,320

*For details see text.
†Myelofibrosis with myeloid metaplasia.
‡Chronic myelocytic leukemia.
§Not calculated since a second wave was not observed.

Table 3. Nucleated erythrocytic (E_{1-5}) cells in cultures

	E_{1-5}: Total cells in chambers			E_{1-5} on day 10: cell inoculum
	On days			
	1	6	10	
MMM*	1:28	1:24	1:8	1:19,551
CML†	1:156	1:1578	1:445	1:850,000
Normal	1:58	1:1284	1:723	1:219,888

*Myelofibrosis with myeloid metaplasia.
†Chronic myelocytic leukemia.

The absolute number of neutrophilic cells from JC II blood cell cultures exhibited two waves of proliferation (Fig. 2). During the first wave all forms of neutrophilic granulocytes from myeloblasts to segmented neutrophils increased in number. Similar increase was noted in neutrophils during the second wave with the exception of that segmented neutrophils did not show any definite increase. Similar results were obtained from JC I and FG studies.

Relative distributions of proliferative [myeloblasts (M_1), promyelocytes (M_2), large myelocytes (M_3) and small myelocytes (M_4)] and non-proliferative [(metamyelocytes (M_5), band forms (M_6) and segmented forms (M_7)] neutrophilic cells during each of the two waves were obtained by averaging the data from all three MMM studies (Table 2). The ratios of proliferative to non-proliferative cells were 1:4·7 and 1:2·9 during the first and second waves respectively. The ratios of proliferative neutrophilic cells (M_{1-4}) during the peaks of the two waves are also shown in Table 2. The ratios of proliferative neutrophilic cells recovered on day 10 to the initial cell inoculum were 1:14,342, 1:19,760 and 1:34,320 for MMM, CML and normal cell cultures respectively (Table 2).

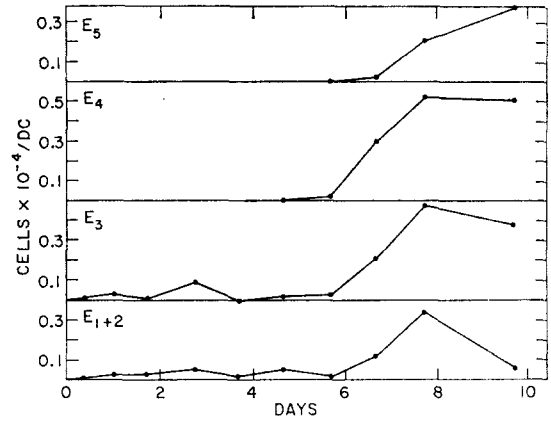


Fig. 3. Recovery of erythrocytic nucleated cells of JC II blood cell cultures at various times following chamber implantation are shown. E_1 = proerythroblast, E_2 = erythroblast, E_3 = basophilic normoblast, E_4 = polychromatophilic normoblast, E_5 = orthochromatic normoblast.

The pattern of nucleated erythrocytic cell (E_{1-5}) proliferation and maturation from JC II cultures are shown in Fig. 3. The cell

number began to increase at about day 6 after implantation, and the elevated levels, with the exception of E_1 fraction, persisted for the rest of the observation period. Observations from JC I and FG studies yielded essentially similar results.

The ratios of nucleated erythrocytic cells (E_{1-5}) to total cells recovered from chambers on days 1, 6 and 10 post-implantation and the ratios of E_{1-5} recovered on day 10 to initial inoculum size along with corresponding data for normal and CML cell cultures are shown in Table 3. With regard to the latter, the values were 1:19,551, 1:850,000 and 1:219,888 for MMM, CML and normal cell cultures respectively.

The relative distributions of proliferative neutrophilic cells (M_{1-4}) and nucleated erythrocytic cells (E_{1-5}) in MMM, CML and normal cell cultures are graphically depicted in Fig. 4. As compared to CML and normal cell cultures, there was a relative increase in E_{1-5} from MMM cultures harvested on days 6 and 10. It is worth noting that whereas all three MMM cultures harvested on day 10 had considerable numbers of nucleated erythrocytes, only 2 out of 6 CML and normal cell cultures contained these cells.

No definite differences in the proliferative patterns of JC I and JC II cultures were observed despite the fact that prior to and during the JC II study, the patient was receiving Melphalan.

DISCUSSION

The proliferative patterns of normal and CML cell cultures have been discussed previously [4, 7]. The results obtained from MMM blood and bone marrow cell culture studies, as presented here, suggest that MMM cells, during the first neutrophilic wave, proliferated and matured in a manner similar to that occurring *in vivo*. Formation of non-proliferative neutrophils (M_{5-7}), especially M_7, during the second wave as compared to the first wave was reduced (Table 2 and Fig. 2). This observation suggests that there is defective maturation and/or curtailed survival of M_7 in the culture.

The first wave of neutrophilic cells is probably due to the proliferation and maturation of the cells in the neutrophilic multiplicative pool (M_{1-4}) with a finite life span. In addition, a minor component is possibly contributed by the stem cells present in the inoculum. The second wave of neutrophilic cells is, however derived primarily from the stem cells as the inoculated cells in the multiplicative pool (M_{1-4}) with a finite life span [9] are thought not to persist for the length of time required to form the second wave. This hypothesis has been confirmed by tritiated thymidine labeling studies in conjunction with diffusion chamber culture of normal marrow cells [4].

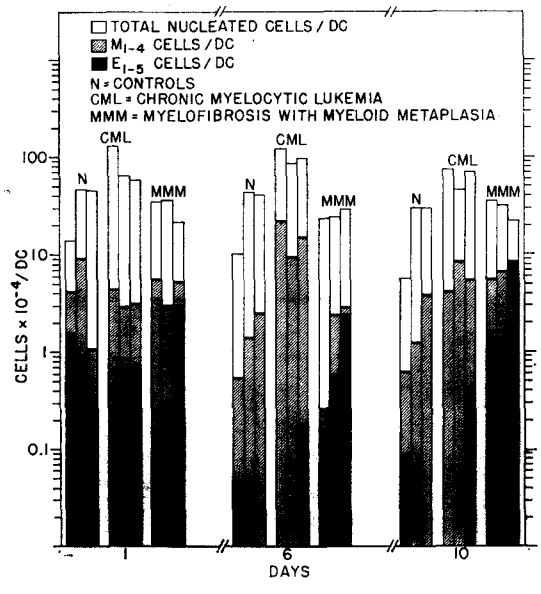


Fig. 4. Cells recovered from cultures on days 1, 6 and 10 post chamber implantation are shown. From baseline to top of open bar, to top of hatched bar and top of solid bar represents total nucleated cells, proliferative pool of neutrophilic cells and nucleated erythrocytic cells respectively.

As noted in Table 2 and Fig. 4. a larger fraction of neutrophilic cells, M_{1-7}, were recovered at day 10 from MMM as opposed to the CML or normal cell cultures. This observation probably reflects the presence of an increased number of neutrophilic progenitors and supports the results obtained for the granulocytic progenitors by an *in vitro* cultures of MMM cells [1].

The recovery of nucleated erythrocytic cells, like the neutrophilic cells, was greater in MMM than in normal or CML cell cultures, especially on day 10 (Fig. 4 and Table 3). This higher recovery of E_{1-5} cells from the MMM cell cultures strongly suggests that the cell inoculum contained an increased number of erythrocytic progenitors.

The mechanism responsible for increased neutrophilic and erythrocytic progenitors in MMM blood and marrow remains to be elucidated. The observations reported could reflect either a compensatory response to diminished marrow space available for hemopoiesis or a repair response to some injury such as is seen following sublethal irradiation

[10]. Another possibility which should be mentioned is that the factors inducing uncontrolled proliferation of marrow fibroblasts might also affect the hemopoietic precursor cells in a similar fashion.

Additional studies are clearly needed to not only confirm these observations but also to culture MMM cells at various stages of the disease in order to gain a better understanding of the pathophysiology of this disease.

Acknowledgements—We thank Mrs. Diane Rider, Miss JoAnn Piccinone, Mrs. Shirley Dunwoody and Mr. L. Cook for technical assistance.

REFERENCES

1. P. A. Chervenick, Increase in circulating stem cells in patients with myelofibrosis. *Blood* **41,** 67 (1973).
2. T. R. Bradley and D. Metcalf, The growth of mouse bone marrow cells *in vitro*. *Aust. J. exp. Biol. med. Sci.* **44,** 287 (1966).
3. B. Pike and W. A. Robinson, Human bone marrow cells in agar gel. *J. Cell Physiol.* **76,** 77 (1970).
4. E. P. Cronkite, A. L. Carsten, G. Chikkappa, J. A. Laissue and S. Öhl, Culture of normal and leukemic cells in diffusion chambers. In *Advances in Biosciences 14.* (Edited by T. M. Fliedner and S. Perry) p. 273, Pergamon Press, Oxford (1973).
5. A. L. Carsten, A. Bøyum and W. Boecker, The culture of human and mouse hemopoietic stem cells in millipore diffusion chambers. In *In vitro Culture of Hemopoietic Cells.* (Edited by D. W. Van Bekkum and K. A. Dicke) p. 279. Radiobiological Institute TNO, Rijswijk (1972).
6. H. B. Benestad, Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. *Scand. J. Haematol.* **7,** 279 (1970).
7. G. Chikkappa, W. R. Boecker, G. Borner, A. L. Carsten, K. Conkling, L. Cook, E. P. Cronkite and S. Dunwoody, Return of alkaline phophatase in chronic myelocytic leukemia cells in diffusion chamber cultures. *Proc. Soc. expt. Biol. Med.* **143,** 212 (1973).
8. A. Bøyum, Separation of leukocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* **21,** Suppl. 97 (1968).
9. E. P. Cronkite and P. C. Vincent, Granulocytopoiesis. *Ser. Haematol.* **II 4,** 3 (1969).
10. D. W. H. Barnes and J. F. Loutit, Effects of irradiation and Antigenic stimulation on circulating hemopoietic stem cells of mouse. *Nature (Lond.)* **213,** 1142, March 18 (1967).

Europ. J. Cancer Vol. 12, pp. 137–141. Pergamon Press 1976. Printed in Great Britain

Effect of Age and Testosterone on the Induction of Hyperplastic Nodules, Carcinomas, and Cirrhosis of the Liver in Rats Ingesting N-2-Fluorenyldiacetamide

MELVIN D. REUBER*

Laboratory of Biology, National Cancer Institute, Bethesda, MD 20014, *U.S.A.*

Abstract—*Hepatic carcinogenesis was studied first in castrated rats and second in castrated male rats given testosterone ingesting* 0·025 *per cent N-2-fluorenyldiacetamide (F-diAA)*†. *Rats were* 12 *or* 52 *weeks of age. The incidence of carcinomas and the number of rats with large carcinomas, multiple carcinomas, and metastases, as well as the degree and incidence of cirrhosis, was highest in the younger castrated male rats given testosterone. Twelve-week-old castrated male rats developed more hyperplastic nodules, carcinomas and cirrhosis than* 52*-week-old castrated male rats. Testosterone increased only slightly the incidence of carcinomas, hyperplastic nodules and cirrhosis in* 52*-week-old castrated male animals. Testosterone is important in F-diAA-hepatic carcinogenesis, particularly in young adult male rats. It is concluded that the restoration of the level of sex hormone in aged rats will not restore the susceptibility to hepatic carcinogenesis comparable to that of younger rats.*

INTRODUCTION

F-diAA has provided a useful model for the study of preneoplastic and neoplastic lesions of the liver in rats [1–3]. The lesions develop slowly over a period of months and can be followed from hyperplastic areas to hyperplastic nodules to small hepatocellular carcinomas and later well-developed large hepatocellular carcinomas, often with metastases. Cirrhosis develops along with the hyperplastic lesions.

Male rats are susceptible to the development of preneoplastic and neoplastic hepatic lesions, whereas females do not develop such lesions [1, 4]. The induction of hyperplastic and neoplastic hepatic lesions is dependent upon the testes, or more specifically endogenous testosterone in male rats, or exogenous testosterone administered to female rats [1]. Thyroidectomized male rats develop fewer carcinomas of the liver and this decrease is probably related to atrophy of the testes [5].

The incidence of carcinomas of the liver and the number of rats with large carcinomas, multiple carcinomas and metastases is highest in intact rats. The incidence and size not only decreases in the animals with one or both testes removed, but the decrease is directly related to the bulk and the weight of the testis or testes in rats [6]. The incidence is lowest in rats with both testes removed. Extensive studies concerning the role of hormones in hepatic carcinogenesis have been carried out with this model [4–10].

Younger adult male rats 12 weeks of age readily develop cirrhosis and carcinomas of the

Accepted 1 September 1975.

*Present address: 11014 Swansfield Road, Columbia, Maryland 21044, U.S.A.

†*Chemical Abstracts*' nomenclature: N, N-diacetyl-2-fluorenamine or N, N-diacetyl-2-aminofluorene.

liver after ingesting F-diAA whereas aged male rats 52 weeks of age with atrophic testes do not [11]. The present experiment was carried out in order to determine whether the restoration of testosterone in aged rats would make them susceptible to cirrhosis and carcinogenesis of the liver.

MATERIAL AND METHODS

Inbred A × C male rats 12 and 52-weeks-old and weighing 175–308 g were used. There were 4 experimental groups of 25 to 27 animals each. The groups consisted of (1) 12-week-old castrated male rats; (2) 12-week-old castrated male rats given testosterone propionate; (3) 52-week-old castrated male rats; and (4) 52-week-old castrated male rats given testosterone propionate. Castrated male rats were used because testosterone propionate in intact animals suppresses pituitary function with atrophy of the testes and actually slightly decreases the incidence of hepatic carcinomas [1].

Gonadectomies were performed and pellets of testosterone propionate were implanted subcutaneously 2 weeks prior to the start of the experiment. The pellets consisted of 1 part of testosterone propionate and 3 parts of cholesterol. The dose was 20 mg of testosterone propionate per 100 g of rat or 80 mg of pellet per 100 g.

Morris diet # 272 contained 0·025% F-diAA [1]. The carcinogenic diet was fed for 4-week periods followed by 1 week on the basal diet until the carcinogen had been administered for 16 weeks. Thereafter the rats ingested Purina Laboratory pellets.

Animals were killed by decapitation at the end of 48 weeks and complete necropsies were carried out. Examination included, in addition to the liver, such organs as brain, eye, pancreas, urinary bladder, prostate, salivary gland, diaphragm, skeletal muscle and endocrine organs.

Tissues were fixed in 4% formaldehyde, stained and sectioned with hematoxylin and eosin. Sections of liver were also stained with periodic acid–Schiff for glycogen and mucin, phosphotungstic acid hematoxylin for canaliculi, Masson trichrome for connective tissue, Hall's for bilirubin, and oil red 0 on frozen section for lipid.

The results were analyzed statistically by a scoring method that was described in detail in an earlier paper [1]. The scoring takes into account the hyperplastic lesions, the number and size of neoplastic lesions, the presence or absence of metastases, and the time required for the development of lesions.

The findings in the livers were classified as (1) no hyperplasia, (2) hyperplasia, (3) hyperplastic nodules, (4) small hepatocellular carcinomas (less than 5 mm in diameter), (5) well-developed (large) hepatocellular carcinomas (greater than 5 mm in diameter), (6) metastases and (7) cirrhosis. These lesions have been described and illustrated by photomicrographs in previous publications [2, 3, 12, 13].

Animals dying before 26 weeks were not included in the results because it has been shown that 26 weeks is needed for the development of a small carcinoma of the liver [1].

RESULTS

Animals, of all groups, lost 20–25 g during the first 4-week period on the carcinogen-containing diet but regained the lost weight



Fig. 1. In the chart only the most advanced lesion in a given chemical is graphed. Cirrhosis is included adjacent to the total carcinomas to make a comparison between the effects on both lesion. The number above each block bar is the total number of animals developing such lesions. Under carcinoma *the white area designates the number of animals with metastases. Total carcinomas and cirrhosis show total number of animals in groups as well as the number with those lesions.*

when they were given the basal diet during the 5th week. Thereafter, they maintained or gained weight, except for minor fluctuations, until the end of the experiment.

Twelve-week-old castrated male rats given testosterone propionate survived for an average of 46 weeks (39 to 48 weeks); whereas rats in the other groups survived for 48 weeks, the duration of the experiment.

Table 1. *Summary of findings at autopsy and range of scores. Average liver weight for* 12*-week-old rats on laboratory pellets is* 10·9 *g, for* 52*-week-old rats* 8·5 *g*

Group	Number of Animals	Average liver weight	Average No. of tumors per liver	Usual tumor diam. (cm)	Range of Scores	Mean ± S. E.
12-week-old Castrated male rats	26	13.7	1	0.5	5-63	44 ± 7.6
12-week-old Castrated male rats plus testosterone	25	37.4	3	up to 7.0 cm	56-523	279 ± 42.4
52-week-old rats Castrated male rats	27	7.8	——	——	3-15	9 ± 0.8
52-week-old Castrated male rats plus testosterone	25	10.6	1	0.5 - 1.5 cm	10-82	22 ± 9.5

The number of animals with lesions of the liver is shown in Fig. 1. Gross findings are summarized in Table 1.

Tumors of the liver

The incidence of carcinomas of the liver in 12-week-old castrated male rats given testosterone propionate was 100%, with 60% of the animals having metastases. There were more than 3 carcinomas per liver and the carcinomas measured up to 7·0 cm in greatest diameter. One-fourth of the rats had poorly and undifferentiated carcinomas, and the remainder well-differentiated hepatocellular carcinomas of the liver. The P value between the 12-week-old castrated male rats given testosterone propionate and rats of the other groups was 0·001.

Fifty-two-week-old castrated male rats given testosterone propionate developed hyperplastic nodules and some small and large carcinomas of the liver. There was 1 carcinoma per liver measuring between 0·5 to 1·0 cm. dia. The carcinomas were well-differentiated hepatocellular that did not invade or metastasize.

Only a small number (3 of 25) of 52-week-old castrated male rats not receiving testosterone had hyperplastic nodules, and none had hepatic carcinomas. On the other hand, approximately one third of the 12-week-old castrated males not given testosterone developed carcinomas, mainly small, and another one-third hyperplastic hepatic nodules. There were no metastases in these animals. The difference between the 12-week-old and 52-week-old castrated male rats was significant at $P = 0{\cdot}05$. The difference between the 52-week-old castrated male rats with and without testosterone was $P = 0{\cdot}001$.

Cirrhosis of the liver

Cirrhosis not only developed in 100% of the 12-week-old castrated male rats given testosterone, but the cirrhosis was severe in degree. The cirrhosis in less than one-half of the 12-week-old castrated male rats not given hormones and in two-thirds of the 52-week-old castrated male rats given testosterone was mild. Cirrhosis of the liver was not present in fifty-two-week-old castrated male rats not receiving hormone.

Lesions in organs other than liver

The large clear vacuoles in the cytoplasm of basophils in the pituitary of the castrated male rats were not present in those animals given testosterone propionate.

The prostrate gland in the 12- and 52-week-old castrated male rats was atrophic. The gland in castrated rats given testosterone propionate had focal hyperplasia of glands.

DISCUSSION

Surgical or chemical castration, thyroidectomy, hypophysectomy and alloxan diabetes all decrease the development of hepatocellular carcinomas and cirrhosis in male rats given F-diAA [1, 4–10]. Exogenous testosterone, norethandrolone or progesterone restore carcinogenesis and cirrhosis in castrated males and induces carcinogenesis and cirrhosis in castrated female rats given the chemicals. The thyroid gland must be present in order for testosterone to be effective [5, 7, 14].

Exogenous testosterone restored F-diAA carcinogenesis and cirrhosis in castrated male rats 12 weeks of age. Male rats 52 weeks of

age do not develop carcinomas and cirrhosis of the liver, presumably because of decreased testicular function. Exogenous testosterone failed to significantly restore carcinogenesis and cirrhosis in castrated older male rats.

The effect of hormones in hepatic carcinogenesis is related to the anabolic activity and specifically to protein synthesis. The more effective hormones in promoting carcinogenesis, norethandrolone and testosterone propionate are strongly anabolic [15, 16]. Less effective hormones, such as progesterone and growth hormone, are weekly anabolic [16–19]. Testosterone, growth hormone, and insulin increase the synthesis of protein in the liver [15, 18–21].

There is a decline with age in protein synthesis in the liver [22, 23]. It would seem that testosterone propionate does not increase protein synthesis in the liver of older rats, hence the lack of development of carcinomas and cirrhosis of the liver in rats given F-diAA. There is probably decreased thyroid function in older rats, so a combination of decreased testicular and thyroid function interfering with hepatic carcinogenesis and cirrhosis cannot be ruled out.

REFERENCES

1. H. I. Firminger and M. D. Reuber, Influence of adrenocortical, androgenic, and anabolic hormones on the development of carcinoma and cirrhosis of the liver in A × C rats fed N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **27,** 559 (1961).
2. M. D. Reuber, Development of preneoplastic and neoplastic lesions of the liver in male rats ingesting 0·025 per cent N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **34,** 697 (1965).
3. M. D. Reuber and H. I. Firminger, Morphologic and biologic correlation of lesions obtained in hepatic carcinogenesis in A × C rats given 0·025 per cent N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **31,** 1407 (1963).
4. M. D. Reuber and H. I. Firminger, Effect of progesterone and diethylstilbestrol on hepatic carcinogenesis and cirrhosis in A × C rats fed N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **29,** 933 (1962).
5. M. D. Reuber, The thyroid gland and N-2-fluorenyldiacetamide carcinogenesis and cirrhosis of the liver in Wistar male rats. *J. nat. Cancer Inst.* **35,** 959 (1965).
6. M. D. Reuber, The importance of testis in the induction of hyperplastic nodules, carcinomas, and cirrhosis of the liver in A × C strain male rats ingesting 0·025 per cent N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **53,** 883 (1974).
7. M. D. Reuber, Importance of thyroid hormones and testosterone in the induction of carcinoma and cirrhosis of the liver in female Wistar rats ingesting N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **36,** 775 (1966).
8. M. D. Reuber, Influence of hormones on N-2-fluorenyldiacetamide induced hyperplastic hepatic nodules in rats. *J. nat. Cancer Inst.* **43,** 445 (1969).
9. M. D. Reuber, Effect of alloxan diabetes on hepatic carcinogenesis and cirrhosis in A × C rats given 2-diacetamidofluorene. *Gann* **62,** 157 (1971).
10. M. D. Reuber, Effect of growth hormone on hepatic carcinogenesis and cirrhosis in A × C rats given N-2-fluorenyldiacetamide. *Cancer Res.* **28,** 2177 (1968).
11. M. D. Reuber, Influence of age and sex on cirrhosis and carcinomas of the liver in A × C strain rats ingesting N-2-fluorenyldiacetamide. *Path. microbiol.*, in press (1975).
12. M. D. Reuber and S. Odashima, Further studies on the transplantation of lesions in hepatic carcinogenesis in rats given 0·025 per cent 2-(diacetoamido)-fluorene. *Gann* **58,** 513 (1967).
13. M. D. Reuber, Histopathology of transplantable hepatic carcinomas induced by chemicals in rats. *Gann Monograph* **1,** 43 (1966).
14. L. Hellman, H. L. Bradlow, B. Zumoff, D. K. Fukushima and T. F. Gallagher, Thyroid-androgen interactions and the hypocholesteremic effect of androsterone. *J. clin. Endocr.* **19,** 936 (1959).
15. C. D. Kockakian, The mechanism of the protein anabolic action of testosterone propionate. In: *A Symposium on Steroid Hormones*, E. S. Gordon (ed.), pp. 113–119. Madison: University of Wisconsin Press (1950).
16. F. J. Saunders and V. A. Drill, Comparative androgenic and anabolic effects of several steroids. *Proc. Soc. expl. Biol. med. (N.Y.)* **94,** 646 (1963).
17. E. Eisenberg and G. S. Gordon, The levator ani muscle of the rat as an index of myotrophic activity of steroidal hormones. *J. Pharmacol exp. Therap.* **99,** 38 (1950).

18. A. KORNER, Growth hormone effects on RNA and protein sysnthesis in liver. *J. cell. comp. Physiol.* **66,** Suppl. 1, 153 (1969).
19. A. KORNER, Growth hormone control of biosynthesis of protein and ribonucleic acid. *Red. Progr. Hormone Res.* **21,** 205 (1965).
20. C. A. SNIPES, Effect of growth hormone and insulin on amino acid and protein metabolism. *Quart. Rev. Biol.* **43,** 127 (1968).
21. I. G. WOOL, Insulin and protein biosynthesis. In: *Action of Hormones in Molecular Processes.* (Edited by G. LITWAK and D. KRITCHEVSKY), pp. 422–469. John Wiley, New York (1964).
22. C. H. BARROWS, JR., The effect of age on protein synthesis. In: *Proc. 8th Inst. Congr. Geront.*, Vol. I, pp. 179–182, Washington D.C. (1969).
23. J. P. HRACHOVEC, The effect of age on tissue proteins. I. Age changes in amino acid incorporation by rat liver purified proteins. *Gerontologia (Basel)* **17,** 75 (1971).

Europ. J. Cancer Vol. 12, pp. 143–149. Pergamon Press 1976. Printed in Great Britain

On the Mode of Decrease of Leukemic Cells during Treatment of Acute Leukemia

TORU MASAOKA, YOSHIHISA HASEGAWA, TAKAAKI UEDA, TAKAYUKI TAKUBO, HIROTOSHI SHIBATA, HIROYUKI NAKAMURA, NORIYUKI TATSUMI, JUNSUKE YOSHITAKE and NOBUYUKI SENDA

Centre for Adult Diseases, Osaka
537 *Higashinari-ku Nakamichi* 1-3-3 *Osaka, Japan*

Abstract—*During the induction treatment of acute leukemia the mode of decrease of leukemic cells in blood and bone marrow and of neutrophile leukocytes in blood were analysed by a computer according to the equation,* $\log z = k_1t^2+k_2t+k_3$.

The parameters of decrease i.e., the maximum rate of decrease, the rate of deceleration and the duration of decrease were examined for each case.

In the case treated with 6-*mercaptopurine, cytosine arabinoside, cyclocytidine and* 838-*D, the maximum rate of decrease and the rate of deceleration were lower and the duration of decrease was longer than in the cases treated with daunomycin, neocarzinostatin,* L-*asparaginase and the combination of daunomycin, methotrexate,* 6-*mercaptopurine and predonisolone.*

Drug-dependent difference in the decrease of leukemic cells in the bone marrow and that of neutrophile leukocytes were also observed.

The present kinetic analysis seems to give important information in the treatment of acute leukemia by providing a numerical estimation of the decrease of leukemic cells and neutrophile leukocytes.

INTRODUCTION

ALTHOUGH the decrease of leukemic cells would be one of the most fundamental requirements in the treatment of acute leukemia, the process of decrease is not clear yet. Nor is it easy to estimate how rapid or how long leukemic cells continue to decrease after administration of certain antileukemic drugs.

The present work was started to obtain information about changes in the number of leukemic cells during treatment. We have already reported that an analysis of the decrease curve using the equation, $\log z = k_1t^2+k_2t+k_3$, is useful to obtain such parameters as the maximum rate of decrease, the rate of deceleration and the duration of decrease [1, 2].

In this paper we shall describe the mode of decrease in leukemic cells observed for each drug by the analysis of decrease curves.

Accepted 26 August 1975.

MATERIAL AND METHODS

During the treatment of acute leukemia the number of leukemic cells in total circulatory blood was plotted against time on semi-logarithmic paper. Blood volume was estimated as 7% of body weight. Each decrease curve was analysed by a computer using the equation, $\log z = k_1t^2+k_2t+k_3$, as described before [1, 2]. The parameters tested were the maximum rate of decrease $(2k_1t_1+k_2)$, the rate of deceleration $(2k_1)$ and the duration of decrease ($t$min).

Figure 1 shows an example of such a decrease curve. In some cases decrease curves of leukemic cells in 1 μl bone marrow or those of neutrophile leukocytes in total circulatory blood were

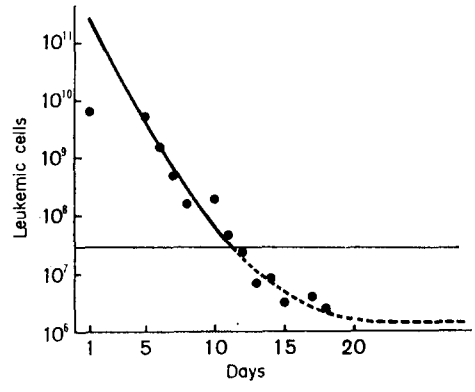


Fig. 1. An example of analysis of decrease curve of leukemic cells in blood. AML, female, 15 years old (case 15).

$log\ z = 0{\cdot}01272t^2 - 0{\cdot}54143t + 11{\cdot}9841$,
$tmin = 21{\cdot}3$ *(days)*, $zmin = 1{\cdot}66 \times 10^6$,
$t_1 = 5$*th day*, $2k_1t_1 + k_2 = -0{\cdot}4143$ $(d/dt\ logz)$,
$2k_1 = 0{\cdot}02542$ $(d^2/dt^2\ logz)$.

tmin is the time and zmin is the number of leukemic cells in total blood when they cease to decrease. t_1 is the day when the examined points begin to fit with the curve. $2k_1t_1 + k_2$ is the maximum rate of decrease. $2k_1$ is the rate of deceleration of decrease. The equation was computed by a computer using the points obtained by differential count of 100 leukocytes. After disappearance of leukemic cells the curve extended according to the equation, coincided with the points obtained by differential count of 5000 leukocytes (broken line). Multiple correlation coefficient between examined points and points estimated from the computed curve was 0·970 indicating a close fitness of the equation to the examined points. This case was treated with daunomycin. Leukemic cells decreased and disappeared as shown in the figure. On the 20th day leukemic cells were less than 5% in the bone marrow and was judged as a complete remission.

also examined. In 27 previously untreated, acute leukemia cases decrease curves were examined as to their differences by drugs.

There were 7 cases of acute lymphatic leukemia, 11 of acute myeologenous leukemia, 2 of blastic crisis of chronic myelogenous leukemia, 5 of acute monocytic leukemia and 2 cases of erythroleukemia (Table 1).

In 11 cases complete remission by the criteria of Kimura [3] was achieved. After the first treatment resistant cases were treated with other drugs. Their length of survival from the start of treatment is shown in Table 1.

The abbreviations for the type of leukemia and drug administered are shown in Table 1.

RESULTS

Decrease curves of 28 courses of treatment in 27 cases of acute leukemia were analyzed. Tables 1 and 2 summarize the cases tested.

All decrease curves fitted well with the number of leukemic cells measured. The multiple correlation coefficients between examined points estimated from each computed curve are shown in Table 2. Multiple correlation coefficients were in a range between 0·850 and 0·999, indicating a fairly close fitness of the examined points to the computed curve.

Mode of decrease of leukemic cells in blood

The maximum rates of decrease of leukemic cells in the blood, the rates of deceleration and the duration of decrease were examined for several drugs. As Table 2 shows, the maximum rates of decrease $(2k_1t_1 + k_2)$ were lower in the cases treated with 6MP, CA, CC and 838-D, than those with DM, NCS, ASP and DAMP.

The rates of deceleration $(2k_1)$ were also lower in the cases treated with 6MP, CA, CC and 838-D than those with DM, NCS, ASP and DAMP.

The duration of decrease (*t*min) was markedly longer in the cases treated with 6MP and 838-D and slightly longer with CA and CC than those treated with DM, NCS, ASP and DAMP.

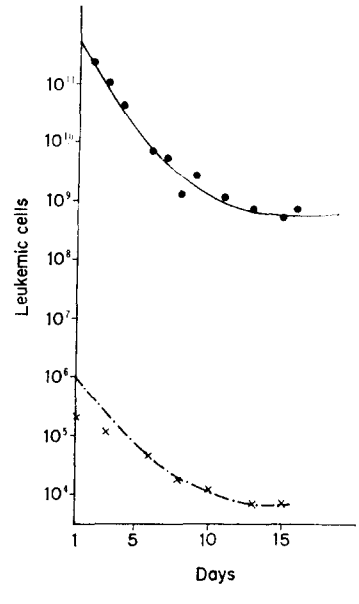


Fig. 2. Decrease curves of leukemic cells in blood and bone marrow AMoL, male, 32 years old (case 16).

For blood,
$logz = 0{\cdot}01718t^2 - 0{\cdot}48333t + 12{\cdot}2162$,
$tmin = 14{\cdot}1$, $zmin = 6{\cdot}61 \times 10^8$,
(z = leukemic cells in total blood).

For bone marrow,
$logz = 0{\cdot}01274t^2 - 0{\cdot}35611t + 6{\cdot}33186$,
$tmin = 14{\cdot}0$, $zmin = 6{\cdot}92 \times 10^3$
(z = leukemic cells/μl of the marrow).

In this case neocarzinostatin was given daily for 8 days. The bone marrow was punctured 3 times a week. Leukemic cells ceased to decrease on the 14th day both in blood and the bone marrow.

Decrease of leukemic cells in the bone marrow

The equation above described was applied to leukemic cells in the bone marrow. The bone marrow was obtained 3 times a week and the decrease curves were drawn. Figure 2 illustrates typical decrease curves both found in blood and bone marrow. The computed curve fitted well with the examined points.

Multiple correlation coefficients between examined points and points estimated from each computed curve are shown in Table 3. Multiple correlation coefficients were in a range between 0·865 and 0·999, indicating a fairly close fitness of the examined points to the computed curve. Table 3 shows that each *t*min value (duration of decrease) of leukemic cells in the bone marrow was similar to that found in blood.

In cases 5, 11 and 26, which were treated with 838-D, DAMP and CC respectively, the maximum rate of decrease of leukemic cells in each was higher in the bone marrow than in blood.

On the other hand, in cases 16, 18 and 28, which were treated with NCS and DM combined with 838-D, the maximum rate of decrease of leukemic cells was higher in blood than in the bone marrow.

Decrease of neutrophile leukocytes

The same procedure was applied to neutrophile leukocytes. Figure 3 shows that the equation can also be applied to the decrease curve of neutrophile leukocytes.

Multiple correlation coefficients between the examined points and the estimated points from

Table 1. *Cases tested. ALL: acute lymphatic leukemia, AML: acute myelogenous leukemia, AMoL: acute monocytic leukemia, EL: erythoroleukemia, CML: blast crisis of chronic myelogenous leukemia. ASP:* 1-*asparaginase* 200μ/*kg daily*, 6*MP:* 6-*mercaptopurine* 2*mg/kg daily, DM: daunomycin* 0·8*mg/kg* 4 *days/week*, 838*:* (838-*D*) *N-Methyl-N, N-bis*(3-*methylsulfonyl oxypropyl*) *amine* 4,4′*biphenyldisulfonate*, (*NSC* 140115) 1 *mg/kg daily* 8–14 *days* [4], *DAMP: combination of daunomycin, amethopterine,* 6*MP and predonisolone, CA: cytosine arabinoside* 1·6 *mg/kg* 5 *days/week, NCS: neocarzinostatin* 0·04 *mg/kg daily* 8–14 *days. before: before treatment. after: after treatment, NCC: Nucleated cell count* ($\times 10^3$), *Survival: from the start of treatment* (*months*), *Cases* 6 *and* 16 *are identical. This case was treated at first with NCS and then with* 838-*D.*

				Leukemic cells					
				Blood		Bone marrow			
Case	Sex Age	Type	Treatment	Before WBC %	After WBC %	Before NCC × 10^3 %	After NCC %	Complete remission	Survival
1	m 56	AML	6MP	19900 73·5	4100 12.0	130 79.9	24 42	no	2
2	f 4	ALL	6MP	12900 44·5	1000 0	960 95·4	96 0·7	yes	55
3	f 14	AML	6MP	3700 48.0	1900 1	34 29·0	32 1·0	yes	25
4	m 6	ALL	838	2400 2·1	200 0	425 69·7	42 0·3	yes	26
5	m 15	AMOL	838	6000 51·0	300 0·4	249 68·5	39 1·7	yes	9
6	m 32	AMOL	838	4400 8·4	700 0	46 58·2	7 1	yes	8
7	m 29	CML	838	168000 55·0	58700 5·0	390 20·0	106 12·0	no	2
8	f 34	CML	CA	16000 19·0	1300 0	120 24·0	5 33·0	no	3
9	m 23	AML	CA	700 13·0	400 2·2	168 86·9	112 79·4	no	21 ~
10	m 29	AMOL	CA	44100 79·0	2000 3·8	42 66·7	12 25·0	no	18 ~
11	f 29	ALL	CC	4200 45·0	400 0·4	357 90·0	12 2·0	yes	18
12	f 16	AML	DM	130000 30·0	1100 0	685 38·8	47 3·4	yes	12
13	m 39	AML	DM	6700 35·0	600 0·2	179 16·9	18 0·2	yes	17
14	m 15	ALL	DM	11200 9·5	1900 0·05	520 86·4	6·5 5·3	no	9
15	f 15	AML	DM	4000 35·0	400 0·1	145 15·0	32 3·0	yes	12
16	m 32	AMOL	NCS	94000 73·0	2600 7·6	289 51·1	38 32·6	no	8
17	f 21	AMOL	NCS	4000 23·0	700 0·1	341 33·0	6 8·0	no	15
18	f 7	ALL	NCS	5300 30·0	600 1·6	33 75·9	35 52·5	no	10
19	m 41	AML	NCS	6600 34·0	3000 4·0	140 38·5	35 7·0	no	6
20	m 32	ALL	ASP	117000 90·0	1000 18·0	490 86·2	132 95·4	no	6·5
21	m 23	ALL	ASP	83400 96·5	2000 60·0	344 94·6	121 62·0	no	2
22	f 17	EL	DAMP	1400 26·6	1000 0	29 52·5	4·5 4·5	yes	8
23	m 37	EL	DAMP	5600 29·0	3200 5·0	180 39·4	59 34·2	no	12
24	f 28	AML	DAMP	200 31·0	200 0·1	135 87·4	11 11·5	no	38
25	m 41	AML	DAMP	5200 52·0	400 0	196 65·3	4 0·8	yes	47 ~
26	m 44	AML	DAMP	9400 96·0	300 47·2	375 76·7	9 58·0	no	5
27	m 33	AMOL	CA.6MP	17800 12·0	1000 3·0	183 54·1	15 14·5	no	4
28	f 49	AML	DM.838	1100 18·6	200 0·15	198 74·0	7 10·0	no	2

Table 2. Maximum rate of decrease ($2k_1t_1+k_2$), *rate of deceleration* ($2k_1$) *and duration of decrease* (t *min*) *of leukemic cells in blood. Multiple correlation coefficient: between examined points and points obtained from the computed curve*

Drug	Case	$2k_1t_1+k_2$ (log/day)	Mean	$2k_1$ (log/day/day)	Mean	*t*min	(days) Mean	Multiple correlation coefficient
6MP	1	−0·1511	−0·1838	0·003320	0·01242	49·8	53·1	0·949
	2	−0·1324		0·001830		82·3		0·953
	3	−0·2678		0·03211		27·3		0·950
838	4	−0·1838	−0·1704	0·01628	0·008390	31·3	42·0	0·999
	5	−0·07445		0·001500		59·9		0·923
	6	−0·2812		0·007840		37·8		0·970
	7	−0·1422		0·007940		38·9		0·859
CA	8	−0·07723	−0·2349	0·002314	0·02962	36·4	22·3	0·949
	9	−0·2032		0·02328		14·7		0·900
	10	−0·4241		0·06326		15·7		0·999
CC	11	−0·2561		0·01726		20·8		0·889
DM	12	−0·4778	−0·5190	0·02900	0·03433	20·5	16·7	0·994
	13	−0·6308		0·05836		12·8		0·976
	14	−0·5531		0·02454		12·3		0·990
	15	−0·4143		0·02542		21·3		0·970
NCS	16	−0·4146	−0·4190	0·03436	0·05462	14·1	12·4	0·970
	17	−0·5990		0·1089		10·5		0·963
	18	−0·3320		0·03338		14·9		0·995
	19	−0·3302		0·04184		9·9		0·981
ASP	20	−0·2481	−0·4936	0·01810	0·1257	14·0	9·1	0·945
	21	−0·7391		0·2332		4·1		0·995
DAMP	22	−0·6200	−0·4126	0·07434	0·06005	10·3	19·1	0·950
	23	−0·4511		0·15012		22·0		0·920
	24	−0·3056		0·02102		15·5		0·850
	25	−0·6017		0·04804		18·5		0·951
	26	−0·08482		0·00672		18·6		0·876
CA 6MP DM 838	27	−0·09956		0·009816		19·1		0·854
	28	−0·4511		0·03758		17·0		0·959

Table 3. Decrease of leukemic cells in blood and bone marrow. Multiple correlation coefficient: between examined points and points estimated from computed decrease curve of leukemic cells in the bone marrow

Drug	Case	$2K_1t_1+K_2$		$2K_1$		*t* min		Multiple correlation coefficient
		Blood	Marrow	Blood	Marrow	Blood	Marrow	
838	5	−0·07445	−0·1095	0·001500	0·002320	59·9	55·3	0·954
DAMP	26	−0·08482	−0·2942	0·006720	0·02242	18·6	19·1	0·965
NCS	16	−0·4146	−0·2287	0·03436	0·02548	14·1	14·0	0·865
NCS	18	−0·3320	−0·03762	0·03338	0·001960	14·9	19·2	0·880
CC	11	−0·2561	−0·6270	0·01726	0·05728	20·8	16·9	0·990
DM838	28	−0·4511	−0·3568	0·03758	0·03804	17·0	17·4	0·915

each computed curve are shown in Table 4. Multiple correlation coefficients were in a range between 0·725 and 0·999, indicating a fairly close fitness of examined points to the computed curves. In 15 cases the curves of the neutrophile leukocytes were compared with those of leukemic cells.

In all cases, except two treated with 838-D, the maximum rate of decrease ($2k_1t_1+k_2$) was higher in leukemic cells than in neutrophile leukocytes.

The ratio of $2k_1t_1+k_2$ of leukemic cells to that of neutrophile leukocytes (b/n of $2k_1t_1+k_2$) was low with 838-D, high with daunomycin and highest with DAMP. This tendency was also seen with the ratio of $2k_1$ of leukemic cells to that of neutrophile leukocytes (b/n of $2k_1$).

Each *t*min value (duration of decrease) for leukemic cells was very similar to that for neutrophile leukocytes, regardless of the drug used.

An example of drug selection

Case 16 AMoL male, 32 years old.

The patient had acute pneumonia by klebsiella. The number of leukemic cells were WBC 96400 with 73% monoblasts and were rapidly increasing. In this case a rapid decrease of leukemic cells in the blood would be necessary to prevent cerebral hemorrhage, but an extremely low level of leukocytes might be dangerous in acute pneumonia.

NCS was selected because of its rapid but short decrease of leukemic cells and low rate of decrease of neutrophile leukocytes. After administration of NCS, leukemic cells in the blood decreased rapidly. They ceased to decrease early on the 14th day at the level of WBC 2600 and leukemic cells $6·6\times10^8$ in total blood (Figure 4). After acute pneumonia was cured by an intensive antibiotic treatment, the patient was again treated with 838-D. 838-D was selected because it may decrease leukemic cells in the blood slow, but over a long period and it tends to decrease leukemic cells in the bone marrow more rapidly than in the blood.

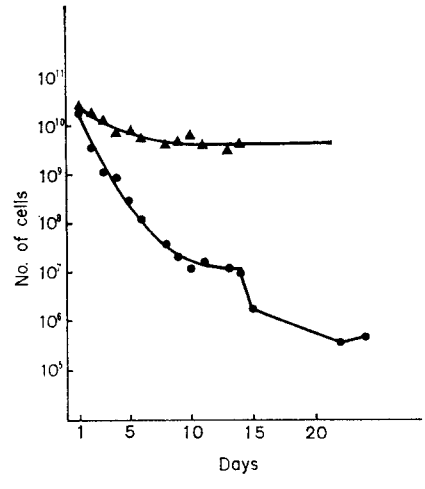


Fig. 3. *Decrease curves of leukemic cells and neutrophile leukocytes.*

ALL, male, 15 years old (case 14).

For leukemic cells,

$logz = 0·2454t^2-0·60222t+11·7503$,

$tmin = 12·3$, $zmin = 1·15\times10^7$.

For neutrophile leukocytes,

$logz = 0·00932t^2-0·18726t+10·5457$,

$tmin = 10·0$, $zmin = 3·99\times10^9$.

In this case daunomycin was given daily for 4 days. Neutrophile leukocytes decreased slower than leukemic cells. Both cells ceased to decrease almost at the same time.

Table 4. *Decrease of leukemic cells and neutrophils. b/n: ratio of leukemic blast cell to neutrophile leukocytes*

Drug	Case	$2k_1t_1+k_2$			$2k_1$			*t*min		Multiple correlation coefficient
		Blasts	Neutrophils	b/n	Blasts	Neutrophils	b/n	Blasts	Neutrophils	
838	4	−0·1838	−0·2515	0·73	0·01628	0·05308	0·31	31·3	35·7	0·956
	5	−0·07445	−0·08949	0·83	0·00150	0·001520	1·00	59·9	69·1	0·989
	7	−0·1422	−0·1013	1·40	0·00794	0·003240	2·50	38·9	46·2	0·990
DM	13	−0·6308	−0·1477	4·30	0·05836	0·01370	4·30	12·8	16·8	0·815
	14	−0·5531	−0·1686	3·30	0·02454	0·01864	1·30	12·3	10·0	0·931
	15	−0·4143	−0·1777	2·30	0·02542	0·01324	1·90	21·3	19·4	0·927
NCS	16	−0·4146	−0·1566	2·60	0·03436	0·01490	2·30	14·1	15·5	0·865
	17	−0·5990	−0·1615	3·70	0·1089	0·01882	5·80	10·5	12·6	0·884
	18	−0·3320	−0·2085	1·60	0·03338	0·05490	0·61	14·9	15·8	0·889
CA	9	−0·2032	−0·1397	1·50	0·02328	0·01620	1·40	14·7	12·6	0·945
	10	−0·4241	−0·1266	3·30	0·06326	0·01790	3·50	15·7	13·1	0·743
CC	11	−0·2561	−0·1753	1·50	0·01726	0·01444	1·20	20·8	19·1	0·941
DAMP	23	−0·4511	−0·04903	9·30	0·15012	0·004380	34·3	22·0	21·2	0·730
	25	−0·6017	−0·03294	18·3	0·04804	0·002460	19·5	18·5	19·4	0·725
DM838	28	−0·4511	−0·4016	1·10	0·03758	0·04906	0·76	17·0	19·2	0·970

A complete remission was achieved on the 68th day.

DISCUSSION

For the effective decrease of leukemic cells it would be necessary to clarify the mode of decrease of leukemic cells after administration of each drug.

By the analysis of decrease curves differences in the mode of decrease of leukemic cells in blood were observed.

In the cases treated with 6MP, CA, CC and 838-D the maximum rates of decrease and the rates of deceleration were higher and the duration of decrease was longer than in the cases treated with DM, NCS, ASP and DAMP.

This means that in the cases treated with daunomycin, leukemic cells decreased rapidly in the early stage of treatment, but the rapid decrease slows down and ceases rather early, while in the cases treated with 838-D leukemic cells decrease slowly but continue to decrease over a longer period with a low rate of decrease slow-down. Although the number of cases tested were few, L-asparaginase, neocarzinostatin and DAMP seem to act like daunomycin, but 6MP, cytosine arabinoside act like 838-D.

The same procedure was found applicable to the decrease curve of leukemic cells in the bone marrow of all six cases tested. Although bone marrow aspirates are not very accurate in quantitating leukemic cells in the bone marrow, frequent aspirations of the marrow seemed to approximate changes in the number of leukemic cells in the marrow, as indicated by high correlation between examined points and points estimated from each computed curve (Table 3).

This preliminary test indicated that the decrease curve of leukemic cells per unit volume of the bone marrow gave a very similar curve as those for leukemic cells in blood. Although these should be tested with many more cases, the kinetic for the decrease of leukemic cells in the marrow seems to be fundamentally similar to that for blood.

In the cases treated with 838-D, DAMP and CC the maximum rate of decrease were higher in the bone marrow than in blood, while in the cases treated with NCS and DM combined with 838 these rates were higher in blood than in the bone marrow.

The drug dependent difference in the rate of decrease of leukemic cells between blood and bone marrow seems very important in order to produce a complete remission, because in many cases leukemic cells disappear in blood but they remain in the marrow.

The same procedure seems also applicable to the decrease of neutrophile leukocytes. The ratio of the rate of decrease of leukemic cells to that of neutrophile leukocytes (b/n ratio of $2k_1t_1+k_2$) was low with 838-D, high with DM and highest with DAMP.

The difference in the rate of decrease between leukemic cells and neutrophile leukocytes seems to indicate difference of side effects of the drugs. It was also found that in many cases leukemic cells ceased to decrease when the decrease of neutrophile leukocytes stopped. Therefore, when neutrophile leukocytes stopped decreasing by special treatment, further decrease of leukemic cells could hardly be expected. This seems helpful in treatments, because neutrophile leukocytes remained in the blood in many cases, while leukemic cells are not demonstrated in blood by successful treatments of leukemia.

For practical use the ideal drug would be that

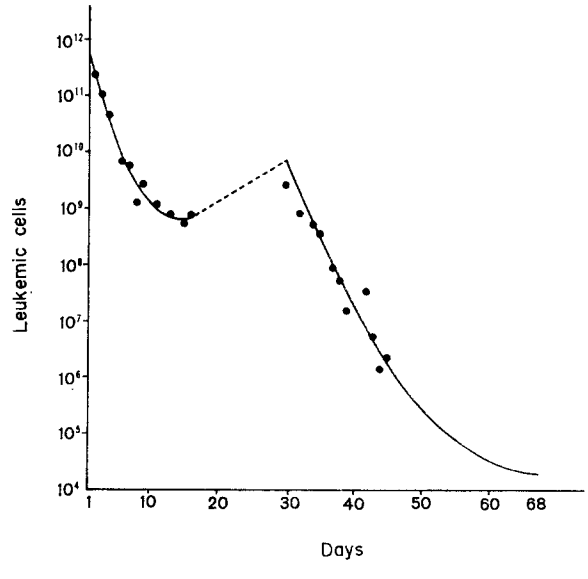


Fig. 4. Decrease curve of leukemic cells of case 16.

AMoL, male, 32 *years old. This patient had acute pneumonia by klebsiella. Before treatment white cell count was* 94000 *with* 73% *monoblasts. This case was treated with neocarzinostatin* 2 *mg daily with intensive antibiotic therapy. Leukemic cells decreased rapidly and ceased to decrease on the* 14*th day. On the* 21*st day pneumonia was almost cured. On the* 28*th day second treatment with* 838-*D was started to produce a complete remission. The left side curve of the figure shows the decrease of leukemic cells in blood during NCS administration.*

$$\log z = 0{\cdot}01718t^2 + 0{\cdot}48333t + 12{\cdot}2163,$$

$$tmin = 14{\cdot}1,\ zmin = 6{\cdot}61 \times 10^8.$$

The decrease ceased on the 14*th day at the level of* $6{\cdot}6 \times 10^8$. *The right side curve of the figure shows the decrease of leukemic cells during* 838-*D administration.*

$$\log z = 0{\cdot}00392t^2 - 0{\cdot}53206t + 22{\cdot}30769,$$

$$tmin = 67{\cdot}8,\ zmin = 1{\cdot}87 \times 10^4.$$

Leukemic cells cease to decrease on the 68*th day after the start of NCS administration at the level of* $1{\cdot}8 \times 10^4$. *On the* 68*th day leukemic cells in the bone marrow was* 1·0%.

which decreases leukemic cells in the bone marrow rapidly and neutrophile leukocytes slowly. For leukemic cells in blood we have already reported that together with the high maximum rate of decrease, long duration of decrease is also a determinant for longer survival of patients.

However, we could not find any drug available which shows all such beneficial modes of decrease of cells. So it would be necessary to select drugs suitable for the condition of patients as seen in Fig. 4, or to combine drugs which would compensate the disadvantages of each other. Although cases tested were few, the mode of decrease of leukemic cells in the bone marrow and that of neutrophile leukocytes was considered to be more favorable in DAMP cases than in other cases without combination.

The kinetic treatment of the decrease curve here described to analyze the change of the number of cells during treatments and to evaluate the mode of action of drugs would be very helpful for planning the treatment of acute leukemia.

REFERENCES

1. T. Masaoka and J. Yoshitake, On the decrease curve of leukemic cells and treatment of acute leukemia. *Acta haem. Jap.* **37,** 351 (1974).
2. T. Masaoka, Y. Hasegawa, N. Tatsumi, J. Yoshitake, T. Ueda, T. Takubo H. Shibata, H. Nakamura and N. Senda, On the analysis of decrease curve of leukemic cells during treatment of acute leukemia. *Acta haem. Jap.* **39,** (1976) (in press).
3. K. Kimura, K. Ota and H. Tsuneyoshi, Supplementary studies of chemotherapy of leukemia. *Gann.* **48,** 496 (1957).
4. M. Hirano, M. Miura, H. Kakizawa, A. Morita, T. Uetani, R. Ohno, K. Kawashima, H. Nishiwaki and K. Yamada, Effect of two new sulfonic acid esters of aminoglycols on chronic myelogenous leukemia. *Cancer Chemother. Rep.* **56,** 47 (1972).

Europ. J. Cancer Vol. 12, pp. 151–157. Pergamon Press 1976. Printed in Great Britain

Detection of Epidermal Growth Factor (EGF)-Binding Sites on HeLa and Corneal Epithelial Cells

R. REVOLTELLA,* M. PEDICONI, M. AMINI†, C. BOSMAN† and L. BERTOLINI

Laboratory of Cell Biology, C.N.R., Rome, Italy

Abstract—*HeLa cells grown in suspension bound, at 2°C, sheep erythrocytes passively coated with Epidermal Growth Factor (EGF) leading to formation of stable rosettes. Rosette formation was similarly obtained with a primary culture of corneal epithelial cells.*

In contrast, only a limited capacity to form rosettes was achieved using normal mouse- or rabbit-spleen lymphocytes, kidney and liver cells, or various tumor-cell lines used as a source of control cells. The binding of EGF occurs at specific binding sites exposed on the cell-surface membrane of the epithelial cells. The reaction can be prevented by a mild cell treatment with proteolytic enzymes; the binding reaction reappears, however, within two hr.

INTRODUCTION

THE EPIDERMAL growth factor (EGF) is a polypeptide isolated from the submaxillary glands of adult, male mice [1, 2], which specifically stimulates the proliferation of various epidermal and epithelial tissues both *in vivo* and *in vitro* [3–5]. The complete amino-acid sequence of EGF has been recently established [2]. The purpose of this investigation was to see whether HeLa cells, which are tumor cells originally derived from an epithelium, or cells in suspension from a primary culture of corneal epithelial cells were capable of binding on their surface membrane EGF-coated erythrocytes leading to the formation of stable rosettes.

The results reported below provide supporting evidence for the existence of high-avidity binding sites for EGF on the surface membrane of both normal and tumor epithelial cells.

Accepted 20 June 1975.

*Correspondence: Roberto Revoltella, M.D. Ph.D.; Cell Biology, C.N.R., Via Romagnosi 18A, 00196, Rome, Italy.

†Department of Pathology, University of Rome, Italy.

MATERIAL AND METHODS

Cells

The following lines of cells were used in the experiments:

(a) murine C 1300 neuroblastoma cells, clone NB/1R, recently developed in our laboratory from an original line received from Dr. F. Jacob, Paris; human neuroblastoma cells, clone NJB, received from Dr. M. Goldstein, St. Louis. Both murine and human neuroblastoma (NB) cells were cultured in suspension in Dulbecco's modified medium (GIBCO) supplemented with 20% fetal bovine serum under 12% CO_2 and air [6].

(b) Murine sarcoma S/180 cells from the American Type Culture Collection. These cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum.

(c) Murine sarcoma 2S/37 cells, a cloned line recently developed in our laboratory. The cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum.

(d) Murine L-929 fibroblasts (NCTC, clone 929). These cells were grown in MEM supple-

mented with 10% fetal calf serum and 10% trypticase soy broth.

(e) HeLa cells. These cells were grown in suspension in Joklik medium supplemented with 5% horse serum, under 56% CO_2 and air.

(f) Suspensions of primary cultures of mouse or rabbit corneal epithelial cells, kidney and liver cells, or spleen lymphocytes, were prepared before use from Swiss Webster mice. Cell suspensions were obtained after incubation of small pieces of these tissues in 0·125% trypsin in Eagle's salt balanced solution, containing 0·005M EDTA, at 37°C for 15 min. The cells were then dispersed by teasing the pieces with a rubber policeman over a 200 μm stainless sieve mesh, washed in phosphate-buffered saline (PBS) pH 7·4 and finally incubated in MEM containing 0·005 M EDTA for three to five hours before use. Cell viability was tested before and after rosette formation by the 0·04% erythrosin dye-exclusion test.

EGF

EGF was prepared from acidified homogenates of the submaxillary glands of adult, albino mice, as previously described [2].

Protein-coating of erythrocytes

Sheep blood red cells (SBRC) in Alsever's solution were washed three times in PBS and then fixed for five minutes at room temperature in 0·125% glutaraldehyde in 0·12 M phosphate buffer pH 7·6 SBRC were then washed three times and 0·5 ml of packed cells were incubated in 5·0 ml of this buffer containing EGF and 10·0 mg of 1-ethyl-3 (3 dietyl amino-propil)-carbodiimide-hydrochloride (CDII) (Otto Chemical Co., Muskegon, Mich.). As control, preparations of SBRC coated with unrelated proteins, such as ferritin (Pentex), ribonuclease and cytochrome-c (Wortington), mouse, rabbit, or human gamma globulins and serum albumin (Mann), were similarly prepared. SBRC were also coated with purified Nerve-Growth-Factor (NGF), prepared from the submaxillary gland of mice as originally described by Bocchini and Angeletti [7]. NGF-coated SBRC were used as source of positive control for rosette formation by the two lines of NB cells which are both capable of binding with specific high-avidity receptor sites the NGF protein [8, 9].

The intensity of protein coating to erythrocytes was tested by the titer of passive hemagglutination tests using specific hyperimmune antibodies. Controls comprised similarly prepared suspensions of SBRC but without addition of any protein.

After one hour of incubation at room temperature, the erythrocytes were washed first in PBS containing 0·1% normal mouse gamma globulins and then in PBS alone, until no protein was detected in the supernatant by optical density readings at 280 nm.

Protein-coated erythrocytes did not spontaneously agglutinate and were stable for about 10–15 days, as tested by the titer of passive hemagglutination tests.

Test of rosette-forming cells (RFC)

The nucleated cells in suspension were washed three times in PBS before use. The viability of the cells used in the experiments was always higher than 95–98%. EGF-coated SBRC (1×10^7) were added to $2{\cdot}5 \times 10^5$ nucleated cells in a final volume of 1·1 ml of cold PBS. The suspension was incubated for five minutes in plastic tubes immersed in an ice bath. The tubes were then centrifuged in the cold for five minutes at $250 \times g$. The cells were resuspended in their own supernatant by a vigorous pipetting and examined microscopically at ×400 magnification for rosette formation. Cells with more than five adherent erythrocytes were considered RFC.

Inhibition of rosette formation

Inhibition of rosette formation was studied by exposing the cells for 30 min at 2°C to the following: normal rabbit, human, and mouse gamma globulin or albumin, ferritin, ribonuclease, cytochorome-c, free EGF, or NGF. Colchicine (Sigma) and vinblastine (Velbe, Lilly) were added at a final concentration of 10^{-5}M.

Cytochalasin-B, dissolved in one or two drops of dimethylsulfoxide and then brought up to volume in PBS, was added at a final concentration of 10 μg/ml. Trypsin (100 μg/ml) was incubated with 5×10^6 cells at 37°C for 15 min in PBS pH 7·4; to terminate the reaction; an equimolar concentration of soybean trypsin inhibitor (Worthington) was added and the temperature lowered to 2°C. Neuraminidase (100 μg/ml) dissolved in calcium buffer pH 6.2 was incubated with the cells at 37°C for 15 minutes, the temperature was then lowered to 2°C and the cells were washed exhaustively. Phospholipases C and D (150 μg/ml) were dissolved in phosphate buffer pH 7·4 at 2°C. After incubation with the inhibitors, cells were washed three times with 5·0 ml of cold PBS and their capacity to form rosettes was tested. Cells were also exposed to ethylendiaminotetracetic acid (EDTA) at 0·01 M final concentration, azide (0·02%), or antimycin

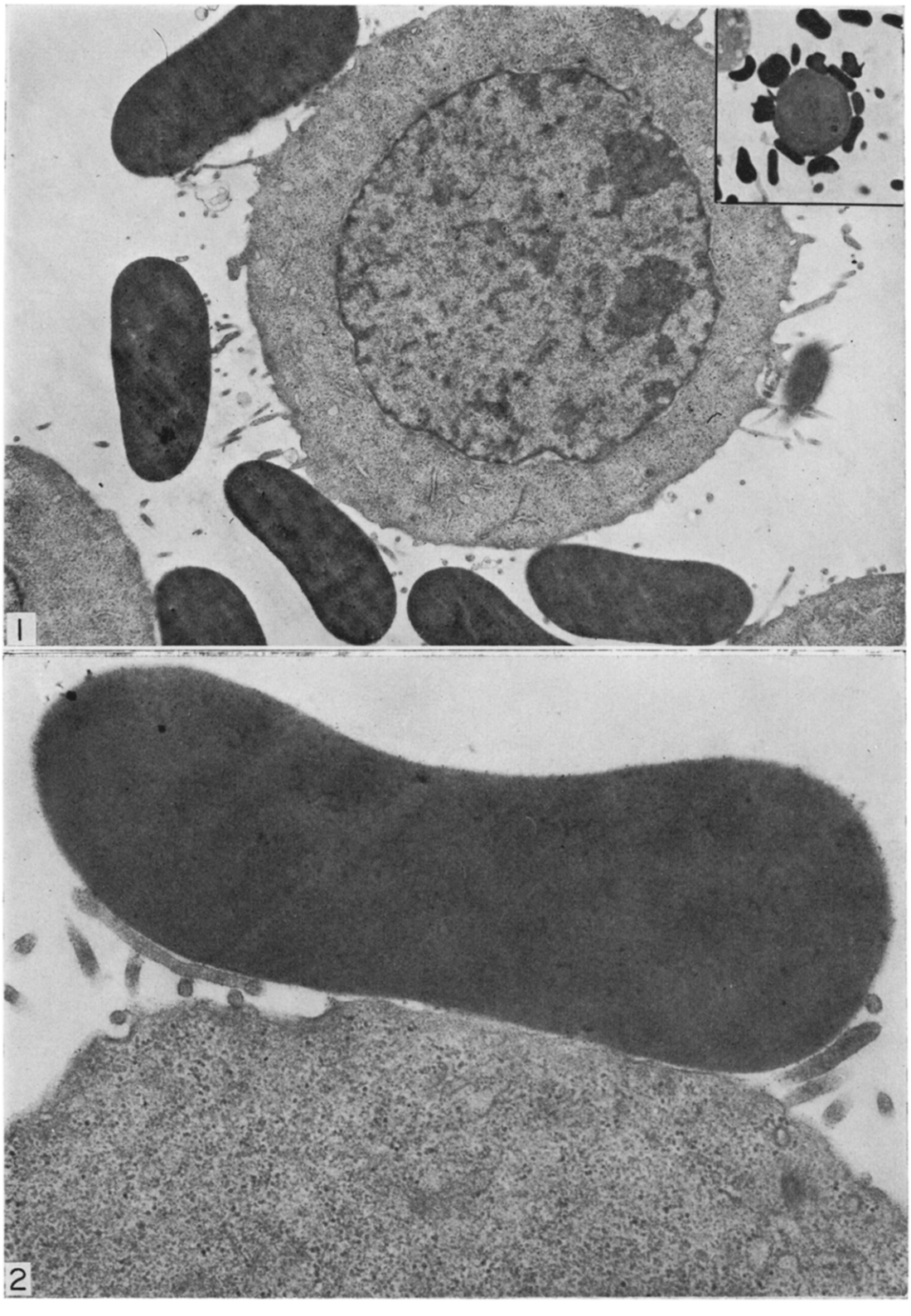


Fig. 1. Typical HeLa rosette-forming cell with EGF-coated erythrocytes at the electron microscope (× 12,500). *In the insert, low magnification of a rosette* (× 1600).

Fig. 2. Detail of a HeLa rosette-forming cell. A large area of contact is visible on a section tangential to the surface membrane of the two adherent cells. Note cytoplasmic processes departing from the HeLa cell towards the EGF-coated erythrocytes (× 39,000).

(to face page 152)

(5 μg/ml), which were added in the medium diluent. At the end of the reaction, the number of viable RFC was counted.

Electron microscopy

The cells in suspension, after reacting with EGF-coated SBRC were fixed in 1% glutaraldehyde in 0·1 M K-phosphate buffer pH 7·4, and then processed for electron microscopy, following the procedure described by Gudat *et al.* [10].

RESULTS

Rosette-forming cells

HeLa cells incubated with EGF-coated SBRC formed rosettes (Figs. 1 and 2). These appeared very stable, since vigorous pipettings did not detach the membrane-bound erythrocytes. Rosette formation with EGF-coated SBRC was similarly obtained with a suspension of primary cultures of rabbit or mouse corneal epithelial cells. Both HeLa and corneal epithelial cells, except with EGF-coated SBRC, did not form stable rosettes with other preparations of protein-coated or uncoated erythrocytes (Tables 1 and 2).

Suspensions of normal kidney or liver cells, spleen lymphocytes, fibroblast and sarcoma cells exhibited a very limited capacity of binding EGF (Table 2). In addition, the few adherent erythrocytes could be easily detached from the membrane surface of these latter cells after

Table 1. *Percentage of rosette formation**

	Ligands coated to SBRC						
Cells	EGF	NGF	Ferritin	Ribonucleases	Cytochrome-c	MCG or RGG	MSA or RSA
HeLa	82 ± 3	4 ± 1	2 ± 1	2 ± 1	3 ± 2	2 ± 1	2 ± 1
Corneal Epithelial:							
Mouse	80 ± 2	6 ± 2	2 ± 1	2 ± 1	3	4 ± 1	2 ± 1
Rabbit	76 ± 1	5 ± 1	2 ± 1	3 ± 1	3 ± 1	2 ± 1	2 ± 1

*arithmetical mean on triplicates
EGF; epidermal growth factor; NGF, nerve-growth factor; MGG; RGG, mouse or rabbit gamma globulin; MSA, RSA, mouse or rabbit albumin.
2×10^5 viable nucleated cells in suspension were incubated at 2°C for five minutes with different protein-coated SBRC. The tubes were centrifuged in the cold at $250 \times g$ for five minutes. The cells were then resuspended by a vigorous pipetting in their own supernatant and the number of rosette-forming cells was counted in a haemocytometric chamber.

Table 2. *Percentage of rosette formation**

	EGF-SBRC				NGF-SBRC			
	Inhibitors				Inhibitors			
Cells	None	EGF	NGF	MGG	None	EGF	NGF	MGG
HeLa	82 ± 1	2 ± 1	83 ± 3	84 ± 2	4 ± 1	2 ± 1	0	2 ± 1
NB C 1300 (NB/1R)	4 ± 2	0	0	5 ± 2	86 ± 5	82 ± 4	4 ± 3	86 ± 5
NJB	4 ± 1	0	0	3 ± 2	88 ± 7	82 ± 5	3 ± 1	78 ± 7
S/180; 2S/37	3 ± 2	0	0	5 ± 2	2 ± 1	3 ± 2	4 ± 1	2 ± 1
Corneal epithelial	78 ± 3	3 ± 1	81 ± 4	76 ± 5	8 ± 1	4 ± 2	0	2 ± 1
Kidney, liver cells; spleen lymphocytes	2 ± 1	0	4 ± 1	3 ± 1	6 ± 3	3 ± 1	0	6 ± 2

*arithmetical mean on triplicates
NGF, nerve-growth factor; EGF, epidermal growth factor
MCG, mouse gamma globulin.
Specificity of rosette formation. 2×10^5 nucleated cells were incubated at 2°C for five min with EGF or, alternatively, NGF-coated SBRC. Inhibition of rosette formation was tested as follows. The nucleated cells were incubated at 2°C for 30 min with 1·0 mg of the inhibitor. The cells were then washed and exposed to the protein-coated erythrocytes. For the preparation of the erythrocytes, 1·0 mg of the ligand (EGF or NGF) was incubated with 0·5 ml of packed SBRC.

one or two pipettings. To control the specificity of rosette formation with the EGF ligand, cells from two clones of mouse and human neuroblastoma cells were incubated with NGF or, alternatively, EGF-coated SBRC. The neuroblastoma cells did not form rosettes with EGF-coated SBRC, whereas with NGF-coated SBRC over 80% of the cells were RFC (Table 2).

Effect on rosette formation of the number of EGF molecules coated onto SERC

The reaction of rosette formation was significantly dependent on the number of EGF molecules coated onto each erythrocytes. With HeLa cells in test, using more intensively EGF-coated SBRC (5 mg EGF/0·5 ml of packed SBRC), 100% RFC was obtained within 10 min of incubation, in the cold. With decreasing numbers of EGF molecules coated per erythrocyte, the time required to form rosettes increased (Fig. 3).

Time-course kinetic study of rosette formation

Using optimal preparations of EGF-coated erythrocytes (5 mg EGF/0·5 ml of packed SBRC), 50% of HeLa cells formed rosettes within five minutes; after 10 min over 80% HeLa cells were RFC. In contrast, under the same experimental conditions, after 5 min of incubation 8–10% fibroblasts and liver, kidney cells were RFC, whereas all the other cells used as controls were only 2–5% RFC. After 30 min of incubation, 31% fibroblasts and 20% kidney cells were RFC, the sarcoma and neuroblastoma cells were 15 to 20% RFC, and the spleen lymphocytes ranged from 2 to 5% RFC (Fig. 4).

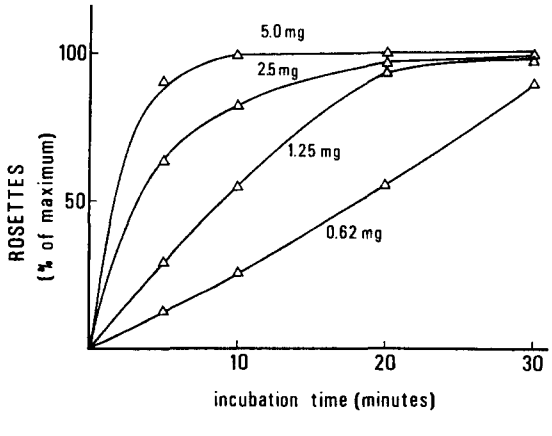


Fig. 3. Rosette formation by HeLa cells incubated with erythrocytes passively coated with EGF. Different concentrations of EGF were used for the erythrocyte preparations. Abscissa: time (minutes); ordinate: per cent of maximum rosette formation.

Inhibition of rosette formation

The effects on rosette formation by HeLa cells of treatment with various types of inhibitors have been reported in Tables 2 and 3, EDTA, azide, or antimycin, added in the medium diluent, did not induce any significant inhibitory effect on EGF-rosette formation. Since EDTA was very convenient for maintaining in suspension primary cultures of cells from normal tissues, EDTA was added to the medium diluent throughout all the experiments. Colchicine, vinblastine, cytochalasin-B, neuroaminidase, and phospholipases did not prevent rosette formation. Trypsin, on the contrary, completely prevented the binding of EGF to the HeLa cell-surface membrane. The binding reaction reappeared, however, within three hours.

No inhibition on rosette formation was induced by normal gamma globulins or serum albumin, ferritin, cytochrome-c or ribonuclease. In contrast, a specific inhibitory effect was obtained using free EGF as inhibitor. Table 2 shows the effect on rosette formation by neuroblastoma cells exposed in the cold to free NGF, or alternatively, EGF, before incubation with NGF- or EGF-coated SBRC. Using NGF as inhibitor, neuroblastoma cells lost their capacity to form rosettes with NGF-coated SBRC. On the contrary, free NGF had no inhibitory effect on the EGF-rosette-forming capacity of HeLa cells. Rosette formation by HeLa cells with EGF-coated SBRC was prevented only after cell pre-treatment with free EGF.

Electron microscopy of RFC

A typical rosette formed by a single HeLa cell in suspension and EGF-coated SBRC, at the electron microscope, is represented in Fig. 1 and 2. The HeLa cell appears as a round cell with a mean diameter of 20–30 μm. The cytoplasm shows many organelles, especially mito-

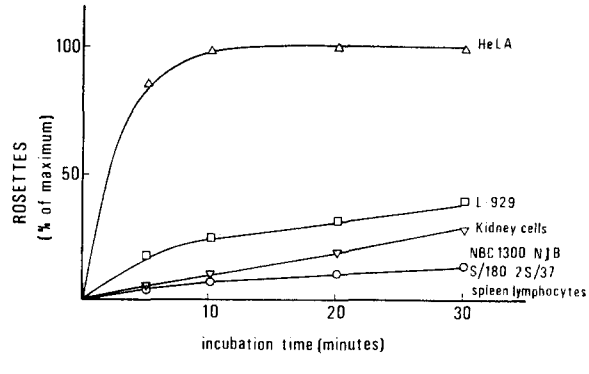


Fig. 4. Curves of rosette formation by HeLa cells and other control cells. Abscissa time (minutes); ordinate: per cent of maximum rosette formation.

Table 3. *Inhibition of rosette formation by HeLa Cells-EGF-SBRC* (*see legend to Table* 2)

	Percentage of inhibition of rosette formation*	Mode of action of inhibitors
EDTA 0·01 M	0	Divalent cations inhibitors
Na-azide (0·02%)	0	Cell respiration inhibitors
Antimycin (5 μg/ml)	2 ± 1	
Colchicine (10^{-5}M)	6 ± 2	Microtubule or microfilaments assembly-disassembly regulators
Vinblastine (10^{5}M)	8 ± 3	
Cytochalasin-B (10 μg/ml)	5 ± 2	
Neuroaminidase (100 μg/ml)	15 ± 2	Membrane-cleavage effects
Phospholipase-C (150 μg/ml)	17 ± 4	
Phospholipase-D (150 μg/ml)	15 ± 3	
Trypsin (100 μg/ml):		
(a) cells tested 10 min after treatment	92	
(b) cells tested 180 min after treatment	92 ± 4	
	8 ± 3	
EGF 1·0 mg/ml	100	Specific or non-specific EGF competitors
0·5 mg/ml	85 ± 4	
0·1 mg/ml	35 ± 5	
NGF 2·0 mg/ml	5 ± 2	
Human gamma globulin		
Mouse gamma globulin		
Rabbit gamma globulin		
Mouse serum albumin (0·1–5·0 mg/ml)	4 ± 3	
Ferritin		
Cytochrome-c		
Ribonuclease		

*arithmetical mean on triplicates

chondria and ribosomes, whose majority appears as monosomes. HeLa cell-erythrocytes contacts, which are seen in most section of rosettes, vary from a small to a large interface (Fig. 2). At other points, the RFC and its surrounding red cells appear separated. This separation may be more apparent than real, resulting perhaps from the angle of section or from shrinkage of cells in preparation. Several cytoplasmic processes departing from the HeLa cell body towards the adherent erythrocytes are frequently visible. These latter cells are not deformed.

DISCUSSION

The data presented show that EGF binds *in vitro* at 2°C with stable bonds to the membrane surface of HeLa and rabbit corneal epithelial cells. The interaction of EGF with these target cells does not depend on divalent cations and is not prevented by the presence in the reaction mixture of inhibitors of the metabolic processing of the cell, such as Na-azide, antimycin, colchicine, vinblastine or cytochalasin-B. On the contrary, evidences are provided which suggest that EGF binds to membrane-structure-receptor sites, most likely of a protein nature. These findings, which confirm and amplify previous works employing other techniques, similarly assessing the presence of EGF receptors on HeLa and epithelial cells [11–13], are in line with the well described capacity of EGF to act *in vivo* and *in vitro* as a potent stimulator of proliferation and keratinization of epithelial cells in the epidermal tissues of several species. A close analysis of the time-course of EGF binding to the cell controls shows, however, an increasing rosette formation simply by prolonging the time of exposure between the cells and the indicator erythrocytes, or by coating the red cells with a larger number of EGF molecules. Such increase was detected, in particular, among murine fibroblasts, which are cells presumably of a mesodermic origin. These findings arise the question as to the specificity of the EGF receptors, in respect to the *in vivo* specificity of the biological effects of the peptide, restricted, as far as it is known, to the epithelial cells. A possibility for such discrepancies is that binding sites for EGF are exposed on the membrane surface of the

epithelial cells at a different extent, compared to other cells. Alternatively, the receptors for EGF on epithelial cells may have a preferential high avidity of binding EGF than the sites exposed onto the surface membrane of other cells. It is of significance at this respect that, in general, those cells which respond best to a promoting ligand, have a substantially greater ability to bind the same ligand compared with cells only minimally responding to it. Evidences in support of a preferential exposure of binding sites for a specific ligand on differentiated cells are known from the literature [14]. For example this is the case for antigen-activated blood lymphocytes [15, 16]. The present report clearly indicates that HeLa and NB cells are capable of originating very specific and stable rosettes when incubated respectively, with EGF- or NGF-coated erythrocytes (cfr. Table 2). Quantitative measurements of EGF bindings to human target cells have revealed that the capacity of these cells of binding *in vitro* EGF molecules exceeded by 5 fold the amount of EGF required for maximal promotion of DNA synthesis [17]. The capacity of chicken or mouse embryo sympathetic cells to bind, also *in vitro* at saturation, NGF, similarly exceeds by 5–7 folds the requirement for maximal emission of neurites [8]. One has to consider, however, that in general, *in vitro* measurements of ligand binding to cells are performed under experimental conditions which may significantly differ one from another and from those which occur *in vivo*. The presence of competitive substances, dotated of opposite biological effects than EGF, but sterically cross-reacting at the level of the same target cell receptors; inhibition of cell transformation by concomitantly membrane interacting and different promoting ligands; modulatory phenomena of the cell membrane during variations of the cell growth cycle; are potential phenomena, among others, which may explain why amplification of receptor expression, far behind the number required for a maximal stimulus *in vitro*, may enable epithelial cells to become easily responders *in vivo* to the EGF effects. On the contrary, other cells, which *in vitro* usually exhibit only a limited expression of membrane receptor sites, *in vivo* may consequently act as low- or non-responders.

In conclusion our present findings strengthen the ability of the test of rosette formation to distinguish between cells which clearly bind EGF and those cells which only show a poor ability of binding. Although corneal epithelial cells respond to EGF by differentiating [5], whether this protein may provoke any metabolic or biological modification in HeLa cells is not yet known.

Acknowledgements—The authors are grateful to Dr. Stanley Cohen for his valuable advice, for reading the manuscript and for a generous gift of EGF.

REFERENCES

1. S. Cohen, Isolation of a mouse submaxillary-gland protein accelerating eruption and eyelid opening in the newborn animal. *J. biol. Chem.* **237,** 1955 (1962).
2. C. R. Savage, L. Tadashi and S. Cohen, The primary structure of epidermal growth factor. *J. biol. Chem.* **247,** 7612 (1972).
3. S. Cohen and G. A. Elliott, The stimulation of epidermal keratinization by a protein isolated from the maxillary gland of the mouse. *J. invest. Dermat.* **40,** 1 (1963).
4. S. Cohen, The stimulation of epidermal proliferation by a specific protein (EGF). *Develop. Biol.* **12,** 394 (1965).
5. R. W. Turkington, The role of epidermal growth factor in mammary-gland development *in vitro*. *Exp. Cell Res.* **57,** 79 (1969).
6. L. Bertolini, L. Diamond, E. Vigneti, M. Rizzoni and R. Revoltella, Variation of phenotypic expression in clones isolated from a murine neuroblastoma cell line. Manuscript in preparation.
7. V. Bocchini and P. U. Angeletti, The nerve-growth factor: purification as a 30,000-molecular-weight product. *Proc. nat. Acad. Sci. (Wash.)* **64,** 787 (1969).
8. R. Revoltella, L. Bertolini and M. Pediconi, Unmasking of nerve-growth factor membrane-specific binding sites in synchronized murine C1300 neuroblastoma cells. *Exp. Cell Res.* **85,** 89 (1974a).
9. R. Revoltella, L. Bertolini, M. Pediconi and E. Vigneti, Specific binding of nerve-growth factor (NGF) by murine C1300 neuroblastoma cells. *J. exp. Med.* **140,** 437 (1974b).

10. F. G. GUDAT, T. N. HARRIS, S. HARRIS and K. HUMMELER, Studies on antibody-producing cells. I. Ultrastructure of 19S and 7S antibody-producing. *J. exp. Med.* **137,** 483 (1970).
11. I. COVELLI, R. ROSSI, R. MAZZI and L. FRATI, Synthesis of bioactive ^{131}I-labeled epidermal growth factor and its distribution in rat tissues. *Eur. J. Biochem.* **27,** 225 (1972).
12. I. COVELLI, R. MOZZI, R. ROSSI and L. FRATI, The mechanism of action of the epidermal growth factor. III. Stimulation of the uptake of labeled precursors in to RNA, DNA, and proteins induced by EGF in isolated tumor cells. *Hormones* **3,** 183 (1972).
13. E. O'KEEFE, M. D. HOLLEMBERG and P. CUATRECASAS, Epidermal growth factor. Characteristics on specific binding in membranes from liver, placenta, and other target tissues. *Arch. Biochem.* **164,** 518 (1974).
14. P. CUATRECASAS, Membrane receptors. *Ann. Rev. Biochem.* **43,** 169 (1974).
15. G. J. V. NOSSAL and G. L. ADA, *Antigens, Lymphoid Cells and the Immune Response.* Academic Press, New York (1971).
16. J. F. R. P. MILLER, Lymphocyte interactions in antibody response. *Int. Rev. Cyt.* **33,** 77 (1972).
17. M. D. HOLLEMBERG and P. CUATRECASAS, Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin. *Proc. nat. Acad. Sci. (Wash.)* **70,** 2964 (1973).

Europ. J. Cancer Vol. 12, pp. 159–160. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Cytotoxicity of Antibody-Phospholipase C Conjugates on Cultured Friend Leukemia Cells

R. A. FLICKINGER and S. R. TROST

Division of Cell and Molecular Biology, State University of New York at Buffalo Buffalo, New York 14214, *U.S.A.*

ANTIBODIES conjugated to enzymes with gluteraldehyde maintain immunological and enzymatic activity [1]. In this study phospholipase C was conjugated to tumor antibodies to ascertain the cytotoxic effect upon the homologous tumor cells.

The polycythaemic strain of Friend leukemia was maintained in inbred DBA/2 mice by intraperitoneal injection of 0·2 ml of a 20% leukemic spleen homogenate. For immunization enlarged tumorous spleens were dissociated into a single cell suspension by passage through a 60 mesh stainless steel screen and the cells were treated with 1/500 neutral buffered formalin for 24 hr at 4°C [2]. Each of 50 DBA/2 mice was injected 9 × intraperitoneally with 1×10^7 cells at 6 day intervals. Four days after the last injection the mice were bled from a brachial vessel and sera collected. The sera were heated at 50°C to inactivate complement and stored at −20°C.

The globulin fractions were obtained from the serum of the immunized DBA/2 mice by two precipitations with 33% saturated ammonium sulfate. Conjugation was performed according to a modification of the method of Siess *et al.* [3]. The precipitated globulins were dissolved in water 1/10th the original serum volume and dialyzed against 0·1 M phosphate buffer, pH 6·8, at 4°C. Fifty mg of phospholipase C (Sigma Chemical Co.) was added to 0·5 ml of globulin, then 0·02 ml of 1% gluteraldehyde (Ladd Research Industries) was added slowly with stirring. Conjugation was allowed to proceed at room temperature for 2 hr, then the mixture was dialyzed against 0·15 M NaCl overnight at 4°C to remove unreacted gluteraldehyde. The conjugates were then precipitated with an equal volume of saturated $(NH_4)_2SO_4$, washed twice with this solution and dialyzed exhaustively against sterile Earle's saline (Grand Island Biological Co.) at 4°C. The solution was sterilized by passage through sterile Swinney filters. Agarose column chromatography [4] of the conjugates revealed three separate peaks, while only two peaks appeared in a mixture of unconjugated globulin-phospholipase C and their position corresponded to the two trailing peaks of the conjugate mixture. However, the conjugates were diluted to a concentration at which free phospholipase C did not affect the cells in the culture experiments. This was done in order to minimize extensive handling of the conjugate.

Mouse spleens were obtained under sterile conditions, minced and the fragments passed through a 60 mesh stainless steel gauge screen into the complete medium (RPMI 1630 and 10% fetal calf serum containing penicillin-streptomycin-fungazone), (Grand Island Biological Supply Co.). A suspension of single cells was obtained by drawing the cells through a 25 gauge needle. After counting, the cells (1×10^6) were distributed to individual small screw-capped vials containing 1 ml of complete medium. The vials were loosely capped and cultured for 24 hr at 37°C in an atmosphere of 95% air–5% CO_2. At the end of the culture period viability was estimated by staining the cells with 2% trypan blue and counting the stained dead cells and unstained living cells.

Accepted 22 September 1975.

Serum from DBA/2 mice immunized with formalin-treated spleen cells and normal serum were each diluted with equal parts of tumor cell suspension (1×10^6 cells) in culture medium and guinea pig complement (Grand Island Biological Supply Co.) and cultured for 24 hr. The average percent viability was 87% for Friend leukemic spleen cells cultured in either normal or immune serum plus guinea pig complement. Antibody-phospholipase C conjugates were prepared using the globulin fractions of DBA/2 mice immunized with formalin-treated Friend leukemic spleen cells. These conjugates were employed in cultures of normal DBA/2 mouse spleen cells and Friend leukemic spleen cells from DBA/2 mice with enlarged tumorous spleens. The normal spleen cells showed 88% viability after 24 hr of culture in normal medium and 84% and 82% viability, respectively, in the presence of 0·6% and 1·2% dilutions of the antibody-phospholipase C conjugate. Leukemic spleen cultures cultured in normal medium displayed 75% viability after 24 hr of cultures, while the level of viability of tumor cells cultured in the antibody-phospholipase C conjugates was 2·1% at a 0·6% concentration of conjugate. No tumor cells were alive after 24 hr of culture with a 1·2% concentration of the antibody-phospholipase C conjugate.

Serum from inbred mice immunized against Friend leukemic spleen cells developed in the same strain was not cytotoxic to the tumor cells *in vitro* in the presence of complement in this study although previous work has demonstrated such cytotoxicity [5, 6]. However, if the globulin fraction is isolated and conjugated to phospholipase C with gluteraldehyde, then the diluted conjugate shows a marked cytotoxicity against Friend leukemic spleen cells, but not against normal spleen cells of the same inbred strain of mice. It might be argued that unconjugated phospholipase C is attacking the tumor cells. However, the fact that the normal cells are unaffected by the diluted conjugate tends to rule out this explanation.

REFERENCES

1. S. Avrameas, Coupling of enzymes to proteins with gluteraldehyde. *Immunochemistry* **6,** 43 (1969).
2. R. A. Steeves and A. A. Axelrad, Neutralization kinetics of Friend and Rauscher leukemia viruses studied with the spleen focus assay method. *Int. J. Cancer* **2,** 235 (1967).
3. E. Siess, O. Wieland and F. Miller, A simple method for the preparation of pure and active γ-globulin-ferritin conjugates using glutaraldehyde. *Immunology* **20,** 659 (1971).
4. G. L. Nicolson and S. J. Singer, Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: Application to saccharides bound to cell membranes. *Proc. nat. Acad. Sci. (Wash.)* **68,** 942 (1971).
5. L. J. Old, E. A. Boyse and F. Lilly, Formation of cytotoxic antibody against leukemias induced by Friend virus. *Cancer Res.* **23,** 1063 (1963).
6. R. A. Steeves, Cellular antigen of Friend virus-induced leukemias. *Cancer Res.* **28,** 338 (1968).

Europ. J. Cancer Vol. 12, pp. 161–162. Pergamon Press 1976. Printed in Great Britain

Erratum

JOACHIM MARK: Two pseudolipid human breast carcinomas studied with G-band technique. *Europ. J. Cancer* **11,** 815 (1975).

Due to a publisher's error the captions to Figs. 1 and 2 were omitted. The figures and their captions are reproduced in full here.

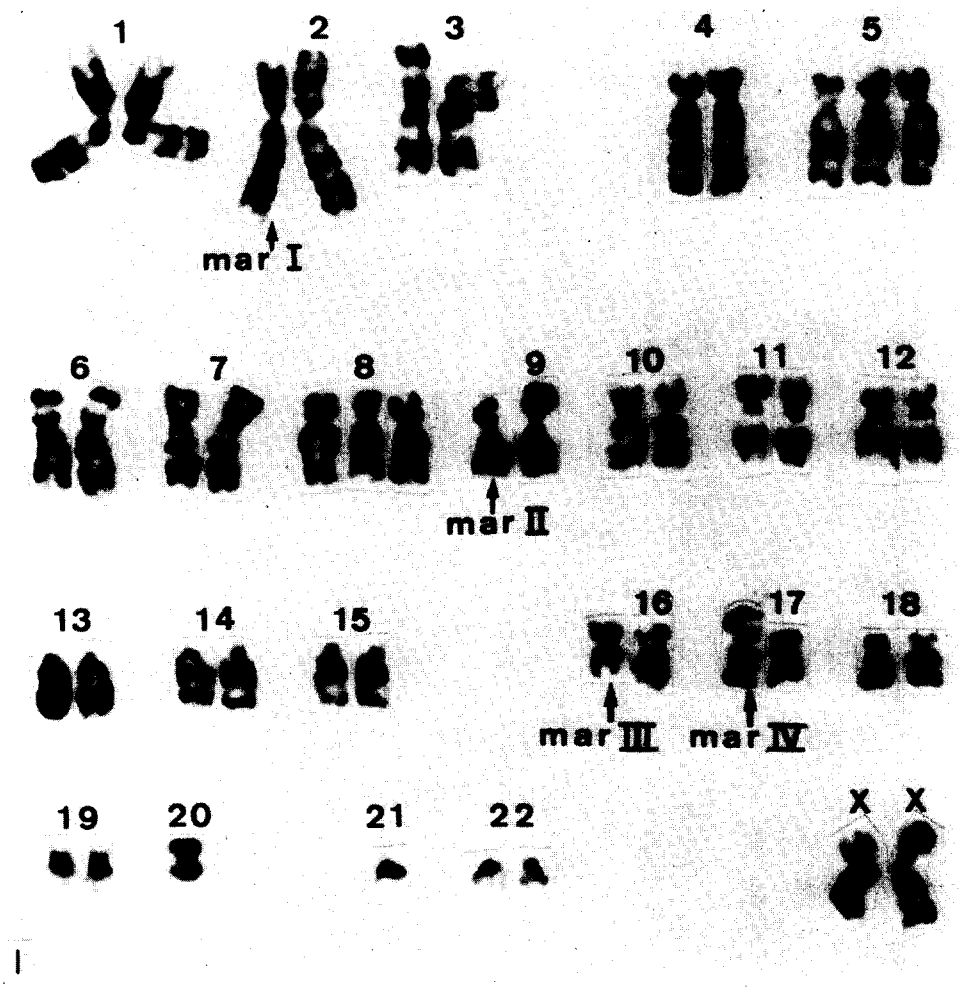


Fig. 1. *The stemline karyotype in case* 1. × 2200.

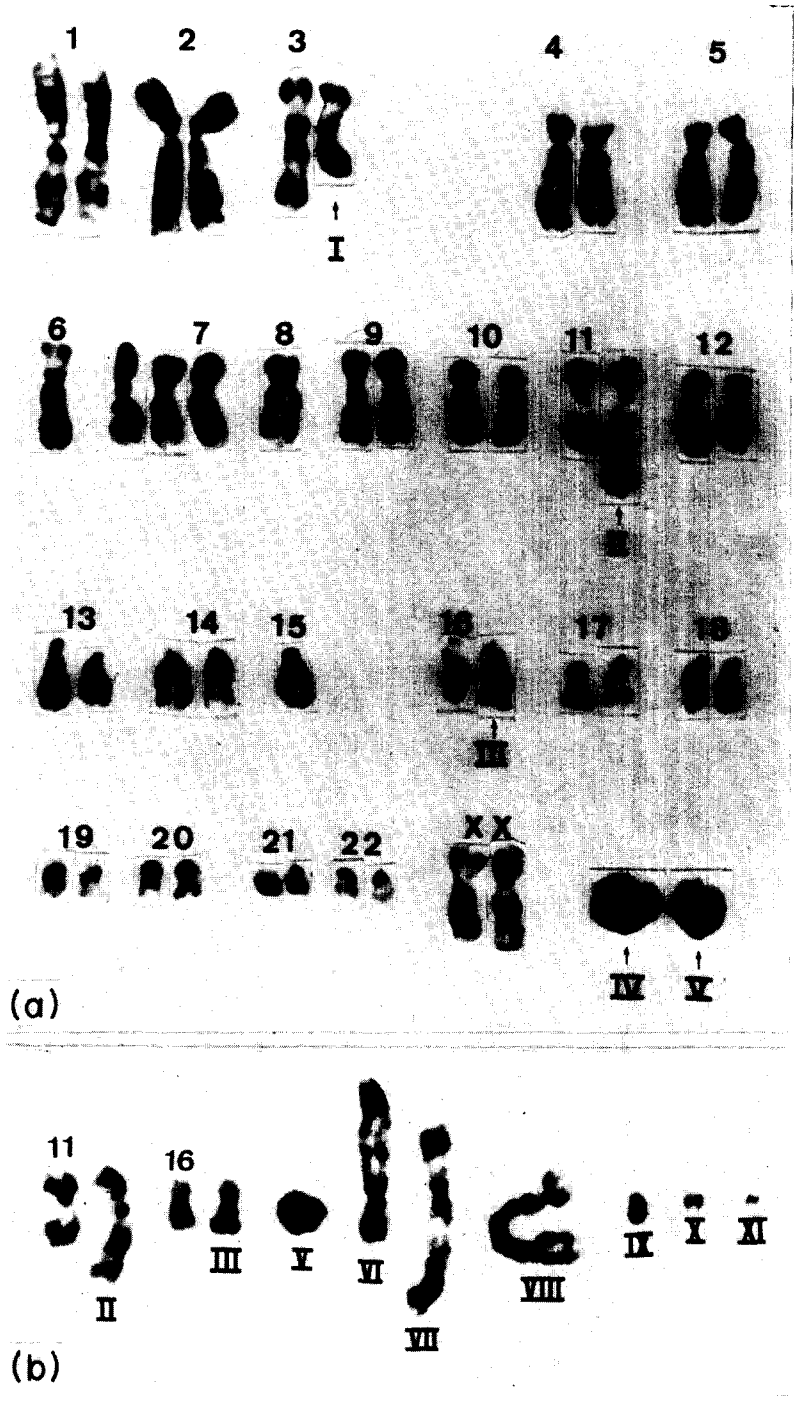


Fig. 2. (*a*) *The stemline karyotype in case* 2; ×2200. (*b*) *Condensed karyotype of a "sideline" (see text) in case* 2; *Roman numerals = marker types and Arabic numerals = normal chromosome types;* × 2200.

Europ. J. Cancer Vol. 12, pp. 163–164. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *February, 1976*

Human Cancer

F. G. Peers, G. A. Gilman and C. A. Linsell: Dietary aflatoxins and human liver cancer. A study in Swaziland.

N. Yamamoto and Y. Hinuma: Clonal transformation of human leukocytes by Epstein–Barr virus in soft agar.

H. S. Smith, R. B. Owens, A. J. Hiller, W. A. Nelson-Rees and J. O. Johnston: The biology of human cells in tissue culture. I. Characterization of cells derived from osteogenic sarcomas.

I. Ernberg, G. Masucci and G. Klein: Persistence of Epstein–Barr viral nuclear antigen (EBNA) in cells entering the EB viral cycle.

K. Rieche, A. Arndt and G. Pasternak: Cellular immunity in mammary cancer patients as measured by the leukocyte migration test (LMT). A follow-up study.

K. O. Fresen and H. zur Hausen: Establishment of EBNA-expressing cell lines by infection of Epstein-Barr virus (EBV)-genome-negative human lymphoma cells with different EBV strains.

B. Armstrong: Recent trends in breast cancer incidence and mortality in relation to changes in possible risk factors.

P. H. Levine, G. B. de Thé, J. Brugere, G. Schwaab, N. Mourali, R. B. Herberman, J. C. Ambrosioni and P. Revol: Immunity to antigens associated with a cell line derived from nasopharyngeal cancer (NPC) in non-Chinese NPC patients.

R. K. Oldham, R. S. Weiner, G. Mathé, J. Breard, M-C. Simmler, P. Carde and R. B. Herberman: Cell mediated immune responsiveness of patients with acute lymphocytic leukemia in remission.

Experimental Cancer

D. R. Black and A. H. Fieldsteel: Incomplete viral synthesis in Friend leukemia virus-induced reticulum cell sarcomas.

J. G. Bowen and R. W. Baldwin: Serum factor levels during the growth of rat hepatoma nodules in the lungs.

J. Keski-Oja, A. Vaheri and E. Ruoslahti: Fibroblast surface antigen (SF): The external glycoprotein lost in proteolytic stimulation and malignant transformation.

B. W. Calnek and L. N. Payne: Lack of correlation between Marek's disease tumor induction and expression of endogenous avian RNA tumor virus genome.

H. Kirchner, M. Glaser, H. T. Holden and R. B. Herberman: Mixed lymphocyte tumor cell interaction in a murine sarcoma virus (Moloney)-induced tumor system. Comparison between lymphoproliferation and lymphocyte cytotoxicity.

M. H. Thompson, H. W. S. King, M. R. Osborne and P. Brookes: Rat liver microsome mediated binding of benzo(a)pyrene metabolites to DNA.

R. Kiessling, G. Petrányi, G. Klein and H. Wigzell: Non-T cell resistance against a mouse Moloney lymphoma.

British Journal of Cancer *February, 1976*

R. Armstrong, A. Garrod and R. Doll: A retrospective study of renal cancer with special reference to coffee and animal protein consumption.

G. M. Taylor, R. Harris and C. B. Freeman: Cell-mediated cytotoxicity as a result of immunotherapy in patients with acute myloid leukaemia.

A. Wolf, K. A. Steele and P. Alexander: Estimation by radioimmunoassay of a specific membrane antigen associated with a murine lymphoma.

M. V. Pimm, M. J. Embleton and R. W. Baldwin: Treatment of transplanted rat tumours with double-stranded RNA (BRL 5907) I. Influence of systemic and local administration.

M. V. Pimm and R. W. Baldwin: Treatment of transplanted rat tumours with double-stranded RNA (BRL 5907) II. Treatment of pleural and peritoneal growths.

Annette B. Wrathmell: The growth patterns of two transplantable acute leukaemias of spontaneous origin in rats.

Annette B. Wrathmell and P. Alexander: Immunogenicity of a rat leukaemia of spontaneous origin (SAL).

Annette B. Wrathmell, C. L. Gauci and P. Alexander: Cross reactivity of an allo-antigen present on normal cells with the tumour-specific transplantation-type antigen of the acute myeloid leukaemia (SAL) of rats.

B. H. Toh, H. K. Muller and W. L. Elrick: Smooth muscle associated antigen in astrocytes and astrocytomas.

J. S. Henderson, R. D. Migliore, and D. Berbrayer: Interference by cortisone with endotoxin's adjuvator action on transplantation of a mouse tumour.

J. Shewell: The effect of methotrexate on spontaneous mammary adenocarcinomas.

T. Webb: Studies on the relationship between concanavalin A and SV40-transformed human fibroblasts.

P. R. M. Thomas, D. Winstanley, M. J. Peckham, D. E. Austin, M. A. F. Murray and H. S. Jacobs: Reproductive and endocrine function in patients with Hodgkin's disease: Effects of oophoropexy and irradiation.

Letter to the Editor

B. J. Smith and D. Kundu: Digoxin does not prevent daunorubicin or adriamycin from binding to rat heart muscle.

Europ. J. Cancer Vol. 12, pp. 165–175. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Fetal Antigens in Human Digestive Tumors

F. MARTIN, M. S. MARTIN and C. BOURGEAUX
Laboratoire d'Immunologie, Centre G.F. Leclerc et I.N.S.E.R.M., Unité 45
Faculté de Médecine, Boulevard Jeanne d'Arc, Dijon, France

CANCER of the digestive organs ranks first as the leading cause of cancer mortality [1]. Immunology may help the physician by providing new diagnostic and therapeutic facilities. Detection of tumor-associated antigens in serum or biological fluids, or the demonstration of immunologic responses in host–tumor systems could simplify and advance the diagnosis of digestive malignancies and improve the results of curative treatment. Ideally, an immunologic test should be simple, safe and reliable enough to be used as a mass screening method in determining a high risk population. More sophisticated methods (radiography, endoscopy, . . .) could then be employed to confirm the presence and localization of the tumor. In patients with proven digestive cancer, immunologic tests may still be useful in evaluating the prognosis or monitoring the effects of treatment. With a few exceptions, surgery is the one curative therapy for digestive malignancies. Its results are limited however by the extension of the tumor. Palliative radiotherapy and chemotherapy are usually disappointing. Therefore an immunological treatment, if it was possible, could be very helpful in destroying residual tumor tissue not removed by surgery.

Immunodiagnosis and immunotherapy are only possible if antigenic difference does exist between the tumor and the corresponding normal digestive tissue. These tumor-associated antigens could be valuable for clinical purposes according to their specificity, immunogenicity and their possible restriction to individual tumors.

The specificity of tumor-associated antigens can be qualitative, if the antigen is completely absent from the non-cancerous tissues. This qualitative tumor-specificity is always questionable, as more sensitive techniques could be able to demonstrate minute amounts of antigens in non-cancerous tissues. For most of the carcino-fetal antigens, the specificity is only quantitative: the antigenic concentration is stronger in cancerous than in non-cancerous tissues.

The immunogenicity of the tumor-associated antigen for the cancer-bearing patient is an important factor, as it is the required condition for immunotherapy. Immunogenicity depends, in part, on the specificity of the antigen. However, an immune reaction can be produced against quantitatively tumor-specific, or even normal antigens.

In experimental systems, tumor-associated antigens can be characteristic of an individual tumor: i.e. cancer induced by chemical agents have individual antigens, detected by transplantation methods, which are different from one tumor to another (even if induced in the same organ by the same carcinogen); virus induced tumors, in contradiction, share common antigens. In human cancer, the practical use of tumor-associated antigens will be fundamentally different if they are common to a group of tumors, or restricted to an individual cancer. Private antigens could only be detected by autochtonous reactions. Common antigens could be identified by reagents raised against other tumors of the same group.

Among the antigens associated with human digestive malignancies, the carcino-fetal antigens are of special importance. Between 1965 and 1966, relationships were demonstrated

between cancer of digestive organs and three carcino-fetal antigens: alpha-fetoprotein [2], carcinoembryonic antigen [3], and fetal sulfoglycoprotein [4]. These three antigens are common to a variety of digestive tumors.

The fetal antigens associated with human digestive cancer and the problem of their specificity and immunogenicity will now be discussed. These antigens are separated into two groups, depending on whether if they are detected by immune reactions in heterologous system, or recognized by an immune response on the tumor-bearing patient.

1. FETAL ANTIGENS DETECTED BY HETEROIMMUNE RESPONSE

The carcino-fetal antigens included in this group are detected by antibodies produced in animals by immunization with cancerous or fetal extracts. In most cases, the heteroantisera have to be made specific by absorption with normal organ extracts. These carcino-fetal antigens are referred to as "antigen" because they are identified by immunological methods. Their capacity to elicit antibodies in animals does not imply that they are immunogenic for the cancerous patient. Alpha-fetoprotein, carcinoembryonic antigen and other carcino-fetal proteins will be considered.

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a major component of fetal serum. Its association with hepatoma, in mice reported by Abelev *et al.* [5] and in humans by Tatarinov [2], classify AFP as a carcino-fetal antigen. General properties, biological significance and clinical value of AFP have been competently reviewed during the last few years [6, 7]. We will therefore limit our survey to recent data and outstanding questions.

Physico-chemical properties of human AFP, an alpha-1 globulin of 70,000 molecular weight which can be isolated on immunoabsorbents [8], are relatively well known. Microheterogeneity seems to be related to differences in sialic acid content, interfering with the electric charge of the molecule [9, 10]. The biological properties and functions of AFP are still questionable. Rat AFP (but not human) is able to bind estrogens, but not other steroid hormones [11]. Human AFP could play a role in enhancement of *in vitro* cell proliferation by human fetal serum, as this activity disappears after absorption of fetal serum by anti-AFP antiserum [12]. AFP does not seem to be antigenic for the tumor-bearing patient: there have been no reports of any immune reaction directed against autogenous AFP. AFP could suppress *in vitro* ^{3}H-thymidine incorporation in human lymphocytes stimulated by phytohemagglutinin, anti-Ig serum or allogeneic lymphocytes [13]. It is not clearly known whether AFP is also immunosuppressive *in vivo* and could play a role in the tolerance of fetus by mother or of tumor by the cancerous patient. Although not auto-immunogenic, AFP can easily induce antibodies by heterospecific immunization. Anti-human AFP antisera are usually produced by immunization with fetal serum or ascitic fluid from patients with hepatoma. It usually has to be extensively absorbed with normal human serum. Even so, there is a cross-reactivity between anti-AFP serum and AFP from various animal species [14].

AFP was first demonstrated in biological fluids, then in fetal and cancerous organs. Concentration of AFP in human serum can vary over a range of more than 10^6 (from 1 ng to more than 5 mg/ml). Hence, AFP detection depends on its concentration and the sensitivity of the method used for its demonstration. The relatively crude double-immunodiffusion method (Ouchterlony) can only detect amounts ranging around 1 μg/ml while the radioimmunoassay has a sensitivity of less than 1 ng/ml [7].

In non-cancerous conditions, serum AFP is detectable in the 6-weeks old embryo, reaching a maximum concentration (mean: 3·4 mg/ml) during the fourth month of the fetal life and then regularly decreasing until birth [15]. AFP can also be found in amniotic fluid between the 14th and 19th weeks of pregnancy; in fetuses with neural tube defects, AFP levels were markedly raised in the amniotic fluid [16]. In the cord serum of the normal newborn, mean AFP concentration is about 50 μg/ml and correlates inversely with gestational age [17]. It decreases at an exponential rate during the first weeks of the post-natal life [18]. In the normal adult, AFP is not found in serum by immunodiffusion, but radioimmunoassay can detect AFP concentrations up to 10 ng/ml [19]. During gestation, AFP from fetal origin can cross the placenta barrier and pass into the maternal serum where it can reach values up to 800 ng/ml [20].

During fetal life, liver is the main source of AFP. Immunofluorescence studies show that AFP can be localized in most fetal hepatocytes. In the neo-natal liver, AFP is detected only in hepatocytes surrounding the hepatic blood vessels [21]. In the early embryo, the yolk sac

and to a lesser extent the gastrointestinal tract contribute to the synthesis of AFP [22].

As shown by Tatarinov [2], AFP can be detected by immunodiffusion in the sera of 50–80% of the patients with primary hepatocellular carcinoma. There has been no reported immunological or biochemical difference between fetal and cancerous AFP. When tested by radioimmunoassay, AFP was found above the upper limit of normal values in nearly all the patients with hepatoma [23, 24]. However, AFP levels are widely distributed in individual patients, ranging from 30 to 5×10^6 ng/ml. These differences can only partially be explained by the volume or pathological features of the tumor, or age, sex and race of the patient [7]. Immunofluorescence studies show large discrepancies in the intensity of the fluorescence from one tumor to another. Even in hepatocellular carcinomas with elevated serum AFP levels, only a few cells are stained (most of them around the vessels) [25]. There seems to be a close relationship between the number of AFP-secreting cells and AFP concentration in the serum. It is not clearly known why only some fetal or cancerous hepatocytes are AFP-secreting. They could be cells at a particular stage of differentiation, whereas more differentiated hepatocytes (or less differentiated hepatoblasts) are unable to produce AFP [6].

Other tumors, besides hepatocellular carcinoma, can elaborate AFP. As shown by Abelev *et al.* [26], AFP can be detected by double immunodiffusion in serum of patients with testicular or ovarian teratoblastoma. Immunofluorescence studies have shown that AFP is synthesized in cells lining small vesicles probably from yolk sac origin [27]. AFP can also be occasionally detected, even by immunodiffusion, in serum from some patients with digestive tumors other than hepatomas. Most of the reported cases were gastric carcinomas (often linitis plastica) associated with liver metastasis [23, 28]. On the other hand, there is no AFP production in cholangiocellular carcinoma.

Elevated levels of circulating AFP can also be found in non-malignant diseases [24, 29, 30]. The most documented example is viral hepatitis, where transient AFP appearance can sometimes be detected even by the crude immunodiffusion methods. More sensitive radioimmunoassay found abnormal AFP levels in most of the cases of viral hepatitis, but also in liver cirrhosis and hemochromatosis. AFP is regularly found to be elevated in tyrosinosis, but not in other metabolic disturbances [31].

The detection of AFP in patients with non-cancerous diseases, and even in normal adults, definitely rules out the specificity of this antigen. However, AFP determination has great value for the diagnostic of hepatocellular carcinoma when levels are above 1 μg/ml. This limit happens to be similar to the lower limit of sensitivity of the double immunodiffusion (Ouchterlony) technique. For practical purposes, the simple, quick and inexpensive immunodiffusion method has the supplementary advantage of an apparent specificity. However, the radioimmunoassay allows a precise quantification of AFP levels and detects its possible variations with the evolution of the tumor.

Carcinoembryonic antigen

The carcinoembryonic antigen was demonstrated in human colonic tumors by Gold and Freedman in 1965 [3]. Rabbits were immunized by saline extracts from colonic carcinoma, and their sera were absorbed with extracts of normal colon and serum. The absorbed antisera reacted with an antigen present in extracts of cancerous and fetal intestine.

Carcinoembryonic antigen (CEA) was later characterized by Krupey *et al.* [32] as a glycoprotein, soluble in 0·6 M perchloric acid, and could be purified by preparative electrophoresis and gel exclusion chromatography. Although extensive studies of the physical properties and chemical composition of CEA have been undertaken [33], two major points have yet to be clarified: the microheterogeneity of CEA and the relationship between its structure and antigenic site. Separative methods such as electrofocusing, ion exchange chromatography and density gradient ultracentrifugation, have proven that conventionally purified CEA contains a variety of molecular species [34, 35], each species being of a different molecular size, electric charge and carbohydrate composition, but sharing the same immunologic reactivity on immunodiffusion. Different sialic acid content of CEA molecules can partly explain this heterogeneity. The tissue distribution and cancer-specificity of the molecular subspecies of CEA have not yet been fully investigated. Data about the chemical structure of the major antigenic site of the CEA molecule(s) are not yet clear [36, 37]. Sialic acid and fucose do not play any role in CEA antigenicity. It has been suggested that linkage between asparagine and N-acetylglucosamine could be of importance in the structure of the antigenic site. Besides the main antigenic determinant, CEA molecule(s) can bear other immunologic sites. Blood group A, B and

Lewis activities are often found associated to purified CEA. It is not yet clear if they are on the CEA molecule itself, or linked to blood group glycoproteins contaminating the CEA preparation [38–40]. The CEA molecule has another determinant distinct from the major antigenic site. This determinant cross-reacts with a glycoprotein of smaller molecular weight, called Non-specific Cross-reacting Antigen (NCA) [41], Normal glycoprotein (NGP) [42] or CEA-X [43]. NCA is present in normal epithelial tissues, but also in normal polymorphonuclear cells, monocytes and macrophages [44]. Anti-CEA sera are often contaminated by anti-NCA antibodies easily removed by absorption with perchloric acid extracts of normal lung or spleen.

Using relatively insensitive methods as immunodiffusion and precipitin–inhibition reaction on unconcentrated extracts, Gold and Freedman [4] were first led to assume that CEA localization was restricted to embryonic, fetal and cancerous digestive tissues. Consequently, CEA was presumed to be a cancer- and tissue-specific antigen. However, more sensitive methods and semi-purified extracts demonstrated CEA in a variety of non-cancerous adult tissues and non-digestive cancer. In specimens of adult colon, CEA could be found by immunodiffusion [45, 46], precipitin-inhibition reaction [45], immunofluorescence [47, 48] and by radioimmunoassay [49–51]. The latter method allows to estimate CEA, found to be 10 to 100 times more concentrated in colo-rectal cancer tissue than in non-cancerous colon. CEA was similarly identified in normal saliva [52], normal liver [53], lung and lactating breast [54], and in carcinomas of the lung [55] and the breast [54]. It has been suggested that CEA detection in non-cancerous tissues was due to antibodies directed against the common determinant shared by CEA and NCA [56]. However, in some of the above-mentioned reports, anti-CEA sera have been extensively absorbed with NCA-containing extracts and completeness of absorption was checked against crude or purified NCA preparations. With these data, CEA cannot be considered as a cancer- or tissue-specific antigen, but only as a glycoprotein common to various epithelial tissues, whose tissue concentration could be quantitatively increased in carcinomas arising from these tissues.

Cellular localization of CEA could explain its increased concentration in digestive malignancies. Using immunofluorescence and immunoferritin techniques, CEA is found in a thin layer (glycocalyx) lining the apical pole of the cancer cells [48, 57, 58]. The same location can be demonstrated in normal mucus cells, suggesting that CEA is a component of the intestinal mucous secretion. Under normal conditions, CEA is excreted in the digestive lumen and detected in feces from normal individuals [59]. In adenocarcinomas, colonic gland excretion can be blocked by cancerous alterations of the normal structure, resulting in the accumulation of CEA-containing mucus in the lumen of the obstructed glands. Such deposits can be observed by immunofluorescence in colo-rectal cancers. The impaired excretion might explain the high levels of circulating CEA in patients with digestive malignancies by escape of CEA into circulating blood.

Immunogenicity of CEA in the cancer-bearing patient has been a questionable point for a long time. Although early data suggested that CEA could be immunogenic [60], more specific methods, such as immunoabsorption [61] or radiolabelled antigen-binding [62], did not confirm the presence of anti-CEA antibodies in serum of patients with digestive malignancies. Immunoglobulins from patients with cancer and normal controls sometimes weakly bind radiolabelled CEA [63, 64]. This binding was explained by natural antibodies directed against blood group determinants on the CEA molecule [64] or auto-immune antibodies against a glycoprotein antigen contaminating the CEA preparation [65]. Consequently, CEA is probably unable to induce immune antibodies in man. Cell-mediated immunity against CEA has not yet been demonstrated either. Lymphocytes from patients with digestive cancer were not transformed *in vitro* when exposed to CEA [66] and no significant inhibition of leukocyte migration was demonstrated when leucocytes of colon cancer patients were incubated with CEA [67]. Purified CEA did not induce delayed skin reactivity in patients with colonic carcinoma delayed skin reactivity in patients with colonic carcinoma [68]. This lack of immunogenicity of CEA makes its use for cancer immunotherapy unlikely.

Using CEA for cancer diagnosis was questioned when Thomson *et al.* [69] developed a radioimmunoassay capable of determining the circulating levels of CEA. In this preliminary work, the antigen was detected in 35 out of 36 patients with colonic or rectal carcinoma, but was absent in normal subjects, pregnant women and in patients with non-cancerous diseases or cancer of non-digestive organs. Yet, data from other laboratories [70–74] has not confirmed

this apparent specificity. Using various radioimmunoassay technics and reagents from different sources, all investigators working in this field have reached conclusions differing from initial results on two major points: 1, high CEA levels can be found in patients with non-digestive cancer or non-malignant digestive diseases. 2, CEA levels can be normal in a high proportion of patients with colo-rectal cancer, chiefly if the tumor is non-metastatic and limited to the intestinal wall. In several recent studies, the proportion of patients with elevated CEA levels is about the same in non-invasive intestinal carcinoma (Duke's A and B) and in non-cancerous diseases such as liver cirrhosis, ulcerative colitis and diverticulitis. In these cases, finding high concentrations of CEA is often referred to as false positive. This term could suggest that radioimmunoassay detects an antigen cross-reacting with, but different from CEA. As a matter of fact, the many attempts to improve the specificity of the test by modifying the method or the reagents have failed until now [75, 76]. Presumably, the failure to distinguish between patients with or without malignancies results from the lack of specificity of the CEA itself, rather than from a methodological defect. However, Edgington and Plow [77] have recently claimed to have dramatically improved the specificity of the assay by using homogeneous isomeric species of CEA (CEA-S) as a radiolabelled antigen in the test. If confirmed by other teams, this result could lead to a revaluation of the diagnostic value of CEA radioimmunoassay. In conventional tests, this value is limited to cases where serum levels are extremely high (for instance, more than 50 ng/ml with Thomson's original method). Most, if not all the patients with CEA levels above this figure, are suffering from cancer, often, but not always, of digestive origin. However, when CEA attains this value, the cancer is usually patent, and very often beyond the possibilities of a curative treatment.

The relation between CEA levels and cancer extension offers a prognostic value to CEA determination. The possibility of curative surgery and the lack of post-operative recurrence are more frequent in patients with preoperative normal CEA concentration [78, 79]. On the other hand, high CEA levels are often correlated with evidence of blood vessel, lymphatic and perineural invasion or distant metastases [80].

After curative resection for colo-rectal cancer, periodic determination of circulating CEA helps to monitor the tumor status of the patients [79, 81–85]. Preoperative elevated CEA levels fall to normal value only if all the cancer tissue has been removed. However, CEA can rise again months or years after surgery, often before clinical symptoms of metastasis or local recurrency. In these cases, CEA determination could be an argument for a second-look operation, in search of potentially curable cancer tissue. A postoperative rise of CEA due to a non-cancerous cause (alcoholic cirrhosis) has been reported [87]. On the other hand, CEA levels can remain normal, even in patients with obvious recurrence of cancer.

Serial CEA determination can be useful in monitoring colo-rectal cancer chemotherapy. Some correlation between objective changes resulting from therapy and CEA levels can be seen, even if exceptions occur [85, 86]. The major limitation seems to be the weak and irregular effectiveness of chemotherapy in digestive cancer at present.

Fetal sulfoglycoprotein antigen

The fetal sulfoglycoprotein antigen (FSA) was detected by Häkkinen [4] by immunizing rabbits with the acid glycoprotein fraction of gastric juice from patients with gastric carcinoma. Antiserum has to be specially prepared to differentiate FSA from other sulfoglycoproteins found in gastric secretion. Immunofluorescence localizes FSA in fetal gut and in most gastric cancers [88]. Detection is also possible in non-tumorous mucosa distal to a cancer and in some specimens of stomach removed for non-cancerous diseases. According to Häkkinen, FSA is a carbohydrate-rich, sulfated glycoprotein. It differs from CEA, although partial crossreactivity has been demonstrated between both antigens [89]. FSA could also be related to the tumor-associated antigen found in gastric cancer secretion by Deutsch *et al.* [90]. Autoantibodies against FSA have never been reported. FSA does not stimulate thymidine incorporation by peripheral lymphocytes in gastric cancer patients.

FSA was found by immunodiffusion in 97% of gastric juice samples from 78 patients with cancer stomach, and in 12% of patients with non-cancerous gastric diseases [91]. A large study of the Finnish population, aged 40 to 65 years, has shown that FSA is positive in 3% to 7%. Follow-up results could evaluate the potential value of FSA test in mass screening for gastric cancer [92].

Alpha-2-H globulin

Alpha-2-H globulin is an iron-containing macroglobulin (molecular weight: 600,000),

which is immunologically related to ferritin. It is a normal componant of fetal liver and serum, and is found in the serum of new-born up to the end of the second post-natal month [93]. Alpha-2-H is detected with serum of rabbit immunized by fetal liver. Using immunodiffusion, it can often be found in the serum of hepatoma-bearing patients, and also in a variety of non-digestive tumors, chiefly pediatric malignancies. Alpha-2-H can also be detected in patients with inflammatory, non-cancerous diseases (liver cirrhosis, myocardic infaction, etc.) and even in healthy adults.

2. FETAL ANTIGENS DETECTED BY IMMUNE RESPONSE IN THE TUMOR-BEARING PATIENT

In animals, onco-fetal antigens can induce an immune response in the tumor-bearing host. This reaction can be detected by various methods: immunofluorescence, complement-dependent or lymphocyte-mediated cytotoxicity. In some [94] but not all the cases [95], onco-fetal antigens protected animals against the tumor growth. The same tumor can contain several varieties of carcinofetal antigens; some were detected only by heteroimmunization and others by inducing an immune response in the animal bearing the tumor [96].

Although rat hepatoma contains onco-fetal antigen(s) inducing antibodies in the tumor-bearing animal [95], there is no report of a similar response in hepatoma-bearing patients. However, the immunogenicity of human colo-rectal cancer is suggested by data from several studies.

Hollinshead *et al.* [97] have shown that the cutaneous delayed hypersensitivity reaction could be elicited in 17 out of 19 patients with colo-rectal cancer by soluble fractions from the membrane of their autologous tumors. The patients did not react when tested with comparable fractions obtained from non-cancerous colon. Positive reactions were obtained with extracts from the digestive tract of first- and second-trimester fetuses. Although all of skin-reactive extracts contain CEA, purified CEA could not induce a positive skin reaction. Furthermore, skin-reactive antigen(s) and CEA could clearly be separated by their migration on polyacrylamide gel electrophoresis [68]. The major limitation of the use of skin reaction in the diagnosis of colon-rectal cancer is the potential hazard of injection of tumor material in human. Therefore it is not known whether normal controls, or patients with non-cancerous diseases will react when tested with colon-cancer extracts.

Hellström and Hellström [98, 99] have found that peripheral blood lymphocytes from patients with colonic carcinoma inhibit the growth of plated colon cancer cells. Significant colony inhibition was obtained with lymphocytes of 16 out of 18 patients with growing tumors and 24 of 27 operated, apparently tumor-free patients. Inhibition was equally obtained with autochtonous and allogeneic tumor cells. Lymphocytes from patients with non-digestive cancer, as well as from normal controls, were not inhibitors. Colo-rectal cancer patients' lymphocytes were without any effect on growth of normal colonic cells, but could inhibit colonies from fetal gut epithelium and not fetal kidney cells. Inhibition of colonic cancer cells could be obtained from patients with gastric or pancreatic carcinoma. Lymphocyte effect could be suppressed by blocking factors present in serum from patients with intestinal cancer. Similar results were found by Baldwin and Embleton [100–102]. They demonstrated that papain-solubilized membrane fractions from colon carcinoma cells could inhibit lymphocyte cytotoxicity, but similar preparations from normal colon or melanoma were without effect. Results of lymphocyte-mediated colony inhibition or cytotoxicity seems to indicate that a carcino-fetal antigen, common to colo-rectal, and probably other digestive carcinoma, is present at the surface of the cancer cells and can elicit an immune response in circulating lymphocytes. However, Tagasuki *et al.* [103], using established lines or shorter term cultures from human colon cancer, could not show a significant difference between lymphocytes from normal controls and colo-rectal cancer patients in a series of more than 400 tests. These results cast a doubt on the practical value of colony inhibition or cytotoxicity reactions. Several factors, among them the availability of suitable target cells, the possible interference of histocompatibility, blood group and organ-specific antigens, and the methods of preparation of circulating lymphocytes have to be further investigated before appreciating the potential usefulness, and even the validity of the test.

Relationship between lymphocyte-sensitising and skin-reactive antigens have not yet been documented. Neither is it known whether antigens associated with colo-rectal cancer and detected by inhibition of leucocyte migration [104, 105] or blastogenic response of lymphocytes [106] are carcino-fetal antigens

and related to antigens detected by other methods.

Even if not clearly defined on the immunochemical point of view, carcino-fetal antigens inducing an immune response may be of special importance for immunodiagnosis and immunotherapy of human digestive cancer. Further information is needed on detection, specificity, distribution and biological role of these antigens. This information could be obtained in animal models (hepatoma, colon carcinoma), in which immuno-reactive carcino-fetal antigens have been demonstrated [95, 107–109].

Acknowledgements—We thank Dr. L. Katz and Dr. S. Jampolis for revision of the manuscript.

REFERENCES

1. J. L. Murray and L. M. Axtell, Impact of cancer: years of life lost due to cancer mortality. *J. nat. Canc. Inst.* **52,** 3 (1974).
2. Y. S. Tatarinov, Content of embryo specific alpha-globulin in the blood of human fetus, new-born and adult man in primary cancer of the liver. *Vop. med. Khim.* **11,** 20 (1965).
3. P. Gold and S. O. Freedman, Specific carcinoembryonic antigens of the human digestive system. *J. exp. Med.* **122,** 467 (1965).
4. I. P. T. Häkkinen, An immunochemical method for detecting carcinomatous secretion from human gastric juice. *Scand. J. Gastroent.* **1,** 28 (1966).
5. G. I. Abelev, S. D. Perova, M. I. Khramkova, Z. A. Postnikova and I. S. Irlin, Production of embryonal alpha-globulin by transplantation of mouse hepatoma. *Transplantation* **1,** 174 (1963).
6. G. I. Abelev, Alpha-fetoprotein as a marker of embryo-specific differentiation in normal and tumor tissues. *Transplant. Rev.* **20,** 3 (1974).
7. R. Masseyeff, Human alpha-fetoprotein. *Path. Biol.* **20,** 703 (1972).
8. S. Nishi, Isolation and characterization of human fetal alpha-globulin from the sera of fetuses and a hepatoma patient. *Cancer Res.* **30,** 2507 (1970).
9. E. F. Zimmermann and M. M. Madapally, Sialyltransferase regulation of alpha-fetoprotein microheterogeneity during development. *Biochem. J.* **134,** 807, (1973).
10. E. Alpert, J. W. Drysdale, J. Isselbacher and P. H. Shur, Human alpha-fetoprotein: isolation, characterization and demonstration of microheterogeneity. *J. biol. Chem.* **247,** 3792 (1972).
11. E. Nunez, L. Savu, F. Engelman, C. Benassayag, O. Crepy and M. F. Jayle, Mise en évidence d'une fraction protéique liant les oestrogènes dans le sérum des rats impubères. *C.R. Acad. Sci. (Paris)* **273,** 242 (1971).
12. M. Page, Activité des protéines foeto-embryonnaires sur la prolifération cellulaire *in vitro* in *L'Alpha-Foetoprotéine*, Edited by R. Masseyeff, p. 457, INSERM, Paris (1974).
13. J. L. Caldwell, C. D. Severson and J. S. Thompson, Human alpha-fetoprotein embryonic alpha-1-globulin with *in vitro* immunosuppressive activity. *Fed. Proc. (Abs)* **32,** 979 (1973).
14. D. Gitlin and M. Boesman, Fetus specific serum proteins in several mammals and their relation to human alpha-fetoprotein. *Comp. Biochem. Physiol.* **21,** 327 (1967).
15. D. Gitlin and M. Boesman, Serum alpha-fetoprotein, albumin and gamma-G-globulin in the human conceptus. *J. clin. Invest.* **45,** 1826 (1966).
16. D. J. H. Brock and R. G. Sutcliffe, Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida. *Lancet* **ii,** 197 (1972).
17. C. G. Bergstrand, B. W. Karlsson, T. Lindberg and H. Ekelund, Alpha-fetoprotein, albumin and total protein levels in blood serum in preterm, term infants and small for gestational infants. *Acta paediat. scand.* **61,** 128 (1972).
18. R. Sananes, F. Martin, J. L. Nivelon, P. Sizaret, C. Bourgeaux, M. Bordes and M. Alison, Etude cinétique de l'alpha-foetoprotéine sérique chez le nouveau né. *Arch. franç. Pediatr.* **30,** 1043 (1973).
19. E. Ruoslahti and M. Seppälä, Alpha-fetoprotein in normal human serum. *Nature (Lond.)* **235,** 161 (1972).
20. M. Seppälä and E. Ruoslahti, Radioimmunoassay of maternal serum alpha-fetoprotein during pregnancy and delivery. *Amer. J. Obstet. Gynec.* **112,** 208 (1972).

21. N. V. Engelhardt, A. I. Goussev, L. Y. Shipova and G. I. Abelev, Immunofluorescent study of alphafoetoprotein in liver and liver tumours. *Int. J. Cancer* **7,** 198 (1971).
22. D. Gitlin, A. Pericelli and G. Gitlin, Synthesis of alpha-fetoprotein by liver, yolk sac and gastrointestinal tract of the human conceptus. *Cancer Res.* **32,** 979 (1972).
23. E. Ruoslahti, M. Seppälä, P. Vuopio, E. Saksela and P. Peltokallio, Radioimmunoassay of alpha-fetoprotein in primary and secondary cancer of the liver. *J. nat. Canc. Inst.* **49,** 623 (1972).
24. J. A. P. Chayvialle, C. Toullon, C. Crozier and R. Lambert, Radioimmunoassay of alpha-fetoprotein in human serum. Clinical value in patients with liver diseases. *Amer. J. digest. Dis.* **19,** 1102 (1974).
25. A. I. Goussev, N. V. Engelhardt, R. Masseyff, R. Camain and B. Basteris, Immunofluorescent study of alpha-fetoprotein in liver and liver tumour. *Int. J. Cancer* **7,** 207 (1971).
26. G. I. Abelev, I. V. Assecritova, N. A. Kraevski, S. D. Perova and N. I. Perevodchikova, Embryonal serum alpha-globulin in cancer patients: diagnostic value. *Int. J. Cancer* **2,** 551 (1967).
27. G. Teilum, R. Albrechtsen and B. Nørgaard-Pedersen, Immunofluorescent localization of alpha-fetoprotein synthesis in endodermal sinus tumor (yolk sac tumor). *Acta path. microbiol. scand.* **82,** 586 (1974).
28. S. Akai and K. Kato, Serum alpha-fetoprotein-positive stomach cancer. *Gann Mono. Cancer Res.* **14,** 149 (1973).
29. G. I. Abelev, Alpha-fetoprotein in ontogenesis and its association with malignant tumors. *Adv. Cancer Res.* **14,** 295 (1971).
30. E. Ruoslahti, M. Seppälä, J. A. Räsänen, P. Vuopio and T. Helske, Alpha-fetoprotein and hepatitis B antigen in acute hepatitis and primary cancer of the liver. *Scand. J. Gastroent.* **8,** 197 (1973).
31. D. Buffe and C. Rimbaut, L'alpha-foetoprotéine dans les atteintes hépatiques et les maladies métaboliques de l'enfant. *Biomedicine* **19,** 172 (1973).
32. J. Krupey, P. Gold and S. O. Freedman, Physicochemical studies of the carcinoembryonic antigens of the human digestive system. *J. exp. Med.* **128,** 387 (1968).
33. W. D. Terry, P. A. Henkart, J. E. Colligan and C. W. Todd, Carcinoembryonic antigen: characterization and clinical applications. *Transplant. Rev.* **20,** 100 (1974).
34. J. E. Colligan, P. A. Henkart, C. W. Todd and W. D. Terry, Heterogeneity of the carcinoembryonic antigen. *Immunochemistry* **10,** 591 (1973).
35. E. F. Plow and T. S. Edgington, Isolation and characterization of a homogeneous isomeric species of carcinoembryonic antigen: CEA-S. *Int. J. Cancer* **15,** 748 (1975).
36. C. Banjo, P. Gold, C. W. Gehrke, S. O. Freedman and J. Krupey, Preparation and isolation of immunologically active glycopeptides from carcinoembryonic antigen (CEA). *Int. J. Cancer* **13,** 151 (1974).
37. J. E. Colligan and C. W. Todd, Structural studies on carcinoembryonic antigen: periodate oxydation. *Biochemistry* **14,** 805 (1975).
38. J. M. Gold and P. Gold, The blood group A-like site on the carcinoembryonic antigen. *Cancer Res.* **33,** 2821 (1973).
39. A. G. Cooper, M. C. Brown, M. E. Kirch and A. H. Rule, Relationship of carcinoembryonic antigen to blood substances A and i: evidence that the antigenic sites are on different molecules. *J. Immunol.* **113,** 1246 (1974).
40. A. M. Holburn, J. P. Mach, D. Macdonald and M. Newlands, Studies of the association of the A, B and Lewis blood group antigens with carcinoembryonic antigen (CEA). *Immunology* **26,** 831 (1974).
41. S. Von Kleist, G. Chavanel and P. Burtin, Identification of a normal antigen that cross-reacts with the carcinoembryonic antigen. *Proc. nat. Acad. Sci.* **69,** 2492 (1972).
42. J. P. Mach and G. Pusztaszeri, Carcinoembryonic antigen (CEA): demonstration of a partial identity between CEA and a normal glycoprotein. *Immunochemistry* **9,** 1031 (1972).
43. D. A. Darcy, C. Turberville and R. James, Immunological study of carcinoembryonic antigen (CEA) and a related glycoprotein. *Brit. J. Cancer* **28,** 147 (1973).

44. M. Bordes, S. Knobel and F. Martin, Carcinoembryonic antigen (CEA) and related antigens in blood cells and hematopoietic tissues. *Europ. J. Cancer* **11,** 783 (1975).
45. F. Martin and M. S. Martin, Demonstration of antigens related to colonic cancers in the human digestive system. *Int. J. Cancer* **6,** 352 (1970).
46. J. Rosai, T. W. Tillack and V. T. Marchesi, Membrane antigens of human colonic carcinoma and non-tumoral colonic mucosa: results obtained with a new isolation method. *Int. J. Cancer* **10,** 357 (1972).
47. P. Burtin, S. Von Kleist, M. C. Sabine and M. King, Immunohistological localization of carcinoembryonic antigen and nonspecific cross-reacting antigen in gastrointestinal normal and tumoral tissues. *Cancer Res.* **33,** 3299 (1973).
48. M. Bordes, R. Michiels and F. Martin, Detection by immunofluorescence of carcinoembryonic antigen in colonic carcinoma, other malignant or benign tumours and non-cancerous tissues. *Digestion* **9,** 106 (1973).
49. F. Martin and M. S. Martin, Radioimmunoassay of carcinoembryonic antigen in extracts of human colon and stomach. *Int. J. Cancer* **9,** 641 (1972).
50. S. K. Khoo, N. L. Warner, J. T. Lie and I. R. Mackay, Carcinoembryonic antigenic activity of tissue extracts: a quantitative study of malignant and benign neoplasms, cirrhotic liver, normal adult and fetal organs. *Int. J. Cancer* **11,** 681 (1973).
51. B. J. Dyce and B. J. Haverback, Free and bound carcinoembryonic antigen in neoplasms and in normal adult and fetal tissue. *Immunochemistry* **11,** 423 (1974).
52. F. Martin and J. Devant, Carcinoembryonic antigen in normal human saliva. *J. nat. Cancer Inst.* **50,** 1375 (1973).
53. H. Z. Kupchik and N. Zamcheck, Carcinoembryonic antigen(s) in liver diseases. Isolation from human cirrhotic liver and serum and from normal liver. *Gastroenterology* **63,** 95 (1972).
54. G. Pusztaszeri and J. P. Mach, Carcinoembryonic antigen (CEA) in non-digestive cancerous and normal tissues. *Immunochemistry* **10,** 197 (1973).
55. P. Sizaret and F. Martin, Carcinoembryonic antigen in extracts of pulmonary cancers. *J. nat. Cancer Inst.* **50,** 807 (1973).
56. J. T. Tomita, J. W. Safford and A. A. Hirata, Antibody response to different determinants on carcinoembryonic antigen (CEA). *Immunology* **26,** 291 (1974).
57. P. Gold, J. Krupey and H. Ansari, Position of the carcinoembryonic antigen of the human digestive system in ultra-structure of tumor cell-surface. *J. nat. Cancer Inst.* **45,** 219 (1970).
58. S. Von Kleist and P. Burtin, Localisation cellulaire d'un antigène embryonnaire de tumeurs coliques humaines. *Int. J. Cancer* **4,** 874 (1969).
59. D. L. J. Freed and G. Taylor, Carcinoembryonic antigen in faeces. *Brit. Med. J.* **i,** 85 (1972).
60. P. Gold, Circulating antibodies against carcinoembryonic antigens of the human digestive system. *Cancer (Philad.)* **20,** 1663 (1967).
61. E. Collatz, S. Von Kleist and P. Burtin, Further investigations of circulating antibodies in colon cancer patients: on the autoantigenicity of the carcinoembryonic antigen. *Int. J. Cancer* **8,** 293 (1971).
62. P. Logerfo, F. P. Herter and S. J. Bennett, Absence of circulating antibodies to carcinoembryonic antigen in patients with gastrointestinal malignancies. *Int. J. Cancer* **9,** 344 (1972).
63. J. M. Gold, S. O. Freedman and P. Gold, Human anti-CEA antibodies detected by radioimmunoelectrophoresis. *Nature (Lond.)* **239,** 60 (1972).
64. J. M. Macsween, The antigenicity of carcinoembryonic antigen in man. *Int. J. Cancer* **15,** 246 (1975).
65. S. Von Kleist, M. King and P. Burtin, Characterization of a normal tissular antigen extracted from human colonic tumors. *Immunochemistry* **11,** 249 (1974).
66. M. C. Lejtenyi, S. O. Freedman and P. Gold, Response of lymphocytes from patients with gastrointestinal cancer to the carcinoembryonic antigen of the human digestive system. *Cancer (Philad.)* **28,** 115 (1971).

67. E. Straus, S. Vernage, H. Janowitz and F. Paronetto, Migration of peripheral leukocytes in the presence of carcinoembryonic antigen. Studies in patients with chronic inflammatory diseases of the intestine and carcinoma of the colon and pancreas. *Proc. Soc. exp. Biol. (N.Y.)* **148,** 494 (1975).
68. A. Hollinshead, C. G. McWright, T. C. Alford, D. H. Glew, P. Gold and R. B. Herberman, Separation of skin-reactive intestinal cancer antigen from the carcinoembryonic antigen of Gold. *Science* **117,** 887 (1972).
69. D. M. P. Thomson, J. Krupey, S. O. Freedman and P. Gold, The radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system. *Proc. nat. Acad. Sci (Wash.)* **64,** 161 (1969).
70. T. L. Moore, H. Z. Kupchik, N. Margon and N. Zamcheck, Carcinoembryonic antigen assay in cancer of the colon and pancreas and other digestive tract disorders. *Amer. J. digest. Dis.* **16,** 1 (1971).
71. D. J. R. Laurence, U. Stevens, R. Bettelheim, D. Darcy, C. Leese, C. Turberville, P. Alexander, E. W. Johns and A. M. Neville. Role of plasma carcinoembryonic antigen in diagnosis of gastrointestinal mammary and bronchial carcinoma. *Brit. med. J.* **iii,** 605 (1972).
72. J. P. Mach, G. Pusztaszeri, M. Dysli, F. Kapp, B. Bierens de Haan, R. M. Loosli, P. Grob and H. Isliker, Dosage radioimmunologique de l'antigène carcinoembryonnaire (CEA) dans le plasma de malades atteints de carcinomes. *Schweiz. med. Wschr.* **103,** 365 (1973).
73. S. N. Booth, J. P. G. King, J. C. Leonard and P. W. Dykes, The significance of elevation of serum carcinoembryonic antigen levels in inflammatory diseases of the intestine. *Scand. J. Gastroenter.* **9,** 651 (1974).
74. H. J. Hansen, J. J. Snyder, E. Miller, J. P. Vandervoorde, O. N. Miller, L. R. Hines and J. J. Burns, Carcinoembryonic antigen (CEA) assay: a laboratory adjunct in the diagnosis and management of cancer. *Hum. Path.* **5,** 139 (1974).
75. H. Z. Kupchik, N. Zamcheck and C. A. Saravis, Immunochemical studies of carcinoembryonic antigens: methodologic considerations and some clinical implications. *J. nat. Cancer Inst.* **50,** 1741 (1973).
76. F. Martin, C. Klepping and J. Guerrin, Dosage radioimmunologique de l'antigène carcinoembryonnaire circulant. Extraction immunoperchlorique du sérum. *Arch. Franc. Mal. App. Digest.* **61,** 725 (1972).
77. T. S. Edgington, CEA-S: a distinctive isomeric variant of carcinoembryonic antigen. *Fed. Proc.* **34,** 845 (1975).
78. N. Zamcheck, T. L. Moore, P. Dhar and H. Kupchik, Immunologic diagnosis and prognosis of human digestive tract cancer: carcinoembryonic antigens. *New Engl. J. Med.* **286,** 83 (1972).
79. S. N. Booth, G. C. Jamieson, J. P. G. King, J. Leonard, G. D. Oates and P. W. Dykes, Carcinoembryonic antigen in management of colorectal carcinoma. *Brit. med. J.* **4,** 183 (1974).
80. N. Zamcheck, W. G. Doos, R. Prudente, B. B. Lurie and L. S. Gottlieb, Prognostic factors in colon carcinoma. Correlation of serum carcinoembryonic antigen level and tumor histopathology. *Hum. Path.* **6,** 31 (1975).
81. J. P. Mach, P. Jaeger, M. M. Bertholet, C. H. Ruegsegger, R. M. Loosli and J. Pettavel, Detection of recurrence of large-bowel carcinoma by radioimmunoassay of circulating carcinoembryonic antigen (CEA). *Lancet* **ii,** 535 (1974).
82. A. M. Mackay, S. Patel, S. Carter, U. Stevens, D. J. R. Laurence, E. H. Cooper and A. M. Neville, Role of serial plasma CEA assays in the detection of recurrent and metastatic colorectal carcinomas. *Brit. Med. J.* **4,** 382 (1974).
83. J. J. Sorokin, P. H. Sugarbaker, N. Zamcheck, M. Pisick, H. Z. Kupchik and F. D. Moore, Serial carcinoembryonic antigen assays, use in detection of cancer recurrence. *J. Amer. med. Ass.* **228,** 49 (1974).
84. A. S. Livingstone, G. L. Rampson, J. Shuster, P. Gold and F. Hinchley, Carcinoembryonic antigen in the diagnosis and management of colorectal carcinoma. *Arch. Surg.* **109,** 259 (1974).
85. M. Ravry, C. G. Moertel, A. J. Schutt and V. L. W. Go, Usefulness of serial serum carcinoembryonic antigen (CEA) determinations during anticancer therapy and long-term follow-up of gastrointestinal carcinoma. *Cancer (Philad.)* **34,** 1230 (1974).

86. A. T. Skarin, R. Delwiche, N. Zamcheck, J. J. Lokich and E. Frei, Carcinoembryonic antigen: clinical correlation with chemotherapy for metastatic gastrointestinal cancer. *Cancer (Philad.)* **33,** 1239 (1974).
87. B. B. Lurie, D. M. Bull, N. Zamcheck, A. M. Steward and R. A. Helms, Diagnosis and prognosis in colon cancer based on a profile of immune reactivity. *J. nat. Cancer Inst.* **54,** 319 (1975).
88. I. Häkkinen, O. Jarvi and J. Grönroos, Sulphoglycoprotein antigens in the human alimentary canal and gastric cancer. An immunohistological study. *Int. J. Cancer* **3,** 572 (1968).
89. I. P. T. Häkkinen, Immunological relationship of the carcinoembryonic antigen and the fetal sulfoglycoprotein antigen. *Immunochemistry* **9,** 1115 (1972).
90. E. Deutsch, C. A. Apffel, H. Mori and J. E. Walker, A tumor-associated antigen in gastric cancer secretion. *Cancer Res.* **33,** 112 (1973).
91. I. Häkkinen and S. Viikari, Occurence of fetal sulphoglycoprotein antigen in the gastric juice of patients with gastric diseases. *Ann. Surg.* **169,** 277 (1969).
92. I. Häkkinen, Foetal sulphoglycoprotein antigen (FSA) as a possible precursor of alimentary canal cancers. *Scand. J. Gastroent.* **7,** 483 (1972).
93. D. Buffe, C. Rimbaut, C. Fuccaro and P. Burtin, Isolement et caractérisation d'une alpha-2-ferroprotéine: la globuline alpha-2-H. *Ann. Inst. Pasteur* **123,** 29 (1972).
94. J. H. Coggin, K. R. Ambrose, B. B. Bellomy and N. G. Anderson, Tumor immunity in hamsters immunized with fetal tissues. *J. Immunol.* **107,** 526 (1971).
95. R. W. Baldwin, D. Glaves and B. M. Vose, Embryonic antigen expression in chemically induced rat hepatomas and sarcomas. *Int. J. Cancer* **10,** 233 (1972).
96. D. M. P. Thomson and P. Alexander, Cross-reacting embryonic antigen in the membrane of rat sarcoma cells which is immunogenic in the syngeneic host. *Brit. J. Cancer* **27,** 35 (1973).
97. A. Hollinshead, D. Glew, B. Bunnag, P. Gold and R. Herberman, Skin-reactive soluble antigen from intestinal cancer-cell membranes and relationship to carcinoembryonic antigens. *Lancet* **i,** 1191 (1970).
98. I. Hellström ,K. E. Hellström and T. H. Shepard, Cell-mediated immunity against antigens common to human colonic carcinomas and fetal gut epithelium. *Int. J. Cancer* **6,** 346 (1970).
99. I. Hellström and K. E. Hellström, Newer concepts of cancer of the colon and rectum: cellular immunity to human colonic carcinomas. *Dis. Colon Rect.* **15,** 100 (1972).
100. R. W. Baldwin, M. J. Embleton, J. S. P. Jones and M. J. S. Langman, Cell-mediated and humoral immune reaction to human tumours. *Int. J. Cancer* **12,** 73 (1973).
101. R. W. Baldwin, M. J. Embleton and M. R. Price, Inhibition of lymphocyte cytotoxicity for human colon carcinoma by treatment with solubilized tumour membrane fractions. *Int. J. Cancer* **12,** 84 (1973).
102. M. J. Embleton, Significance of tumour associated antigens on human colonic carcinomata. *Brit. J. Cancer* **28,** Suppl. I, 142 (1973).
103. M. Tagasuki, M. R. Mickey and P. I. Terasaki, Reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer Res.* **33,** 2898 (1973).
104. P. J. Guillou and G. R. Giles, Inhibition of leucocyte migration by tumour-associated antigens of the colon and rectum. *Gut* **14,** 733 (1973).
105. D. M. Bull, J. R. Leibach, M. A. Williams and R. A. Helms, Immunity to colon cancer assessed by antigen induced inhibition of mixed mononuclear cell migration. *Science* **181,** 957 (1973).
106. G. M. Mavligit, U. Ambus, J. U. Guterman, E. M. Hersh and C. M. McBride, Antigen solubilized from human solid tumors. Lymphocyte stimulation and cutaneous delayed hypersensitivity. *Nature New Biol.* **243,** 188 (1973).
107. G. Steele and H. O. Sjögren, Cross-reacting tumor-associated antigen(s) among chemically induced rat colon carcinomas. *Cancer Res.* **34,** 1801 (1974).
108. G. Steele and H. O. Sjögren, Embryonic antigens associated with chemically induced colon carcinomas in rats. *Int. J. Cancer* **14,** 435 (1974)
109. F. Martin, S. Knobel, M. Martin and M. Bordes, A carcinofetal antigen located on the membrane of cells from rat intestinal carcinoma in culture. *Cancer Res.* **35,** 333 (1975).

Europ. J. Cancer Vol. 12, pp. 177–180. Pergamon Press 1976. Printed in Great Britain

General Papers

Treatment of Experimental Liver Tumors by Continuous Intra-arterial Chemotherapy

H. H. COTINO and A. ZWAVELING
Department of Surgery, University Hospital, Leiden, The Netherlands

Abstract—*A system for continuous infusion into the hepatic artery in rats is described. Rats were intrahepatically inoculated with Walker* 256 *carcinoma cells and then treated by the administration of* 5*-fluorouracil through the canula. Rats so treated developed significantly smaller tumours than did control animals.*

This system allows free movement of the animals, does not affect the liver blood supply and permits prolonged administration of drugs. This method of investigation can also be applied to other research programs.

INTRODUCTION

THERE IS no consensus on the value of continuous intra-arterial infusion of chemotherapeutic agents for the treatment of primary carcinoma of the liver or liver metastases. According to some authors, this method gives better results than administration via the intravenous or oral route [1–7] but other are less convinced of its superiority [8, 9].

Adequate evaluation of the published results is difficult, because of the wide variation in the ages of the patients, the stage of the disease, and the lack of comparable control groups. Furthermore, the assumed longation of survival times often amounts to weeks or months, which makes statistical evaluation extremely difficult.

When our experiments were performed, no models were available for intra-arterial infusion of rat livers *in vivo*. Later, Leivestad [10] described such a model. Our work concerned an attempt to develop an adequate experimental model, the main requirement being a system for rats permitting infusion via the hepatic artery over a period of several days without too much hindrance to the animal's movements. With such a model we could try to find a suitable and appropriate chemotherapeutic agent for a comparative study of the effect on tumor growth of continuous intra-arterial infusion, intravenous and oral administration.

Accepted 5 November 1975.

MATERIAL AND METHODS

Inbred male Wistar rats with an average weight of 225 g were inoculated intrahepatically with about 2 million live Walker 256 carcinosarcoma cells. These rats were randomly separated into 5 groups of five animals each. One group was not treated and served as controls, three groups were treated for 7 successive days after the 4th post-inoculation day with 5-fluorouracil (5-FU) given intra-arterially, intravenously, or orally (one group each), and the remaining group was given an intra-arterial infusion with saline during the same 7-day period. The procedures were as follows:

For the intra-arterial administration of the drug an infusion pump (Braun Unita I, Meslungen, Germany) with a flow rate of 5·7 ml/day was used. The basic solution consisted of physiological saline with heparin 40 U per rat per day.

Under Nembutal® anesthesia (administered intraperitoneally), a polyethylene canula (No. 10) with the tip elongated over a flame was introduced into the gastroduodenal artery and led s.c. to the surface of the neck, where it was drawn through a steel tube (1 mm dia) leading to the infusion pump. The tube was fixed to the animal's neck by a collar made of polythylene tubing (No. 100). Freedom of movement was provided by the use of a swivel consisting of a hooded needle turning in a polyethylene tube.

Intravenous administration was performed by injection into a tail vein, oral administration via a gastric canula. The animal was kept in a cage measuring $20 \times 20 \times 20$ cm. The food consisted of standard pellets, and water was provided *ad libidum*.

On the basis of a preliminary study, the intravenous and oral doses were fixed at 12·5 mg/kg of body weight, daily, for 7 days. This dose, which gave only a slight toxicity, resulted in some loss of weight and a small reduction in the number of leukocytes and thrombocytes. Higher doses caused marked toxicity.

The intra-arterial dose was fixed at 25 mg/kg of body weight daily for 7 days. There were no symptoms of toxicity, the liver function tests showed no abnormality, and there were no histological changes. Because good results were obtained with this dose, higher doses were not evaluated.

Tumor implantation was performed in the median lobe of the liver by the injection (25 ga. needle) of 0·03 ml of a cell suspension containing about 2 million viable cells. After retraction of the needle, the injection site was coagulated.

The cell suspension was prepared under sterile conditions, and a small portion was used to count the viable cells in a hemocytometer after dilution with eosin to stain the damaged, nonviable cells. On the 11th day after inoculation, the animals were killed, the livers removed and the tumors compared.

For technical reasons the groups were not treated simultaneously: each experiment was done in 5 animals, one from each group. A fresh tumor suspension was made for each series.

The groups were as follows:

Group I: tumor implantation, on the 4th day followed by intra-arterial infusion of 5-FU 25 mg/kg body weight daily for 7 days.

Group II: tumor implantation, on the 4th day followed by intra-arterial infusion of heparinized saline alone for 7 days.

Group III: tumor implantation, on the 4th day followed by intravenous injection of 5-FU 12·5 mg/kg body weight daily for 7 days.

Group IV: tumor implantation, on the 4th day followed by oral administration of 5-FU 12·5 mg/kg body weight daily for 7 days.

Group V: tumor implantation without further treatment.

For groups I and II, after termination of the infusion the rats were anesthetized with sodium pentobarbital given intraperitoneally and a laparotomy was performed. As soon as the infusion system was disconnected from the pump, methylene blue was injected via the canula to check the potency.

The livers were then extirpated and measured in three directions for calculation of the volume.

RESULTS

The tumor sizes are plotted logarithmically in Fig. 1. The Friedman test showed significance at the 5% level. Tumor growth was distinctly lower in the animals treated by intra-arterial infusion of 5-FU than in the other groups (i.v. and oral administration; controls). There was no distinct difference between intravenously- and orally-treated groups on the one hand and the control groups on the other.

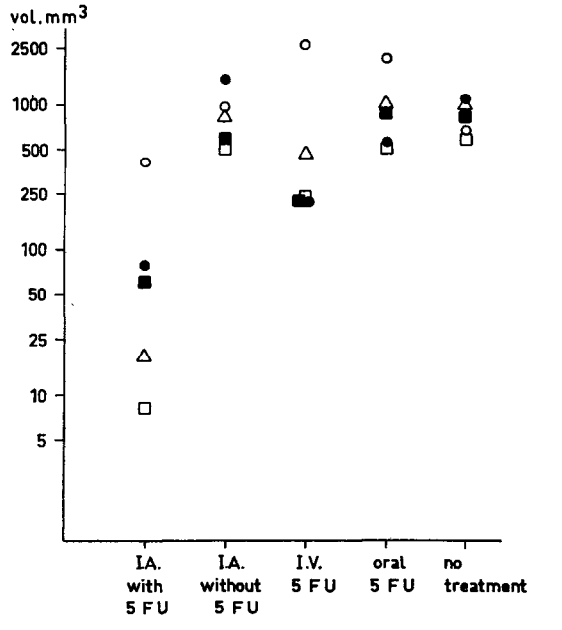


Fig. 1. Tumor growth, according to treatment. (On the horizontal axis, 5 groups of 5 rats each, killed on the 11th day after inoculation; on the vertical axis, the volume of the tumor is plotted in natural logarithms.) The intra-arterial treatment with 5-FU gave significantly better results than the other methods ($P < 0{\cdot}05$).

The intra-arterial treatment gave very few technical difficulties. On a few occasions the canula became clogged by a clot. The animals remained quiet and showed no sign of being restricted by the system. Since the weights of the rats in groups II and V were similar both before and after treatment, it may be concluded that the system itself did not cause loss of weight.

Unfortunately, our tumor proved unsuitable for the determination of the influence of the treatment on survival times, because all of the animals died of metastases, mainly in the retroperitoneal lymph nodes but in a few cases in the lungs. This mortality pattern was demonstrated by 9 experiments comprising 5 animals each, as before, but in this series the animals were kept alive and the measurements were performed at autopsy after spontaneous death. From Fig. 2, which shows the tumor volumes at death, it is evident that the animals treated with 5-FU intra-arterially again had smaller tumors than the others ($P < 0{\cdot}05$). The survival times are shown in Table 1. In this series the intra-arterial group did not have appreciably longer survival times, probably due to the early occurence of the metastases which were the cause of death.

DISCUSSION

The model described above was developed for long-term, stress-free, intra-arterial infusion in rats. The animals tolerate this treatment very well, as shown by the absence of any change in body weight as compared with untreated control rats.

Leivestad [10] had already developed an intra-arterial infusion system for the hepatic artery of the rat, but with his system the animal is under restraint.

This is avoided in our system, which permits free movement of the animals during infusion, thus eliminating a source of stress.

The technique described here can be expected to prove useful in other experimental situations, e.g. biochemical liver studies and stress-free sampling of blood. Our results show that with this method intra-arterial treatment of liver tumors of rats is superior to systemic application.

With respect to possible applications in move, the following may be said. The treatment of human liver tumors, both primary and

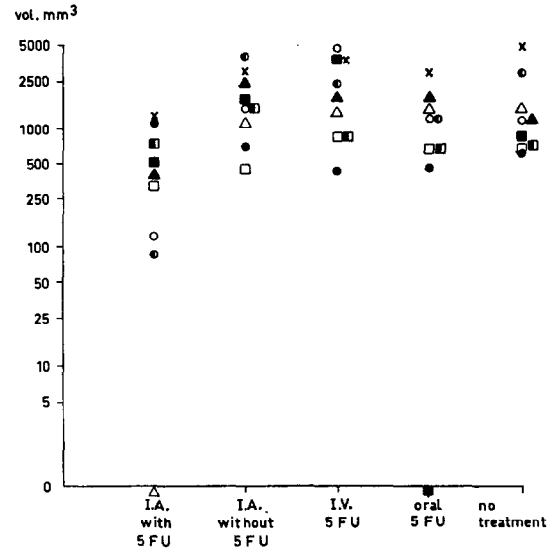


Fig. 2. Tumor growth, according to treatment. (On the horizontal axis, 5 groups of 9 rats each, allowed to die from the disease; on the vertical axis, the volume of the tumor at death.) The intra-arterial administration of 5-FU gave significantly better results than the other methods ($P < 0{\cdot}05$).

Table 1. Survival time from the day of inoculation, according to treatment

Exp. No.	i.a. with 5-FU	i.a. without 5-FU	i.v. 5-FU	Oral 5-FU	No treatment
1	20	17	26	23	19
2	26	23	33	>42	21
3	19	19	22	19	20
4	29	25	34	21	19
5	29	18	20	28	25
6	25	26	25	25	25
7	34	23	23	34	32
8	24	25	25	25	23
9	22	19	19	19	17
Mean survival	25	21	25	24	22

secondary, still presents great problems. The circulation in the liver is such that it should be very susceptible to intra-arterial treatment, especially with 5-FU. The normal liver receives 70–80% of its blood supply through the portal vein [11, 12], and a large number of liver tumors are known to be supplied mainly arterially. This means that intra-arterially applied 5-fluorouracil would go mainly to the tumor and only a small proportion to the healthy tissues. The poor results still reported in patients are possibly to be attributed to the fact that, so far, the treatment only has been applied in a very advanced stage of the disease.

REFERENCES

1. F. J. Ansfield, G. Ramirez, J. L. Skibba, G. T. Bryan, H. L. Davis, Jr. and G. W. Wirtanen, Intra-arterial infusion with 5-Fluorouracil. *Cancer (Philad.)* **28,** 1147 (1971).
2. B. Cady and R. A. Oberfield, Infusion chemotherapy of liver metastases from large bowel cancer. *Lahey Clin. Found. Bull.* **21,** 89 (1972).
3. B. Clarkson, Ch. Young, W. Dierick, P. Kuehn, M. Kim, A. Berret, P. Clapp and W. Lawrence, Jr., Effects of continuous hepatic artery infusion of antimetabolites on primary and metastatic cancer of the liver. *Cancer (Philad.)* **15,** 472 (1962).
4. I. E. Fortuny, Th. Gides and B. J. Kennedy, Cancer of the liver; intra-arterial infusion. *Minn. Med.* **55,** 197 (1972).
5. H. A. Freckman, Intra-arterial therapy for neoplasms. *J. Amer. med. Ass.* **201,** 891 (1967).
6. D. B. Rochlin and Ch. R. Smart, An evaluation of 51 patients with hepatic artery infusion. *Surg. Gynecol. Obstet.* **123,** 535 (1966).
7. R. D. Sullivan and W. Z. Zurek, Chemotherapy for liver cancer by protracted ambulatory infusion. *J. Amer. med. Ass.* **194,** 481 (1965).
8. S. Tindel, Intra-arterial chemotherapy for recurrent neoplasms. *J. Amer. med. Ass.* **200,** 913 (1967).
9. O. Creech, Jr., Intra-arterial chemotherapy for recurrent neoplasms. *J. Amer. med. Ass.* **200,** 983 (1967).
10. Ø. Leivestad and R. A. Malt, Continuous infusion into the hepatic artery and vena cava of the rat. *Surgery* **74,** 401 (1973).
11. A. M. Rappaport and M. Knoblauch, The hepatic artery, its structural, circulatory and metabolic functions. *T. Gastroenterology Suppl.* 10^{B} (1967).
12. H. Baily and A. Love, *A Short Practice of Surgery* 14th ed. p. 791. London, Lowis & Co. 1968.

Europ. J. Cancer Vol. 12, pp. 181–188. Pergamon Press 1976. Printed in Great Britain

Combination Chemotherapy with 4'Demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside), VP 16-213 (NSC 141540) in L1210 Leukemia*

PER DOMBERNOWSKY and NIS I. NISSEN
Department of Internal Medicine, Finsen Institute, DK-2100 Copenhagen, Denmark

Abstract—*The epipodophyllotoxin derivative VP 16–213 (NSC 141540), an antineoplastic agent of high activity against transplantable leukemia in mice and of proven activity in human tumours, was studied in combination therapy with actinomycin D, 1,3-bis(2-chloro-ethyl)-1-nitrosourea (BCNU), cyclophosphamide (CTX), cytosine arabinoside, daunorubicin, 5-fluorouracil, 6-mercaptopurine, methotrexate or vincristine against L1210 leukemia in mice.*

With one treatment on day 1 after transplantation, the therapeutic results of the combination of VP 16–213 with actinomycin D, cytosine arabinoside, daunorubicin, 5-fluorouracil, 6-mercaptopurine, methotrexate and vincristine, were in the same range or inferior to the effect of the single agents. Combination of VP 16–213 with CTX or BCNU led to remarkably good results, which were of the "more than additive" type. Thus, in equitoxic doses, for example, the combination of VP 16–213+CTX (ratio 1:1) at the 0·5 LD_{10} level gave 75% cures compared with 5% for VP 16–213 alone and 0% for CTX.

When treatment was started on day 6 after transplantation, no advantage of the VP 16–213+CTX combination could be demonstrated, while the VP 16–213+BCNU combination still proved superior to the single agents, with respect to increase in life-span and cure rate.

INTRODUCTION

AMONG the podophyllotoxin derivatives tested for antitumour activity, 4'demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside) VP 16-213 has shown the most promising properties. With certain schedules it cures mice with L1210 leukemia in a very high percentage [1, 2], and it has been ranked as one of the most effective plant compounds against the L1210 tumour in the National Cancer Institute screening program.

VP 16-213 has reached phase I clinical trials, in which antitumour activity has been demonstrated [3–7] and phase II studies are now in progress.

During the past decade the development in clinical chemotherapy has been from single-

Accepted 24 October 1975.

*This investigation was supported by grants from the Danish Hospital Foundation for Medical Research, Region of Copenhagen, and the Hasselbalch Foundation.

Correspondence to: P. Dombernowsky M.D., Finsen Institute, Department of Internal Medicine, DK-2100 Copenhagen, Denmark.

agent therapy to multiple-agent combination chemotherapy, and numerous reports have emphasized the corresponding, major improvement in the results [8, 9].

The next step in the clinical evaluation of a new compound, such as VP 16-213, is therefore an assessment of its value in combination chemotherapy. To obtain some guidance as to which combinations it might be fruitful to test clinically, we undertook a study of the experimental therapeutic activity of VP 16-213 in 2-drug combinations with a number of cytostatic agents. The therapeutic activity was investigated in the L1210 leukemia which has proved a good indicator for active antitumour agents in man [9, 10].

MATERIAL AND METHODS

Mice and leukemia

First generation hybrids of female random-bred Swiss mice and male inbred DBA/2 mice (N/D mice) were used in all experiments. All mice weighed 18–22 g. An inoculum of 10^5 L1210 leukemia cells in 0·2 ml given i.p. was used in all experiments as described previously [11].

Drugs and drug administration

VP 16-213 was supplied from Sandoz Ltd., Basle, Switzerland, in vials containing 100 mg active substance in a solvent composed of tween 80 20 mg, polyethylene glycol 300 600 mg, benzyl alcohol 30 mg, citric acid (anhydrous) 2 mg and absolute alcohol ad 1 ml for each 20 mg VP 16-213.

All drugs were freshly prepared and dissolved to make an isotonic solution. Thereafter they were diluted with 0·9% NaCl until the dose/g of mouse was contained in 0·01 ml. The mice were weighed before each injection, and the appropriate dose was administered i.p. With combination therapy the 2 drugs were injected separately in each flank with an interval of few seconds and without preference of which drug to be given first.

Toxicity experiments

Non-tumour-bearing mice were randomized into groups of 10. 5–8 groups of mice were treated once with doses that caused, during an observation period of 40 days, a lethality ranging from 0% to 100%. For the drugs singly (except cytosine arabinoside), and for BCNU and CTX combined with VP 16-213 in different ratios, the lethal dose for 10% (LD10) and 50% (LD50), with confidence limits, was calculated by a computerized program of logit analysis [12]. For Ara-C a dose of 1000 mg/kg, corresponding to about 1/3 of LD_{10} [13], was used.

Drug toxicity was evaluated also in therapy experiments where non-tumour-bearing mice were given the same drug combinations and concentrations as used for therapy.

Therapy experiments

After transplantation, mice were randomized into a control group of 18 mice, and treatment groups of 10 mice. The animals were treated only once 24 hr or 6 days after transplantation. Mice in the control group received 0·2 ml isotonic NaCl. The mean survival time after inoculation of 10^5 cells was, as previously determined, 7·9 days [11]. If the mean survival time of a control group differed from 7·9 days by more than 0·5 day, the whole experiment was rejected. The therapy experiments were done twice and the data pooled. The antitumour effect of a drug was evaluated by comparing the median survival time (MST) in treated and untreated tumour-bearing mice, and % increase in life-span (% ILS) was calculated [14]. Sixty-day survivors from the start of an experiment were defined as "cures", as only 1 of 50 mice observed for 100 days died after that day with persistent tumour. In no instance did an untreated control animal survive.

The Wilcoxon two-tailed test for two samples was used for the statistical evaluation [15].

RESULTS

Table 1 shows LD_{10} and LD_{50} in mg/kg for one treatment of N/D mice with actinomycin D, BCNU, CTX, daunorubicin, 5-fluorouracil, 6-mercaptopurine, methotrexate, vincristine and VP 16-213.

Table 2 gives % ILS and fraction of cures for single-drug treatment and 2-drug combination chemotherapy with VP 16-213 on day 1 after 10^5 L1210 cells i.p. The drug concentrations used are expressed as fractions of LD_{10}: 0·1 LD_{10}, 0·5 LD_{10}, and 1·0 LD_{10} respectively.

Treatment once, on day 1, with LD_{10} VP 16-213 gave an ILS of 125% and 20% cures as compared with an ILS from 25% to 56% and no cures for one treatment on day 1 with LD_{10} of actinomycin D, daunorubicin, 5-fluorouracil, 6-mercaptopurine and methotrexate and 0·3 LD_{10} of cytosine arabinoside. BCNU and CTX treatment resulted in an ILS of more than 650% and cure rate of 17/20 mice and 11/20 mice.

The combination of actinomycin D, cytosine arabinoside, daunorubicin, 5-fluorouracil, 6-

Table 1. LD_{10} *and* LD_{50} *for Single-agent chemotherapy once in N/D mice*

Drug	LD_{10}			LD_{50}		
Actinomycin D	0·42	mg/kg	(0·34–0·51)	0·69	mg/kg	(0·62–0·77)
BCNU	44	—	(41–48)	50	—	(48–53)
CTX	328	—	(280–384)	443	—	(404–484)
Daunorubicin	2·7	—	(2·3–3·1)	3·9	—	(3·6–4·2)
5-Fluorouracil	141	—	(117–171)	255	—	(228–285)
6-Mercaptopurine	166	—	(128–215)	274	—	(239–314)
Methotrexate	43	—	(34–55)	135	—	(117–155)
Vincristine	1·9	—	(1·5–2·4)	3·4	—	(3·1–3·8)
VP 16–213	47	—	(37–61)	78	—	(67–91)

Values in brackets indicate 95% confidence limits.

mercaptopurine or methotrexate with VP 16-213 in different ratios afforded no definite therapeutic advantage over treatment with VP 16-213 alone, and with the combination of VP 16-213 and vincristine the results proved inferior to VP 16-213 alone.

VP 16-213 combined with CTX entailed at many ratios an impressive increase in ILS and in the fraction of cures as compared with CTX alone; e.g., with 0·5 LD_{10} VP 16-213 + 0·5 LD_{10} CTX 80% cures were found as compared with 55% and 20% for single-agent treatment with LD_{10} of CTX or VP 16-213, respectively.

Combination of VP 16-213 and BCNU also resulted in a high fraction of cures in many ratios. However, due to the good results obtained with BCNU alone, it was not possible to demonstrate a statistically significant superiority of the combination in these first experiments.

The promising results with VP 16-213 and CTX or BCNU made it necessary to determine the LD_{10} and LD_{50} for combinations of these drugs in different ratios. Comparison of therapeutic efficiency at equitoxic doses would then better answer the question concerning a possible synergistic effect of the combinations compared with single-drug treatment.

Table 3 shows the LD_{10} and LD_{50} for the combinations of VP 16-213 and BCNU or CTX in the ratios 1:1, 1:2, 2:1, 1:5, and 5:1.

Therapy experiments with the 1:1 ratio of VP 16-213 and BCNU or CTX, for early and advanced L1210 leukemia, are illustrated in Fig. 1 and 2. With treatment day 1 after transplantation at the 1:1 ratio VP 16-213 + CTX (Fig. 1) in the LD_{10} dose cures 18/20 mice as compared with 9/20 and 4/20 for CTX and VP 16-213 alone, respectively ($P < 0·01$). Treatment using doses below LD_{10} demonstrated even more clearly the superiority of the combination to single-drug treatment. At the 0·5 LD_{10} level CTX + VP 16-213 cured 15/20 mice and the ILS was > 60 days compared with 6/20 and 3/20 cured and ILS of 21·0 and 16·5 days respectively, for CTX and VP 16-213 as single agents ($P < 0·01$). With 1:2, 2:1, 1:5 and 5:1 ratios no further advantage was observed from the combination therapy.

Treatment of advanced L1210 leukemia (day 6 after transplantation) with VP 16-213 + CTX in the ratio 1:1 showed no advantage of the combination as compared with single-agent treatment (Fig. 1). The results were inferior to treatment on day 1, with no cures and a MST of 21·5 days.

Figure 2 presents the results of treatment on day 1 for VP 16-213 + BCNU in the ratio 1:1. A cure rate of 18/20 for the combination, compared with 19/20 mice with BCNU alone, was obtained at LD_{10}. At 0·5 LD_{10} the combination cured 9/20 mice and the ILS was 30·0 days as compared with 3/20 cures and an ILS of 15·0 days for 0·5 LD_{10} BCNU ($P < 0·05$).

With the high cure rate at LD_{10} it was difficult to demonstrate a possible difference between single-drug treatment and combination chemotherapy. Further exploration of the VP 16-213 and BCNU combinations was performed in mice with advanced L1210 leukemia.

Figure 2 sets out the results of treatment with VP 16-213 and BCNU on day 6 after transplantation in the ratio 1:1. Doses from 1·25 LD_{10} to 0·75 LD_{10} led to a clearcut increase in both life-span and fraction of cures with combination chemotherapy as compared with single-agent treatment. At the LD_{10} level the cure rates were 11/20, 5/20, and 0/20, and the ILS > 60 days, 23·5 days, and 12·5 days for VP 16-213 + BCNU, BCNU and VP 16-213 respectively ($P < 0·02$ and 0·01).

Combination chemotherapy with BCNU and VP 16-213 in the ratios 1:2, 2:1, 1:5, and 5:1 showed no further advantage.

Table 2. Effect of combination chemotherapy with VP 16–213 on survival time of N/D mice transplanted with early L1210 leukemia

Agent(s)	Dosage in terms of LD_{10}	Median % increase in life-span over controls (including survivors) (ILS)	Fraction of "cures"	Fraction of lethality in concurrent toxicity controls
VP 16–213	1·0	125	36/180	—
	0·5	81	13/180	—
	0·1	38	0/180	—
Actinomycin D	1·0	38	0/20	—
	0·5	19	0/20	—
	0·1	6	0/20	—
VP 16–213 + actinomycin D	1·0 + 1·0	56	2/20	8/20
	1·0 + 0·5	44	2/20	0/20
	1·0 + 0·1	31	5/20	0/20
	0·5 + 1·0	56	0/20	6/20
	0·5 + 0·5	81	3/20	3/20
	0·5 + 0·1	56	2/20	0/20
	0·1 + 1·0	50	0/20	2/20
	0·1 + 0·5	50	1/20	0/20
	0·1 + 0·1	69	0/20	0/20
BCNU	1·0	> 650	17/20	—
	0·5	88	2/20	—
	0·1	25	0/20	—
VP 16–213 + BCNU	1·0 + 1·0	0	3/20	20/20
	1·0 + 0·5	0	6/20	11/20
	1·0 + 0·1	100	6/20	2/20
	0·5 + 1·0	225	9/20	11/20
	0·5 + 0·5	> 650	19/20	0/20
	0·5 + 0·1	100	4/20	0/20
	0·1 + 1·0	> 650	17/20	1/20
	0·1 + 0·5	250	9/20	0/20
	0·1 + 0·1	63	0/20	0/20
CTX	1·0	> 650	11/20	—
	0·5	88	0/20	—
	0·1	13	0/20	—
VP 16–213 + CTX	1·0 + 1·0	0	2/20	19/20
	1·0 + 0·5	175	7/20	4/20
	1·0 + 0·1	488	11/20	1/20
	0·5 + 1·0	> 650	12/20	3/20
	0·5 + 0·5	> 650	16/20	0/20
	0·5 + 0·1	> 650	13/20	0/20
	0·1 + 1·0	> 650	15/20	0/20
	0·1 + 0·5	> 650	14/20	0/20
	0·1 + 0·1	63	0/20	0/20
Cytosine arabinoside	0·3	44	0/20	—
VP 16–213 + Cytosine arabinoside	1·0 + 0·3	75	3/20	4/20
	0·5 + 0·3	94	0/20	0/20
	0·1 + 0·3	38	0/20	0/20
Daunorubicin	1·0	25	0/20	—
	0·5	19	0/20	—
	0·1	6	0/20	—
VP 16–213 + daunorubicin	1·0 + 1·0	94	5/20	6/20
	1·0 + 0·5	106	4/20	4/20
	1·0 + 0·1	125	7/20	0/20
	0·5 + 1·0	100	6/20	4/20
	0·5 + 0·5	94	2/20	0/20
	0·5 + 0·1	88	3/20	0/20
	0·1 + 1·0	44	0/20	0/20
	0·1 + 0·5	31	0/20	0/20
	0·1 + 0·1	31	0/20	0/20
5-Fluorouracil	1·0	56	0/20	—
	0·5	31	0/20	
	0·1	19	0/20	—

Table 2.—Continued

Agent(s)	Dosage in terms of LD_{10}	Median % increase in life-span over controls (including survivors) (ILS)	Fraction of "cures"	Fraction of lethality in concurrent toxicity controls
VP 16–213 + 5-fluorouracil	1·0 + 1·0	94	1/20	13/20
	1·0 + 0·5	113	4/20	2/20
	1·0 + 0·1	144	6/20	0/20
	0·5 + 1·0	94	2/20	2/20
	0·5 + 0·5	106	2/20	0/20
	0·5 + 0·1	81	0/20	0/20
	0·1 + 1·0	81	1/20	0/20
	0·1 + 0·5	69	1/20	0/20
	0·1 + 0·1	44	1/20	0/20
6-Mercaptopurine	1·0	44	0/20	—
	0·5	31	0/20	—
	0·1	6	0/20	—
VP 16–213 + 6-mercaptopurine	1·0 + 1·0	69	2/20	7/20
	1·0 + 0·5	56	2/20	4/20
	1·0 + 0·1	56	2/20	1/20
	0·5 + 1·0	94	1/20	2/20
	0·5 + 0·5	119	1/20	0/20
	0·5 + 0·1	94	0/20	0/20
	0·1 + 1·0	69	1/20	2/20
	0·1 + 0·5	69	2/20	0/20
	0·1 + 0·1	75	1/20	0/20
Methotrexate	1·0	44	0/20	—
	0·5	44	0/20	—
	0·1	19	0/20	—
VP 16–213 + methotrexate	1·0 + 1·0	0	3/20	7/20
	1·0 + 0·5	56	2/20	5/20
	1·0 + 0·1	106	1/20	2/20
	0·5 + 1·0	119	3/20	4/20
	0·5 + 0·5	94	2/20	0/20
	0·5 + 0·1	81	1/20	0/20
	0·1 + 1·0	69	0/20	0/20
	0·1 + 0·5	69	0/20	0/20
	0·1 + 0·1	56	0/20	0/20
Vincristine	1·0	38	0/20	—
	0·5	13	0/20	—
	0·1	13	0/20	—
VP 16–213 + vincristine	1·0 + 1·0	31	0/20	9/20
	1·0 + 0·5	44	0/20	3/20
	1·0 + 0·1	81	2/20	6/20
	0·5 + 1·0	25	0/20	10/20
	0·5 + 0·5	56	2/20	0/20
	0·5 + 0·1	25	0/20	0/20
	0·1 + 1·0	25	0/20	2/20
	0·1 + 0·5	25	0/20	0/20
	0·1 + 0·1	25	0/20	0/20

The data were pooled from 2 or more experiments in each combination and concentration. Median survival time of untreated control mice was from 7·4 to 8·4 days. Cured mice were recorded as having lived 60 days.

DISCUSSION

The combination of 2 drugs may prove (1) less active than either drug alone, (2) equal in activity, or (3) superior in activity. In the latter case, the effect may be evaluated as subadditive, additive, or more than additive, the usual endpoint of therapeutic research [16].

Inherent in the results of combination chemotherapy are factors such as the toxicological spectra of the drugs, optimal use of drug schedules of each of the drugs, and optimal use of scheduling the drugs when given together, again partly dependent on the mechanism of action in the cell cycle for each of the drugs.

To facilitate direct comparisons of the single

Table 3. LD_{10} *and* LD_{50} *for combination chemotherapy once with VP 16-213 in different ratios*

Drugs	Ratio	LD_{10}*	LD_{50}*
VP 16–213+CTX	1+1	0·59 (0·55–0·63)+0·59 (0·55–0·63)	0·56 (0·53–0·59)+0·56 (0·53–0·59)
	1+2	0·30 (0·24–0·38)+0·60 (0·48–0·76)	0·30 (0·27–0·33)+0·73 (0·65–0·82)
	2+1	0·69 (0·58–0·83)+0·35 (0·29–0·42)	0·67 (0·61–0·74)+0·42 (0·38–0·46)
	1+5	0·16 (0·13–0·20)+0·80 (0·65–0·96)	0·16 (0·14–0·18)+0·96 (0·85–1·08)
	5+1	1·05 (0·91–1·22)+0·21 (0·18–0·24)	0·87 (0·79–0·96)+0·21 (0·19–0·24)
VP 16–213+BCNU	1+1	0·59 (0·52–0·68)+0·59 (0·52–0·68)	0·66 (0·62–0·71)+0·66 (0·62–0·71)
	1+2	0·41 (0·36–0·46)+0·82 (0·72–0·92)	0·32 (0·30–0·34)+0·93 (0·86–1·00)
	2+1	0·65 (0·56–0·76)+0·33 (0·28–0·38)	0·54 (0·50–0·58)+0·40 (0·37–0·42)
	1+5	0·19 (0·17–0·22)+0·95 (0·85–1·06)	0·15 (0·14–0·16)+1·10 (1·01–1·19)
	5+1	0·88 (0·75–1·04)+0·18 (0·15–0·21)	0·75 (0·68–0·81)+0·22 (0·20–0·24)

*LD_{10} and LD_{50} are expressed in terms of LD_{10} and LD_{50} respectively for the single agents used (see Table 1). Values in brackets indicate 95% confidence limits.

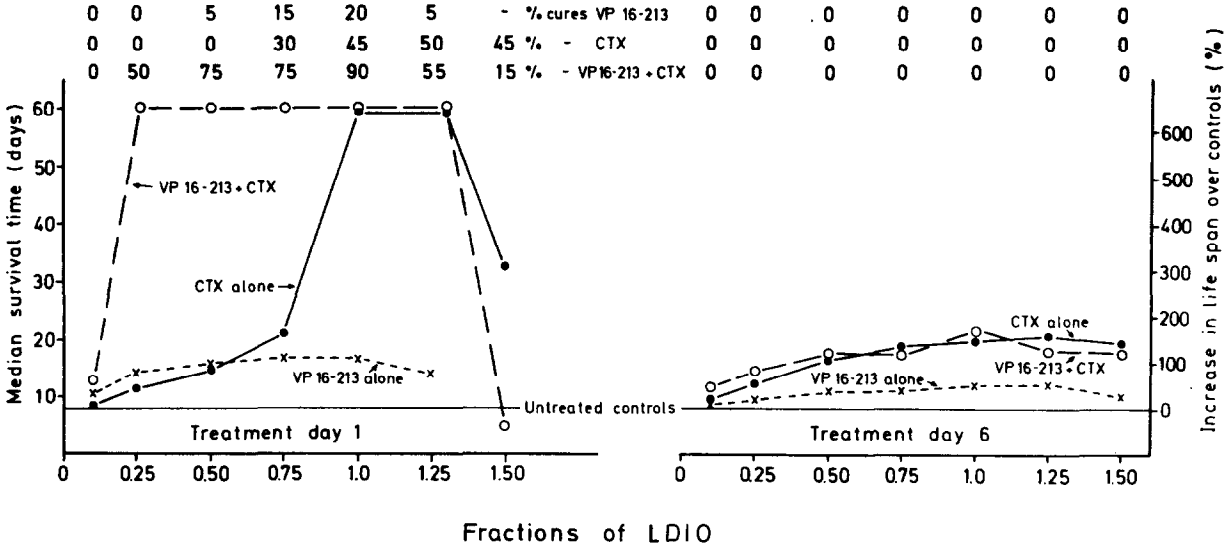


Fig. 1. Effect of treatment with VP 16–213, CTX and VP 16–213+CTX in the ratio 1:1 on survival time and % cures of leukemic (L1210) mice. The inoculum was 10^5 cells i.p., and the animals were treated only once 24 hours and 6 days after tumour inoculation. Twenty mice were used in the treated groups and 36 mice in the control group. For the combination the LD_{10} expresses the experimentally determined combined toxicity of the 2 drugs.

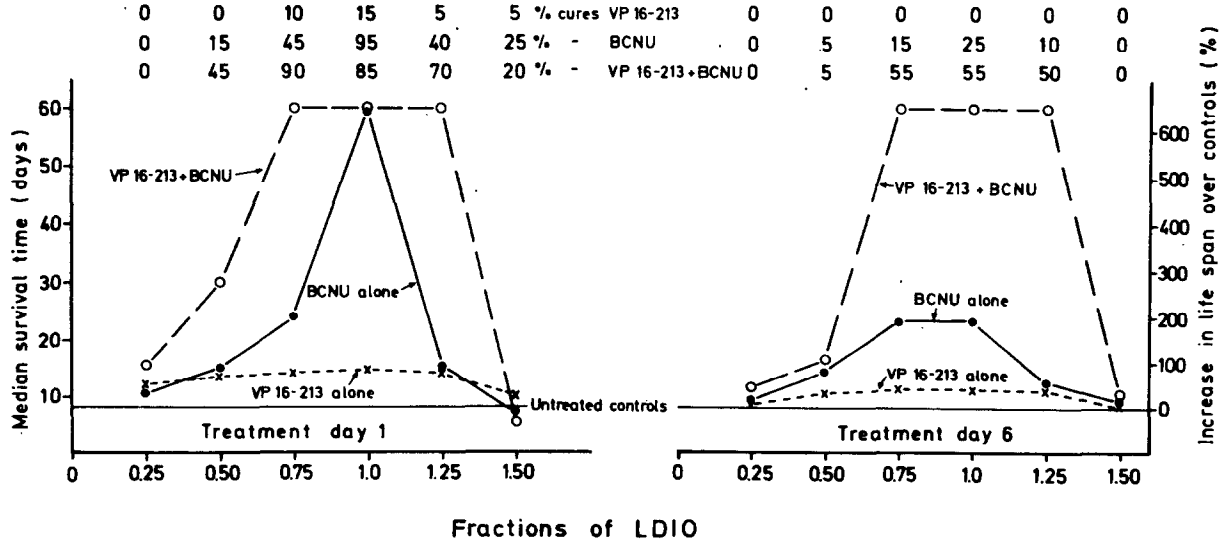


Fig. 2. Effect of treatment with VP 16–213, BCNU and VP 16–213+BCNU in the ratio 1:1 on survival time and % cures of leukemic (L1210) mice. Experimental conditions as described in Fig. 1.

agents in this study, we expressed all doses as fraction of LD_{10}. For most of the combinations we arrived at the equitoxic dose of the combination by adding the decimal fractions of the LD10 of the individual agents. For CTX and BCNU combinations with VP 16-213, the more correct procedure of a direct determination of the toxicity of the combination was undertaken in separate experiments. This showed the combinations to be less toxic (toxicity less than additive) than indicated by adding the single-drug toxicities, and it confirmed the overall result: that BCNU and CTX in combination with VP 16-213 showed a consistent superiority over the single components of the "more than additive" category.

The VP 16-213 + BCNU combination showed both with early and late L1210 leukemia high fractions of cures. However, advantage of the VP 16-213 + CTX combination could only be demonstrated with the early L1210 leukemia. This is probably due to the fact that VP 16-213 and CTX in contrast to BCNU have only minimal effect on intracerebral L1210 leukemia, which is present in the advanced stage of disease [2, 17, 18].

For 6 other drugs (actinomycin D, cytosine arabinoside, daunorubicin, 5-fluorouracil, 6-mercaptopurine and methotrexate), the combination proved inferior to or in the same range as the single agents. One drug combination, VCR + VP 16-213, was consistently less active than either single drug alone. These results were obtained by a simple schedule, viz. one treatment on day 1 after transplantation and this does not preclude that other results could not be reached with a number of other schedules. Several studies have demonstrated VP 16-213 to be a cell cycle active agent (main effect in late S or G_2) [19, 20]. Our previous results [1], demonstrating a superior effect of divided treatment (every 3 hr) on day 1, compared with one single injection, also points in this direction, and the possibility should be considered that the lack of improvement in results using VP 16-213 in simultaneous combination with antimetabolites as in our schedule could be due to an interference of the 2 drugs in the sensitive $S + G_2$ part of the cell cycle.

Using the relative simple schedule of simultaneous treatment day 1, 3 and 7 in L1210 leukemia, Rivera [21] could demonstrate some advantage to the combination of VP 16-213 + Ara-C as compared to single agent therapy. This finding underlines the need for further studies of VP 16-213 in combination therapy using not only various schedules but also various sequences of drug therapy.

The studies of Rivera [21] and our present studies have been performed with the L1210 leukemia. In the main part of its development this tumour is characterized as being composed mainly of cells in cell cycle [22], an unusual finding in human tumours. To obtain more valid prediction for the clinical situation studies using tumours with lower growth fractions should also be performed.

VP 16-213 is a new drug with significant antitumour effect in experimental tumours systems and with proven (but so far moderate) clinical activity. The present results indicate that it might be of value clinically in combination chemotherapy with drugs such as BCNU or CTX.

Acknowledgements—The authors are indebted to Miss J. Andersen, Mrs. G. Arnved, Mrs. N. Arnkær and Mrs. I. Kobbernagel for technical assistance.

REFERENCES

1. P. Dombernowsky and N. I. Nissen, Schedule dependency of the antileukemic activity of the podophyllotoxin-derivative VP 16–213 (NSC 141540) in L1210 Leukemia. *Acta path. microbiol. scand.* Sect. A. **81,** 715 (1973).
2. H. Stähelin, Activity of a new glycosidic lignan derivative (VP 16–213) related to podophyllotoxin in experimental tumors. *Europ. J. Cancer* **9,** 215 (1973).
3. F. Cavalli, H. J. Ryssel, K. Batz, R. W. Sonntag and K. W. Brunner, Erste Resultate mit dem Epipodophyllotoxin-Derivat VP 16–213 bei der Behandlung akuter Leukämien. *Schweiz. med. Wschr.* **105,** 250 (1975).
4. P. J. Creaven, S. J. Newman, O. S. Selawry, M. H. Cohen and A. Primack, Phase I clinical trial of weekly administration of 4′-demethylepipodophyllotoxin 9-(4,6-0-2-ethylidene-β-D-glucopyranoside) (VP 16–213, NSC 141540). *Cancer Chemother. Rep.* **58,** 901 (1974).
5. European Organization for Research on the Treatment of Cancer, Clinical Screening Group. Epipodophyllotoxin VP 16–213 in treatment of acute leukaemias, haematosarcomas and solid tumours. *Brit. med. J.* **3,** 199 (1973).

6. G. Mathé, L. Schwarzenberg, P. Pouillart, R. Oldham, R. Weiner, C. Jasmin, C. Rosenfeld, M. Hayat, J. L. Misset, M. Musset, M. Schneider, J. L. Amiel and F. De Vassal, Two epipodophyllotoxin derivatives, VM 26 and VP 16213, in the treatment of leukemias, hematosarcomas, and lymphomas. *Cancer (Philad.)* **34,** 985 (1974).
7. N. I. Nissen, V. Larsen, H. Pedersen and K. Thomsen, Phase I clinical trial of a new antitumor agent, 4'demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside) (NSC 141540; VP 16–213). *Cancer Chemother Rep.* **56,** 769 (1972).
8. V. T. DeVita and P. S. Schein, The use of drugs in combination for the treatment of cancer. *New Engl. J. Med.* **288,** 998 (1973).
9. A. Goldin, J. S. Sandberg, E. S. Henderson, J. W. Newman, E. Frei and J. F. Holland, The chemotherapy of human and animal acute leukemia. *Cancer Chemother. Rep.* **55,** 309 (1971).
10. A. Goldin, A. A. Serpick and N. Mantel, Experimental screening procedures and clinical predictability value. *Cancer Chemother. Rep.* **50,** 173 (1966).
11. P. Dombernowsky, The proliferation kinetics of L1210 ascites tumour. *Acta path. microbiol. scand.* Sect. A. **80,** 603 (1972).
12. P. Armitage, *Statistical Methods in Medical Research*, p. 359, Blackwell, Oxford, 1971.
13. H. E. Skipper, F. M. Schabel, Jr. and W. S. Wilcox, Experimental evaluation of potential anticancer agents. XXI. Scheduling of arabinosylcytosine to take advantage of its S-phase specificity against leukemia cells. *Cancer Chemother. Rep.* **51,** 125 (1967).
14. R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher and B. J. Abbot, Protocols for screening chemical agents and natural products against animal tumors and other biological systems (Third Edition). *Cancer Chemother. Rep.* **3,** 1 (1972).
15. Documenta Geigy. *Scientific Tables* (Sixth Edition). J. R. Geigy S.A., Basle, Switzerland, p. 191, 1962.
16. S. K. Carter, Planning Combined Therapy—The interaction of experimental and clinical studies. *Cancer Chemother. Rep.* **4,** 3 (1974).
17. H. E. Skipper, F. M. Schabel, Jr. and W. S. Wilcox, Experimental evaluation of potential anticancer agents XIII. On the criteria and kinetics associated with curability "of experimental leukemia". *Cancer Chemother. Rep.* **35,** 1 (1964).
18. H. E. Skipper, F. M. Schabel, Jr., W. S. Wilcox, W. Russel Laster, Jr., Mary W. Trader and S. A. Thompson, Experimental evaluation of potential anticancer agents XVIII. Effects of therapy on viability and rate of proliferation of leukemic cells in various anatomic sites. *Cancer Chemother. Rep.* **47,** 41 (1965).
19. A. Grieder, R. Maurer and H. Stähelin, Effect of an epipodophyllotoxin derivative (VP 16–213) on macromolecular synthesis and mitosis in mastocytoma cells *in vitro*. *Cancer Res.* **34,** 1788 (1974).
20. C. C. Huang, Y. Hou and J. J. Wang, Effects of a new antitumor agent, epipodophyllotoxin, on growth and chromosomes in human hematopoietic cell lines. *Cancer Res.* **33,** 3123 (1973).
21. G. Rivera, Combination Chemotherapy with epipodophyllotoxins and arabinosylcytosine (Ara-C). *Proc. Amer. Ass. Cancer Res.* **15,** 108 (1974).
22. P. Dombernowsky and N. R. Hartmann, Analysis of variations in the cell population kinetics with tumor age in the L1210 ascites tumor. *Cancer Res.* **32,** 2452 (1972).

Europ. J. Cancer Vol. 12, pp. 189–194. Pergamon Press 1976. Printed in Great Britain

Changes in Morphology and Hormone Dependency Following Transplantation of Rat 9,10-Dimethyl-1,2-benzanthracene-Induced Mammary Adenocarcinoma*

H. HORN, I. ERLICHMAN, A. GEIER and I. S. LEVIJ†
Departments of Endocrinology and Pathology†
Hebrew University–Hadassah Medical School, Jerusalem, Israel

Abstract—*The hormone dependency of three DMBA-induced mammary adenocarcinomas ("I", "II" and "III") and of three transplanted generations derived from them was examined in an inbred line of female rats (Lewis). Tumour "I" and its three successively transplanted generations did not regress after castration. Tumour "II" regressed after castration and so did 7 out of 8 tumours in the first transplanted generations; in the second and third transplanted generations all tumours became hormone independent. Tumour "III" regressed after castration and progressed after oestradiol-17β administration; however, the three transplanted generations were not hormone dependent.*

In addition to becoming hormone independent, the transplanted tumours also underwent histological changes namely the appearance of cellular stroma and fibrosarcoma in what were originally carcinomatous tumours. In order to examine the possibility that the histological changes observed in the transplants from tumours "I", "II" and "III" were the result of castration or of oestradiol-17β administration, two other DMBA-induced adenocarcinomas (tumours "IV" and "V") were transplanted without any hormonal treatment. Since cellular stroma with small areas of fibrosarcoma appeared in one of the transplants of these tumours it seems that the above histological changes were not caused by hormonal treatment.

INTRODUCTION

9,10-Dimethyl-1,2-benzanthracene (DMBA)—induced mammary tumours in rats are variable with regard to hormone dependency [1–3]. Since this interferes with the study of hormone dependency of experimental breast cancer, Segaloff induced mammary adenocarcinoma with DMBA in an inbred line of female Fischer rats with subsequent transplantation of the tumours into successive generations of the same strain. However, there was only one DMBA-induced tumour in this study which grew in a number of consequent transplantations. Under the influence of oestradiol-17β the transplanted adenocarcinomas developed a cellular stroma, but in no case did a fibrosarcoma appear [1]. This was in contrast to the findings of several investigators [4–8] who observed transition from adenocarcinoma to

Accepted 24 October 1975.

*This study was supported by grant 13/73 from the Israel Cancer Association.

Address for correspondence: H. Horn, Ph.D., Department of Endocrinology, Hebrew University–Hadassah Medical School, P.O. Box 1172, Jerusalem, Israel.

sarcoma in transplanted spontaneous breast adenocarcinoma in mice.

The present paper reports on successful transplantations of 5 mammary adenocarcinomas induced with DMBA in an inbred line of female Lewis rats and on the hormonal dependency and histological appearance of up to three generations of the transplanted tumours.

MATERIAL AND METHODS

Animals

All animals were female inbred rats, 50 days old, from the Lewis strain (Lew/FMAI) grown in the Hebrew University Hadassah Medical School, Jerusalem, Israel. The animals were maintained on rat pellets and water *ad libitum*.

Carcinogen

DMBA (9,10-dimethyl-1,2-benzanthracene) obtained from Sigma (St. Louis, Mo., U.S.A.) was dissolved in olive oil (30 mg/ml) and shaken overnight. One hundred and eighty rats were kept fasting for 4 hr and then fed with 30 mg of DMBA through an intragastric tube [9]. Three to 10 months later palpable tumours appeared in breast tissue in 43 out of the 103 rats which survived the feeding. Tumours were used for study when they reached a diameter of 15–25 mm and at least 4 weeks after they first appeared.

Hormone dependency

Tumour carrying animals were castrated under ether anaesthesia. The effect of castration on regression or progression of a tumour was estimated by measuring its diameter with a caliper twice a week during 4–6 weeks. When a tumour regressed, i.e., its mean diameter decreased 50% or more, the animal was injected with oestradiol-17β (obtained from Ikapharm, Ramat-Gan, Israel) s.c., 5 μg daily for 3–4 weeks. Hormone dependency was regarded to exist if a tumour decreased in size after castration and grew again after oestradiol-17β administration. A tumour which did not decrease in size after castration was regarded as hormone-independent. Animals with hormone-independent tumours were not injected with oestradiol-17β.

Transplantation

Five DMBA-induced tumours from 5 animals were used for transplantation studies. Each tumour was cut into fragments about 2 mm^3 and transplanted subcutanously at the upper part of the milk line, one on each side. Transplantation into the following generations was carried out by the same procedure into 6–10 intact animals.

Histopathological examination

Samples of all tumour lines obtained by partial or total excision were fixed in a 4% solution of neutral buffered formalin. After paraffin embedding multiple sections of 6 μ were cut and stained with haematoxylin and eosin. In all cases histological examination included different parts of the tumour, but from each animal only one tumour was examined.

RESULTS

Tumour "I"

The results of successive transplantations from a DMBA-induced adenocarcinoma in a rat are shown in Fig. 1. Eight months after DMBA feeding, the animal was castrated and the tumour was found to be hormone-independent. Fragments of this tumour were transplanted into 12 animals (F_1) and the rest was taken for histological examination which revealed an adenocarcinoma with fibrous stroma and a normal number of elongated fibroblasts with small nuclei, without pleomorphism (Fig. 2).

Tumours appeared in all F_1 animals, and in six of them the tumours were found to be hormone-independent. Fig. 1 shows only the 7 tumours in the F_1 generation which were examined histologically and for hormone dependency. Five tumours were adenocarcinomas with normal stroma. One tumour (animal No. 1) was an adenocarcinoma with cellular stroma, containing an increased number of fibroblasts with plump nuclei, prominent nucleoli and a small number of mitoses (Fig. 3). One tumour (animal No. 3) was a fibrosarcoma consisting of bundles of elongated cells with plump irregular nuclei, prominent nucleoli and numerous mitoses; in this tumour multiple sections did not reveal carcinomatous structures (Fig. 4).

Tumours from the 6 castrated animals of the F_1 generation were successfully transplanted, each into 6–7 animals of the F_2 generation. From each group at least one animal was castrated and the tumours in all castrated animals were found to be hormone-independent. Histological examination of tumour samples of

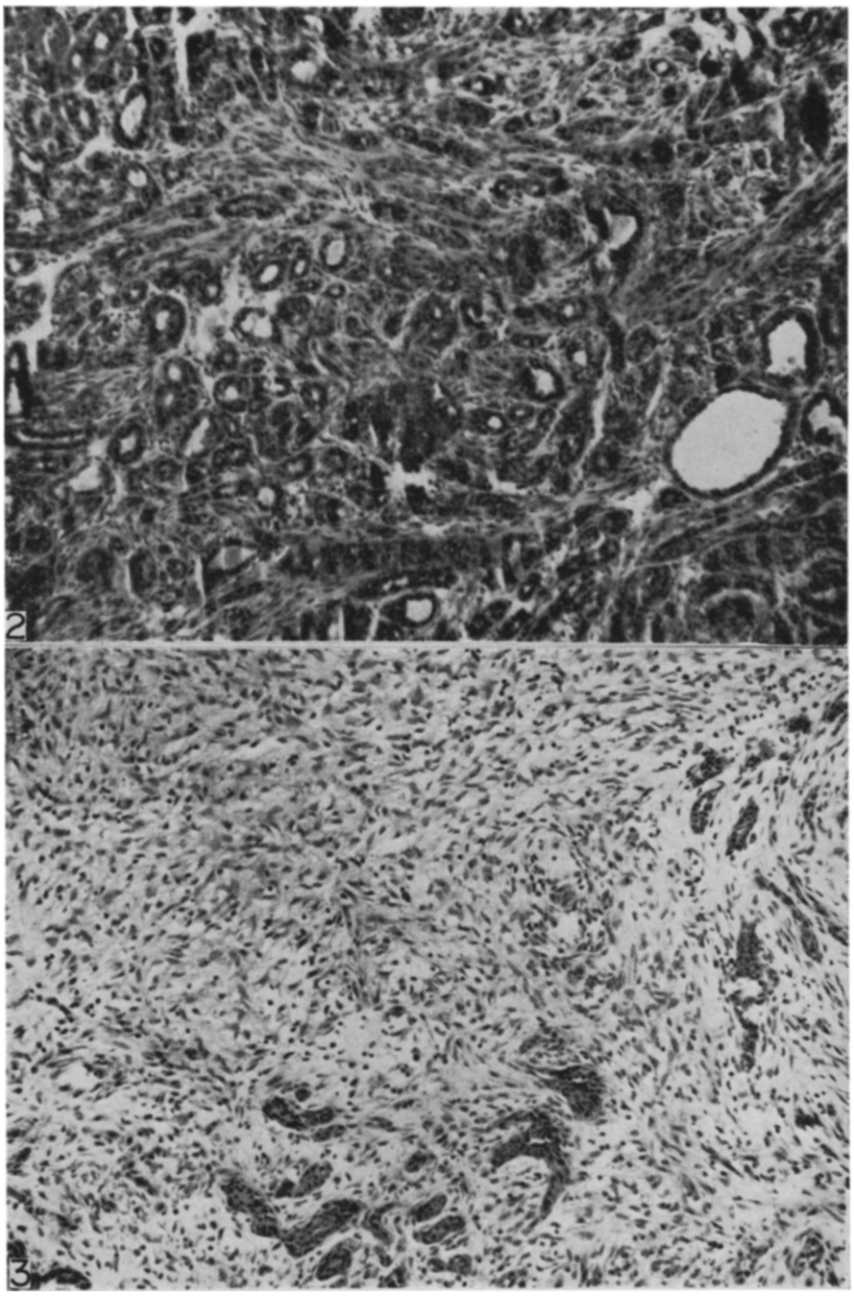


Fig. 2. Histological section of DMBA-induced rat mammary tumour, showing pattern of adenocarcinoma (Haematoxylin and eosin, × 260).

Fig. 3. Histological section of transplanted rat mammary adenocarcinoma, with area of cellular stroma in upper part of figure, and tumourous glandular structures in the lower part. The stroma contains many small fibroblasts with a benign appearance (Haematoxylin and eosin, × 105).

(to face page 190)

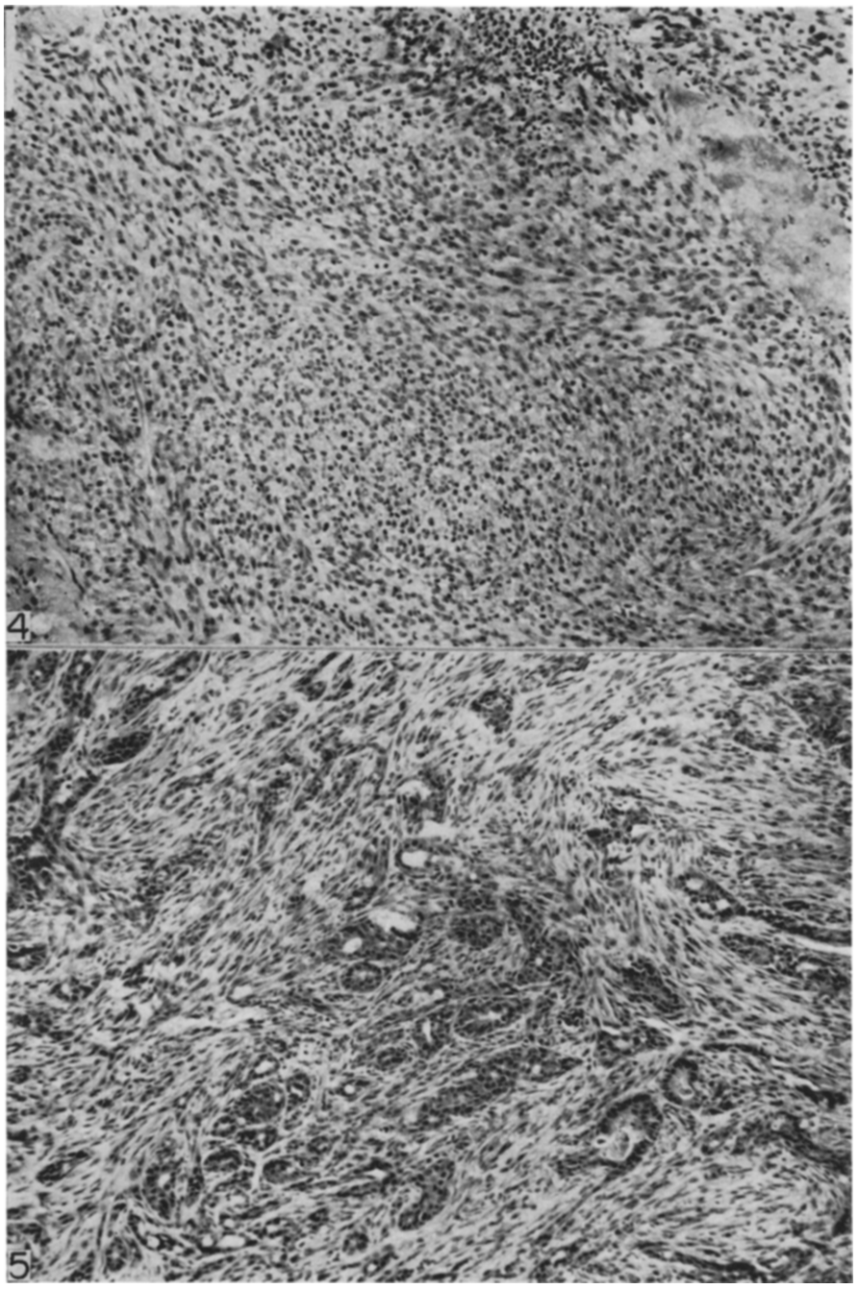


Fig. 4. *Histological section of a transplanted rat mammary tumour. This tumour was derived from an adenocarcinoma, but after transplantation no malignant glandular structures were found, and all tumour tissue showed a fibrosarcomatous pattern.* (*Haematoxylin and eosin,* × 105).

Fig. 5. *Histological section of a transplanted rat mammary tumour showing a combination of adenocarcinoma with a fibrosarcomatous stroma.* (*Haematoxylin and eosin,* × 105).

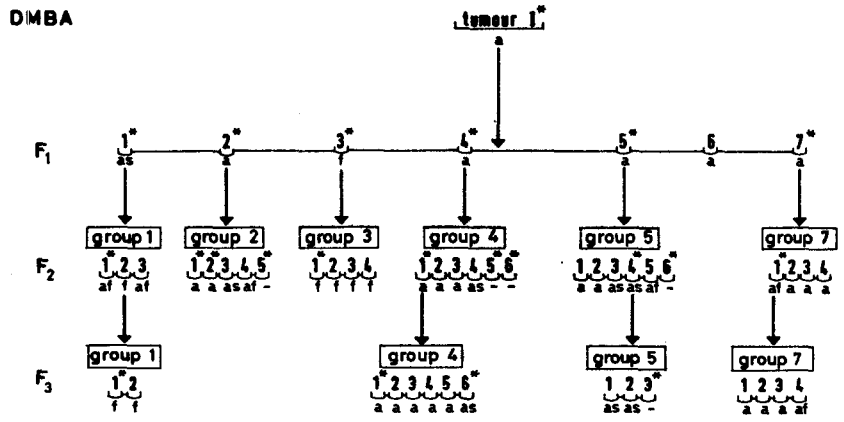


Fig. 1.

F_1, F_2, F_3	*first, second and third transplanted generation.*	—	*histology not examined.*
a	*adenocarcinoma.*	*	*castration showed hormone-independent tumour.*
as	*adenocarcinoma with cellular stroma.*	+	*tumour regressed after castration.*
af	*adenocarcinoma with fibrosarcoma.*	⊕	*hormone-dependent tumour (castration followed*
f	*fibrosarcoma.*		*by oestradiol injection).*
aq	*adenocarcinoma with squamous cell carcinoma.*		

Numbers not marked indicate non-castrated animals.

the F_2 generation, as listed in Fig. 1, revealed more adenocarcinoma with cellular stroma and fibrosarcoma than in the F_1 generation. All tumours derived from the fibrosarcoma (animal No. 3) in the F_1 generation remained fibrosarcomas, and those derived from the tumour with cellular stroma (animal No. 1) became fibrosarcomas. In one of these tumours no carcinomatous structures were found, but in the other two tumours there was a combination of areas with a purely fibrosarcomatous pattern and fields containing adenocarcinoma (Fig. 5). Not all tumours of the F_2 generation transplanted from adenocarcinomas with normal stroma (animals 2, 4, 5 and 7 of the F_1 generation) retained this character: a tendency was noted to develop either cellular stroma or fibrosarcomatous areas in the adenocarcinoma.

Three tumours from castrated animals and one tumour from a non-castrated animal of the F_2 generation were successfully transplanted, each into 6 animals of the F_3 generation. The results in the F_3 generation as far as histology and hormone-independency are concerned were similar to those obtained in the F_2 generation, except for the tumours derived from animal No. 1 in group 7 where the fibrosarcomatous part of the tumour was recognized in only one of the examined transplants.

Tumour "II"

The results of successive transplantations from another DMBA-induced adenocarcinoma are shown in Fig. 6. Three months after DMBA

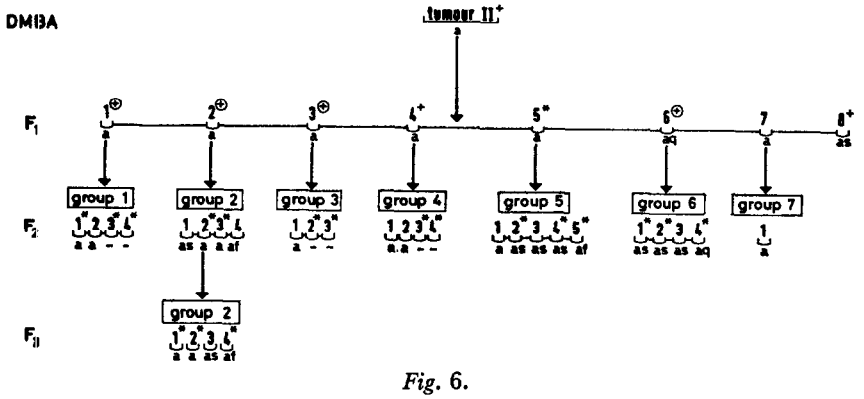


Fig. 6.

F_1, F_2, F_3	*first, second and third transplanted generation.*	—	*histology not examined.*
a	*adenocarcinoma.*	*	*castration showed hormone-independent tumour.*
as	*adenocarcinoma with cellular stroma.*	+	*tumour regressed after castration.*
af	*adenocarcinoma with fibrosarcoma.*	⊕	*hormone-dependent tumour (castration followed*
f	*fibrosarcoma.*		*by oestradiol injection).*
aq	*adenocarcinoma with squamous cell carcinoma.*		

Numbers not marked indicate non-castrated animals.

feeding, the animal was castrated and the tumour regressed. No oestradiol-17β was given to this animal. Five weeks after castration the tumour was removed and successfully transplanted into 10 animals. Histological examination of this tumour revealed a poorly differentiated adenocarcinoma with a small amount of fibrous stroma with normal cellularity.

Eight animals of the F_1 generation were castrated and in all except 1 (animal No. 5) the tumours regressed. Four of the animals where the tumours had regressed (animals No. 1, 2, 3, 6) were injected with oestradiol-17β and the others (animals no. 4 and 8) served as controls. During the first month the tumours of the oestradiol-17β injected animals progressed while the tumours of the controls remained unchanged. Afterwards the control tumours also started to grow. Transplantation from the F_1 tumours was performed after progression of the tumours (either under the influence of oestradiol-17β or spontaneously). Histology of the F_1 generation tumours showed adenocarcinoma with normal stroma in 6 cases, 1 adenocarcinoma with cellular stroma and 1 adenocarcinoma with squamous cell carcinoma. Seven tumours were further transplanted into the F_2 generation. All transplantations were successful except for group No. 7 where only 1 tumour out of 10 transplantations appeared, but most of the tumour used for transplantation was necrotic.

In the F_2 generation half the animals from each group were castrated and all tumours were found to be hormone-independent. Histological examination of 2–3 tumours from each group, as listed in Fig. 6, showed relatively more adenocarcinoma with cellular stroma than in the F_1 generation and in 2 cases (in group No. 2 and in group No. 5) fibrosarcoma appeared together with adenocarcinoma. One adenocarcinoma from group No. 2 of the F_2 generation removed from a castrated animal was further transplanted.

Three animals of the F_3 generation were castrated and found to be hormone-independent. Histological examination revealed 2 adenocarcinomas with normal stroma, 1 with cellular stroma and 1 with areas of fibrosarcoma.

Tumour "III"

The results of transplantation from a third DMBA-induced adenocarcinoma are shown in Fig. 7. The animal was castrated 4 months after DMBA feeding and the tumour was found to be hormone-dependent according to the criteria given in "Material and Methods". Following oestradiol-17β injection the tumour was removed and transplanted into 12 animals. Histological examination of the tumour revealed a papillary adenocarcinoma with necrosis. Tumours appeared only in 5 animals of the F_1 generation, probably due to the partial necrosis of the DMBA-induced tumour. Fig. 7 shows only the tumours which were examined histologically and/or for hormone dependency.

Two animals of the F_1 generation were tested for hormone dependency and the tumours were found to be hormone-independent. Unfortunately we could not examine more animals for hormone dependency because 2 animals of this generation died from pneumonia.

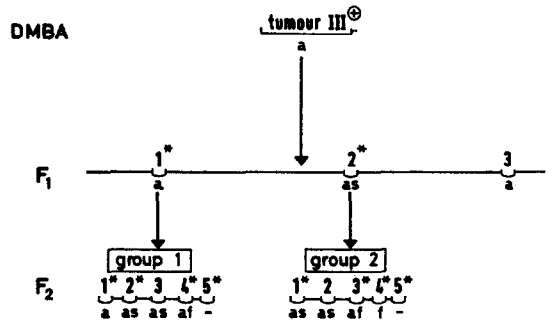


Fig. 7.

F_1, F_2, F_3	*first, second and third transplanted generation.*
a	*adenocarcinoma.*
as	*adenocarcinoma with cellular stroma.*
af	*adenocarcinoma with fibrosarcoma.*
f	*fibrosarcoma.*
aq	*adenocarcinoma with squamous cell carcinoma.*
—	*histology not examined.*
*	*castration showed hormone-independent tumour.*
+	*tumour regressed after castration.*
⊕	*hormone-dependent tumour (castration followed by oestradiol injection).*

Numbers not marked indicate non-castrated animals.

Histology of the F_1 generation tumours showed 2 adenocarcinomas with cellular stroma. The 2 tumours from the castrated animals were further transplanted into the F_2 generation

All transplantations into the F_2 generation were successful. In the F_2 generation 4 animals from each group were castrated and the tumours were found to be hormone-independent. Histological examination of 8 tumours of this generation showed only 1 adenocarcinoma with normal stroma. The other 7 samples were adenocarcinoma with cellular stroma or with fibrosarcoma, and 1 was pure fibrosarcoma.

*

In order to study whether the histological changes observed in tumours "I", "II" and "III" were the result of castration, with or without oestradiol-17β treatment, 2 other

DMBA-induced adenocarcinomas were transplanted without any hormonal treatment. These were designated tumours "IV" and "V".

Tumour "IV"

Ten months after DMBA-feeding tumour "IV" was transplanted into 20 intact animals without examining its hormone dependency. Histological examination revealed an adenocarcinoma with normal stroma. Tumours appeared in 16 animals. Histological examination of tumours of the F_1 generation revealed 5 adenocarcinomas with normal stroma and 1 adenocarcinoma with cellular stroma and small areas of fibrosarcoma.

Tumour "V"

Seven months after DMBA-feeding tumour "V" was transplanted into 20 intact rats without examining its hormone dependency. The primary tumour was found to be an adenocarcinoma with normal stroma. Transplantation was successful in 11 animals. Histological examination of 3 tumours of the F_1 generation revealed adenocarcinoma with normal stroma.

DISCUSSION

The purpose of the present study was to obtain hormone-dependent and hormone-independent mammary adenocarcinoma in the rat. It seems from the results of our experiments that transplantation favours the development of hormone-independent tumours. A DMBA-induced adenocarcinoma which was found to be hormone independent (tumour "I") retained this character in three transplanted generations. Another DMBA-induced adenocarcinoma (tumour "III") which was found to be hormone dependent (it regressed after castration and progressed after oestradiol-17β administration), was further transplanted. Two of the transplanted tumours were examined and found to have become hormone-independent. The tumours remained hormone independent following subsequent transplantations. A third DMBA-induced adenocarcinoma (tumour"II") which regressed after castration was further transplanted without injecting oestradiol-17β. Six out of the seven transplanted tumours which were examined regressed after castration. However, in the following generation all examined tumours became hormone-independent.

The question arises whether the difference in hormone dependency between the tumours transplanted from tumour "II" and those transplanted from tumour "III" was due to oestradiol-17β administration to the animal which carried tumour "III". This can not be answered with certainty because of the limited number of tumours examined. Even if we assume that the administration of oestradiol-17β had an effect on the transformation of the hormone-dependent tumours, this still does not explain the loss of hormone-dependency in the second generation of transplanted tumours, also in those which were not injected with oestradiol-17β (from animal No. 4, Fig. 6).

In addition to the loss of responsiveness to castration we have noted another change in the transplanted tumours, i.e., the appearance of cellular stroma and fibrosarcoma. The phenomenon of sarcomatous changes in spontaneous mouse adenocarcinoma during transplantations *in vivo* was first reported in 1905 by Ehrlich and Apolant [10]. Following Ehrlich and Apolant [10, 11] other investigators reported similar observations in mouse mammary tumours *in vivo* and *in vitro* [4–8]. The question whether the spindle cells of the tumour are altered epithelial cells from the original adenocarcinoma or whether they are derived from the host connective tissue cells under the influence of the malignant epithelial cells, can not be answered with certainty [7]. Ehrlich and Apolant [10, 11] favoured the interpretation that the host connective tissue cells may have undergone sarcomatous changes under the influence of the malignant epithelial cells. Ewing [4], on the other hand, suggested that the spindle cells might be altered epithelial cells.

There are only a few papers describing sarcomatous changes in transplanted mammary adenocarcinoma in rats [12, 13], but none of these tumours was DMBA-induced. Segaloff [1] succeeded in transplantation of a DMBA-induced rat adenocarcinoma into only one immature female Fischer rat. This adenocarcinoma was not altered following further transplantations without hormonal treatment, but the author noted that histological changes did appear in the tumours of hormone-treated animals: after oestradiol-17β administration the adenocarcinoma had a large amount of stroma whereas practically no stroma was present in the solid adenocarcinomas of animals treated with testosterone propionate.

In the present study, there was no difficulty in transplantation of DMBA-induced adenocarcinomas into Lewis rats and in further propagation of the transplanted tumours with

the exception of tumour "III" (Fig. 7) and tumour 7 of the F_1 generation of tumour "II" (Fig. 6) where partially necrotic tumour tissue was used for transplantation.

It is interesting to note that in our experiment sarcomatous changes took place as early as the first passage in tumours "I" (Fig. 1) and "IV", and the second generation in tumours "II" and "III" (Figs. 6 and 7). The question arises whether there exists a correlation between castration and oestradiol-17β administration on the one hand, and sarcomatous changes on the other hand. It is generally agreed that steroid hormones stimulate molecular processes at the transcriptional level [14]. This might be related to the appearance of sarcomas. However, in our experiment this does not seem to have been the case since also in a "control" tumour (No. "IV"), where the animal was not castrated nor injected with oestradiol-17β, there appeared an adenocarcinoma with cellular stroma and areas of fibrosarcoma in the F_1 generation. The appearance of increasingly cellular stroma and even fibrosarcoma also had no direct relation to the disappearance of hormone dependency since the latter occurred also in tumours which remained adenocarcinomas with normal stroma.

Genetic factors might influence the rate of sarcomatous changes as was described by Van Rijssel *et al.* [6] for different strains of mice. This could explain the difference between our results and those of Segaloff [1] who worked with Fischer rats. In that strain of rats the tumour remained adenocarcinoma throughout numerous transplantations and was not transformed into fibrosarcoma.

REFERENCES

1. A. Segaloff, Hormones and breast cancer. *Rec. Progr. Hormone Res.* **22,** 351 (1966).
2. R. Hilf, A. Segaloff and L. J. Lerner, Influence of hormonal treatment of Fischer rats on the biochemistry of a transplantable dimethylbenz(a)anthracene induced mammary carcinoma. *Cancer Res.* **28,** 1550 (1968).
3. W. L. McGuire and C. C. Chamness, Studies on the Estrogen Receptor in Breast Cancer. In: Receptors for Reproductive Hormones. *Advanc. Exp. Med. Biol.* (Edited by B. W. O'Malley and A. R. Means) New York, Plenum Press, **36,** 113 (1973).
4. J. Ewing, Pathological aspects of some problems of experimental cancer research, *J. Cancer Res.*, **1,** 71 (1916).
5. A. Taylor and N. Carmichael, Stromal malignancy in mouse-grown transplants of egg-cultivated mouse mammary carcinoma. *Cancer Res.* **7,** 78 (1947).
6. G. Van Rijssel, R. Van Nie, O. Muhlbock and M. W. De Bruyn, *Sarcomatoide Transformatie van Overgeente Carcinomen.* Vierde Jaarboek van Kankeronderzoek en Kankerbestrijding in Nederland, 17 (1954).
7. A. Goldfeder and F. Nagasaki, Spontaneous transformation from carcinomatous to sarcomatous-like growth. *Cancer Res.* **14,** 267 (1954).
8. J. Ringsted, *In vitro* study of a mouse mammary carcinoma stimulating carcinosarcoma and sarcomatous outgrowth during transplantation. *Acta. path. et microbiol. scand.* **40,** 373 (1957).
9. C. Huggins, L. C. Grand and F. P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* **189,** 204 (1961).
10. P. Ehrlich and H. Apolant, Beobachtungen uber maligne Mausetumoren. *Berl. klin. Wschr.* **42,** 871 (1905).
11. P. Ehrlich and H. Apolant, Zur kenntnis der Sarcomentwickelung bei Carcinom—Transplantationen. *Zentr. allg. Path. Anat.* **17,** 513 (1906).
12. W. O. Dunning, M. R. Curtis and M. E. Maun, Spontaneous malignant mixed tumors of the rat and the successful transplantation and separation of both components from a mammary tumor. *Cancer Res.* **5,** 644 (1945).
13. H. P. Morris, T. B. Dunn and C. S. Dubnik. Two transplantable rat carcinomas induced with N-acetyl-2-aminofluorene. *J. nat. Cancer Inst.* **9,** 225 (1949).
14. B. W. O'Malley, G. C. Rosenfeld, J. P. Comstock and A. R. Means, Induction of specific translatable messenger RNA's by oestrogen and progesterone. In: Gene Transcription in Reproductive Tissue, *Karolinska Symposia on Research Methods in Reproductive Endocrinology.* Edited by E. Diczfalusy). Karolinska Institute Stockholm, p. 381 (1972).

Europ. J. Cancer Vol. 12, pp. 195–198. Pergamon Press 1976. Printed in Great Britain

Phase I Clinical Trial of a 3-Day Divided Dose Schedule of Ifosfamide (NSC 109724)

R. L. NELSON,* P. J. CREAVEN, M. H. COHEN and B. E. FOSSIECK, Jr.
NCI-VA Medical Oncology Section and Medical Service, Veterans Administration Hospital, Washington, D.C. 20422, *U.S.A.*

Abstract—*Twenty patients with unresectable cancer received ifosfamide as a* 30 *min i.v. infusion daily for* 3 *days. Dose limiting toxicity was seen in* 6 *patients and was genitourinary in* 4, *hematologic in* 1, *and CNS in* 1. *Leukopenia was seen in* 10 *patients, and microscopic hematuria in all except* 4 *patients. Divided dose administration enabled a larger total dose of drug to be given per treatment course, but did not change the genitourinary nature of the dose limiting toxicity when compared with a high single dose regimen. Total urinary alkylating activity was slightly less following the divided dose administration. For the* 3 *day divided dose schedule, a daily dose of* 2400 *mg/m² appears to be the maximally tolerated dose.*

INTRODUCTION

THE CYCLOPHOSPHAMIDE analog ifosfamide (NSC 109724) has demonstrated greater activity against several experimental tumors than the parent compound, and has shown clinical effectiveness in certain human neoplasms [1–4]. The human dose limiting toxicity of the two drugs differs significantly, ifosfamide displaying greater bladder and renal toxicity and less hematologic toxicity than cyclophosphamide, when the two drugs are compared in a high single dose regimen [5]. Bladder toxicity, manifested by hemorrhagic cystitis, can be prevented by bladder irrigation with acetylcysteine (1% solution, 2000 ml/day), and renal toxicity is largely alleviated by careful attention to hydration status [6]. Recently, Brock and others in Germany have investigated a daily for five days divided dose schedule, and demonstrated that higher total dosages (8000–11,000 mg/m²) may be given with moderate marrow depression and tolerable bladder toxicity if bladder irrigation is employed [7]. Since bladder irrigation would virtually exclude use of the drug in an outpatient population, we have studied the tolerance to a daily for three days divided dose regimen employing vigorous hydration but no bladder irrigation.

Accepted 22 October 1975.

*Address reprint requests to: Robert L. Nelson, M.D., Clinical Pharmacologist, The Lilly Laboratory for Clinical Research, William N. Wishard Memorial Hospital, Indianapolis, Indiana 46202, U.S.A.

MATERIAL AND METHODS

Patients and laboratory studies

Patients with histologically confirmed advanced cancer not amenable to conventional therapy were eligible for inclusion in this study, provided they had adequate renal, hepatic, and bone marrow function (BUN < 22 mg%, bilirubin < 3·0 mg %, WBC > 4000/mm³, platelets > 100,000/mm³), and had received no cytotoxic chemotherapy in the preceding four weeks. Of the 20 patients entered (mean age 54, range 44–72), 7 had carcinoma of the lung (2 adenocarcinoma, 2 epidermoid carcinoma, 2 large cell anaplastic, and 1 small cell anaplastic carcinoma), 4 had adenocarcinoma of the GI tract, 2 had epidermoid carcinoma of the tonsil, 2 had epidermoid carcinoma of the esophagus, 2 had adenocarcinoma of the kidney, 1 had epidermoid carcinoma of the anus, 1 had epidermoid carcinoma of the penis, and one had transitional cell carcinoma of the urinary bladder. Most of these patients had received extensive prior cytotoxic chemotherapy or local radiotherapy. Those patients having

measurable lesions were evaluated for objective antitumor response (decrease of at least 50% in product of greatest and least diameters) by appropriate roentgenograms or direct caliper measurements of accessible tumors on physical examination every 3 weeks. Determinations of hematocrit, leukocyte count, platelet count, serum electrolytes, blood urea nitrogen, creatinine, creatinine clearance, bilirubin, alkaline phosphatase, and glutamic–oxalacetic transaminase (SGOT) were performed pre-treatment and weekly thereafter. Urinalysis including sediment microscopy was performed twice daily during drug administration, and weekly thereafter. In 3 patients all urine was collected for 5 days beginning at the start of drug treatment on day 1 for determination of total urinary alkylating activity.

Drug administration

All patients were hospitalized during treatment. Intravenous hydration using either 5% dextrose in water or 0·45% saline was begun 18 hr prior to treatment at a rate of 100 ml/hr per m^2 body surface area, and continued at least 24 hr after the last drug infusion. Oral intake of fluid was also encouraged. Ifosfamide (1600 mg/m^2 or 2400 mg/m^2) was given in 50 ml of 0·9% saline as a 30 min intravenous infusion each morning for three days. Patients were observed after the infusion for mental status changes and oliguria (urine flow rate < 50 ml/hr). If urine flow rate dropped below 30 ml/hr, furosemide (20–40 mg) was administered i.v. to alleviate the antidiuresis. Blood pressure was measured at 15 min intervals during drug administration, and hourly for 2 hr post infusion. All patients received a second 3-day course of ifosfamide in three weeks unless they had unacceptable toxicity during the first course, or expired. Thereafter, treatment was continued every 3 weeks only in those patients who continued to show no serious toxicity, and had either no change in tumor size or documented tumor regression. The dose was not escalated in any patient.

RESULTS

Eight patients were entered at a dose range of 1600 mg/m^2 per day for 3 days, and 12 patients were entered at a dose range of 2400 mg/m^2 per day for 3 days. The toxicity is summarized in Table 1. Hematologic toxicity, defined by leukocyte count $< 4000/mm^3$ or platelet count $< 100{,}000/mm^3$, was seen in only one-half of patients and was mild (mean day to nadir 11 days, mean nadir 2900 leukocytes). One patient who had extensive prior chemotherapy was treated at the high dose range, and developed prolonged thrombocytopenia (platelets $< 45{,}000/mm^3$) for 12 days; he was subsequently treated at the low dose level without serious toxic effect. Alopecia was a fairly predictable side effect, as was nausea and vomiting, which was usually manageable with parenteral anti-emetics. An antidiuretic effect similar to that seen with high doses of cyclophosphamide was observed in some patients, and about one-half of these required furosemide to maintain urine flow rate above 30 ml/hr. Response to furosemide usually occurred within an hour. Visibly bloody urine was not observed at the 1600 mg/m^2 dose, but was seen at 2400 mg/m^2. The appearance of numerous transitional cells in the urine usually preceded both gross and microscopic hematuria. Cumulative urinary tract toxicity was not observed in those patients who were able to tolerate more than one course of treatment. Central nervous system toxicity, manifested by mild confusion and/or excitement, lasted from 1–8 hr and was spontaneously reversible.

There was 1 death in the present study which could have been related to drug administration. A patient with a past history of cerebrovascular

Table 1. *Toxicity of a daily* × 3 *schedule of ifosfamide*

	1600 (mg/m^2 per day)	2400 (mg/m^2 per day)
Number entered	8	12
Leukopenia (WBC < 4000)	4 (50%)	6 (50%)
Thrombocytopenia (plts < 100,000)	1 (12%)	0
Nausea and vomiting	5 (62%)	11 (92%)
Lethargy/confusion	2 (25%)	6 (50%)
Alopecia (No./evaluable)	3/4(75%)	5/7(71%)
Oliguria	2 (25%)	3 (25%)
Microscopic hematuria (alone)	7 (88%)	5 (42%)
Gross hematuria	0	4 (33%)
Acute tubular necrosis	0	1 (8%)

disease who received 2400 mg/m^2 for 3 days had a subarachnoid hemorrhage on day 4 of therapy, subsequently developed acute tubular necrosis, oliguric renal failure, shock, and expired on day 7; drug induced nephrotoxicity may have contributed to the cause of death.

Toxicity was considered dose limiting in 1/8 patients at the lower dose level (psychotic reaction in a patient with past history of psychosis), and 5/12 patients treated at the high dose (1 prolonged thrombocytopenia, 1 acute tubular necrosis, 3 gross hematuria). Of the pre-treatment clinical and laboratory parameters examined, no single factor nor combinations of factors consistently predicted which patients would have dose limiting toxicity. The patients' level of physical activity was unrelated to any parameter of toxicity in this study.

Of the 15 patients evaluable for antitumor effect, only one partial regression was documented in a patient with adenocarcinoma of the lung treated at the low dose range. In this patient, the cross product of the diameters of the mass on chest roentgenogram was observed to decrease by 50% over the course of 5 weeks. The duration of this partial regression was 4 months. Three other patients (1 small cell anaplastic carcinoma of lung, 1 large cell anaplastic carcinoma of lung, and 1 epidermoid carcinoma of the anus) treated at the high dose range were classified as stable (no change in tumor size) after 2 courses of therapy. In the remaining patients, treatment was stopped after two courses because of clearly defined progression of disease.

Total urinary alkylating activity, measured by the 4-nitrobenzylpyridine reaction [8], was 1692·8 and 1751·0 μmoles mechlorethamine equivalent in 2 patients after 1600 mg/m^2 per day × 3 days, and 3010·8 μmoles in 1 patient after 2400 mg/m^2 per day × 3 days. Results in these same patients after a second course of drug 21 days later at the same dosages were 1610·9, 1572·5, and 3159·2 μmoles mechlorethamine equivalent respectively.

DISCUSSION

Early clinical trials with ifosfamide given in a single dose regimen demonstrated that the dose limiting toxicity of the drug was primarily on the urinary tract. Common clinical manifestations included both microscopic and gross hematuria from hemorrhagic cystitis. More rarely, nephrotoxicity with acute renal tubular damage was observed [1, 5]. In an attempt to obviate the urinary tract toxicity, which has been shown to correlate with levels of urinary alkylating activity [9], Brock and others have investigated several divided dose schedules by giving the drug daily for 2–5 days. Using vigorous intravenous hydration and continuous bladder irrigation they were able to give more drug per treatment course compared to the single dose schedule, with minimal urinary tract toxicity and predictable moderate leukopenia [7].

The present study describes tolerance to a daily for 3 days divided dose schedule without bladder irrigation. Such a schedule might be suited for outpatient therapy in certain selected patients who could maintain a high oral fluid intake. For this 3 day divided dose schedule not employing bladder irrigation, a daily dose of 2400 mg/m^2 appears to be the maximally tolerated dose and is recommended for phase II trials. Patients with compromised renal function should probably be started at a lower dose in view of the nephrotoxicity of the drug. Care must also be taken to alleviate the prominent antidiuretic effect of ifosfamide seen in some patients by appropriate use of furosemide when necessary, since decreased urine flow rates correlate with urinary tract toxicity [9].

Previous studies by our group using a high single dose schedule reported the maximally tolerated dose per treatment course to be 5000 mg/m^2 [6]. Gross hematuria was seen at this dose, but was not observed in the present study in 8 patients who received 4800 mg/m^2 over 3 days. Divided dose administration enabled a larger total dose of drug to be given per treatment course (7200 mg/m^2 vs 5000 mg/m^2), but was ineffective in changing the nature of the dose limiting toxicity which was mainly urinary tract toxicity in both regimens.

Total urinary alkylating activity after 5000 mg/m^2 as a single dose was previously reported by our group to be 2188·7 μmoles mechlorethamine equivalent [9]. Mean total urinary alkylating activity in 2 patients who received 1600 mg/m^2 per day × 3 days (total dose = 4800 mg) in this study was 1656·8 μmoles mechlorethamine equivalent. Thus for a total dose per treatment course of 5000 mg/m^2, total excretion of alkylating activity was somewhat less following divided dose administration.

REFERENCES

1. J. J. Van Dyk, H. C. Falkson, A. M. Van der Merwe, and G. Falkson, Unexpected toxicity in patients treated with isophosphamide. *Cancer Res.* **32,** 921 (1972).
2. W. Scheef, Problems, experience, and results of clinical investigations with isophosphamide. *Proc. VII Int. Cong. of Chemotherapy, Prague,* 1971. Urban & Schwarzenberg, München (1972).
3. D. L. Ahmann, H. F. Bisel, and R. G. Hahn, Phase II clinical trial of isophosphamide (NSC 109724) in patients with advanced breast cancer. *Cancer Chemother. Rep.* **58,** 861 (1974).
4. D. N. Bremner, J. St. C. McCormick, and J. W. W. Thomson, Clinical trial of isophosphamide (109724)—results and side effects. *Cancer Chemother. Rep.* **58,** 889 (1974).
5. M. H. Cohen and A. Mittleman, Initial clinical trials of isophosphamide. *Proc. Amer. Ass. Cancer Res.* **14,** 64 (1973).
6. M. H. Cohen, P. J. Creaven, F. Tejada, H. H. Hansen, F. Muggia, A. Mittleman, and O. S. Selawry, Phase I clinical trial of isophosphamide (NSC 109724). *Cancer Chemother. Rep.* **59,** 751 (1975).
7. Milan Slavik, Personal communication, Investigational Drug Branch, National Institutes of Health, Bethesda, Maryland, U.S.A.
8. O. M. Friedman and E. Boger, Colorimetric estimation of nitrogen mustards in aqueous media. *Anal. Chem.* **33,** 906 (1961).
9. P. J. Creaven, L. M. Allen, D. A. Alford, and M. H. Cohen, Clinical pharmacology of isophosphamide. *Clin. Pharmacol. Therap.* **16,** 77 (1974).

Europ. J. Cancer Vol. 12, pp. 199–202. Pergamon Press 1976. Printed in Great Britain

Surface Morphology of Normal and Chronic Lymphocytic Leukaemia Lymphocytes*

G. COHNEN,† K. FISCHER,† H. LUDWIG‡ and G. BRITTINGER†
†*Division of Haematology, Department of Medicine, and* ‡*Department of Gynaecology and Obstetrics, University of Essen, Essen, Germany*

Abstract—*Peripheral blood lymphocytes from normals and patients with chronic lymphocytic leukaemia (CLL) were studied by scanning electron microscopy. The surface morphology of normal lymphocytes showed considerable heterogeneity. Most cells had a small or moderate number of stub- or finger-like projections. In contrast, CLL lymphocytes exhibited a rather homogeneous surface architecture indicating the monoclonal nature of the neoplastic population. The dominating cell type either had sparse microvilli or was moderately to markedly villous in 9 of the 10 cases studied. Lymphocytes from one patient displayed a smooth, albeit slightly irregular, surface. The abnormality of leukaemic lymphocytes was further indicated by their reduced cell size.*

INTRODUCTION

SCANNING electron microscopy has been applied in previous studies to investigate the surface morphology of various human blood cells [1–4]. Recently, the surface architecture of normal human lymphocytes has been examined by this method, and differences have been described between thymus-dependent (T) and bone marrow-dependent (B) cells [5].

In chronic lymphocytic leukaemia (CLL), lymphocytes are proliferating and accumulating which exhibit membrane-bound immunoglobulins, a surface marker for B cells [6–9]. As seen by transmission electron microscopy, CLL lymphocytes differ from normal cells by a reduced size [10–12] and by a diminished number of lysosomes [13]. Furthermore, various membrane abnormalities have been found in the leukaemic cells [14–17]. In order to define possible alterations of their surface morphology, we studied peripheral blood lymphocytes from CLL patients in comparison with normal cells using scanning electron microscopy.

Accepted 15 October 1975.
*This work was supported by Ministerium für Wissenschaft und Forschung, Nordrhein-Westfalen, Deutsche Forschungsgemeinschaft (LU 118–6), and Alfred und Cläre Pott-Stiftung, Essen, Germany.

MATERIAL AND METHODS

Lymphocytes were separated from heparinized venous blood of 8 normal donors and of 10 patients with CLL (lymphocyte counts ranging from 16,000 to 130,000/μl) by centrifugation over a Ficoll-sodium metrizoate solution (D = 1·077) at 400 *g* for 40 min. Viability as judged by dye exclusion test using erythrosin B was > 95%. Differential counts of stained smears revealed that preparations from normals contained > 95% mononuclear cells with 70–80% lymphocytes. Purity of the CLL lymphocyte suspensions was > 95%. At light microscopy, no morphological differences were observed between the cells from the different CLL patients.

As determined by spontaneous rosette formation with sheep red blood cells (SRBC), 68–80% of normal lymphocytes and 1–12% of CLL lymphocytes were T cells. About 80–90% of the blood lymphocytes from the CLL patients studied have previously been shown to bear B cell markers by immunofluorescent

staining of membrane-bound immunoglobulins [7].

For scanning electron microscopy, the lymphocytes were fixed at room temperature in 2% redistilled glutaraldehyde in phosphate buffer (pH 7·4; 320 mosm) and were kept in the fixative for at least 12 hr at 4°C. Then, the cells were layered on polycarbonate membrane filters of 0·8 μm porosity (Nuclepore; Thomas Co., Philadelphia, Pa., U.S.A.) by aspiration–filtration. Premature drying of the cells was always carefully avoided during the subsequent preparatory procedure. After rinsing with phosphate buffer (pH 7·4), the cells were dehydrated in graded concentrations of aethyl alcohol followed by a graded series of amyl acetate/absolute alcohol and finally absolute amyl acetate each for 1 hr at 22°C. After critical point drying in carbon dioxide (Polaron E 3000; Polaron Equipment Ltd., London, England), portions of the filters were attached to specimen holders using double-sided sticky tape. Then, the cells were coated with a thin layer of gold by a sputtering method in a vacuum apparatus (Balzers AG, Liechtenstein) and were examined at an angle of 30° in a Cambridge Stereoscan S 4–10 scanning electron microscope (Scientific and Medical Instruments Ltd., Cambridge, England) which was used at an accelerating voltage of 20 kV and with a 200 μm dia illuminating aperture. The resolution of the microscope was in the order of 150 Å. Hundreds of cells were scanned on the screen at direct magnifications of from 1000 to 20,000 to obtain an evaluation of cell size and surface morphology of the entire population before recording micrographs which were used for more detailed measurements.

RESULTS

Normal peripheral blood lymphocytes showed different kinds of membrane morphology. About 80% of the cells which ranged from 4·0 to 6·0 μm (mean 4·8 μm) dia had a small to moderate number of surface projections (Fig. 1, 2). These were either stub-like (70–200 nm broad) or short microvilli (up to 400 nm long, 100–300 nm broad). Their number varied from cell to cell between 10 and 50 (mean 35) per exposed half of the surface. About 15% of the lymphocytes (mean dia 5·5 μm) were characterized by a more complex surface architecture with 50–150 (mean 85) microvilli per exposed part of the cell (Fig. 2). These projections varied in length from 0·5 to 1·0 μm and in breadth from 100 to 300 nm. Lymphocytes with microvilli covering almost the entire membrane were rarely seen. Single cells were completely devoid of surface digitations. Monocytes were identified by their ruffled membranes and ridge-like profiles.

Similar to normal cells, all CLL lymphocytes were spherical with the method used. Cells from 7 patients had a diameter ranging from 3·8 to 5·0 μm (mean 4·2 μm) and showed a small or moderate number of surface projections (up to 50 microvilli per exposed surface) which was almost constant on the majority of lymphocytes in each of the cases (Fig. 3, 4). The size of the microvilli was similar to that on corresponding normal cells. In 2 patients the lymphocytes had a dia of 4·2–5·2 μm (mean 4·8 μm) and displayed a more villous surface with about 80 processes per exposed part of the cell (Fig. 5). Cells from one patient which ranged from 3·7 to 4·4 μm (mean 4·0 μm) dia had a smooth surface with some degree of irregularity (Fig. 6). In each of the patients studied more than 90% of the lymphocytes belonged to the same morphological type. Only single lymphocytes were observed which differed in their surface morphology from most other cells (Fig. 6). The 2 smooth cells shown in Fig. 3 may be interpreted as lymphocytes which are completely devoid of microvilli. However, the possibility cannot be excluded that they represent spherocytes.

DISCUSSION

Our observations indicate that most normal human blood lymphocytes display a small to moderate number of stub- or finger-like surface projections. Previous studies have shown that T cells possess smaller numbers of microvilli and are generally smoother than the more villous B cells [5]. Similarly, we found about 80% smoother cells among preparations of normal peripheral blood lymphocytes which contained up to 80% SRBC rosette-forming T cells. However, despite this coincidence it is difficult to accurately identify T and B lymphocytes merely on the basis of these morphological criteria, since there exists an overlapping population with intermediate surface architecture [18]. Furthermore, temperature [19], cell cycle [20], and cellular contact and interaction *in vitro* [21–23] have been demonstrated to influence the surface appearance of the cells. Thus, the possibility has to be considered that the morphological features may reflect different functional states of the lymphocytes rather than specific membrane properties of either T or B cells.

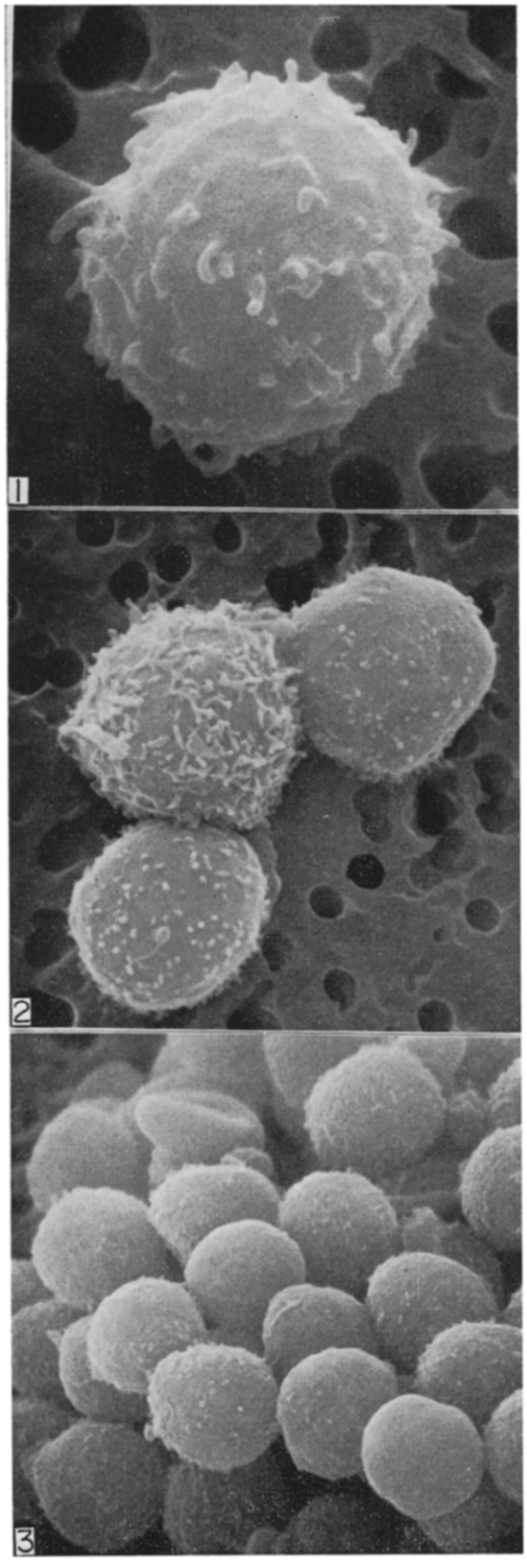


Fig. 1. Spherical normal lymphocyte showing a moderate number of microvilli (Mag. × 10,930).

Fig. 2. Two normal lymphocytes with a small number of stub-like projections and one cell with a markedly villous surface (Mag. × 5670).

Fig. 3. Spherical CLL lymphocytes showing a small number of microvilli. Furthermore, two cells with a smooth surface and an erythrocyte are seen (Mag. × 4050).

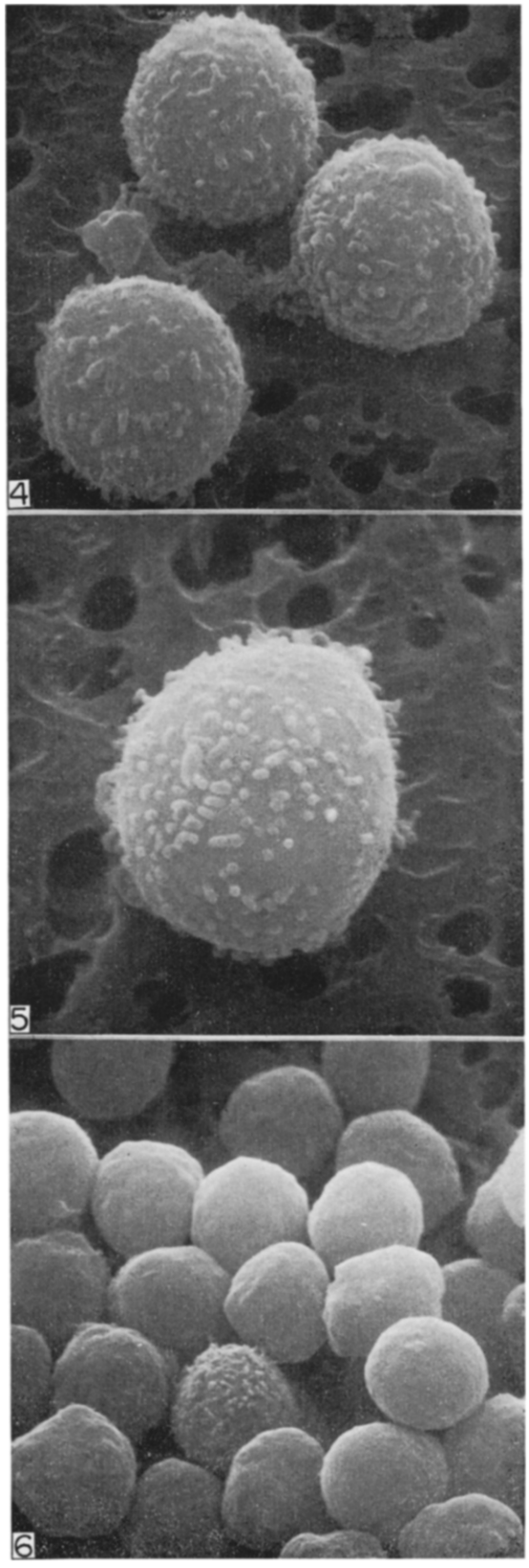


Fig. 4. CLL lymphocytes with moderate numbers of microvilli. Two thrombocytes are also present (Mag. × 5670).

Fig. 5. Lymphocyte from another CLL patient showing many stub-like projections (Mag. × 8100).

Fig. 6. CLL lymphocytes having smooth surface. One cell displays many microvilli (Mag. × 4050).

(to face page 200)

The surface morphology of cells may depend on the preparatory procedures used. Thus, the attachment of live cells to the filter membranes prior to fixation alters their surface structure [24]. In the present study, the lymphocytes have therefore been first fixed in suspension and then layered on the filters. Although the adherence of these prefixed cells is reduced [24], there is no evidence as yet that the resulting cell loss is selective. Moreover, the morphological features of the cells may be influenced by the osmotic pressure of the fixative solution [25] or by some of the methods used to collect and separate the lymphocytes [18].

It is now well established that the majority of blood lymphocytes from most patients with CLL bear membrane-bound immunoglobulins which are restricted to one heavy and light chain, usually IgM κ or IgM λ, suggesting a monoclonal proliferation of the leukaemic cells [6, 8, 9]. This concept is further supported by our morphological findings. Thus, in contrast to normal cells, CLL lymphocytes from the individual patients represent a rather homogeneous population with regard to their surface architecture. Those single lymphocytes which differ in their surface morphology from the dominating (leukaemic) cells most likely belong to the residual population of normal lymphocytes. In most cases the predominant cell type is characterized either by a moderately or by a markedly villous surface. The presence of microvilli on leukaemic lymphocytes has recently also been described by Polliack *et al.* [5, 18] and Catovsky *et al.* [26]. However, despite the uniformity of B cell-like membrane markers the spectrum of the morphological features may vary from patient to patient and striking differences may exist in the surface architecture of CLL lymphocytes. Thus, as demonstrated in the present study, in single cases with B-type CLL the lymphocytes are completely smooth without obvious projections. Since all cells have been prepared under standardized conditions, this morphological diversity appears not to result from variations in the technique, but rather seems to reflect membrane alterations and/or different stages of differentiation of the leukaemic cells.

The abnormality of CLL lymphocytes is further indicated by the observation that their diameter is reduced in comparison with the corresponding normal cell type. Previous planimetric measurements have shown that this diminution of cell size is attributable to a decreased cytoplasmic content [10–12]. It should be mentioned that the diameters of both normal and CLL lymphocytes at scanning electron microscopy are smaller than those obtained at light or transmission electron microscopy. This finding may be caused by a certain degree of cell shrinkage resulting from the critical point drying procedure [24, 27] or by osmotic effects during fixation [25].

Evidence from our findings suggests that the surface morphology of B cell-like CLL lymphocytes cannot be used as a typical example for that of normal B cells and that scanning electron microscopy does not contribute to the determination of the T or B cell nature of leukaemic cells. Further studies are necessary to relate certain patterns of surface structure to the clinical course of the disease and to define possible variations of the morphological features which may occur during progression of the disease or as a consequence of therapy.

Acknowledgements—We are grateful to Miss H. Metzger and Mrs. M. Funke for excellent technical assistance.

REFERENCES

1. M. Bessis, *Living Blood Cells and their Ultrastructure.* (Edited by M. Bessis) Springer Verlag, Berlin-Heidelberg-New York (1973).
2. J. A. Clarke and A. J. Salsbury, Surface ultramicroscopy of human blood cells. *Nature (Lond.)* **215,** 402 (1967).
3. L. W. McDonald and T. L. Hayes, Correlation of scanning electron microscope and light microscope images of individual cells in human blood and blood clots. *Exp. mol. Path.* **10,** 186 (1969).
4. T. W. Michaelis, N. R. Larrimer, E. N. Metz and S. P. Balcerzak, Surface morphology of human leukocytes. *Blood* **37,** 23 (1971).
5. A. Polliack, N. Lampen, B. D. Clarkson and E. De Harven, Identification of human B and T lymphocytes by scanning electron microscopy. *J. exp. Med.* **138,** 607 (1973).
6. A. C. Aisenberg, K. J. Bloch and J. C. Long, Cell-surface immunoglobulins in chronic lymphocytic leukemia and allied disorders. *Amer. J. Med.* **55,** 184 (1973).

7. G. Cohnen, W. Augener, A. Buka and G. Brittinger, Rosette-forming lymphocytes in normals and patients with malignant lymphomas. *Acta haemat. (Basel)* **51,** 65 (1974).
8. S. S. Fröland, J. B. Natvig and P. Stavem, Immunological characterization of lymphocytes in lymphoproliferative diseases. Restriction of classes, subclasses, and Gm allotypes of membrane-bound Ig. *Scand. J. Immunol.* **1,** 351 (1972).
9. J. L. Preud'homme and M. Seligmann, Surface bound immunoglobulins as a cell marker in human lymphoproliferative diseases. *Blood* **40,** 777 (1972).
10. G. Cohnen, S. D. Douglas, E. König and G. Brittinger, Pokeweed mitogen response of lymphocytes in chronic lymphocytic leukemia: A fine structural study. *Blood* **42,** 591 (1973).
11. R. Shrek, Ultrastructure of blood lymphocytes from chronic lymphocytic and lymphosarcoma cell leukemia. *J. nat. Cancer Inst.* **48,** 51 (1972).
12. H. R. Schumacher, T. K. Maugel and K. D. Davis, The lymphocyte of chronic lymphatic leukemia. I. Electron microscopy-onset. *Cancer (Philad.)* **26,** 895 (1970).
13. S. D. Douglas, G. Cohnen, E. König and G. Brittinger, Lymphocyte lysosomes and lysosomal enzymes in chronic lymphocytic leukemia. *Blood* **41,** 511 (1973).
14. P. J. L. Holt, S. G. Pal, D. Catovsky and S. M. Lewis, Surface structure of normal and leukaemic lymphocytes. I. Effects of mitogens. *Clin. exp. Immunol.* **10,** 555 (1972).
15. C. Huber, G. Michlmayr, H. Braunsteiner and H. Huber, Redistribution of immunoglobulin determinants on human lymphocytes in lymphoproliferative disorders. *Europ. J. Cancer* **10,** 517 (1974).
16. S. Kornfeld, Decreased phytohemagglutinin receptor sites in chronic lymphocytic leukemia. *Biochim. biophys. Acta* **192,** 542 (1969).
17. H.-D. Menne and H.-D. Flad, Membrane dynamics of HL-A-anti-HL-A-complexes of human normal and leukaemic lymphocytes. *Clin. exp. Immunol.* **14,** 57 (1973).
18. A. Polliack and E. De Harven, Surface features of normal and leukemic lymphocytes as seen by scanning electron microscopy. *Clin. Immunol. Immunopath.* **3,** 412 (1975).
19. P. S. Lin, D. F. H. Wallach and S. Tsai, Temperature-induced variations in the surface topology of cultured lymphocytes are revealed by scanning electron microscopy. *Proc. nat. Acad. Sci. (Wash.)* **70,** 2492 (1973).
20. K. Porter, D. Prescott and J. Frye, Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. *J. Cell Biol.* **57,** 815 (1973).
21. M. M. Kay, B. Beloiradsky, K. Yee, J. Vogel, D. Butcher, J. Wybran and H. H. Fudenberg, Cellular interactions: Scanning electron microscopy of human thymus-derived rosette-forming lymphocytes. *Clin. Immunol. Immunopath.* **2,** 301 (1974).
22. P. S. Lin, A. G. Cooper and H. H. Wortis, Scanning electron microscopy of human T-cell and B-cell rosettes. *New Engl. J. Med.* **289,** 548 (1973).
23. A. Polliack, S. M. Fu, S. D. Douglas, Z. Bentwich, N. Lampen and E. De Harven, Scanning electron microscopy of human lymphocyte-sheep erythrocyte rosettes. *J. exp. Med.* **140,** 146 (1974).
24. B. Wetzel, G. B. Cannon, E. L. Alexander, B. W. Erickson Jr. and E. W. Westbrook, A critical approach to the scanning electron microscopy of cells in suspension. In *Scanning Electron Microscopy (Part III)*. Proceedings of the Workshop on Advances in Biomedical Applications of the SEM, p. 581. IITRI, Chicago (1974).
25. P. B. Bell, Jr., U. Brunk, P. Collins, N. Forsby and B.-A. Fredriksson, SEM of cells in culture: Osmotic effects during fixation. In *Scanning Electron Microscopy (Part I)*. Proceedings of the Eighth Annual Scanning Electron Microscope Symposium, p. 380. IITRI, Chicago (1975).
26. D. Catovsky, B. Frisch and S. Van Noorden, B, T and "null" cell leukaemias. Electron cytochemistry and surface morphology. *Blood Cells* **1,** 115 (1975).
27. A. Boyde, R. A. Weiss and P. Vesely, Scanning electron microscopy of cells in culture. *Exp. Cell Res.* **71,** 313 (1972).

Europ. J. Cancer Vol. 12, pp. 203–210. Pergamon Press 1976. Printed in Great Britain

The Effect of BCG on B_{16} Mouse Melanoma: A Comparison of Routes of Administration on Tumour Growth at Different Anatomical Sites

J. W. PROCTOR, B. G. AUCLAIR and M. G. LEWIS
McGill University Cancer Research Unit, McIntyre Medical Sciences Building, 3655 *Drummond Street, Montreal, P.Q., Canada H3G 1Y6.*

Abstract—*A comparison was made of the effects of BCG administered by different routes on the growth of B_{16} mouse melanoma at varying anatomical locations. A standard single dose (0·015 mg/g) of BCG was given intra-orally i.o.), intradermally (i.d.), subcutaneously (s.c.), intraperitoneally (i.p.), or intravenously (i.v.) at different time points before or after, either s.c., i.p., or i.v. injection with B_{16} mouse melanoma.*

In general, if BCG was given 10 days before tumour challenge (immunoprophylaxis) a significant resistance to tumour growth was observed, regardless of the site of tumour challenge, or the route of BCG administration. This effect was less obvious when BCG was given after tumour implantation (immunotherapy), and unexpectedly BCG was more effective against an s.c. challenge with higher, rather than lower numbers of tumour cells. In one experiment, suppression of bloodborne metastatic spread was observed in the absence of detectable effects against s.c. tumour growth. Facilitation of tumour growth in the lung occurred when s.c. BCG and i.v. tumour were given on the same day and in general this dose of BCG given s.c. or i.o. were less effective than BCG given by other routes.

Thus, in the B_{16} mouse melanoma system, immunization with BCG can be beneficial to the host animal, the outcome depending in part on the route and timing of administration of the BCG.

INTRODUCTION

A NUMBER of previous reports on a wide variety of animal tumour models indicate that BCG, like other adjuvants is more effective, firstly when given before [1, 2] rather than after [3, 4] tumour implantation and secondly when injected into the tumour itself, rather than when administered at sites distant from it [5–7]. However, i.d. immunization with BCG, particularly into tumour deposits, produces a variety of side effects in human cancer patients [8, 9]. In an attempt to avoid these side effects, some patients are now being treated with oral BCG, and to date no harmful side effects have been observed [10]. The present study was designed, therefore, to compare the effects of a standard single dose of BCG given at various time points by different routes, including the oral route, on tumour growth following s.c., i.p., or i.v. injection of tumour cells.

Accepted 17 September 1975.

MATERIAL AND METHODS

Mice

Conventionally housed 8 week old inbred male C57Bl/6J mice were obtained from Jackson Laboratories and maintained on rat cake and tap water *ad libitum* in all experiments.

Tumour

The B_{16} melanoma which arose spontaneously in the above strain, and which has been

maintained by long-term transplantation, metastasizes reproducibly to the lung in 100% of animals when grown as an s.c. implant in the right hind limb in this laboratory.

BCG

A standard dose of 0·015 mg/g total body weight of BCG (Institut de Microbiologie et Hygiene, Montreal) was injected under ether anaesthesia, in a 0·1 ml volume of saline, either i.p., s.c., or i.d. in the left hind limb, i.o. via a fine feeding tube into the stomach, or i.v. via a lateral tail vein. In each of the experiments to be described, groups of 5–10 mice were immunized at day −10, day 0, or day +10.

Tumour challenge

A single suspension of greater than 95% viability, as assessed by phase microscopy, was obtained by enzyme digestion of small pieces of tumour incubated at 37°C for half an hour, in 25 ml of MEM medium containing 0·13 g of trypsin, 0·15 g of collagenase and a trace amount of DNA'ase (all Type 1 Sigma, England). The resulting single cell suspension was washed thoroughly and adjusted to relevant concentrations for injection. On Day 0 in each experiment, either 10^5, 10^4, or 10^3 tumour cells were injected in 0·2 ml volumes, i.p., i.o., or i.v., or in 0·025 ml volumes s.c., or i.d. using a 0·25 ml Hamilton syringe.

RESULTS

The effect of BCG of s.c. tumour growth (Table 1)

Groups of 5 mice were immunized with BCG on day −10, day 0, and day +10, by the routes previously described, and together with a control group of 8 mice, were injected s.c. into the right hind limb with either 10^5 or 10^4 tumour cells on day 0. The animals were killed on day +30, the tumours carefully dissected and weighed to the nearest 0·1 g.

The leg tumour weights from animals injected with 10^5 cells 10 days after BCG immunization were significantly lower following treatment i.p., s.c., i.v., or i.o. than the controls or those treated i.d. Those groups challenged with 10^4 cells showed protection only following i.p. and i.v. immunization.

Of the groups immunized at day 0 significantly lower tumour weights were found in

Table 1. The effect of BCG immunization on subcutaneous tumour growth

No. of tumour cells injected s.c. on Day 0	Day of BCG immunization	Mean weight (grams) tumour 30 days after subcutaneous injection: Normal controls 8 mice/group	Route of BCG immunization: i.p.	i.d.	i.v.	i.o.	s.c. (C)	s.c. (I)
					5 mice/group			
	−10		3.6[b] (1.8–4.3)	4.5 (3.0–5.4)	1.6[a] (0.9–2.2)	3.4[b] (3.1–3.6)	2.5[b] (1.5–3.9)	N.T.
10^5	0	4.8* (3.6–5.6)**	4.0 (3.8–4.6)	5.1 (3.6–6.2)	2.9[b] (2.3–2.7)	4.7 (3.8–5.6)	3.9 (3.0–5.1)	N.T.
	+10		3.1[b] (2.5–4.3)	3.1[b] (1.7–4.9)	3.5[b] (3.1–4.3)	4.6 (3.4–5.6)	3.7[c] (3.1–4.7)	N.T.
	−10		2.5[a] (1.4–2.9)	3.1 (2.4–3.7)	1.8[b] (1.1–2.5)	2.8 (1.9–3.9)	3.0 (2.1–4.7)	N.T.
10^4	0	3.6 (2.6–4.5)	2.9 (2.3–3.7)	2.6 (1.9–3.4)	3.4 (2.6–4.6)	2.7 (1.9–3.6)	3.5 (2.0–4.6)	N.T.
	+10		3.6 (2.8–4.3)	3.6 (2.5–4.4)	3.1 (2.4–3.8)	3.2 (2.1–4.6)	3.6 (2.7–4.3)	N.T.
		10 mice/group			10 mice/group			
	−10		1.9 (0.6–3.1)	1.5 (0.4–3.0)	1.5 (0.2–1.7)	2.4 (1.1–3.7)	2.1 (0.9–3.7)	1.2 (0.2–2.6)
10^3	0	1.7 (0.6–2.3)	2.1 (1.0–3.3)	1.8 (0.8–2.9)	1.8 (0.4–2.6)	2.0 (1.2–2.6)	1.7 (0.7–2.8)	1.8 (0.7–3.4)
	+10		1.5 (0.2–2.9)	1.8 (0.5–2.7)	1.4 (0.6–2.9)	2.4 (1.1–4.4)	1.9 (0.5–3.4)	1.3 (0.4–2.3)

s.c. (C) Subcutaneous immunization in contralateral limb to tumour
s.c. (I) Subcutaneous immunization in ipsilateral limb to tumour

* Single figures represent mean values
** Figures in brackets represent range of values

Statistical analysis (Mann–Whitney)

[a] $P < 0.001$
[b] $P < 0.01$ Suppression compared to normal controls
$P < 0.05$

only that group immunized i.v. and challenged with 10^5 tumour cells.

Mice immunized i.p., i.d., s.c., or i.v. on day +10 after injection with 10^5 cells were significantly protected compared to those immunized by the oral route or to the control animals. No effect was seen in the immunized groups challenged with 10^4 tumour cells.

In a second similar experiment, 10^3 tumour cells were injected in the right hind limb of groups of 10 animals immunized with BCG as above, together with a control group of 15 animals; a further group of 10 animals was immunized s.c. with BCG in the same limb as the tumour, though at a site separate from the tumour itself. These animals were killed on day +30, the tumours dissected and weighed, and the lungs carefully examined and the spontaneous metastases counted.

No significant protection against s.c. tumour was observed in any of the groups irrespective of the route and timing of BCG administration. There was, however, a significant reduction in bloodborne metastatic spread irrespective of the time of BCG immunization or the site of BCG administration, except in all those groups immunized with oral BCG and that immunized i.d. 10 days before tumour challenge (see Fig. 1).

The effects of BCG on the outgrowth of lung nodules following i.v. injection (Tables 2 and 3)

Groups of 5 animals were given BCG by the five routes described previously on day −10, day 0, and day +10, and these groups together with a control group of 8 animals were injected with either 10^5 or 10^4 tumour cells via the lateral tail vein on day 0. The animals were killed on day 34, and bled out, the lungs carefully dissected and the tumour nodules counted.

The incidence of outgrowth of lung tumour nodules was 100% in virtually all the groups. There were significantly fewer tumour nodules in all groups immunized at day −10 irrespective of the number of tumour cells injected at day 0 except in those groups immunized s.c. and challenged with 10^4 tumour cells, and the group immunized i.o. and challenged with 10^5 tumour cells.

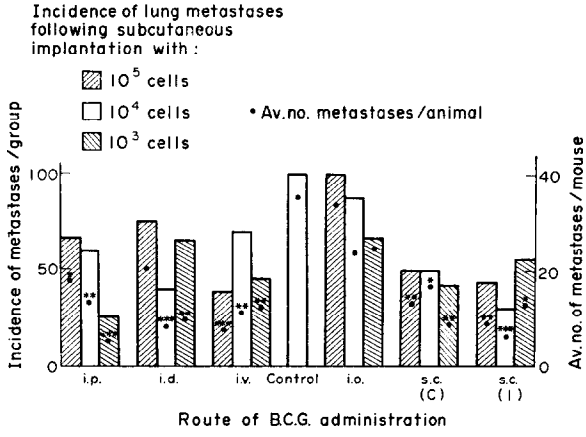


Fig. 1. The effect B.C.G. on spontaneous blood borne metastatic spread to the lung from a subcutaneous tumour.

In contrast, when animals were immunized on the day of tumour inoculation only the groups immunized i.v. and challenged with 10^5 cells and that immunized i.d. and challenged with 10^4 tumour cells showed a significant degree of protection compared to controls. Of interest was the finding of significant facilitation of lung tumour outgrowth in both of the groups immunized s.c. on day 0.

When animals were immunized 10 days after tumour injection, only the group immunized i.d. and challenged with 10^4 cells were found to have significantly fewer lung tumour nodules than the control group.

Table 2. The effect of BCG immunization on intravenously injected tumour cells

No. of tumour cells injected i.v. on Day 0	Day of BCG immunization	Incidence of lung tumour growth: Untreated controls	Route of BCG administration: i.p.	i.d.	i.v.	i.o.	s.c.
10^5	-10	8/8	4/5	5/5	5/5	5/5	5/5
	0		5/5	5/5	5/5	5/5	5/5
	+10		5/5	5/5	5/5	5/5	5/5
10^4	-10	8/8	4/5	5/5	5/5	5/5	5/5
	0		5/5	5/5	5/5	5/5	5/5
	+10		5/5	5/5	5/5	5/5	5/5
10^3	-10	14/15	5/10	5/10	6/10	6/10	7/10
	0		7/10	10/10	8/10	9/10	10/10
	+10		10/10	10/10	9/10	10/10	10/10

Table 3. The effect of BCG immunization on intravenously injected tumour cells

No. of tumour cells injected i.v. on Day 0	Day of BCG immunization	Normal controls	Mean number of lung tumour nodules: Route of BCG immunization i.p.	i.d.	i.v.	i.o.	s.c.
10^5	-10	37.5* (17-112)**	7.0[a] (0-13)	8.3[a] (8-10)	5.2[a] (1-9)	15.5 (3-40)	11.0[b] (6-15)
	0		21.3 (7-38)	34.0 (5-54)	3.5[a] (2-5)	28.5 (20-40)	63.5[d] (25-122)
	+10		19.6 (13-29)	21.6 (15-29)	27.8 (7-49)	31.8 (12-52)	43.0 (10-111)
10^4	-10	17.2 (5-48)	3.4[a] (0-6)	8.5[b] (6-11)	12.2[c] (8-16)	9.6[c] (2-18)	15.4 (12-21)
	0		21.0 (7-48)	13.0 (5-22)	16.8 (10-36)	13.0 (7-23)	31.7[d] (25-38)
	+10		22.0 (18-26)	11.0[c] (4-15)	18.2 (10-23)	20.0 (12-37)	15.2 (6-28)
10^3	-10	2.6 (0-10)	0.7[b] (0-2)	0.7[b] (0-2)	1.2[c] (0-3)	1.4 (0-6)	1.8 (0-6)
	0		1.7 (0-5)	2.1 (1-5)	3.4 (0-9)	2.7 (0-6)	6.7[d] (1-15)
	+10		2.6 (1-4)	2.3 (1-6)	2.5 (0-6)	2.8 (1-6)	2.3 (1-6)

* Single figures represent mean values
** Figures in brackets represent the range of values

Statistical analysis (Mann–Whitney)

[a] $P < 0.001$
[b] $P < 0.01$ Suppression compared to normal controls
[c] $P < 0.05$
[d] $P < 0.05$ Facilitation compared to normal controls

Table 4. The effect of BCG immunization on intraperitoneal tumour growth

No. of tumour cells injected i.p. on Day 0	Day of BCG immunization	Normal controls 35 mice/group	Survival in days: Route of BCG immunization i.p.	i.d.	i.v. (5 mice/group)	i.o.	s.c.
10^5	-10	18.1* (15-21)**	22.8[a] (21-25)	17.8 (17-19)	21.4[b] (21-22)	16.6 (16-17)	17.0 (15-19)
	0		16.2 (15-17)	18.0 (17-21)	17.0 (16-18)	16.2 (15-17)	17.4 (16-19)
	+10		18.2 (18-19)	18.0 (17-19)	16.4 (15-17)	17.4 (16-18)	17.8 (17-19)
10^4	-10	20.2 (17-24)	26.4[a] (24-28)	20.4 (19-22)	22.8[b] (22-25)	20.2 (18-22)	20.2 (17-22)
	0		19.0 (18-21)	19.2 (16-21)	19.0 (16-21)	19.0 (18-20)	19.2 (16-22)
	+10		18.8 (16-22)	21.8 (19-24)	20.2 (18-23)	19.4 (16-21)	19.0 (18-20)
10^3	-10	20 mice/group 28.1 (26-30)	39.4[a] (29-56)	30.2[c] (28-35)	36.3[a] (31-49)	30.2[c] (27-33)	30.5[c] (27-43)
	0		32.7[b] (28-45)	27.7 (26-32)	29.1 (26-34)	28.9 (24-33)	28.6 (26-35)
	+10		29.2 (25-36)	28.3 (24-33)	29.1 (27-34)	28.5 (25-33)	28.9 (24-34)

* Single figures represent mean values
** Figures in brackets represent range of values

Statistical analysis (Mann–Whitney)

[a] $P < 0.001$
[b] $P < 0.01$ Suppression compared to normal controls
[c] $P < 0.05$

In a second experiment, similarly immunized groups of 10 animals and a control group of 15 animals were challenged with 10^3 tumour cells i.v. and killed on day 29 when the lung tumour nodules were counted.

Of the groups immunized 10 days before tumour challenge, significant protection resulted from BCG administered i.p., i.d., or i.v. No obvious differences was detected between experimental groups immunized i.o. or s.c. and the control group. Animals immunized on day 0 or day +10 were similarly not protected by BCG given by any of the routes used. As in the previous experiment, BCG injected s.c. at day 0 produced significant facilitation of the outgrowth of lung tumour nodules.

The effects of BCG on i.p. tumour growth (Table 4)

Groups of 5 animals were immunized with BCG by the five routes described previously on day −10, day 0, or day +10 and challenged together with control groups of 35 animals with either 10^5 or 10^4 tumour cells i.p. on day 0.

The day of death was recorded and survival times used as an index of protection by BCG.

All control groups died within a 5–8 day time span and of those groups challenged with 10^5 or 10^4 tumour cells, only those immunized i.p. or i.v. 10 days previously showed a significant degree of protection.

Those groups immunized on day 0 or day +10 by any route developed no resistance to challenge.

In a second similar experiment, groups of 10 immunized animals and a group of 20 control animals were challenged with 10^3 tumour cells. All the groups injected with 10^3 tumour cells 10 days after immunization lived significantly longer than the controls and those immunized i.p. or i.v. showed a greater degree of protection than those immunized by other routes.

With one exception, the group of animals immunized i.p. at day 0, no significant alteration in survival times of any group was detected following immunization either at day 0 or at day +10, compared to the controls.

DISCUSSION

These observations are in agreement with several previous studies [1–4] and demonstrate that in general immunoprophylaxis is better than treatment at the time of, or following tumour implantation, irrespective of the route of BCG administration or the site of tumour challenge.

Also, regardless of the site of tumour challenge, i.o. or s.c. administration of this dose was less effective than i.p. or i.v. administration. Intradermal immunization produced an intermediate effect. These findings are reminiscent of those of Rosenthal [11], who showed that when BCG was given to guinea pigs i.v. or by aerosol spray, a more widespread systemic stimulation was observed histologically than when the i.o. or s.c. routes were used. Intradermal administration produced an intermediate effect.

The results with oral BCG were disappointing as in the human situation this route has not led to the various side effects reported following intratumoral or i.d. administration of BCG [8, 9]. It should be stressed, however, that a single arbitrary dose of BCG was used at one time point in these experiments. It is quite conceivable that relatively larger doses given over a prolonged period of time would be needed to produce a systemic effect by this route, as it is likely that many of the viable organisms are destroyed in the gut, and that others could pass through the intestine without exerting an effect. However, if larger i.o. doses did produce an effect systemically, it is entirely possible that the incidence of side effects might also increase, as the vascular drainage of the gut is to the liver.

The superior effects of i.p. and i.v. BCG administration are of doubtful significance to the human situation, as the incidence of serious side effects using these routes of administration could be anticipated to increase greatly. However, should non-bacterial adjuvants such as Glucan [13] prove as useful as BCG in its antitumour effects and should it be possible to give its i.p. or i.v., this might constitute a more successful approach than the more conventional routes of administration of such an agent.

Our finding that only in the case of s.c. tumour was an effect produced by immunization 10 days after tumour implantation was unexpected. The lung and peritoneal cavities are well endowed with macrophages and/or histiocytes in comparison to the s.c. tissue and it might be expected that BCG therapy is more effective against tumour at these two sites rather than at s.c. sites.

A significant degree of protection was evident in the lung in the presence of s.c. tumours as suppression of spontaneous metastatic spread occurred in the absence of an effect on the s.c. tumours themselves. These

metastases do not start to develop until 15–20 days after tumour implantation (Proctor, unpublished results) and thus in this experiment some 5–10 days after the last BCG administration.

Thus the intial implantation of the metastatic tumour cells occurred at a time when the immune response to the BCG could be expected to be well developed and at a time point when the lung was shown to be protected in previous experiments, e.g., after immunization with BCG ten days prior to i.v. injection.

Secondary tumour deposits are thought to give rise to further metastases by tertiary spread [14]. An inhibition of tertiary spread, even in the absence of a significant effect on existing secondary lesions, might constitute an important contribution to increasing the longevity of the patient.

There was no obvious difference in our experiments between the effects of s.c. BCG immunization in the ipsilateral limb compared to immunization in the limb contra-lateral to the tumour implant. It should be noted, however, that care was taken to inject the BCG at a separate site from the tumour when using the ipsilateral limb.

The facilitation of lung tumour growths observed when the tumour was injected on the day of s.c. immunization is in agreement with observations made by others [15–18]. This could be ascribed to the phenomenon of immunostimulation [19], as suggested by Chee and Bodurtha [18] to explain their findings, or to antigenic competition [20].

The putative mechanisms by which a non-specific stimulation of the reticulo endothelial system might lead to tumour specific effects revolve around the role of the macrophage in specific immunity. This class of cells have been implicated in the processing of antigen [21] and might clear colloidal particles such as antigen antibody complexes from the bloodstream as suggested by Proctor *et al.* [22]. Such complexes have been postulated to abrogate the effector arm of the immune response [23]. Further, macrophages have been demonstrated to lyse tumour cells *in vitro* non-specifically following specific arming [24]. All such mechanisms might be aided by stimulation of the reticulo endothelial system and thus augment a specific antitumour immune response.

There was some evidence, albeit indirect, that specific antitumour effects occurred in one of our experiments. Namely the finding that immunization with BCG prior to s.c. challenge was effective by most of the routes used against implantation with 10^5 tumour cells compared to only a negligible effect on local tumours induced by 10^3 cells. A purely nonspecific effect of BCG could be expected to produce the opposite result, as indeed was seen in the case of i.p. tumour growth, where only in the groups challenged with 10^3 tumour cells was immunization by the s.c. and i.d. routes effective while there was no such effect against challenges with 10^5 or 10^4 tumour cells.

Intravenous injection of tumour cells leads in the B_{16} model [25] to a rapid holdup in the lungs with a rapid destruction of the majority of tumour cells within 48 hr. A similar situation has been observed by us in a rat sarcoma model: when radio-labelled tumour cells are injected i.v., 90% of them are destroyed within 24 hr while a less rapid destruction occurs following intramuscular (i.m.) injection where 90% of the injected cells die within 48 hr (Proctor and Rudenstam, in preparation).

A rapid rise in circulating tumour antigen levels occurs within the bloodstream of rats during the first 24–48 hr following i.m. implantation of this particular sarcoma [26], and it is likely that injection of 10^5 cells would produce higher levels of circulating antigen than would 10^3 tumour cells injected at the same site. Thus, it is conceivable that following BCG immunization increased numbers of macrophages could process relatively more antigen after implantation with 10^5 cells leading to better lymphocyte stimulation and thus a more effective specific antitumour immunity than would be produced following implantation with the lower cell dose.

Previous work [22] has demonstrated that spontaneous metastatic spread could be suppressed by immunization with a combination of a specific number of irradiated cells, which by themselves facilitated such spread, with a dose of BCG which was ineffective on its own. It is unlikely that these effects were mediated solely by non-specific antitumour mechanisms.

While the results from the study, at best only demonstrate a modest effect of BCG, this must be considered in the light of the fact that only a single immunization was used at arbitrary time points. The dose of BCG used is critical in terms of suppressing or facilitating growth from a standard challenge with tumour [18], in much the same way as the effect of immunization with irradiated tumour cells on spontaneous metastatic spread has been shown to be critically dependent on the number of tumour cells used, and on the amount of tumour present [22, 27]. More recent work (Proctor *et al.*, in preparation) has shown that

different doses of BCG, Glucan, Levamisole or irradiated tumour cells produce variable effects on spontaneous bloodborne metastatic spread from the B_{16} model, and that these effects are also dependent on the amount of tumour present. Thus, the arbitrary dosage of BCG used in this study was not likely to be optimal.

Our results support the concept that the effect of BCG varies considerably depending on the route of administration, and that in addition to nonspecific effects, tumour specific effects may be produced.

Acknowledgements—This work was supported by grants from the National Cancer Institute of Canada and from the Cancer Research Society Inc., Quebec.

We thank Miss Leontyna Stokowski for technical support, and Dr. Martin Jerry, M.D., Ph.D. for help in preparing the manuscript.

REFERENCES

1. E. N. Halpern, G. Biozzi, C. Stiffel and D. Mouton, Effect de la stimulation du système réticulo-endothélial par l'inoculation du bacille de Calmette–Guérin sur le développement de l'epithelioma atypique T-8 de Guérin chez le rat. *C.R. Soc. Biol. (Paris)* **153,** 919 (1959).
2. L. J. Old, D. A. Clark and B. Benacerraf, Effect of Bacillus Calmette–Guerin infection on transplanted tumors in the mouse. *Nature (Lond.)* **184,** 291 (1959).
3. I. Parr, Response of syngeneic murine lymphomas to immunotherapy in relation to the antigenicity of the tumor. *Brit. J. Cancer* **26,** 174 (1972).
4. S. C. Bansal and H. O. Sjögren, Effects of BCG on various facets of the immune response against polyoma tumors in rats. *Int. J. Cancer* **11,** 162 (1973).
5. R. W. Baldwin and M. V. Pimm, Influence of BCG infection on growth of 3-methyl cholanthrene-induced rat sarcomas. *Europ. J. clin. biol. Res.* **16,** 875 (1971).
6. B. Zbar, I. D. Bernstein, G. L. Bartlett, M. G. Hanna, Jr. and H. J. Rapp, Immunotherapy of caner: Regression of intradermal tumours and prevention of growth of lymph node metastases after intralesional injection of living *Myocabacterium bovis. J. nat. Cancer Inst.* **49,** 119 (1972).
7. R. C. Bast, Jr., B. Zbar, T. Borsos and H. Rapp, BCG and cancer. *New Engl. J. Med.* **290,** 1413 (1974).
8. A. Z. Bluming, BCG: A note of caution. *New Engl. J. Med.* **289,** 860 (1973).
9. F. C. Sparks, M. J. Silverstein, J. S. Hunt, G. M. Haskell, Y. H. Pilch and D. L. Morton, Complications of BCG immunotherapy in patients with cancer. *New Engl. J. Med.* **289,** 827 (1973).
10. M. G. Lewis, L. M. Jerry and H. Shibata, Prospective of specific immunotherapy. in *Proc. of 11th Int. Cancer Cong. Florence.* October, 1974 (in press).
11. S. R. Rosenthal, BCG and the lympho-reticulo-endothelial system. *Nat. Cancer Inst. Monogr.* **39,** 91 (1973).
12. S. J. Riggi and N. R. DiLusio, Identification of a reticulo endothelial stimulating agent in zymosan. *Amer. J. Physiol.* **200,** 297 (1961).
13. R. A. Willis, In: *The Spread of Tumours in the Human Body.* Butterworths, London (1952).
14. J. Stjernswärd, Immune status of the primary host toward its own methylcholanthrene-induced sarcomas. *J. nat. Cancer Inst.* **40,** 13 (1968).
15. W. F. Piessens, F. L. Lachapelle, N. Legros and J.-C. Heuson, Facilitation of rat mammary tumor growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).
16. P. Lemonde, Dual effect of BCG on tumour and tumour immunity. *Proc. Amer. Assoc. Cancer Res.* **15,** abstract 163 (1974).
17. D. D. Chee and A. J. Bodurtha, Facilitation and inhibition of B_{16} melanoma by BCG *in vivo* and by lymphoid cells from BCG-treated mice *in vivo. Int. J. Cancer* **14,** 137 (1974).
18. R. T. Prehn, The immune reaction as a stimulator of tumor growth. *Science* **176,** 170 (1972).
19. P. Liacopoulos, J. Couderc and M. F. Gille, Competition of antigens during induction of low zone tolerance. *Europ. J. Immunol.* **1,** 359 (1971).
20. E. R. Unanue, B. R. Askonas and A. C. Allison, A role of macrophages in the stimulation of immune responses by adjuvants. *J. Immunol.* **103,** 71 (1969).
21. J. Proctor, C. M. Rudenstam and P. Alexander, Increased incidence of lung metastases following treatment of rats bearing hepatomas with irradiated tumour cells and the beneficial effect of *Corynebacterium parvum* in this system. *Biomedicine (Express)* **19,** 248 (1973).

22. R. W. Baldwin, M. R. Price and R. A. Robins, Blocking of lymphocyte mediated cytotoxicity for rat hepatoma cells by tumour specific antigen-antibody complexes. *Nature New Biol.* **238,** 185 (1972).
23. R. Evans and P. Alexander, Cooperation of immune lymphoid cells with macrophages in tumour immunity. *Nature (Lond.)* **228,** 620 (1970).
24. I. J. Fidler, Metastasis: Quantitative analysis of distribution and fate of tumor emboli labelled with ^{125}I-5-iodo-2′-deoxyuridine. *J. nat. Cancer Inst.* **45,** 773 (1970).
25. D. M. P. Thomson, V. Sellens, S. Eccles and P. Alexander, Radioimmunoassay of tumour specific transplantation antigen of a chemically induced rat sarcoma: Circulating soluble tumour antigen in tumour bearers. *Brit. J. Cancer* **28,** 277 (1973).
26. R. R. Vanwijck, E. A. Godrick, H. G. Smith, J. Goldweitz and R. E. Wilson, Stimulation or suppression of metastases with graded doses of tumor cells. *Cancer Res.* **31,** 1559 (1971).

Europ. J. Cancer Vol. 12, pp. 211–218. Pergamon Press 1976. Printed in Great Britain

Purification of a Protein Inhibitor of DNA Synthesis in Cells of Hepatic Origin*

B. VINET and W. G. VERLY

Department of Biochemistry, University of Montreal, Montreal, Canada
Biochimie, Faculté des Sciences, Université de Liége, Belgique.

Abstract—*A 40,000 dalton protein has been isolated from rabbit liver which inhibits DNA synthesis in Novikoff cells and isolated hepatocytes. It has little action on cells which have not a hepatic origin that have been tested.*

SAETREN [1] found in crude liver extract a high molecular weight factor which reduced the mitotic index specifically in regenerating liver. Verly *et al.* [2] have partially purified from rabbit liver a low molecular weight peptide which decreased the incorporation of thymidine into DNA of regenerating rat liver slices; the purified factor was specific for cells of hepatic origin. It was later shown by Deschamps and Verly [3] to really inhibit DNA synthesis, and by Simard *et al.* [4] to block the G_1-S transition specifically in hepatocytes, both *in vitro* and *in vivo*.

The purified inhibitor of Deschamps and Verly [3] had an action on thymidine incorporation into DNA of Novikoff cells. Although this action was lower than that on regenerating rat liver slices, we decided to use it as a much simpler assay in an attempt to completely purify the hepatic inhibitor. Our endeavour was successful: a pure inhibitor of DNA synthesis in Novikoff cells has been isolated, but this inhibitor is a 40,000 dalton protein with very little action on regenerating rat liver slices. It has however an action on thymidine incorporation into DNA of cultured hepatocytes and very little action on the cultured cells of non-hepatic origin that were tested.

Accepted 1 September 1975.
*This work was supported by grants from the National Cancer Institute of Canada and from the Fonds Cancérologique de la Caisse générale d'Epargne et de Retraite Belge. B.V. was a recipient of M.R.C. studentship.

MATERIAL AND METHODS

Assay on Novikoff cells

About 50 millions Novikoff cells (Children Cancer Research Foundation, Boston) were injected intraperitoneally to a 150–175 g male Sprague–Dawley rat and the ascitic fluid collected one week later. This fluid was diluted with 4 times its volume of Hanks' solution (Grand Island Biological Co.) and centrifuged at 120 *g* for 1 min; the sediment was washed several times with Hanks' solution to remove the red blood cells and finally suspended in Hanks' solution. The cells were counted with an hemacytometer.

To 0·7 ml of cell suspension (2 million cells) were added 0·1 ml of Hanks' solution containing 4 μCi of ^{3}H(methyl)-thymidine (New England Nuclear; 6 Ci/mmol) and the volume brought to 1 ml with various buffers containing the liver preparation (assay) or not (control). The incubation was carried out at 37°C for 1 hr under an O_2–CO_2 (95:5; v:v) atmosphere with gentle shaking; it was stopped by cooling at 0°C and adding 5 ml of cold 0·15 M NaCl containing 100 μg/ml of unlabelled thymidine (Baker Chemical Co.). The cell suspension was filtered on a fiber glass Reeve Angel, grade 934AH, filter of 24 mm dia; the filter was washed with 5 ml of 0·15 M NaCl containing 100 μg/ml of unlabelled thymidine, then three times with 5 ml of 10% trichloracetic acid also containing 100 μg/ml of unlabelled thymidine.

The filter was dried and introduced in a counting vial. After addition of 10 ml of toluene containing 4% of PPO and 0·1% of dimethyl-POPOP, the radioactivity was measured with a Packard Tri-Carb scintillation spectrometer; the results, corrected for background, are expressed in counts/min (c.p.m.) per 2 million cells.

Other cells and tissue slices

Ehrlich cells were a gift of Dr. V. Fredette from the Institut de Microbiologie, Laval-des-Rapides, Montreal. HeLa cells came from Dr. T. P. Brent, McGill Cancer Unit, Montreal, who received them, in 1963, from Dr. H. Harris, then at the John Innes Institute, Hertford, England.

About 10 million Ehrlich cells were inoculated intraperitoneally to 20–25 g male Swiss white mice and the ascitic fluid collected one week later. HeLa cells were cultured in Spinner flasks, under an atmosphere of air:CO_2 (95:5; v:v), in Gibco-199 medium supplemented with bicarbonate (2·2 g/l) and 5% horse serum; the cells were used after a 3-day incubation. Hepatocytes from dissociated liver of newborn rats (RLC) were cultured as monolayers by Dr. A. Simard of the Institut du Cancer de Montréal. All these cells were treated as the Novikoff cells and the assay on them carried out in exactly the same way.

The method used for the regenerating rat liver and other tissue slices has been described by Verly *et al.* [2], except that the incubation time was restricted to 1 hr.

Preparation of a crude liver extract

The liver of an adult, 5–7 pound albino male rabbit was homogenized in four parts of water at 4°C using a Potter–Elvehjem apparatus. After two successive centrifugations, one at 10,000 ***g*** for 2 hr (Sorvall RC-2B with rotor SS-34), the other, on the collected supernatant, at 105,000 ***g*** for 16 hr (Beckman L2-65B with rotor 30), the final supernatant was filtered through a Whatman no. 1 paper, the filtrate lyophilized and the resulting powder kept at −20°C.

Chromatographies on Sephadex G-200

Ten grams of Sephadex G-200 superfine (Pharmacia Fine Chemicals Co.) were soaked for 5 hr at 90°C, in 800 ml of water or 0·01 M NaCl, 0·005 M Tris–HCl, pH 7·4 buffer. Two 2·5 × 40 cm columns were then prepared and equilibrated at 4°C with either of the solvents. After application of the sample, the elution with the chosen solvent was at a rate of 30 ml/hr; 2·5 ml fractions were collected.

DEAE Sephadex chromatography

Five grams of Sephadex A-25 (Pharmacia Fine Chemicals Co.) were soaked in 500 ml of 0·05 M NaCl, 0·005 M Tris–HCl, pH 9·6, for 24 hr at room temperature. After two washings with 500 ml of the same buffer, the pH of the suspension was 7·8. A 1·3 × 15 cm column was then formed at 4°C on which the sample in 10 ml of 0·01 M NaCl, 0·005 M Tris–HCl, pH 7·8 buffer was placed. The elution was carried out with the same buffer increasing the NaCl concentration by steps: 20 ml at 0·05 M, 15 ml at 0·15 M, 15 ml at 0·3 M and 15 ml at 1 M. Fractions of 2·5 ml were collected.

Polyacrylamide gel electrophoresis

Gels containing 7·5% of acrylamide (w:v) were used and the electrophoresis carried out in Tris–glycine, pH 8·3 buffer following the method described by Zweig and Whitaker [5].

Protein concentration

It was measured by the technique of Folin [6] using bovine serumalbumin as standard.

Chromatography on Dowex-50

A 1 × 26 cm column of 200–400 mesh Dowex AG 50w-X2 (Calbiochem) was equilibrated with 0·1 M ammonium formate–formic acid, pH 3·2 buffer. After applying the 2 ml radioactive sample, the elution was carried out with the same buffer at room temperature and 3 ml fractions were collected. To 1 ml aliquots were added 9 ml of a scintillation cocktail made of 1 part of Triton X-100 and two parts of toluene containing 0·4% (w:v) PPO and 0·01% (w:v) of dimethyl-POPOP; a ^{3}H-hexadecane standard was used and the radioactivity, corrected for background and yield, is expressed in disintegrations per minutes (dis/min).

EXPERIMENTS AND RESULTS

Action of the crude extract on the incorporation of ^{3}H-thymidine into the TCA-insoluble fraction of Novikoff cells

Novikoff cells (2 millions) have been incubated in a total volume of 1 ml of Hanks' solution with 4 μCi of ^{3}H-thymidine at 37°C for various periods of time. Figure 1(a) shows

a linear incorporation of radioactivity in the TCA-insoluble fraction during a period not exceeding 2 hr. A one-hour incubation time was thus chosen for the assay.

The lyophilized powder from the rabbit liver supernatant was extracted with water. A 0·2 ml aliquot of this crude extract (20 mg/ml proteins) was added to the Novikoff cell suspension before incubation with 4 μCi of

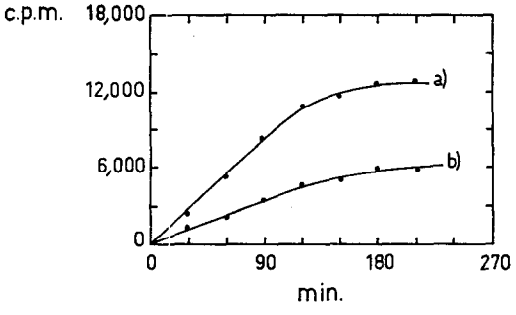


Fig. 1. ^{3}H-thymidine incorporation into the TCA-insoluble fraction of Novikoff cells.

(a) Two million Novikoff cells were incubated at 37°C with 4 μCi of ^{3}H-thymidine (6 Ci/mmol) in 1 ml of Hanks' solution for the indicated time (min.) and the TCA-insoluble radioactivity determined (c.p.m.).

(b) Same experiment with 0·2 ml of rabit liver supernatant in the incubation medium.

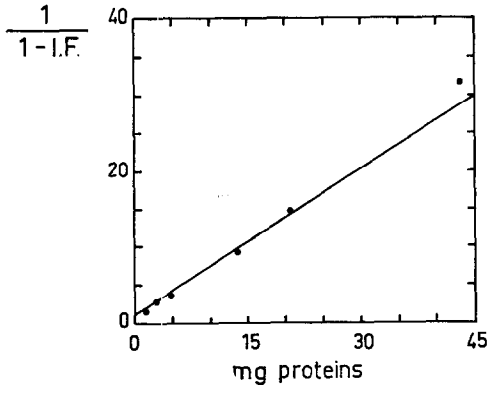


Fig. 2. Dose–effect relationship.

Two million Novikoff cells and 4 μCi of ^{3}H-thymidine (6 Ci/mmol) were incubated for 1 hr at 37°C in Hanks' solution containing rabbit liver supernatant (1 ml total volume). The inhibited fraction (I.F.) of tritium incorporation into the TCA-insoluble fraction was measured as a function of the amount of supernatant (mg of proteins).

^{3}H-thymidine in a total volume of 1 ml. Figure 1(b) shows that the liver extract reduced the incorporation of tritium in the TCA-insoluble fraction.

Novikoff cells were incubated, for 1 hr at 37°C, with various amounts of the crude liver extract and 4 μCi of ^{3}H-thymidine. The fractional decrease (I.F. = inhibited fraction) of ^{3}H-thymidine incorporation into the TCA-insoluble fraction was calculated in each case:

$$\text{I.F.} = \frac{\text{control} - \text{assay}}{\text{control}}$$

where "control" and "assay" are the radioactivities incorporated in the TCA-insoluble fractions of 2 million cells in the control and the assay respectively. We define the Novikoff inhibitory unit as the amount of inhibitor necessary to reduce by 50% the radioactivity incorporated in the TCA-insoluble fraction in our standard assay. Figure 2 shows a linear relationship between $1/(1-\text{I.F.})$ and the amount of crude liver extract. It follows that the number of Novikoff inhibitory units (U) is given by the equation:

$$U = \frac{\text{I.F.}}{1 - \text{I.F.}}$$

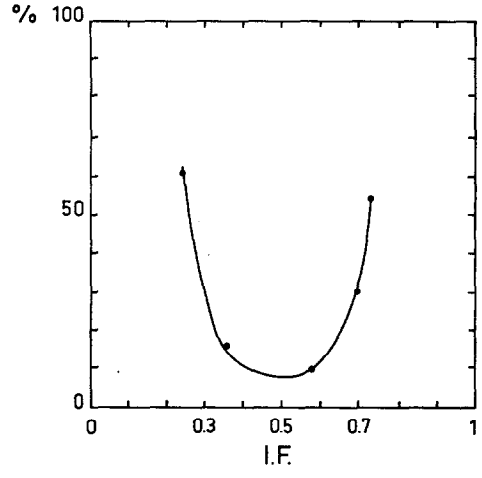


Fig. 3. Error on the estimation of the number of inhibitory units.

Using as reference the average of five controls without liver extract, the inhibited fraction (I.F.) of tritium incorporation into the TCA-insoluble fraction of Novikoff cells incubated with ^{3}H-thymidine, in the standard assay conditions, was measured for different amounts of rabbit supernatant; the corresponding number of inhibitory units (U) was calculated. Five samples were prepared for each dose of supernatant; the mean value of U and the standard error were calculated. The figure gives the relative error in U (%) as a function of I.F.

In another experiment, five samples have been prepared for each dose of the crude extract. The I.F. value was calculated in each case using as control value the average of five determinations made in absence of the crude extract. The individual U values and the standard deviation for each dose were then estimated. Figure 3 shows that the relative error in U is minimum and below 10% when I.F. is around 0·50, and does not exceed 30% as long as I.F. is between 0·30 and 0·70.

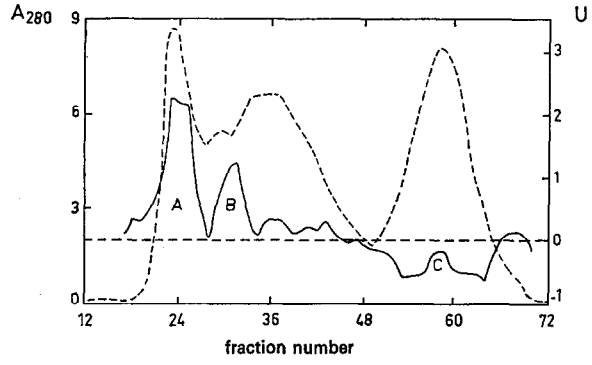


Fig. 4. Chromatography of the crude liver extract on Sephadex G-200.

The lyophilized powder of the rabbit liver supernatant (0·6 g) was extracted with 1·5 ml of 0·01 M NaCl, 0·005 M Tris–HCl, pH 7·4, and chromatographed on a 2·5 × 40 cm column of Sephadex G-200, with the same buffer, at 4°C. The dotted line gives the absorbance at 280 nm, while the continuous line gives the number of inhibitory units (U) for each 2·5 ml fraction collected.

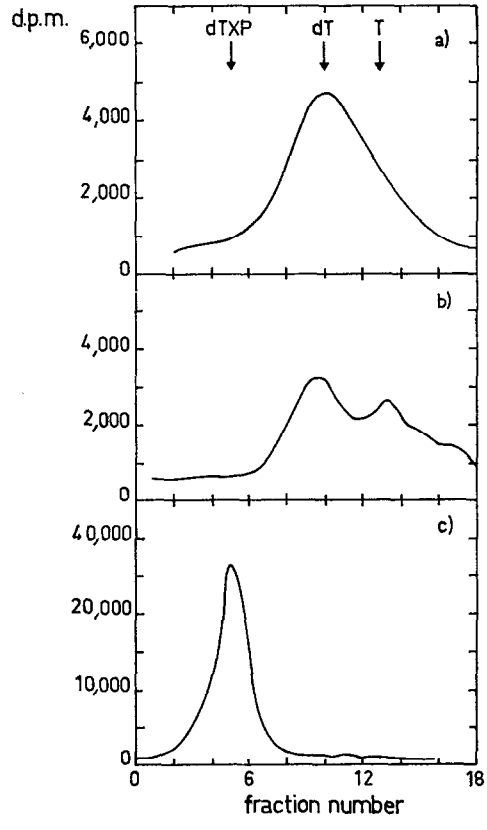


Fig. 5. Action of inhibitors A and B on thymidine radioactivity in the incubation medium and on thymidine phosphate radioactivity in the Novikoff cells.

The inhibitors were added to the incubation medium containing ^{3}H-thymidine in the standard assay. After 1 hr at 37°C, the Novikoff cells were separated from the incubation medium. The medium was chromatographed on Dowex-50 and the radioactivity (d.p.m.) measured on each 3 ml fraction; (a) experiment with inhibitor A, and (b) with inhibitor B. In the case of inhibitor A, the TCA-soluble fraction of the Novikoff cells was also chromatographed (c). The arrows indicate the position of the thymidine phosphates (dTXP), thymidine (dT) and thymine (T).

Purification of the rabbit liver inhibitor

A lyophilized powder of rabbit liver supernatant was prepared as described under Methods. A 0·6 g portion of this powder was extracted with 1·5 ml of 0·01 M NaCl, 0·005 M Tris–HCl, pH 7·4, then centrifuged at 10,000 ***g***. The supernatant was applied onto the appropriate Sephadex G-200 column and the elution performed with the same buffer at 4°C. Figure 4 indicates three regions where the fractions had an action on Novikoff cells. Peaks A and B contained substances which inhibited, while region C contained substances which stimulated ^{3}H-thymidine incorporation into the TCA-insoluble fraction.

Table 1. Action of different inhibitors on ^{3}H-thymidine in the incubation medium, and the radioactivity in the TCA-soluble and TCA-insoluble fractions of the Novikoff cells

Inhibitor	Radioactivity (% control)		
	Medium	Novikoff cells	
	Thymidine	dTXP	DNA
A	100	160	15
B	70		68
A-II-G_w		130	8

The inhibitor was added to the standard assay mixture; after 1 hr at 37°C, medium and Novikoff cells were separated. The incubation medium and the TCA-soluble fraction of the Novikoff cells were chromatographed on Dowex-50 (Fig. 5); the TCA-insoluble radioactivity of the Novikoff cells was also measured. Controls without inhibitor were run in parallel. The residual ^{3}H-thymidine in the incubation medium, the radioactivity of the intracellular thymidine phosphates (dTXP) and the tritium incorporated in the cellular TCA-insoluble fraction (DNA) are given in percents of the corresponding values in the control.

An aliquot of the pooled fractions of each peak A or B was incubated with Novikoff cells in the presence of ^{3}H-thymidine in the usual way. After separation, by centrifugation, of the cells and the incubation medium, the latter was chromatographed on Dowex 50. Figure 5 shows that, after incubation with inhibitor A, all the radioactivity of the medium was still in thymidine molecules; on the contrary, after incubation with inhibitor B, part of the radioactivity was found in thymine molecules. Table 1 indicates that the conversion of labelled thymidine into thymine seemed to explain most of the decrease of tritium incorporation in the TCA-insoluble fraction caused by inhibitor B;

the latter thus seems to be composed mostly of enzymes that convert thymidine into thymine. The Novikoff cells incubated with inhibitor A were washed twice with 5 ml of Hanks' solution containing 100 μg/ml of unlabelled thymidine, before being shaken with 5 ml of 5% TCA on a Vortex mixer. Centrifugation enabled to separate the acid-soluble and the acid-insoluble

Table 2. Action of different inhibitors on various cells and tissues

Cell or tissue	I.F. (%) A	A-I	A-II		A-II-G_w	
Novikoff	60	70	35	43	52	92
R.L.C.				31		
HeLa	14	1	0		4	
Ehrlich					15	
regenerating liver	27	32	7			6
spleen	6	6	0			
kidney	2					

In each experiment (vertical column of data), the same amount of inhibitor was incubated with different kinds of cells or tissue slices and the incorporation of ^{3}H-thymidine in the TCA-insoluble fraction compared to that of a control without inhibitor; an inhibited fraction (I.F.) of tritium incorporation in DNA was calculated in each case. The tissue slices (regenerating liver, spleen, kidney) were from subtotally hepatectomized 80 g male rats sacrificed 24 hr after the operation.

R.L.C. = culture of dissociated rat liver cells.

fractions. The acid-soluble fraction was chromatographed on Dowex 50: the radioactivity was found exclusively in the position of thymidine phosphates (Fig. 5). Table 1 shows that inhibitor A, while reducing the radioactivity incorporated into the TCA-insoluble fraction, actually increased the radioactivity in thymidine phosphates; this was interpreted as an indication of true inhibition of DNA synthesis. Table 2 shows that inhibitor A had a greater effect on Novikoff cells and regenerating rat liver slices than on cells of non-hepatic origin.

The pooled fractions (10 ml) of peak A were applied onto the DEAE Sephadex column and the elution carried out with Tris–HCl, pH 7·8 buffer using a NaCl discontinuous gradient. Inhibitor A was separated in two peaks: A-I and A-II (Fig. 6). Both inhibitors A-I and A-II were rather specific for liver cells (Table

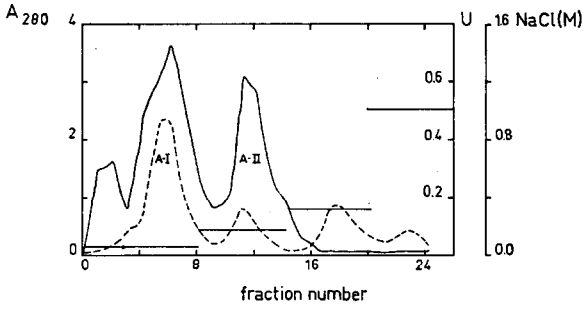


Fig. 6. Chromatography of inhibitor A on DEAE-Sephadex.

Inhibitor A in pH 7·8 Tris–NaCl buffer was chromatographed on a 1·3 × 15 cm DEAE-Sephadex column using a NaCl discontinuous gradient (horizontal lines). Absorbance at 280 nm (dotted line) and number of inhibitory units on Novikoff cells (U; continuous line) were measured on each 2·5 ml fraction.

2). Inhibitor A-II had little action on regenerating rat liver slices, but reduced ^{3}H-thymidine incorporation into the TCA-insoluble fraction of isolated rat liver cells approximately to the same extent as with Novikoff cells.

Because inhibitor A-I had a proteolytic effect on bovine serumalbumin that was absent in inhibitor A-II, and because the specific activity of A-I on Novikoff cells (units/mg of proteins) was much lower than for A-II, the latter inhibitor was chosen for further purification.

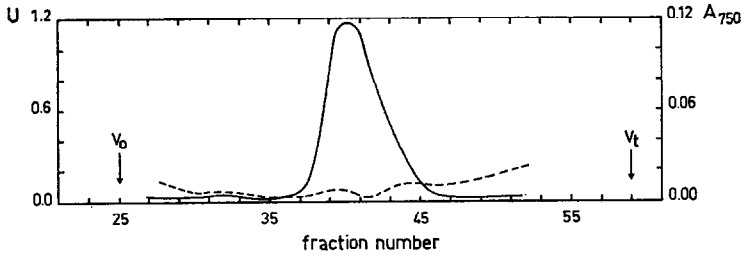


Fig. 7. Chromatography of inhibitor A-II on Sephadex G-200.

Inhibitor A-II was applied on a 2·5 × 40 cm Sephadex G-200 column and the elution carried out with water at 4°C. Absorbance at 750 nm given by Folin's reagent (dotted line) and number of inhibitory units (U; continuous line) were measured on each 2·5 ml fraction.

V_0 = void volume; V_t = total volume.

With the aim of concentrating inhibitor A-II, the pooled fractions of the A-II peak (5 ml) were desalted by filtration on Sephadex G-200 in water. Figure 7 shows that the inhibitor appeared as a single peak (A-II-G_w) between the void volume and the total volume of the column; the amount of protein associated with this peak was very low.

The inhibitor A-II-G_w

The pooled fractions of peak A-II-G_w were lyophilized and the residue taken in 0·15 M NaCl, 0·005 M Tris–HCl, pH 7·8. An aliquot was submitted to gel electrophoresis and the gel stained with Coomassie blue. As shown in the densitometric record of Fig. 8(a), a single protein band was observed.

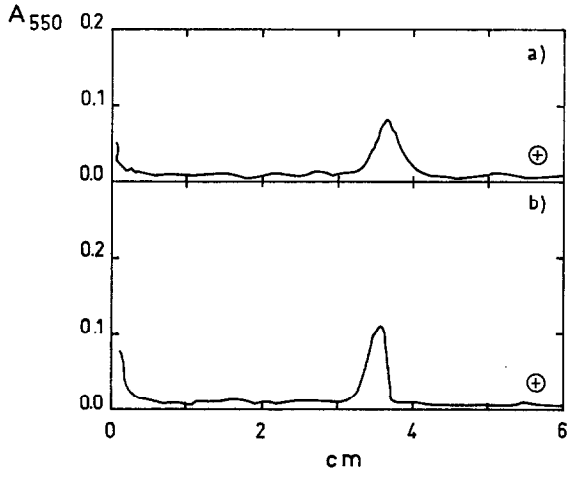


Fig. 8. Polyacrylamide gel electrophoresis of the inhibitor A-II-G_w.

(a) The fractions of peak A-II-G_w (Fig. 7) were lyophilized together, then submitted to polyacrylamide gel electrophoresis in Tris–glycine pH 8·3 buffer, with a 3 mA current per tube, at 4°C for 2 hr. After staining with Coomassie blue, the absorbance at 550 nm was recorded.

(b) Electrophoresis of an aliquot from a pool of five A-II-G_w peaks prepared independently.

Inhibitor A-II-G_w and four proteins of known molecular weights were successively chromatographed on the same Sephadex G-200 column; comparison of their K_{av} enabled to calculate a mass of approximately 40,000 daltons for the inhibitor (Fig. 9).

100 μg of pronase (Calbiochem), bovine pancreatic ribonuclease A (Sigma Chemical Co.; protease and salt-free), deoxyribonuclease (Worthington Biochemicals) or 50 units of neuraminidase (General Biochemical Co.) were added to inhibitor A-II in 0·2 ml of the Tris–NaCl buffer, and incubated at 37° for 30 min.; the incubation mixture was then tested on Novikoff cells in the usual assay. Two controls were carried out where enzyme or inhibitor A-II had been omitted. Only pronase and ribonuclease decreased the inhibitory effect of A-II. In order to know whether this result was due to the enzyme action on the inhibitor or on the cells, the mixture of A-II and enzyme (pronase or ribonuclease), after 30 min at 37°C, was chromatographed on Sephadex G-200 in water and the collected fractions tested on Novikoff cells; a control without enzyme was carried out parallelly. The A_{280} curves in Fig. 10 give the enzyme position since no absorbance at 280 nm was observed when A-II was chromatographed alone; the enzyme was, in every case, well separated from the A-II-G_w peak so that it could no longer interfere with the assay on Novikoff cells. Incubation of A-II alone for 30 min at 37°C did not suppress the A-II-G_w inhibitory peak and the same result was obtained when A-II had been incubated with ribonuclease; the A-II-G_w inhibitory peak however disappeared when A-II had been incubated with pronase (Fig. 10).

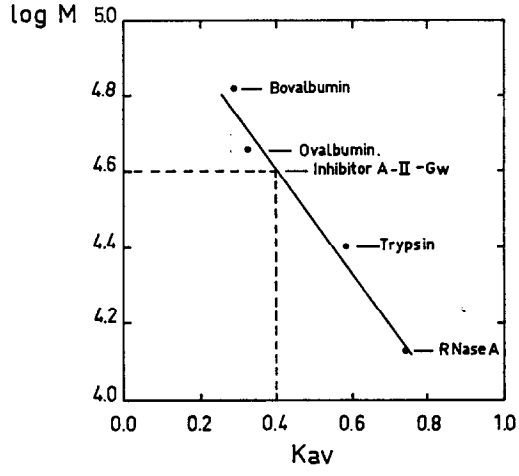


Fig. 9. Molecular weights of inhibitor A-II-G_w.

Inhibitor A-II-G_w and four proteins of known molecular weights were chromatographed in water on the same Sephadex G-200 column; the elution volume (V_e) was determined in each case. $K_{av} = (V_e - V_0)/(V_t - V_0)$ is given as a function of the logarithm of the molecular weight (log M). V_0 = void volume; V_t = total volume.

To check the reproducibility of the isolation procedure, the preparation was repeated five times and the fractions of the A-II-G_w peaks pooled together. Analysis by gel electrophoresis indicated the presence of a single protein band (Fig. 8(b)). The final product was shown to block the conversion of thymidine phosphates into DNA (Table 1) and to be rather specific for Novikoff cells (Table 2). Table 3 indicates a purification of about 2000

times and an overall yield of 5%; these values must be taken as minima since reference was made to the crude extract which contained several, specific and non-specific, inhibitors.

DISCUSSION

We have purified a protein which inhibits ^{3}H-thymidine incorporation into the DNA of Novikoff cells. Its protein nature is shown by its sensitivity to pronase. As judged by polyacrylamide gel electrophoresis, this protein appears to be pure after only three purification steps: two gel filtrations on Sephadex G-200 separated by an ion-exchange chromatography. It is worthwhile to note that, on the first chromatography on Sephadex G-200, the inhibitor appeared with the void volume, i.e. with molecules bigger than 200,000 daltons, while, on the second chromatography on Sephadex G-200, it appeared as a 40,000 dalton protein. The most probable explanation is that, in the crude extract, the inhibitor was part of a high molecular weight complex which was subsequently dissociated; this peculiar behaviour would explain why the inhibitor was so easily purified.

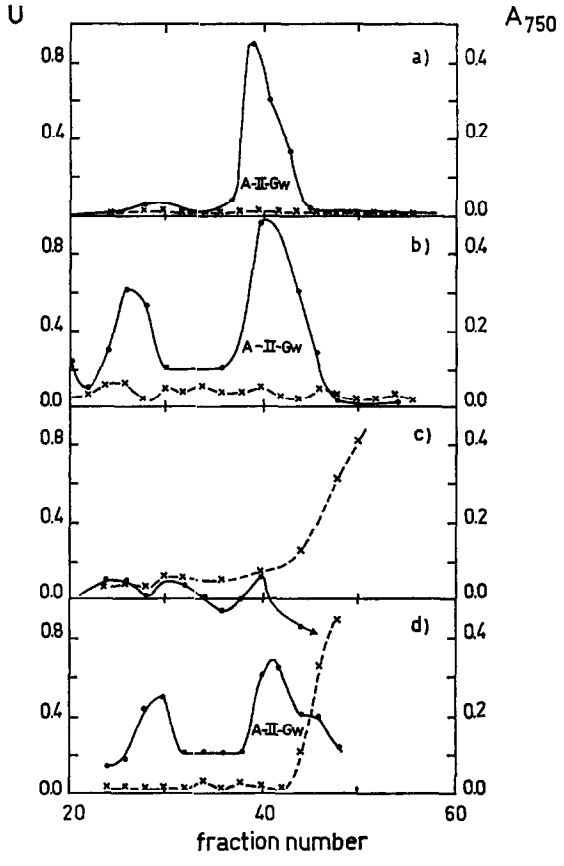


Fig. 10. *Action of pronase and ribonuclease on inhibitor A-II-G_w.*

The inhibitor A-II in Tris–NaCl, pH 7·8 buffer, was incubated with 500 *μg/ml of either pronase or ribonuclease for* 10 *min at* 37°*C, before being chromatographed on Sephadex-G-200 in water; a control without enzyme was run in parallel. The absorbance at* 750 *nm given by Folin's reagent (A_{750}; dotted line) and the action on Novikoff cells (U; continuous line) were measured on each* 3 *ml fraction (after lyophilisation and redissolution in Tris–NaCl, pH* 7·8 *buffer).*

(a) non-incubated A-II; (b) A-II incubated without enzyme; (c) A-II incubated with pronase; (d) A-II incubated with ribonuclease.

We have shown that the protein inhibitor truly interferes with DNA synthesis: it blocks the conversion of thymidine phosphates into DNA. The purified inhibitor has little action on cells of non-hepatic origin, such as HeLa or Ehrlich cells; it is active on separated cells of hepatic origin, either Novikoff cells or hepatocytes from rat liver.

It is however surprising that our protein inhibitor had so little action on DNA synthesis in regenerating rat liver slices. It is also surprising that, when the inhibition of DNA synthesis in regenerating liver slices is used for the assay, a small molecular weight peptide is followed (Verly *et al.*; Deschamps and Verly) [2, 3], while, when the inhibition of DNA synthesis in Novikoff cells is used for the assay, a 40,000 dalton protein is isolated. During the first step of our purification, the small molecular weight compound would have been in the region of the stimulatory substances; indeed, in this region, a small peak of less stimulation can be seen (Fig. 4) which could be due to the inhibitor found by Deschamps and Verly [3]. On the other hand, we rationalize that a high molecular weight compound must not diffuse easily through the layers of cells of a tissue slice; this would explain why our purified protein has so little action on regenerating liver slices: to see its effect, one must separate the cells so that they can be exposed directly to the incubation medium.

Table 3. *Data on the purification of a rabbit liver factor which inhibits DNA synthesis in Novikoff cells*

Fraction	Units/mg prot.	Purification factor	Yield (%)
Supernatant	0·6	1	100
Peak A	1·8	3	18
Peak A-II	150	250	8
Peak A-II-G_w	1100	1800	5

The purification factor is the ratio of the specific biological activity (units/mg of protein) in the fraction and in the supernatant. The yield is the total number of inhibitory units in the fraction divided by the number of units in the starting supernatant.

Because it is isolated from liver and because it seems to inhibit DNA synthesis specifically in cells of hepatic origin, the protein purified in this work is a candidate to what Bullough [6] has called "chalones". We ought however to show that the inhibitory protein is secreted in the blood, but this is not too unlikely since the hepatocytes produce most of the plasma proteins. We ought moreover to prove that it is a regulator of liver growth; we have not so far been able to isolate a sufficient amount of this protein inhibitor to test its action on liver regeneration after a subtotal hepatectomy.

We wonder whether the protein that we have isolated is the same as the high molecular weight factor which inhibits the growth of hepatoma cells described by Aujard *et al.* (1973) [7]; this factor seems to appear in the perfusion fluid from an isolated rat liver (Molimard *et al.*, 1976) [8]. It could also be the α_1-globulin shown by Nadal and Boffa (1974) [9] to block the G_1-S transition in hepatocytes.

REFERENCES

1. H. A. Satren. A principle of auto-regulation of growth. Production of organ-specific mitose-inhibitors in kidney and liver. *Exp. Cell Res.* **11,** 229 (1956).
2. W. G. Verly, Y. Deschamps, J. Pushpathadam and M. Desrosiers. The hepatic chalone. I—Assay method for the hormone and purification of the rabbit liver chalone. *Canad. J. Biochem.* **49,** 1376 (1971).
3. Y. Deschamps and W. G. Verly. The hepatic chalone. II—Chemical and biological properties of the rabbit liver chalone. *Biomed.* **22,** 195 (1975).
4. A. Simard, L. Corneille, Y. Deschamps and W. G. Verly. Inhibition of cell proliferation in the livers of hepatectomized rats by a rabbit hepatic chalone. *Proc. nat. Acad. Sci. (U.S.)* **71,** 1763 (1974).
5. G. Zweig and J. R. Withaker. Electrophoresis in stabilizing media, in *Paper Chromatography and Electrophoresis,* vol. I, Academic Press, New York and London (1967).
6. W. S. Bullough. The control of mitotic activity in adult mammalian tissues. *Biol. Rev.* **37,** 307 (1962).
7. C. Aujard, E. Chany and C. Frayssinet. Inhibition of DNA synthesis of synchronized cells by liver extracts acting *in vitro*. *Exp. Cell Res.* **78,** 476 (1973).
8. R. Molimard, G. Pietu, E. Chany, G. Trincal and C. Frayssinet. An inhibitor of hepatoma cell multiplication in the efferent fluid from isolated perfused rat livers. *Biomedicine* **23,** 434 (1976).
9. C. Nadal and G. A. Boffa. Activité inhibitrice des α_1-globulines du sérum de rat sur la multiplication des hépatocytes *in vivo*. *C. R. Acad. Sc. (Paris)* **278** (série D), 1071 (1974).

Europ. J. Cancer Vol. 12, pp. 219–225. Pergamon Press 1976. Printed in Great Britain

The Effect of Carcinogens on Mitotic Frequency in Mouse Lung Primordia *In Vitro*

J. R. W. MASTERS*
Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C.2., Great Britain.

Abstract—*The chronology and extent of alterations in epithelial and mesenchymal mitotic indices induced by three carcinogens—3-methylcholanthrene (MCA), 3,4-benzo(a)pyrene (BP) and urethan were studied using a model system, mouse embryonic lung in organ culture. At certain concentrations MCA and BP increased epithelial cell mitotic frequency but reduced mesenchymal cell mitotic frequency. In contrast, the alterations in the frequencies of epithelial and mesenchymal cell mitosis were similar in the presence of urethan, a substance carcinogenic* in vivo *but not* in vitro. *These results provide further evidence that the epithelium and mesenchyme exhibit a differential response to carcinogens, and it is suggested that the observed changes in mitotic frequency may have been the result of modification of epithelial–mesenchymal interactions controlling cell growth.*

INTRODUCTION

ORGAN development is controlled by a series of tissue interactions [1, 2], and there is evidence that such interactions play a role during teratogenesis and carcinogenesis [3–5].

If one of the primary changes during carcinogenesis is a modification of cellular interrelationships, then it is reasonable to propose that treatment with carcinogens would result in characteristic changes in the rates of epithelial and mesenchymal cell division. Evidence that carcinogens do interfere with normal growth control mechanisms has been provided by organ culture studies [6]. For example, treatment of organ cultures of mouse prostate tissue with 3-methylcholanthrene resulted in epithelial hyperplasia, while in contrast the frequency of mesenchymal cell mitosis was reduced [7]. Similar changes were observed in organ cultures of mouse embryonic lung tissue treated with 3,4-benzo(a)pyrene and, furthermore, these changes were not induced by a non-carcinogenic hydrocarbon, pyrene [8].

The purpose of the present study was to investigate further the effect of carcinogens on mitotic frequency in mouse lung primordia *in vitro*. Particular reference was made to the relationship between epithelial and mesenchymal cell mitosis, following treatment with three chemical carcinogens—3-methylcholanthrene (MCA), 3,4-benzo(a)pyrene (BP) and urethan.

MATERIAL AND METHODS

Left lung rudiments from C57BL Icrf a^t mice were used. These were dissected from 11-day embryos, day 0 of gestation being calculated as the morning on which a vaginal plug was found.

The organ culture system consisted of a 30 mm dia optically flat Anumbra petri dish (Jensen Scientific Limited) containing a flat stainless steel grid. A sheet of cellulose dialysis tubing (Visking Scientific Instrumentation Company,

Accepted 23 October 1975.
*Present address: Department of Clinical Surgery, Royal Infirmary, Edinburgh, EH3 9YW.

London) coated with a thin layer of 1% Difco Noble agar in Hank's buffered salt solution was placed on the grid, providing a tissue support. 1·2 ml of Waymouth's MB 752/1 medium (Wellcome Laboratories Ltd., Beckenham, England), supplemented with 10% foetal calf serum (Flow Laboratories Limited, Irvine, Scotland) and 25 mM Hepes (Sigma, London) were added to each dish. The cultures were incubated at 37°C in a water-saturated atmosphere of 95% air/5% CO_2.

Five groups of explants were set up using the following media:

(1) medium alone

(2) medium containing 5 μl/ml acetone

(3) medium containing 2·5, 5·0, 7·5 or 10·0 μg/ml 3-methylcholanthrene (Schuschardt, Germany), dissolved in acetone to produce a final acetone concentration of 5 μl/ml medium.

(4) medium containing 1·0, 2·0 or 5·0 μg/ml 3,4-benzo(a)pyrene (Schuschardt, Germany), dissolved in acetone to produce a final acetone concentration of 5 μl/ml medium.

(5) medium containing 0·1, 0·2, 0·5 or 1·0% urethan (Sigma), dissolved directly in the medium.

The tissue was cultured for 24, 48 or 72 hr. Three explants were cultured for each time period at each concentration of carcinogen. Four hours prior to fixation the explants were transferred to medium containing 5 μg/ml colchicine (Sigma, London) in order to arrest cells in metaphase. The tissues were fixed in 2·5% glutaraldehyde in 0·1 M Sorenson's buffer, post-fixed in 1% osmium tetroxide and embedded in Araldite. Araldite embedment was used in preference to paraffin embedment for the production of replicate sections of accurate thickness.

Two 3 μM sections, a minimum of 100 μM apart, were cut from each explant using a glass knife. The sections were stained for two minutes at 65°C on a hot-plate in 1% basic fuchsin in 50% alcohol.

The total number of epithelial and mesenchymal metaphase and interphase cells were counted in each section. The number of cells counted per explant were: (a) epithelium, 189–1398, average 595; (b) mesenchyme 419–2223, average 1357. The mitotic index (MI) was calculated on the basis of the number of metaphases/100 cells per 4 hr.

RESULTS

The lung primordia developed *in vitro* in a manner comparable to that *in vivo*. During the three day culture period, the single epithelial tube of the 11-day mouse embryo left lung rudiment branched to produce an arboreal system of ducts, consisting of 4 or 5 second generation branches and 9 to 15 terminal buds formed by third and fourth generation branches. The ducts were lined by columnar and cuboidal epithelium, and the duct system was surrounded by mesenchyme containing a small number of capillaries. Thus, the morphological development *in vitro* was similar to that described by previous authors [8, 9].

A comparison was made of the effects of three carcinogens on three aspects of cell division in mouse lung primordia in organ culture, the three aspects being (a) epithelial cell mitotic index (MI), (b) mesenchymal cell MI, and (c) ratio of the epithelial: mesenchymal cell MIs.

(a) 3-*Methylcholanthrene* (*MCA*)

The effects of MCA on the three aspects of cell division are summarised in Table 1 and illustrated in Fig. 1.

Depending on the concentration of MCA used, three different patterns of epithelial cell mitosis were observed: (a) in the presence of 2·5 μg/ml, the MIs were similar to those of the control group, (b) 5·0 and 7·5 μg/ml caused an increase in the MIs at 48 and 72 hr, and (c) 10 μg/ml caused both an increase and a decrease in the frequency of mitosis, depending on the culture period (Fig. 1a).

The frequency of mesenchymal cell mitosis was reduced after 24 hr in the presence of 2·5, 5·0 or 7·5 μg/ml MCA, although the MIs were similar to those of the controls after 48 and 72 hr *in vitro* (Fig. 1b). This pattern of cell division was in contrast to the variable effects produced by 10 μg/ml MCA, which caused a reduction of the MI at 24 hr, an increase at 48 hr and a further reduction at 72 hr. Thus, the alterations in the mitotic frequencies produced by 10 μg/ml MCA were similar in both the epithelium and the mesenchyme.

As a result of these alterations in the MIs the ratios of the epithelial:mesenchymal cell MIs were raised above those of the control group at each time period and at each concentration of carcinogen, with the single exception of the group of explants grown for 24 hr in the presence of 10 μg/ml MCA (Fig. 1c).

(b) 3,4-*Benzo(a)pyrene* (*BP*)

The same pattern of epithelial cell division

Table 1. *The mitotic indices of the epithelium (E) and mesenchyme (M) and the ratio of the epithelial: mesenchymal MIs (E/M) of* 11 *day mouse embryo lung grown for* 24, 48 *or* 72 *hr in the presence of* 2·5, 5·0, 7·5 *or* 10·0 *μg.* 3-*MCA/ml medium or* 5 *μl acetone/ml medium. S.D. = Standard Deviation*

MEDIUM CONTAINING	CULTURE PERIOD HOURS								
	24			48			72		
	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.
5μl Acetone/ml. (control)	7.30±0.37	3.55±0.38	2.06±0.16	3.65±0.32	2.46±0.77	1.61±0.59	2.47±0.62	2.25±0.79	1.13±0.14
2.5 μgms.3MCA/ml.	7.15±0.51	3.08±0.45	2.45±0.19	3.95±0.44	2.35±0.43	1.69±0.13	2.46±0.36	1.90±0.19	1.31±0.28
5.0 μgms.3MCA/ml.	7.27±0.62	2.15±0.13	3.35±0.23	5.01±1.00	2.64±0.26	1.91±0.39	2.73±0.30	2.00±0.30	1.36±0.06
7.5 μgms.3MCA/ml.	7.34±0.68	2.65±0.55	2.88±0.82	6.41±0.90	2.93±0.39	2.19±0.24	4.24±0.59	2.46±0.24	1.73±0.22
10 μmgs.3MCA/ml.	4.66±0.36	2.36±0.35	2.00±0.30	6.85±0.33	3.89±0.90	1.82±0.39	2.09±0.13	1.37±0.18	1.53±0.14

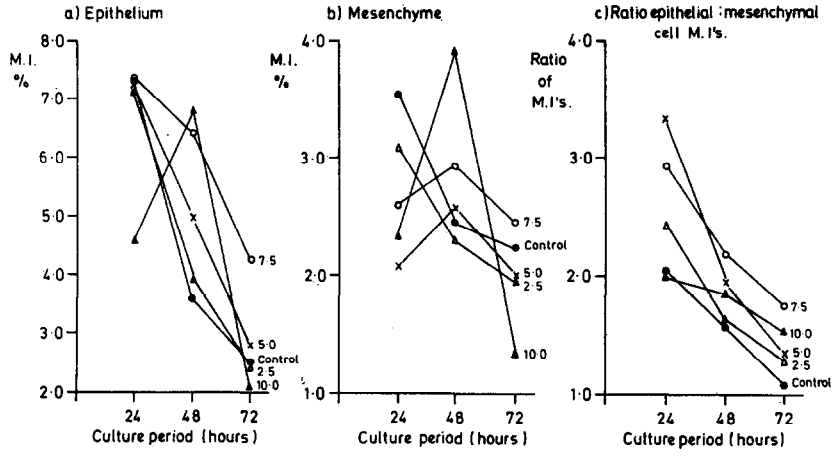


Fig. 1. *The mitotic indices (MI) of the (a) epithelium, (b) mesenchyme, and (c) the ratio between the epithelial: mesenchymal MIs of* 11 *day mouse embryo lung after* 24, 48 *and* 72 *hr in organ culture on control medium containing* 5 *μl/ml acetone, and on similar medium containing* 2·5, 5·0, 7·5 *or* 10 *μg/ml* 3-*methylcholanthrene.*

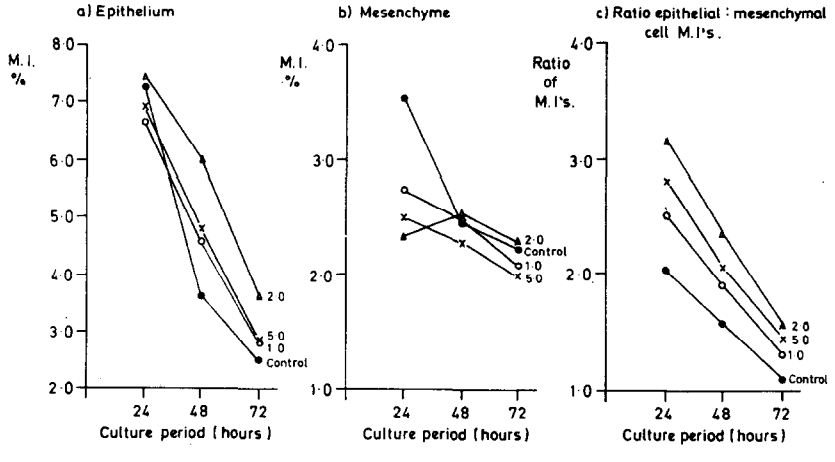


Fig. 2. *The mitotic indices (MI) of the (a) epithelium, (b) mesenchyme, and (c) the ratio between the epithelial: mesenchymal MIs of* 11 *day mouse embryo lung after* 24, 48 *and* 72 *hr in organ culture on control medium containing* 5 *μl/ml acetone, and on similar medium containing* 1·0, 2·0 *or* 5·0 *μg/ml* 3,4-*benzo(a)pyrene.*

Table 2. The mitotic indices of the epithelium (E) and mesenchyme (M) and the ratio of the epithelial: mesenchymal MIs (E/M) of 11 day mouse embryo lung grown for 24, 48 or 72 hr in organ culture in the presence of 1·0, 2·0 or 5·0 μg BP/ml medium or 5 μl acetone/ml medium. S.D. = Standard Deviation

MEDIUM CONTAINING	CULTURE PERIOD HOURS								
	24			48			72		
	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.
5μl Acetone/ml. (control)	7.30±0.37	3.55±0.38	2.06±0.16	3.65±0.32	2.46±0.77	1.61±0.59	2.47±0.62	2.25±0.79	1.13±0.14
1μgm.BP/ml.	6.62±0.12	2.71±0.57	2.51±0.49	4.54±0.70	2.49±0.62	1.89±0.45	2.78±0.33	2.10±0.14	1.32±0.12
2μgms.BP/ml.	7.43±0.75	2.33±0.18	3.18±0.10	6.01±0.46	2.55±0.58	2.42±0.41	3.65±1.42	2.33±0.64	1.61±0.70
5μgms.BP/ml.	6.94±0.27	2.50±0.37	2.81±0.33	4.78±0.57	2.34±0.47	2.12±0.58	2.79±0.23	2.00±0.43	1.47±0.49

Table 3. The mitotic indices of the epithelium (E) and mesenchyme (M) and the ratio of the epithelial: mesenchymal MIs (E/M) of 11 day mouse embryo lung grown for 24, 48 or 72 hr in the presence of 0·1, 0·2 or 0·5% urethan or on control medium. S.D. = Standard Deviation

MEDIUM CONTAINING	CULTURE PERIOD HOURS								
	24			48			72		
	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.	E±S.D.	M±S.D.	E/M±S.D.
Control	7.02±0.47	3.52±0.15	2.00±0.17	3.54±1.03	2.17±0.53	1.60±0.17	2.35±0.53	2.18±0.58	1.10±0.10
0.1% Urethan	6.23±1.06	2.95±0.34	2.11±0.24	5.30±0.16	2.62±0.27	2.03±0.19	3.60±1.17	2.25±0.43	1.63±0.54
0.2% Urethan	4.54±0.79	2.07±0.09	2.19±0.34	4.59±0.44	2.53±0.35	1.82±0.09	3.57±0.15	2.36±0.12	1.52±0.14
0.5% Urethan	3.52±0.60	2.26±0.14	1.57±0.37	4.62±0.94	2.64±0.50	1.83±0.68	4.22±0.39	2.72±0.25	1.56±0.07

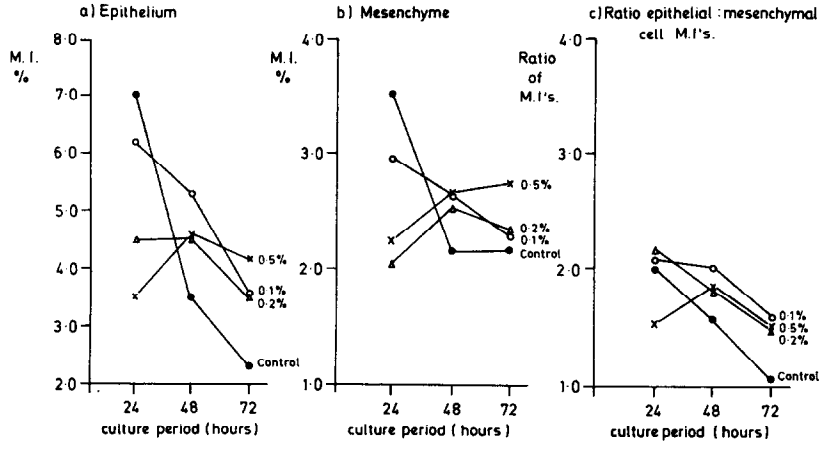


Fig. 3. The mitotic indices (MI) of the (a) epithelium, (b) mesenchyme, and (c) the ratio between the epithelial: mesenchymal MIs of 11 day mouse embryo lung after 24, 48 and 72 hr in organ culture on control medium and on similar medium containing 0·1, 0·2 or 0·5% urethan.

was observed in the presence of each concentration of BP used—0·1, 0·2 and 0·5 μg/ml (see Fig. 2 and Table 2). Thus, the epithelial cell MIs were similar to those of the control group at 24 hr, but were higher after 48 and 72 hr *in vitro* (Fig. 2a). In addition, the same pattern of mesenchymal cell division was observed irrespective of the concentration of BP used. However, in contrast to epithelial cell division, the mesenchymal cell MIs were lower than those of the controls at 24 hr, but were similar after 48 and 72 hr *in vitro* (Fig. 2b). The effect of these alterations was to raise the ratios of the epithelial:mesenchymal cell MIs above those of the controls at each time period and at each concentration of carcinogen (Fig. 2c).

(c) *Urethan*

The patterns of epithelial and mesenchymal cell division were similar in the presence of 0·1, 0·2 and 0·5% urethan. After 24 hr *in vitro* the MIs were reduced, whereas the MIs of the 48 and 72 hr cultures were higher than those of the control group (see Table 3 and Fig. 3a and 3b). The net effect of these alterations was to raise the ratio of the epithelial:mesenchymal cell MIs above those of the controls, except for the group of explants grown for 24 hr in the presence of 0·5% urethan (Fig. 3c).

A study was also made on the effect of 1% urethan on embryonic lung development in organ culture. No mitotic figures were observed in either the epithelium or mesenchyme during the three day culture period and, furthermore, no morphogenetic development occurred. While the epithelium appeared to be relatively well maintained, extensive necrosis was observed in the mesenchyme.

DISCUSSION

A study has been made on the chronology and extent of changes induced by carcinogens in the frequencies of epithelial and mesenchymal cell mitosis, using mouse embryonic lung tissue in organ culture as a model system. 3-methylcholanthrene (MCA) and 3,4-benzo-(a)pyrene (BP), at certain concentrations, increased the frequency of epithelial cell mitosis, while in contrast reduced the frequency of mesenchymal cell mitosis. These changes resulted in an increase in the ratios of the epithelial:mesenchymal mitotic indices (MIs). The ratios were higher at each time period and at each concentration of MCA and BP than those of the controls, with the single exception of the cultures grown for 24 hr in the presence of 10 μg/ml MCA. It is considered that such changes in mitotic frequency could lead to an abnormal growth pattern, and may play a primary role in carcinogenesis.

Although such detailed studies of the chronology and extent of changes in mitotic frequency induced by carcinogens in organotypic cultures have not previously been published, these results confirm and extend previous observations. For instance, a potential carcinogen (a cigarette smoke fraction) was shown to stimulate epithelial cell mitosis in organ cultures of human foetal lung [10] and mouse trachea [11], and the proliferative activity continued following removal of this substance from the culture medium. Using a similar system to that described in the present study, it was shown that 2 μg/ml BP stimulated epithelial cell mitosis, but reduced the frequency of mesenchymal cell mitosis [8]. These studies indicate that carcinogens can selectively stimulate epithelial cell mitosis, and it has been suggested that such changes, resulting in morphological evidence of epithelial hyperplasia, are indicative of preneoplasia [7]. The validity of this hypothesis could be proven by transplanting the carcinogen-treated tissue, and showing that tumours develop [3, 12–15].

It has been suggested that organ culture is a degenerating system, and not comparable to the *in vivo* situation. However, with regard to the present study, the epithelial cell MI of the 11 day mouse lung primordia was not significantly different from that of 14 day mouse lung primordia, determined using the same *in vitro* method (2·35 ± 0·53 and 1·76 ± 1·01 respectively), while the mesenchymal cell MI *in vitro* was higher than that of 14 day mouse lung primordia (2·18 ± 0·58 and 0·9 ± 0·19 respectively) [16]. Therefore, it is considered that the *in vitro* findings in the present study are of relevance to the *in vivo* situation.

It has been suggested that the changes in the mitotic frequencies of the epithelium may be initiated by a loss of a regulatory ability of the mesenchyme [8]. In the absence of carcinogens the frequency of epithelial cell mitosis was proportional to the amount of associated mesenchyme, in that a reduction in the amount of mesenchyme resulted in a decrease in the rate of epithelial cell division [9, 16]. In contrast, in the present study, certain concentrations of MCA and BP caused a decrease in the frequency of mesenchymal cell mitosis, which was followed by an increase in the frequency of epithelial cell mitosis. There are two alternative explanations for such changes, either (a) the carcinogens altered the normal growth control mechanisms operating between the epithelium and mesenchyme or, (b) the two tissues responded independently and differentially to the carcinogens.

Carcinogenesis is not restricted to epithelial cells, yet the changes considered to be indicative of preneoplasia were restricted to the epithelium. No adequate explanation can be given for the differential sensitivity of the epithelium and mesenchyme, although it is pertinent to note that explants of foetal and adult mouse lung tissue treated *in vitro* with MCA may develop carcinomas, but not sarcomas, following transplantation to isologous mice [12–14]. Furthermore, MCA and BP usually induce pulmonary carcinomas rather than sarcomas *in vivo* [17].

The patterns of epithelial and mesenchymal

cell division observed in the presence of 10 μg/ml MCA differed from those observed in the presence of BP and lower concentrations of MCA. Both a decrease and an increase in the frequency of cell mitosis were observed, depending on the culture period. Carcinogens have two effects on cell division, a stimulatory effect which may increase the mitotic frequency, and a toxic effect which may reduce the mitotic frequency [19]. The mitotic frequencies observed following treatment with 10 μg/ml MCA are probably the net result of these two effects on cell division. Furthermore, such carcinogens have been shown to be metabolised in culture systems [18], and therefore the concentrations may have altered during the culture period, thereby altering the balance between the toxic and stimulatory effects.

In contrast with the explants grown in the presence of MCA or BP, those cultures grown in the presence of 0·1, 0·2 or 0·5% urethan exhibited a decrease in the frequency of both epithelial and mesenchymal cell mitosis at 24 hr, but an increase after 48 and 72 hr *in vitro*. This pattern of cell division might be expected in the presence of a toxic substance [19], in that an initial decrease in the mitotic frequency was followed by a recovery and corresponding increase in the mitotic frequency. Although urethan is carcinogenic *in vivo*, it does not induce tumour development *in vitro*, and the results indicated that urethan is metabolised to a carcinogenic derivative *in vivo* [15]. If mouse lung tissue *in vitro* cannot form this derivative, this may explain the differences between the *in vitro* effects of urethan and those of MCA and BP.

The observation that the addition of 1% urethan to the culture medium inhibited lung growth and morphogenesis concurs with previous findings [20]. In addition, it was shown that the epithelium from urethan-treated lung explants is still able to respond to inductive stimuli (pulmonary or salivary mesenchyme) by undergoing morphogenetic development [20]. This latter finding concurs with the observation in the present study that the integrity of the epithelium appeared to be maintained, whereas extensive necrosis was observed in the mesenchyme.

In summary, it has been shown that MCA and BP induced similar changes in the mitotic frequency in embryonic lung tissue in organ culture, and resulted in an increase in the ratios of the epithelial:mesenchymal cell MIs. Further experiments are necessary in order to determine whether other carcinogens induce similar alterations in mitotic frequencies, in order to determine whether such changes are a characteristic effect of carcinogens. There is experimental evidence to show that continued epithelial–mesenchymal interaction is necessary during carcinogenesis [3], and it may be possible to demonstrate that modification of such interactions may play a primary role during carcinogenesis.

Acknowledgements—I gratefully acknowledge the help and advice of Dr. G. Hodges and wish to thank Dr. W. Miller for reading, and Mrs. Beck for typing the manuscript.

REFERENCES

1. G. Hodges, Stromal-epithelial interactions. In *Biology of the Periodontium*, p. 27. (Edited by T. Melcher) Academic Press, New York (1969).
2. K. Kratochwil, Tissue interactions during embryonic development: general properties. In *Tissue Interactions in Carcinogenesis*, p. 1. (Edited by D. Tarin) Academic Press, New York (1972).
3. C. J. Dawe, Changes in cell inter-relationships during epithelial carcinogenesis. In *Topics in Chemical Carcinogenesis*, p. 401. (Edited by W. Nakahara, S. Takayama, T. Sugimura and S. Odashima) Univ. Park Press, Baltimore (1972).
4. J. W. Orr and A. R. Spencer, Transplantation studies of the role of the stroma in epidermal carcinogenesis. In *Tissue Interactions in Carcinogenesis*, p. 291. (Edited by D. Tarin) Academic Press, New York (1972).
5. L. Saxen and J. Rapola, *Congenital Defects*, p. 78. Holt, Rinehart & Winston, New York (1969).
6. C. Heidelberger, Chemical oncogenesis in culture. *Adv. Cancer Res.* **18,** 317 (1973).
7. I. Lasnitzki, The effect of carcinogens, hormones and vitamins on organ cultures. *Int. Rev. Cytol.* **VII,** 79 (1958).
8. P. C. Chan, F. K. Sanders, and E. L. Wynder, Effect of 3,4-benzo(a)pyrene on mouse lung primordia *in vitro*. *Nature (Lond.)* **223,** 847 (1969).

9. T. Alescio and M. Di Michele, Relationship of epithelial growth to mitotic rate in mouse embryonic lung developing *in vitro*. *J. Embryol. exp. Morph.* **17,** 213 (1968).
10. I. Lasnitzki, The effect of a hydrocarbon-enriched fraction of cigarette smoke condensate on human fetal lung grown *in vitro*. *Cancer Res.* **28,** 510 (1968).
11. I. Lasnitzki, The effect of a hydrocarbon-enriched fraction from cigarette smoke on mouse tracheas grown *in vitro*. *Brit. J. Cancer* **22,** 105 (1968).
12. J. O. Laws and A. Flaks, Pulmonary adenomata induced by carcinogen treatment in organ culture. *Brit. J. Cancer* **20,** 550 (1966).
13. A. Flaks and J. O. Laws, Pulmonary adenomata induced by carcinogen treatment in organ culture. Influence and duration of treatment. *Brit. J. Cancer* **22,** 839 (1968).
14. R. F. Davies, I. R. Major and E. R. Aberdeen, Pulmonary adenomata induced by carcinogen treatment in organ culture. Influence of increasing amounts of carcinogen. *Brit. J. Cancer* **24,** 785 (1970).
15. S. Rogers, Studies of the mechanism of action of urethan in initiating pulmonary adenomas in mice. *J. nat. Cancer Inst.* **15,** 1675 (1955).
16. J. R. W. Masters, Epithelial–mesenchymal interaction in development and carcinogenesis in the mouse lung. Ph.D. Thesis, University of London (1974).
17. R. A. Willis, *Pathology of Tumours*, 4th ed., p. 28. Butterworth, London (1967).
18. H. V. Gelboin, N. Kinoshita and F. J. Wiebel, Microsomal hydroxylases: induction and role in polycyclic hydrocarbon carcinogenesis and toxicity. *Fed. Proc.* **31,** 1928 (1972).
19. M. F. Rajewsky, Proliferative parameters of mammalian cell systems and their role in tumor growth and carcinogenesis. *Z. Krebsforsch.* **78,** 12 (1972).
20. A. Globerson and R. Auerbach, *In vitro* studies on thymus and lung differentiation following urethan treatment. In *Methodological Approaches to the Study of Leukemias*, p. 3. (Edited by V. Defendi) Wistar Institute Symposium Monograph 4 (1964).

Europ. J. Cancer Vol. 12, pp. 227–235. Pergamon Press 1976. Printed in Great Britain

The Metabolism of 6-Aminochrysene in the Rat

PRESTON H. GRANTHAM, NGUYEN-BA-GIAO, LETITIA C. MOHAN, TIMOTHY BENJAMIN, PETER P. ROLLER and ELIZABETH K. WEISBURGER

Carcinogen Metabolism and Toxicology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD. 20014, U.S.A.

Abstract—*The absorption, distribution and excretion of 6-aminochrysene-5, 6-^{14}C were investigated in rats. The highest concentration of radioactivity in the organs at 48 hr after an i.p. dose were in the liver and kidneys. Only 12% of the administered radioactivity was recovered in the urine and 56% from the feces during a ten day period. Up to 42% of the dose was excreted in the bile during 48 hours. The major biliary metabolite of 6-aminochrysene was the N-glucuronide of 6-aminochrysene. 12-Hydroxy-6-acetylaminochrysene was present in the urine as a glucuronide and in unconjugated form in the feces.*

INTRODUCTION

IT HAS been reported that 6-aminochrysene (6-AC), also called "Chrysenex," is a carcinostatic agent with low or no general cytotoxic activity [1–3]. Some of the reported biological actions are: chemotherapeutic reducing effects on splenomegaly [4, 5], depressing effect on leukocytosis in leukemic patients [6, 7], and carcinostatic effect on certain spontaneous and experimental tumours [2, 8, 9]. 6-AC has also been shown to be a strong inducing agent for the synthesis of microsomal enzymes in experimental animals [1, 10]. More recently Roe *et al.* [11] reported that 6-AC produced hepatic and pulmonary tumours when injected subcutaneously into neonatal mice during the first three days of life. Lambelin *et al.* [12] have since confirmed the carcinogenicity of 6-AC in mice.

Due to the lack of information on the metabolism of 6-AC, we began a study several years ago on this problem, using initially 6-AC-12^{3}H as a tracer. The initial results (Table 1) indicated that at least 60% of urinary radioactivity was due to $^{3}H_2O$ and not to 6-AC metabolites. Therefore, ring-^{14}C labeled 6-AC was synthesized for more definitive metabolism studies. This report details our findings.

Accepted 17 October 1975.

MATERIAL AND METHODS

Synthesis of 6-AC-5,6-^{14}C

Chrysene-5,6-^{14}C (0·14 mCi, 10·9 mCi/mM) was purchased from Mallinckrodt, St. Louis, Missouri. A benzene solution of the ^{14}C-compound and 504 mg of unlabeled chrysene was taken to dryness on a water bath under a stream of nitrogen. The compound was suspended with mechanical stirring in 14 ml of acetic acid at 40°C [13], and a solution of 0·4 ml of concentrated nitric acid, 0·5 ml of concentrated sulfuric acid, and 4 ml of acetic acid was added all at once. After several minutes, the temperature rose and the suspension became yellow. After an additional 20 min of reaction time, the precipitate was filtered off and washed three times with 7 ml of 50% acetic acid. Upon drying the yield was 516 mg of 6-nitrochrysene-5,6-^{14}C, m.p. 210–212°C.

6-Nitrochrysene-5,6-^{14}C (516 mg) was dissolved in 100 ml of hot ethyl acetate. After cooling, 100 mg of palladium on powdered charcoal (Matheson, Coleman & Bell) was added to the solution. The mixture was pressurized at 44 lb/in^2 with H_2, shaken for 7 hr

Table 1. Summary of metabolism studies with 6-AC-12-^3H

Average urinary excretion* % of dose†			Fecal excretion % of dose		Biliary excretion % of dose‡		
Days	Single injection	Repeated dosing	Days	Single injection	Hours	I.V.	I.P.
1	1.25	1.60	3	12.65	5	14.2	
2	0.77	1.25	7	4.20	17		13.1
3	0.59	1.01	10	2.42	20	4.5	
			Total	19.27	24		2.4
4	0.59	0.84			44		3.4
5	0.56	0.84			67		0.9
6		0.60					
7	0.54	0.39					
8	0.42	0.46					
9	0.65	0.47					
10	0.49	0.55					
Totals	6.01	8.01					

* A total of 17 three month old male rats (250 g body wt) were used for this study. The dose was 100 mg/kg body wt of 6-AC-12-^3H (2.3 μCi/mg). For the repeated dosing study 10 rats were fed 0.05% 6-AC in the diet for 2 weeks, followed by a single i.p. injection of 6-AC-12-^3H.

† Distillation of the various urines from the single injection or repeated dosing experiment showed that 60 and 64%, respectively, of the radioactivity present was due to ^3H_2O.

‡ Distillation of bile (i.v. injection) showed that 2% of the activity was due to ^3H_2O.

and allowed to stand overnight under pressure. The catalyst was filtered off and the solvent was evaporated under a stream of N_2. A benzene solution of the product was passed through a column (2 × 10 cm) of aluminium oxide (Merck), the benzene eluate was evaporated to dryness, and the residue was further purified by recrystallization from benzene–petroleum ether. The yield of 6-AC-5,6-^{14}C was 96%, m.p. 209–211°C (Kofler).

The compound was chromatographed on silica gel thin layer chromatographic (TLC) plates in the solvent system ether-dimethylformamide (95:5). A single radioactive spot was obtained, Rf 0·72, which accounted for 98% of the applied radioactivity. The specific activity of the eluted 6-AC-5,6-^{14}C was 60·0 μCi/mmole.

Reference compounds

The reference compounds 12-hydroxy-6-aminochrysene and 12-hydroxy-6-acetylaminochrysene were prepared by the method of Thang *et al.* [14]. *N*-Hydroxy-6-acetylaminochrysene was prepared by partial hydrogenation and concurrent acetylation of 6-nitrochrysene in ethyl acetate, triethylamine and acetic anhydride with palladium–carbon catalyst according to the method of Miller *et al.* [15]. The m.p. was 184–187°C (ethanol–water). Further details are given in Table 2.

Table 2. Ultraviolet, Rf, mass spectra and HPLC data of 6-AC derivatives and metabolites

Reference compounds	Ultraviolet absorption ε (nm) EtOH	Max.	Min. (Shoulder)	Rf Values in solvents† A	D	Mass spectral data‡ M/e	High pressure liquid chromatograph retention times (min.)
6-Aminochrysene	66,100	277 230	252 205	0.72	0.34	243(100) 215(36) 213(13) 202(4) 200(4) 189(7) 121.5(14) 107.5(14)	5.7
6-Acetylaminochrysene	91,800	272 227	247 205	0.50	0.28	285(20) 243(37) 215(22) 44(41) 18(100)	8.4
N-OH-6-acetylaminochrysene	89,100	271 220	235 (267,242) 206	0.22	0.31	301(52) 285(19) 259(78) 242(100) 228(27) 215(92) 213(23) 202(8)	----
12-OH-6-aminochrysene*		277 227	252 205	0.45	0.16	259(100) 258(13) 231(13) 230(35) 202(8) 130(32)	14.2
12-OH-6-acetylaminochrysene	-----	275 227	250 205	0.20	0.10	301(59) 259(100) 258(28) 231(31) 230(36) 215(10) 202(38)	17.2
Urinary Metabolites:							
Glucuronide fraction (12-OH-6-acetylaminochrysene)		275 ---	246 ---	0.20	0.10	301(80) 259(100) 258(38) 231(41) 230(36) 215(10) 202(50)	17.2

* Very unstable in ethanol

† Solvents: [A] DMF:Ether (5:95) silica gel plates; [D] Hexane:$CHCl_3$: Dioxane:MeOH (120:40:25:15) Baker Flex-Silica Gel 1B-F plates

‡ Spectra were obtained with a JMS-01SG-2 mass spectrometer, solid probe, 70 eV ionizing voltage.

Animals

Male Fischer rats (235–255 g) were injected intraperitoneally with a suspension of 6-AC-5,6-^{14}C (5·8 μCi) in steroid suspending vehicle* at a dose of 100 mg/kg body weight.

Collection of urine, feces, or bile

The rats were housed in stainless steel metabolism cages (Acme Research Products, Cincinnati, Ohio) designed for separate collection of urine and feces. The urines were collected in ice-cold containers and frozen until used. The feces were first dried in air and then *in vacuo*. For collection of bile the rats were anesthesized with ether and the common bile duct was isolated and cannulated with polyethylene tubing (PE 10). After recovery four rats were intubated with 5 ml of physiological saline solution and placed in restraining cages. 6-AC-5,6-^{14}C (3·1 μCi) was administered intraperitoneally about 30 min later; food and water (saline) were available *ad libitum*.

After 48 hr some of the animals were sacrificed under light ether anesthesia by withdrawal of blood from the abdominal aorta into a heparinized syringe. Selected organs were excised, weighed, and aliquots were homogenized with 4 volumes of water. The isotopic content was determined as described below.

Determination of radioactivity

The radioactivity was measured by liquid scintillation counting, in a Beckman LS-250, with automatic external standardization. Urine, bile and organic extracts were counted in 15 ml of 30% methanol in toluene scintillator solution containing 2,5-diphenyloxazole (PPO) (3 g) and 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP) (0·1 g) per liter.

The feces (5–10 mg, ground) and tissue homogenates (100 μl) were digested in 3 ml of hyamine by shaking overnight at 40–50°C. The samples were counted after the addition of 12 ml of a mixture containing PPO (5 g) and POPOP (50 mg) per liter of toluene.

Thin-layer chromatography

Silica gel plates (Eastman No. 6060 and Baker 1B-E) were used with the following solvent systems: (a) ether-dimethylformamide (95:5); (b) ethyl acetate–methanol–water and acetic acid (60:30:9:1); (c) ethyl acetate–methanol–water–ammonium hydroxide (60:30:9:1), and (d) hexane–chloroform–*p*-dioxane–methanol (120:40:25:15). The t.l.c. were examined under u.v. light and the bands which appeared as dark u.v. absorbing spots were removed, cut out, eluted with methanol and counted by liquid scintillation counting.

High pressure liquid chromatography

A Spectra-Physics Model 3500 chromatograph equipped with a Lichrosorb (10 μm) column (25 cm) was used. Column elution was with a gradient system consisting of (a) isooctane and (b) methylene chloride containing 30% methanol. The gradient was started at 10% B, held for 10 min, followed by 10–40% B in 15 min. The pressure was 160 psi and the flow rate was 0·8 ml/min. Monitoring was by ultraviolet absorption at 280 nm. The peaks of interest were collected and the solvent removed with a stream of nitrogen. The resulting materials were used for mass spectrometer analysis by direct probe, for u.v. spectra and t.l.c.

Extraction and isolation of urinary metabolites

The methods used were adapted from those used in our previous studies on aromatic amine derivatives [16, 17]. Urines were adjusted to pH 6 with dilute HCl, buffered with 0·1 M sodium acetate, and extracted 5 times with equal volumes of ether to remove unconjugated metabolites. The aqueous phase was incubated overnight with β-glucuronidase (400 units/ml) (Sigma, Type II) and again extracted with ether ro remove the liberated metabolites (glucuronides). The resulting aqueous phase was adjusted to pH 1 with concentrated HCl and refluxed for 15 min, cooled, then adjusted to pH 6 with solid $NaHCO_3$. Ether extraction of the mixture removed the compounds which were liberated by this treatment. The ether extracts were taken to dryness on a water bath under a stream of N_2. The residues were dissolved in ethanol and aliquots were applied to t.l.c. plates for separation of metabolites.

Extraction of metabolites from feces

The metabolites in the feces (9 g) were extracted 4 times by mechanical stirring with 80% aqueous ethanol (60 ml). The combined extracts were reduced to a small volume *in vacuo*. The residue was diluted with 30 ml H_2O and 10 ml 0·1 M acetate buffer, pH 6, and fractionated as described for the urine.

Extraction of metabolites from bile

The bile was diluted with 3 volumes of

*Steroid suspending vehicle contains: Polysorbate 80, 0·4%; carboxymethylcellulose, 0·5%; benzyl alcohol, 0·9% in physiological saline solution (0·9% NaCl).

water, adjusted to a pH of 6 with 1 N HCl, and extracted 5 times with an equal volume of ether to remove unconjugated metabolites. The remaining aqueous phase was divided into three portions and treated as follows: (1) incubated with β-glucuronidase at 37°C for 2 hr, (2) incubated without added enzyme at 37°C for 2 hr, (3) the pH was adjusted to 1 with 6 N HCl and the mixture let stand at room temperature for 1/2 hr. After treatments (1–3) the mixtures (where needed) were adjusted to pH 6 and extracted with ether to remove liberated metabolites. Additionally, biles were applied directly to t.l.c. plates for separation of unaltered conjugated metabolites.

Table 3. *Distribution of radioactivity in tissues of rats* 48 *hr after intraperitoneal injection of* 6-*AC*-5, 6-^{14}*C*

Tissues	Tissue concentration (μg/g wet wt.)	Tissue content (% of dose)
Liver	22.4 ± 3.2	0.94 ± 0.1
Kidney	15.2 ± 0.6	0.11 ± 0.01
Spleen	7.9 ± 2.2	0.02 ± 0.01
Heart	2.3 ± 0.4	
Lungs	7.4 ± 1.8	0.04 ± 0.01
Blood	6.6 ± 0.3*	
Plasma	5.0 ± 0.7*	
Stomach contents		0.11 ± 0.03
Intestinal contents		1.0 ± 0.09
Cecum contents		5.9 ± 1.2

The mean values of 4 animals ± the standard error are given.

* Values are expressed as μg/ml.

RESULTS

Table 3 shows the distribution of isotope in selected tissues 48 hr after intraperitoneal injection of 6-AC-5,6-^{14}C. The liver and kidneys showed the highest specific activities, the spleen and lungs showed intermediate values while the blood and plasma had the lowest levels. The total isotopic content of the liver represented 0·94% of the dose while that of the kidneys amounted to only 0·11% of the dose. The ^{14}C-contents of the stomach, intestine and cecum accounted for 0·1, 1·0 and 5·9% of the dose, respectively.

Excretion of metabolites in the urine and feces

During the first 48 hr after a single intraperitoneal injection of 6-AC-5,6-^{14}C, only 6·3% of the dose was excreted in the urine (Table 4). Even after 10 days, only 12% of the dose appeared by this route, indicating that the blood–kidney route is a minor pathway for clearance from the body.

Within the first day after intraperitoneal injection, only about 3% of the dose appeared in the feces (Table 4), while during the second day 22·3% was excreted. Thereafter through 10 days, the output of the isotope decreased, but a total of 56% of the dose was finally excreted via the feces, representing the major route of excretion.

Table 4. *Excretion of radioactivity into urine and feces after intraperitoneal injection of* 6-*AC*-5, 6-^{14}*C*

Days	Number of rats	Urine (% of dose)	Feces (% of dose)
1	6	3.3 ± 0.3	3.2 ± 1.6
2	6	3.0 ± 0.4	22.3 ± 1.7
3	2	1.7 (1.2, 2.1)	9.5 (7.2, 11.5)
4	2	0.9 (0.8, 0.9)	5.3 (4.4, 6.1)
5-6	2	1.1 (1.0, 1.2)	4.5 (3.3, 5.4)
7-8	2	1.1 (1.0, 1.1)	7.3 (5.7, 8.6)
9-10	2	0.9 (0.8, 1.0)	3.9 (3.6, 4.2)
		12.0	56.0

The data are expressed as percent of dose ± standard error for the first 2 days. The means and actual values for 2 animals are given in parentheses for days 3–10.

Excretion of metabolites in the bile

Excretion of 6-AC metabolites in the bile amounted to 16·2 ± 0·5% of the dose in 4 hr; 17·4 ± 2·5% from 4–24 hours; 8·5 ± 0·9% for 24–48 hr. Excretion was fairly rapid during the first 4 hr. However, although the rate slowed appreciably thereafter, the total output of radioactivity in bile over a 48 hr period amounted to 42·1 ± 2·6% of the dose.

Urinary and fecal metabolites

The metabolites in the urine were further classified as shown in Table 5. Free, uncon-

Table 5. *Fractionation of urinary and fecal metabolites*

	% of urinary or fecal ^{14}C				
	Extractable	Free	Glucuronides	Sulfates	Water-soluble
Urine*	---	8.8 ± 0.3	22.7 ± 2.9	31.4 ± 3.4	35.0 ± 3.1
Feces†	53.9 ± 4.1	43.9 ± 3.6	0.48 ± 0.03	2.6 ± 0.3	6.1 ± 0.2

* A 48-hr collection of urine was used. Values are means ± standard errors of 6 rats.

† Feces from the second day of collection from 6 rats were used.

jugated metabolites, accounted for 8·8% of the urine-^{14}C, the glucuronide conjugates were 22·7%, and 31·4% was acid-hydrolyzable (sulfates). A significantly large amount, 35% was non-hydrolyzable or non-extractable "water-soluble" metabolites. The glucuronide fraction was applied to t.l.c. plates in solvents A and D, and revealed a major metabolite with Rf 0·20 and Rf 0·10 respectively (Table 2). High pressure liquid chromatography of the glucuronide fraction showed the presence of one major peak with a retention time of 17·2 min. Mass spectrometry of this metabolite showed a molecular ion at M/e 301 with major fragment

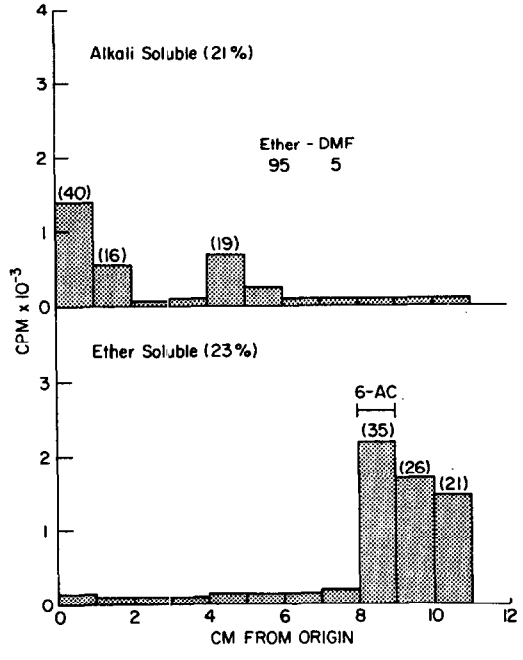


Fig. 1. *Radiogram from t.l.c. plate of extractable unconjugated fecal metabolites of* 6-*AC* (*solvent system A*).

ions at M/e 259, 231 and 215 (Table 2). The spectrum and retention time on HPLC were identical to that of synthetic 12-hydroxy-6-acetylaminochrysene.

Extraction with 80% aqueous ethanol rendered 53·9% of the fecal-^{14}C extractable; almost 44% was composed of free metabolites (Table 5). Glucuronides and sulfates accounted for only 0·48 and 2·6%, respectively, and "water-soluble" metabolites for 6·1%. The free metabolites were fractionated further into ether and alkali-soluble compounds. A t.l.c. radiogram is shown in Fig. 1. The ether-soluble fraction showed 3 major components which accounted for (a) 35, (b) 26 and (c) 21% of the activity. The Rf of component A agreed precisely with that of authentic 6-AC (Rf 0·72). The two faster moving components in fractions 10 and 11, Rf 0·80 and 0·87, respectively, have not been identified.

The alkali soluble fraction showed the presence of three major components which were present in fractions 1, 2 and 5. The metabolite in fraction 5 was further characterized by elution of the band from t.l.c. plates with methanol. T.L.C. in solvent A gave a single spot with Rf 0·20. The mass spectrum showed the molecular ion peak at M/e 301 and major fragment ions at M/e 259, 231 and 215 which corresponded to a 12-hydroxy-acetylamino derivative. Further t.l.c. confirmed the identity of this material with synthetic 12-hydroxy-6-acetylaminochrysene.

Biliary metabolites

Aliquots of bile from the various collections were applied to t.l.c. plates of silica gel and developed with solvent system C. The major metabolite, Rf 0·20, accounted for 76 and 73% of the radioactivity in the 0–4 and 4–8 hour collections, respectively (Table 6), but during the 8–24 hour period it decreased to 42%.

Table 6. *Major biliary metabolites of* 6-*AC*-5, 6-^{14}*C*

Rf values	Collection time (h) 0-4	4-8	8-24	0-24	Reference compound	Naphthoresorcinol reagent
	% of ^{14}C					
0.81	---	---	----	----	6-AC	
0.20	76	73	42	58		blue
0.06	4.8	5.6	13	6.9		--
origin	3.1	4.3	23	27		--

T.L.C. was carried out in solvent C as described in Methods. The glucuronide was detected by spraying the developed chromatograms with naphthoresorcinol, 1% (wv) solution in acetone diluted ×4 with 10% phosphoric acid immediately before use and heating the t.l.c. plate at 120°C for 10 min.

This metabolite gave a blue color with naphthoresorcinol indicating the presence of a glucuronic acid conjugate. Two other spots with significant radioactivity, Rf 0·06 and at the origin, increased with time. Bile at the various time periods did not show any detectable free 6-AC (Rf 0·81). A combined collection from 0–24 hr showed that the glucuronide, Rf 0·20, now represented only 58% of the ^{14}C, while the amount of isotope at the origin was increased, accounting for 27% of the radioactivity.

Bile from 0–24 hr was used to check the degree of hydrolysis of biliary metabolites under various conditions (Table 7). At pH 1 and at pH 4 almost 50% of the ^{14}C was ether-extractable. However, less was extractable as

*Table 7. Hydrolysis, extractability and identity of biliary metabolites**

pH	1	4	6	7	7	7	
Time (hr.)	0.5	0.5	0.5	0.5	2	2	
Temperature	25	25	25	25	37 (with glucuronidase)	37	
% Ether extractable	49	43	12	2.4	25	16	
Ether-soluble metabolites	% of radioactivity						
Rf values							Identity
0.80	3.8	2.6	---	---	19	6	
0.72	83	80	---	---	54	72	6-AC
origin	4.8	8.4			12	10	

* A 24-hr collection of bile was used. T.L.C. was carried out in solvent A.

the pH increased so that at pH 7 only 2% was ether soluble. Incubation of samples of bile with and without β-glucuronidase at 37°C for 2 hr rendered 25 and 16% of the ^{14}C ether-extractable, respectively. A sizable portion of the material extractable at pH 1 or pH 4 showed a spot, Rf 0·72, on t.l.c. in solvent A, that corresponded exactly to that of authentic 6-AC (Table 7). The sample without β-glucuronidase showed that 72% of the extractable material was 6-AC, comparable to results of the pH 1 and 4 groups. However, after β-glucuronidase treatment, only 54% of the ether-soluble material corresponded to 6-AC. A second spot, Rf 0·80, which accounted for 19% of the fraction may possibly have been an oxidation product of 6-AC or another metabolite. Under all conditions, there was also some radioactivity which remained at the origin.

Facile oxidation of 6-aminochrysene

Allowing methanol or ethanol solutions of 6-AC to stand at room temperature and exposed to light overnight led to formation of a red crystalline compound, m.p. 233–235°C, Rf 0·75, on 0·25 mm t.l.c. silica gel plates (solvent A). Mass spectral data showed a molecular ion of 258 with major fragments at 230 (base peak) and 202. The spectrum and melting point are compatible with that of 5,6-chrysenedione, reported melting point 235°C [18]. The high resolution mass spectrum (solid probe, 160°C, 70 eV) showed that although the major component was the chrysenedione ($C_{18}H_{10}O_2$), there was also a small amount of a quinoneimine present ($C_{18}H_{11}NO$).

DISCUSSION

Our preliminary study with 6-AC-12-^{3}H (Table 1) showed that about 60% of the urinary radioactivity was due to $^{3}H_2O$, indicating that a fairly substantial exchange of the 12-^{3}H with body water occurred. Thus the value of 6-AC-12-^{3}H as a tracer for metabolism experiments lasting more than a few hours was lost. Loss in biliary water, reflecting the initial pass through the liver was only 2%. However, loss of urinary activity in $^{3}H_2O$, a measure of further passes through the systemic circulation, and perhaps metabolism by intestinal flora, was very large, introducing a high degree of uncertainty. For these reasons our more definitive work was done with the ring labeled material.

From the definitive study, it appeared that the principal route of excretion of 6-AC-5,6-^{14}C after an i.p. dose was by the feces. Although most of the dose was thus recovered in 3 days, significant levels of radioactivity appeared in the feces for 10 days, finally accounting for 56% of the dose. It was quickly evident that 6-AC is not solubilized readily in the body. This water-insoluble nature and consequently more lipid-soluble characteristic may perhaps explain why 6-AC is somewhat active against breast cancer [8, 9]. Due to its lipid-solubility it would presumably deposit in tissues containing fair amounts of fat, such as the breast. The relatively slow rate of excretion might be due either to a slow rate of absorption from the intraperitoneal cavity or extensive enterohepatic circulation of metabolites. The latter explanation may be more logical since biliary execretion of isotope in rats with cannulated bile ducts accounted for 42% of the dose during the first 48 hr.

The fecal metabolites were more numerous than those in bile, and they were predominantly unconjugated in contrast to those in bile which were chiefly glucuronides. Besides 6-AC several unidentified more polar metabolities were present. One of these has been character-

ized as 12-hydroxy-6-acetylaminochrysene. It is of interest that the feces showed significant amounts of hydroxylated and acetylated metabolites while bile contains predominantly the *N*-glucuronide of 6-AC. This could suggest possible sites of hydroxylation and acetylation within the gastrointestinal tract. Further studies on the intraduodenal and intracecal metabolism of 6-AC and its *N*-glucuronide may help to resolve these questions. In addition, the contribution of the intestinal flora should not be overlooked.

In the bile, one metabolite which represented 75% of the biliary radioactivity, apparently was a glucuronic acid conjugate which yielded 6-AC after incubation with and without β-glucuronidase or on standing at room temperature at an acid pH. Because there are neither hydroxyl- nor carboxyl groups available for O-glucuronide conjugation, only *N*-glucuronide formation would account for these observations.

N-Glucuronides as metabolites have been well documented in the literature [19–22]. The enzymatic synthesis of aniline *N*-glucuronide by microsomal preparations in the presence of uridine diphosphate glucuronic acid was demonstrated [23]. At 37°C the aniline conjugate was extensively hydrolyzed within 30 min at pH 5 and 6; in addition, in the presence of mammalian or bacterial β-glucuronidase, the rate of hydrolysis was no greater than that with boiled enzymes.

The glucuronic acid conjugate in the bile of 6-AC injected rats showed similar properties. Due to the insolubility of 6-AC we were unable to synthesize the *N*-glucuronide by the standard techniques and variations thereon which were efficacious for other amines [24, 25]. The presence of other more polar metabolites in bile was evident by the radioactivity which remained at the origin. In addition, there was an unidentified β-glucuronidase sensitive metabolite (Rf 0·80) which appeared to be conjugated with glucuronic acid.

The urine was a relatively minor pathway for the excretion of 6-AC metabolites. There was no evidence for the presence of the *N*-glucuronide in urine, which is to be expected in view of the compound's lability at the pH of normal urine. Small quantities of free 6-AC were found which may be a reflection of this fact. Other conjugated metabolites, glucuronides and sulfates, were present in urine. 12-Hydroxy-6-acetylaminochrysene was present in the glucuronide fraction as shown by chromatography and by mass spectrometry. The molecular ion peak at M/e 301 and the major fragment ions at M/e 259, 231 and 202 were similar to those of the compound found in feces. No evidence for hydroxylation on the nitrogen was found as has been described for *N*-2-fluorenylacetamide and other aromatic amines [26].

It is possible that with repeated dosing the levels of an N-hydroxy metabolite might increase to detectable levels. Repeated administration of carcinogenic aromatic amines usually causes an appreciable increase in the levels of N-hydroxy derivatives [27] presumed to be activated metabolic intermediates. On the other hand, 6-AC may be carcinogenic in mice because of its polynuclear aromatic structure, more like a hydrocarbon than the usual aromatic amine.

Overall, relatively few metabolites of 6-AC were excreted, compared with the large number reported from the metabolism of other aromatic amines such as 2-naphthylamine or *N*-2-fluorenylacetamide. Based on analogy with other amines, there could be at least 4 separate hydroxylated metabolites (at the N, 5, 9 and 12 positions) if all the more reactive positions were attacked. The relative insolubility of 6-AC may be a partial explanation.

Work with 6-AC was complicated by the facile oxidation of the parent compound to a quinone, presumably 5,6-chrysenedione. Previously Brill and Radomski [28] had identified an aminonaphthoquinone-naphthylimine as a photo-oxidation product of 2-naphthylamine. To our knowledge, however, there are no previous reports on complete removal of the amino group of an aromatic amine under such mild conditions as occurred with 6-AC. The ^{15}N-labeled material would be required for definitive studies on the kinetics of this photo-oxidation reaction.

Acknowledgements—The encouragement and co-operation of Dr. G. Lambelin and Dr. R. Roncucci of Continental Pharma Research Laboratories in the initiation of this project were invaluable. Tritiated 6-AC for the initial study was provided by them. The technical and editorial efforts of Mrs. A. Parker and Mrs. F. Williams are gratefully acknowledged.

REFERENCES

1. N. P. Buu-Hoi, D-P. Hien and P. Mabille, Some biological properties of 6-aminochrysene and its derivatives, a family of carcinostatic compounds devoid of cytotoxic action. In *Cancer Chemotherap. Proc. Takeda Int. Conf., Osaka*, p. 71. (Edited by A. Goldin). Maruzen Co., Tokyo, Japan (1966).

2. J. Gelzer and P. Loustalot, Chrysenex in experimental advanced mammary cancer. *Europ. J. Cancer* **3,** 79 (1967).
3. G. Rudali, N. P. Buu-Hoi and A. Lacassagne, Sur quelques effets biologiques du 2-amino-chrysene. *C. R. Acad. Sci.* **236,** 2020 (1953).
4. G. Lambelin, G. Mees and N. P. Buu-Hoi, Chronic toxicity studies of 6-aminochrysene in the rat. *Arzneim.-Forsch. (Drug. Res.)* **17,** 1117 (1967).
5. M. Payet, M. Sankale, O. Bao, A. Bourgeade and A. M. Sow, Le traitement des splenomegalies chroniques communes de l'Africain par le 6-aminochrysene. Premiers resultats. *Bull. Acad. Nat. Med. Paris* **147,** 279 (1963).
6. N. P. Buu-Hoi, Bui-Quoc-Huong and Tran-Lu-Y, Action leucopeniante du 6-aminochrysene dans les leucemies myeloides chez l'homme. *Chemotherapia (Basel)* **4,** 413 (1962).
7. R. Hugonot, Place du 6-aminochrysene dans le traitement des leucemies chroniques. In *Proceedings 3rd Internatl. Congr. Chemotherapy, Stuttgart, Germany, July* 22–27, 1963. (Edited by H. P. Kuemmerle and P. Preziosi) Vol. II, p. 1068. Georg Thieme, Stuttgart (1964).
8. H. J. Tagnon, A. Coune, S. Garattini, R. Rosso, G. Lambelin, M. Gautier and N. P. Buu-Hoi, The antitumoral activity of some derivatives of 6-aminochrysene. *Europ. J. Cancer* **6,** 81 (1970).
9. Groupe Europeen du Cancer du Sein, Induction par le 6-aminochrysene de remission du cancer du sein en phase avancee chez la femme. *Europ. J. Cancer* **3,** 75 (1967).
10. M. G. Donelli and S. Garattini, Stimulatory effect of 6-aminochrysene and congeners on zoxazolamine metabolism. *Biochem. Pharmacol.* **20,** 2049 (1971).
11. F. J. C. Roe, R. L. Carter and S. Adamthwaite, Induction of liver and lung tumours in mice by 6-aminochrysene administered during the first 3 days of life. *Nature (Lond.)* **221,** 1063 (1969).
12. G. Lambelin, J. Roba, R. Roncucci and R. Parmentier, Carcinogenicity of 6-aminochrysene in mice. *Europ. J. Cancer* **11,** 327 (1975).
13. M. S. Newman and J. A. Cathcart, The orientation of chrysene. *J. org. Chem.* **5,** 618 (1940).
14. Do-Cao Thang, Cao-Xuan, N. P. Buu-Hoi and P. Jacquignon, Synthese de metabolites possibles de l'amino-6-chrysene, substance cancerostatique. *C. R. Acad. Sci. Paris* **272,** 1570 (1971).
15. E. C. Miller, J. A. Miller and H. A. Hartmann: N-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased activity in the rat. *Cancer Res.* **21,** 815 (1961).
16. P. H. Grantham, T. Matsushima, L. Mohan, E. K. Weisburger and J. H. Weisburger, Changes in the metabolism of labelled acetanilide and binding of isotope to serum and liver macromolecules during chronic administration. *Xenobiotica* **2,** 551 (1972).
17. P. H. Grantham, J. H. Weisburger and E. K. Weisburger, Effect of the antioxidant butylated hydroxytoluene (BHT) on the metabolism of the carcinogens N-2-fluorenylacetamide and N-hydroxy-N-2-fluorenylacetamide. *Fd. Cosmet. Toxicol.* **11,** 209 (1973).
18. C. Graebe and F. Hönigsberger, Ueber die Oxydationsproducte des Chrysenes. *Annalen* **311,** 257 (1900).
19. E. Boyland, D. Manson and S. F. D. Orr, The biochemistry of aromatic amines. 2. The conversion of arylamines into arylsulphamic acids and arylamine-N-glucosiduronic acids. *Biochem. J.* **65,** 417 (1957).
20. D. V. Parke, Studies in detoxication. 84. The metabolism of [^{14}C]aniline in the rabbit and other animals. *Biochem. J.* **77,** 493 (1960).
21. J. W. Bridges, M. R. Kirby and R. T. Williams, The structure of the glucuronide of sulphadimethoxine formed in man. *Biochem. J.* **96,** 829 (1965).
22. H. Tsukamoto, H. Yoshimura and K. Tatsumi, Metabolism of drugs. XXXV. Metabolic fate of meprobamate. 3. A new metabolic pathway of carbamate group—the formation of meprobamate N-glucuronide in animal body. *Chem. pharm. Bull. (Tokyo)* **11,** 421 (1963).
23. J. Axelrod, J. K. Inscoe and G. M. Tomkins, Enzymatic synthesis of N-glucosyluronic acid conjugates. *J. biol. Chem.* **232,** 835 (1958).
24. L. Berger and J. Lee, Arylamine-N-glycosides. Part II. Arylamine-N-pentosides and complex salt formation studies. *J. org. Chem.* **11,** 84 (1946).
25. M. Ishidate, S. Takitani and T. Kishi, Arylamine N-glucuronide and its structure. *Chem. pharm. Bull. (Tokyo)* **7,** 291 (1959).

26. J. H. Weisburger and E. K. Weisburger, Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Revs.* **25,** 1 (1973).
27. J. A. Miller, J. W. Cramer and E. C. Miller, The N- and ring-hydroxylation of 2-acetylaminofluorene during carcinogenesis in the rat. *Cancer Res.* **20,** 950 (1960).
28. E. Brill and J. L. Radomski, 2-Amino-1, 4-naphthoquinone-N^4, 2-naphthylimine. A photo-oxidation product of 2-aminonaphthalene. *Experientia* **21,** 368 (1965).

Europ. J. Cancer Vol. 12, pp. 237–238. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

The Interaction of Daunomycin with Normal and Leukaemic Leukocyte DNA

P. J. GRAY and D. R. PHILLIPS
Biochemistry Department, La Trobe University, Bundoora, Victoria 3083, *Australia.*

LEUKAEMIC DNA differs from normal leukocyte DNA in a number of respects. Baxt *et al* [1] have identified DNA sequences in human leukaemic cells that cannot be detected in normal DNA. Silber *et al.* [2] have detected increased methylation of some of the bases of DNA (5-methyl cytosine has been specifically identified) from leukaemic leukocyte DNA. Other differences have also been observed e.g., a higher frequency of oligomeric forms (circular dimers and catenated dimers) of mitochondrial DNA in leukaemic leukocytes [3] and a greater variability in the asymmetry of buoyant density bands of leukaemic DNA [4]. As the antibiotic daunomycin, known to be particularly effective in the treatment of leukaemia, exerts its effect by binding to DNA [5], it is possible that the extent of daunomycin binding to leukaemic DNA may differ from the amount bound to normal DNA because of the differences between leukaemic and normal DNA.

Leukocytes were isolated from an acute myeloblastic leukaemic patient (AML), an acute lymphoblastic patient (ALL) and a normal donor by the use of a continuous flow cell separator. The DNA was extracted by the method of Marmur [6], modified by including a pronase treatment after the first ethanol precipitation. No significant differences were detected between any of the DNAs when characterised according to their buoyant density in CsCl ($1{\cdot}698_7$–$1{\cdot}699_4$ g.cm^{-3}), melting temperature, melting breadth or hyperchromicity.

The binding of daunomycin to DNA was followed by the spectrophotometric method of Blake and Peacock [7], using 5 cm cells. The apparent association constants were determined from regression analysis of data points on the linear section of Scatchard plots (only those data points having similar residuals of consecutive groups of three points were used). This region corresponds to the strong intercalation mode of binding [8]. The binding data obtained in 0·15M NaCl, pH 7, 25°C are tabulated below.

DNA	Number of sites per nucleotide	Apparent association constant (l.mol^{-1})
Normal	$0{\cdot}169 \pm 0{\cdot}009$	$(7{\cdot}4 \pm 1{\cdot}3) \times 10^6$
A.L.L.	$0{\cdot}167 \pm 0{\cdot}009$	$(7{\cdot}3 \pm 1{\cdot}9) \times 10^6$
A.M.L.	$0{\cdot}166 \pm 0{\cdot}006$	$(9{\cdot}0 \pm 3{\cdot}7) \times 10^6$
Calf thymus	$0{\cdot}158 \pm 0{\cdot}003$	$(7{\cdot}2 \pm 1{\cdot}2) \times 10^6$

There is no significant difference between either the number of binding sites or the association constant for any of the DNAs studied. The reason why differences in binding were not detected may be related to the differences existing between the various DNAs. The major differences are the presence of some base sequences unique to leukaemic DNA [1], and the increased methylation of leukaemic DNA [2]. The increased methylation would be anticipated to contribute most to any differences in binding, as the methyl groups protrude into the major and minor grooves of the DNA and would prevent the approach of daunomycin to the intercalation sites. Because no alteration of binding was observed, this suggests that the methylation, although several times

Accepted 21 October 1975.

greater in leukaemic DNA than in normal DNA [2], is not sufficient to alter the binding characteristics by an amount detectable by the method used. It must also be concluded that the base sequences unique to leukaemic leukocyte DNA are not significantly different from the normal leukocyte DNA with respect to their ability to interact with daunomycin, or, if they do bind daunomycin selectively, that they constitute only a minor amount of the total DNA isolated.

The fact that the binding parameters for calf thymus DNA are identical to the leukocyte DNA parameters is direct evidence to support the use of calf thymus DNA as a good representation of leukocyte DNA for future studies of daunomycin–DNA interactions.

Acknowledgements—The daunomycin used was a gift from Farmitalia, Italy and is gratefully acknowledged, as is the use of the continuous flow cell centrifuge at the Peter MacCallum Clinic, Melbourne, Australia. This work was carried out during the tenure of a grant from the Anti-Cancer Council of Victoria.

REFERENCES

1. W. G. Baxt and S. Spiegelman, Nuclear DNA sequences present in human leukaemic cells and absent in normal leukocytes. *Proc. nat. Acad. Sci. (Wash.)* **69,** 3737 (1972).
2. R. Silber, E. Berman, B. Goldstein, H. Slein, G. Farnham and J. R. Bertino, Methylation of nucleic acids in normal and leukaemic leukocytes. *Biochim. biophys. Acta.* **123,** 638 (1966).
3. D. A. Clayton and J. Vinograd, Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leukocytes. *Nature (Lond.)* **216,** 652 (1967).
4. G. Corneo, P. Bianchi, E. Ginelli and E. Polli, Heterogeneity of base composition of DNA extracted from human leukaemic leukocytes. *Europ. J. Cancer* **2,** 307 (1966).
5. J. Bernard, R. P. M. Boiron and U. J. R. Maral, *Rubidomycin*, Springer-Verlag, New York (1969).
6. J. Marmur, A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. mol. Biol.* **3,** 208 (1961).
7. A. Blake and A. Peacock, The interaction of aminoacridines with nucleic acids. *Biopolymers* **6,** 1225 (1968).
8. F. Zunino, R. Gambetta, A. DiMarco and A. Zaccara, Interaction of daunomycin and its derivatives with DNA. *Biochim. biophys. Acta.* **277,** 489 (1972).

Europ. J. Cancer Vol. 12, pp. 239–240. Pergamon Press 1976. Printed in Great Britain

E.O.R.T.C. News

Definition and Function of E.O.R.T.C.

H. J. TAGNON
President E.O.R.T.C., Professor of Oncology,
Service de Médecine et d'Investigation Clinique, Institut Jules Bordet, Brussels, Belgium

THE European Organization for Research on Treatment of Cancer (E.O.R.T.C.) was created to stimulate and coordinate therapeutic research on cancer in Western Europe. It has an administration board, coordinating office, data center and cooperative research groups active in laboratory and clinical research. It is a non-governmental multinational body which is supported by private donations and can function effectively because of financial support from the Euratom to some of its laboratories but also thanks to a grant from the National Cancer Institute, Bethesda, Md., which covers administrative and coordinating activities and has been an indispensible factor of the success achieved so far.

The European Organization for Research on Treatment of Cancer is a European and not a national organization. While it is true that each country of Western Europe has adequate research talents as well as good hospital and laboratory facilities, one important factor for success in many research areas is lacking: this factor is the continental, as opposed to the national, dimension of cancer research as exemplified for instance in the United States. National responsibility for research may be quite enough for certain programs but by and large, training in clinical medicine and research is more successful when carried out in a broader environment. The occasional exchanges of personnel and techniques as they take place among our small European nations are useful but insufficient. At the other extreme, world wide organizations have their special usefulness which is not primarily the training of research personnel or the production of original data and new concepts. In contrast, the rapid circulation of people and exchange of ideas with the resulting cross fertilization of minds and elimination of "parochial" types of planning and working is characteristic of the continental dimension as is well illustrated by medical science in the United States. As the situation is now, much of the research potential of Europe is badly underused. Some of the reasons are the following: the limited resources of a small or medium size nation may not be sufficient for the acquisition of certain types of equipment; despite meetings and symposia, there remains a basic deficiency of communications among scientists, leading to needless duplication of identical research in adjoining countries supported by distinct national foundations. Pharmacological and clinical research require large financial investments, access to several well equipped hospitals, extensive experimental support and early availability of results to achieve speedy progress. The continental dimension of medical performance as well as of training for research has lasting educational value and improves the general level of medical care throughout the continent, thereby making available everywhere the highest quality of medical care.

These factors have been invoked to explain the spectacular progress of medical research and especially clinical medical research in the United States which contribute over 75% of the medical publications of the world. But another factor is the integration and continuous confrontation of multiple disciplines.

This is why the E.O.R.T.C. is committed to the promotion of "biomedical research" understood as a synthetic approach to laboratory and clinical science. Despite many declarations to the contrary, the distinction between basic and applied research appears to be entirely artificial. Progress in medicine results from clinical observation and experimentation as well as from laboratory work. The history of

medicine demonstrates that new important data and concepts originate as often in the clinic as in the laboratory. While it is true that the majority of clinicians are not scientists, there is, nevertheless, a class of clinicians who have developed a scientific approach to clinical medicine and have acquired an experimental turn of mind. These are as scientific and basic in their approach to disease than the laboratory scientist. This type of clinician should be distinguished from the ordinary practitioner and be identified as a "clinical scientist". It is quite useless to oppose laboratory and clinical scientists as is too often done. The truth is that they should work together in biomedical research for better and quicker results. This is why the E.O.R.T.C. promotes the association of laboratory and clinical scientists in definite programs and research projects.

In cancer research, the ultimate aim is to find a treatment for the disease. This will come about more rapidly by constant communication among the workers at all levels than by exclusive reliance on one type of research. Ultimately advances in biology and in medicine are utilized for the treatment of patients and this should be carried out in a strictly scientific manner, with a specific and sophisticated methodology, and considerable mathematical and statistical support. The *clinical trial*, a big and difficult undertaking, represents the culmination of the research effort which mobilizes all biological sciences. From the clinical trial, results of significance flow back to the laboratory worker bringing him new ideas and orientation.

This free flow of communications needs developing in Europe where laboratory workers as well as clinical scientists do not always have the modesty to realize that little can be achieved by proud isolation and dogmatic pronouncements on who will discover the cure of cancer and how it will come about. Actually, the cure of cancer is being discovered every day and the most important contributions so far have come from workers who ignored the distinction between "basic" and "applied" research.

The E.O.R.T.C. is fulfilling a third function: by promoting therapeutic clinical research as a scientific discipline, it responds to the ethical obligation to advance knowledge and improve patient's care. This ethical obligation is binding on the whole medical profession and more especially on clinical scientists working in institutions and hospitals supported by public funds since these make available to them the tools of research.

Europ. J. Cancer Vol. 12, pp. 241–243. Pergamon Press 1976. Printed in Great Britain

The Practice of Cooperative Clinical Trials

M. STAQUET

EORTC Coordinating and Data Center, Institut Jules Bordet, Bruxelles, Belgium and Service de Statistique Médicale, Faculté de Médecine, Université Libre de Bruxelles, Belgium

THERE are more and more multicenter trials being undertaken in the clinical research field because most researchers are now aware of the necessity of carrying out clinical trials with randomized or historical control. Until recently, only 21% of the published clinical trials in cancer therapy were randomized with the inclusion of a control group [1]. The main reason for resorting to historical control or to no control at all is that both the stratified randomization procedure and the treatment of a group with a standard drug or with a placebo are time consuming. Indeed, unless a new treatment is dramatically better than the standard one, the number of patients required to test the null hypothesis of no difference between treatments is so large that it is not possible to run a controlled trial within a reasonable period of time. It is therefore necessary to resort to multicenter trials, where the required number of patients will be available quickly. For rare diseases, there is no other alternative than cooperative trials. In cancer therapy, the multi-institutional approach is even more justified where potentially active drugs, surgery, radiotherapy, immunotherapy and their combinations need rapid and adequate assessment.

MOTIVATION

Successful completion of a multicenter trial rests to a very large extent on the officers (chairmen and secretaries) of the cooperative groups. They must have the ability to assemble investigators with similar interests, skills and facilities. They have to devote a large amount of time to the scientific and administrative tasks required in the functioning of a group. A multicenter trial is not a personal project which will lead to personal publication. A cooperative trial will create an extra burden for senior clinicians who are generally overloaded with their patients and their own research. In the case of EORTC, no reward is available for the participating investigators in terms of a financial grant to his institution. On the contrary, each participant has to provide his own secretarial help and sometimes even the drugs. Participation in a medically important, well planned scientific experiment is the main motivation of EORTC investigators.

PROBLEMS

The two main problems encountered in the work of an international clinical cooperative group are the development of effective protocols and forms and the functioning of a managerial organization [2–9].

Protocol design is usually a complicated undertaking. Most investigators participating in a cooperative trial have their own ideas based on past experience on selection of patients and on modalities of treatment. They usually differ from each other. There are only two attitudes to take in this case. One is to consider all ideas of the participants and decide on a compromise plan incorporating the best aspects of each. This will result in an extremely complex protocol which cannot be easily implemented. Such protocols are self-destroying since more and more participants will ignore the most complex rules as time passes. The other is to restrict the study to less ambitious goals, but to define them with great precision. This will indeed result in some clinicians refusing to participate, but it will provide clear and quick answers to simple, well formulated questions.

The managerial organization is at the present time provided by the EORTC Coordinating and Data Center. The center provides services to the groups at three levels: in the design of the trial, in the follow-up of the patients and in the final analysis of the data.

DIFFICULTIES

The presence in a cooperative group of people from different disciplines sometimes makes the definition of the goal of the study too vague or too ambitious. This can lead to protocols with too many stratifications and too many treatments and imposition of impractical rules ("octopus protocols").

Careful management is required because cooperative trials are difficult to carry out due to the complexities of the logistics. There are long distances between investigators and language barriers which will easily disrupt communication between them. Patients must be handled in a uniform manner in order to obtain unbiased results. Table 1 shows the difficulties which might arise at each step of the implementation of a protocol.

Table 1. *Difficulties in EORTC cooperative trials*

Trials' path	Difficulties	
EORTC Clinical Cooperative Group	Language	
↓	Multidisciplinarity	
Group's Protocol Committee and Coordinating and Data Center	"Octopus" protocol	
	Leadership	
↓	Drug availability	
	Feasibility	
EORTC review committee	Time lag	
↓		
Trial participants	Motivation	Ethics
	Time	Cohesion
↓	Power	Qualification
	Accrual rate of patients	
EORTC Coordinating and Data Center	Rejection of data	
↓	Resources	
Publication	Authorship	

At the group level, chairmen and secretaries should be elected on the basis of enthusiasm and scientific quality rather than prominence. Nothing is more deleterious for a group than members considering them as little learning societies where to report personal experience with a few cases.

As time passes, senior investigators may become uninterested in the trial and give the task of including patients to young, less experienced investigators. This may cause a lack of uniformity in reporting and a "chronology bias" [8] due to a decrease in accrual rate. Delay in sending patients reports to the Data Center is frequent and frequently leads to loss of data.

The rejection of data by the Coordinating and Data Center is usually a difficult decision. It implies the objective judgment not only of the statisticians but also of qualified clinical oncologists.

ADVANTAGES

Clinical cooperative trials, when well prepared and executed, will produce important data on therapies. They will also help to raise the standards of therapy and clinical research because good work attracts good scientists and new ideas. Successful clinical trials will facilitate the multidisciplinary approach within institutions. The self-discipline imposed by a common protocol and by a controlled study will contribute to the medical education of the participants and their colleagues. One of the major disadvantages of cooperative trials is that they minimize credit in the scientific literature since cooperative clinical trials are often reported as group effort.

PROSPECTS

Cooperative clinical trials have contributed to cancer therapy in many fields [9]: substantial progresses have been made in acute lymphocytic and myelogenous leukemia,

Hodgkin's disease, metastatic breast cancer, prostatic cancer, and Wilm's tumor. Many negative but important cooperative studies have been also published, especially in the field of adjuvant chemotherapy. With the increasing complexities of cancer treatment, the development of cooperative groups to test new modalities of treatments has become a necessity. In solid tumors, for instance, there is a lack of adequate data for making logical therapeutic decisions [10]. Large numbers of trials will be necessary to choose the best treatment among the many possible combinations of drugs, surgery and radiation therapy.

REFERENCES

1. T. C. Chalmers, J. B. Block and S. Lee, Controlled studies in clinical cancer research. *New Engl. J. Med.* **287,** 75 (1972).
2. B. G. Greenberg, Conduct of cooperative field and clinical trials. *Amer. Statist.* **13,** 13 (1959).
3. L. Herrera, The organization of cooperative effort by multiple hospitals, exemplified by testing of therapeutic agents in rheumatoid arthritis. *Meth. Inform. Med.* **1,** 6 (1962).
4. J. W. Kuzma, Planning and management aspects of cooperative trials. *J. clin. Pharmacol.* **10,** 2 (1970).
5. B. G. Greenberg, Maintaining interest among members of cooperative clinical study group. *Cancer Chemother. Rep.* **14,** 189 (1970).
6. M. J. R. Healy and R. Saracci, Report of a symposium sponsored by the UICC on principles and practice in clinical trials, *Int. J. Cancer* **8,** 541 (1971).
7. L. V. Foye, Personal communication.
8. A. R. Feinstein, Sources of "transition bias" in cohort statistics. *Clin. Pharmacol. Ther.* **12,** 704 (1971).
9. W. C. Levin, D. J. Fink, S. Porter, T. Hall and V. Loeb, Cooperative clinical investigation. A modality of medical science. *J. Amer. med. Ass.* **227,** 1295 (1974).
10. S. K. Carter and W. T. Soper, Integration of chemotherapy into combined modality treatment of solid tumors. *Cancer Treat. Rev.* **1,** 1 (1974).

Europ. J. Cancer Vol. 12, pp. 245–246. Pergamon Press 1976. Printed in Great Britain

The Data Center of the European Organization for Research on Treatment of Cancer

STEPHEN L. GEORGE
EORTC Data Center, Institut Jules Bordet, Rue Héger-Bordet, 1, 1000 *Bruxelles, Belgium*

I. HISTORY

ALTHOUGH the European Organization for Research on Treatment of Cancer (EORTC) has been in existence since 1962, there was originally no official organization to which member groups could turn for statistical advice and assistance. Each of the cooperative groups was forced to make arrangements for data collection and processing and statistical analyses as best it could. As a result, although several of the groups were fortunate enough to locate competent statistical centers, many other groups had inadequate or non-existent statistical support. Thus it was felt that the establishment of a central Data Center with a competent professional staff and with responsibility *solely* to the EORTC, would provide for efficient statistical services in the most economical manner possible. A similar concept of statistical support of cooperative clinical group activity in the United States led, in the 1950s, to the establishment of centralized statistical centers to provide support for the statistical needs of each regional group. Since the EORTC has structures and purposes similar to the regional cooperative oncology groups in the United States, an attempt was made to model the EORTC Data Center after the statistical centers of these groups, hopefully adapting the better aspects and programs of the best centers to the special needs of the European organizations. An obvious advantage of this approach is that over twenty years of experience and expertise at similar operations can be exploited, and with little cost, to the advantage of the EORTC.

The EORTC Data Center was originally conceived as an extension of the activities of the EORTC Coordinating Office of Brussels, which has been in existence since 1969, headed by a "cordinator", Dr. Maurice Staquet, but which was too small to provide the extensive statistical services required. In October 1972, an application for a grant renewal of the existing research grant funding the Coordinating Office was submitted to the U.S. National Cancer Institute (NCI) by Dr. Henri Tagnon, principal investigator on the original grant, and currently, the President of the EORTC. The renewal application was essentially a proposal to expand the Coordinating Office by providing for the establishment of an EORTC Data Center to "receive, review, process, and analyze data for all clinical studies conducted by all EORTC cooperative groups." There was a site visit by members of the Cancer Clinical Investigation Review Committee (CCIRC) in March, 1973, and the grant renewal application was subsequently approved by the NCI Council for two years, with funding to start on November 1, 1973. In June, 1975, another renewal application was approved, extending the funding through October 31, 1978, after which time support will come entirely from European sources.

The EORTC Data Center actually began operation on January 15, 1974, with the appointment of three persons: a director, a statistician, and a data clerk/secretary. Two positions (data manager, computer analyst) were filled later in the year. The approval of the new grant request with its expanded budget will allow for three new positions to be filled (statistician, programmer, computing assistant) bringing the total staff to eight persons by the end of the year.

II. OBJECTIVES

The purpose of the EORTC Data Center is to make available to the cooperative research groups of the EORTC a wide range of statistical and data processing expertise, including, but not limited to, advice on the design of studies, assistance with the writing of protocols, development of data handling and processing procedures, as well as the statistical analysis and reporting the results to these groups. Its goal is to improve the quality of the studies conducted by the groups that avail themselves of its services by becoming involved in the activities of the group from the early planning stages of a study to the final report of the results of the study.

In the planning stage of a study, the Data Center assists in the selection of an appropriate experimental design, estimates the required size and duration of the study, collaborates on the writing of protocols, designs data forms, and works out procedures for collecting and forwarding the data.

Once the study has begun, the Data Center provides for central registration and randomization (if required), receives and edits the data forms, maintains an up to date computer file on each patient, sends requests for missing or overdue forms, and produces periodic administrative reports and statistical analysis of the study. It should be noted here that all patient records are kept strictly confidential and no report or analysis of any study are released without the express consent of the group involved.

III. CURRENT STATUS

Throughout 1974 and through the first half of 1975, there was a rapid increase in the number of cooperative groups availing themselves of the services of the Data Center. Today, there are thirteen EORTC cooperative groups working with the Center: ten with currently active clinical trials and three other groups with trials in the planning stage. There are twenty three different active clinical trials (eight of which were started prior to 1974), one trial which has been closed, and one tumour registry which was started in 1968. Five additional trials and one registry are still in the planning stage. Planning is also underway for replacement studies of many of the currently active studies.

There has been a total of approximately 3100 patients registered on these studies: 1500 on one of the 23 clinical trials and 1600 on the one tumour registry. Approximately 1800 of the 3100 patients are still on study and under active follow-up. The current new patient entry rate onto these studies is approximately 1000 patients per year and is certain to increase once the new trials are activated.

IV. SUMMARY

In the 24 months that the EORTC Data Center has been in operation, the groundwork has been laid for the provision, by an organization devoted full-time to the EORTC, of a wide range of statistical and data processing capabilities oriented to the special problems arising from clinical data. There have been the usual "growing pains" experienced by any new and expanding organization, but these have been minimized by a borrowing of experience, "hardware" and "software" from existing statistical centers.

Perhaps the primary achievement of the EORTC Data Center has been the development of working relationships with the thirteen cooperative groups. The total cost and effort involved in developing similar capabilities for each group at thirteen different centers would have been far in excess of the actual cost through the Data Center.

The goal of improving the quality of the studies of these groups has been partially realized through the planning of studies and the provision of efficient data processing and sophisticated statistical analysis. Further improvements are anticipated through an increased awareness, on the part of clinical investigators, of the value of a reliable and experienced data center devoted to the special problems arising in clinical research.

Europ. J. Cancer Vol. 12, pp. 247–248. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *March, 1976*

Human Cancer

J. E. de Vries and P. Rümke: Tumor-associated lymphocyte cytotoxicity superimposed on 'spontaneous' cytotoxicity in melanoma patients.

H. L. Sulit, S. H. Golub, R. F. Irie, R. K. Gupta, G. A. Grooms and D. L. Morton: Fetal calf serum- and human serum-grown human tumor cells; Influences on the tests for lymphocyte cytotoxicity, serum blocking and serum arming effects.

M. M. Urist, A. W. Boddie, Jr., E. C. Holmes and D. L. Morton: Capillary tube leukocyte adherence inhibition: An assay for cellular-mediated immunity in cancer patients.

C. H. Law, N. E. Day and K. Shanmugaratnam: Incidence rates of specific histological types of lung cancer in Singapore Chinese dialect groups and their aetiological significance.

S. Tokudome and M. Kuratsune: A cohort study on mortality from cancer and other causes of death among workers at a metal refinery.

F. K. Nkrumah and I. V. Perkins: Relapse in Burkitt's lymphoma.

G. W. Bornkamm, H. Stein, K. Lennert, F. Rüggeberg, H. Bartels and H. zur Hausen: Attempts to demonstrate virus-specific sequences in human tumors: IV. EB viral DNA in European Burkitt's lymphoma and immunoblastic lymphadenopathy with excessive plasmacytosis.

B. Christenson and Å. Espmark: Long-term follow-up studies on herpes simplex antibodies in the course of cervical cancer. II. Antibodies to surface antigen of herpes simplex virus infected cells.

P. Goss and P. G. Parsons: Temperature-sensitive DNA repair of ultraviolet damage in human cell lines.

Experimental Cancer

E. Ruoslahti, E. Engvall, M. Vuento and H. Wigzell: Monkey antisera with increased specificity to carcinoembryonic antigen (CEA).

J. Bruce, N. A. Mitchison and G. R. Shellam: Studies on a Gross-virus-induced lymphoma in the rat. III. Optimisation specificity and applications of the *in vitro* immune response.

S. O. Prather and R. N. Lausch: Kinetics of serum factors mediating blocking, unblocking, and antibody-dependent cellular cytotoxicity in hamsters given isografts of PARA-7 tumor cells.

T. Sairenji, T. Katsuki and Y. Hinuma: Cell-killing by Epstein–Barr virus: Analysis by colony inhibition procedure.

R. McDonald, B. Thakkar, L. G. Wolfe and F. Deinhardt: Characteristics of three strains of feline fibrosarcoma virus grown in cat and marmoset monkey cells.

E. L. Springer, A. J. Hackett and W. A. Nelson-Rees: Alteration of the cell membrane architecture during suspension and monolayer culturing.

P. Nettesheim and M. L. Williams: The influence of vitamin A on the susceptibility of the rat lung to 3-methylcholanthrene.

British Journal of Cancer *March, 1976*

H. B. Hewitt, E. R. Blake and A. S. Walder: A critique of the evidence for active host defence against cancer based on personal studies of 27 murine tumours of spontaneous origin.

M. G. Lewis, J. W. Proctor, D. M. P. Thomson, G. Rowden and T. M. Phillips: Cellular localisation of immunoglobulin within human malignant melanomas I, 2.

Sudha G. Gangal, B. P. Gothoskar, Chitra S. Joshi and S. H. Advani: Demonstration of cellular immunity in chronic myeloid leukemia migration inhibition assay.

A. T. Ichiki, K. L. Wenzel, Y. P. Quirin, R. D. Lange and J. Eveleigh: Immunochemical studies of carcinoembryonic antigen (CEA) variants.

N. Matthews, P. J. Chalmers, G. R. Flannery and R. C. Nairn: Characterization of cytotoxic spleen cells and effects of serum factors in a syngeneic rat tumour system.

S. Menard, M. I. Colnaghi and M. Boiocchi: Relationship between antigenicity and morphology of murine lung adenomata.

J. S. Senn, P. H. Pinkerton, G. B. Price, T. W. Mak and E. A. McCulloch: Human preleukaemia cell culture studies in siderblastic anaemia.

J. S. Wishnok and M. C. Archer: Structure–activity relationships in nitrosamine carcinogenesis.

John Meier, Mary S. Coleman and John J. Hutton: Adenosine deaminase activity in peripheral blood cells of patients with hematologic malignancies.

P. R. Twentyman and N. M. Bleehen: The sensitivity to cytotoxic agents of the EMT6 tumour *in vivo* comparative response of lung nodules in rapid exponential growth and of the solid flank tumour.

R. L. Fenichel, F. J. Gregory and H. E. Alburn: Antitumor and antimetastatic activity of 3-(P-chlorophenyl)-2, 3-dihydro-3-hydroxy-thiazolo (3, 2-*a*) benzimidazole-L-acetic acid (Wy-13, 876).

H. Horn, I. Erlichman and I. S. Levij: Antitumour efficacy of calusterone against DMBA-induced rat mammary adenocarcinoma *in vivo* and in organ culture.

Brief Communication

J. R. W. Masters, K. Sangster, R. A. Hawkins and A. A. Shivas: Elastosis and estrogen receptors in human breast cancer.

Letters to the Editor

P. Alexander: Surveillance against neoplastic cells—is it mediated by macrophages?

M. Kawai, J. Rosenfield, P. McCulloch and B. L. Hillcoat: Blood levels of 5-fluorouracil during intravenous infusion[1].

R. H. Whitehead: Implications of the osmolalities of some commonly used tissue culture media.

C. S. Potten: Cellular interrelationships and tissue homeostasis—Report on Proceedings of the Tenth Paterson Symposium: September 21/23 1975.

Europ. J. Cancer Vol. 12, pp. 249–254. Pergamon Press 1976. Printed in Great Britain

Effects of *cis*-Dichlorodiammine Platinum (II) on DNA Synthesis in Kidney and Other Tissues of Normal and Tumour-Bearing Rats

DAVID M. TAYLOR, KENNETH D. TEW and JULIE D. JONES
Radiopharmacology Department, Biophysics Division, Institute of Cancer Research, Sutton, Surrey SM2 5PT, U.K.

Abstract—*The induction of renal tubular damage is the dose-limiting toxic effect in the treatment of human cancer with* cis-*dichlorodiammine platinum (II)* (cis-*DDP*). *The effects of* cis-*DDP on the rate of DNA synthesis in the renal tubular epithelium has been studied in normal rats and in animals in which the rate of renal tubular DNA synthesis has been stimulated by administration of folic acid. In normal kidneys the rate of DNA synthesis is reduced to one twentieth of the control rate at 24 hr after 4 mg* cis-*DDP/kg but thereafter recovers rapidly and at 3 and 7 days after treatment the rate of DNA synthesis is about 16 times greater than normal. Administration of* cis-*DDP concurrently with, or up to 12 hr after, folate almost completely abolishes the folate-stimulated increase in renal DNA synthesis. Administration of the drug between 1 and 9 days before folate administration reduces the folate response to one quarter to one half the level observed in non-drug treated animals. Studies of the effect of the drug on DNA synthesis in a transplanted tumour and in intestinal mucosa of non-folate treated rats shows that in all tissues there is a five to seven fold reduction in the rate of DNA synthesis at 24 hr after 4 mg* cis-*DDP/kg but by 72 hr the rate of DNA synthesis in intestinal mucosa is increased above normal level while that in the tumour is still markedly depressed. There appears to be no simple correlation between the effect on DNA synthesis and the uptake of* cis-*DDP in the tissues.*

INTRODUCTION

THE INVESTIGATION of the anti-tumour activity of the platinum coordination compound *cis*-dichlorodiammine platinum (II) (*cis*-DDP) in animals and man has shown that the most serious toxic side effects are damage to the renal tubules, which is the dose-limiting tissue in man, the gastrointestinal epithelium and the bone marrow [1]. It is, therefore, of considerable interest to obtain further information concerning the comparative effects of the drug on events related to cell proliferation in the tumour cell and in these critical normal tissues. The object of this paper is to describe the results of studies of the effects of *cis*-DDP on DNA synthesis in kidney, gastrointestinal mucosa, bone marrow and liver of normal and tumour-bearing rats. An important factor in relation to the dose-limiting toxic effects on a critical normal tissue is the ability of the tissue to repair the drug-induced damage by cell proliferation or other means. A sharp burst of cell proliferation is induced in the renal tubular epithelium of experimental animals by administration of large doses of folic acid [2] and this system can be used to study the effects of *cis*-DDP, or other cytotoxic agents, on the ability of the kidney to respond to a subsequent proliferative stimulus.

This paper describes the results of studies of the effects of *cis*-DDP on the ability of folic acid to stimulate DNA synthesis in the kidneys of both normal and tumour-bearing rats. The effects of the drug on DNA synthesis in the tumour and some normal tissues has also been investigated and attempts have been made to correlate the effects observed with the uptake and retention of *cis*-DDP in the various tissues studied.

Accepted 26 November 1975.

MATERIAL AND METHODS

Animals

The animals used were rats of the inbred August strain which were 4–6 months old at the start of each experiment. Some animals carried a non-metastasizing, transplantable carcinoma of renal origin (BICR-A.15) which had been implanted subcutaneously into the flank 14–21 days before the start of the experiment. Tumour size was assessed *in situ* by measuring three diameters with a vernier caliper and converted to tumour weight by means of an experimentally determined calibration curve.

Folate induction of DNA synthesis

To initiate a burst of DNA synthesis in the renal tubules, normal or tumour-bearing rats were given a single intraperitoneal injection of 250 mg folic acid per kg body weight as a solution containing 50 mg folate/cm^3 0·3M sodium bicarbonate. The rate of DNA synthesis in the kidney was measured at a standard time of 26 hr after folate injection [3].

Measurement of DNA synthesis

For the measurement of the rate of DNA synthesis each animal received 500 μCi/kg of ^{3}H-thymidine (Radiochemical Centre, Amersham, England: Specific activity 20–30 Ci/mmole) by i.p. injection and was killed 60 minutes later. Tissues for analysis were dissected out, frozen in solid CO_2 and subsequently homogenised in 0·2M perchloric acid. Samples of intestinal mucosa were obtained from approximately 15 cm lengths of small intestine which were washed free of contents with ice-cold isotonic saline, opened longitudinally and the mucosa scraped off with a scalpel. DNA was extracted by a modified Schmidt–Thannhauser procedure described previously [3] and the DNA concentration was measured by the method of Dische as modified by Giles and Myers [4]. Radioactivity due to ^{3}H was measured by liquid scintillation counting using an emulsion scintillant (3), each sample being counted for 10 min or until at least 10^4 counts had been recorded.

The specific activity of DNA, expressed as the percentage of the administered ^{3}H per mg DNA, one hr after injection of ^{3}H-thymidine was used as an indicator of DNA synthesis.

Cis-*DDP*

The drug in these studies was administered intraperitoneally as a freshly prepared solution in isotonic saline. In the experiments in which the drug was administered more than 72 hr before the measurement of DNA synthesis, a non-drug treated, pair-fed control group of animals was included in the series. These animals received the same weight of food each day as was consumed by the drug treated group on the previous day. DNA synthesis was measured in the pair-fed group 24 hr later than in the other groups in the experiment.

RESULTS

In normal animals the administration of 250 mg folate/kg produces an approximately twentyfold increase in the rate of DNA in the renal tubules when this is measured 26 hr later [2]. In tumour-bearing rats the response

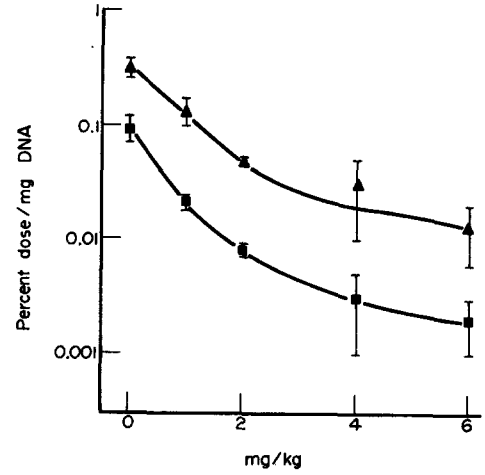


Fig. 1 The effects of different doses of cis-*DDP on the rate of folate-stimulated DNA synthesis 26 hr after folate administration in the kidneys of normal and tumour bearing rats. The ordinate represents DNA specific activity expressed as per cent administered ^{3}H-thymidine per mg DNA, the abscissa shows the dose of drug in mg per kg body weight.* cis-*DDP and folate administered simultaneously.*

▲ *normal rats*
■ *tumour bearing treated rats*

Symbols represent mean of 5 *rats* ± *SEM*

to folate is less marked with the rate of DNA synthesis increasing to only about ten times the level observed in untreated control animals. The effects of *cis*-DDP at dose levels of 1, 2, 4 and 6 mg/kg, administered simultaneously with folate, on the enhancement of the rate of DNA synthesis in rat kidney 26 hr later are illustrated in Fig. 1. These results show that, in both normal and tumour-bearing animals, the folate response is almost completely suppressed by *cis*-DDP at dose levels greater than 4 mg/kg and is markedly reduced at the other dose

levels tested. Histological and autoradiographic studies showed that the changes in DNA synthesis measured at 26 hr after folate were associated with similar reductions in mitotic index and the percent of labelled mitoses observed 48 hr after folate (Table 1).

Table 1. *Effects of* cis-*DDP* (2 *mg/kg*) *on mitotic index, percentage of labelled mitoses and rate of DNA synthesis in folate stimulated rat kidney following simultaneous administration of folate and the drug*

	Folate only	cis-DDP + Folate
Mitotic index %*	1·7 ± 0·1	0·5 ± 0·1
% Labelled mitoses*	60	20
DNA synthesis %†	100 ± 2	11 ± 2

*Mitotic Index and percentage of labelled cells measured at 48 hr post-folate (10–15,000 cells examined).
†DNA specific activity measured at 26 hr post-folate.

In order to study the effect of *cis*-DDP on the ability of the kidney to respond to the folate stimulus when the drug was given before or after folate, non-tumour-bearing rats were given 4 mg *cis*-DDP/kg at time intervals ranging from 1 hr to 10 days prior to measurement of the rate of DNA synthesis at 26 hr after folate administration. The results of this experiment are illustrated in Fig. 2. Virtually complete suppression of the folate-induced stimulation of DNA synthesis is observed when the drug is given concomitantly with, or up to 12 hr after, folate administration. At longer time intervals there is some recovery of the folate response but even when the drug is given 9 days prior to folate, that is 10 days prior to measurement of DNA synthesis, the folate-induced stimulation of DNA synthesis is only

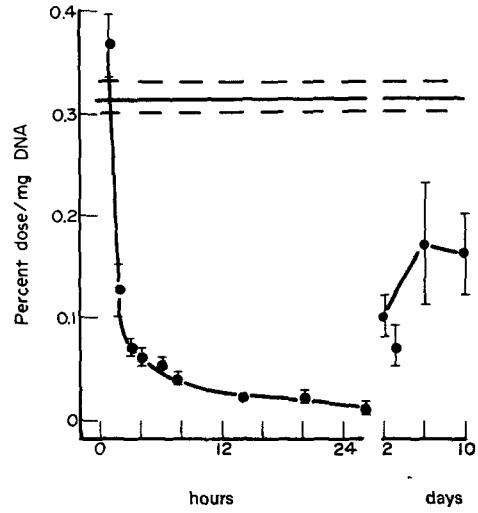


Fig. 2. *The effects of* 4 *mg/kg* cis-*DDP on the stimulation of DNA synthesis in rat kidney. The ordinate represents specific activity of DNA expressed as per cent administered* ^{3}*H-thymidine per mg DNA. The abscissa shows the time of the exposure to the drug before measurement of the rate of DNA synthesis. DNA synthesis was measured at a standard time of* 26 *hr after folate administration, thus the scale marked in hours shows the effects of the drug administered at the same time as, or at various times after folate injection. The scale marked in days shows the effects produced when the drug was given* 1,2,5 *or* 9 *days before folate. The horizontal lines represent the rate of DNA synthesis in the kidneys of rats receiving only folate. Error bars represent* ± 1 *SEM.*

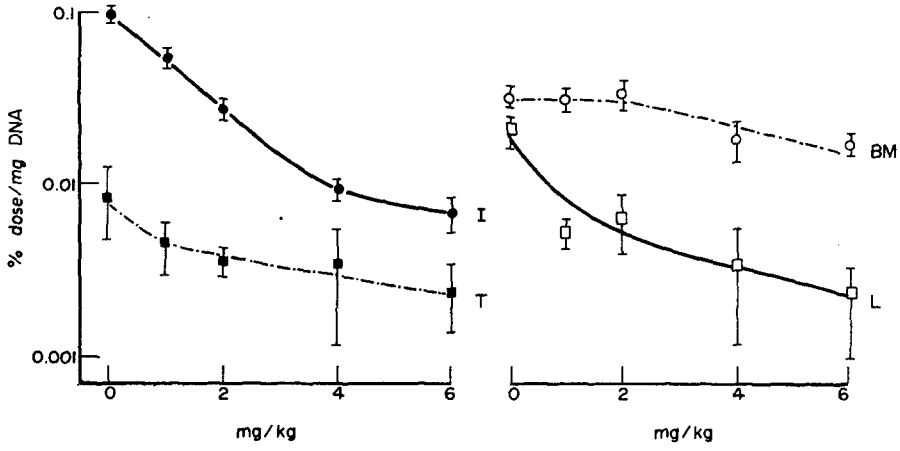


Fig. 3. *Effects of* cis-*DDP on the rate of DNA synthesis in tumour (T), liver (L), intestinal mucosa (I) and bone marrow (BM) of folate treated rats. The ordinate shows DNA specific activity as per cent administered* ^{3}*H-thymidine per mg DNA and the abscissa the dose of drug in mg per kg body weight. Error bars represent* ± *SEM.*
cis-*DDP* LD_{50} = 13 *mg/kg*

about one half of that observed in the control, or pair-fed control, animals. A similar pattern of response to 4 mg *cis*-DDP/kg was observed in the kidneys of folate-treated, tumour-bearing rats.

The effect of *cis*-DDP on DNA synthesis in the tumour and some normal tissues was also studied. The dose response curve for the tumour, gastrointestinal mucosa, bone marrow and liver of the tumour-bearing rats is shown in Fig. 3. The most pronounced effect is seen in the intestine where the rate of DNA synthesis is reduced to one tenth, or less, of the control level 26 hr after treatment with 6 mg *cis*-DDP/kg body weight. In the tumour the rate of DNA synthesis is reduced to between one third and one sixth of the control level by this dose of drug. A single dose of 4 or 6 mg *cis*-DDP/kg produces an approximately 50% reduction in tumour mass six days after treatment but thereafter the tumour regrows at the control rate, Fig. 4.

In some experiments the tumour-bearing animals were not given folate in order that the effects of the drug on the unstimulated kidney could be assessed. The effects of *cis*-DDP (4 mg/kg) on the rate of DNA synthesis in the tumour, liver, intestinal mucosa and kidney of non-folate treated rats are illustrated in Fig. 5. In all the tissues studied the greatest reduction in the rate of DNA synthesis is observed 24 hr after drug administration and

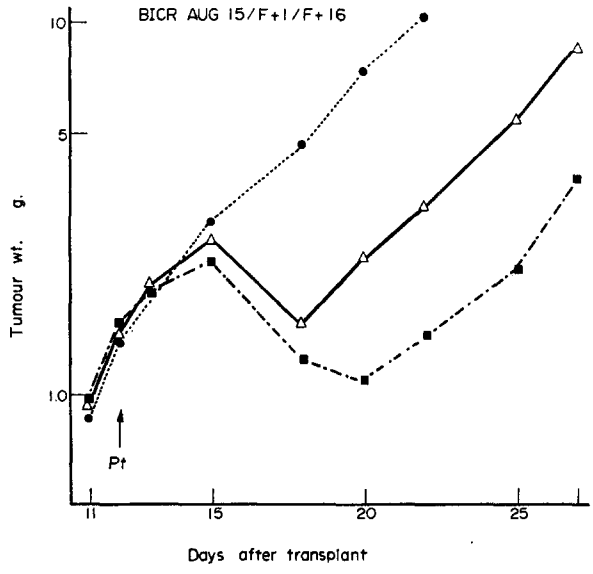


Fig. 4. The effect of single doses of 4 *or* 6 *mg* cis-*DDP/kg body weight on the growth of the BICR-A.*15 *tumour.* 3 *rats per group.*

▲——▲ 4 *mg/kg*
■–·–·–■ 6 *mg/kg*
●·····● *Control*

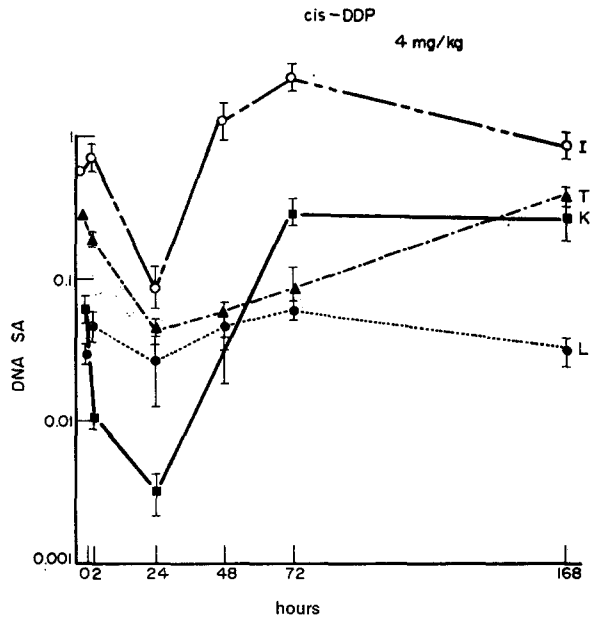


Fig. 5. The effects of 4 *mg* cis-*DDP/kg body weight on the rate of DNA synthesis in tumour (T), liver (L), kidney (K) and intestinal mucosa (I) of normal rats at various times after a single dose of drug.* 5 *rats per group. Error bars represent* ± 1 *SEM.*

the largest effects are observed in the tumour, intestinal mucosa and kidney. However, by 72 hr after drug administration the rates of DNA synthesis in the gastrointestinal mucosa and kidney are greater than the control levels whereas that in the tumour is still reduced. By 7 days the rate of DNA synthesis in the tumour, liver and intestinal mucosa has returned to normal levels but that in the kidney is still about 16 times greater than normal. A similar pattern of response was seen in the tumour, liver and intestinal mucosa of folate-treated rats.

DISCUSSION

The results presented here show that single doses of *cis*-DDP can produce marked depression of DNA synthesis in tumour, kidney and intestinal mucosa *in vivo*. This is in accord with the observations by other workers [5, 6] that *cis*-DDP depressed DNA synthesis in tumour cells in culture. Only a slight depression of DNA synthesis was observed in the bone marrow of these animals and the liver showed variable results ranging from no significant depression to a six fold reduction in the rate of DNA synthesis.

The effects of *cis*-DDP on the kidney are particularly interesting. In the kidneys of non-folate treated rats there is a dramatic reduction in the rate of DNA synthesis to reach a nadir, at about one twentieth of the

control rate, at 24 hr after drug administration. This is followed by a rapid recovery and by 3 days after drug treatment the rate of renal DNA synthesis is 16 times greater than control levels, this very high rate being still maintained at 7 days. This stimulation of renal DNA synthesis is probably a regenerative response to the tubular damage which *cis*-DDP is known to cause [1, 7]. Increased rates of DNA synthesis have also been observed in the kidneys of animals exposed to other agents which cause tubular damage, such as lead or uranium, although with these agents the increased rate of DNA synthesis occurs more rapidly and is less prolonged than that produced by *cis*-DDP [4, 8].

Another interesting observation relative to folate induced stimulation of renal DNA synthesis is that the response produced in tumour bearing rats is only about one third of that of normal rats, Fig. 1; however, the response to *cis*-DDP treatment appears to be similar in both groups of rats.

In normal rats the effects of *cis*-DDP on the rate of DNA synthesis 24 hr after injection appear to show little relationship to the amount of the drug retained in the various tissues. In the tumour, kidney and intestine there is a five to sevenfold reduction in the rate of DNA synthesis at 24 hr, but studies with ^{195m}Pt-*cis*-DDP [8] have shown that the retention of the drug at 24 hr is 0·37, 3·25 and 0·22% dose/g wet tissue respectively. The retention of the drug in the tissues is fairly prolonged, the half-times of the retention in tumour, intestine and kidney being 2·8, 6·0 and 8·4 days respectively [8]. Cell fractionation studies suggest that the drug is retained in the tissue in bound form, combined with tissue proteins, nucleic acid bases or other tissue components [8, 9] and this raises the question whether in bound form the drug retains its cytotoxicity. Studies of the effect of complexes of *cis*-DDP with serum proteins, or guanine, on the folate stimulation of renal DNA synthesis suggest that the complexes are less effective than the free drug (Table 2)—similar observations of reduced cytotoxicity of *cis*-DDP-serum complexes towards L5178Y cells in culture have been reported previously [9].

Studies in experimental animals have shown high retention of *cis*-DDP and other platinum compounds in the kidney [9–11], but little is known concerning the retention of the drug in human kidneys. In studies using ^{195m}Pt labelled *cis*-DDP, Lange *et al.* [11] were able to demonstrate the kidneys by radioactive scanning of the abdomen in one of their patients but the concentration of radioactivity in the kidney appears to be less than that in the liver. In a similar study, using ^{195m}Pt-*cis*-DDP, Smith and Taylor [12] failed to show clear images of the kidneys of two patients although the liver was clearly demonstrated on the scans. Smith and Taylor [12] estimated that the liver uptake of *cis*-DDP in their patients was 10–13% of the injected dose, or about 7×10^{-3}% dose/g of wet liver, at 2 hr after injection. The scanning data suggest that the concentration of ^{195m}Pt-*cis*-DDP in the human kidney is considerably less than that in the liver; thus in these two patients it seems likely that the total uptake of the drug in the kidneys was considerably less than 2% of the administered dose. This compares with an uptake of 6–8% in the kidneys of rats [8]. If the total uptake of *cis*-DDP in the human kidney is very much lower than that in the rat, the severity of the renal damage produced may be less than would be predicted on the basis of the observations in rats. However, the presence of malignant disease may produce a decreased renal function [13] which may serve to exacerbate the damage produced by *cis*-DDP.

Table 2. Effects of cis-*DDP complexes on folate stimulation of DNA synthesis in rat kidney 26 hr after simultaneous administration of folate and the drug complex*

Complex	DNA specific activity % dose/G DNA
cis-DDP only	30 ± 19
cis-DDP—Rat Serum	70 ± 26
cis-DDP—Guanine	155 ± 26
No Drug	315 ± 15

*Five rats per group (error bars represent ± 1 standard deviation).

Comparison of the patterns of recovery of DNA synthesis in the various tissues after a single dose of *cis*-DDP, Fig. 5, shows that in the two most sensitive normal tissues, kidney and intestinal mucosa, the rate of DNA synthesis has returned to normal levels by 48 hr whereas that in the tumour is still markedly depressed 72 hr after drug administration. If a similar differential between the recovery of the tumour and critical normal tissues also occurs in man, this would suggest that in the treatment of human cancer with *cis*-DDP the risks of producing severe renal dysfunction should be reduced, without diminution of the effect on the tumour, if the interval between successive doses of the drug was considerably longer than 24 hr.

REFERENCES

1. B. Rosenberg, Platinum Coordination Complexes in Cancer Chemotherapy. *Naturwissenschaften* **60,** 339 (1973).
2. G. Threlfall, D. M. Taylor and A. T. Buck, The effect of folic acid on growth and deoxyribonucleic acid synthesis in the rat kidney. *Lab. Invest.* **15,** 1477 (1966).
3. G. Threlfall and D. M. Taylor, Modification of folic acid-induced changes in renal nucleic acid and protein synthesis by Actinomycin D and cycloheximide. *Europ. J. Biochem.* **8,** 591 (1969).
4. J. B. Greig, D. M. Taylor and J. D. Jones, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on stimulated DNA synthesis in the liver and kidney of the rat. *Chem. Biol. Interactions,* **8,** 31 (1974).
5. H. C. Harder and B. Rosenberg, Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein synthesis in mammalian cells *in vitro*. *Int. J. Cancer* **6,** 207 (1970).
6. J. A. Howle and G. R. Gale, *Cis*-dichlorodiammine platinum II persistent and selective inhibition of deoxyribonucleic acid synthesis *in vivo*. *Biochem. Pharmac.* **19,** 2757 (1970).
7. B. J. Leonard, E. Eccleston, D. Jones, P. Todd and A. Walpole, Antileukaemic and Nephrotoxic properties of Platinum compounds. *Nature (Lond.)* **234,** 43 (1971).
8. D. M. Taylor, unpublished observations.
9. D. M. Taylor, J. D. Jones and A. B. Robins, Metabolism of platinum ^{14}C-ethylendiamine dichloride in the rat. *Biochem. Pharmac.* **22,** 833 (1973).
10. J. A. Berman, F. K. V. Leh and W. Wolf, Radiopharmacokinetics of antineoplastic platinum compounds: Evaluation of ^{195m}Pt-*cis*-dichloro-bis-cyclopentylamine Platinum II. *J. nucl. Med.* **15,** 478 (1974).
11. R. C. Lange, R. P. Spencer and H. C. Harder, The antitumor agent *cis*-Pt(NH_3) Cl_2: Distribution studies and dose calculations for ^{193m}Pt and ^{195m}Pt. *J. nucl. Med.* **14,** 191 (1973).
12. P. H. S. Smith and D. M. Taylor, Distribution and retention of the antitumor agent ^{195m}Pt-cis-dichlorodiammine platinum II in man. *J. nucl. Med.* **15,** 349 (1974).
13. M. Fichman and J. Bethune, Effects of neoplasms on renal electrolyte function. *Ann. N.Y. Acad. Sci.* **230,** 448 (1974).

Europ. J. Cancer Vol. 12, pp. 255–262. Pergamon Press 1976. Printed in Great Britain

Preparation and Properties of Antisera Directed Against Antigens of the P-815 Mastocytoma Cell not Shared by its Syngeneic Host, the DBA/2 Mouse*

M. BERTSCHMANN, K. J. CLEMETSON and E. F. LÜSCHER

Theodor Kocher Institute, University of Berne, Berne, Switzerland

Abstract—*P-815 mastocytoma cells, upon intradermal injection, induce an immune response in the syngeneic host, the DBA/2-mouse, in the course of which the tumor may be rejected due to the presence of immunocompetent cells. No evidence for the production of humoral, cytotoxic antibody has as yet been found. However, such antibodies, directed specifically against antigen(s) of the P-815 cell can be produced by immunizing C3H mice or sheep and by subsequent* in vivo *absorption of the allo- or xeno-antisera in the DBA/2 mouse. By* in vitro *absorption of such* in vivo *absorbed anti-P-815-antisera with P-815 cells or DBA/2-spleen cells, respectively, it can be demonstrated that the specificity of the* in vivo *absorbed antiserum is exclusively determined by antigenic determinants of the tumor cell. As expected, immunization of the C3H mouse with normal DBA/2-tissue induces an antiserum, which, after* in vivo *absorption in the DBA/2 mouse has no cytotoxic effect against the P-815 cell.*

The injection of the in vivo *absorbed anti P-815 antiserum in DBA/2 mice which are challenged with P-815 cells, leads to a delay in the development of the tumor but not to a complete depression of tumor growth. Full inhibition of tumor growth was achieved, however, when complement (guinea-pig) was injected at the same time.*

The possible relationship of the P-815 specific antigen(s) inducing a humoral response and the antigen(s) inducing rejection in the syngeneic system is still unclear.

INTRODUCTION

DBA/2 MICE acquire immunity against the syngeneic P-815 mast cell tumor after the temporary growth and spontaneous rejection of an intradermally (i.d.) applied tumor cell inoculum. An immune reaction in the syngeneic host is only observed when cells are given i.d. whereas all attempts to immunize animals by subcutaneous (s.c.) or intraperitoneal (i.p.) injection of treated or untreated tumor cells, tumor cell homogenates or tumor cell membranes were unsuccessful [1]. It is possibly this feature and the weak immunogenicity which led some authors to the conclusion that the P-815 tumor cell is free from new antigenic specificities [2].

Other experiments demonstrate or at least indicate as well that specific antigens may exist on the P-815 cell. Sedlacek *et al.* [3] have produced evidence that immune reactions participate to a certain degree in prolongation of the survival time of DBA/2 mice when P-815 tumor cells had been treated with neuraminidase before injection. Greenberg *et al.* [4] describe a cytotoxic effect, which is specific for the P-815 cell (P-815Y line), of normal spleen cells of the NZB strain of mice. They therefore suggest the presence of an antigen common to P-815 and the NZB strain of mice, against which the NZB animals have lost their tolerance. Hawrylko *et al.* [5] confirmed our observation of a weak immunogenicity of the P-815 tumor. In addition they were able to potentiate the immune response by BCG. Immunopotentiation of the P-815 tumor could also be arrived at using Corynebacterium parvum [6]. However, both authors worked with B6D2 F_1

Accepted 21 November 1975.

*This work was supported by grants from the Swiss National Foundation for Scientific Research.

hybrids and the heterozygosity in the Ir locus possibly adds to the recognition of otherwise unapparent antigenic differences [7]. With the Mishell–Dutton culture method a primary cellular immune response to P-815 cells was inducible in the syngeneic system [8].

In our experiments an immune reaction could clearly be shown after the spontaneous tumor rejection, as animals were able to suppress, without any visible tumor growth, a challenge with tumor cells which was 100 times higher than the minimal cell dose for 100% tumor takes. It was also demonstrated [9] that the immunity could be adoptively transferred with spleen cells but not with serum from regressor animals. Attempts to demonstrate humoral antibody failed. Neither progressor nor regressor sera were cytotoxic *in vitro*, nor could the fixation of antibody on the P-815 cell be demonstrated by indirect immunofluorescence. These findings are not exceptional as the presence of a cellular and the absence of a humoral immune response is known for other tumor–host systems.

Although it is conceivable that with more sophisticated methods the presence of humoral antibody will be shown, such methods will probably not be simple or fast enough to use in following antigenic activity during solubilization and purification procedures. A test that uses the inhibition of a cytolytic antibody by the corresponding antigen, comparable to that used by many authors for following histocompatibility antigens, would be highly desirable. We therefore tried to prepare a P-815-specific antibody by absorbing out the alloantibodies (H-2 and non H-2) from a C3H anti P-815 and the xenoantibodies from a sheep anti P-815 antibody. As the normal counterpart of the P-815 cell is the normal mast cell, which is not available in reasonable amounts, we used the *in vivo* absorption method as described by Kitano *et al.* [10] for removal of the unwanted antibodies. We present in this paper the characteristics of an allo- and a xenoantibody that are rendered P-815 specific by means of *in vivo* absorption in DBA/2 mice.

MATERIAL AND METHODS

Animals

C3H ♀ ♀ and DBA/2 ♀ ♀, 4–6 months old purchased from Bomholtgard, Ry, Denmark were used throughout the experiments. The xenoantibody was prepared in a sheep.

Tumor cells

P-815 tumor cells were obtained in 1962 from R. Schindler, Institute of Pathology, University of Berne. They were cultivated *in vitro* in the medium described by Schindler *et al.* [11] or in DMEM medium (Dulbecco's modification of the minimum essential medium) containing 10% horse serum. For comparison two additional P-815 tumor lines, separated from ours for different periods of time were used. They had the following characteristics: (a) line P-815/L, cultivated separately from our line since at least 1965 at the Swiss Institute for Experimental Cancer Research, Lausanne; (b) line P-815/SK, the line maintained at the Memorial Sloan Kettering Cancer Center, New York, separated before 1958. All cell lines were conserved in liquid nitrogen.

Preparation of antibody

The method of Munoz [12] described for the immunization of mice with chicken serum albumin was adapted for the immunization with tumor cells. *In vitro* cultivated cells were washed three times with phosphate buffered saline pH 7·4 (PBS), suspended in PBS and emulsified with an equal amount of complete Freund's adjuvant. Animals of the C3H line of mice were then injected i.p. with $2 \cdot 10^7$ P-815 cells in a volume of 0·4 ml of emulsion. Injections were repeated four times at weekly intervals. Then animals were allowed to accumulate ascitic fluid and they were tapped when the abdomens appeared swollen. After several tappings immunization was continued and tappings repeated until the animals no longer produced ascitic fluid. The sheep anti P-815 antibody was prepared by the i.v. injection of 10^9 cells in PBS (4 injections in 2 weeks) followed by repeated bleedings and immunizations.

Titration of antibody

The cytolytic effect of antibody was measured by the release into the supernatant of ^{51}Cr from previously labeled cells [13,14]. The reaction mixtures contained equal amounts (20 μl) of cell suspension ($1 \cdot 25 \cdot 10^6$ cells/ml), antibody dilution and a mixture of 10 parts of freeze dried guinea pig and 4 parts of freshly frozen rabbit serum as a source of complement. For titration of sera containing large amounts of antibody (before *in vivo* absorption) the complement was diluted 1:3, but for titration of sera after *in vivo* absorption a 1:1·5 dilution was used in order to overcome the well known inhibition of complement activity by mouse

serum [15]. The reaction mixtures were incubated for 1 hr at 37° in an atmosphere of 95% air and 5% CO_2. The reaction was stopped by the addition of 100 µl of PBS containing 1 mM EDTA. After centrifugation the radioactivity of 100 µl aliquots of the supernatant was determined using a Nuclear Chicago γ-spectrometer. Results were corrected for the complement control according to the following formula: specific ^{51}Cr-release (%) =

$$\frac{\text{count/min of sample} - \text{count/min of c-control}}{\text{count/min of labeled cells} - \text{count/min of c-control}} \cdot 100$$

The specific ^{51}Cr-release (%) was then converted to percentage of dead cells. Maximum release (100% dead cells) was obtained using a control antibody known to kill all cells and corresponds to 65–75% of the total radioactivity contained in the cells. The serum dilution killing 50% of the cells was chosen as antibody titer (1 LD_{50}).

In vivo *absorption of antibody*

DBA/2 mice were injected i.p. with varying amounts of antibody. They were bled from 4–12 hr later depending on the experiment and the amount of antibody injected. The blood was taken from the heart of ether anaesthetized animals, pooled, the serum collected and frozen at −20°C until titrated.

In vitro *absorption of antibody*

P-815 and normal DPA/2 spleen cells were used for *in vitro* pre-absorption of *in vivo* absorbed antibody. Tumor cells were washed 3 times with PBS and suspended at a concentration of $3 \cdot 10^6$ cells per 50 µl of antiserum dilution containing approximately 5 LD_{50} of antibody. Spleen cells were separated from contaminating erythrocytes by Ficoll–Hypaque-flotation [16], washed and suspended at a 3 times higher concentration to compensate for the smaller size and therefore smaller surface of these cells. After absorption for 20 min at 37°C and an additional 20 min at 4°C, cells were pelleted by centrifugation and the supernatant antiserum titrated for residual cytotoxicity.

RESULTS

DBA/2 mice were injected with 2 ml of a C3H anti P-815 antibody of a titer of 1:800. When bled 4 hr later, the serum of these animals had a residual cytotoxic titer against P-815 target cells of 1:50 (Fig. 1).

Several control experiments were carried out in order to demonstrate that this residual titer was in fact P-815 specific and not simply due to an overloading of the absorbing organism with H-2 (and non H-2) alloantibodies. Firstly: a C3H anti DBA/2 spleen cell antiserum was adjusted to the same cytotoxic strength against P-815 target cells as the C3H anti P-815 antiserum. When this antiserum was *in vivo* absorbed in DBA/2 mice, no residual cytotoxic effect whatsoever could be shown (Fig. 1). Secondly: the cytolytic activity of the *in vivo* absorbed alloantibody could be removed by absorption *in vitro* with P-815 cells, but not by absorption with normal DBA/2 spleen cells (Fig. 2).

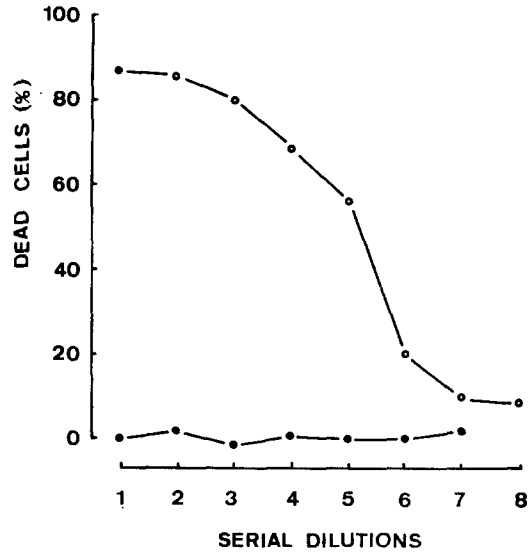


Fig. 1. Residual cytotoxicity of alloantisera after in vivo *absorption in DBA/2 mice.* ○——○ *C3H anti P-815,* ●——● *C3H anti DBA/2 spleen cells. Cytotoxicity measured by the release of* ^{51}*Cr from previously labelled P-815 target cells. Dilution steps: twofold, starting dilution:* 1:2.

The same type of experiments were also done with the sheep anti P-815 xenoantibody. The results were essentially the same, although xenoantibody of equal strength (as titrated *in vitro* against P-815 target cells) appeared to be more injurious to the DBA/2 animals, when passed *in vivo*, than the alloantibody. The resulting antibody, however, also fulfilled the criteria of P-815 specificity when absorbed *in vitro* with P-815 and DBA/2 spleen cells respectively (Fig. 2). It appeared to have a better avidity for the P-815-specific antigen(s) than the mouse antibody, as it was generally completely removed by *in vitro* absorption with P-815 cells, whereas with the mouse antibody pre-absorption *in vitro* with P-815

cells was not always complete (Fig. 2). A better binding capacity of the xenoantibody compared to the mouse antibody was also observed when it was used to detect solubilized P-815 specific antigen(s) [17].

As the P-815 tumor represents a permanent tumor line that has been transplanted and cultivated *in vitro* for many years, opportunity for changes in the antigenic make up of the membrane were manifold. It is true however that cells were conserved in the frozen state over long periods of time and that they had not changed their main characteristics. Nevertheless two additional P-815 lines were tested for

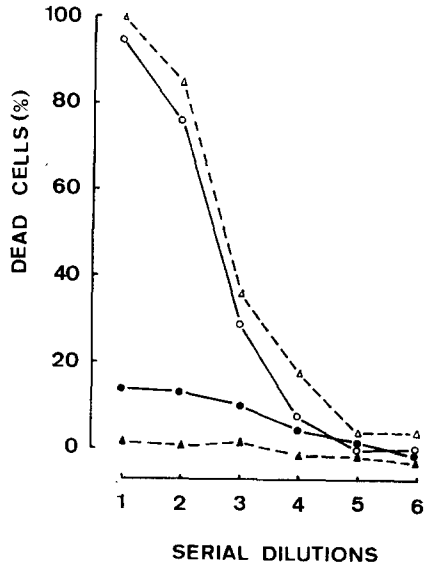


Fig. 2. Residual cytotoxicity of in vivo *absorbed allo- and xenoantibody after in vitro absorption.* ●——● *C3H anti P-815* in vivo *absorbed in DBA/2,* in vitro *absorbed with P-815* ($3 \cdot 10^6$ *cells/50 µl*). ○——○ *C3H anti P-815* in vivo *absorbed in DBA/2,* in vitro *absorbed with DBA/2 spleen cells* ($9 \cdot 10^6$ *cells/50 µl*). ▲——▲ *sheep anti-P-815* in vivo *absorbed in DBA/2,* in vitro *absorbed with P-815* ($3 \cdot 10^6$ *cells/50 µl*). △——△ *sheep anti P-815* in vivo *absorbed in DBA/2,* in vitro *absorbed with DBA/2 spleen cells* ($9 \cdot 10^6$ *cells/50 µl*). *Cytotoxicity measured by the release of* ^{51}Cr *from previously labelled P-815 target cells. Dilution steps: twofold, starting dilution* 1 : 10.

crossreactivity with the P-815 cell line maintained in our laboratory. Although separated from our cell cultures since at least 1958 or 1965 respectively, both lines were sensitive to lysis by P-815 specific antibody raised against our cell line (Table 1). For comparison, titers of the sheep anti P-815 and the C3H anti P-815 antibody are also given in the table. They suggest that the difference in titers are rather due to a general difference in sensitivity to lysis, than to quantitative differences in P-815 specific antigens.

In an attempt to obtain P-815 specific antisera of higher titers, three groups of 6 DBA/2 mice were given 2, 4 and 6 ml of alloantibody each, i.e., all mice were injected with 2 ml at time zero. After 4 hr 6 animals were bled and the remaining two groups were further treated with 2 ml of antibody per mouse. A second group was bled 12 hr after the first injection and the last group was again injected with 2 ml of antibody each and bled 24 hr after the beginning of the experiment. The resulting *in vivo* absorbed antibodies had titers of 1:50, 1:110 and 1:160 respectively: that is the titers were directly related to the quantity of injected alloantibody. These results further indicate that the *in vivo* absorbed antibody cannot simply be unabsorbed alloantibody left behind after overloading the hosts.

In analogous experiments the xenoantibody behaved similarly to the alloantibody. When 0·5, 1·5 and 2·0 ml of sheep anti P-815 gammaglobulin (ammonium sulfate precipitated) of a titer of 1 : 2000 was injected, the proportion of unabsorbed antibody remained constant, although this proportion was lower in the case of the xenoantibody compared to the allosystem. In both cases even the highest titered antisera proved to be P-815 specific when tested by *in vitro* absorption with P-815 and normal DBA/2 spleen cells.

The substantial reduction of cytotoxic titers of antibody when passed *in vivo*, is not only due to the removal of antibody directed against antigens of normal DBA/2 tissue, but also to losses intrinsic to the technique of *in vivo* absorption. In order to get an estimate of the proportion of antibody, which is lost by the technique as such, 2 ml of the C3H anti P-815 antibody was passed in C3H animals as well as in DBA/2. A direct comparison of the resulting titers (on one day in the same experiment) showed that when 2 ml of antibody with a titer of 1:800 were passed in the antibody producer (C3H) the resulting titer was 1:230 whereas the titer was reduced to 1:25 when passed in the absorbing DBA/2 animals. This result indicates that approximately 10% of the total C3H anti P-815 antibody is directed against antigens present on P-815 but not on normal DBA/2 cells.

Once the *in vitro* effect of the *in vivo* absorbed antibody was established, the question arose as to its effectiveness, especially its cytotoxic effect *in vivo*. Hence groups of 6 DBA/2 animals were injected i.v. on day −1 with 0·1 or 0·4 ml of *in vivo* absorbed antibody or normal mouse serum. On day 0 all mice were injected i.d.

Table 1. *Cytotoxic titers of allo- xeno- and P-815 specific antisera with P-815 of different sublines as target cells*

Antiserum	Target cells P-815/B*	P-815/L*	P-815/SK*
sheep anti P-815	1 : 2000	1 : 700	1 : 2600
C3H anti DBA/2-spleen cells	1 : 400	1 : 150	1 : 800
sheep anti P-815 *in vivo* abs. in DBA/2	1 : 100	1 : 30	1 : 140
C3H anti P-815 *in vivo* abs. in DBA/2	1 : 50	1 : 30	1 : 200

Cytotoxicity measured by the release of ^{51}Cr from previously labelled cells.
Titer: Dilution of antibody killing 50 % of cells (1 LD_{50}).
*P-815/B: subline from Berne; P-815/L: subline from Lausanne; P-815/SK: subline from the Memorial Sloan Kettering Cancer Center.

with 10^4 P-815 tumor cells. All control animals showed tumor growth six days after injection as expected, but all experimental animals were still free of visible tumors. They developed tumor nodules with a delay of 1–2 days and one animal remained indefinitely tumor free (Table 2). Although this is only a single case, on which not too much emphasis may be placed, it is remarkable because this animal was the only one among several hundreds that remained free after the i.d. injection of 10^4 P-815 cells.

The survival rate results were not impressive, as in the control group 3/6 animals rejected their tumors spontaneously, in the 0·1 ml antibody group only one did so and in the 0·4 ml antibody group 4/6 animals survived for more than 6 months. An attempt to get clearer results by injecting the antiserum on days −1, 1 and 3 (0·2 ml each time) were no more successful: again there was a regular delay in the time when tumors first appeared, but the final outcome was not clearly different for control and experimental groups.

The DBA/2 line of mice are described as being genetically deficient in the C5 component of complement [18]. For the line raised by Bomholtgard this deficiency still exists (Dr. Friis, personal communication). This, and possibly also the well-known poor lytic activity of the complement system of the whole mouse species could possibly be a crucial point in the incomplete destruction of the tumor cells even in the presence of potentially lytic antibody. We therefore injected groups of 6 DBA/2 ♀♀ i.d. with P-815 cells that were coated with P-815 specific antibody (undiluted, *in vivo* absorbed C3H anti P-815 antibody) or with normal C3H serum. After the coating the cells were washed once and suspended in PBS for the injection. Two hours later the various groups were injected i.v. with 0·4, 0·2 and 0·1 ml of lyophilized and reconstituted guinea pig complement. The control group was treated with 0·4 ml of complement. The results are shown in Fig. 3. They clearly show that P-815 specific antibody is able to produce a complete complement mediated cell destruction, as no animal that received 0·4 ml of guinea pig serum showed any detectable tumor. Smaller amounts of complement were ineffective as was

Table 2. In vivo *effect of P-815 specific* (in vivo *absorbed*) *C3H anti P-815 antiserum in P-815 tumor-bearing DBA/2 mice*

Treatment*	P-815 cells†	Tumor takes day 6	day 8	Tumor diameter, mm day 10	Survival rate
normal mouse serum, 0·4 ml	10^4	6/6	6/6	6·2 ± 0·34‡	3/6
P-815 specific antiserum 0·1 ml	10^4	0/6	6/6	5·2 ± 0·48	1/6
P-815 specific antiserum 0·4 ml	10^4	0/6	5/6	2·9 ± 0·61	4/6

*Antibody injected i.v. on day 1.
†Tumor cells injected i.d. on day 0.
‡Standard error.

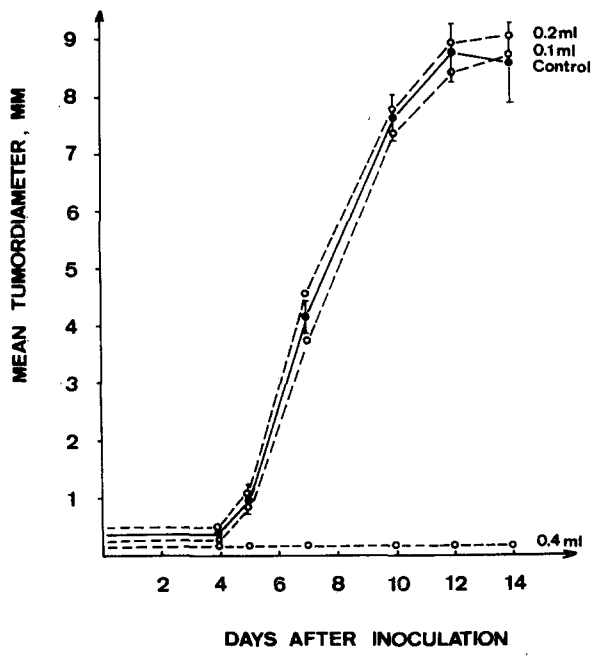


Fig. 3. Growth of P-815 tumor cells in DBA/2 mice after the i.d. injection of 10^4 cells and the i.v. injection of guinea pig serum as a source of complement. O- - -O *cells coated with C3H anti P-815 antiserum,* in vivo *absorbed. Quantities of guinea pig serum as indicated.* ●——● *control group, cells coated with normal mouse serum; 0·4 ml of guinea pig serum. Standard error indicated for the control group only, as standard errors of the experimental groups were of the same order of magnitude.*

coating of the cells with normal serum instead of antibody.

DISCUSSION

Although no P-815 specific humoral antibody could be detected so far in syngeneic tumor bearing hosts nor in animals which had successfully rejected P-815 tumors, these cells are able to induce a humoral immune response in both an allogeneic and a xenogeneic system. Obviously the main part of this antibody is directed against allogeneic (H-2 and non H-2) or xenogeneic antigens. Nevertheless, there is a component in the allogeneic as well as in the xenogeneic antibody, which could not be absorbed by the antigens of the normal DBA/2 organism, and which accounts for approximately 10% of the total activity in the allosystem and for somewhat less in the xenosystem. This non-absorbable component was only present when the alloantibody was induced with P-815 tumor cells, but not when normal DBA/2 spleen cells were the immunogen, indicating that it is directed specifically against antigenic structures typical of the P-815 cell. The P-815 specificity was further demonstrated by *in vitro* absorption of the *in vivo* absorbed antiserum. The cytolytic activity could be removed with P-815 but not with normal DBA/2 spleen cells. The question why a humoral immune response is induced in the allosystem, but not in the syngeneic host, is as yet unanswered. A mechanism such as that proposed by Schierman and McBride [19, 20], could be effective, that is the H-2 antigens of the P-815 cell would act as adjuvants or carrier antigens in the allogeneic C3H animals, whereas in the syngeneic system the tumor antigens behave as haptenic groups. Fischer-Lindahl *et al.* [21] also raise the question whether tumor specific antigens can be recognized unless they are associated with or modify SD (serologically defined) antigens of the major histocompatibility complex. Their experiments were concerned, however, with the effective phase of cellular immunity, not with humoral reactions. Finally, it is possible that with more sophisticated methods antibody or antigen-antibody complexes might be demonstrated in syngeneic progressor or regressor hosts.

Our experiments on the *in vivo* effect of the P-815 specific antibody have demonstrated that humoral antibody can partially inhibit the tumor growth. Either a cytostatic [22] or a cytotoxic effect could be responsible. If we assume a cytotoxic effect, the observed delay of 1–2 days in the first appearance of visible tumors could mean that up to 90% of the cells were killed, as this delay corresponds to the delay which is observed when the cell inoculum is reduced to one tenth (Bertschmann, unpublished results). The additional injection of guinea pig serum as a source of complement resulted in the complete destruction of the tumor cells in all animals. This experimental situation is certainly somewhat remote from the natural one, but we would like to emphasize that it is a generally accepted method of demonstrating cytolytic antibody *in vitro* by adding complement of foreign origin to the system. In our experiments this procedure seemed particularly justified because the DBA/2 strain of mice lack C5 [18] and inhibitory factors for complement in the mouse species in general are well known [15].

Another question is, whether more than one P-815 specific antigen could be involved, that is one inducing rejection in the syngeneic host and the other inducing a humoral response in the allogeneic host.

Further experimental work to answer this question is in progress and seems especially important, as there is accumulating evidence that in many chemically induced tumors various different antigens may be expressed, such as the individual, generally stronger,

antigens inducing a rejection reaction ("tumor-specific" antigens) and the weaker, cross-reacting antigens of the carcino-embryonic type ("tumor-associated" antigens) which are not invariably regarded as being relevant *in vivo* [23,24].

In the DBA/2 line of mice an antigen of the crossreacting type has been described which is present in spontaneous mammary tumours and in many chemically-induced leukemias as well (ML antigen) [25]. As it is similarly demonstrated by the cytolytic effect of an *in vivo* absorbed alloantibody, special attention will have to be paid to the possible relationship of the ML antigen to the antigen we have shown on the P-815 tumor cell. The ML antigen is however described as being present in strains which carry the mammary tumor agent, whereas P-815 was induced and has been transplanted in DBA/2 fostered mice, which no longer carry this agent.

The cross-reactions of the P-815/L (Lausanne) and the P-815/SK (Sloan Kettering) lines with our line demonstrate that the antigen detected by the *in vivo* absorbed antibody is not an antigen that has been recently induced by some casual event, but was already present a relatively short time after or possibly coincidentally with tumor induction and may therefore represent a true tumor specific or tumor associated antigen. The presence of the same antigen in different P-815 sublines also seems especially important because established *in vitro* cell lines are often regarded as being inappropriate for work on tumor antigens.

Acknowledgements—The skilful technical assistance of Miss Y. Litzistorf is gratefully acknowledged.

We are indebted to Drs. K. T. Brunner and J. C. Cerottini, Swiss Institute for Experimental Cancer Research, Lausanne and to Dr. J. Burchenal, Memorial Sloan Kettering Cancer Center, New York, for providing us with the different P-815 sublines.

REFERENCES

1. M. Bertschmann, M. W. Hess, E. F. Lüscher, Eine Methode zum Nachweis der Antigenität des P–815 Mastozytoms in der histokompatiblen (syngeneischen) Maus. *Schweiz. med. Wschr.* **102,** 1197 (1972).
2. A. A. Nordin, J. C. Cerottini, K. T. Brunner, The antibody response of mice to allografts as determined by a plaque assay with allogeneic target cells. *Europ. J. Immunol.* **1,** 55 (1971).
3. H. H. Sedlacek, F. R. Seiler, Dose dependency of the effect of syngeneic, vibrio-cholerae-neuraminidase-treated mastocytoma cells on the life span of DBA f/2 mice. *Behring Inst. Mitt.*, **55,** 343 (1974).
4. A. H. Greenberg, J. H. L. Playfair, Spontaneously arising cytotoxicity to the P-815Y-mastocytoma in NZB mice. *Clin. exp. Immunol.* **16,** 99 (1974).
5. E. Hawrylko, G. B. MacKaness, Immunopotentiation with BCG. III. Modulation of the response to a tumorspecific antigen. *J. nat. Cancer Inst.* **51,** 1677 (1973).
6. M. T. Scott, Corynebacterium parvum as a therapeutic agent in mice. II. Local injection. *J. nat. Cancer Inst.* **53,** 861 (1974).
7. B. Benacerraf, Immune response genes. *J. Immunol.* **3,** 381 (1974).
8. R. L. Lundak, D. J. Raidt, Cellular immune response against tumor cells. I. *In vitro* immunization of allogeneic and syngeneic mouse spleen cell suspension against DBA/2 mastocytoma cells. *Cell. Immunol.* **9,** 60 (1973).
9. M. Bertschmann, E. F. Lüscher, Immune reactions of the syngeneic host after the intradermal injection of tumor cells and microsomes. In *Microenvironmental Aspects of Immunity*, (Edited by B. D. Jankovic and K. Isakovic), Plenum, New York, p. 513 (1973).
10. M. Kitano, E. Mihich, D. Pressman, Antigenic differences between leukemia L1210 and a subline resistant to methyl-glyoxal-bis (guanyl-hydrazone). *Cancer Res.* **32,** 181 (1972).
11. R. Schindler, M. Day, G. A. Fischer, Culture of neoplastic mast cells and their synthesis of 5-hydroxytryptamine and histamine *in vitro*. *Cancer Res.* **19,** 47 (1959).
12. J. Munoz, Production in mice of large volumes of ascites fluid containing antibody. *Proc. Soc. exp. Biol. Med.* **95,** 757 (1957).
13. A. R. Sanderson, Quantitative titration, kinetic behaviour, and inhibition of cytotoxic mouse isoantisera. *Immunology* **9,** 287 (1965).
14. H. Wigzell, Quantitative titration of mouse H-2 antibodies using ^{51}Cr-labelled target cells. *Transplantation* **3,** 423 (1965).

15. W. D. Terry, T. Borsos, H. J. Rapp, Differences in serum complement activity among inbred strains of mice. *J. Immunol.* **92,** 576 (1964).
16. A. Böyum, Separation of leucocytes from blood and bone marrow. *Scand. J. clin. lab. Inv.* **21,** Suppl. 97, 1 (1968).
17. K. J. Clemetson, A. Gerber, M. Bertschmann, E. F. Lüscher, Solubilization of histocompatibility and tumour-associated antigens of the P-815 murine mastocytoma cell. *Europ. J. Cancer* **12,** 263 (1976).
18. U. R. Nilsson, H. J. Müller-Eberhard, Deficiency of the fifth component of complement in mice with an inherited complement defect. *J. exp. Med.* **125,** 1 (1967).
19. L. W. Schierman, R. A. McBride, Adjuvant activity of erythrocyte isoantigens. *Science* **156,** 658 (1967).
20. K. Rajewsky, V. Shirrmacher, S. Nase, N. K. Jerne, The requirement of more than one antigenic determinant for immunogenicity. *J. exp. Med.* **129,** 1131 (1969).
21. K. Fischer-Lindahl, F. H. Bach, Human lymphocytes recognise mouse alloantigens. *Nature (Lond.)* **254,** 607 (1975).
22. P. H. Cleveland, C. F. McKhann, K. Johnson, S. Nelson, A micro-assay for humoral cytotoxicity demonstrating sublethal effects of antibody. *Int. J. Cancer* **14,** 417 (1974).
23. R. C. Rees, M. R. Price, R. W. Baldwin, L. P. Shah, Inhibition of rat lymph node cell cytotoxicity by hepatoma-associated embryonic antigen. *Nature (Lond.)* **252,** 751 (1974).
24. I. Hellström, K. E. Hellström, Cytotoxic effect of lymphocytes from pregnant mice on cultivated tumor cells. I. Specificity, nature of effector cells and blocking by serum. *Int. J. Cancer* **15,** 1 (1975).
25. B. B. Stück, E. A. Boyse, L. J. Old, E. A. Carswell, ML: A new antigen found in leukemias and mammary tumours of the mouse. *Nature (Lond.)* **203,** 1033 (1964).

Europ. J. Cancer Vol. 12, pp. 263–270. Pergamon Press 1976. Printed in Great Britain

Solubilization of Histocompatibility and Tumour-associated Antigens of the P-815 Murine Mastocytoma Cell*

K. J. CLEMETSON, A. GERBER, M. BERTSCHMANN and E. F. LÜSCHER
Theodor Kocher Institute, University of Berne, Berne, Switzerland

Abstract—*The distribution of tumour-associated and histocompatibility antigens from P-815 murine mastocytoma cells was determined during fractionation of homogenized cells. Using inhibition of the cytotoxicity of anti P-815 allo- and xenoantisera rendered specific for P-815 by absorption* in vivo *it was possible to follow the tumour-associated antigen and to compare its behaviour with that of the H-2 antigens. Both antigens could be solubilized in high yield from a crude membrane preparation using 1·2 % DOC. Unlike the anti P-815 xenoantiserum, the anti P-815 alloantiserum appeared to be directed to a large extent against a specific conformation of the tumour antigen which could only be detected on intact cells and not after homogenization. Both the H-2 and the tumour-associated antigens on gel filtration in DOC had an approximate molecular weight of 90,000–100,000 Daltons.*

INTRODUCTION

ONE OF the major problems in the effort to establish the antigenic and structural properties of membrane antigens as molecular entities is to obtain their release from the membrane in a high yield and in an active and water-soluble form which is amenable to further biochemical separation.

Histocompatibility antigens have been solubilized by treating crude membrane fractions of cells with papain [1–5] and using hypertonic salt solutions, especially 3*M* KCl [6]. The papain method gives soluble antigen though in low yield. There has been some controversy over how the 3*M* KCl acts and the nature of the solubilized antigen [7–9].

A wide range of detergents has been used in attempts to solubilize membrane antigens [10–15]. Some destroy antigenic activity, presumably by causing irreversible conformational changes. Where this does not happen, often a major problem is the removal of the detergent while maintaining the antigen in a water-soluble state. Where detergents have been successfully used the main advantage is that the antigen is solubilized in an intact, undegraded form and in high yield.

Tumour antigens have been solubilized using papain [16,17], trypsin or 3*M* KCl [18], by autolysis followed by sonication and papain digestion [19], or using β-glucosidase [20]; detergents have not yet been extensively used [21].

P-815 is a transplantable, mast cell tumour of the DBA/2 mouse originally induced by methylcholanthrene [22] which can be cultured *in vitro*. It is characterized by a high malignancy and a weak immunogenicity (for a more detailed description see [23]).

Recently, by immunization in allogeneic and xenogeneic systems, followed by absorption of these antisera *in vivo* in DBA/2 mice, we have been able to produce antisera which are directed specifically against P-815 and are neither cytotoxic to nor are absorbed by normal DBA/2 cells [23]. In this report we describe the use of these antisera in following the solubilization and characterization of

Accepted 21 November 1975.

*This work was supported by the Swiss National Foundation for Scientific Research.

P-815 associated antigen(s) and compare these with the H-2 antigens of this cell.

MATERIAL AND METHODS

Culture of P-815 cells

P-815 cells were grown either in P-815 medium [24] or in Dulbecco's modification of minimum essential medium (DMEM) (Flow Laboratories) containing 10% horse serum. For membrane isolation, cells were grown in batches of 10 l to a density of $\sim 10^6$ cells/ml in the latter medium.

Preparation of crude membrane fraction

This was carried out essentially by the method of Wallach and Kamat [25]. The cell suspension (10 l) was centrifuged for 20 min at 400 × **g** and 4°C and washed twice in 0·15*M* NaCl, 0·005*M* tris-(hydroxymethyl)-amino-methane(tris)/HCl and once in 0·25*M* sucrose, 0·005*M* tris/HCl, pH 7·4. The wet cell weight of the final pellet was determined and the cells resuspended in ten times this amount of 0·25*M* sucrose, 0·005*M* tris/HCl, pH 7·4. The cells were disrupted twice by decompression from 60 atmospheres of nitrogen. The efficiency of this process was checked by examination of the homogenate under the microscope. The homogenate was made 0·001*M* in ethylenediaminetetraacetic acid (EDTA) by adding 0·1*M* solution and was centrifuged at 2500 × **g** for 30 min. The supernatant from this was centrifuged for 1 hr at 100,000 × **g**. The sediment from this final centrifugation was resuspended in an equal volume of 0·15*M* NaCl, 0·005*M* tris/HCl, pH 7·4 and represents the crude membrane fraction. It was stored frozen at −70°C.

Solubilization of crude membrane fraction using sodium deoxycholate

The crude membrane fraction was thawed quickly and solid sodium deoxycholate (DOC) added to give the required concentration. The mixture was stirred rapidly until the DOC dissolved, then more slowly for a further 30 min and was then centrifuged at 100,000 × **g** for 1 hr. The pellet was resuspended to the original volume in 0·05*M* phosphate buffer, pH 7·6 and both the pellet and the supernatant were dialysed separately against this buffer for 72 hr with at least four changes of buffer.

Column chromatography in DOC

Sephadex G-150 was equilibrated with 0·05*M* phosphate buffer, pH 7·6 containing 0·6% DOC and was packed in a column (2·5 × 80 cm) while gradually increasing the DOC concentration to 1·2%. This is necessary to avoid aggregation of the dextran beads in the detergent. After application of the 100,000 × **g** supernatant, from the P-815 membranes solubilized in 1·2% DOC, to the column, it was eluted with 0·05*M* phosphate buffer, pH 7·6 containing 1·2% DOC, at a flow-rate of 14 ml/hr, collecting 3 ml fractions. Fractions were dialysed against 0·05*M* phosphate buffer, pH 7·6 for at least 72 hr with four buffer changes and were stored at −20°C. All buffers used contained 0·05% NaN_3 except the final dialysis buffer.

Estimation of molecular weight was made by comparison with ovalbumin, bovine serum albumin and bovine gamma globulin (Sigma) run on the same column under the same conditions. The excluded volume was determined using Dextran Blue 2000 (Pharmacia) and the total volume using $Na_2\ ^{51}CrO_4$, as markers. Protein was determined using the Folin reagent [26]. Lipid was determined by extraction [27] followed by thin layer chromatography on silica gel [28]. This method also extracts and detects DOC.

Antisera

Allo- and xenoantisera were prepared and titrated as described in [23]. The C3H anti DBA/2 alloantiserum recognizes principally H-2 and probably also non-H-2 differences between these mouse lines. The C3H anti P-815 recognizes these differences and also antigen specific for P-815 and referred to here as P-815 associated antigen. The presence of more than one type of P-815 associated antigen cannot be excluded. The sheep anti P-815 recognizes these differences and also other, xenoantigens. After absorption of anti P-815 antisera *in vivo* in DBA/2 mice they recognize only the P-815 associated antigen.

Inhibition of cytolysis assay

The cytotoxicity of 20 μl of antibody containing 10 LD_{50} units (10 times the quantity necessary to lyse 50% of 50,000 cells) of antibody was assayed after incubation with a serial, twofold dilution of 20 μl of antigen. P-815 cells labelled with ^{51}Cr were added (20 μl of a suspension of $2·5 \times 10^6$ cells/ml) followed by 20 μl of complement (see 23 for details). The cells were incubated with this mixture for 1 hr at 37°C in an atmosphere of 5% CO_2, 95% air. The cytolysis was stopped by addition of 100 μl of an ice-cold solution of phosphate buffered saline, 0·001*M* in EDTA.

Table 1. *Fractionation of homogenized P-815 membranes: Distribution of H-2 and tumour-associated antigens*[1]

	H-2 Antigen[2] %				Tumour-associated antigen[2] %			
	2500 × **g**		100,000 × **g**		2500 × **g**		100,000 × **g**	
Batch no.	Supernatant	Sediment	Supernatant	Sediment	Supernatant	Sediment	Supernatant	Sediment
1	87	11	—	66	110	17	—	20
2	85	—	—	85	100	10	—	35
3	93	11	24	69	57	9	—	17
4	96	—	17	89	43	8	—	18
5	69	—	26	61	52	5	12	54
6	100	4	20	74	30	4	—	37
7	64	3	12	52	44	4	20	20
8	94	8	9	84	55	4	3	41
9	125	13	26	66	132	9	—	89
Mean	90·3	5·6	14·9	71·7	69·7	7·8	3·9	36·7
Standard error of the mean	5·9	1·8	3·4	4·1	11·8	1·4	2·4	7·7

[2]H-2 determined with C3H anti DBA/2, tumour-associated antigen with C3H anti P-815 *in vivo* absorbed antiserum:
[1]Homogenate taken as 100% (— = Low activity/No titre).

After centrifugation (300 × **g**, 10 min) the radioactivity in 100 μl aliquots of the supernatant was determined using a Nuclear Chicago gamma spectrometer. Results were corrected for the complement control according to the formula: Percentage specific ^{51}Cr release =

$$\frac{\text{count/min of sample} - \text{count/min complement control}}{\text{count/min antibody control} - \text{count/min complement control}} \cdot 100$$

The dilution of antigen giving 50% inhibition of cytolysis is taken as the titre of the antigen and the specific activity of the antigen preparation measured in this assay is defined as:

$$\frac{\text{number of LD}_{50}\text{ of antibody used}}{\text{titre} \times \text{volume of antigen taken (in ml)} \times \text{mg protein/ml}} \quad \text{units/mg}$$

One unit of antigen activity is the quantity which absorbs 1 LD_{50} of antibody.

RESULTS

The distribution and yield of tumour-associated and histocompatibility antigens at the various stages of preparation of the crude membrane fraction were determined and compared for nine batches of cells (Table 1) using C3H anti DBA/2 and C3H anti P-815 *in vivo* absorbed (abs.) antisera. The yield of tumour-associated antigen was similarly determined for a further five batches using sheep anti P-815 (abs.) antiserum (Table 2). Yields before and after homogenization were also compared using C3H anti DBA/2 for the H-2 and both C3H anti P-815 (abs.) and sheep anti P-815 (abs.) for the tumour-associated antigens (Table 3).

The relative area of plasma and inner (rough and smooth endoplasmic reticulum) membranes was estimated, in the intact cells, to be about 1:3·4, using a morphometric method [29].

Preliminary experiments using the detergents sodium dodecyl sulphate, Triton X-100 and

Table 2. *Fractionation of homogenized P-815 cells: Distribution of tumour-associated antigen**

	% Yield			
	2500 × **g**		100,000 × **g**	
	Supernatant	Sediment	Supernatant	Sediment
Mean of 5 batches of cells	88	12	—	42
Standard error of the mean	9·4	2·0	—	5·1

*Homogenate taken as 100%; Antigen determined with sheep anti P-815 *in vivo* absorbed antiserum.

sodium deoxycholate to solubilize antigens from the crude membrane preparation of P-815 soon showed that only DOC gave a satisfactory yield of active material and efforts were then concentrated on optimizing this yield.

*Table 3. Yield of H-2 and tumour-associated antigens after homogenization**

Antiserum	% Yield in homogenate
C3H anti DBA/2	99
C3H anti P-815 *in vivo* absorbed	16
Sheep anti P-815 *in vivo* absorbed	92

*Whole cells taken as 100%; H-2 antigen determinations: Mean of 7 batches of cells. Tumour-associated antigen determinations: Mean of 3 batches of cells.

A range of concentrations of DOC was examined (0·5–3·0%) and distribution of protein and antigenic activity between the supernatant and pellet, after centrifugation at 100,000 × **g** for 1 hr, was determined (Table 4). It was found that the solubilized protein increased from 69% at 0·5% DOC to a plateau at a maximum of about 80% at 1·5% DOC. Solubilization of H-2 antigens increased between 0·5 and 0·9% DOC and then remained constant. At the highest concentrations of DOC (2·0 and 3·0%) the yield dropped somewhat. The differences were less pronounced with the tumour-associated antigen but a slight improvement in solubilization was found up to 1·2% DOC. Based on this, 1·2% DOC was taken as standard for further experiments.

The optimum ionic strength for stability of H-2 antigens was determined by dialysing solubilized antigen against different buffer concentrations in the range 0·002–0·15*M* phosphate buffer, pH 7·6. Below 0·03*M* the yield dropped, reaching 62% at 0·002*M* but between 0·03 and 0·15*M* little difference was seen, the yield averaging 89% of the non-solubilized material.

The optimum pH for H-2 antigen stability was found by dialysing solubilized antigen against 0·05*M* phosphate buffers of different pH in the range 6–10, and lay in the range 7·5–8, the yield falling off sharply below 6·5 and above 9. Further experiments were carried out working under these optimized conditions.

The molecular weight distribution of protein, lipid and antigens was examined by gel filtration on Sephadex G-150 either after removal of DOC by dialysis or in the presence of DOC. In the second case aliquots were dialysed to remove DOC before assay for antigen activity. Where the DOC was removed from the solubilized material before gel filtration the antigen activity was associated with a high molecular weight protein peak excluded from the gel. In the presence of DOC this excluded protein peak was still present, but the antigenic activity was now included in the gel and appeared as a separate peak (Fig. 1a and 1b).

Analysis in detail of fractions from columns run in 1·2% DOC, for various membrane components showed that lipid (at a K_{av} of 0·6–0·7) was well separated from the excluded protein peak and from the antigens with a K_{av} of 0·2–0·3.

Assay for antigenic activity of fractions was carried out with the following antibodies: C3H anti DBA/2, C3H anti P-815 (abs.), sheep anti P-815 and sheep anti P-815 (abs.). With both the C3H anti DBA/2 and the C3H anti P-815 (abs.), column fractions inhibited

*Table 4. Distribution of protein and antigens between supernatant and pellet after 100,000 × g for 1 hr at various DOC concentrations**

DOC concentration %	0·5	0·7	0·9	1·2	1·5	2·0	3·0
Protein in supernatant %	69	71	76	77	78	78	78
H-2 antigen in supernatant %	38	61	88	89	86	80	75
H-2 antigen in pellet %	15	6	—	—	—	—	—
Tumour antigen in supernatant %	75	83	92	92	91	ND	ND
Tumour antigen in pellet %	26	20	16	8·5	—	ND	ND

*H-2 antigen determined with C3H anti DBA/2 antiserum, tumour-associated antigen determined with sheep anti P-815 *in vivo* absorbed antiserum: Crude membrane fraction taken as 100%: — = Low activity/No titre
ND = Not determined.

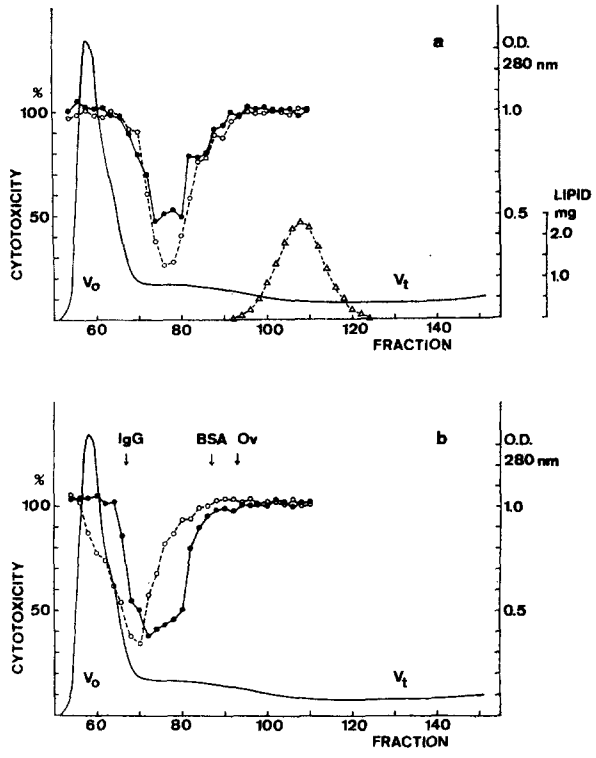


Fig. 1. *Chromatography of DOC solubilized P-815 membranes on Sephadex G-150 in DOC containing buffer. (a), H-2 antigens determined by the capacity of fractions to neutralize C3H anti DBA/2 antiserum* O- - -O *and tumour-associated antigen using C3H anti P-815 in vivo absorbed antiserum* ●——●. —— *Protein (O.D. 280 nm). Lipid, semi-quantitative (see "Material and Methods")* △- - -△. V_o, *excluded volume;* V_t, *total volume. (b) Antigens detected by sheep anti P-815 antiserum* O- -O *and tumour-associated antigen detected by sheep anti P-815 in vivo absorbed antiserum* ●——●. —— *Protein (O.D. 280 nm). Marker proteins: bovine gamma globulin (IgG), bovine serum albumin (BSA) and ovalbumin (Ov).*

cytotoxicity in the same region (Fig. 1a). The sheep anti P-815 was inhibited by fractions eluting in a higher molecular weight region but when sheep anti P-815 (abs.) antiserum was used, the fractions inhibiting cytotoxicity were about the same as for the alloantisera, though the maximum was shifted to a slightly higher molecular weight.

Fractions containing antigenic activity were pooled, dialysed and concentrated by ultrafiltration on a Diaflo PM 10 membrane. Recovery of antigenic activity was about 20% with a purification of about eight times in terms of antigenicity/protein. Concentration of antigenic fractions by freeze-drying gave improved yields of H-2 antigens (>40%) but relatively poor yields (< 10%) of tumour-associated antigens.

When antigen-containing fractions were pooled, dialysed to remove DOC, and concentrated by ultrafiltration, and were afterwards analysed by gel filtration on Sephadex G-150 in the absence of DOC, the K_{av} remained 0·2–0·3. By comparison of the elution volume of the antigen with that of proteins of known molecular weight it was possible to estimate the molecular weight of the H-2 antigen as about 90,000–100,000 Daltons and the tumour antigen as detected with C3H anti P-815 (abs.) or sheep anti P-815 (abs.) antisera as within this range but slightly larger.

DISCUSSION

These studies indicate that both histocompatibility and tumour-associated antigens of P-815 murine mastocytoma cells are present on the plasma membrane and can be solubilized in high yield using DOC.

When the cells are homogenized, the yields of H-2 antigens and of tumour-associated antigen (as determined using the *in vivo* absorbed xenoantiserum) in the homogenate are virtually quantitative. This would exclude the possibility that these antigens are expressed on internal membranes since, as these have a surface area some 3–4 times greater than the plasma membrane, then the yield in the homogenate should be greater than that originally present on whole cells.

Using the *in vivo* absorbed alloantiserum to determine tumour-associated antigen in the homogenate a very much lower yield was found (16% of that on whole cells). There are two possible explanations for this: the tumour-associated antigen may be solubilized during the homogenization process and the *in vivo* absorbed xenoantiserum may recognize this soluble species to a much greater extent than does the similarly treated alloantiserum. However, in following the cell fractionation there does not appear to be much difference in the distribution of antigen between supernatant and pellet as detected by either antiserum. A difference would be expected if higher solubility were the explanation. The alternative explanation is that the *in vivo* absorbed alloantiserum detects a particular conformation or arrangement of the tumour-associated antigen on the intact cell and cannot detect this after homogenization. The *in vivo* absorbed xenoantiserum does not differentiate between these situations, perhaps due to its being directed against more antigenic determinants.

If the activity in the homogenate is taken as 100% then the yields of tumour-associated antigen in the crude membrane fraction (sediment 100,000 × **g**) are about the same (40%) when assayed by both antisera.

Yields of H-2 antigens did not change between whole cells and homogenate, and during cell fractionation over 90% of the original antigenic activity could be accounted for. In the case of the tumour-associated antigen, only about 50% of the activity of the homogenate could be detected in the fractionated material. This lower yield is most likely accounted for by loss of tumour-associated antigen due to factors such as conformational change during fractionation or enzymatic degradation. The difference in ability of the absorbed alloantiserum to recognize the tumour-associated antigen after cell homogenization is probably also responsible for the higher variation in the determination of antigen during cell fractionation (Table 1). Both types of antigen are readily soluble in DOC and at the optimum concentration of DOC virtually no antigen remains associated with membrane or higher molecular weight material; all the activity is included on a Sephadex G-150 filtration, run in the presence of DOC.

When the DOC is removed from the solubilized material by dialysis, the antigens are reincorporated into larger molecular entities which are excluded from Sephadex G-150 but which retain their antigenicity. This reincorporation seems to be due to the presence in detergent-solubilized membrane antigens of a lipophilic region which has been removed in antigens solubilized with papain [10].

When the antigens are separated from the bulk of the excluded protein and from lipid, then this reintegration does not occur on removal of DOC. After some time (days) at 4°C, or after freezing and thawing, aggregation does occur and is accompanied by a loss of antigenic activity.

The molecular weight of both antigens appeared to lie in the range 90,000–100,000 Daltons, as determined by gel filtration in the presence of DOC, with the tumour-associated antigen slightly higher than the H-2. These molecular weights may be exaggerated due to binding of DOC to lipophilic regions of the antigens compared to the hydrophilic proteins used as standards, since this phenomenon is known to occur. However, on gel filtration of partially purified antigen in the absence of DOC the same K_{av} was observed.

This molecular size is similar to that reported for DOC-solubilized HL-A antigens (88,000 Daltons) [11] or for DOC-solubilized Ag-B antigens (100,000 Daltons) [14] or for part of the H-2 antigen solubilized by NP-40 and determined by polyacrylamide gel electrophoresis (88,000 Daltons) [30], although histocompatibility antigens solubilized by other methods [4] give molecular weights in the 35,000–45,000 Daltons range.

Data on the molecular weights of undegraded tumour specific/associated antigens are largely lacking; Davies *et al.*, [19] have shown that the tumour-specific antigen of EL4 cells is somewhat larger than the H-2 antigens but did not estimate the molecular weights. A molecular weight of 55,000 Daltons has been reported [16] for papain solubilized tumour antigen from an aminoazo dye-induced rat hepatoma, although probably the intact molecule is larger.

These studies show that DOC is a good solubilizing agent for this tumour-associated antigen and can give both H-2 and tumour antigens in an "intact" form which can be separated from the bulk of other membrane components.

Although the antigenic activity detected by *in vivo* absorbed allo- and xenoantisera specific for P-815 cells has been ascribed to a "tumour-associated" antigen, it has not yet been established whether this (or these) antigen(s) are tumour-specific transplantation antigens or if they are other immunologically specific determinants expressed by the P-815 cell. It has been shown in the preceding paper that these antigens are typical of the P-815 cell and are not due to antigenic drift or modification of our particular cell line.

Acknowledgements—We would like to thank Miss S. Widmer for excellent technical assistance.

REFERENCES

1. F. Lilly and S. G. Nathenson, Solubilization of the H-6.A isoantigen of the mouse. In *Advance in Transplantation.* (Edited by J. Dausset, J. Hamburger and G. Mathé) p. 279. Munksgaard, Copenhagen (1968).
2. A. Sanderson, HL-A substances from human spleens. *Nature (Lond.)* **220,** 192 (1968).
3. D. L. Mann, G. N. Rogentine, J. L. Fahey and S. Nathenson, Human lymphocyte membrane (HL-A) alloantigens: Isolation, purification and properties. *J. Immunol.* **103,** 282 (1969).

4. P. Cresswell, R. J. Robb, M. J. Turner and J. L. Strominger, Papain-solubilized HL-A antigens: Chromatographic and electrophoretic studies of the two subunits from different specificities. *J. biol. Chem.* **249,** 2828 (1974).
5. M. Hess and D. A. L. Davies, Basic structure of mouse histocompatibility antigens. *Europ. J. Biochem.* **41,** 1 (1974).
6. R. A. Reisfeld, M. A. Pellegrino and B. D. Kahan, Salt extraction of soluble HL-A antigens. *Science* **172,** 1134 (1971).
7. D. L. Mann, The effect of enzyme inhibitors on the solubilization of HL-A antigens with 3*M* KCl. *Transplantation* **14,** 398 (1972).
8. S. K. Oh, M. A. Pellegrino and R. A. Reisfeld, Hypertonic salt extraction of HL-A antigens: Assessment of protease activity. *Proc. Soc. exp. Biol. Med. (N.Y.)* **145,** 1272 (1974).
9. M. Prat, G. Tarone and P. M. Comoglio, Antigenic and immunogenic properties of membrane proteins solubilized by sodium deoxycholate, papain digestion or high ionic strength. *Immunochemistry* **12,** 9 (1975).
10. T. A. Springer, J. L. Strominger and D. Mann, Partial purification of detergent-soluble HL-A antigen and its cleavage by papain. *Proc. nat. Acad. Sci. (Wash.)* **71,** 1539 (1974).
11. D. Snary, P. Goodfellow, M. J. Hayman, W. F. Bodmer and M. J. Crumpton, Subcellular separation and molecular nature of human histocompatibility antigens (HL-A). *Nature (Lond.)* **247,** 457 (1974).
12. J. R. Dawson, J. Silver, L. B. Sheppard and D. B. Amos, The purification of detergent-solubilized HL-A antigens by affinity chromatography with the hemagglutinin from *Lens culinaris*. *J. Immunol.* **112,** 1190 (1974).
13. I. Bernier, A. Dautigny, J. Columbani and P. Jollès, Detergent-solubilized HL-A antigens from human platelets: A comprehensive study of various purification techniques. *Biochim. biophys. Acta* **356,** 82 (1974).
14. M. Letarte-Muirhead, R. T. Acton and A. F. Williams, Preliminary characterization of Thy-1.1 and Ag-B antigens from rat tissues solubilized in detergents. *Biochem. J.* **143,** 51 (1974).
15. D. Sauser, C. Anckers and C. Bron, Isolation of mouse thymus-derived lymphocyte specific surface antigens. *J. Immunol.* **113,** 617 (1974).
16. R. W. Baldwin, J. R. Harris and M. R. Price, Fractionation of plasma membrane-associated tumour-specific antigen from an aminoazo dye-induced rat hepatoma. *Int. J. Cancer* **11,** 385 (1973).
17. R. Billing and P. I. Terasaki, Human leukemia antigen. II. Purification. *J. nat. Cancer Inst.* **53,** 1639 (1974).
18. R. S. Metzgar, T. Mohanakumar, R. W. Green, D. S. Miller and D. P. Bolognesi, Human leukemia antigens: Partial isolation and characterization. *J. nat. Cancer Inst.* **52,** 1445 (1974).
19. D. A. L. Davies, V. S. G. Baugh, S. Buckham and A. J. Manstone, Separation of the specific antigen of a mouse lymphoma from histocompatibility antigens. *Europ. J. Cancer* **10,** 781 (1974).
20. R. W. Baldwin, J. G. Bowen and M. R. Price, Solubilization of membrane-associated tumour-specific antigens by β-glucosidase. *Biochim. biophys. Acta* **367,** 47 (1974).
21. E. Kedar, A. Aaronov and D. Sulitzeanu, Analysis of cell membrane antigens using radioiodinated, purified antibodies. I. Solubilization and preliminary fractionation of membrane antigens of cells transformed by SV_{40} virus. *Israel J. med. Sci.* **9,** 266 (1973).
22. T. B. Dunn and M. Potter, A transplantable mast-cell neoplasm in the mouse. *J. nat. Cancer Inst.* **18,** 587 (1957).
23. M. Bertschmann, K. J. Clemetson and E. F. Lüscher, Preparation and properties of antisera directed against antigens of the P-815 mastocytoma cell not shared by its syngeneic host, the DBA/2 mouse. *Europ. J. Cancer* **12,** 255 (1976).
24. R. Schindler, M. Day and G. A. Fischer, Culture of neoplastic mast cells and their synthesis of 5-hydroxytryptamine and histamine *in vitro*. *Cancer Res.* **19,** 47 (1959).
25. D. F. H. Wallach and V. B. Kamat, Preparation of plasma-membrane fragments from mouse ascites tumor cells. In *Methods in Enzymology*. (Edited by E. F. Neufeld and V. Ginsburg) Vol. VIII, p. 164. Academic Press, New York (1966).

26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
27. O. Renkonen, T. U. Kosanen and O. V. Renkonen, Extraction of serum inositides and other phosphatides. *Ann. Med. exp. Biol. Fenniae* (Helsinki), **41,** 375 (1963).
28. H. Jatzkewitz, Eine neue Methode zur quantitativen Ultramikrobestimmung der Sphingolipoide aus Gehirn. *Z. physiol. Chem.* **326,** 61 (1961).
29. E. R. Weibel, G. S. Kistler and W. F. Scherle, Practical stereological methods for morphometric cytology. *J. Cell. Biol.* **30,** 23 (1966).
30. B. D. Schwartz, K. Kato, S. E. Cullen and S. G. Nathenson, H-2 histocompatibility alloantigens. Some biochemical properties of the molecules solubilized by NP-40 detergent. *Biochemistry* **12,** 2157 (1973).

Europ. J. Cancer Vol. 12, pp. 271–276. Pergamon Press 1976. Printed in Great Britain

Biogenesis of Steroid Hormones by Human Mammary Gland *In Vivo* and *In Vitro**

N. DESHPANDE, PAMELA CARSON, L. DI MARTINO† and A. TARQUINI†

Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX and †Department of Surgery, Istituto Oncologico Regionale, Cagliari, Sardinia, Italy

Abstract—*The ability of human breast tumours to synthesise steroid hormones was studied by perfusion of the human breast* in situ *and incubation of the neoplasms* in vitro. *The results obtained indicate that these tumors are capable of converting pregnenolone and dehydroepiandrosterone to progesterone and androstenedione respectively. Other enzymes normally associated with androgen and oestrogen synthesis were absent in these neoplasms. The significance of these findings is discussed.*

INTRODUCTION

ALTHOUGH it is generally accepted that an abnormal endocrine environment may be one of the factors responsible for the induction of malignancy in the human breast, measurement of protein or steroid hormones in body fluids have failed to show any major differences between patients with early breast cancer and non-cancerous controls [1, 2]. As far as the relation between the endocrine environment and the clinical course of the disease is concerned, approximately one third of patients with metastatic disease respond temporarily to endocrine ablation whereas the remainder appear to have hormone independent metastases [1]. Thus, if hormones are needed for the maintenance and growth of these neoplasms and the tumour is unresponsive to endocrine ablation, it could be postulated that the independence may be due to the ability of the malignant breast tissue to synthesise the necessary hormones. In the case of steroid hormones such a hypothesis can be examined, as the metabolic pathways by which the hormones are synthesised by the endocrine glands are well established. The incubation of human breast tumours with various precursors of steroid hormones has shown that these neoplasms have the ability to synthesise most of the hormones normally associated with the endocrine glands [3–7]. However, the ability to metabolise a given substrate *in vitro* does not necessarily indicate that the same phenomenon takes place *in vivo*. Griffiths, Jones, Cameron, Gleave and Forrest [8] perfused human breast tissue during mastectomy with dehydroepiandrosterone sulphate and androstenedione and were able to detect radioactivity in dehydroepiandrosterone, androstenedione, testosterone and 5α-dihydrotestosterone. However, the anatomy of the breast is such that it is not absolutely certain that the injected substrate has not escaped into the general circulation and the metabolism taken place elsewhere. Therefore, an attempt was made to perfuse the human breast *in situ* and to compare steroid metabolism *in vivo* with incubation of neoplasms *in vitro*. This paper reports on our findings.

Accepted 13 November 1975.

*This work was presented to the 11th International Cancer Congress, Florence, October 1974.

MATERIAL AND METHODS

Chemical

All solvents were of analytical grade and were redistilled before use.

The radioactive steroid substrates were purchased from the Radiochemical Centre, Amersham, England, and were purified by paper chromatography in A and B systems of Bush [9]. The manufacturers' specific activities were not checked. The specific activities of ^{3}H-labelled compound expressed as Ci/mmole were as

follows:—cholesterol 5·0; pregnenolone 20·0; progesterone 1·0; 17α-hydroxypregnenolone 12·6; 17α-hydroxyprogesterone 49·2; dehydroepiandrosterone 22·0; androstenedione 0·0031; oestrone 40·0; oestradiol 41·0; oestriol 58·0. The specific activities of ^{14}C-labelled compounds expressed as mCi/mmol were:—cholesterol 55·2; pregnenolone 53·0; progesterone 60·7; 17α-hydroxyprogesterone 61·0; dehydroepiandrosterone 52·0; androstenedione 60·0; testosterone 52·2; oestrone 50·0; oestradiol 31·8; oestriol 53·0.

The non-radioactive steriods used were purchased from Steraloids Ltd., Croydon, Surrey, England, and were recrystallised from methanol. 2α-Cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one (cyanoketone) was purchased from the Sterling Winthrop Institute, New York, U.S.A. Nicotinamide-adenine dinucleotide (NAD+) and nicotinamide-adenine dinucleotide phosphate (reduced, NADPH) were purchased from British Drug Houses Ltd., Poole, Dorset, England.

Clinical

Perfusion of the human breast in situ. All the patients studied were women with early breast cancer awaiting mastectomy, whose informed consent was obtained. No drugs were administered prior to the perfusion of the breast.

The surgical technique used to perfuse human breast *in situ* does not present any particular difficulties as it amounts to the first step of radical or simple mastectomy. First a racket-shaped incision is made circumscribing the breast and then, without damaging its distal ramifications, the main internal artery of the gland where it emerges from the internal mammary artery is located (i.e. at the second intercostal space). Once this vessel and its corresponding vein has been isolated, all the remaining mammary vessels—central and lateral, including those few which reach the gland through its lower facing are cut and tied: in this way, with the exception of the vascular "stalk" mentioned, the breast is completely isolated from the underlying muscles and the surrounding anatomic structures. Two polyvinyl cannulae (2–3 mm diam.) are then introduced into the two isolated vessels. Five μCi of the radioactive steroid to be infused is dissolved in 0·1 ml of ethanol, further diluted with 0·5 ml of 0·9% NaCl solution and injected into the arterial cannula. Following this, the blood from the vein is collected for 10 min and a peripheral blood sample is taken from one of the basilical veins to test whether the injected steroid has escaped into general circulation. At the end of the infusion the cannulae are removed, the isolated vessels are cut and tied and the mastectomy is carried out. In all, the procedure adds an extra 15 min to the total time of mastectomy.

After the mastectomy, the tumour was removed; frozen immediately and transferred to the laboratory for identification of radioactive metabolites.

After thawing, the tumour was cleared of surrounding fat and connective tissue, weighed and cut into small pieces. A small amount of radioactive carrier steroid [^{14}C-labelled if the substrate was tritium labelled and vice versa (5000 dis/min)] corresponding to the one to be isolated was added, to account for losses during extraction and isolation procedures. Radioactivity from the tumour was extracted after homogenization in ethanol as described previously [10]. Blood radioactivity was extracted with ethanol.

The dried tumour and blood extracts were re-dissolved in 10 ml of 70% aqueous methanol, left at −20°C overnight and centrifuged in a refrigerated centrifuge at 4°C for 20 min. The aqueous methanol was decanted and evaporated to dryness. The dried extracts were then partitioned between water (5 ml) and ethyl acetate (3 × 10 ml) and the organic solvent evaporated to dryness in readiness for paper chromatography.

Incubation of human breast tumours in vitro. Benign and malignant breast tumours were obtained from a series of patients and the diagnosis was confirmed by histological examination. One gram of neoplasm was then weighed, cut into small pieces and homogenized in 0·25 M sucrose solution. The homogenate was adjusted to a concentration of 100 mg/ml and centrifuged at 800 g for 20 min at 4°C in a refrigerated centrifuge. The supernatant was used as a source of the enzymes, mentioned in the results section, to study the biosynthetic capabilities of breast tumours. One ml of cytosol, equivalent to 100 mg of tumour tissue, and appropriate blanks were incubated in duplicate in 0·15 M phosphate buffer, pH 8, for 30 min according to the procedure of Yates and Deshpande [11]. Obligatory co-factors such as NAD (0·30 μmoles) or NADPH (0·24 μmoles) were added to incubates at the start and at 10 min intervals. The reaction was stopped by the addition of 5 ml of ethanol and steroids were extracted from these incubates as described elsewhere [11].

Isolation and identification of steroids from blood, tumour and incubates. Extensive paper chromatography was used to identify these steroids

synthesized by the tumour *in vivo* and *in vitro*. The procedures are described elsewhere [12].

Further characterisation of the steroids synthesized was obtained by isotope dilution studies in which 50 mg of non-radioactive compound was added to the isolated metabolite and crystallised to constant specific activity so that the difference between crystals and mother liquor was less than 5%.

RESULTS

The biosynthetic capabilities of breast tumours were investigated by studying the following enzyme systems.

(*a*) *NADPH dependent*

1. 20-22R-desmolase (cholesterol→ pregnenolone)
2. 17–20 lyase (17α-hydroxypregnenolone →dehydroepiandrosterone and 17α-hydroxyprogesterone →Δ^4-androstenedione).
3. Aromatizing enzymes (androstenedione →oestrone and testosterone → oestradiol-17β).
4. 16α-hydroxylase (oestradiol-17β → oestriol).

(*b*) *NAD-linked systems*

3β-hydroxysteroid dehydrogenase; Δ^{5-4}-isomerase (dehydroepiandrosterone →Δ^4-androstenedione and pregnenolone →progesterone).

At the outset it was decided that in order to confirm the presence of a particular enzyme, the following criteria would have to be fulfilled:—

In perfusion studies,

(*a*) there must be only a minimal amount of radioactive metabolite found in the peripheral circulation;

(*b*) the same metabolite must be present in larger amounts in mammary vein blood than in the peripheral blood;

and furthermore,

(*c*) the tumours must show the presence of both substrate and transformed product.

Finally, all *in vivo* conversions must be confirmed by incubation of these tumours *in vitro*.

Using the above criteria, it was observed that human breast tissue has the ability to convert pregnenolone to progesterone and dehydroepiandrosterone to androstenedione both *in vivo* and *in vitro*. The results obtained in these experiments are shown in Tables 1 and 2. When tumour cytosol fraction was incubated in the presence of a known specific inhibitor of 3β-hydroxysteroid dehydrogenase complex i.e., cyanoketone it was observed that the inhibitor reduced both progesterone and androstenedione synthesis by about 20% (see Table 3) .Table 4 shows the characterization of progesterone and androstenedione by isotope dilution studies after the incubation of tumours with pregnenolone and dehydroepiandrosterone, respectively.

DISCUSSION

By using a combined approach of *in situ* perfusion and *in vitro* incubation it has been possible to study the biosynthetic capabilities of human breast tumours. The perfusion

Table 1. *Metabolism of various steroids by human breast* in situ. *Five μCi of each substrate was injected into the internal mammary artery and the metabolites were identified in the mammary vein blood. The results are expressed as disintegrations per minute per ml of blood and gram of tumour tissue*

Substrate	No. of patients	Metabolites	Peripheral blood	Mammary blood	Tumour
^{3}H-Cholesterol	3	Pregnenolone	0	0	0
		Dehydroepiandrosterone	0	0	0
^{3}H-Pregnenolone	2	Progesterone	0	400	300
			0	2460	250
^{14}C-Progesterone	3	17α-Hydroxyprogesterone	0	0	0
^{3}H-17α-Hydroxyprogesterone	3	Δ^4-Androstenedione	0	0	0
^{14}C-Denydroepiandrosterone	3	Δ^4-Androstenedione	10	80	70
			10	210	70
			10	200	230
^{3}H-Δ^4-Androstenedione	3	Oestrone	0	0	0
^{3}H-Oestradiol-17β	3	Oestriol	40	1970	160
			120	1740	240
			670	1900	250

Table 2. The metabolism of various steroids by human breast tumours in vitro. *One ml of tumour cytosol fraction in duplicate was incubated with various substrates for 30 min (0.15 M phosphate buffer, pH 8.0) and the metabolites were isolated by paper chromatography. The molar values were obtained from the amount of radioactivity associated with the metabolite and the specific activity of its substrate.*

B = Benign tumours
M = Malignant tumours

Substrate	Amounts incubated μCi	No. of tumours	Metabolite isolated	Conversion μmoles × 10^{-4}/g/30 min
^{14}Cholesterol	0·5	3	Pregnenolone	0
^{14}C-Pregnenolone	0·5	M_1	Progesterone	2·11
		M_2		2·69
		M_3		2·39
		B_1	Progesterone	0·00
		B_2		1·30
		B_3		1·30
^{14}C-Progesterone	0·5	3	17α-Hydroxyprogesterone	0
^{3}H-17α-Hydroxypregnenolone	0·8	3	Dehydroepiandrosterone	0
^{3}H-17α-hydroxyprogesterone	1·3	3	Δ^4-Androstenedione	0
^{14}C-Dehydroepiandrosterone	0·5	M_1	Δ^4-Androstenedione	2·07
		M_2		3·60
		M_3		2·78
		B_1	Δ^4-Androstenedione	0·00
		B_2		1·79
		B_3		1·71
^{3}H-Androstenedione	0·64	3	Oestrone	0
^{14}C-Testosterone	0·5	3	Oestradiol	0
^{3}H-Oestradiol-17β	2·04	3	Oestriol	0

technique used shows that only a negligible amount of radioactivity escapes into general circulation. Although we have so far attempted to detect only the important enzyme systems, the results show that many of the key enzymes involved in hormone biogenesis are not present in the tumours examined in this study. However, these results are not in agreement with those of Adams and Wong [13]. Failure to observe conversion of cholesterol to pregnenolone suggests that unlike other steroid synthesising endocrine glands, human breast is incapable of initiating hormone synthesis. Furthermore, inability to convert 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to dehydroepiandrosterone and androstenedione respectively means that breast tumours appear to lack lyases normally associated with side chain cleavage. This rules out androgen synthesis from C_{21} precursors. Similarly, breast tumours failed to aromatise androstenedione or testosterone to oestrone and oestradiol respectively, which indicates that these neoplasms were

Table 3. The effect of 5 μg of cyanoketone on the conversion of pregnenolone to progesterone and dehydroepiandrosterone to Δ^4-androstenedione by human breast tumours in vitro. *One ml of tumour cytosol was incubated in duplicate with 0.5 μCi of ^{14}C pregnenolone or ^{14}C dehydroepiandrosterone for 30 minutes (0.15 M phosphate buffer, pH 8.0). The results are expressed as μmol × 10^{-4}/g/30 minutes. The molar values were calculated from the amount of radioactivity in progesterone and androstenedione and the specific activities of ^{14}C pregnenolone and dehydroepiandrosterone*

	Progesterone	Δ^4-Androstenedione
Control	3·40	2·90
Cyanoketone	2·60	2·40
% Inhibition	24	17

Table 4. Crystallisation of progesterone and androstenedione to constant specific activity after the addition of 50 mg of non-radioactive steroids. The compounds were isolated after the incubation of pregnenolone and dehydroepiandrosterone, respectively, with human breast tumours

	Progesterone cpm/mg	Δ^4-Androstenedione cpm/mg
Pool	321	90
Cr (1)	323	90
M1 (1)	325	108
Cr (2)	306	95
M1 (2)	301	96

incapable of synthesising oestrogens. Oestradiol-17β was converted to oestriol by the breast in *in situ* perfusion. However, when the tumours were incubated with the substrate *in vitro* we failed to isolate oestriol from the incubates. Thus, the evidence for the presence of oestrogen specific 16α-hydroxylase remians inconclusive.

In our studies conclusive evidence has been obtained to show the presence of 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase in human breast tumours. The human breast is capable of converting pregnenolone to progesterone and dehydroepiandrosterone to androstenedione both *in vivo* and *in vitro*. Furthermore, as in the case of the 3β-hydroxysteroid dehydrogenase complex from the adrenal cortex [14, 15] the synthesis can be inhibited by cyanoketone, indicating that the tumour enzyme complex is the same, or very similar, to that found in adrenal glands. However, it remains to be seen whether the synthesis occurs because these hormones are necessary for cell proliferation and maintenance.

The role of progesterone in the induction of mammary tumours in laboratory animals is known [16] and, furthermore, both androgens and progesterone have a stimulatory effect on the organ cultures of human breast tissue [17]. Whether the same phenomenon takes place *in vivo* is not known. In females the androgens act as immediate precursors for oestrogen synthesis but since we have been unable to detect any oestrogen formation in these studies, it is reasonable to exclude androstenedione synthesis for that purpose.

The molecular studies related to the mechanism of action of steroid hormones indicate that they act by specific binding to cytosol and nuclear receptor proteins and furthermore, studies on progesterone binding proteins show that some of the breast tumours have the ability to bind progesterone [18]. In the case of androgens, newly synthesized androstenedione may be converted to testosterone and 5α-dihydrotestosterone for which a specific high affinity, low capacity cytosol receptor has been found in human breast tumours [7, 19]. Thus, assuming that these two hormones have a biological role in the human breast, the breast cells have the ability to synthesize the hormones and thus become partially independent of hormonal environment. However, inability to synthesize both the precursors (i.e., pregnenolone and dehydroepiandrosterone) means that they still depend on a peripheral supply of these steroids.

Although it is impossible to study the biosynthetic capabilities *in vivo* in normal women or patients with benign disease, the finding that benign breast tumours are also capable of synthesizing the same hormones *in vitro* as malignant tissue indicates that the ability is not acquired as a result of the induction of malignancy and non-malignant epithelial cells may show the same phenomenon. The acellular nature of the human breast has made it difficult to study the synthesis due to lack of sufficient material and attempts are now being made to maintain these cells in cell culture.

In conclusion, although the ability to synthesize biologically active hormones such as progesterone and androstenedione from inactive precursors gives the human breast a paraendocrine status, the observation that benign breast tumours also demonstrate the same phenomenon suggests that there are no transcriptional changes in the hormone synthesizing enzymes as a result of transformation. It remains to be seen whether the amounts synthesized in responsive and unresponsive tumours shows any quantitative differences.

Acknowledgement—The authors are indebted to Dr. R. D. Bulbrook for his continued interest and to Mr. J. L. Hayward, FRCS, of the Breast Clinic at Guy's Hospital, for the supply of tumours.

REFERENCES

1. R. D. Bulbrook, Hormone assays in human breast cancer. In *Vitamins and Hormones.* (Edited by R. S. Harris, I. G. Wool and J. A. Loraine) vol. 23, p. 329. N.Y. Academic Press (1965).
2. D. Y. Wang and M. C. Swain, Hormones and breast cancer. In *Biochemistry of Women, Methods for Clinical Investigation.* (Edited by A. S. Currie and J. B. Hewitt) p. 192. C.R.C. Press, Cleveland, Ohio (1974).
3. J. B. Adams and M. S. F. Wong, Paraendocrine behaviour of human breast carcinoma: *in vitro* transformation of steroids to physiologically active hormones. *J. Endocr.* **41,** 41 (1968a).
4. J. B. Adams and M. S. F. Wong, Enzymic synthesis of steroid sulphates. VI. Formation of cholesteryl-3 35_s-sulphate in incubation of human breast carcinoma extracts with adenosine-3′-phosphate-5′-phospho-35_s sulphate. *Steroids* **11,** 313 (1968b).

5. D. Jones, E. H. D. Cameron, K. Griffiths, E. N. Gleave and A. P. M. Forrest, Steroid metabolism by human breast tumours. *Biochem. J.* **116,** 919 (1970).
6. T. L. Dao and P. R. Libby, Conjugation of steroid hormones by normal and neoplastic tissues. *J. clin. Endocr.* **28,** 1431 (1968).
7. W. R. Miller, A. P. M. Forrest and T. Hamilton, Steroid metabolism by human breast and rat mammary carcinoma. *Steroids* **23,** 379 (1974).
8. K. Griffiths, D. Jones, E. H. D. Cameron, E. N. Gleave and A. P. M. Forrest, Transformation of steroids by mammary cancer tissue. In *Estrogen Target Tissue and Neoplasia.* (Edited by Thomas L. Dao) p. 151. University of Chicago Press, Chicago and London (1972).
9. I. E. Bush, Methods of paper chromatography applicable to the study of steroids in mammalian blood and tissue. *J. biol. Chem.* **50,** 370 (1952).
10. F. Ellis, J. R. Parker, R. D. Bulbrook and N. Deshpande, The uptake of radioactivity by normal and neoplastic human breast tissues after administration of tritiated testosterone. *Brit. J. Surg.* **52,** 54 (1965).
11. J. Yates and N. Deshpande, Kinetic Studies of the enzymes catalysing the conversion of 17α-hydroxyprogesterone and dehydroepiandrosterone to androstenedione in human adrenal gland *in vitro*. *J. Endocr.* **60,** 27 (1974).
12. N. Deshpande, V. Jensen, R. D. Bulbrook and E. Boesen, *In vitro* steroidogenesis by an undifferentiated embryonal cell sarcoma of the ovary. *J. Endocr.* **43,** 437 (1969).
13. J. B. Adams and M. S. F. Wong, Paraendocrine behaviour of human breast cancer. In *Estrogen Target Tissues and Neoplasia.* (Edited by Thomas L. Dao), p. 125. University of Chicago Press, Chicago and London (1972).
14. A. S. Goldman, Stoichiometric inhibition of various 3β-hydroxysteroid dehydrogenases by a substrate analogue. *J. clin. Endocr.* **27,** 325 (1967).
15. A. M. Neville and L. L. Engel, Inhibition of α and β-hydroxysteroid dehydrogenases and steroid Δ-isomerase by substrate analogues. *J. clin. Endocr.* **28,** 49 (1968).
16. G. Ropcke and L. M. Boot, Prolactin and ovarian hormones in carcinoma of the mammary gland in mice. Proc. IVth International Congress, Series No. 273, *Endocrinology* p. 1232 (1972).
17. O. Mioduszewska, T. Koszarowski and C. Gorski, The influence of hormones on breast cancer *in vitro* in relation to the clinical course of the disease. In *Prognostic Factors in Breast Cancer.* (Edited by A. P. M. Forrest and P. B. Kunkler), p. 347. E. & S. Livingstone, Edinburgh & London (1968).
18. L. Terenius, Estrogen and progestogen binders in human and rat mammary carcinoma. *Europ. J. Cancer* **9,** 291 (1973).
19. R. K. Wagner, L. Gorlich and P. W. Jungblut, Dihydrotestoterone receptor in human mammary cancer. *Acta Endocr. Suppl.* **173,** 65 (1973).

Europ. J. Cancer Vol. 12, pp. 277–282. Pergamon Press 1976. Printed in Great Britain

Leucocyte Migration in Response to P.P.D. in Patients with Trophoblastic Tumours

P. A. M. WALDEN*, C. R. PENTYCROSS†‡, R. LEYTON, P. BROWNE†, J. DENT and P. VERNON

Department of Medical Oncology, Charing Cross Hospital (Fulham), London, W6 8RF

Abstract—*Migration inhibition studies using 3 different concentrations of purified protein derivative (P.P.D.) was performed on 36 patients successfully treated for choriocarcinoma, 36 subjects who had spontaneously aborted a hydatidiform mole and 36 normal controls. In both the patient and mole groups a considerable number showed increased migration instead of inhibition with the two lower doses of antigen. No significant difference could be found between the two groups. By contrast a much smaller number in the control group showed this phenomenon, although with two even lower doses, increased migration tended to occur in the control group. The difference between the results of this group and those of the other two groups is significant. There appeared to be no correlation between increased migration in those who had initially presented with metastatic disease and those who had not. The significance of these phenomena and other aspects of tumour-host interaction is discussed.*

INTRODUCTION

BETWEEN 1957 and 1973, 258 women were successfully treated by chemotherapy for gestational trophoblastic tumours in the Department of Medical Oncology at Charing Cross Hospital (Fulham). A group of these patients has been studied to determine whether any long-term deleterious effects attributable to cancer chemotherapy could be detected. The achievement of a pregnancy more than 3 yr after completion of treatment formed the basis of selection of the study group. A second group, consisting of women who had spontaneously aborted a hydatidiform mole but who had received no cytotoxic chemotherapy, was also studied. Although the risk of choriocarcinoma following hydatidiform mole is about 1000 times greater than after normal pregnancy the risk of this for the patient who has a mole is only about 3%.

Depression of the immune response is an important and potentially lethal complication of cancer [1], radiotherapy [2], or chemotherapy [3]. The last report refers specifically to patients having gestational trophoblastic tumours. In addition both oestrogen [4, 5] and progesterone [6] have been implicated as immune suppressors in pregnancy and factors with a similar effect have been shown to be generated in culture systems in both pregnancy and malignant trophoblastic disease [7]. Against this background the immune profile of the subjects in each group formed an integral part of the study and we here report the results of tests of cell-mediated immunity as assessed by the Leucocyte Migration Test (L.M.T.) in the presence of purified protein derivative (P.P.D.).

Accepted 18 September 1975.

*In receipt of an M.R.C. grant during part of the period.

†Currently in receipt of a grant from the Cancer Research Campaign.

‡Correspondence to Dr. C. R. Pentycross, Department of Medical Oncology, Charing Cross Hospital (Fulham) London, W6 8RF.

MATERIAL AND METHODS

Patient details

(a) *Treated trophoblastic tumour group.* There were 36 patients in each of the study groups. The ages in the treated group ranged from 21–43 (mean 29 yr). A histological diagnosis of choriocarcinoma had been established in 4 instances and invasive mole in 4 others. A histological diagnosis was not obtained in the other 28 patients and the evidence for a trophoblastic tumour included in all instances a persistently raised human chorionic gonadotrophin (hCG) titre several months after evacuation of a hydatidiform mole or an abortion. In addition 11 patients had pulmonary metastatic disease, 6 of these with widespread metastases. Eighteen patients received treatment with methotrexate and folinic acid alone; 10 were treated with a combination of methotrexate, folinic acid and actinomycin-D and 4 with a combination of 6-mercaptopurine, methotrexate and folinic acid. The patients were studied 2–10 yr after completion of chemotherapy.

(b) *Untreated hydatidiform mole group.* The untreated mole group consisted of 36 women who had spontaneously aborted a hydatidiform mole and had received no chemotherapy; their ages ranged from 18 to 42 (mean 27 yr). The intervals between the evacuation of the mole and the time of study ranged from 4 to 22 months.

(c) *Control group.* The control group were drawn mainly from the female staff at the Charing Cross Hospital. Their ages ranged from 19 to 54 (mean 29 yr). None of them had ever had a hydatidiform mole or cancer, none were pregnant at the time of testing although six were taking various contraceptive agents. These were all of moderate or low oestrogen content.

Every subject in all three groups was Mantoux positive on skin testing.

Experimental details

The leucocyte migration technique using purified protein derivative (P.P.D.) was a modification of that described elsewhere [8].

Sterility was maintained throughout. Migrations were set up in a laminar air flow (Bassaire) cabinet following preliminary exposure of the polystyrene migration chambers (Sterilin Ltd., Richmond, Surrey) and other implements and cover-slips to u.v. irradiation for 45 min.

At initial presentation, 20 ml of blood was collected in a sterile 20 ml syringe to which 0·2 ml of sterile mucous heparin B.P. had been added. The syringe was then placed in the vertical position and attached to the side wall of the Bassaire cabinet and the blood was allowed to settle for one hour at 33°C; the plasma was then decanted by attaching a No. 1 needle (S.W.A.) to the syringe and bending it to an acute angle and compressing the plunger. The plasma was transferred to a centrifuge tube (Sterilin) and spun in an M.S.E. centrifuge at 1000 rev/min for 5 min; the button of cells obtained was then mixed with 10 ml of 10% Hank's Balanced Salt Solution (HBSS). The cell suspension was agitated and then returned to the centrifuge and spun again at 1000 rev/min for 5 min; this washing procedure was repeated three times in all.

After the final washing, 2 ml of Eagles minimum essential medium containing 10% horse serum was added to the button of cells; the cell suspension was mixed thoroughly and the suspension was aspirated with 12 sterile plain haematocrit tubes (Gillman-Hawksley) to three quarters of their length; the tubes were then sealed at the open end in a low flame and spun in the M.S.E. centrifuge at 3000 rev/min for 10 min, resulting in a small column of buffy coat cells 2–3 mm long at the bottom of the centrifuge tube.

The haematocrit tubes containing the cell columns were then cut at the cell-fluid interface and the portions containing the cells were immediately transferred to the tissue culture chambers of a sterile, disposable polystyrene tray (Sterilin) and the closed ends were then anchored to a silicone grease plug attached to the side of the culture chamber. Eagles minimum essential medium containing 10% horse serum was carefully added to fill each of three control chambers. Tuberculin without preservative in Eagles minimum essential medium plus 10% horse serum was then added to each of the other nine chambers at three different concentrations; 500 μg/ml was added to three of the chambers, 120 μg/ml to the second group of three chambers and the concentrations of 30 μg/ml was added to the last three chambers.

A thin layer of silicone grease was applied to the rim of each chamber; these were then sealed with a circular coverslip and transferred to an incubator at 37°C. After 20 hr incubation, clear-cut round areas of migrating cells were apparent surrounding the open end of each haematocrit tube. The migration area of each haematocrit tube was studied by focussing through a microscope without a condenser using a powerful light source and a right-angle

reflecting lens attached to the eye-piece. The image was then projected onto graph paper and the outline carefully traced out. The circular areas were than cut out and each disc of graph paper was weighed, the results being expressed in milligrams. The quantitative value of the migrations was calculated from the average weight of the discs from 3 identically treated cultures.

Final results were expressed as the migration index (*MI*) as a percentage. This was calculated by dividing the average migration of culture containing antigen as represented by the weight of each disc of graph paper from these cultures (*Mx*) by the average migration of normal control cultures represented by the weight of the discs from these cultures (*Mc*); the formula is shown below:

$$MI = \frac{Mx}{Mc} \times 100$$

RESULTS

The results are shown in Tables 1 and 2 and Fig. 1 and 2. Table 1 summarised the migration inhibition and migration increase findings in each group, giving for each dose the number and percentage of subjects in whom inhibition of or increased leucocyte migration was found, the mean inhibition or increase in migration and the standard deviation. The difference between the patient and mole groups is not significant. However, there is a significant difference between both of these groups and the control group in terms of the numbers of people showing the increased migration phenomenon in response to the two lower doses. ($P = < 0{\cdot}001$).

In the treated-patient group 6 patients showed increased migration of leucocytes in response to the medium dose of antigen (120 μg/ml of P.P.D.), 2 responded by an inhibition to the low dose (30 μg/ml of P.P.D.); 2 further patients revealed more migration with the medium dose than with the low dose and the remaining two showed the reverse.

In the normal control group only 2 subjects showed this phenomenon, one of them exclusively to the lowest dose of P.P.D. and the other to all three doses.

However, when the dose was lowered to 15 μg/ml, out of nine subjects, one showed an increase in migration and two showed no response either way; with a dose of 3 μg/ml 4/8 (50%) of normal subjects showed increases while the rest showed inhibition.

Table 2 shows the relationship between migration inhibition or increase and the presence of metastases, both pulmonary and vaginal. In the group who revealed inhibition of leucocyte migration, one patient had presented with both pulmonary and vaginal metastases; similarly in the groups who showed increases there was one patient with both pulmonary and vaginal metastases. The number of subjects involved is too small to allow a statistical analysis to be made.

Figure 1 presents graphically the urinary 24 hr hCG excretion and peripheral blood lymphocyte count in 54 patients with choriocarcinoma at initial presentation. There is no

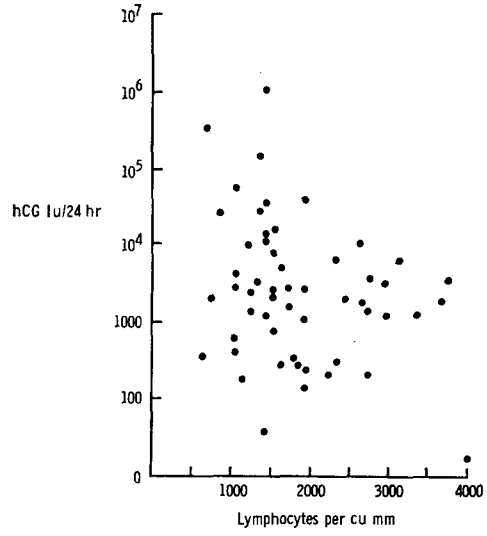


Fig. 1. Choriocarcinoma: hCG (Iu/24 hr urine excretion) and blood lymphocyte count/cmm at presentation.

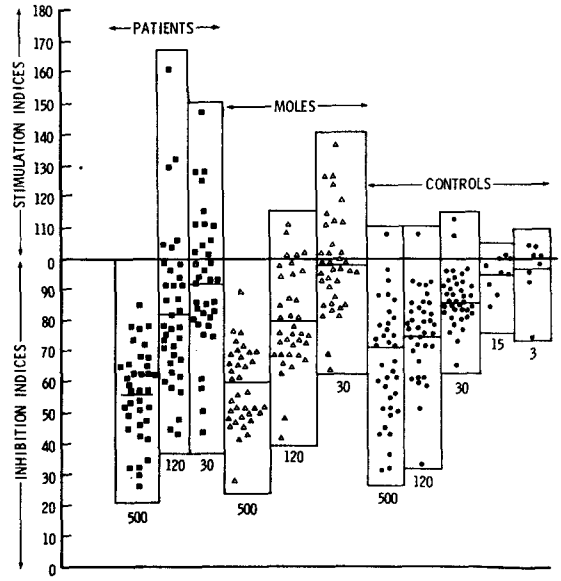


Fig. 2. Leucocyte migration inhibition and stimulation with P.P.D. at various concentrations (μg/ml).

Table 1. *Migration stimulation*

	Concentration of P.P.D. (μg/ml)	No. of subjects	Mean inhibition or stimulation	Standard deviations
Treated Patient Group	30	24 (67%)	78·4 (I)	7·3
		12 (33%)	115·7 (S)	13·1
	120	30 (83%)	68·7 (I)	9·5
		6 (17%)	122·9 (S)	20·7
	500	36 (100%)	50·4 (I)	11·6
Untreated Mole Group	30	22 (61%)	87·5 (I)	4·3
		14 (39%)	113·9 (S)	10·9
	120	31 (86%)	68·7 (I)	9·5
		5 (14%)	104·7 (S)	4·3
	500	36 (100%)	51·9 (I)	11·3
Control Group	30	34 (94·5%)	86·4 (I)	3·8
		2 (5·5%)	110·0 (S)	3·0
	120	35 (97·3%)	75·5 (I)	8·0
		1 (2·7%)	108·0 (S)	—
	500	35 (97·3%)	71·9 (I)	10·6
		1 (2·7%)	108·0 (S)	—

I = Inhibition
S = Stimulation

Table 2. *Proportion of patients with pulmonary or vaginal metastases showing inhibition or increases in migration*

Metastasis	Total number	Inhibition	Increase
Pulmonary	11	8	3
	—	—	—
	36	22	14
Vaginal	4	3	1
	—	—	—
	36	22	14

significant association between high hCG levels and the degree of lymphopenia.

Figure 2 summarises diagrammatically the inhibition and increase indices in all groups and with all doses of P.P.D.

DISCUSSION

In the Leucocyte Migration Test described by Søborg and Bendixen [8], the leucocytes of tuberculin positive patients showed inhibition of migration in the presence of P.P.D. Our observations on normal subjects confirm this but the leucocytes of about one-third of a group of patients previously treated for trophoblastic tumours and a similar proportion of a group of patients who had had hydatidiform moles but no chemotherapy, responded to P.P.D. with increased rather than diminished leucocyte migration compared with antigen-free control cultures. It has to be borne in mind that migration inhibition or enhancement with a particular antigen in a given individual will not necessarily be precisely reproducible on serial testing.

Søborg [9] observed a range of responses to Brucella Abortus Bang antigen including no responses, increased migration and inhibition of migration and he associated these findings with increasing sensitization. However Lockshin [10] was unable to confirm that increased migration occurred. It has been demonstrated that the inhibition of migration depends upon the release of a "Lymphokine" by lymphocytes [11] which has been given the name Migration Inhibition Factor (M.I.F.).

The observations on our patients with trophoblastic tumours were made 3–10 yr following completion of treatment by chemotherapy and all had had at least one pregnancy. The mole group were studied 4–22 months after evacuation of the hydatidiform mole; none of these patients had received chemotherapy. Although 5 of the 36 subjects in the control group had previously been pregnant, none were pregnant at the time of this study so that an effect of pregnancy itself can be excluded. Six of the control subjects were taking oral contracept-

ives. Some of these preparations might be expected to produce similar findings but in our tests, with the P.P.D. doses used on the treated and mole patients, one normal subject who showed enhancement was taking Minovlar, an oral contraceptive of moderate oestrogen content and the other subject who showed enhancement was not taking any form of oral contraception.

Also, the possibility that other diseases, including non-malignant conditions, may be associated with migration enhancement rather than inhibition has not been excluded. Bailey *et al.* [12] found increased leucocyte migration to homologous *E. coli* suspensions in some patients with *E. coli* urinary tract infections; the increased migration was associated with infection localised to the upper urinary tract. These authors concluded that the ability of the homologous infecting organism to increase migration might be an index of defective cell-mediated immunity which, in its turn, could be a significant factor in the pathogenesis of renal parenchymal infection. The concept that increased migration might represent a low level of acquired cell-mediated immunity was suggested by Smith *et al.* [13], based on studies with kidney homogenates in the M.I.F. test applied to renal transplant patients.

The defect could well be a partial one in which M.I.F. production is uniquely defective while other parameters of cell-mediated immunity, including the production of other lymphokines, remains normal [14].

Recently a new lymphokine has been characterised [15] and has been termed Migration Enhancement Factor (M.E.F.). However these authors stressed the biological attributes of this substance rather than attempting to discuss its clinical significance.

Those patients who had clinically evident metastatic disease when treated, showed no greater excess of enhanced responses than those who had not, but the significance of this is limited by the time interval which lapsed between treatment and the time of making the observations reported here.

Clearly, we do not know whether the observed difference in our studies antedated the occurrence of the tumour or mole in our patients and we cannot therefore make any conclusions about its significance as a cause or consequence of the disease process.

Acknowledgements—We would like to thank Professor K. D. Bagshawe and Dr. D. C. Dumonde for helpful advice with this manuscript and all the people who donated blood.

REFERENCES

1. M. G. Whittaker and C. G. Clark, Depressed lymphocyte function in carcinoma of the breast. *Brit. J. Surg.* **58,** 717 (1971).
2. J. Stjernswäld, M. Jondal, F. Vanky, H. Wigsell and R. Sealy, Lymphopenia and change in distribution of human B and T lymphocytes in peripheral blood induced by irradiation for mammary carcinoma. *Lancet* **i,** 1352 (1972).
3. K. D. Bagshawe, Recent observations related to the chemotherapy and immunology of gestational choriocarcinoma. *Advanc. in Cancer Res.* **18,** 231 (1973).
4. S. R. Waltman, R. M. Burde and J. Berrios, Prevention of corneal homograft rejection by estrogens. *Transplantation* **II,** 194 (1971).
5. D. T. Purtilo, H. M. Hallgren and E. J. Yunis, Depressed maternal lymphocyte response to phytohaemagglutinin in human pregnancy. *Lancet* **i,** 769 (1972).
6. J. S. Munroe, Progesteroids as immunosuppressive agents. *J. reticuloend. Soc.* **9,** 361 (1971).
7. A. Maráz and I. B. Petri, Intrinsic alteration of lymphocyte reactivity in women with normal pregnancy or tumor of placental origin. *Cell. Immunology* **10,** 496 (1974).
8. M. Søborg and G. Bendixen, Human lymphocyte migration as a parameter of hypersensitivity, *Acta med. Scand.* **181,** 247 (1967).
9. M. Søborg, *In vitro* migration of peripheral human leucocytes in cellular hypersensitivity. *Act med. Scand.* **184,** 135 (1968).
10. M. D. Lockshin, Failure to demonstrate leukocyte migration inhibition in human tuberculin hypersensitivity. *Proc. Soc. exp. Biol. (N.Y.)* **132,** 928 (1969).
11. D. C. Dumonde, R. A. Wolstencroft, G. S. Panayi, M. Matthew, J. Morley and W. T. Howson, "Lymphokines." Non-antibody mediators of cellular immunity generated by lymphocyte activation, *Nature (Lond.)* **224,** 38 (1969).
12. R. R. Bailey, A. P. Roberts, B. Rowe and D. C. Dumonde, The leucocyte migration test in urinary tract infection. *New Zeal. med. J.* **80,** 10 (1974).

13. M. G. M. Smith, A. L. W. F. Eddleston, J. A. Dominguez, D. B. Evans, M. Bewick and R. Williams, Changes in leucocyte migration after renal transplantation. *Brit. med. J.* **4,** 275 (1969).
14. D. C. Dumonde, "Lymphokines": molecular mediators of cellular immune responses in animals and man. *Proc. Roy. Soc. Med.* **63,** 899 (1970).
15. R. H. Weisbart, R. Bluestone, L. S. Goldberg and C. M. Pearson, Migration Enhancement Factor: A new lymphokine. *Proc. nat. Acad. Sci.* (*Wash.*) **71,** 875 (1974).

Europ. J. Cancer Vol. 12, pp. 283–290. Pergamon Press 1976. Printed in Great Britain

Tumour Immunity in Tetraparental Mouse Chimaeras—A Review

R. D. BARNES
Clinical Research Centre, Harrow, Middlesex, HA1 3UJ, Great Britain

Abstract—*Mouse chimaeras derived by aggregation of early embryos have been used for a variety of purposes including a study of tumour development which is discussed here.*

Whereas in certain chimaeras the innate susceptibility associated with one of the component strains is unaltered, perhaps more importantly, in other chimaeras, tumour resistance appears dominant. How tumour resistance is conferred in the latter situation is discussed in relation to the evidence that is available at the present time.

INTRODUCTION

CHIMAERAS derived by aggregation of early embryos [1] are obtained generally by techniques pioneered by Tarkowski [2] and Mintz [3]. More recently, a more sophisticated microsurgical technique has been introduced [4]. Such EEA-derived chimaeras have been used for a variety of purposes, including a study of tumour development which is discussed here. In this field, Mintz has played a major role in exploiting the research potential of such chimaeras [5, 6]. Upon the basis of her findings Mintz has assumed that tumour susceptibility was unaltered in tumour susceptible Ⓢ ↔ tumour resistant Ⓡ chimaeras [5, 6]. We have since disputed this assumption [7, 8] and now have attempted to resolve these differences.

TUMOURS INVESTIGATED

(1) *Mammary tumours*

One of the first tumours to be studied by Mintz in the EEA chimaera model was mammary carcinoma [5, 6]. The following strains, each with a varying but fixed incidence of mammary tumours, were used to obtain EEA chimaeras:

C3Hf	—	C57BL/6
(43%; 15 months)		(0%)
C3H/HeNicr	—	C57BL/6*
(100%; 10 months)		(40%; 14 months)

(Age—Average age of tumour onset.)
*—milk fostered on C3H.

Accepted 6 May 1975.

The incidence of mammary tumours in the resultant Ⓢ ↔ Ⓡ C3Hf ↔ C57BL/6 chimaeras was 50% (5/10); (18 months) and in the C3H/HeNicr ↔ C55BL/6 was 85% (23/27); (10 months).

With the possible exception of the latter combination, little effect could be attributed, therefore, to the relatively resistant C57BL/6 component.

Isozymal analysis (malate dehydrogenase) revealed subsequently that although the majority of tumours was derived from the susceptible strain, there were exceptions (4/42).

One out of six tumours in the C3Hf ↔ C57BL/6 and 3/41 tumours in the C3H/HeNicr ↔ C57BL/6 Ⓢ ↔ Ⓡ chimaeras were noted to have originated from the resistant C57BL/6 strain. Apart from this, 5/41 tumours appeared to have originated from both Ⓢ and Ⓡ strain components in the C3H ↔ C57BL/6 group. The same was true in a smaller series of C3H ↔ BABL/cⓈ ↔ Ⓡ chimaeras, where a C3H and BALB/c carcinoma was detected [5, 6].

The presence of malignancy in the strain that is normally tumour-resistant has to be explained. In this respect, we suggest that Mintz cannot conclude that neither "susceptibility nor resistance is transmitted between cells" [6]. Again, but in the context of resistance, the slightly delayed incidence of mammary tumours in the C3Hf ↔ C57BL/6 and reduced incidence in the C3H/HeNicr ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras suggest a possible influence attributed to the Ⓡ resistant strain.

Mintz extended her investigation of mammary tumour development by studying the fate of transplanted pre-malignant hyperplastic

nodules. These nodules obtained from the C3Hf ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras were found to grow in both Ⓢ and Ⓡ parental strain recipients. This suggested the presence of both parental strain cell populations in the pre-malignant nodule. However, the growth patterns in the two situations were different. Whereas the nodule remained hyperplastic in the Ⓢ (susceptible) strain recipient, it became normal in the Ⓡ (resistant) recipient. Although Mintz concluded that the cellular genome of the mammary epithelium was of primary importance [6], the influence of other host factors in the resistant strain must be considered. In light of our current knowledge concerning the effect of various host-determined genetic factors upon virus-associated mammary tumour development such factors cannot be dismissed without consideration.

(2) *Hepatomas*

The development of spontaneous hepatomas was studied by Mintz in chimaeras derived from:

C3H ♂ (69%; 24 months) — C57BL/6 ♂ (5%; 25 months)
C3Hf ♂ (33%; 20 months)

Whereas the incidence in the C3H ↔ C57BL/6Ⓢ ↔ Ⓡ situation was 45% (13/29 at an average of 24 mos) in the C3Hf ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras the incidence was 30% (3/10 at an average age of 23 months). In both cases the incidence was less than in the corresponding Ⓢ parental strain.

Isoenzymal analysis (NAPD-dependent malate dehydrogenase) showed that hepatomas were not confined to the Ⓢ strain [6, 9]. Of the 25 hepatomas seen in the C3H ↔ C57BL/6-Ⓢ ↔ Ⓡ chimaeras, one tumour was found to be a C3H + C57BL/6Ⓢ + Ⓡ mosaic insofar as both isoenzymes were detected. One of the four tumours in the C3Hf ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras was also found to have originated from the C57BL/6Ⓡ strain component. Tumour development in the Ⓡ strain component of the chimaeras was subsequently confirmed by assaying β-glucuronidase activity [9]. Levels of this enzyme are known to be controlled by allelic differences at a single locus in the mouse [10]. Using this marker, 2/12 hepatomas in a group of C3H ↔ C57BL/6 chimaeras were found to be a Ⓢ + Ⓡ mixture [6].

From the data available, two facts are apparent: Firstly, the incidence of hepatomas is generally reduced in the Ⓢ ↔ Ⓡ chimaeras. Secondly, tumours were noted to have developed in the Ⓡ strain cellular component.

Another observation should be noted. Analysis of normal hepatic tissue (using the β-glucuronidase assay) showed that the Ⓢ strain usually predominated. The same appears to be true in the case of the lymphoma Ⓢ ↔ Ⓡ AKR ↔ CBA/H-T6 chimaeras [11, 12]. In both situations, the Ⓢ cell component was found to predominate in the tissue destined for tumour development—prior to the development of malignancy. Again, and in both cases, tumours were seen to arise in the relatively minor Ⓡ cell population [6, 12]. This will be discussed later; meanwhile, it should again be stressed that the overall incidence of hepatomas in the Ⓢ ↔ Ⓡ chimaeras was less than in the parental Ⓢ strain.

(3) *Lung tumours*

BALB/cAnNicr ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras were investigated by Mintz for the incidence of lung tumours [6]. Again, the overall incidence of 36% (8/22) was less than in the Ⓢ parental strain where the incidence was 56% (11/19). As before, tumours were not confined to the Ⓢ strain. Analysis (glucose phosphate isomerase g.p.i.) of the marker showed that 4/8 tumours possessed the corresponding Ⓡ C57BL/6 marker. Although contamination cannot be excluded with certainty, actual C57BL/6Ⓡ cell tumour development cannot be excluded.

The reduced incidence of tumours in the chimaeras points to the probable influence of the Ⓡ strain component and certainly *not* to the conclusion "that cell extrinsic to the lung are necessarily of little consequence" [6].

(4) *Reticulum cell sarcomas*

Reticulum cell sarcoma-prone NZB ↔ CFW and NZB ↔ BALB/cⓈ ↔ Ⓡ were recently investigated by us [13, 14]. Whereas approximately 60% of NZB mice develop reticulum cell sarcomas [15], such tumours have yet to be seen in our CFW and BALB/c sublines, (unpublished observation).

Two facts emerged from the study of these reticulum cell sarcoma Ⓢ ↔ Ⓡ chimaeras. Firstly, the incidence of tumours was much less than in the susceptible NZB, since sarcomas were only identified in two out of a total of six NZBⓈ ↔ Ⓡ chimaeras; both in the group of four NZB ↔ CFW chimaeras [13, 14]. This was at an age that was greater than the normal life expectancy of the NZB [14].

One tumour appeared particularly interesting since tumour cells were observed in peripheral blood. "Spill over" into the peripheral blood is uncommon in the NZB [15], that is, except when treated with immunosuppressive agents [16]. Evidence has since suggested that a form of immunosuppression may have occurred in these chimaeras as a consequence of graft-versus-host (GVH)-like activity [17, 18]. Since GVH is known to cause immunosuppression [19], it would appear that the GVH-like reaction may have been responsible for the acute progression of the tumour. This is only mentioned to emphasise one possible factor that may be involved in a study of tumour development in any Ⓢ ↔ Ⓡ chimaera.

(5) *Plasmacytomas*

Mintz studied plasmacytomas (myelomas) induced by mineral oil in BALB/c ↔ C57BL/6 Ⓢ ↔ Ⓡ chimaeras and Wegmann and Phillips investigated *spontaneous* plasmacytomas in SJL/J ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras.

The induced plasmacytomas may not have been restricted to the Ⓢ (susceptible) BALB/c component since it seemed that certain of the tumours may have originated from the C57BL/6 Ⓡ component.

Although the data is preliminary, Wegmann and Phillips have shown both a delayed and reduced incidence of spontaneous plasmacytomas in a group of 23 EEA derived SJL/J ↔ C57BL/6Ⓢ ↔ Ⓡ chimaeras, (personal communication). It was interesting to note that the only tumour was in a mouse that was subsequently noted to have features of GVH-like disease. It is interesting to speculate upon the possible association of the tumours and GVH-like disease in light of the earlier-mentioned finding, where we noted acute progression of another tumour seen in one of the NZB ↔ CFW chimaeras where GVH-like activity was also demonstrated.

(6) *Lymphomas*

Like Mintz [6] we have also investigated EEA derived lymphoma-prone AKRⓈ ↔ Ⓡ chimaeras [7, 8]. Results upon first examination would appear to conflict. Whereas tumour susceptibility of the AKR was unaltered in Mintz's three AKR ↔ C3H chimaeras [6] we recorded a markedly reduced and delayed incidence of lymphomas in our AKR ↔ CBA/H-T6 chimaeras [7, 8]. Before attempting to explain the difference between these two sets of results we should note certain similarities. Apart from a 100% incidence of lymphomas in both sublines of parental AKR, AKR cells were found to predominate in each situation prior to the stage of lymphoma development [6, 11, 12].

Our overall findings in the AKR ↔ CBA/H-T6 chimaera were also similar to those of Mintz but only in the case of non-lymphomatous tumours [6]. The fact that one tumour was seen in the Ⓡ (resistant) CBA/H-T6 suggests an Ⓢ ↔ Ⓡ influence [12]. Perhaps more important was the fact that the incidence of tumours was generally less in the Ⓢ ↔ Ⓡ chimaeras than in the corresponding Ⓢ parental strain. This suggests an Ⓡ → Ⓢ reaction. The findings in our AKR ↔ CBA/H-T6 are now discussed in light of the two areas of interstrain activity, namely Ⓢ → Ⓡ and more important Ⓡ → Ⓢ reactivity.

CBA mice retain their innate resistance to lymphoma even when they are derived following embryo transplantation from AKR mothers [20]. In contrast, one of the six lymphomas seen in the AKR ↔ CBA/H-T6 chimaeras was identified cytogenetically as being CBA/H-T6 in origin [12]. Although the mechanism still remains to be determined, this clearly points to a Ⓢ → Ⓡ activity.

In contrast to the findings in the lymphoma prone AKR ↔ C3HⓈ ↔ Ⓡ situation investigated by Mintz [6], the delayed and reduced incidence of lymphomas in our AKR ↔ CBA/H-T6 chimaeras is similar to the finding in Mintz's other non-lymphomatous tumour studies [6]. This suggests Ⓡ → Ⓢ reactivity. Before attempting to explain this difference, it seems important firstly to justify Ⓡ → Ⓢ activity and then attempt to see how this may have been achieved.

The incidence of lymphomas in the AKR ↔ CBA/H-T6 chimaeras was undoubtedly both delayed [7] and diminished [8]. Although it might be considered that a reduction in incidence from 100% to 30% is only relative, the findings detailed below reduce the significance of this argument. It could be argued that the surviving mice may have subsequently developed lymphomas if left to live out their natural life span. However, this possibility must be considered in light of the fact that the majority of chimaeras lived and were then investigated well beyond the average life span of our parental AKR [21]. Accepting Ⓡ → Ⓢ activity it remained to determine the mechanism involved. Hopefully the following facts, will clarify the issue.

(i) In respect of mixed coat colour composition and in the proportion of the functional

gametes, the chimaeras were roughly "balanced" (neither one of the two types was always dominant) [8].

(ii) In spite of (i) there was an overwhelming preponderance of AKR cells in:

(*a*) PHA stimulated blood cultures [11]

(*b*) lymphomyeloid [12] and

(*c*) somatic tissue [12]

(iii) This AKR predominance was also matched at a cell product level;
e.g.

(*a*) red cell isoenzymes [22]

(*b*) serum allotype [22]

(iv) Numerous type-C murine leukaemia virus-like particles were found to be present throughout every tissue [23] and

(v) Gross antigen specificity was subsequently confirmed [24].

(vi) Surprisingly, and in view of (v), there was, in marked contrast to the AKR, very little evidence of renal antibody–antigen complexes [24].

(vii) Absence of complexes has now been shown to be due to absence of anti-Gross antibody activity [25, 26].

(viii) This in turn raised the possibility that absence of masking antibody–viral antigen complexes has enabled "normal" tumour immunity to be effected in the chimaeras [27]. The remaining question is whether the breakdown of tolerance to the Gross virus is the primary defect of the AKR in terms of tumour susceptibility [28].

This issue aside, let us remember that although the initial results showed an overwhelming excess of AKR T cells in the PHA-stimulated peripheral blood cultures [11], analysis upon the basis of θ surface antigenic determinants showed roughly equal numbers of AKR and CBA T cells [29]. These two findings therefore appeared paradoxical, although this apparent conflict could be resolved if it could be shown that AKR T cells had been processed with θ^{CBA}. That is, θ processing had occurred independent of the cellular genome. This view has some support since following treatment of their lymphoid cell suspensions with anti θ^{AKR} (in the presence of C′) and subsequent selective T cell mitogenic stimulation (CON. A) a significant number of AKR cells were identified cytogenetically [30]. If θ processing has occurred independent of the cellular genome then the process might be analogous to acquisition of certain red cell blood groups in both man and sheep. In both situations red cell surface antigens are acquired from a source other than the red cell or its precursor. The human Lewis blood group [31] and in sheep [32] the R and O red cell groups [32] are both "painted" from a source external to the red cell. Conceivably, θ processing may occur in a similar manner.

Although θ processing independent of the cellular genome has yet to be proven, two related situations concerning other cell surface antigens should be noted. When a TL+ (thymus leukaemia) leukaemia is transplanted into syngeneic mice preimmunised against TL antigens for example, the phenotype of leukaemia cells is changed (modulated) from $TL^+ \rightarrow TL^-$, whilst the anti-TL antibody is present [33]. The same is true for the Gross (G)-virus-associated cell-surface-antigen GCSA. This again becomes (modulated) in animals preimmunised with $GCSA^+$ cells [34]. Whether similar modulation has occurred in the AKR $\leftrightarrow$ CBA chimaeras in respect of θ determinants remains to be proven. At this stage, that there is an AKR T cell population present that survives treatment with anti θ^{AKR} is all that can be stated.

Although C-type murine leukaemia virus-like particles were identified in all the chimaeras, their numbers varied [23]. Similarly, although Gross (gs-1) antigen specificity was confirmed in each case, the titre varied [26]. Curiously enough, in each case, viral replication appeared related to coat colour composition. (Viral replication is used here in context of numbers of C-type particles and levels of gs-1 antigen). Although it was not surprising to note that viral replication was maximum in the chimaeras that were predominantly albino (AKR) what is remarkable is the fact that viral replication was notably less in the more agouti (CBA) chimaeras—*mice that were still predominantly AKR at a cellular level* [11, 12]. In other words, oncogenic viral replication in cytogenetically-defined AKR cells varied in relation to coat colour composition of the chimaeras. Re-examination of our earlier findings [29] has since showed that the proportion of the two θ defined cell populations also paralleled coat colour composition.

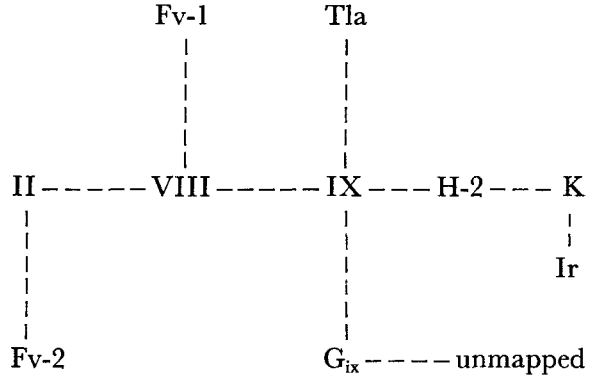
Like skin, the stroma of the thymus is also derived from ectoderm. In chimaeras derived by aggregation of very early undifferentiated embryos, it might therefore be anticipated that the composition of ectodermally-derived thymic stroma might parallel that of the coat. Although yet to be proven, this assumption does not appear unreasonable. One is therefore left to consider how the coat/thymic stromal

composition can influence oncogenic viral replication, and furthermore, in what now appears to be genotypically essentially an AKR chimaera. It must be presumed that the influence is manifest through some soluble factor. In this context we were interested to note a recent report [35] defining the influence of a soluble factor that diminished *in vitro* infectivity of the N-associated Gross virus. The question remains, however, whether we are seeing the effect of the same or similar factor in the chimaeras *in vivo*. Although there are obvious differences between the *in vitro* and *in vivo* conditions, if the phenomenon were the same, the *in vivo* situation offers distinct advantages in further investigation. Isolation, definition and determination of the source of the factor appear tremendously important in respect to influencing oncogenic viral replication and therefore, tumour development in general. The possibility that the source of this factor being stromal elements of the thymus has to be considered.

Since our experiments were commenced, the field of murine tumour genetics has emerged. Whereas it is now known that the Fv-1 locus determines permissiveness of cells to infection with endogenous murine leukaemia virus, the Fv-2 locus control infectivity with the Friend group of oncogenic viruses. The two alleles defined at the Fv-1 locus are Fv-1^n and Fv-1^b, the latter being dominant [36]. At the Fv-2 locus, the alleles controlling susceptibility to Friend-induced leukaemia viral infection, are Fv-2^s and Fv-2^r (s-susceptibility, r-resistant)—again the latter allele being dominant [37]. As mentioned above, both loci control *infectivity* to the oncogenic murine leukaemia viruses.

Another and major H-2 histcompatibility locus has been associated with virus induced *tumour susceptibility* since H-2^k mice are notably susceptible to virus associated lymphoma [38]. It therefore was not surprising to find the C3H/He in Mintz's Ⓢ ↔ Ⓡ lymphomatous chimaeras were like the AKR, Fv-1^n, Fv-2^s and H-2^k positive. What is surprising, is that this is *also* the situation in the lymphoma-resistant CBA. This has argued for the existance of *another and furthermore dominant factor in the CBA* that leads to lymphoma resistance [21]. It is presumed that this factor is responsible for Ⓡ → Ⓢ activity in the AKR ↔ CBA/H-T6 chimaeras. Whether it is the same factor(s) that influences oncogenic Gross viral replication or maintains tolerance still remains to be determined.

The theoretical association of these three and the other related loci with linkage groups II, VIII and IX are shown in the following diagram:



The Tla and G_{ix} loci are both closely associated with mouse leukaemia and like H-2, are both related to linkage group IX.

As mentioned previously, there is no obvious difference between the AKR, C3H and CBA at either Fv-1, Fv-2 or at the major H-2 (H-2^k) locus—the same is true at the Tla locus [39]. There is, however, a difference at the G_{ix} locus [40, 41]. Whereas both the AKR and the C3H/He (the subline used by Mintz) [6] are similar and show high titres of the corresponding G_{ix} cell surface antigen, no G_{ix} antigen is detected in the CBA/H-T6 [40, 41]. It has been suggested that G_{ix} might represent the viral gene [39] and in this context it is interesting to note that two unlinked genes are required for G_{ix} antigen expression. One is semi-dominant and is associated with linkage group IX, whereas the more important fully dominant gene remains unmapped. One is left with the possibility that this might be where the advantage is confirmed in the AKR ↔ CBA situation. In retrospect, it would have been interesting to see if G_{ix}^{-ve} became G_{ix}^{+ve} in the AKR ↔ CBA chimaeras that developed lymphomas, especially the one that was shown to have a CBA lymphoma. Regarding G_{ix} and its influence on Gross virus cell surface antigen, one is naturally left to consider whether this is how tolerance to the corresponding Gross virus is maintained in the AKR ↔ CBA/H-T6 chimaeras.

Whereas we initially started comparing our AKR and CBA, it now seems important to consider all the differences between our ⓇCBA and Mintz's ⓈC3H. In respect to tumour resistance, one possible area of interest is the Ir (immune response) complex which is linked to the K end of H-2 region [41]. It seems important to consider this region as another possible area where there may be a difference between the C3H (and AKR) and the CBA.

In terms of comparing the immune response of the CBA and C3H it was interesting to note a recent report that clearly suggested differences between CBA and C3H [42–44].

Initially, new-born CBA thymus cells were shown to react against syngeneic spleen cells in mixed lymphocyte culture [42, 43]. This self thymus → spleen CBA reactivity, however, is short-lived and is lost within the first few days of life [44]. What is equally interesting was the failure to demonstrate similar self reactivity in the closely-related C3H/He—the same subline as used by Mintz [6]. Furthermore, whereas reactivity of CBA thymocytes against syngenic spleen cells was short-lived, the reaction of C3H thymocytes against CBA spleen cells could be demonstrated for a much longer period without any "switching off" of such activity. For the purpose of this discussion it only appears important to note that there were clear differences between the immunogenicity of the CBA and C3H. The question remains, does this explain the difference in tumour susceptibility between Mintz's C3H Ⓢ → Ⓡ and our CBA Ⓡ → Ⓢ chimaeras. In this context one is left to query whether the remarkable property in "switching off" self reactivity in the CBA contributed to the tumour resistance of the AKR ↔ CBA chimaeras. Here it is interesting to note that the AKR are notably "in-tolerant" to Gross virus infection, since from the age of three months antibody against Gross virus associated antigens can be demonstrated [45]. In spite of early acquisition upon the germ line, tolerance is not maintained and one is left to consider whether this is due to the late exposure of corresponding viral antigens. The CBA, with its remarkable ability to recognise and "switch-off" self reactivity might well be relevant and could have conceivably also switched off anti-Gross activity in the AKR ↔ CBA chimaeras. Thus, in the absence of masking anti-Gross viral antigenic complexes, tumour immunity can be effected. It is known that Gross antigens can only be detected relatively late in the *in-utero* life of the AKR [46] but conceivably this is still early enough for the CBA to recognise the corresponding antigens as "self" and for tolerance to be maintained. Meanwhile, this interesting chimaera model continues to be tested in attempts to determine:

(i) the source and nature of factors that influence oncogenic viral replication,

(ii) whether θ processing occurs independent of the cellular genome,

(iii) how tolerance to the oncogenic virus is maintained, and

(iv) location of the genetic control of the whole process(es), all in respect of learning how the AKR ↔ CBA chimaeras remains relatively lymphoma resistant.

REFERENCES

1. R. D. Barnes, Tetraparental animals and their uses. *Proc. II. Int. Conf. of Immunology*. In *Progress in Immunol.* **2,** 397 (1974). North Holland Publishers.
2. A. K. Tarkowski, Mouse chimaeras developed from fused eggs. *Nature (Lond.)* **190,** 857 (1961).
3. B. Mintz, Experimental study of the developing mammalian egg: Removal of the zona pellucida. *Science* **138,** 594 (1962).
4. R. Gardner, Manipulations on the blastocyst. *Adv. Bioscience* **6,** 279 (1970).
5. B. Mintz, Genetic Concepts and Neoplasia. *Proc. 23rd Ann. Symp. Fundamental Cancer Research*, M. D. Anderson Hospital and Tumour Inst. p. 477 Williams & Wilkins, Baltimore (1970).
6. B. Mintz, R. P. Custer and J. Donnelly, Genetic defects in allophenic mice. In *Int. Rev. of Exp. Path.* Edited by G. W. Richter and M. A. Epstein. Vol. 10 p. 143 Acad. Press. New York and London (1972).
7. R. D. Barnes, M. A. Tuffrey and J. Kingman, The delay of leukaemia in tetraparental ovum fusion-derived AKR chimaeras. *Clin. Exp. Immunol.* **12,** 541 (1972).
8. R. D. Barnes, M. A. Tuffrey and C. E. Ford, Suppression of lymphoma development in tetraparental AKR mouse chimaeras derived from ovum fusion. *Nature New Biol.* **244,** 282 (1973).
9. H. Condamine, R. P. Custer and B. Mintz, Pure-strain and genetically mosaic liver tumours histochemically identified with the β-glucuronidose marker in allophenic mice. *Proc. nat. Acad. Sci. (Wash.)* **68,** 2032 (1971).
10. K. Paigen, The genetic control of enzyme activity during differentiation. *Proc. nat. Acad. Sci. (Wash.)* **47,** 1641 (1961).

11. M. TUFFREY, R. D. BARNES, E. P. EVANS and C. E. FORD, Dominance of AKR mitoses in tetraparental AKR ↔ CBA/H-T6T6 chimaeras. *Nature New Biol.* **243,** 207 (1973).
12. C. E. FORD, E. P. EVANS, M. D. BURTENSHAW, H. CLEGG, R. D. BARNES and M. TUFFREY, Marker chromosome analysis of chimaeras: Dominance of AKR mitoses in tetraparental AKR ↔ CBA-T6 mice. *Differentiation* **2,** 257 (1974).
13. R. D. BARNES, M. TUFFREY, J. KINGMAN, C. THORNTON and M. W. TURNER, The disease of the NZB mouse I: Examination of ovum fusion derived tetraparental NZB: CFW chimaeras. *Clin. exp. Immunol.* **11,** 605 (1972).
14. R. D. BARNES, M. TUFFREY and E. J. WILLS, The disease of the NZB mouse II. Further studies on ovum fusion derived tetraparental NZB ↔ CFW chimaeras. *J. Immunogenet.* **2,** 195 (1975).
15. J. EAST, Immunopathology and neoplasms in New Zealand Black (NZB) and SJL/J mice. *Progr. exp. Tumour Res.* **13,** 84 (1970).
16. T. P. CASEY, Azathioprime (Imuran) administration and the development of malignant lymphomas in NZB mice. *Clin. exp. Immunol.* **3,** 305 (1968).
17. R. D. BARNES and M. TUFFREY, Tolerance in ovum fusion derived tetraparental mouse chimaeras. In *Microenvironmental Aspects of Immunity*, Edited by B. D. JANKOVIC and K. ISAKOVIC, p. 427, Plenum Publishing Corp., N.Y. (1972).
18. R. D. BARNES, J. HOLLIDAY and M. TUFFREY, Immunofluorescence and elution studies in tetraparental NZB ↔ CFW chimaeras and graft-versus-host diseased NZB. *Immunology* **26,** 1195 (1974).
19. M. KOLTAY, R. G. KINSKY, B. G. ARNASON and J. B. SCHAFFER, Immunoglobulins and antibody formation in mice during the graft-versus-host reaction. *Immunology* **9,** 58 (1965).
20. R. D. BARNES and M. TUFFREY, Absence of lymphomas in CBA mice derived by embryo transfer and born from lymphoma-prone AKR mice. *Europ. J. Cancer* **10,** 575 (1974).
21. R. D. BARNES and M. TUFFREY, Lymphoma susceptibility of the AKR mouse strain acquired before the stage of implantation. *Brit. J. Cancer* **26,** 400 (1974).
22. R. D. BARNES, M. TUFFREY, L. DRURY and D. CATTY, Unequal rates of cell proliferation in tetraparental mouse chimaeras derived by fusion of early embryos. *Differentiation* **2,** 257 (1974).
23. E. J. WILLS, M. TUFFREY and R. D. BARNES, Type-C murine leukaemia virus particles in lymphoma resistant AKR ↔ CBA chimaeras. *J. clin. exp. Immunol.* (1975).
24. R. D. BARNES, M. TUFFREY, J. HOLLIDAY, J. H. M. HILGERS and T. SOUISSI, Gross viral antigen in tumour resistant tetraparental AKR ↔ CBA/H-T6 chimaeras. *Brit. J. Cancer* (In press).
25. R. D. BARNES, M. TUFFREY and J. HOLLIDAY, Failure to detect antibody against Gross virus in tetraparental AKR ↔ CBA chimaeras. *Brit. J. Cancer* **3,** 1 (1975).
26. R. D. BARNES, M. TUFFREY and R. C. BOURNE, Failure to detect anti MuLV-gs antibody in tetraparental AKR ↔ CBA chimaeras. *Cancer Res.* **35,** 2699 (1975).
27. R. D. BARNES, Leukaemia—an immune deficiency? *Adv. Bioscience* **12,** 160 (1974).
28. R. D. BARNES, The use of embryo transfer and embryo fusion to study the immune response to virus infection in mice. *Proc. W.H.O. Int. Symp. on Infection and Immunology in Rheumatic Diseases.* Blackwell, Oxford (1975).
29. C. A. BONA, M. TUFFREY and R. BARNES, Distribution of theta antigens in ovum fusion derived AKR ↔ CBA/H-T6 tetraparental mouse chimaeras. *Tissue Antigens* **4,** 31 (1974).
30. R. D. BARNES, M. TUFFREY, E. P. EVANS, M. D. BURTENSHAW and C. E. FORD, Possible acquisition of theta antigen independent of the cellular genome (Submitted for publication).
31. R. R. RACE and R. SANGER (Eds), In *Blood Groups in Man.* Blackwell, Oxford (1968).
32. J. RENDEL, Further studies on some antigenic characters of sheep blood determined enistatic action of genes. *Acta. Agric. Scand.* **1,** 224 (1957).
33. E. A. BOYSE, E. STOCKERT and L. J. OLD, Modification of the antigenic structure of the cell membranes by thymus leukaemia (TL antibody) *Proc. nat. Acad. Sci. (Wash.)* **58,** 954 (1967).

34. T. Aoki and P. A. Johnson, Suppression of Gross leukaemia cell-surface antigens; a kind of antigenic modulation. *J. nat. Cancer Inst.* **49,** 183 (1972).
35. R. W. Tennant, B. Schluter, Wen Kuang Yang and A. Brown, Reciprocal inhibition of mouse leukaemia virus infection by Fv-1 allele cell extracts (gene action cell culture/virus host range/restriction mechanism). *Proc. nat. Acad. Sci. (Wash.)* **11,** 4241 (1974).
36. J. W. Hartley, W. P. Rowe and R. J. Huebner, Host range restrictions of murine leukaemia viruses in mouse embryo cell cultures. *J. Virol.* **5,** 221 (1970).
37. F. Lilly, Fv-2: Identification and location of a second gene governing the spleen focus response to Friend leukaemia in mice. *J. nat. Cancer Inst.* **45,** 163 (1970).
38. F. Lilly, Mouse leukaemia: A model of a multiple-gene disease. *Bibl. haematol. (Basel)* **36,** 213 (1970).
39. E. A. Boyse and L. J. Old, Some aspects of normal and abnormal cell surface genetics. *Ann. Rev. Genetics* **3,** 269 (1969).
40. E. Stockert, L. J. Old and E. A. Boyse, The G_{ix} system: A cell surface alloantigen associated with murine leukaemia virus: Implications regarding chromosomal integration of the viral genome. *J. exp. Med.* **133,** 1334 (1971).
41. E. A. Boyse, L. J. Old and E. Stockert, The relation of linkage group IX to leukaemogenesis in the mouse. In *RNA Viruses and Host Genome in Oncogenesis.* Edited by P. Emmelot and P. Bentvelzen, p. 171. North-Holland, Amsterdam (1972).
42. M. L. Howe, A. L. Goldstein and J. R. Battisto, Isogeneic lymphocyte interaction: Recognition of self antigens by cells of the neonatal thymus. *Proc. nat. Acad. Sci. (Wash.)* **67,** 613 (1970).
43. H. V. Boehmer and W. J. Byrd, Responsiveness of thymus cells to syngeneic and allogeneic lymphoid cells. *Nature New Biol.* **235,** 50 (1972).
44. J. Carter and T. Wegmann, Mendelian segregation of a tolerance-inducing self-antigen in the spleen. *Cellular Immunol.* **7,** 402 (1973).
45. M. B. A. Oldstone, T. Aoki and F. J. Dixon, The antibody response of mice to murine leukaemia virus in spontaneous infection: Absence of classical immunological tolerance. *Proc. nat. Acad. Sci. (Wash.)* **69,** 134 (1972).
46. J. Hilgers, A. Declève, J. Galesloot and H. S. Kaplan, Murine leukaemia virus group-specific antigen expression in AKR mice. *Cancer Res.* **34,** 2553 (1974).

Europ. J. Cancer Vol. 12, pp. 291–297. Pergamon Press 1976. Printed in Great Britain

The Effect of Daunomycin on Cell Proliferation and Protein Synthesis of Human Leukemic Blast Cells*

GUNHILD LANGE WANTZIN, HANS KARLE, PREBEN PHILIP and SVEN-AAGE KILLMANN

Division of Hematology, Department of Medicine A, Rigshospitalet, University Hospital of Copenhagen, Denmark

Abstract—*The effect of daunomycin (DMC) (about 1·5 mg/kg body weight) on cytokinetic parameters and ^{3}H-leucine incorporation of leukemic blast cells in bone marrow and blood was studied at intervals up to 96 hr after DMC in 5 patients, 4 with AML and 1 with AUL.*

In all cases the MI showed a decrease 6–12 hr after DMC. The ^{3}H-TDR values declined at various times (12–96 hr after) DMC. The depression of the MI and the ^{3}H-TDR LI was long-lasting and pretreatment values were not yet reached by 72–96 hr after DMC.

The incorporation of ^{3}H-leucine into human leukemic blast cells was shown to be inhibited by DMC late (72–96 hr) after drug administration in four out of five patients. The late decrease in leucine incorporation suggests that DMC besides an immediate direct killing effect seen from the decrease in the number of circulating blast cells in the peripheral blood also has a late inhibiting effect on LBC. The late effect suggests that protein synthesis is reduced secondary to effects on DNA and/or RNA synthesis.

INTRODUCTION

DAUNOMYCIN has been shown to inhibit the synthesis of DNA and RNA *in vivo* and *in vitro* [1–4]. Presumably the drug forms a stable complex with preformed DNA by intercalation between adjacent base pairs of the double helix [5]. It is believed that this action is responsible for the observed inhibition of DNA polymerase and RNA polymerase [4].

During the last few years DMC has been shown to be active in the treatment of acute leukemia given as a single drug [6–9] and in combination therapy with other drugs [10–13].

Previous studies of the cytokinetic effects of DMC on leukemic blast cells in human acute leukemia have demonstrated an inhibitory action on the progression of cells through G_2 phase [14], a depression of DNA synthesis [14,15] and a general lytic action on leukemic cells [15].

The purpose of this study was to examine possible long-term effects of a single dose of DMC on the kinetics of human LBC and to demonstrate a possible inhibition of DMC on protein synthesis in LBC as estimated from ^{3}H-leucine incorporation.

MATERIALS AND METHODS

Studies were done in five adult patients, four with AML and one with AUL. The patients and some pertinent data are registered in Table 1.

DMC was given as a single i.v. "flash" injection except in patient HA (AML), who received two doses of DMC at a 24-hr interval. Bone marrow and blood samples were taken immediately before and at intervals up to 96 hr after DMC.

Accepted 8 December 1975.

*Work supported by the Danish Medical Research Council, The Danish Cancer Society and P. Carl Petersen's Foundation.
Work performed under the auspices of the European Organization for the Treatment of Cancer.

List of abbreviations:
^{3}H-TDR: ^{3}H-thymidine, ^{3}H-LEU: ^{3}H-leucine, LI: labeling index, MI: mitotic index, MGC: mean grain count, "U-cells": cells with a DNA content corresponding to cells in S phase which do not label with ^{3}H-TDR. AML: acute myeloblastic leukemia, AUL: acute unclassifiable leukemia, DMC: daunomycin, ARA-C: cytosine arabinoside, BM: bone marrow, B: blood, LBC: leukemic blast cells.

Table 1. *Clinical and therapeutic data on patients studied*

Patient	Diagnosis	Sex/Age (yr)	Body weight	Previous treatment	Drug given during study
HA	AML-relapse	F 43	58·0	160 mg ARA-C + 120 mg thioguanine for 18 days, 7½ months before study and one dose of 160 mg ARA-C 5 days before study	DMC 100 mg repeated after 24 hr
AS	AML	F 70	56·4	NIL	DMC 100 mg
SC	AUL	M 46	75·2	NIL	DMC 110 mg
NMS	AML	M 53	73·0	NIL	DMC 110 mg
AJE	AML	M 65	68·8	NIL	DMC 105 mg

Table 2. *Changes in cytokinetic parameters of leukemic blast cells after daunomycin. ND: not done. x): DMC repeated after* 24 *hr. xx): No LBC present in the blood, hence the rather low values cannot be due to blood contamination*

Patient	Parameter	BM/B	Hours after DMC								
			0	6	12	24	36	48	72	84	96
HA	^{3}H-TDR LI	BM	3·4	2·6	1·8	2·5		2·8	3·5		2·1
x)	^{3}H-TDR LI	B	0·1	0·1	0·2	0·1		0	0		0·1
	MI	BM	0·20	0·05	0·10	0·10		0·10	0·15		0·20
	% G_2 cells	BM	0	0	0	1		0	1		0
	% "U cells"	BM	0	0	1	0		1	2		2
	Blood blasts/µl	B	61·750	60·140	70·312	39·990		20·352	22·506		20·376
AS	^{3}H-TDR LI	BM	15·5		16·0		8·9			7·1	
	^{3}H-TDR LI	B	3·7		4·5		2·3			1·3	
	MI	BM	0·60		0·00		0·45			0·10	
	% G_2 cells	BM	1		7		18			6	
	% "U cells"	BM	0		2		8			2	
	Blood blast/µl	B	49·140		41·832		9·450			1·976	
SC	^{3}H-TDR LI	BM	2·6 xx)	4·5	4·9	0·9		0·6	0·5		0·6
	MI	BM	0·15	0·00	0·00	0·00		0·05	0·00		0·10
NMS	^{3}H-TDR LI	BM	7·4	6·1	4·1	3·2		4·1	4·2		4·9
	MI	BM	0·10	0·00	0·07	0·15		0·10	ND		ND
	Blood blast/µl	B	112	70	60	36		23	15		7
AJE	^{3}H-TDR LI	BM	6·0	7·6	8·3	9·8		7·9	8·1		4·9
	MI	BM	0·20	0·00	0·05	0·25		0·25	0·23		0·25
	Blood blasts/µl	B	96	36	30	30		43	36		38

Part of the blood and marrow aliquot was smeared without delay on glass slides and stained with May–Grünwald–Giemsa. The total number of blast cells per µl of venous blood was determined from blood leucocyte counts and a differential count of 200 cells at intervals as shown in Table 2. From the marrow smears the percentage of blast cells in mitosis from late prophase to late telophase was determined from a count of at least 5000 blast cells.

The remaining part of the sample was divided into two; one half was incubated with ^{3}H-thymidine (specific activity 2·0 Ci/mM, final concentration about 1 µCi/ml marrow or blood) for one hour at 37°C; the other half was incubated with ^{3}H-leucine (specific activity 30–50 Ci/mM, final concentration about 2 µCi/ml marrow or blood) for two hours at 37°C. Autoradiographs were prepared using Kodak NTB-2 dipping emulsion and an exposure time of 1 week. The percentage of labeled blast cells was determined from a count of at least 1000 blast cells when incubated with ^{3}H-TDR and at least 800 blast cells when incubated with ^{3}H-LEU. After evaluation of background, cells with $\geqq 5$ grains were considered labeled. In the ^{3}H-LEU studies the mean grain count was

determined from at least 100 labeled blast cells. For technical reasons, cells with > 100 grains were recorded as having 100 grains.

In case HA and AS, the distribution of 25 labeled and 100 unlabeled blast cells with respect to their DNA content was determined by ultramicrospectrophotometry (Zeiss UMSP-I) after Feulgen staining as previously described by Ernst & Killmann [16].

RESULTS

1. *The number of circulating blast cells* in the peripheral blood decreased after DMC, as seen from Table 2.

2. *Mitotic index and ^{3}H-TDR labeling index.* The effect of DMC on MI and ^{3}H-TDR LI of bone marrow blast cells is also shown in Table 2. The initial MI values varied from 0·10 to 0·60 %. In all five cases an initial decrease in the MI was observed 6–12 hr after the injection. Pretreatment levels were regained within 24 hr in two patients (NMS, AJE). In the remaining three patients, the MI remained below the pretreatment value for 72 to 96 hr. No definite overshoot of MI was observed in any of the patients.

Pretreatment ^{3}H-TDR LI in bone marrow varied from 2·6% to 15·5%. Without exceptions a decrease in ^{3}H-TDR LI of LBC was seen. The nadir was reached at widely different time intervals. In 3 patients, a definite decline in TDR LI first became apparent at 24–96 hr after DMC. In all patients the ^{3}H-TDR LI was below the pretreatment level in the last sample taken, i.e., 72–96 hr after DMC. In AJE, a moderate temporary rise in ^{3}H-TDR LI was seen but this was not accompanied by a rise in MI.

3. *DNA content in the nuclei of marrow blast cells.* Table 2 shows data from the study of *unlabeled* blast cells with respect to their DNA content in patient HA and AS. In patient HA no accumulation of cells in G_2 after DMC was observed, whereas in patient AS a pronounced accumulation of cells in G_2 took place. Figure 1 shows that the most pronounced accumulation of cells in G_2 is observed 36 h after DMC, and is declining by 84 hr after DMC. Some "U-cells" (i.e., cells with a DNA content $> 2n < 4n$, not labeled by ^{3}H-TDR), were observed and are listed in Table 2. "U-cells" reached a maximum at 36 hr in AS. No change was observed in the *labeled* cells after DMC with respect to their distribution of DNA content (data not shown).

4. *^{3}H-LEU labeling index and mean grain counts of marrow blast cells* (*Table* 3). In 3 patients the ^{3}H-LEU LI was about 90% whereas in the other 2 patients the values were 57·6 and 78·0%, respectively. No initial decrease in ^{3}H-LEU LI after DMC was seen in any of the patients. In 3 patients (HA, NMS, AJE) a pronounced drop in ^{3}H-LEU LI was observed 72–96 hr after DMC (Fig. 2). In the remaining 2 patients (AS, SC) the ^{3}H-LEU LI was not influenced by DMC. In all patients, the initial MGC values were in the range from 11·6 to 35·1. The 2 patients (AS, NMS) with the highest initial MGC values showed a marked decrease 84–96 hr after DMC. In the remaining 2 patients no clearcut pattern was observed.

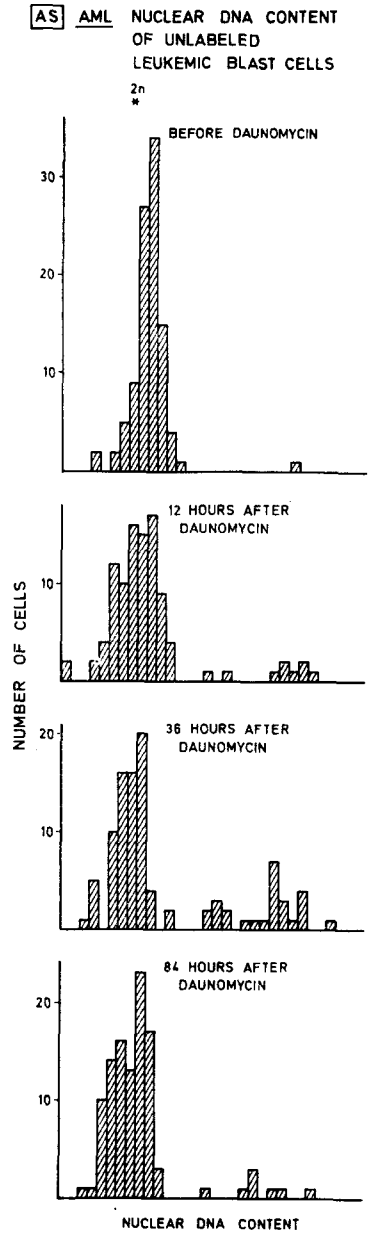


Fig. 1. *Distribution of unlabeled blast cells with respect to their DNA content. Notice accumulation of cells in G_2-phase* 36 *hr after DMC* (*case AS*).

Table 3. Changes in ^{3}H-leucine incorporation of leukemic blast cells after daunomycin. For abbreviations, see legend of Table 2

Patient	Parameter	BM/B	Hours after DMC								
			0	6	12	24	36	48	72	84	96
HA	^{3}H-LEU LI	BM	57·6	45·0	48·4	51·6		54·7	28·5		26·0
x)	^{3}H-LEU LI	B	24·3	ND	ND	ND		ND	ND		ND
	^{3}H-LEU MGC	BM	11·6	9·7	9·4	13·4		13·3	13·7		12·9
	^{3}H-LEU MGC	B	8·5	ND	ND	ND		ND	ND		ND
AS	^{3}H-LEU LI	BM	96·6		95·9		97·5			92·4	
	^{3}H-LEU LI	B	49·0		ND		ND			ND	
	^{3}H-LEU MGC	BM	35·1		27·8		25·1			15·5	
	^{3}H-LEU MGC	B	9·3		ND		ND			ND	
SC	^{3}H-LEU LI	BM	90·7	91·0	87·3	92·2		93·1	91·8		88·4
	^{3}H-LEU MGC	BM	16·7	24·4	13·5	30·0		13·4	21·3		16·4
NMS	^{3}H-LEU LI	BM	87·1	71·0	84·3	79·9		75·3	53·4		59·7
	^{3}H-LEU MGC	BM	25·7	13·0	17·3	21·5		18·9	19·6		11·9
AJE	^{3}H-LEU LI	BM	78·0	88·5	69·9	79·9		74·9	73·1		56·6
	^{3}H-LEU MGC	BM	17·1	21·1	11·2	14·0		16·3	12·0		10·9

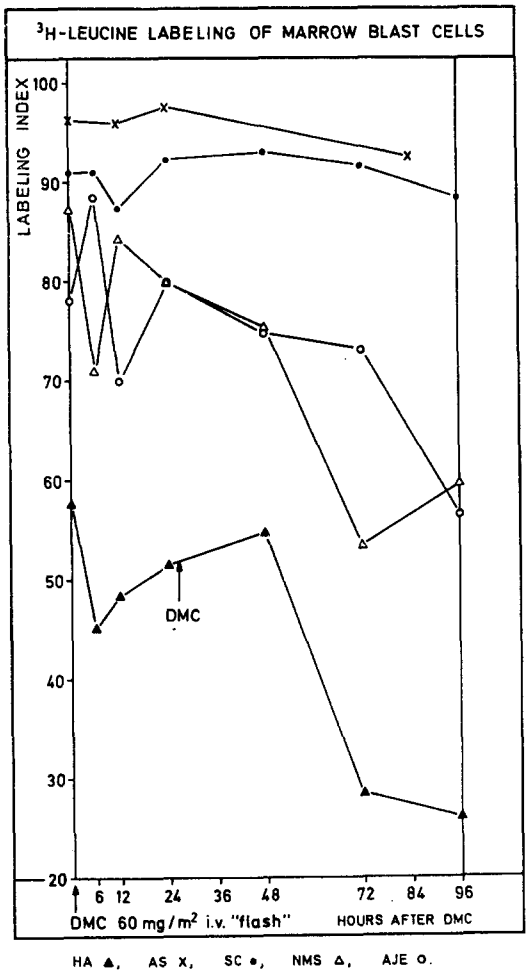


Fig. 2. Variations in ^{3}H-LEU LI of leukemic blast cells during treatment with daunomycin.

5. *^{3}H-LEU labeling index and mean grain counts of blood blast cells.* Before DMC, the ^{3}H-LEU LI in the blood blast cells was clearly below the values of the marrow blast cells in the two patients studied (HA, AS) (Table 3), as also reported previously [17].

DISCUSSION

After DMC, circulating LBC, which are believed to be mainly "quiescent cells" [18] fell markedly, as also observed by Ernst [14] and Stryckmans *et al.* [15]. Stryckmans *et al.* [15] have shown that the only explanation of the decline in blood LBC after DMC is a lytic effect of DMC, particularly because they found no return of blood blasts to the marrow such as observed by Killmann *et al.* [19] after methotrexate.

In all cases, the MI dropped to practically zero 6–12 hr after DMC which is consistent with previous studies [14], in which the MI was followed for up to 25 hr after the drug. The present data show that the depression of the MI in three of the patients was long-lasting; in fact pretreatment values were not yet reached by 72–96 hr after DMC. Thus, DMC has both a rapid and a prolonged effect on the MI.

The depression of the MI has probably two reasons, namely a block in G_2 and a reduction of DNA-synthesis. A block in G_2 has been demonstrated earlier [14] and was also seen in patient AS in this study, with a maximum pile-up in G_2 as late as 36 hr after DMC. A G_2-block was not seen in patient HA; no explanation for this can be offered although it should be noted that this patient had received ARA-C 5 days previously (Table 1). ARA-C has been shown to have a long-lasting effect on LBC [17]. The G_2-block probably accounts for the early low MI-values as

compared to the simultaneous ^{3}H-TDR LI which in general does not decrease the first 12 hours after DMC. It is also apparent from the short term data of Ernst [14] that the LI remains constant for approximately 20 hr after DMC. A continued G_2-block, as seen in patient AS, may also contribute to the longer-lasting depression of MI, probably due to cell death in arrested G_2.

In the present study, the ^{3}H-TDR values declined at various times (12–96 hr) after DMC; this presumed reduced flux through S-phase would then be expected to yield low MI-values at later time points. It should be noted that the decrease in ^{3}H-TDR LI persists for a long time, in the present study until discontinuation of the study, i.e. 72–96 hr after DMC. Stryckmans *et al.* [15] found the same in 2 out of 4 patients thus studied. The block in S is not necessarily complete; indeed, Stryckmans *et al.* [15] have observed that some mitoses after DMC + ^{3}H-TDR labeling *in vivo* are labeled. In the present study it was also noted that mitotic activity was only severely reduced during part of the study period.

Although many LBC cells are probably killed in G_1 as referred to above, this does not preclude that cells can enter from G_1 into S after DMC. The evidence that this happens is that after DMC, a fraction of LBC can still be labeled with ^{3}H-TDR in spite of the fact that there is a loss of cells from S, cf. the pile-up in G_2 and the persistence of some mitoses. The difficulty in fully analysing the cytokinetic perturbations induced by DMC is that the parameters studied are static in nature which allow some qualitative deductions to be made but do not permit exact cell flux estimates.

The DNA-synthesis time after DMC may remain unaltered; if DMC should influence DNA-synthesis time, one would expect a prolongation; the presence of "U-cells" after DMC would fit such a notion. An unimpaired influx from G_1 to S with a reduced loss through M would result in an increased ^{3}H-TDR LI, particularly if S was prolonged. This was not observed. The lack of an increase in ^{3}H-TDR LI may be explained by loss of cells to G_2 and/or cells dying in S, e.g., as "U-cells". With these latter unknowns, it cannot be decided whether the influx from G_1 to S was normal or decreased. The distribution of labeled cells with regard to DNA-content was not changed at any time and thus at least argues against an increased influx into S; in that case, the distribution of labeled cells would have been shifted to the left. The late decrease in LI favors but does not prove a late decrease in the transition from G_1 to S.

Others [20–22] have previously studied amino acid incorporation into malignant cells *in vitro* or *in vivo* as a function of a cytostatic therapy but these data are not helpful in the interpretation of the present data. The main aim in studying ^{3}H-leucine incorporation into leukemic blast cells after DMC was an attempt to estimate the duration of the cellular effect of the drug which might well be longer than usually assumed. A general discussion of the problems involved in evaluating protein synthesis from the incorporation of ^{3}H-LEU has been presented previously [17].

The incorporation of ^{3}H-leucine into human leukemic blast cells was shown to be inhibited by DMC surprisingly late (72–96 hr) after drug administration in four out of five patients. In 3 patients (HA, NMS, AJE) a pronounced decrease was observed in the ^{3}H-LEU LI whereas in patient AS the decrease was seen mainly in the MGC. Except from this, some transitory minor alterations were seen during the first day in patient SC. The main pattern is distinctly different from what is observed after a single dose of ARA-C which induces a decrease in ^{3}H-leucine incorporation with a nadir at 6 –24 hr after ARA-C, and pretreatment levels being reached again within the study period of 96 hr [17]. The late decrease in leucine incorporation suggests that DMC besides an immediate direct killing effect (drop in blood LBC-count), as also observed by others [14,15], has a late inhibiting effect on LBC. From knowledge of LBC kinetics in general [18] and the reduced division activity after DMC (Table 2), it appears likely that a fair amount of the decrease in LEU-LI or LEU-MGC took place in cells that had not undergone cell division during the period after DMC was given.

The data provide no evidence whether DMC effects protein synthesis directly or indirectly. The late effect suggests an indirect action via template activity of DNA and/or RNA. Several reports indicate that DMC inhibits the synthesis of DNA and RNA [1–4, 23].

Previous leucine incorporation data after ARA-C suggested that this drug entered non-S-cells. The present data concerning the rapid fall of the LBC-count suggest but does not prove that DMC also enters non-S-cells. In contrast, it is very clear that the action of DMC is long-lasting (up to $\geqq$ 96 hr). This residual damage is worth keeping in mind when chemotherapy is planned.

REFERENCES

1. J. Whang-Peng, B. G. Leventhal, J. W. Adamson and S. Perry, The effect of daunomycin on human cells *in vivo* and *in vitro*. *Cancer (Philad.)* **23,** 113, (1969).
2. A. Rusconi and A. DiMarco, Inhibition of nucleic acid synthesis by daunomycin and its relationship to the uptake of the drug in HeLa cells. *Cancer Res.* **29,** 1507 (1969).
3. W. Wilmanns and K. Wilms, DNA synthesis in normal and leucemic cells as related to therapy with cytotoxic drugs. *Enzyme* **13,** 90 (1972).
4. N. S. Mizuno, B. Zakis and R. W. Decker, Binding of daunomycin to DNA and the inhibition of RNA and DNA synthesis. *Cancer Res.* **35,** 1542 (1975).
5. E. Calendi, A. DiMarco, M. Reggiani, B. Scarpinato and L. Valentini, On physico-chemical interactions between daunomycin and nucleic acids. *Biochem. biophys. Acta* **103,** 25 (1965).
6. C. Jacquillat, Y. Najean, M. Weil, J. Tanzer, M. Boiron and J. Bernard, Traitement des leucémies aiguës lymphoblastiques par la rubidomycine. *Path. et Biol.* **15,** 913 (1967).
7. M. Boiron, C. Jacquillat, M. Weil, J. Tanzer, D. Levy, C. Sultan and J. Bernard, Daunorubicin in the treatment of acute myelocytic leukaemia. *Lancet* **i,** 330 (1969).
8. M. Weil, O. J. Glidewell, C. Jacquillat, R. Levy, A. A. Serpick, P. H. Wiernik, J. Cuttner, B. Hoogstraten, L. Wasserman, R. R. Ellison, S. Gailani, K. Brunner, R. T. Silver, V. B. Rege, M. R. Cooper, L. Lowenstein, N. I. Nissen, F. Haurani, J. Blom, M. Boiron, J. Bernard and J. F. Holland, Daunorubicin in the therapy of acute granulocytic leukemia. *Cancer Res.* **33,** 921 (1973).
9. P. H. Wiernik, Recent clinical trials for acute nonlymphocytic leukemia at the Baltimore Cancer Research Center. In *Modern Trends in Human Leukemia II. Biochemical, Biological and Immunological Aspects of Cell Differentiation.* (Edited by R. Neth.) Proceedings of Workshop, Einhof, Wilsede, Lüneburger Heide, June 23–27 (1975). In press.
10. E. Gluckman, A. Basch, B. Varet and B. Dreyfus, Combination chemotherapy with cytosine arabinoside and rubidomycin in 30 cases of acute granulocytic leukemia. *Cancer (Philad.)* **31,** 487 (1973).
11. The Finnish Leukaemia Group, (Chairman: E. Ikkala), Combination chemotherapy of acute leukaemia in adults. *Scand. J. Haemat.* **12,** 341 (1974).
12. The Medical Research Council's Working Party on Leukaemia in Adults. (Chairman: Sir Richard Doll), Treatment of acute myeloid leukaemia with daunorubicin, cytosine arabinoside, mercaptopurine, L-asparaginase, prednisone and thioguanine: Results of treatment with five multiple-drug schedules. *Brit. J. Haemat.* **27,** 373 (1974).
13. M. Eppinger-Helft, S. Pavlovsky, A. Suarez, F. S. Muriel, G. Hidalgo, A. Pavlovsky and G. Vilaseca, Sequential therapy for induction and maintenance of remission in acute myeloblastic leukemia. *Cancer (Philad.)* **35,** 347 (1975).
14. P. Ernst, Perturbation of generation cycle of human leukaemic blast cells *in vivo* by daunomycin. *Scand. J. Haemat.* **11,** 13 (1973).
15. P. A. Stryckmans, J. Manaster, F. Lachapelle and M. Socquet, Mode of action of chemotherapy *in vivo* on human acute leukemia. *J. clin. Invest.* **52,** 126 (1973).
16. P. Ernst and S.-Aa. Killmann, Perturbation of generation cycle of human leukemic blast cells by cytostatic therapy *in vivo*: Effect of corticosteroids. *Blood* **36,** 689 (1970).
17. G. Lange Wantzin, H. Karle and S.-Aa. Killmann, Cell proliferation and protein synthesis in human leukaemic myeloblasts after cytosine arabinoside therapy. *Brit. J. Haemat.* **32,** 289 (1976).
18. S.-Aa. Killmann, Kinetics of leukaemic blast cells in man. *Clin. Haemat.* **1,** 95 (1972).
19. S.-Aa. Killmann, H. Karle, P. Ernst and V. Andersen, Return of human leukemic myeloblasts from blood to bone marrow. *Acta med. scand.* **189,** 137 (1971).

20. S. B. NADLER, H. J. HANSEN, C. C. SPRAGUE and H. SHERMAN, The effect of 6-mercaptopurine on the incorporation of labeled amino acids into cellular protein of chronic granulocytic leukemia leukocytes. *Blood* **18,** 336 (1961).
21. R. I. FRESHNEY, J. PAUL and I. M. KANE, Assay of anti-cancer drugs in tissue culture: Conditions affecting their ability to incorporate ^{3}H-leucine after drug treatment. *Brit. J. Cancer* **31,** 89 (1975).
22. P. A. FRENGLEY, W. A. PECK and M. A. LICHTMAN, Inhibition of time-dependent enhancement of amino acid transport by leukemic leukocytes: A possible index of the sensitivity of cells to drugs. *Blood Cells* **1,** 139 (1975).
23. L. E. CROOK, K. R. REES and A. COHEN, Effect of daunomycin on HeLa cell nucleic acid synthesis. *Biochem. Pharmacol.*, **21,** 281 (1972).

Europ. J. Cancer Vol. 12, pp. 299–303. Pergamon Press 1976. Printed in Great Britain

Metabolic Controls in Precancerous Liver–VII. Time Course of Loss of Dietary Feedback Control of Cholesterol Synthesis During Carcinogen Treatment*

JOHN R. SABINE

Department of Animal Physiology, Waite Agricultural Research Institute, University of Adelaide, S.A., Australia

Abstract—*A technique is described, involving continuous low-level feeding of AY*9944 (*trans*-1,4-bis(2-*chlorobenzyl-aminomethyl*) *cyclohexane dihydrochloride*), *whereby the dietary feedback status of hepatic cholesterol synthesis in individual rats can be monitored at frequent intervals.*

Use of this technique has shown that the hepatocarcinogens ethionine (0·25 % *in feed*) *and methylazoxymethanol acetate* (*MAM-acetate*) (50 *mg/kg of body wt. i.p.*) *cause a rapid and sudden loss of feedback inhibition of hepatic cholesterol synthesis at approximately* 7–9 *and* 11–14 *days respectively. Loss of control is not caused by the non-carcinogenic hepatotoxin carbon tetrachloride.*

INTRODUCTION

PREVIOUS studies in this laboratory [1–4] have shown clearly that loss of dietary "feedback" control of hepatic cholesterol synthesis, a metabolic defect characteristic of all fully-developed hepatomas [5,6] is also a consistent consequence of hepatocarcinogen treatment. This loss of metabolic control during chemical carcinogenesis occurs weeks or months before liver tumours appear and has been observed, both here and elsewhere, with all four carcinogens tested so far: N-2-fluorenylacetamide [1,2], aflatoxin [2,7,8], ethionine [3] and 3′-methyl-4-dimethylaminoazobenzene [4].

Because this loss of control over cholesterol metabolism appears to be a necessary prerequisite for hepatoma development (although hepatomas do not inevitably follow once control is lost) we have been trying lately to discern as accurately as possible for any individual animal that point in time, following the onset of carcinogen treatment, when a significant number of hepatic cells first lose control. Although our previous work indicated loss as early as within 2–4 weeks, we could not define this time more accurately than *plus or minus* 2 weeks. We are now using a modification of our previously-published technique for vival assay of hepatic cholesterogenesis [9], by which means we can monitor the cholesterol-feedback status of the liver of any rat at least daily, and more frequently if necessary.

In this paper I am reporting our first results with this technique, using primarily ethionine as the hepatocarcinogen. I am also reporting an extension of our previous results, in that loss of control is shown with yet another hepatocarcinogen, methylazoxymethanol acetate [10], and perhaps more importantly that control is not lost with just any hepatotoxin—results with rats treated with carbon tetrachloride, which is usually regarded as non-carcinogenic, did not differ from those with rats not treated at all.

Accepted 28 November 1975.

*Supported by a grant of the Australian Research Grants Committee.

MATERIAL AND METHODS

Animals, diets and treatments

We used only male rats (Hooded-Wistar), aged 2–3 months at the beginning of each experiment, and obtained from this University's Central Animal House. They were housed in a windowless but well-ventilated room, under the 12 hr light, 12 hr darkness standard, and unless stated otherwise were offered *ad libitum* water and a standard laboratory diet (Charlicks M164 mouse cubes).

Experimental animals were fed *ad libitum* either powdered regular diet (controls) or this diet with the following substances added—in varying combinations and sequences, depending upon the particular experiment (see RESULTS): 0·005% AY9944 (trans-1,4-*bis*)-2-chlorobenzylaminomethyl cyclohexane dihydrochloride (Ayerst, Montreal); 5% cholesterol (Pierce); 0·25% D-L-ethionine (Koch-Light). Other substances administered were the carcinogen MAM-acetate (methylazoxymethanol acetate, Mann Research Labs. 50 mg/kg of body wt. i.p.); carbon tetrachloride and sodium phenobarbital (50 mg/kg feed). Every experimental and every control group contained 4 rats.

Vival assay of dietary-feedback inhibition of hepatic cholesterogenesis

The drug AY9944 inhibits the enzymatic conversion of 7-dehydrocholesterol to cholesterol [11], resulting in a build-up of the precursor, i.e., 7-dehydrocholesterol, in any tissue synthesizing cholesterol, as well as in the plasma. This compound is normally present only in very low or negligible concentration.

Thus, first the Ayerst group [e.g., 12,13], and then Horton *et al.* in this laboratory [9] were able to show that the amount of 7-dehydrocholesterol present following a standard dose of AY9944 is an accurate, relative measure of a tissue's active cholesterogenesis. Since in the rat the liver is the major source of plasma cholesterol, the amount of 7-dehydrocholesterol present in the plasma 24 hr after a standard dose of AY9944 is an accurate measure of the cholesterogenic activity of the liver [9]. In our previous studies [9] we showed that this technique can measure effectively hepatic cholesterogenesis at weekly intervals. Dietary feedback of this synthesis can be assessed every second week.

In these present studies we have modified this procedure, in that the AY9944 was mixed with the animals' food and fed continuously at a low level (50 mg/kg of feed). At appropriate times (sometimes daily) accurate samples of whole blood were collected from the tip of the tail vein, saponified in 30% KOH for 60 min at 90°C, extracted with *n*-hexane, and the hexane evaporated and the 7-dehydrocholesterol measured as "fast-acting" sterols (2 min) with the stable Leibermann-Burchard reagent of Kim and Goldberg [14].

RESULTS

In order to simplify the presentation of results, in every figure in this paper not all sampling times are shown—only those necessary to illustrate the particular point required. Those values omitted did not differ significantly from values found and shown, just before and/or just after.

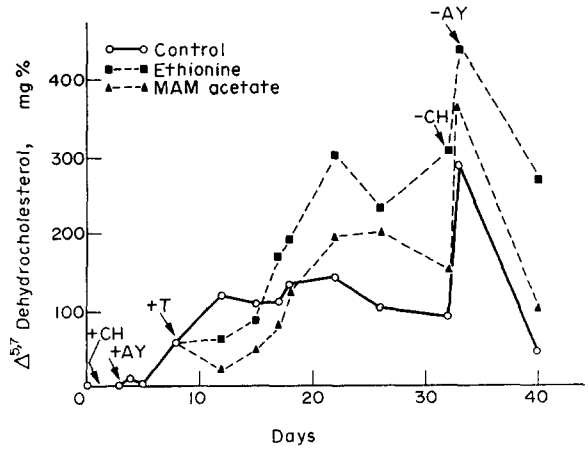


Fig. 1. *Loss of dietary inhibition of hepatic cholesterol synthesis following carcinogen treatment. Blood levels of 7-dehydrocholesterol, produced by dietary AY*9944 (+*AY*) *but minimised by cholesterol feeding* (+*CH*), *were elevated following carcinogen treatment* (*T*). *Values shown are averages for groups of* 4 *rats following treatment, and for all* 12 *rats prior to treatment.*

The basic application of this technique is shown in Fig. 1. Twelve rats were fed cholesterol for 2–3 days, in order to establish an effective feedback suppression of hepatic cholesterogenesis. AY9944 was then added to the diet, and blood 7-dehydrocholesterol monitored from then on at frequent intervals.

When a baseline had been established (day 8) the rats were divided into 3 groups—one without further treatment, i.e., as controls, and the other two treated with carcinogens—one with ethionine added to the diet, and one with MAM-acetate injected. Figure 1 shows clearly that the blood levels of 7-dehydrocholesterol for the ethionine and MAM-acetate treated rats first diverged from the control levels at

approximately days 17 and 22 respectively, i.e., 9 and 14 days following carcinogen treatment, and remained significantly different for the duration of the experiment. I interpret these results as showing the times at which the dietary cholesterol failed to continue to suppress hepatic cholesterol synthesis, i.e., cholesterol-negative feedback inhibition was lost by a significant number of hepatic cells. I am impressed by the suddenness with which this occurred. When cholesterol was removed from the diet (day 32) the three groups responded with increased cholesterol synthesis. When AY9944 was removed (day 33) plasma levels of 7-dehydrocholesterol declined rapidly—and at approximately the same rate for all groups.

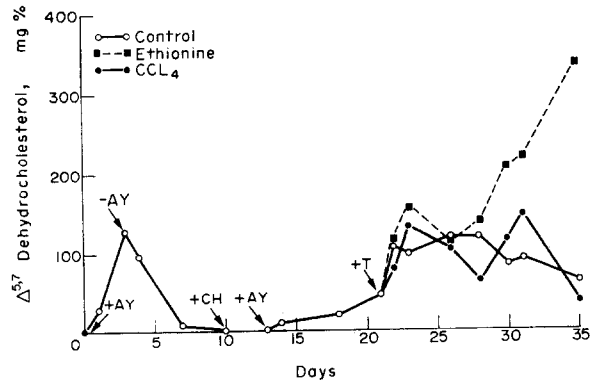


Fig. 2. Loss of dietary inhibition of hepatic cholesterol synthesis following treatment by the carcinogen ethionine but not by acute carbon tetrachloride. Experimental and control groups each contained 4 animals, and values shown are group averages. The technique used and abbreviations shown are as in Fig. 1.

The experiment shown in Fig. 2 illustrated some of the same points, but also provided additional information. The early part of this figure shows the very rapid and substantial rise in blood 7-dehydrocholesterol that occurred when AY9944 was fed without dietary cholesterol, i.e., without an inhibited hepatic cholesterogenesis. The feedback imposed by dietary cholesterol (after day 10) was again broken by the carcinogen ethionine, and again at approximately 9 days after treatment began.

But Fig. 2 shows another important observation. Carbon tetrachloride is a commonly used liver poison, but is generally regarded as not being carcinogenic for the liver. The dose rate used in this study (1·0 ml/kg of body wt. i.p.) was high enough to eventually kill 3 out of the 4 animals before the experiment finished, but during this time (14 days) the blood 7-dehydrocholesterol values for the treated animals did not differ from control values.

Similar observations were made in the next experiment (Fig. 3)—dietary feedback of hepatic cholesterol synthesis was broken by the carcinogen ethionine (this time at approx. 7 days), but not by the non-carcinogen, CCl_4. In this case the CCl_4 was administered

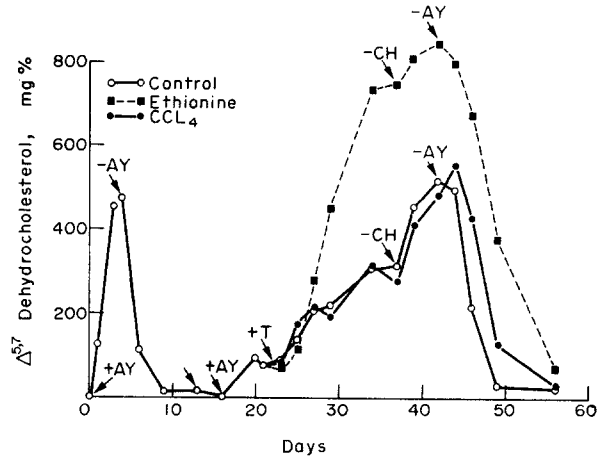


Fig. 3. Loss of dietary inhibition of hepatic cholesterol synthesis following treatment by the carcinogen ethionine, but not by chronic carbon tetrachloride. The technique used, abbreviations and animal numbers are as in Fig. 1 and 2.

chronically (as a 50% solution in corn oil, 1·5 ml/kg of body wt., s.c. twice weekly) which was enough to seriously depress body weight, but did not kill the animals.

The final experiment (Fig. 4) again illustrated the time course of loss of feedback control of hepatic cholesterogenesis following either continuous feeding with ethionine (approx. 8 days) or a single injection of MAM-acetate (approx. 11 days). But another significant point is also shown in this figure.

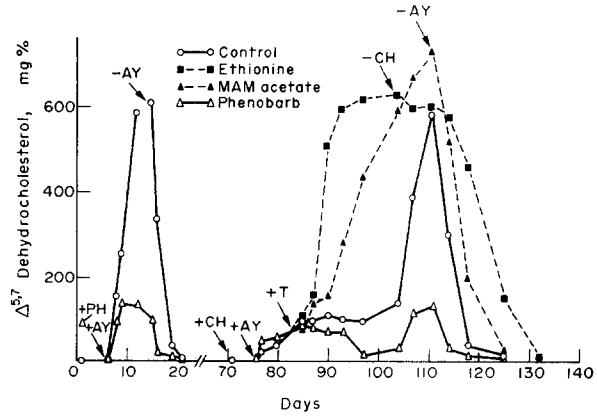


Fig. 4. Loss of dietary inhibition of hepatic cholesterol synthesis following treatment by the carcinogens ethionine and MAM-acetate, but not by sodium phenobarbital. Values shown are averages for groups of 4 animals, or for 12 animals prior to carcinogen treatment. The technique used and abbreviations shown are as in the previous figures.

The continuous assay system reported in this paper is obviously ineffective in monitoring feedback control in any animal so treated that its ability to metabolize and/or eliminate plasma 7-dehydrocholesterol is either impaired or enhanced. Phenobarbital (Fig. 4) is apparently one such treatment. Although this has been reported to increase hepatic cholesterogenesis [15], in this experiment it also apparently greatly increased the metabolism of 7-dehydrocholesterol, as shown by the inability of phenobarbital-treated rats to build up significant levels of 7-dehydrocholesterol in their plasma during AY9944 feeding, either with or without concurrent cholesterol feeding.

In this same context, an impaired metabolism of 7-dehydrocholesterol could not be invoked as a reason for the results seen during carcinogenesis. Figures 3 and 4, as did Fig. 1, show a rapid and uniform decline in 7-dehydrocholesterol levels following removal of AY9944 from the diet, for both experimental and control animals.

One remaining point from this work is illustrated in Fig. 5, which shows blood 7-dehydrocholesterol levels for individual rats, not the averages for groups of 4, as in other figures. Since the important use of the technique described here is to monitor continuously individual animals for their feedback status, a varied selection of individual responses is shown in Fig. 5. We have already commented [2, 4] that some 10% of the young, male Hooded-Wistar rats from our Animal House show little or no feedback control of hepatic cholesterol synthesis, and this is illustrated by the results for one of the control rats and possibly one of the ethionine-treated rats. In subsequent experiments such animals were eliminated before carcinogen treatment was commenced.

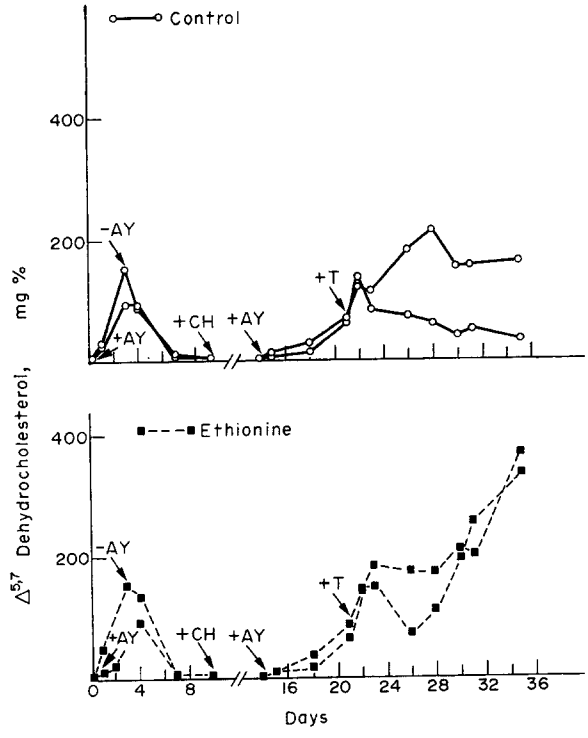


Fig. 5. Dietary feedback control of hepatic cholesterol synthesis for 4 individual rats, taken from among those shown previously. Blood levels of 7-dehydrocholesterol following dietary AY9944 are shown not depressed and depressed by dietary cholesterol, and elevated by ethionine treatment despite dietary cholesterol.

The two ethionine-treated rats shown in Fig. 5 displayed individual responses to the carcinogen. Their levels of 7-dehydrocholesterol were still not distinguishable from control values at 7 days for one and 10 days for the other, i.e., post-treatment. But by 10 and 14 days respectively these levels had diverged considerably from normal, i.e. in the 3–4 days between the times given the livers of these animals had acquired a characteristic of all liver tumours, i.e., they were then truly "pre-cancerous".

Although loss of feedback control is a necessary prerequisite for hepatoma development, not all livers that lose control necessarily develop hepatomas. This was shown in these experiments by histological examination of the livers of all surviving rats twelve months after carcinogen treatment. The ethionine livers were barely distinguishable from the controls, although at the time of treatment the animals had severely lost weight. On the other hand the MAM-acetate-treated animals all showed liver abnormalities—predominantly either early hepatomas or cholangiomas.

DISCUSSION

The primary aim of the experiments described in this paper was to narrow to within a few days the detection of the time when carcinogen-treated livers first lose dietary feedback control of cholesterol synthesis. This object was achieved, and any individual animal can now be monitored closely during this critical time.

As a consequence of this, two important further developments are now possible. First, by an examination of livers taken just before and just after control is lost we should be able more readily to determine the cause of this loss—since other differences might be minimal. From other work in this laboratory [16] we now believe that in the normal liver cholesterol

feedback control is mediated via physicochemical changes in the microsomal membrane supporting the operative enzyme, i.e. β-hydroxy-β methyl glutaryl co-enzyme A reductase [5]. Thus the next step is to determine whether these membrane changes, including primarily a cholesterol ester-induced alteration in membrane fluidity, fail to occur in the carcinogenic state.

The second development that should now be possible is to relate in time the loss of feedback control to other cellular changes occurring during carcinogenesis. But since the loss of feedback control, a critical feature of all subsequent hepatomas [5,6], occurs within days of commencing carcinogen treatment, it is the earliest recognizable sign of subsequent tumour development. Determination of the cellular basis for this loss will thus be a critical advance in our knowledge of hepatic tumourigenesis.

Acknowledgements—I thank Miss Dianne Gibson for expert technical assistance, Dr. Morris Givner (Ayerst, Montreal) for the gift of AY9944, Dr. Radimir Rac (Institute of Medical and Veterinary Science, Adelaide) for the histology.

REFERENCES

1. B. J. Horton and J. R. Sabine, Metabolic controls in precancerous liver: defective control of cholesterol synthesis in rats fed N-2-fluorenylacetamide. *Europ. J. Cancer* **7,** 459 (1971).
2. B. J. Horton, J. D. Horton and J. R. Sabine, Metabolic controls in precancerous liver—II. Loss of feedback control of cholesterol synthesis, measured repeatedly *in vivo*, during treatment with the carcinogens N-2-fluorenylacetamide and aflatoxin. *Europ. J. Cancer* **8,** 437 (1972).
3. B. J. Horton and J. R. Sabine, Metabolic controls in precancerous liver—IV. Loss of feedback control of cholesterol synthesis and impaired cholesterol uptake in ethionine-fed rats. *Europ. J. Cancer* **9,** 11 (1973).
4. B. J. Horton, J. D. Horton and J. R. Sabine, Metabolic controls in precancerous liver—V. Loss of control of cholesterol synthesis during feeding of the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene. *Europ. J. Cancer* **9,** 573 (1973).
5. J. R. Sabine, Defective control of lipid biosynthesis in cancerous and precancerous liver. *Progr. biochem. Pharmacol.* **10,** 269 (1975).
6. M. D. Siperstein, Regulation of cholesterol biosynthesis in normal and malignant tissues. *Curr. Top. cell. Reg.* **2,** 65 (1970).
7. M. D. Siperstein, Feedback control of cholesterol synthesis in normal, malignant and pre-malignant tissues. *Proc. Canad. Cancer Conf.* **7,** 152 (1967).
8. M. D. Siperstein, Deletion of the cholesterol negative feedback system in precancerous liver. *J. clin. Invest.* **45,** 1073 (1966).
9. B. J. Horton, J. D. Horton and J. R. Sabine, Repeated estimation of liver cholesterol synthesis *in vivo*, using the inhibitor AY-9944. *Biochem. biophys. Acta* **239,** 475 (1971).
10. M. S. Zedeck, S. S. Sternberg, R. W. Poynter and J. McGowan, Biochemical and pathological effects of methylazoxymethanol acetate, a potent carcinogen. *Cancer Res.* **30,** 801 (1970).
11. D. Dvornik, M. Kraml, J. Dubuc, M. Givner and R. Gaudry, A novel mode of inhibition of cholesterol biosynthesis. *J. Amer. chem. Soc.* **85,** 3309 (1963).
12. D. Dvornik, M. L. Givner and J. G. Rochefort, AY-9944, an inhibitor of cholesterol biosynthesis, as a tool in the estimation of the rate of cholesterogenesis. *Circulation* **32,** Supp. II, 10 (1965).
13. M. L. Givner, J. G. Rochefort and D. M. Dvornik, Tool for the estimation of the rate of cholesterol biosynthesis. *Circulation* **34,** Supp. III, 12 (1966).
14. E. Kim and M. Goldberg, Serum cholesterol assay using a stable Lieberman–Burchard reagent. *Clin. Chem.* **15,** 1171 (1969).
15. A. L. Jones and D. T. Armstrong, Increased cholesterol biosynthesis following phenobarbital induced hypertrophy of agranular endoplasmic reticulum in liver. *Proc. Soc. exp. Biol. (N.Y.)* **119,** 1136 (1965).
16. J. R. Sabine and M. J. James, The intracellular mechanism responsible for dietary feedback control of cholesterol synthesis. *Life Sci.* In press (1976).

Europ. J. Cancer Vol. 12, pp. 305–311. Pergamon Press 1976. Printed in Great Britain

Plasma FSH and LH in Post-menopausal Women with Breast Cancer

D. Y. WANG,* P. R. GOODWIN,* R. D. BULBROOK* and J. L. HAYWARD†

**Department of Clinical Endocrinology, Imerial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX and †Breast Cancer Unit, Guy's Hospital, London, SE1 9RT*

Abstract—*FSH and LH have been measured in the plasma of normal post-menopausal women and in patients with early breast cancer (before and after mastectomy) and advanced breast cancer. The mean levels of plasma FSH or LH in women with early breast cancer are the same as those found in normal women. There was no alteration in this mean level associated with mastectomy.*

In women with advanced breast cancer, the mean LH level was the same as in normal subjects although the mean FSH concentration was statistically sub-normal. This difference is probably due to the hormonal treatment that most of these patients were receiving.

Plasma FSH or LH measured at the time of recurrence of the disease showed no differences from those determined shortly after mastectomy.

Patients with early breast cancer with low FSH or LH, measured either before or after mastectomy, had a faster recurrence rate than patients with high plasma gonadotrophin levels. However, these differences were not statistically significant.

INTRODUCTION

THERE is evidence that the secretion of gonadotrophins in women with breast cancer may be abnormal in that there have been reports that the incidence of amphophil hyperplasia is increased in the anterior pituitaries of women with breast cancer compared to normal controls [1, 2]. The gonadotrophic potency of pituitary extracts was observed to parallel approximately the number of amphophil cells present [3]. Also it has recently been reported that both LH and FSH are found in the same cell population [4]. In accord with these findings measurements of urinary gonadotrophins indicate that the excretion of these hormones is raised [5, 6].

In post-menopausal women with advanced breast cancer it has been claimed that the amounts of gonadotrophin excreted in the urine are related to response to endocrine treatment but both high and low urinary gonadotrophin titres have been reported to be associated with response to treatment [5, 7–10].

These studies were carried out with bioassays of urinary extracts. The availability of radioimmunoassay techniques sensitive enough to make it possible to measure the concentration of circulating plasma gonadotrophin led us to re-examine the problem of gonadotrophin secretion in breast cancer. Thus, the aims of this study were to determine plasma levels of FSH and LH in post-menopausal subjects with early or advanced breast cancer, to compare these with those found in normal women and to assess whether the amounts of plasma gonadotrophins in women with early breast cancer have any relation to recurrence rates.

MATERIAL AND METHODS

Subjects

All subjects studied were post-menopausal and aged between 50 and 65 years. Blood was

Accepted 13 November 1975.

collected from 40 normal women, 49 patients with early breast cancer one day before mastectomy, 51 women 10–14 days after surgery, and 26 women with advanced and progressing breast cancer.

In this study there were 23 patients in whom the disease had recurred 4–28 months after mastectomy. FSH measurements were made on 14 and 17 of these subjects before and after surgery, respectively. The levels of plasma LH were assayed in 17 and 21 of these patients before and after mastectomy, respectively. Furthermore, FSH and LH assays were done on blood samples taken from 12 patients at the time of recurrence.

The blood was collected into heparinized tubes, the plasma separated after centrifugation and stored at $-20°C$ until assayed.

Method for determining LH and FSH

The method for estimating LH and FSH was based on a double-antibody radioimmunoassay. The antiserum to FSH was kindly supplied by Dr. W. R. Butt and the antiserum to LH was obtained from the National Institute of Health, Bethesda. LH (MRC 71/53) and FSH (MRC 69/104) were supplied by the Medical Research Council and iodination of these carried out as described by Hunter, Greenwood and Glover [11]. The iodinated hormones were separated from the reaction mixture by chromatography on a Sephadex G 25 column (10×1 cm). The iodinated LH was purified further immediately before use on CF11 cellulose. All samples were assayed at three concentrations to check parallelism with the standard curve.

The concentrations were expressed in terms of the MRC standards for LH (MRC 68/40) (77 IU/ampoule) and FSH (68/39) (32·8 IU/ampoule).

RESULTS

(a) Plasma LH and FSH levels in patients with breast cancer

The plasma concentrations of LH and FSH in post-menopausal women with breast cancer are indistinguishable from those found in normal healthy controls, with the sole exception of a sub-normal level of FSH in women with advanced metastatic disease (Table 1).

The normal geometric mean LH level is 25·1 mIU/ml; in pre-mastectomy patients the concentration was 24·8, and after mastectomy a geometric level of 23·7 mIU/ml was found. In the advanced disease, the geometric mean level was 17·4 mIU/ml (Table 1 and Fig. 1). None of these differences is significant.

The geometric mean level of FSH in normal post-menopausal women is 19·1 mIU/ml. In patients with early breast cancer the levels are 20·8 before, and 19·6 mIU/ml after mastectomy. There is, however, a significant ($P < 0·01$; $t = 3·386$) diminution of the mean FSH level in patients with advanced breast cancer, where a geometric mean level of 10·8 mIU/ml was found (Table 1, Fig. 1).

Table 1. *Plasma LH and FSH concentrations in normal women and in patients with breast cancer*

Clinical status	LH (m IU/ml)	FSH (m IU/ml)
Normal	1·399 ± 0·167 (39)	1·282 ± 0·191 (40)
Early breast cancer		
(*a*) Pre-mastectomy	1·394 ± 0·201 (46)	1·318 ± 0·220 (46)
(*b*) Post-mastectomy	1·374 ± 0·228 (51)	1·293 ± 0·208 (48)
Advanced breast cancer	1·241 ± 0·465 (26)	1·033 ± 0·402 (26)

Results are expressed as logarithmic mean ± S.D. with number of subjects in parentheses.

(b) Effect of mastectomy on plasma LH and FSH levels

The geometric mean plasma gonadotrophin levels are unaffected by mastectomy (Table 1). In a large proportion of cases studied, both pre- and post-operative plasma samples were available from the same patient. The data on these patients are shown in Fig. 2, where pre-operative levels have been plotted against post-operative gonadotrophin levels. Although in a few patients mastectomy is associated with large falls in plasma gonadotrophin concentrations, the majority of patients show no appreciable change. The calculated regression lines shown in Fig. 2 are statistically indistinguishable from the 45° line.

(c) Relation between plasma LH and FSH levels and recurrence rates

In the series of patients studied there were 12 subjects where blood specimens were obtained at the time of recurrence. A com-

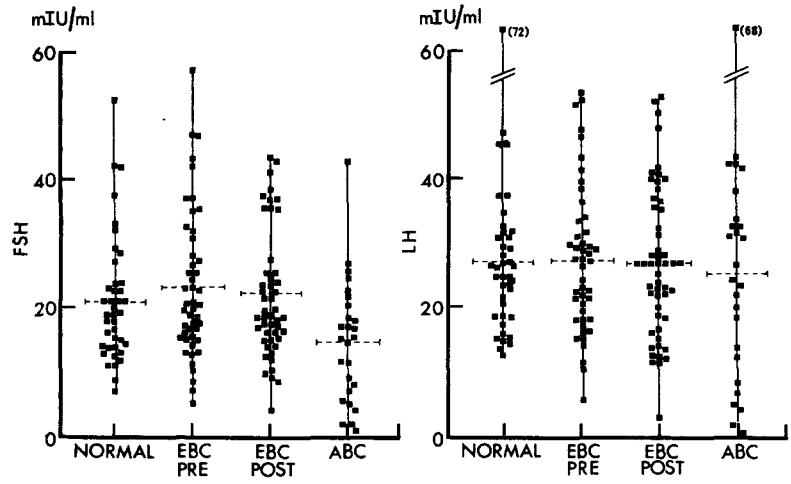


Fig. 1. *Plasma FSH and LH in normal women and patients with breast cancer.*

EBC-PRE and EBC-POST refer to patients with early breast cancer in whom gonadotrophin has been determined before and after mastectomy, respectively. ABC refers to women with advanced breast cancer. Horizontal broken lines show the mean values.

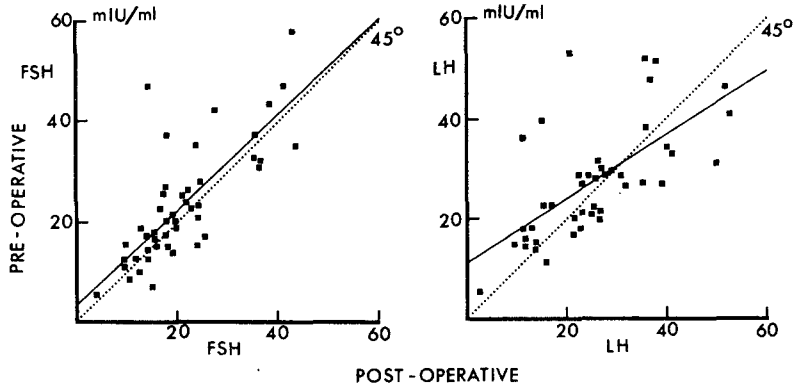


Fig. 2. *Comparison of plasma FSH and LH measured before and after mastectomy.*

The pre-operative levels of FSH or LH are plotted along the ordinate and the post-operative levels along the abscissa. The continuous lines are the calculated regression lines for FSH and LH. The regression equations are, for FSH $y = 3{\cdot}50 + 0{\cdot}94x$ ($r = 0{\cdot}78$; $N = 46$) and for LH, $y = 11{\cdot}18 + 0{\cdot}63x$ ($r = 0{\cdot}64$; $N = 43$). y and x refer to the pre- and post-operative levels respectively, r represents the regression coefficients and n is the number of subjects. The dotted line is the 45° line.

parison of the level of FSH or LH soon after mastectomy and at this time (Table 2) shows that although the two measurements sometimes differ considerably, the direction of this change is not consistent and the mean levels remain relatively constant.

To determine whether FSH or LH levels might be of prognostic value, the patients in this investigation have been divided into two groups; those with plasma levels of gonadotrophin above the median value for the whole group and those with levels below this median value. The recurrence rates in the two groups were then computed and the results are shown in Figs. 3 and 4. Although in the four categories examined (LH or FSH, before or after mastectomy) women in the low-gonadotrophin group consistently had a faster recurrence rate than the corresponding high-gonadotrophin group, the recurrence rates are statistically indistinguishable.

DISCUSSION

The results of this study show that plasma gonadotrophin levels are identical in normal women and in patients with breast cancer. The only significant abnormality found in this study was a depressed plasma FSH level in women with the advanced disease. This result needs to be treated with caution since most of

Table 2. FSH and LH levels at time of recurrence

	FSH (m IU/ml)		LH (m IU/ml)		Time to recurrence (months)
	Post	Rec	Post	Rec	
	17·2	20·1	22·7	28·7	5
	35·5	38·0	19·6	38·4	6
	4·0	25·5	2·7	2·8	9
	18·4	5·4	13·8	15·3	10
	36·7	15·6	27·8	32·1	10
	25·4	9·6	26·7	20·4	11
	35·6	49·3	42·9	37·0	12
	9·8	23·8	11·2	13·5	16
	40·8	15·4	15·5	18·1	18
	16·4	19·2	26·2	16·8	21
	13·9	20·0	28·0	23·2	22
	38·4	17·9	36·5	30·5	28
Arithmetic mean	24·3	21·7	22·8	23·1	
Geometric mean (logarithm to the base 10)	1·310	1·274	1·282	1·293	

POST refers to the gonadotrophin levels measured 7–10 days after mastectomy.
REC refers to levels found at the time of recurrence. Recurrence Time is taken from the date of mastectomy to the date of recurrence of the disease.

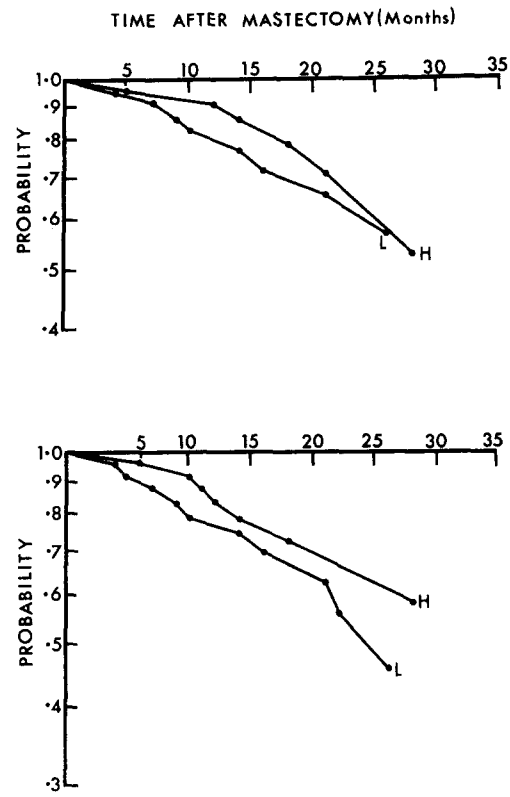


Fig. 3. Plasma FSH levels and recurrence rate.
The upper figure refers to FSH levels measured before mastectomy and the lower figure to post-operative FSH levels. *L* represents the category of patient with FSH concentration below the median for the total group and *H* for patients with FSH levels higher than the median level. The ordinates are probability of non-recurrence.

the patients had been previously treated with steroids. Franchimont has shown that both oestrogen or androgen administration can depress plasma FSH concentrations without a concomitant diminution of plasma LH levels, as appears to be the case in this study [12]. Also the mean levels of FSH and LH when measured at the time of recurrence of the disease are normal.

The results are not in accord with those reported by Marmoston [6] who found that the urinary gonadotrophin residue, as isolated by the benzoic acid–tungstic acid method of Butt [13] was elevated in women with early breast cancer, nor with those of Loraine *et al.* [5] who also found elevated levels in the advanced disease. Both groups used bioassay methods for determining the urinary gonadotrophin concentration.

It is clear from our results that measurement of plasma LH or FSH levels is of no value in predicting recurrence rates in post-menopausal women. The contradictory nature of published results makes it impossible to assess the value of gonadotrophin concentrations in predicting endocrine response to treatment of the advanced disease. Boyland *et al.* [8] and Hayward *et al.* [9] both found that patients with high gonadotrophin tended not to respond to endocrine ablation. Loraine *et al.* [5] reported to the contrary; that patients with high gonadotrophin responded to oophorectomy and adrenalectomy. Paradoxically, they also reported that women with high gonadotrophin excretion did not respond to oestrogen treatment but this finding was not substantiated by Martin [10] or Segaloff *et al.* [7] who observed that patients who responded favourably to oestrogen treatment tended to have high levels of urinary gonadotrophins.

The finding of essentially normal gonadotrophin levels in patients with breast cancer is in accord with other work indicating that pituitary function as a whole is normal in this disease.

Greenwood *et al.* [14] using insulin sensitivity tests on 10 patients with breast cancer before and 7 days after mastectomy found their growth hormone response to be normal. How-

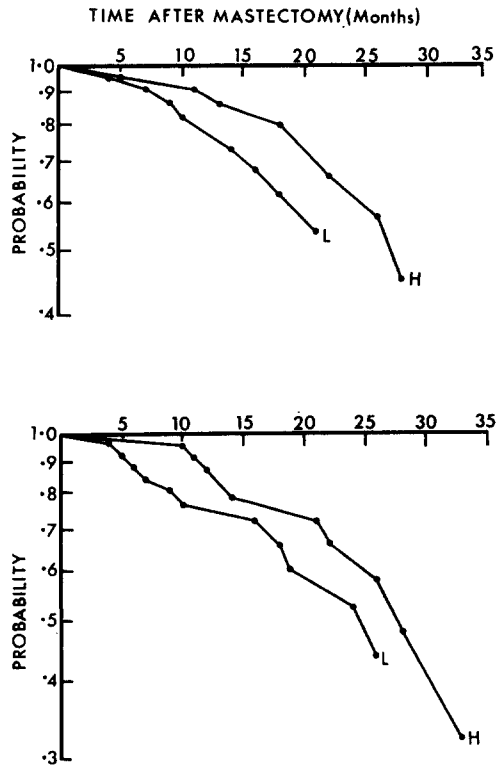


Fig. 4. *Plasma LH levels and recurrence rate.*

The upper figure refers to LH levels measured before mastectomy and the lower figure to post-operative LH levels. *L* represents the category of patients with LH concentration below the median for the total group and *H* for patients with LH levels higher than the median level. The ordinates are probability of non-recurrence.

ever, the patients had higher resting levels of growth hormone at both times. In a second series of 21 patients tested with oral glucose an anomalous rise in growth hormone was observed. Pearson *et al.* [15] also found an abnormal rise in growth hormone after oral glucose in 9 out of 23 women tested. Although both groups observed changes in carbohydrate metabolism, they concluded that this could not be explained by abnormalities in growth hormone secretion.

In certain animal models it is clear that prolactin governs the incidence and growth rates of breast tumours [16, 17]. In the clinical management of breast cancer hypophysectomy can bring about a remission of the disease [18] but whether this is due to a reduction in plasma prolactin is uncertain since stalk section resulting in an increased plasma prolactin concentration [19], can also bring about a remission.

Plasma prolactin levels in women with breast cancer have been shown by several workers to be normal [20–22], although two groups of workers claimed a significantly higher prolactin level [23, 24]. Recently Kwa and his colleagues [25] have reported that although there was no difference in plasma prolactin levels between normal women and patients with breast cancer there was a significant difference in subjects from families with a high frequency of breast cancer.

Although there are several reports implicating thyroid dysfunction as an aetiological factor in breast cancer the results are not conclusive [26–29]. A recent investigation of plasma TSH concentration, measured by radioimmunoassay, has indicated elevated levels in some patients [30]. However, other evidence shows plasma lipid patterns in women with breast cancer consistent with hyperthyroidism [31] and this discrepancy needs to be resolved.

As far as ACTH is concerned, no direct measurements are available but since abnormalities in adrenal steroids do appear to be related to the aetiology and clinical course of breast cancer [32], a study of this hormone might be useful.

In conclusion, our results indicate that plasma gonadotrophin levels are normal at diagnosis and furnish no information about the subsequent clinical course of the disease.

REFERENCES

1. A. S. Burt and B. Castleman, Some histological effects of estrogens and castration on the anterior pituitary in women with carcinoma of the breast. *Cancer (Philad.)* **6,** 236 (1953).
2. S. C. Sommers, Endocrine abnormalities in women with breast cancer. *Lab. Invest.* **4,** 160 (1955).
3. A. B. Russfield, Combined bioassay and histological study of 73 human hypophyses. *Cancer (Philad.)* **13,** 790 (1960).
4. R. F. Phifer, A. R. Midgley and S. S. Spicer, Immunohistologic and histologic evidence that follicle-stimulating hormone and luteinizing hormone are present in the same cell type in the human pars distalis. *J. clin. Endocr.* **36,** 125 (1973).
5. J. A. Loraine, J. A. Strong and M. Douglas, The value of pituitary gonadotrophin assays in patients with mammary carcinoma. *Lancet* **ii,** 575 (1957).

6. J. Marmoston, Urinary hormone metabolite levels in patients with cancer of the breast, prostate and lung. *Ann. N.Y. Acad. Sci.* **125,** 959 (1966).
7. A. Segaloff, D. Gordon, R. A. Carabasi, B. N. Horwitt, J. V. Schlosser and P. J. Murison, Hormonal therapy in cancer of the breast. VII. Effect of conjugated estrogens (equine) on clinical course and hormonal excretion. *Cancer (Philad.)* **7,** 758 (1954).
8. E. E. Boyland, B. Goldsmark, W. P. Greening, P. Rigby-Jones, J. J. Stevenson and M. A. M. Abul-Fadl, The effect of irradiation of the pituitary on gonadotrophin excretion in women with advanced mammary cancer. In *Endocrine Aspects of Breast Cancer.* (Edited by A. R. Currie), p. 170. E. & S. Livingstone, Edinburgh (1958).
9. J. L. Hayward, R. D. Bulbrook and F. C. Greenwood, Hormone assays and prognosis in breast cancer. *Mem. Soc. Endocrin.* **10,** 144 (1961).
10. F. I. R. Martin, Urinary gonadotrophins in post-menopausal women with breast cancer. *Brit. med. J.* **ii,** 351 (1964).
11. F. C. Greenwood, W. M. Hunter and J. S. Glover, The preparation of I^{131}-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89,** 114 (1963).
12. P. Franchimont, The regulation of follicle stimulating hormone and luteinizing hormone secretion in humans. In *Frontiers in Neuroendocrinology,* (Edited by L. Martini and W. F. Ganong), p. 331. Oxford University Press, New York (1971).
13. W. R. Butt, The extraction of gonadotrophins from urine by the combined use of benzoic acid and tungstic acid. *J. Endocr.* **17,** 143 (1958).
14. F. C. Greenwood, V. H. T. James, B. F. Meggitt, J. D. Miller and P. H. Taylor, Pituitary function in breast cancer. In *Prognostic Factors in Breast Cancer.* (Edited by A. P. M. Forrest and P. B. Kunkler), p. 409. E. & S. Livingstone, Edinburgh and London (1968).
15. O. H. Pearson, O. Llerena, N. Samaan and D. Gonzalez, Serum growth hormone and insulin levels in patients with breast cancer. In *Prognostic Factors in Breast Cancer.* (Edited by A. P. M. Forrest and P. B. Kunkler), p. 421. E. & S. Livingstone, Edinburgh and London (1968).
16. O. H. Pearson, O. Llerena, L. Llerena, A. Molina and T. Butler, Prolactin-dependent rat mammary cancer: A model for man? *Trans. Assoc. Am. Physns. (Philadelphia)* **82,** 225 (1969).
17. A. R. Boyns, R. Buchan, E. N. Cole, A. P. M. Forrest and K. Griffiths, Basal prolactin blood levels in three strains of rats with differing incidence of 7,12-dimethylbenz(a)anthracene induced mammary tumours. *Europ. J. Cancer* **9,** 169 (1973).
18. H. J. B. Atkins, M. A. Falconer, J. L. Hayward and K. S. Maclean, Adrenalectomy and hypophysectomy for advanced cancer of the breast. A comparative study. *Lancet* **i,** 489 (1957).
19. C. Y. Bowers, H. G. Friesen, P. Hwang, H. J. Guyda and K. Folkers, Prolactin and thyrotropin release in man by synthetic pyroglutamyl-histidyl-prolinamide. *Biochem. biophys. Res. Commun.* **45,** 1033 (1971).
20. A. R. Boyns, E. N. Cole, K. Griffiths, M. M. Roberts, R. Buchan, R. Wilson and A. P. M. Forrest, Plasma prolactin in breast cancer. *Europ. J. Cancer* **9,** 99 (1973).
21. S. Franks, D. N. L. Ralphs, V. Seagroatt and H. S. Jacobs, Prolactin concentration in patients with breast cancer. *Brit. Med. J.* **iv,** 320 (1974).
22. I. Mittra, J. L. Hayward and A. S. McNeilly, Hypothalamic pituitary-prolactin axis in breast cancer. *Lancet* **i,** 889 (1974).
23. R. M. L. Murray, G. Mozaffarian and O. H. Pearson, Prolactin levels with L-Dopa treatment in metastatic breast carcinoma. In *Prolactin and Carcinogenesis.* (Edited by A. R. Boyns and K. Griffiths), p. 158. Alpha Omega Alpha, Cardiff (1972).
24. R. M. L. Murray and G. Sarfaty, Prolactin in early and advanced breast carcinoma. *Proc. 11th Int. Cancer Congress Abstract* **2,** 122 (1974).
25. H. G. Kwa, M. de Jong-Bakker, E. Engelsman and F. J. Cleton, Plasma-prolactin in human breast cancer. *Lancet* **i,** 433 (1974).
26. R. W. Repert, Breast carcinoma study: Relation to thyroid disease and diabetes. *J. Mich. med. Soc.* **51,** 1315 (1952).

27. A. A. Loeser, A new therapy for prevention of post-operative recurrences in genital and breast cancer. A six year study of the prophylactic thyroid treatment. *Brit. med. J.* **ii,** 1380 (1954).
28. E. L. Wynder, I. J. Bross and T. Hirayama, A study of the epidemiology of cancer of the breast. *Cancer (Philad.)* **13,** 559 (1960).
29. R. D. Bulbrook, B. S. Thomas and J. Utsunomiya, Urinary 11-deoxy-17-oxosteroids in British and Japanese women with reference to the incidence of breast cancer. *Nature (Lond.)* **201,** 189 (1964).
30. I. Mittra and J. L. Hayward, Hypothalamic-pituitary-thyroid axis in breast cancer. *Lancet* **i,** 885 (1974).
31. E. B. Feldman and A. C. Carter, Circulating lipids and lipoproteins in women with metastatic breast carcinoma. *J. clin. Endocr.* **33,** 8 (1971).
32. D. Y. Wang, M. C. Swain, J. L. Hayward and R. D. Bulbrook, Hormones in the aetiology and clinical course of breast cancer. *Rec. Res. Cancer Res.* **39,** 177 (1972).

Europ. J. Cancer Vol. 12, pp. 313–319. Pergamon Press 1976. Printed in Great Britain

Effects of Different Dusts on Respiratory Carcinogenesis in Hamsters Induced by Benzo(a)pyrene and Diethylnitrosamine*

A. SELLAKUMAR†, F. STENBÄCK‡ and J. ROWLAND§

†*New York University, Department of Environmental Medicine, Tuxedo, New York* 10987, *U.S.A. and* ‡§*The Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska* 68105, *U.S.A.*

Abstract—*The modes of interaction between (a) subcutaneously injected diethylnitrosamine (DEN) and intratracheally instilled ferric oxide (Fe_2O_3); and (b) benzo(a)pyrene (B(a)P) and dusts; namely, Fe_2O_3, aluminium oxide (Al_2O_3) or carbon (C) intratracheally instilled, were studied in Syrian golden hamsters. The objective was to determine whether or not an initiation–promotion type relationship existed. DEN alone induced laryngeal and tracheal papillomas and, when followed by Fe_2O_3, elicited lung tumors. These tumors may be auto-tranplanted tumors. Fe_2O_3 followed by DEN treatment, and simultaneous DEN/Fe_2O_3 treatment produced tumors of the larynx and trachea in the same way as DEN alone. Single applications of DEN and of Fe_2O_3 induced 1 tracheal papilloma. A number of forestomach papillomas, as well as other tumors, such as adrenal cortex adenomas, were seen in treated groups and in similar numbers in the untreated controls. Repeated applications of B(a)P alone and Fe_2O_3 given before or after B(a)P failed to induce more than a few laryngeal and tracheal tumors. A significant number of tumors occurred only when both agents, B(a)P and dust, were administered simultaneously. Thus, no significant initiating-promoting action by dusts on DEN or B(a)P-induced lung tumorigenesis in the respiratory tract of Syrian golden hamsters was observed. It is concluded that Fe_2O_3 acts as a carrier agent, enhancing the effects of B(a)P.*

INTRODUCTION

THE concept of initiation and promotion as two distinct stages in tumor formation was originally proposed by Berenblum and Shubik based upon their studies in mice skin using 3,4-benzo(a)pyrene (B(a)P) followed by croton oil [1, 2]. Initiating action is brought about quickly, perhaps instantaneously. It is irreversible and results from administration of certain chemical compounds in a limited number of species and tissues [3–5]. The progression of these dormant tumor cells depends on the continued treatment, i.e., promotion. This is a slow-acting phenomenon [3] and partly reversible [4]. It may be induced by a number of chemical and physical agents [6, 7]. Some investigators using other chemical compounds have repeated these tests with varying results [8–10].

Diethylnitrosamine (DEN) has proven an effective tumor inducer in Syrian golden hamsters [11–17]. Intratracheal instillation (i.t.) of B(a)P absorbed on Fe_2O_3 particles, have produced a number of respiratory system tumors in hamsters [18, 19]. Fe_2O_3 has also been reported as enhancing the tumor-inducing effect of dimethylnitrosoamine (DMN) given subcutaneously (s.c.) [20]. Carbon (C), aluminum oxide (Al_2O_3) and magnesium oxide (MgO) alone failed to exhibit carcinogenic activity [21]. Given, i.t., in combination with

Accepted 13 November 1975.

*Supported by Contract No. NIH-PHS-43-68-959 from the National Cancer Institute.

DEN treatment, these dusts increased the number of respiratory tumors [21, 22].

The present study was undertaken to investigate the role of different dusts, namely Fe_2O_3, C, Al_2O_3 in two experimental models. The relationship between the carcinogen and carrier dust in respiratory tumor induction was also investigated. Accordingly, in the first experiment we attempted to find whether or not Fe_2O_3 acted as a promoting agent to DEN given s.c. in the same fashion as croton oil as a promotor in two-stage carcinogenesis [1, 2, 6] or as an initiating agent. In our second experiment, we tested the role of Fe_2O_3 as initiator or promotor when given i.t. with B(a)P. We also investigated the promoting effects of C and Al_2O_3 with B(a)P.

MATERIAL AND METHODS

Randomly bred 8–9 week-old male Syrian golden hamsters of the Eppley colony were housed 6 per plastic cage on sterilized San-i-cel bedding. They were fed Wayne animal pelleted diets and given water *ad libitum*. DEN, Fe_2O_3, Al_2O_3, and C were obtained from the Fisher Scientific Co., Fairlawn, New Jersey; B(a)P from Aldrich Chemical Co., Milwaukee, Wisconsin; and saline (0·9% sodium chloride in water, sterile, non-pyrogenic) from Baxter Laboratories Inc., Morton Grove, Illinois. The purity of B(a)P, determined by thin-layer chromatography, was 99%. The particle sizes for Fe_2O_3, < 10 μm (98%), < 5 μm (94%), and < 1 μm (24%); C, < 10 μm (82%), < 5 μm (77%), and < 1 μm (19%), and Al_2O_3, < 25 μm (100%), < 10 μm (99%), < 5 μm (97%), < 1 μm (39%).

For simulataneous instillation of B(a)P and Fe_2O_3, equal weights were transferred to a mullite mortar where they were mixed and then ground together for about 8 hr, yielding a fine, homogenenously distributed dust containing 50% B(a)P and Fe_2O_3 by weight. This combined dust was then ball-milled for 17 hr.

The present study details results from two different experimental groups. In experiment A, the animals were divided into 7 groups, 1 mg DEN in 0·25 ml saline was injected s.c., and Fe_2O_3 in 0·2 ml saline i.t., by the method of Saffiotti [19]. The groups were treated as follows: Group 1, DEN once weekly for 6 weeks, left untreated for 6 weeks, followed by 3 mg Fe_2O_3 once weekly for 15 weeks; Group 2, 3 mg Fe_2O_3 once weekly for 15 weeks, left untreated for 6 weeks, followed by DEN for 6 weeks; Group 3, 2 mg Fe_2O_3 once every second week (15 times), left untreated for 6 weeks, followed by DEN once weekly for 6 weeks; Group 4, DEN once followed the second week by 3 mg Fe_2O_3 once; Group 5, 3 mg Fe_2O_3 once weekly for 15 weeks, and then DEN once weekly for 6 weeks beginning with the fourth experimental week; Group 6 received DEN alone, once weekly for 6 weeks. Group 7 consisted of untreated animals.

In the second set of experiments, experiment B, 8 hamster groups received the following treatments, i.t., in 0·2 ml saline: Group 1, 3 mg B(a)P once weekly for 10 weeks; Group 2, 3 mg Fe_2O_3 once weekly for 10 weeks; Group 3, 3 mg Fe_2O_3 and 3 mg B(a)P together once weekly for 10 weeks; Group 4, 3 mg B(a)P once weekly for 10 weeks, left untreated for 4 weeks, and then 3 mg Fe_2O_3 once weekly for 10 weeks; Group 5, 3 mg Fe_2O_3 once weekly for 10 weeks, no treatment for 4 weeks, followed by 3 mg B(a)P once weekly for 10 weeks; Group 6, 10 weekly instillations of 3 mg B(a)P, left untreated for 4 weeks, then 3 mg C once weekly for 10 weeks; Group 7, 10 weekly instillations of 3 mg B(a)P, left untreated for 4 weeks, then 3 mg Al_2O_3 once weekly for 10 weeks. Group 8 was the untreated control.

Animals were weighed weekly and those in poor condition were isolated and allowed to die spontaneously or killed when moribund. All animals were completely autopsied, except for a few lost through cannibalism. At autopsy, the trachea was ligated and lungs removed *en bloc*, while still fully expanded, and fixed in 10% neutral buffered formalin. Histological sections were prepared from each lung lobe and also from the larynx, trachea, stem bronchi, and other organs showing gross pathological alterations. Sections were stained with hematoxylin and eosin and special stains as required.

RESULTS

The survival rates of the animals in experiment A are given in Table 1. By the 60th week, Groups 5 and 6 had lower survival rates and no animals survived past the 80th week.

In Group 1, 30 of 48 animals (63%) showed tumors (Table 2). The first tracheal papilloma occurred at 26 weeks. Thirteen laryngeal papillomas, 34 tracheal papillomas, 1 lung adenoma and 11 adenosquamous lung tumors occurred in addition to tumors of other organs.

When treatment was reversed (Group 2), there were only 15 animals with tumors of the upper respiratory tract (Table 2). The first tracheal papillomas appeared much later than with Group 1, at 53 weeks, as expected. A

Table 1. Experiment A: Survival rates in Syrian golden hamsters treated with DEN and Fe_2O_3

Group	Treatment[a]	Initial no. hamsters	Survivors at weeks 20	40	60	80	100	120
1	DEN, 6x[b]; *I*[c]; 3 mg Fe_2O_3,15x	48	48	33	19	6	2	0
2	3 mg Fe_2O_3, 15x; *I*, DEN, 6x	48	42	28	20	14	1	0
3	2 mg Fe_2O_3 every 2nd wk, 15x; *I*; DEN, 6x	36	31	15	11	4	0	0
4	DEN, 1x followed by 3 mg Fe_2O_3, 1x	35	33	22	13	6	0	0
5	3 mg Fe_2O_3, 15x; DEN, 6x beginning at 4th experimental week	48	44	19	5	0	0	0
6	DEN, 6x	48	44	27	8	0	0	0
7	Untreated control	48	47	38	20	6	3	0

a) 1 mg DEN in 0.25 ml saline by subcutaneous injection given once weekly; Fe_2O_3 in 0.2 ml saline instilled intratracheally once weekly, except for group 3.

b) x = times.

c) *I* = interval of 6 weeks without treatment.

Table 2. Experiment A: Number and types of tumors induced by DEN and Fe_2O_3

Group	Treatment[a]	Total # animals	Total[b] # resp. TBA (%)	Total # resp. tumors	Total number of tumors: Larynx papilloma	Trachea papilloma	Lung: adeno sq.	Lung: adenoma	Other tumors
1	DEN,6x[c]; *I*[d];3 mg Fe_2O_3,15x	48	30(63)	59	13	34	11	1	11[e]
2	3 mg Fe_2O_3,15x; *I*; DEN,6x	48	15(31)	22	7	15	--	-	2[f]
3	2 mg Fe_2O_3 every 2nd week, 15x; *I*; DEN, 6x	36	11(31)	18	3	14	1	-	1[g]
4	DEN, 1x followed by 3 mg Fe_2O_3, 1x	35	1(3)	1	--	1	-	-	3[h]
5	3 mg Fe_2O_3,15x; DEN, 6x beginning at 4th experimental week	48	13(27)	22	5	16	-	1	-
6	DEN, 6x	48	28(58)	36	7	28	-	1	1[i]
7	Untreated Control	48	--	--	--	--	-	-	6[j]

a) 1 mg DEN in 0.25 ml saline by subcutaneous injection given once weekly; Fe_2O_3 in 0.2 ml saline instilled intratracheally once weekly, except for group 3.
b) TBA = tumor bearing animals.
c) x = times.
d) *I* = interval of 6 weeks without treatment.
e) 10 forestomach papillomas; 1 adrenal cortical adenoma.
f) 2 forestomach papillomas.
g) 1 forestomach papilloma.
h) 2 forestomach papillomas; 1 adrenal cortical adenoma.
i) 1 forestomach papilloma.
j) 3 forestomach papillomas; 2 lymphomas; 1 salivary gland undifferentiated carcinoma.

lower dose of Fe_2O_3 and longer treatment period in Group 3 (2 mg every 2nd week for 30 weeks, as opposed to 3 mg/week for 15 weeks in Group 2) produced a similar percentage (31) of tumors, with the first laryngeal papillomas seen at 66 weeks.

Simultaneous application of Fe_2O_3 and DEN (Group 5) induced laryngeal and tracheal papillomas in 13 of 48 animals (27%) and 1 lung adenoma. The first tracheal papilloma appeared at 27 weeks and 16 papillomas were seen in 10 animals. Single applications of DEN and of Fe_2O_3 (Group 4) induced a minimal neoplastic response, i.e., a single tracheal papilloma out of 35 animals seen at 43 weeks.

Six once weekly subcutaneous injections of DEN alone (Group 6) caused laryngeal and tracheal papillomas in 58% of the animals and 1 lung adenoma. The first tumor was in an animal dying 20 weeks after the beginning of the treatment. No respiratory tumors were found in the untreated animals (Group 7).

The tumors observed (See Table 2) were histologically similar in all groups. Those of the larynx and trachea were papillomas similar to those previously described in DEN-treated animals [21]. They showed a papillary configuration with papillae lined by cuboidal or squamous cells. No evidence of invasive growth occurred. Grossly, the lung tumors were whitish nodules of varying sizes. Histologically, they were adenosquamous tumors composed of monomorphic polyhedral squamoid cells arranged in concentric layers. The lesions were

clearly delineated without signs of infiltrations or metastases. Dust accumulations were also seen in all groups, accompanied by accumulations of dust laden macrophages and fibrosis.

Incidence of tumors in non-respiratory organs was not significant when compared to the untreated controls (Table 2). The exception was Group 1 which had a higher incidence of forestomach papillomas.

Survival rates for experiment B are given in Table 3. At 40 weeks, Group 3 had the lowest rate of survival and at 80 weeks, none survived. This is explained by the high incidence of tumors (71%) in this group.

In Group 1, 15% of the animals had benign tumors of the upper respiratory tract, including 1 lung adenoma (Table 4). In Group 2, animals given Fe_2O_3 alone, no respiratory tract tumors were found. Group 3 (B(a)P and Fe_2O_3 given simultaneously for 10 weeks) had the highest number of TBA (71%) with neoplasms in the larynx, trachea and bronchi, as expected (Table 4). Several animals had more than one tumor, which resulted in a total number of 66 tumors. Of these tumors, 55% were squamous cell carcinomas originating from the trachea and bronchus. Three adenosquamous and 1 adenocarcinoma of the lung were also induced. Thirty-seven per cent of the respiratory tumors found were of the benign type.

The instillation of B(a)P followed by Fe_2O_3 (Group 4) compared to Fe_2O_3 followed by B(a)P (Group 5) showed no difference in tumor incidence (13%) (Table 4). The exception was the single squamous cell carcinoma of the

Table 3. Experiment B: Survival rates in Syrian golden hamsters after intratracheal instillation of B(a)P with/without dusts

Group	Treatment[a]	Initial no. hamsters	Survivors at weeks 20	40	60	80	100	120
1	B(a)P 10x[b]	48	47	47	31	16	2	0
2	Fe_2O_3 10x	48	47	47	33	14	3	0
3	B(a)P and Fe_2O_3 10x	48	45	28	4	0	0	0
4	B(a)P 10x; *I*[c]; Fe_2O_3 10x	48	42	36	26	10	1	0
5	Fe_2O_3 10x; *I*; B(a)P 10x	48	47	39	27	13	4	0
6	B(a)P 10x; *I*; Al_2O_3 10x	48	43	34	19	6	2	0
7	B(a)P 10x; *I*; C 10x	48	44	39	24	11	2	0
8	Untreated control	48	47	38	20	6	3	0

a) 3 mg B(a)P with and without 3 mg dust in 0.2 ml saline instilled intratracheally once weekly.

b) x = times

c) *I* = interval of 4 weeks without treatment.

Table 4. Experiment B: Number and types of tumors induced by B(a)P with/without dusts

Group	Treatment[a]	Total no. animals	Total # resp. TBA[b] (%)	Total # resp. tumors	Larynx pap.	Larynx sq.ca.	Trachea pap.	Trachea sq.ca.	Lung adenoma	Lung adenosq.	Lung adca.	Lung sq.ca.	Other tumors
1	B(a)P, 10x[e]	48	7(15)	7	2	-	4	-	1	--	-	-	26[e]
2	Fe_2O_3, 10x	48	0(0)	-	-	-	-	-	-	-	-	-	1[f]
3	B(a)P and Fe_2O_3, 10x	48	34(71)	66	7	1	14	19	4	3	1	17	1[g]
4	B(a)P,10x;*I*[d];Fe_2O_3,10x	48	6(13)	6	-	-	6	-	-	-	-	-	23[h]
5	Fe_2O_3,10x;*I*;B(a)P,10x	48	6(13)	7	1	-	5	1	-	-	-	-	20[i]
6	B(a)P,10x;*I*;Al_2O_3,10x	48	3(6)	7	1	-	2	-	4	-	-	-	22[j]
7	B(a)P,10x;*I*; C,10x	48	3(6)	3	2	-	1	-	-	-	-	-	22[k]
8	Untreated control	48	0(0)	-	-	-	-	-	-	--	-	-	6[l]

a) 3 mg B(a)P with and without 3 mg dust in 0.2 ml saline instilled intratracheally once weekly.
b) TBA = tumor bearing animals.
c) x = times.
d) *I* = interval of 4 weeks without treatment.
e) 21 forestomach papillomas; 1 skin melanoma; 1 liver hemangioma; 1 adrenal cortical adenoma; 2 adrenal cortical carcinomas.
f) 1 spleen hemangioma.
g) 1 spleen hemangioma.
h) 23 forestomach papillomas.
i) 20 forestomach papillomas.
j) 1 spleen hemangioma; 1 adrenal cortical adenoma; 1 malignant lymphoma; 18 forestomach papillomas; 1 forestomach squamous carcinoma.
k) 21 forestomach papillomas; 1 adrenal cortical adenoma.
l) 3 forestomach papillomas; 2 lymphomas; 1 anaplastic carcinoma.

trachea in Group 5 appearing at the 69th week. Replacing Fe_2O_3 with Al_2O_3 or C (Groups 6 and 7) resulted in approximately 50% fewer tumor-bearing animals: 13% with Fe_2O_3; 6% with Al_2O_3 and 6% with C. Those seen were all benign type tumors. No respiratory tract tumors were seen in untreated controls, Group 8.

All groups treated with B(a)P showed higher incidences of forestomach papillomas as compared to untreated controls (Table 4). The exception was in Group 3. This is explained by the fact that most of the animals in this group died early with respiratory tract tumors.

DISCUSSION

The present results demonstrate different carcinogenic effects in various segments of the respiratory tract of hamsters when DEN alone is administered, s.c., or B(a)P, i.t., with or without dusts. The results of these experiments confirm the findings of others [21, 23, 24]. DEN injected at the dose given produced 35 papillomas in 28 animals in the upper respiratory tract, but no significant number of tumors in the lower segment. B(a)P given with Fe_2O_3 induced higher incidences of tumors, in both upper (41 tumors in 34 animals) and in lower segments (25 tumors in 34 animals).

Previous studies have shown that the tumor yield is increased when DEN is given s.c. followed by Fe_2O_3, i.t., alone or instilled together with B(a)P [21, 23]. Montesano *et al.* [23] suggested that the effect was linked to the cellular reaction to the instillation of the particulate material. They also showed that DEN, administered after the instillation of a subeffective dose of B(a)P mixed with Fe_2O_3, increased the number of squamous cell carcinomas at those sites which are particularly susceptible to the carcinogenic action of only higher doses of B(a)P. Intratracheal instillation of Fe_2O_3 after DEN treatment shortened the the latent period of bronchio-alveolar adenomas and presented a marked increase in the tumor count in the lower respiratory tract. They concluded that Fe_2O_3 might be considered as a cocarcinogen.

Administration of Fe_2O_3 followed by DEN (Experiment A, Group 2) showed no differences in the percentage of TBA as compared to Group 5, where DEN and Fe_2O_3 were given simultaneously. A lower dose (2 mg) and longer period of treatment with Fe_2O_3, followed by DEN (Group 3) also showed no difference in percentage of TBA. These results do not correlate with those of others [22, 23]. This may be due to the level of doses and/or routes of administration. A single application of DEN followed by Fe_2O_3 produced 1 tracheal papilloma. We found an increased number of adenosquamous tumors of the lung in Group 1. Each of the animals in Group 1 with adenosquamous tumors also had tracheal papillomas. This strongly suggests that these adenosquamous tumors may be auto-transplanted tumors. In a series of studies performed in our laboratory tumor cells were taken from DEN-induced tracheal papillomas in hamsters, trypsinized and instilled intratracheally into Syrian golden hamsters. This produced adenosquamous tumors in the lower respiratory tract which were morphologically similar to those found in the present study. Histochemical [21] as well as ultrastructural studies [22] have shown remarkable morphological similarities between tracheal papillomas and adenosquamous tumors of the lower respiratory tract.

The fact that simultaneous applications of Fe_2O_3 and DEN induced a lower number of upper respiratory tract tumors, than when Fe_2O_3 was administered after DEN, suggests a possible promoting effect of Fe_2O_3 on DEN-induced tumor formation. Whether this is an initiation–promotion type process, comparable to that in mouse skin (whereby a single application of a subeffective dose of a carcinogen followed by a non-carcinogen, such as croton oil, is effective in producing tumors) remains to be seen. With regard to our studies using B(a)P followed by Fe_2O_3 in saline (Experiment B, Group 4), Fe_2O_3 did not act as a promoter, since the tumor incidence proved approximately the same as that observed when B(a)P was administered alone (Experiment B, Group 1). This lack of effect, however, was not specific to Fe_2O_3, since it was also seen in Experiment B, Groups 6 and 7, using C and Al_2O_3 dusts. The increased number of tumors in Experiment B, Group 3 with B(a)P and Fe_2O_3 given simultaneously support the contention that Fe_2O_3 appears to act as a cocarcinogen. Harris *et al.* [25, 26] reported basal cell hyperplasia of the upper respiratory tract when Fe_2O_3 alone or with pyrene was administered. These authors also found hyperplasia and metaplasia when Fe_2O_3 was administered with B(a)P.

Still other reasonable explanations of the effect of Fe_2O_3 include the possibility that the dust exerts a modifying effect on the metabolism as well as penetration and retention of DEN [16]. These speculations, however, are not supported by the findings of a low tumor yield, when both chemicals were given simultaneously (Experiment A, Group 5), as compared to s.c.

injections of DEN alone (Experiment A, Group 6).

The suggestions that Fe_2O_3 enhances B(a)P's action was not supported by our results. Repeated Fe_2O_3 application, prior to B(a)P treatment (Experiment B, Group 5), failed to elicit a significant tumor response; i.e., only 1 squamous cell carcinoma of the trachea was encountered.

The only experiment wherein a significant neoplastic response occurred when the carcinogen and the dust were given simultaneously, involved Group 3, Experiment B. The probable explanation was given by Saffiotti *et al.* [27], who demonstrated an increased retention of B(a)P when applied simultaneously with Fe_2O_3. Other possibilities, such as the effect on penetration of B(a)P [28], surface activity, particle size and shape, and localization, need further study.

Acknowledgements—The authors gratefully acknowledge the excellent technical assistance of R. Theis; the editorial assistance of Ms. A. L. Verde and the typing of Ms. D. Mackay and Ms. S. Steinberg.

REFERENCES

1. I. Berenblum and P. Shubik, The role of croton oil applications associated with a single painting of a carcinogen in tumor induction of the mouse's skin. *Brit. J. Cancer* **1,** 379 (1947).
2. I. Berenblum and P. Shubik, A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. *Brit. J. Cancer* **1,** 383 (1947).
3. M. H. Salaman and R. H. Gwynn, The histology of cocarcinogenesis in mouse skin. *Brit. J. Cancer* **5,** 252 (1951).
4. P. Shubik, The growth potentialities of induced skin tumors in mice. The effect of different methods of chemical carcinogenesis. *Cancer Res.* **10,** 713 (1950).
5. F. Stenbäck, Promotion in the morphogenesis of chemically inducible skin tumors. *Acta Path. microbiol. scand. Suppl.* **1208,** 1 (1969)
6. I. Berenblum, Carcinogenesis and tumor pathogenesis. *Adv. Cancer Res.* **2,** 129 (1954).
7. U. Saffiotti and P. Shubik, The effects of low concentrations of carcinogen in epidermal carcinogenesis. A comparison with promoting agents. *J. nat. Cancer Inst.* **16,** 961 (1956).
8. A. W. Pound, Further observations concerning the influence of preliminary stimulation by croton oil and acetic acid on the initiation of skin tumors in mice by urethane. *Brit. J. Cancer* **20,** 385 (1966).
9. A. W. Pound and J. R. Bell, The influence of croton oil stimulation on tumor initiation by urethane in mice. *Brit. J. Cancer* **16,** 690 (1962).
10. A. C. Ritchie, Epidermal carcinogenesis in the mouse by intraperitoneally administered urethane followed by repeated applications of croton oil. *Brit. J. Cancer* **11,** 206 (1957).
11. W. Dontenwill and U. Mohr, Carcinoma des Respirationstractus nach Behandlung von Goldhamstern mit Diäthylnitrosamine. *Z. Krebsforsch.* **64,** 305 (1961).
12. W. Dontenwill and U. Mohr, Vergleichende Untersuchungen an metaplastischen und malignen Epithelwucherungen des Respirationstraktes im Tierexperiment. *Z. Krebsforsch.* **65,** 168 (1962).
13. K. M. Herrold, Induction of olfactory neuroepithelial tumors in Syrian hamsters by diethylnitrosamine. *Cancer (Philad.)* **17,** 114 (1964).
14. K. M. Herrold, Effect of route of administration on the carcinogenic action of diethylnitrosamine (n-nitrosodi-ethylamine). *Brit. J. Cancer* **18,** 763 (1964).
15. R. Montesano, Systemic carcinogens (n-nitroso compounds) and synergistic additive effects in respiratory carcinogenesis. *Tumori* **56,** 335 (1970).
16. R. Montesano and U. Saffiotti, Carcinogenic response of the hamster respiratory tract to single subcutaneous administrations of diethylnitrosamine at birth. *J. nat. Cancer* **44,** 413 (1970).
17. R. Montesano and U. Saffiotti, Carcinogenic response of the respiratory tract of Syrian golden hamsters to different doses of diethylnitrosamine. *Cancer Res.* **28,** 2197 (1968).
18. U. Saffiotti, Experimental respiratory tract carcinogenesis. In *Progress in Experimental Tumor Research.* (Edited by F. Homburger) Vol. 11, p. 302. Karger, New York/Karger (1969).

19. U. Saffiotti, F. Cefis and L. Kolb, A method for the experimental induction of bronchogenic carcinoma. *Cancer Res.* **28,** 104 (1968).
20. F. Stenbäck, A. Ferrero, R. Montesano and P. Shubik, Synergistic effect of ferric oxide on dimethylnitrosamine carcinogenesis in the Syrian golden hamster. *Z. Krebsforsch.* **79,** 31 (1973).
21. F. Stenbäck, A. Ferrero and P. Shubik, Synergistic effects of diethylnitrosamine and different dusts on respiratory carcinogenesis in hamsters. *Cancer Res.* **33,** 2209 (1973).
22. J. W. Feron, E. Emmelot and T. Vossenaar, Lower respiratory tract tumors in Syrian golden hamsters after intratracheal instillations of diethylnitrosamine alone and with ferric oxide. *Europ. J. Cancer* **8,** 444 (1972).
23. R. Montesano, U. Saffiotti and P. Shubik The role of topical and systemic factors in experimental respiratory carcinogenesis. In *Inhalation Carcinogenesis.* (Edited by M. G. Hanna, Jr., H. M. Nettesheim and J. R. Gilbert). USAEC Symposium Series 18, p. 353. Oak Ridge Tennessee (1970).
24. D. A. Creasia and P. Nettesheim, Respiratory cocarcinogenesis studies with ferric oxide: a test case of current experimental models. In *Experimental Lung Cancer Carcinogenesis and Bioassays.* (Edited by E. Karbe and J. Park), p. 234. Springer-Verlag, Frankfurt, Germany (1974).
25. C. C. Harris, M. B. Sporn, D. G. Kaufman, J. M. Smith, F. E. Jackson and U. Saffiotti, Histogenesis of squamous metaplasia in the hamster tracheal epithelium caused by vitamin A deficiency or benzo(a)pyrene-ferric oxide.
26. C. C. Harris, M. B. Sporn, D. G. Kaufman, J. M. Smith, M. S. Baker and U. Saffiotti, Acute ultrastructural effects of benzo(a)pyrene and ferric oxide on the hamster tracheobronchial epithelium. *Cancer Res.* **31,** 1977 (1971).
27. U. Saffiotti, Experimental respiratory tract carcinogenesis and its relation to inhalation exposure. In *Inhalation Carcinogenesis.* (Edited by M. G. Hanna, P. Nettesheim and J. R. Gilbert), USAEC Symposium Series 18, p. 27. Springfield, Virginia (1970).
28. A. R. Kennedy and J. B. Little, Localization of polycyclic hydrocarbon carcinogens in the lung following intratracheal instillation in gelatin solution. *Cancer Res.* **35,** 1563 (1975).

Europ. J. Cancer Vol. 12, pp. 321–323. Pergamon Press 1976. Printed in Great Britain

Lack of Interference in Antigen Production *In Vitro* Between B-Type and C-Type Oncornaviruses as Determined by Double Stain Immunofluorescence

FRIDA G. VAN PELT*, ADA HOUWELING*, A. M. AARSSEN† and P. BENTVELZEN†

**Institute for Experimental Gerontology TNO*, †*Radiobiological Institute TNO*, 151 *Lange Kleiweg, Rijswijk, The Netherlands*

Abstract—*By means of immunofluorescence using two different fluorochromes antigens of B-type and C-type oncornavirus could be detected simulataneously in mammary tumour cultures. No mutual interference between both kinds of viruses could be detected.*

A MAJOR problem with murine mammary tumour culture lines is the production of endogenous C-type oncornavirus after passage *in vitro* [1–3]. To avoid contamination of the mammary tumour virus (MTV) isolates from tissue culture fluid with murine leukaemia virus (MuLV) early detection of the latter is necessary. For that purpose, electron microscopy is an insensitive and cumbersome technique. The present immunofluorescence study was undertaken not only for the development of a simple sensitive monitor system, but also for obtaining some information about interactions between the two kinds of viruses. We were specifically interested in finding out whether a cell could produce antigens of both viruses simultaneously, and whether there would be mutual interference. It has been suggested that *in vivo* such interference would exist [4].

Antiserum to Rauscher murine leukaemia virus (RLV) was prepared in WAG/Rij inbred rats by immunizations with cells from a transplantable syngeneic lymphosarcoma induced by RLV. The specificity of this antiserum has been discussed elsewhere [5].

Accepted 13 November 1975.

For immunization of rabbits, B-type particles (MTV virions) were isolated from BALB/cfC3H mammary tumours by the technique of Calafat and Hageman [6]. A rabbit was immunized 4 × at weekly intervals with 0·2 mg purified virus, emulsified in complete Freund's adjuvans. Antibodies to host materials were first removed by immunoadsorbent column chromatography using BALB/c lactating mammary gland extract coupled to glutaraldehyde. Thereafter, the rabbit serum was injected into 6-week-old BALB/c male mice (1 ml i.p.). The animals were bled 16 hr later. The rabbit globulins were separated from the mouse serum by passing the serum over an immunoadsorbent column of goat-antirabbit-IgG antiserum. The rabbit globulins were eluted with 0·1 M glycine–HCl buffer, pH 2·4.

The absorbed rabbit antiserum does not react in the indirect immunofluorescence test with secondary embryonic fibroblast cultures from any mouse strain whether infected with RLV or not. It reacts with every cell line producing a MTV.

The C3H mammary tumour cell line, Mm5mt obtained from Dr. D. L. Fine, NCL Frederick Cancer Research Center, U.S.A. [7] and the BALB/c transformed kidney cell line,

A3 obtained from Dr. J. Links, Netherlands Cancer Institute [3], were cultured in NCTC 135 medium + 10% newborn calf serum + antibiotics. Primary cultures of mammary tumours were prepared as follows. Tumours were minced in Hanks balanced salt solution and then pushed through a nylon gauze, producing an almost monocellular suspension. The suspension was washed 3 times. Then, 8×10^5 cells/ml NCTC 135 medium + 15% newborn calf serum were plated in large Petri dishes containing 6 microscopic slides. After 2 days, the medium was changed to one containing 10% serum.

The cells were washed and fixed in cold acetone and thereafter the slides were washed thrice in PBS. Four spots on each slide were used for incubation. Spot 1 was incubated with the rat anti-RLV serum, followed by a second incubation with a goat antiserum to rat IgG labeled with fluorescein isothiocyanate (FIFC) (dil. 1:20). Spot 2 was incubated with the anti-MTV serum (dil. 1:40) and thereafter with a goat antiserum to rabbit IgG labeled with tetramethylrhodamine isothiocyanate (TRITC) diluted 20 times. Spot 3 was incubated with both antivirus sera simultaneously and thereafter with the two conjugates (Nordic, Tilburg, the Netherlands). Spot 4 was incubated with the two conjugates only. The incubations were done for 30 min at 37°C in a moist chamber. After each incubation, the slides were thoroughly washed three times in PBS. Finally, coverslips were mounted with elvanol [8].

The preparations were viewed under a Leitz Orthoplan microscope with the Ploemopak 2·1, with the filter systems L and N. Filter system L contains narrow-band excitation and emission filters for FITC; 2 × KP 490 nm, 1 mm GG 455 nm, TK 510 nm (interference dividing plate) and K 515 nm + SAL 525 nm as barrier filters. Filter system N is used for TRITC fluorescence and consists of 2 mm BG 36 + KP 560 nm + K 530 nm as excitation filters with the dichromic mirror TK 580 nm, and K 580 nm + KP 610 nm as emission filters. The narrow-band excitation and emission filters for FITC (system L) eliminate contaminating fluorescence of TRITC at 510 nm.

From the results (Table 1) with two cell lines and primary cultures of BALB/c mammary tumour induced by MTV-B, it is obvious that a great percentage of the cells produce both kinds of viral antigens (see also Fig. 1). There is no statistical indication that either type of virus interfered with the production of antigens from the other. During subsequent passage of the cell lines, we often noted an enhanced production of C-type particles, as determined by electron microscopy, but this did not lead to the inhibition of MTV release. The supposed *in vivo* interference is probably not due to intracellular competion between both viruses in mammary cells.

Table 1. *Immunofluorescence detection of oncornaviral antigens*

Cultures	Number of cells examined	Percentage of cells producing intracytoplasmic antigens of			
		MTV- MuLV-	MTV+ MuLV-	MTV- MuLV+	MTV+ MuLV+
Mm5mt	273	47	32	5	16
A3	60	6	43	8	43
BALB/MT-B/Ry	115	35	15	16	34

Acknowledgements—This investigation was supported in part by Public Health Service, Contract NOI-CP43328 with Division of Cancer Cause and Prevention, Viral Oncology, of the National Cancer Institute, U.S.A.

REFERENCES

1. E. Y. Lasfargues, B. Kramarsky, N. H. Sarkar, J. C. Lasfargues, N. Pillsbury and D. H. Moore, Stimulation of mammary tumor virus production in a mouse mammary tumor cell line. *Cancer Res.* **30,** 1109 (1970).
2. J. Hilgers, W. C. Williams, W. B. Myers and L. Dmochowski, Detection of antigens of the mouse mammary tumor (MTV) and murine leukemia virus (MuLV) in cells of cultures derived from mammary tumors of mice of several strains. *Virology* **45,** 470 (1971).
3. J. Calafat, F. Buijs, P. C. Hageman, J. Links, J. Hilgers and A. Hekman, Distribution of virus particles and mammary tumor virus antigens in mouse mammary tumors, transformed BALB/c mouse kidney cells, and GR ascites leukemia cells. *J. nat. Cancer Inst.* **53,** 977 (1974).
4. F. Squartini, M. Olivi, J. B. Bolis, R. Ribacchi and G. Giraldo, Reciprocal interference between mouse mammary tumour virus and leukemia virus. *Nature (Lond.)* **214,** 730 (1967).

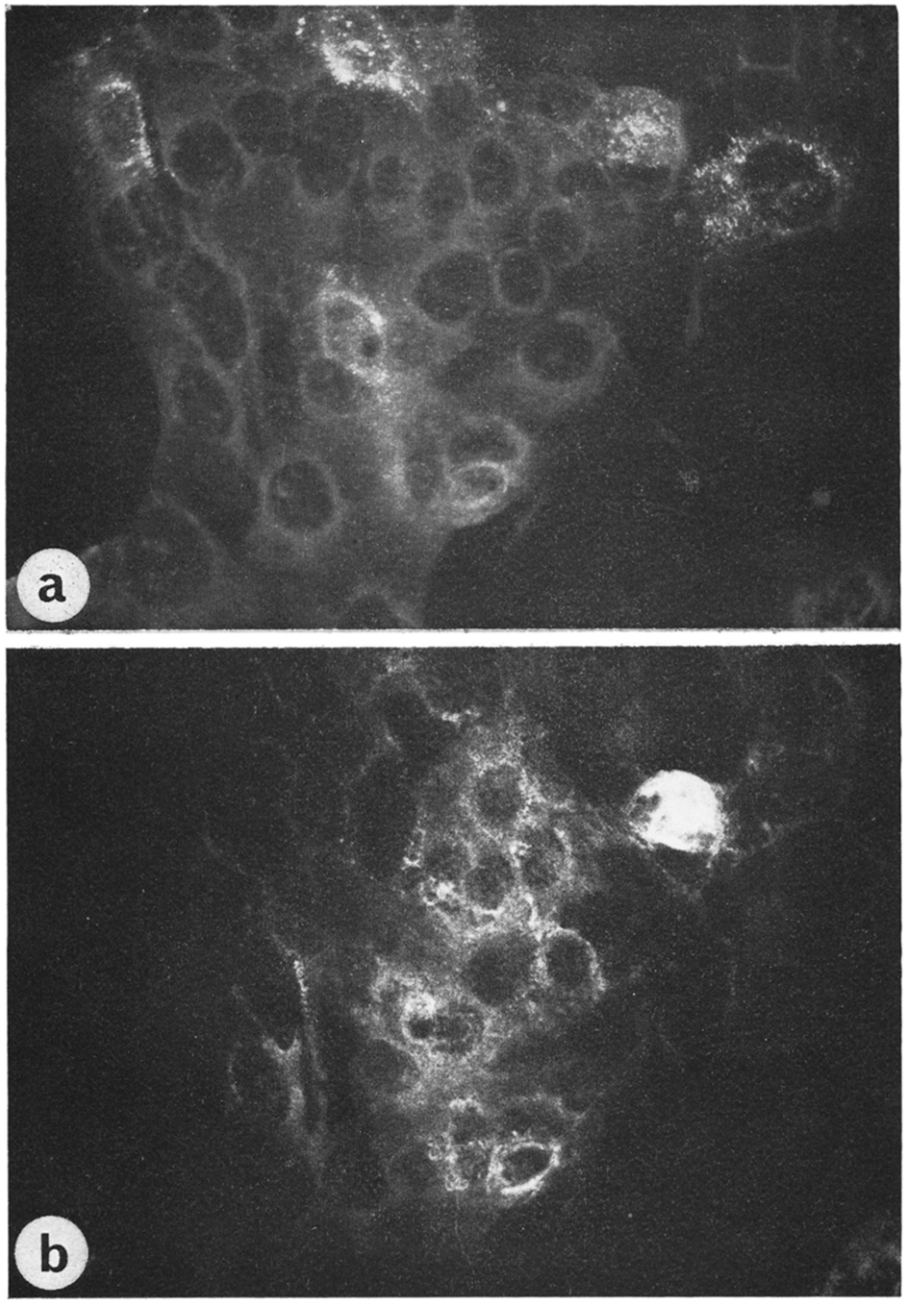


Fig. 1. *Mammary tumour cell line BALB/MT-B/Ry.*
(*a*) *Cells stained red for the demonstration of MTV antigens.*
(*b*) *Identical cells stained green for the demonstration of MuLV antigens.*
Note that some cells are stained for one antigen and other cells are stained for two antigens. × 575.

(to face p. 322)

5. C. ZURCHER, J. BRINKHOF, P. BENTVELZEN and J. C. H. DE MAN, C-type virus antigens detected by immunofluorescence in human bone tumour cultures. *Nature (Lond.)* **254,** 457 (1975).
6. J. CALAFAT and P. C. HAGEMAN, Binding of Concanavalin A to the envelope of two murine RNA tumour viruses. *J. gen. Virol.* **14,** 103 (1972).
7. D. L. FINE, J. K. PLOWMAN, S. P. KELLEY, L. O. ARTHUR and E. A. HILLMAN, Enhanced production of mouse mammary tumor virus in dexamethasone-treated, 5-iododeoxyuridine-stimulated mammary tumor cell cultures. *J. nat. Cancer Inst.* **52,** 1881 (1974).
8. J. RODRIQUEZ and F. DEINHARDT, Preparation of a semipermanent mounting medium for fluorescent antibody studies. *Virology* **12,** 316 (1960).

Europ. J. Cancer Vol. 12, pp. 325–326. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *April, 1976*

Human Cancer

T. Matsuo, K. Yamamoto and T. Osato: Differential induction of Epstein–Barr virus-related antigens in human lymphoblastoid cells by virus-mediated cell-to-cell contact.

K. J. Ranadive, S. V. Gothoskar, A. R. Rao, B. U. Tezabwalla and R. Y. Ambaye: Experimental studies on betelnut and tobacco carcinogenicity.

T. Hakulinen and L. Teppo: The increase in working years due to elimination of cancer as a cause of death.

A. B. Miller, J. Lindsay and G. B. Hill: Mortality from cancer of the uterus in Canada and its relationship to screening for cancer of the cervix.

C. L. M. Olweny, E. Katongole-Mbidde, A. Kaddu-Mukasa, I. Atine, R. Owor, S. Lwanga, W. Carswell and I. T. Magrath: Treatment of Burkitt's lymphoma: Randomized clinical trial of single agent versus combination chemotherapy.

H. G. Kwa, F. Cleton, M. de Jong-Bakker, R. D. Bulbrook, J. L. Hayward and D. Y. Wang: Plasma prolactin and its relationship to risk factors in human breast cancer.

Yu. S. Tatarinov, D. M. Falaleeva and V. V. Kalashnikov: Human pregnancy-specific $beta_1$-globulin and its relation to chorioepithelioma.

H. Ben-Bassat, N. Goldblum, S. Mitrani, G. Klein and B. Johansson: Concanavalin A receptors on the surface membrane of lymphocytes from patients with African Burkitt's lymphoma and lymphoma cell lines.

Experimental Cancer

G. Berry and J. C. Wagner: Effect of age at inoculation of asbestos on occurrence of mesotheliomas in rats.

N. T. Patel and V. Holoubek: RNA associated with nonhistone chromosomal proteins in rat liver and in ascites hepatoma.

A. C. Minson, M. E. Thouless, R. P. Eglin and G. Darby: The detection of virus DNA sequences in a herpes type 2 transformed hamster cell line (333–8–9).

C. D. Copple and J. K. McDougall: Clonal derivatives of a herpes type 2 transformed hamster cell line (333–8–9): Cytogenetic analysis, tumorigenicity and virus sequence detection.

N. D. Kon, J. T. Forbes and P. A. Klein: Ability of delayed-type hypersensitivity reactions to distinguish tumor associated antigens and histocompatibility antigens in soluble extracts from murine fibrosarcomas.

C. Mazurek, H. Chalvet, C. Stiffel and G. Biozzi: Study of the mechanism of *Corynebacterium parvum* anti-tumour activity. I. Protective effect on the growth of two syngeneic tumours.

J. K. Reddy, M. S. Rao and M. V. Jago: Rapid development of hyperplastic nodules and cirrhosis in the liver of rats treated concurrently with thioacetamide and the pyrrolizidine alkaloid lasiocarpine.

A. Watanabe, M. Miyazaki and K. Taketa: Prompt elevation of serum α-fetoprotein by acute liver injury following a single injection of ethionine to rats.

Y. Yoshikawa, K. Yamanouchi, A. Fukuda and M. Hayami: Characterization of an early cytotoxicity-inducing factor in sera of Japanese quails after inoculation with Rous sarcoma virus.

W. Leibold, G. Huldt, T. D. Flanagan, M. Andersson, M. Dalens, D. H. Wright, A. Voller and G. Klein: Tumorigenicity of Epstein–Barr virus (EBV)-transformed lymphoid line cells in autologous squirrel monkeys.

British Journal of Cancer *April, 1976*

G. T. Rogers, F. Searle and K. D. Bagshawe: Carcinoembryonic antigen: Isolation of a sub-fraction with high specific activity.

L. Levin, J. E. McHardy, T. A. Poulton, C. M. Curling, M. J. Kitau, A. M. Neville and C. N. Hudson: Tumour associated immune responses and isolated carcinoembryonic antigen and alpha feto-protein levels related to survival in ovarian cancer patients.

P. R. Johnson and P. Hersey: Anti leukaemia activity as a bystander effect of graft versus host reactions.

Shin Chiyoda, Hideake Mizoguchi, Shigetaka Asano, Fuminaro Takaku and Yasusada Miura: Influence of leukaemic cells on the colony formation of human bone marrow cells *in vitro* II. Suppressive effects of leukaemic cell extracts.

Syed H. Abid and Newton Khoobyarian: Antitumour activity of adenovirus 12 structural proteins against Moloney sarcoma tumours in mice.

F. R. Westwood and E. Longstaff: Stimulation of cellular ingestion by basic proteins *in vitro* .

F. R. Balkwill and R. T. D. Oliver: Diagnostic and prognostic significance of peripheral blood cultural characteristics in adult acute leukaemia.

W. R. Miller: *In vitro* effects of oestrogen on 5a reduction of testosterone in hormone dependent rat mammary carcinomas.

Gerd Reznik, Ulrich Mohr and Norbert Kmoch: Carcinogenic effects of different nitroso-compounds in Chinese hamsters. I. Dimethylnitrosamine and D-diethylnitrosamine.

Frank M. Hallinan and Norman MacLean: DNA polymerase activity in plasma from leukemic guinea pigs. I.

B. Terracini, M. C. Testa, J. R. Cabral and L. Rossi: The role of age at treatment and dose in carcinogenesis in C3Hf/Dp mice with a single administration of N-nitroso-N-methylurea.

B. R. Rabin: The effect of aflatoxins on the incorporation of RNA and protein precursors by isolated hepatocytes.

Irena Szumiel and A. H. W. Nias: The effect of combined treatment with a platinum complex and ionizing radiation on Chinese hamster ovary cells *in vitro*.

P. R. Twentyman: The effect of bleomycin and pentamycin in combination on the survival of EMT6 mouse tumour cells *in vitro* and *in vivo*.

G. G. Ribeiro : The results of diethylstilboestrol therapy for recurrent and metastatic carcinoma of the male breast.

Brief Communication

M. Baum and M. Breese: Antitumour effect of *Corynebacterium parvum*: Possible mode of action.

Europ. J. Cancer Vol. 12, pp. 327–340. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Oncogenic Viruses: Implications for Human Diseases (Part I)

J. M. PREVOST
Service de Médecine et Laboratoire d'Investigation Clinique, Institut Jules Bordet,
1000 *Bruxelles, Belgium.*

DESPITE rapid advance in research and knowledge on the biology of animal tumor viruses [1–4], human cancer viruses remain elusive. The status of the results is still confusing and frustrating, mainly because the observation of viruses in tumor cells by electron microscopy or by the demonstration of constituents or products of the activity of some viral genome in the cell does not allow one to draw a positive conclusion establishing the causal relationship between viruses and human tumors. Nevertheless, a *constant association* throughout these studies has convinced many oncologists and virologists that viruses are indeed linked to human cancers, as exemplified by the Epstein–Barr virus case [5] and the recent demonstration of RNA tumor viruses in breast carcinoma [6] and acute myelogenous leukemia [7].

This review will be divided into two parts: the experimental basis for the search of tumor viruses and the putative human cancer viruses. These parts correspond to two main basic questions: Is it possible to transpose the knowledge from animal models to human malignant illnesses and what are the biochemical and biological bases for these extrapolations? and What evidences do we have at the present time for the role of viruses in human cancer?

Four main approaches are currently in use to try to assess the contention that viruses may cause cancer in man: (i) *in vivo* and *in vitro* analogies with observations in other species on indigenous tumor linked viruses or artificial models (viruses oncogenic in species other than the natural host, e.a. human adenoviruses); (ii) epidemiological and serological studies for the definition of particular associations, mode of transmission, time and geographic case clustering and antecedent infection. It is apparent now that viruses inducing cancer are ubiquitous agents, either spreading horizontally with a low efficiency of oncogenic activity or transmitted vertically from parent to progeny [8]. They induce latent or chronic subclinical infections long before the development of the tumor, which might require some inducing or triggering agent as a cofactor; (iii) biochemical and molecular approaches with technics designed for the study of nucleic acids (DNA, RNA) and their products and through the knowledge of the biophysical and structural properties of viruses [9–12], and (iv) immunological methods for the detection of viral components such as virus induced antigens and immune reactions against virus associated processes [13–15]. Each of these methods has its own problems and limitations; one of the most important appears to be the source of virus but this problem might be partially circumvented by the similarities and homologies with animal models.

With this background, it must be remembered that cancer is a complex problem. Even if viruses are among the main etiological factors in these diseases, tumors arise through the subtle interactions of these agents at the level of the cell and its genetic make-up. Multiple endogenous (hormones, immunity . . .) and exogenous factors (chemicals, radiations . . .) interplay with each other in determining the outcome of the cancerous process.

RNA TUMOR VIRUSES IN ANIMALS

Among viruses with a RNA genome, the RNA tumor virus group, often called leuko-

viruses or oncornaviruses, are the only known representatives with oncogenic potential, as opposed to the wide distribution of potential tumorigenic activity in different genera of DNA viruses. They form a fairly homogeneous group and are widely distributed in vertebrates. They are associated with naturally occurring tumors in wild animals and laboratory inbred or randombred animals.

Morphological observations [16–19] lead to the division of the group into two subgroups, based on the fine architecture of the viral particles and the mode of budding at the cell membrane: the *C-type* particle associated with leukemia and sarcomas and the *B-type*, found essentially in mammary tumors. A third type of morphologic entity is represented by the *A-particles* either intracytoplasmic or intracisternal. They are often seen in cells producing other types of particles, but the relationships between the two is at present unresolved: A-type particles might be immature forms of the complete C- or B-viral types or precursors, or alternatively byproducts of the viral infection.

The general biophysical, biochemical and biological properties of the RNA tumor viruses are outlined in Table 1 [1, 2, 20–24]. The nucleoid contains a nucleoprotein complex and has a higher density (1·25–1·27 g/ml) than the complete particle. The genome of all oncornaviruses consists of a single stranded RNA with a high molecular weight, around 10×10^6 daltons; after denaturation [25, 26] by heat or treatment with dimethylsulfoxide (DMSO) or formamide in order to disrupt hydrogen bonds, the apparent molecular weight falls to 3×10^6, suggesting that the genome is in fact segmented into 2 to 4 subunits [25–27]. The question of haploidy (each subunit is unique) or polyploidy (assembly of identical units) is still unresolved. These facts explain the relative high frequency of recombination in case of infection by two different viruses [1]. The RNA subunits of purified, cloned transforming and nontransforming viruses have different mobilities under electrophoresis in cross-linked acrylamide gels suggesting different length or molecular weight; in the avian system [28, 29], the RNA subunits of transforming viruses migrate slowly, are longer and appear to contain additional information, in relationship to the RNA of nontransforming leukemia viruses or transformation defective sarcoma virus [30]. In the murine situation, the relative length of the subunits of transforming and nontransforming viruses are the opposite [31]; this is best explained by the fact that transforming viruses in mice are defective for replication, and are therefore unable to form complete, infectious particles without the cooperation of a helper virus or leukemia virus. Smaller nucleic acid molecules are present in the genome of oncornaviruses, but they appear to be picked up by the virus during the maturation process in the cell: 18 and 28S molecules, 8S and 4–5S species, the latter having amino acid acceptor functions (transfer RNA properties) [32] and probably primer activity for the reverse transcriptase [1, 33, 34], are also present in the core.

Polypeptides of C-type particles have been extensively studied and purified. The envelope contains 2 glycopeptides and the core 4–6 polypeptidic components, along with minor

Table 1. Properties of RNA tumor viruses

(*a*)	MORPHOLOGY: Spherical particles, 80–100 nm dia, enveloped with surface knobs or spikes; dense core surrounded by a shell; central core in *C-type* and eccentrical in *B-type* particles. Released by budding through cell membranes.
(*b*)	BIOPHYSICAL PROPERTIES: Buoyant density in sucrose: 1·16–1·18. Genome: High molecular weight, single stranded RNA, containing an unusual long stretch of poly(A). Sedimentation: 60–70 S and MW: 10×10^6 daltons and after denaturation, 35 S with a MW: $3–4 \times 10^6$ daltons and other low MW RNA.
(*c*)	BIOCHEMICAL PROPERTIES: 2 envelope glycoproteins and 4 internal proteins—lipids in the envelope (35 % dry weight). Enzymes: reverse transcriptase associated with the core of the virion (MW: 70,000 d.) —RNAse H and other activities (ligases, kinases, methylases).
(*d*)	CRITERIA FOR TAXONOMIC CLASSIFICATION: (Species of origin)* (i) Nucleic acid relatedness between viruses belonging to different species or families (Molecular hybridization). (ii) Nucleic acid relatedness between viral RNA and host species DNA (Molecular hybridization). (iii) Immunological determinants on reverse transcriptase (enzyme immune inhibition assay). (iv) Immunological characteristics of viral glycoproteins and polypeptides (type-, species-, and interspecies-specific determinants) (Radioimmunoassay and others).

*See references [20–24].

proteins notably with enzymatic activities (reverse transcriptase). Three types of immunological determinants have been defined on these molecules: *type-specific* antigens, carried only by a particular strain or isolate, *group-specific* or *species specific*, common to viruses from one animal species and *interspecies* determinants, common to mammalian or avian viruses. Each of the polypeptide or glycopeptide has its own immunologic complexity and specificities [13, 23, 24, 35].

The life cycle of a typical RNA tumor virus is schematically presented in Fig. 1. This

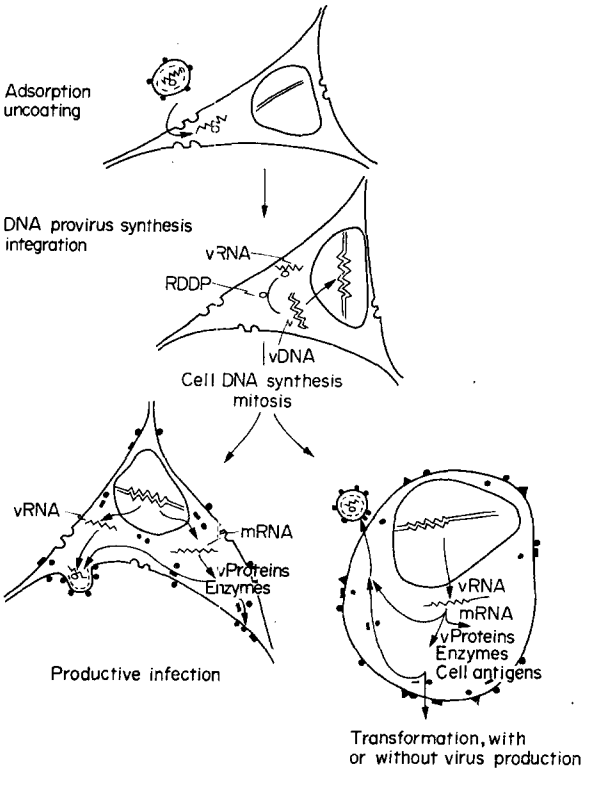


Fig. 1. Schematic diagram of the life cycle of RNA tumor viruses. Abbreviations used: vRNA, viral ribonucleic acid genomes; vDNA, viral deoxyribonucleic provirus; RDDP, RNA-dependent-DNA-polymerase or reverse transcriptase; mRNA, virus specific messenger RNA; vProteins, viral proteins.

scheme is derived from experiments with *non-defective*—as far as replication is concerned—strains of C-type viruses, the so-called *leukemia* viruses which are unable to transform fibroblasts *in vitro* but induce leukemia-lymphomas when inoculated into appropriate animals. It can be assumed to be of general relevance for all oncornaviruses; the replication of *defective sarcoma* viruses follow the same pattern, with active complementation of some function(s) by helper leukemia viruses or products from endogenous or integrated viral genes. Two peculiarities differentiate these viruses from other known RNA viruses: first, the *noncytocidal* interaction between viruses and cells and, secondly, the establishment of a *stable* relationship between viruses and cells, resulting eventually in malignant transformation. These two facts, associated with the stable properties of transformed cells suggest that viral genetic information is constantly present in the host cell, probably in the chromosomes. In addition, experimental evidences [1, 20] notably the inhibition of initiation of viral replication and transformation in the presence of inhibitors of DNA synthesis and actinomycin D, the dependence of viral production upon cell DNA synthesis and mitosis, led to the prediction for the requirement of a DNA intermediate in the replication of oncornaviruses. An enzyme allowing the transcription of RNA into DNA was postulated to be present in the virus and demonstrated independently by Temin and Baltimore and coworkers in 1970 [36]. Later, molecular hybridization studies [37, 38] demonstrated sequence homologies between nucleic acids from the virus and the infected or transformed cells. Furthermore, by transfection experiments with nucleic acids (DNA) extracted from infected or transformed cells, Hill and Hillova were able to recover complete, infectious virus indistinguishable from the virus used in the initial transforming or infecting event [39].

As proposed by Temin and others [1, 26, 36] DNA copies (DNA provirus) of the viral RNA are made, probably early after penetration and uncoating in the cytoplasm [40], migrate into the nucleus and are covalently integrated within cellular DNA. Thereafter, the viral information is under strict control and regulation by the cell as with other cellular genes in the chromosomes. But these controls for the transcriptional and translational events are poorly understood, in normal, infected or transformed cells. Proteins and glycoproteins are synthesized in a coordinated sequence in infected cells resulting in the assembly of complete, infectious viruses [13]; on the other hand, cells transformed by oncornaviruses express various antigens (virus specific TSSA or TSTA, viral glycoproteins and polypeptides, fetal antigens) in an uncoordinated pattern, eventually associated to some virus production [13, 41–44].

Virus specific DNA polymerase or the *reverse transcriptase* has been detected in every member of the leukovirus group and appears to be coded for by the viral genome [1, 3, 36]. The properties and specific requirements of the enzyme are summarized in Table 2, as

defined by Spiegelman, Gallo, Baltimore, Temin and other groups [36, 45–48]. This allows the specific identification of the activity and differentiation between viral and cellular DNA polymerases [49, 50]. In addition to the arguments listed above, three other orders of experimental facts have demonstrated the absolute dependence of virus functions on the enzyme for replication and transformation: inhibition of the enzyme by chemicals like the rifampicin derivatives [51] and thermosensitive mutants of the Rous Sarcoma virus, one of

Table 2. Criteria for identification of reverse transcriptase activity (RNA-dependent-DNA-polymerase)

1.	Enzyme present in RNA tumor viruses. Associated with the Visna virus and Foamy agent.
2.	DNA polymerase associated with the core of the particle or with a cellular particulate fraction.
3.	Requirements for maximum activity: low concentration of non-ionic detergents (NonIdet P40 or Triton X-100), all four deoxyribonucleotide triphosphates, divalent cations (Mn^{2+} or Mg^{2+}) and a reducing agent.
4.	Enzyme able to catalyse an *endogenous reaction*, without added template-primer, through the primer and template activity of the RNA of the virus particles (Heteropolymeric RNA).
5.	Stimulation of the reaction by an exogenous primer-template such as synthetic homopolymers (poly-r(A)—oligo-d(T) or poly-r(C)—oligo-d(G)) or other natural RNA or RNA–DNA hybrids.
6.	Response to oligo-d(T)—poly-r(A)>>>> oligo-d(T)—poly-d(A).
7.	RNAse sensitivity of the reaction.
8.	The product of the reaction exists at least as hybrid RNA–DNA on the particulate RNA.
9.	The DNA product, copied in the presence or absence of actinomycin D must be able to hybridise specifically to the template RNA used.
10.	Immunological criteria: specific determinants on the molecules allowing the differentiation from other cellular enzymes. Carry group—and interspecies-specific determinants.

See references in Text [36, 45–50].

which exhibits a thermosensitive defect in its polymerase with correlated effects on replication and transformation [52] and noninfectious mutants of avian sarcoma virus, lacking reverse transcriptase activity [53].

Two mechanisms are proposed to account for the transforming or tumorigenic properties of oncornaviruses; they are not mutually exclusive and experimental arguments support the two possibilities. The first theory holds that viral oncogenesis is accomplished by the introduction of new sets of genetic information in the cell (*Provirus*) [12, 54]. The second one postulates that the viral genes are residing in the chromosome of every normal cell (*Virogene–Oncogene* hypothesis of Huebner-Todaro) [55]. These genes are normally repressed but when activated, their oncogenic potential is sometimes expressed (Oncogene) or virus is produced (Virogene). It must be stressed, in connection to the general distribution of viral genetic information in normal cells, that the level at which the phenotypic expression is controlled, is actually unknown; studies with virus conditional mutants, revertant clones of transformed cells and metabolic inhibitors, suggest that the regulator mechanism is translational under cellular and/or viral control. The *protovirus* hypothesis of Temin [54, 56] supposes a continuous flow of information from DNA to RNA and back to DNA by reverse transcription. This mechanism is perhaps necessary for gene amplification, duplication and modification, by genetic mutation or recombination. If at a time in these cycles a specific set of genes related to cell control mechanisms or viral information becomes distinct from the chromosomal corresponding DNA, the result would appear sometimes as neoplastic transformation.

These mechanisms have their counterpart in the modes of transmission known for the oncornaviruses group [1, 57]: The *horizontal mode*, from one host to another by contact or proximity and the *vertical* mechanism, with its two modalities, which imply very different molecular mechanisms and biological controls, such as congenital infection (virus released by the mother and infects the offspring) and *genetic transmission* (sequences integrated into the chromosomal DNA, in the germ cells). The discovery of *endogenous* viruses [55, 58] substantiates further the importance of viral genetic information in normal cells. As a rule, this class of viruses can be differentiated from the laboratory isolates or "infectious" forms; they do not grow in tissues from the host species of origin, hence the denomination *xenotropic* [59] viruses—as opposed to the *ecotropic* and *infectious* forms—have clearly distinctive antigenic properties and nucleic acid sequences and are unable to transform cells *in vitro*, so classified as leukemia viruses. They were recognized and activated by chemical induction (inhibitors of DNA and protein synthesis, chemical carcinogens, etc.), irradiation or by cocultivation of tissues with xenogeneic cells [60–63], but sometimes they appear spontaneously after long term tissue culture.

In contrast, some endogenous viruses are able to replicate in tissues of the host species from which they were activated (ecotropic viruses). Their distribution is a general fact in a particular species as observed by activation [58], molecular hybridization [63] or serological studies (mouse) [64–66], probably, every species has its own (or sometimes, more than one, as in the mouse situation) [67, 68] endogenous virus. Nevertheless, the role of these viruses in neoplastic processes remains to be demonstrated [58, 69, 70] and recent observations suggest that they are able, probably, to infect species other than the natural host and produce tumors in these new situations [71]. These studies also indicate that this viral genetic information has its own role in the process of normal cell differentiation and evolution [72, 73]. As corollary to the presence of viral genes in normal cells, viral antigens are expressed often in an uncoordinate manner without virus production.

Interactions between exogenous (infectious) and endogenous viruses [1, 58] may occur in different conditions, notably through genetic recombination or phenotypic mixing. These hypotheses and mechanisms led to attempts to rescue defective viral genomes in tumor cells, notably sarcoma viruses by complementation or xenotropic viruses by host range modification. These assays were often unsuccessful; in other cases an endogenous virus was activated such as the AT-124 and RD-114 viruses [74, 75]. Other RNA viruses such as Vesicular stomatitis virus (VSV) are able to interact with RNA tumor viruses [76]. Furthermore, these kinds of interactions have been demonstrated between DNA and RNA containing viruses in the *Herpes simplex–VSV* system, for example, which led to an interesting hypothesis for oncogenic mechanisms through genetic or non-genetic interactions between two different viruses [77].

Endogenous viruses are linked in laboratory animals to noncancerous pathologic conditions, which have their counterpart in the human situation. In some strains of mice (NZB, NZW), their expression is associated with a strong immune response leading to autoimmune disease, similar to S.L.E. in man [78], and to immune complex deposits in the kidney, notably, with membranous glomerulonephritis [79]. Other mouse strains or the wild mouse exhibit subclinical or minimal renal immune damages, with accumulation of viral antigens, antibodies and complement components in the glomerulus [80]. These findings lead to the recognition that, rather than immune tolerance to endogenous leukemia virus associated antigens, a chronic autogenous immune response to a variety of viruses and virus associated antigens exists in some, if not all, strains or species [64, 81]. It also appears that an inverse relationship exists between the "natural" immune states and the incidence of leukemia [82, 83]. In addition, typical C-type murine viruses isolated from feral mice have sometimes neurotropic properties [84] and are associated with neuromuscular degenerative diseases, characterized by degeneration of lower motor neurons [85].

B-type particles are classically linked to epithelial tumors of the mammary gland in two species, mice [86] and rhesus monkeys (Mason–Pfizer monkey virus) [87]. Their structure and general biology is similar to the C-type viruses but differ in some points such as morphology (eccentric nucleoid, surface spikes), mode of budding, polypeptidic architecture and immunological properties. Various strains have been described for the mouse/mammary tumor virus, classified according to natural or reference host strain, virulence or oncogenicity, mode of transmission, hormone dependence and type of the tumor induced. Two routes of transmission from parent to offspring are known [86]: an exogenous route via the milk (the classical Bittner milk factor or MMTV-S strain) and genetic transmission (endogenous virus) through the gametes, as an integrated provirus. Sometimes infectious virus is associated with blood cellular elements, but cells infected and replicating MMTV can be found throughout the whole body, particularly in liver and kidney parenchymal cells, spleen and genital ducts of the male.

The interaction of the virus with the cell and its host is under multiple regulation mechanisms, most of which are not very well understood because of the lack of an *in vitro* assay for the virus: genetic, immunologic and hormonal mechanisms [86]. Nucleic acid hybridization studies have, nevertheless, demonstrated that mice of strains with high or low tumor incidence of mammary tumors have the same amounts of MMTV DNA in their genome, but that controls are probably present at the level of transcription or even at later stages [1, 88, 89]. Using various immunologic methods, two main groups of antigen specificities have been recognized in the MMTV systems: the major one is related directly to the presence of the virus and seems to be related to the glycoproteins of the virus [86, 90, 91] and the second system is tumor associated and independent from virus structural poly-

peptides. Private antigens, specific for one tumor, are also present in some tumors [92].

DNA TUMOR VIRUSES IN EXPERIMENTAL MODELS

DNA containing oncogenic viruses undergo two types of interactions with cells: (i) *productive infections* in permissive cells or *replicative cycle of the virus*, culminating in the release of thousands of virus particles and cell death, and (ii) *cell transformation* in which the virus does not multiply and is allowed to express only some functions resulting in morphological and biological alterations partly under viral genetic control. Among the Papovavirus group, polyoma virus, SV-40 and 3 groups of adenoviruses (the human groups particularly) have been extensively studied during the past ten years. Comparisons of the mechanisms of the replicative cycle and transformation have led to a good understanding of the molecular events governing these two types of cell–virus interactions [1, 92]. They are schematically depicted in Fig. 2. It emphasizes the distinction between early versus late functions of the viral genome and transforming and virogenic functions. It is generally admitted that the cycle of DNA viruses might be divided into two parts, with respect to the replication of the viral DNA. The *early* functions do not require viral DNA synthesis for their expression as they are expressed before viral DNA synthesis begins and result from transcription of parts of the incoming viral DNA strands. The *late* functions require new viral DNA synthesis and result in the more or less complete transcription of the virus sequences, part of which are expressed earlier in the cycle. Papovaviruses and adenoviruses induce *de novo* synthesis of cellular DNA during both productive and incomplete infections (abortive cycles or transformation) [93–95]; this phenomenon is dependent on the expression of part of the viral genome (an early function) [96], but its mechanism and significance for virus replication and transformation is not understood. It might be speculated to be a primary requirement for viral integration. Cells transformed by these viruses express only some of the early functions as detected by molecular hybridization and immunological analysis of virus specific antigens or antibodies or tumor specific determinants induced by the virus [1, 92] (see Table 3). They do not produce virus spontaneously [1, 2, 4] but complete particles are rescued by cocultivation or fusion with permissive cells and by chemical induction

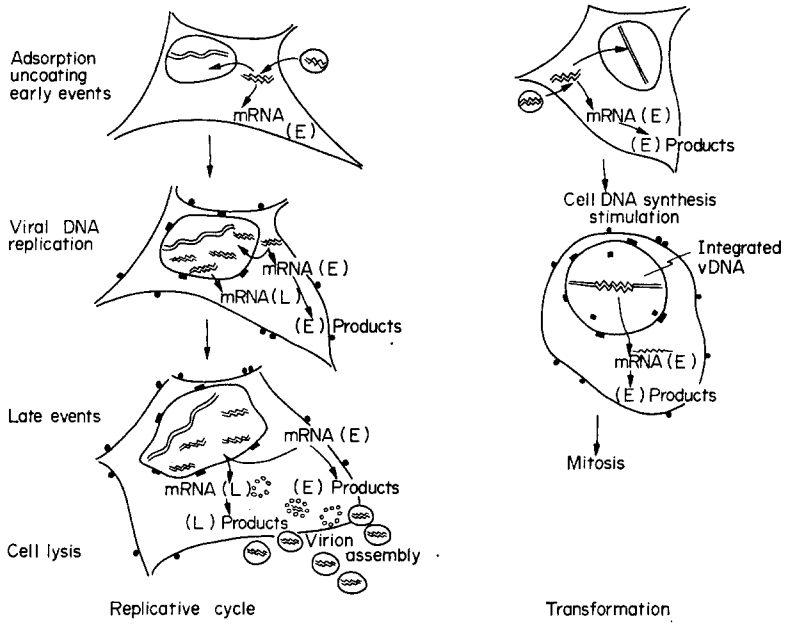


Fig. 2. Schematic outline of the interactions of DNA tumor viruses (especially Papovaviruses and adenoviruses) with the cell. Abbreviations used: vDNA, viral deoxyribonucleic acid genome; mRNA (E), early messenger viral RNA; mRNA (L) late messenger RNA, virus specific; (E) and (L) Products, products translated from (E) and (L) mRNA.

Table 3. Summary of Biological Properties of DNA Tumor Viruses and Associated Tumors

	PAPOVAVIRUSES		ADENOVIRUSES	HERPES VIRUSES
	Polyoma	SV 40		
Virus—Structure	Nonenveloped, 45 nm dia 2–3 structural proteins (+host cell histones)		Nonenveloped, 80 nm dia —8 polypeptides	Enveloped, 180 nm dia 24 polypeptides (at least)
—DNA	3×10^6 MW, circular, supercoiled		20–25 $\times 10^6$ MW, linear	100×10^6 MW, linear with nicks, redundancy and inversions
in vitro Transformation	Infectious & transforming +(Non-permissive hosts)		Infectious & transforming +(Non-permissive host)	Infectious +(*H. simplex*; EBV; human CMV)
Virus rescue	—(rarely +)	+	Always—	—
Virus DNA	Integrated, covalently complete sequences		Integrated, covalently only part of viral sequences	Present (state?)
Virus functions	Early (mRNA) (T-antigen, TSTA, S)		Early (mRNA) (T-antigen, TSTA)	Virus specific mRNA Virus antigens structural or non-structural
Tumors in Animals	Sarcomas and solid tumors in non-natural hosts Papillomas		Sarcomas, in non-natural hosts	Lymphomas—leukemias, Adenocarcinomas or sarcomas, sometimes in natural hosts
Virus	No rescue	Virus rescue +	No virus rescue	Virus rescue +/—
Antibodies	Specific for T-antigen		Specific for T-antigen	Virus specific antibodies
Implications in Human Cancer	No	Yes? (warts, others)	No	Yes { EBV—Burkitt's lymphomas —Nasopharyngeal carcinomas *H. simplex* and cervix Carcinomas (?)

from SV-40 transformed cells, which occurs only occasionally in polyoma and never for adenovirus tumor cells. Virus DNA is covalently integrated in the cellular genome of stably transformed cells [97, 98], often in multiple copies but sometimes only as a small fragment of the viral molecule [99–102], again suggesting that the activity of only a specific fraction of the viral nucleic acid is required to initiate and maintain the transformed state. Furthermore, recent studies on the *in vitro* transforming activity of purified DNA molecules confirm these findings and allowed for the isolation of a specific fragment bearing the transforming function(s), through the use of DNA fragmentation at random [103] or with sequence specific endonucleases, the restriction enzymes, combined with electrophoretic analysis of the fragments [101–103]. From these studies on adenovirus type 2 and 5, it was determined that the minimum size of the transforming or T fragment is around $1{\cdot}5 \times 10^6$ daltons, about 6–7% of the total DNA size and is located, and has been mapped physically, at the left or G-C rich end of the DNA molecule [103]. These findings open a new era in the understanding of viral carcinogenesis, which is a prerequisite to the complete evaluation of the role of viruses in cancer and to the dissection of underlying mechanisms.

Other approaches have furthermore demonstrated the role of some viral genes in the oncogenic activity of DNA viruses for initiation as well as maintenance of the transformed state. These include analysis of host range mutants, temperature sensitive mutants, hybrid viruses and isolation of revertant cells from the neoplastic to the normal state. The conditions under which transformed characteristics are or are not expressed can be manipulated experimentally, allowing for the identification of specific events which eventually correlated with malignancy and its regulation by viral or cellular genes [104,105]. In the case of polyoma virus, for example, two genes appear to control transformation. One of these functions is essential for the induction of transformation but not for its maintenance [106]. It is suggested that this mutation, called the ts-a mutation for polyomavirus, affects some essential step (a virus coded protein?) in the replication of viral DNA, probably at the level of integration into cellular DNA. The other mutation affects the interaction between virus functions and cellular growth mechanisms. For example, the ts-3 mutant [4, 107] is unable to induce cellular DNA synthesis and also fails to maintain characteristic transformed cell surface changes (lectin agglutination) in the restrictive conditions. These facts were correlated to changes

in the concentration of cAMP in the cells, suggesting a close interaction between viral genes, cell growth control mechanisms and cell surface modifications. However, at present the molecular aspects of these mutations and their products (proteins) are not understood. These genetic experiments demonstrate further that viral transformation is a multistep mechanism with regulatory controls at various levels, from the viral as well as the cell component of the system. They also form the principle tenet we have so far that products coded for by the viral genome are needed continuously for the maintenance of transformed phenotypes.

In contrast to the artifactual situation presented by Papovaviruses and Adenoviruses, Herpesviruses [108] are the only known DNA viruses group with oncogenic properties in the natural species they infect: the Lucké frog carcinoma of the kidney, Marek's disease lymphoma in chickens, and some other lymphotropic herpesviruses (viruses of the rabbit, and perhaps the guinea pig). *Herpesvirus saimiri* and Herpes ateles virus infect naturally one species (squirrel monkeys, for *Herpesvirus saimiri*) and have oncogenic properties in others (marmosets, owl monkeys for *H. saimiri*) [109]. All these viruses are able to induce a wide spectrum of host–virus interactions from chronic or latent infections to malignant disease; various mechanisms and factors might be involved in the determinism of the clinical expression of the infection [110]. Marek's disease [112] is a highly contagious lymphomatosis in chickens caused by a typical member of the Herpes group and transmitted from an unusual source for infectious viruses, the feather follicles and the superficial layers of the squamous epithelium. The virus is able to induce either self-limiting diseases, progressive lymphoma with a typical neural involvement or no disease at all. Many factors are involved in the type of disease developed: genetic resistance, various immune factors, etc., but the underlying mechanisms are not known. Two recent observations are important in relation to this model. First, the target cell for transformation is a thymus-derived lymphoid cell [112] and complete virus replication occurs at the level of epithelial cells, showing that two different types of cells with different embryologic origin behave differently as far as virus–cell interaction is concerned. Secondly, vaccination against the disease was achieved with a remarkable success through the use of attenuated Marek's disease Herpes viruses or an antigenically related virus isolated from turkeys (Herpesvirus turkey), the mechanism of which is barely understood [108]. Furthermore, *in vitro* [113] as well as *in vivo* experiments [114] suggest that interactions between avian leukoviruses (or endogenous viruses) and MDHV may be determinant in the outcome of infections by the two agents; the relevance of these observations to the wild situation is actually difficult to evaluate but even if it is so and if the "cofactor" model for herpesviruses is accepted, prevention is nevertheless very effective by the removal of this cofactor by immune mechanisms.

Herpes Saimiri is a lymphotropic agent, also of the T-lymphocyte, producing lymphoproliferative diseases in various monkeys (marmosets, capuchin monkey) other than the natural host (the squirrel monkey), which harbor the virus in thymus derived lymphocytes without apparent illness [109, 115, 116]. Various studies have demonstrated that different hosts have different immune responses to the viral infection. It is suggested that immune mechanisms are important determinants for the expression of the malignant properties of the virus in these models [117]. In this connection, demonstration of lymphoma induction in squirrel monkeys infected as newborns is particularly relevant as is the success of vaccinations by using killed virus preparations, against a viral challenge but not against the transplantation of tumor cells [118]. No evidences have been presented so far for leukovirus–herpesvirus interaction in the Saimiri system [116,119].

These two models are very reminiscent of the EBVirus situation in man and are particularly important for providing evidences on the involvement of herpesviruses in oncogenesis at the molecular, cellular and host biological level as well as models for treatment and prevention.

REFERENCES

1. J. Tooze, The molecular biology of tumour viruses. *Cold Spring Harbor Lab. Monogr. Series*, Cold Spring Harbor, New York (1973).
2. F. Fenner, B. R. MacAuslan, C. A. Mims, J. Sambrook and D. O. White, *The Biology of Animal Viruses*. 2nd edition, Academic Press, New York (1973).
3. In *The Tumor Viruses*. Cold Spring Harbor Symp. Quant. Biol. Vol. 39, Cold Spring Harbor, New York (1974).

4. R. DULBECCO, Cell transformation by viruses and the role of viruses in cancer. *J. gen. Microbiol.* **79,** 1 (1973).
5. G. KLEIN, The Epstein–Barr virus. In *The Herpes Viruses.* (Edited by A. S. KAPLAN), p. 521. Academic Press, New York (1973).
6. C. MACGRATH, P. M. GRANT, H. D. SOERLE, T. GLANCY and M. A. RICH, Replication of oncornavirus like particles in human breast carcinoma cell line MCF-7. *Nature (Lond.)* **252,** 247 (1974).
7. R. E. GALLAGHER and R. C. GALLO, Type C RNA tumor viruses isolated from cultured human acute myelogenous leukemia cells. *Science* **187,** 350 (1975).
8. L. GROSS, Facts and theories on viruses inducing cancer and leukemia. *Proc. nat. Acad. Sci. Wash.* **71,** 2013 (1974).
9. M. GREEN, The molecular basis for the attack on cancer. *Proc. nat. Acad. Sci. Wash.* **69,** 1035 (1972).
10. E. WINOCOUR, The investigation of oncogenic viral genomes in transformed cells by nucleic acid hybridization. *Advanc. Cancer Res.* **14,** 37 (1971).
11. J. M. BISHOP, N. QUINTRELL, E. MEDEIROS and H. E. VARMUS, Of birds and mice and men: comments on the use of animal models and molecular hybridization in the search for human tumour viruses. *Cancer (Philad.)* **34,** 1421 (1974).
12. S. SPIEGELMAN, R. AXEL, W. BAXT, D. KUFE and J. SCHLOM, Human cancer and animal viral oncology. *Cancer (Philad.)* **37,** 1406 (1974).
13. H. BAUER, Virion and tumor cell antigens of C type RNA tumor viruses. *Adv. Cancer Res.* **20,** 275 (1974).
14. E. W. LAMON, The immune response to virally determined tumor associated antigens. *Biochim. biophys. Acta (Reviews on Cancer)* **355,** 149 (1974).
15. L. J. OLD, E. A. BOYSE, G. GEERING and H. F. OETTGEN, Serologic approaches to the study of cancer in animals and man. *Cancer Res.* **28,** 1288 (1968).
16. W. BERNHARD, Electron microscopy of tumor cells and tumor viruses, a review. *Cancer Res.* **18,** 491 (1958).
17. E. DEHARVEN, Remarks on the ultrastructure of type A, B, and C virus particles. *Advanc. Virus Res.* **19,** 221 (1974).
18. N. H. SARKAR, D. H. MOORE and R. C. NOWINSKI, Symmetry of the nucleocapsid of the oncornaviruses. In *RNA Viruses and Host Genome in Oncogenesis.* (Edited by P. EMMELOT and P. BENTVELZEN) p. 71. North-Holland, Amsterdam (1972).
19. W. BERNHARD. In *Ultrastructure of Animal Viruses and Bacteriophages.* (Edited by A. J. DALTON and F. HAGUENAU) p. 283. Academic Press, New York (1973).
20. R. M. RUPRECHT, N. C. GOODMAN and S. SPIEGELMAN, Determination of natural host taxonomy of RNA tumor viruses by molecular hybridization: application to RD-114, a candidate human virus. *Proc. nat. Acad. Sci. Wash.* **70,** 1437 (1973).
21. D. GILLESPIE and R. C. GALLO. RNA processing and RNA tumor virus origin and evolution. *Science* **188,** 802 (1975).
22. S. R. TRONICK, J. R. STEPHENSON, S. A. AARONSON and T. G. KAWAKAMI, Antigenic characterization of type C RNA isolates of Gibbon apes. *J. Virol.* **15,** 115 (1975).
23. R. C. NOWINSKI, E. FLEISSNER and N. H. SARKAR, Structural and serological aspects of the oncornaviruses. In *Perspectives in Virology.* (Edited by M. POLLARD) Vol. VIII, p. 31 (1973).
24. M. STRAND and J. T. AUGUST, Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants of the major internal proteins and envelope glycoproteins. *J. Virol.* **13,** 171 (1974).
25. P. K. VOGT, The genome of avian tumor viruses, A discussion of four models. In *Possible Episome in Eukaryotes.* (Edited by L. G. SILVESTRI) p. 35. North-Holland, Amsterdam (1973).
26. H. TEMIN, Mechanisms of cell transformation by RNA tumor viruses. *Ann. Rev. Microbiol.* **25,** 609 (1971).
27. J. P. BADER, T. L. STECK and K. KAKEFUDA, The structure of RNA containing viruses. *Curr. Top. Microbiol. Immunol.* **51,** 106 (1970).
28. P. H. DUESBERG and P. K. VOGT, Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular RNA. *J. Virol.* **72,** 594 (1973).

29. P. H. Duesberg and P. K. Vogt, RNA species obtained from clonal lines of avian sarcoma and from avian leukosis virus. *Virology* **54,** 207 (1973).
30. G. S. Martin and P. H. Duesberg, The a-subunit in the RNA of transforming avian tumor viruses. I.—Occurrence in different virus strains. II.—Spontaneous loss resulting in non-transforming variants. *Virology* **47,** 494 (1972).
31. J. Maisel, V. Klement, M. M-C. Lai, W. Ostertag and P. Duesberg, Ribonucleic acid components of murine sarcoma and leukemia viruses. *Proc. nat. Acad. Sci. Wash.* **70,** 3536 (1973).
32. M. Travnicek, RNA with amino acid acceptor activities isolated from an oncogenic virus. *Biochim. biophys. Acta* **166,** 757 (1968).
33. E. Canaani and P. Duesberg, Role of subunits of 60-70S avian tumor virus RNA in its template activity for the viral deoxyribonucleic acid polymerase. *J. Virol.* **10,** 23 (1972).
34. A. J. Faras and N. A. Dibble, RNA directed DNA synthesis by the DNA polymerase of Rous Sarcoma Virus: structural and functional identification of 4S primer RNA in uninfected cells. *Proc. nat. Acad. Sci. Wash.* **72,** 859 (1975).
35. D. P. Bolognesi, G. Huper, R. W. Grenn and T. Graf, Biochemical properties of oncornavirus polypeptides. *Biochim. biophys. Acta (Reviews on Cancer)* **355,** 220 (1974).
36. H. M. Temin and D. Baltimore, RNA directed DNA synthesis and RNA tumor viruses. *Advanc. Virus Res.* **17,** 129 (1972).
37. J. M. Bishop, N. Jackson, W. E. Levinson, E. Medeiros, N. Quintrell and H. E. Varmus, The presence and expression of RNA tumor virus genes in normal and infected cells: detection by molecular hybridisation. *Amer. J. clin. Pathol.* **60,** 31 (1973).
38. M. Hill and J. Hillova, RNA and DNA forms of the genetic material of C-type viruses and the integrated state of the DNA in the cellular chromosome. *Biochim. biophys. Acta (Reviews on Cancer)* **355,** 7 (1974).
39. M. Hill and J. Hillova, Recovery of temperature sensitive mutants of Rous sarcoma virus from chicken cells exposed to DNA extracted from hamster cells transformed by the mutant. *Virology* **49,** 309 (1970).
40. G. Lovinger, H. P. Ling, R. A. Klein, R. V. Gilden and M. Hatanaka, Unintegrated murine leukemia viral DNA in newly infected cells. *Virology* **62,** 280 (1974).
41. R. V. Gilden and S. Oroszlan, Group specific antigens of RNA tumor viruses as markers for subinfectious expression of the RNA tumor virus genome. *Proc. nat. Acad. Sci. Wash.* **69,** 1021 (1972).
42. J. A. Bilello, M. Strand and J. T. August, Murine sarcoma genome expression: transformants which express viral envelope glycoproteins in the absence of the major internal protein and infectious particles. *Proc. nat. Acad. Sci. Wash.* **71,** 3234 (1974).
43. B. C. DelVillano, B. Nave, B. P. Crocker, R. A. Lerner and F. J. Dixon, The oncornavirus glycoprotein gp69/71: a constituent of the surface of normal and malignant thymocytes. *J. exp. Med.* **141,** 172 (1975).
44. M. Strand, F. Lilly, J. T. August, Host control of endogenous murine leukemia virus gene expression: Concentration of viral proteins in high and low leukemia mouse strains. *Proc. nat. Acad. Sci. Wash.* **71,** 3682 (1974).
45. S. Spiegelman, A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson, Characterization of the products of RNA directed DNA polymerases in oncogenic viruses. *Nature (Lond.)* **227,** 563 (1970).
46. P. S. Sarin and R. C. Gallo, RNA directed DNA polymerase. In *International Reviews of Science.* (Edited by K. Burton) Vol. VI, Nucleic acids. Butterworth, Oxford (1973).
47. I. M. Verma and D. Baltimore, Purification of the RNA dependent polymerase of AMV and its assay with polynucleotide templates. In *Methods in Enzymology.* (Edited by L. Grossman and K. Moldave) Vol. XXIX, p. 125. Academic Press, New York (1973).
48. A. S. Dion, A. B. Vaidya and G. S. Fout, Cation preferences for poly(rC)-oligo(dG) directed DNA synthesis by RNA tumor viruses and human milk particulates. *Cancer Res.* **34,** 3509 (1974).
49. N. C. Goodman and S. Spiegelman, Distinguishing reverse transcriptase of a RNA tumor virus from other known DNA polymerases. *Proc. nat. Acad. Sci. Wash.* **68,** 2203 (1971).

50. E. M. Scolnick, W. P. Parks and G. J. Todaro, Reverse transcriptase of primate viruses as immunological markers. *Science* **177,** 1119 (1972).
51. R. C. Gallo, Y. S. Yang, J. Whang-Peng, R. C. Ting, S. S. Yang, and J. W. Abrell, RNA tumor viruses, DNA polymerases and oncogenesis: Some selective effect of rifampicin derivatives. *Medicine* **51,** 159 (1972).
52. I. M. Verma, W. S. Mason, S. D. Drost and D. Baltimore, DNA polymerase activity of two ts-mutants of Rous sarcoma virus is termolabile. *Nature (Lond.)* **151,** 27 (1974).
53. H. Hanafusa, D. Baltimore, D. Smoler, K. F. Watson, A. Yaniv, and S. Spiegelman, Absence of polymerase protein in virions of alfa-type Rous sarcoma virus. *Science* **177,** 1188 (1972).
54. H. M. Temin, Introduction to virus caused cancers. *Cancer (Philad.)* **37,** 1347 (1974).
55. G. J. Todaro, R. J. Huebner, The viral oncogene hypothesis. New evidences. *Proc. nat. Acad. Sci. Wash.* **69,** 1009 (1972).
56. H. M. Temin, The protovirus hypothesis: Speculations on the significance of RNA directed DNA synthesis for normal development and for carcinogenesis. *J. nat. Cancer Inst.* **46,** 3 (1971).
57. M. B. Gardner, Avian and murine RNA tumor viruses: Mode of transmission. In *Biohazards in Biological Research.* (Edited by A. Hellman, M. N. Oxman and R. Pollack), p. 143. Cold Spring Harbor Laboratory, New York (1973).
58. G. J. Todaro, Detection and characterization of RNA tumor viruses in normal and transformed-cells. In *Perspectives in Virology.* (Edited by M. Pollard) Vol. VIII, p. 81. Academic Press, New York (1973).
59. J. A. Levy, Xenotropic viruses: Murine leukemia viruses associated with NIH Swiss, NZB and other mouse strains. *Science* **182,** 1151 (1973).
60. D. R. Lowy, W. P. Rowe, N. Teich and J. W. Hartley, Murine leukemia virus: High frequency activation *in vitro* by 5-iododeoxyuridine and 5-bromodeoxyuridine. *Science* **174,** 155 (1971).
61. J. S. Greenberger and S. A. Aaronson, Cycloheximide induction of xenotropic type C viruses from synchronized mouse cells: Metabolic requirement for virus activation. *J. Virol.* **15,** 64 (1975).
62. R. A. Weiss, R. P. Friis, E. Katz and P. K. Vogt, Induction of avian tumor viruses in normal cells by physical and chemical carcinogens. *Virology* **46,** 920 (1971).
63. G. J. Todaro, Endogenous type C viruses in cell cultures. In *Biohazards in Biological Research.* (Edited by A. Hellman, M. N. Oxman and R. Pollack) p. 114. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1973).
64. J. C. Lee, M. G. Hanna, J. N. Ihle and S. A. Aaronson, Autogenous immunity to endogenous RNA tumor viruses: Differential reactivity of IgM and IgG to virus envelope antigens. *J. Virol.* **14,** 773 (1974).
65. S. A. Aaronson and J. R. Stephenson, Widespread natural occurrence of high titers of neutralizing antibodies to a specific class of endogenous mouse type C viruses. *Proc. nat. Acad. Sci. Wash.* **71,** 1957 (1974).
66. R. C. Nowinski and S. L. Kaehler, Antibody to leukemia virus: Widespread occurrence in inbred mice. *Science* **185,** 869 (1974).
67. J. R. Stephenson, S. A. Aaronson, P. Arnstein, R. J. Huebner and S. R. Tronick, Demonstration of two immunologically distinct xenotropic type C viruses of mouse cells. *Virology* **61,** 56 (1974).
68. R. E. Benveniste, M. M. Lieber and G. J. Todaro, A distinct class of inducible murine type C viruses that replicate in the rabbit SIRC cell line. *Proc. nat. Acad. Sci. Wash.* **71,** 602 (1974).
69. J. R. Stephenson, J. S. Greengerger and S. A. Aaronson, Oncogenicity of an endogenous C type virus chemically activated from mouse cell cultures. *J. Virol.* **13,** 237 (1974).
70. H. Meier, B. A. Taylor, M. Cherry and R. J. Huebner, Host gene control of type C-RNA tumor virus expression and tumorigenesis in inbred mice. *Proc. nat. Acad. Sci. Wash.* **70,** 1450 (1973).
71. R. E. Benveniste and G. J. Todaro, Evolution of C type viral genes: Inheritance of exogenously acquired viral genes. *Nature (Lond.)* **252,** 456 (1974).

72. R. E. Benveniste and G. J. Todaro, Evolution of type C viral genes. I. Nucleic acid hybridization with baboon type C virus as a measure of divergence among primate species. *Proc. nat. Acad. Sci. (Wash.)* **71,** 4513 (1974).
73. R. E. Benveniste, R. Heineman, G. L. Wilson, R. Callahan and G. J. Todaro, Detection of baboon type C viral sequences in various primate tissues by molecular hybridization. *J. Virol.* **14,** 56 (1974).
74. R. M. MacAllister, M. Nicolson, M. B. Gardner, R. W. Rongey, S. Rasheed, P. Sarma, R. Huebner, M. Hatanaka, S. Oroszlan, R. V. Gilden, A. Kabigting and L. Vernon, C Type virus released from cultured human rhabdomyosarcoma cells. *Nature New Biology* **235,** 3 (1972).
75. G. J. Todaro, P. Arnstein, W. P. Parks, E. Lennette and R. J. Huebner, A type C virus in human rhabdomyosarcoma cells after inoculation into antithymocytes serum treated NIH Swiss mice. *Proc. nat. Acad. Sci. Wash.* **70,** 859 (1973).
76. D. Boettiger, D. N. Love and R. A. Weiss, Virus envelope markers in mammalian tropism of avian RNA tumor viruses. *J. Virol.* **15,** 108 (1974).
77. A. S. Huang, E. L. Palma, N. Hewlett and B. Roizman, Pseudotype formation between enveloped RNA and DNA viruses. *Nature (Lond.)* **252,** 743 (1974).
78. R. C. Mellors, Autoimmune disease and neoplasia of NZB mice—implication of murine leukemia-like virus. In *Perspectives in Virology.* (Edited by M. Pollard) Vol. VI, p. 239 (1968).
79. T. Yoshiki, R. C. Mellors, M. Strand and J. T. August, The viral envelope glycoperotein of murine leukemia virus and the pathogenesis of immune complex glomerulonephritis of New Zealand mice. *J. exp. Med.* **140,** 1011 (1974).
80. M. B. Oldstone, Virus neutralization and virus induced immune complex disease: Virus antibody reaction resulting in immuno-protection or immunological injury—two different sides of the same coin. *Progr. med. Virology* **19,** 123 (1974).
81. R. J. Huebner and H. J. Igel, Immunological tolerance to gs antigen as evidence for vertical transmission of non infectious C-type RNA virus genomes. In *Perspectives in Virology.* (Edited by M. Pollard) Vol. VII, p. 35 (1971).
82. J. Yuhas and N. H. Clapp, Incidence of leukemias and non lymphatic tumors in mice with glomerulosclerosis and allied disorders. *J. nat. Cancer Inst.* **48,** 367 (1972).
83. M. G. Hanna, R. W. Tennant, J. M. Yuhas, N. H. Clapp, N. K. Batzing and M. J. Sneldgrass, Autogenous immunity to endogenous RNA tumor virus antigens in mice with a low natural incidence of lymphoma. *Cancer Res.* **32,** 2226 (1972).
84. J. E. Officer, Isolation of a neurotropic type C virus. *Science* **181,** 945 (1973).
85. J. M. Andrews and M. B. Gardner, Lower motor neuron degeneration associated with C type RNA tumor virus infections in mice, neuropathological features. *J. Neuropathol. exp. Neurol.* **33,** 285 (1974).
86. P. Bentvelzen, Host virus interaction in murine mammary carcinogenesis. *Biochim. biophys. Acta (Reviews on Cancer)* **355,** 236 (1974).
87. M. M. Mason, A. E. Bogden, V. Ilievski, H. J. Esther, J. R. Baker and H. C. Chopra, History of rhesus monkey adenocarcinoma containing virus particles resembling oncogenic RNA viruses. *J. nat. Cancer Inst.* **48,** 1323 (1972).
88. J. M. Bishop, N. Jackson, N. Quintrell and H. E. Varmus, Transcription of RNA tumor virus genes in normal and infected cells. In *Possible Episomes in Eukaryotes.* (Edited by L. G. Silvestri) p. 61. North-Holland, Amsterdam (1973).
89. W. P. Parks and E. M. Scolnick, Murine mammary tumor cell clones with varying degrees of virus expression. *Virology* **55,** 163 (1973).
90. J. H. Hilgers, G. J. Theunis and R. van Nie, Mammary tumor virus antigens in normal and mammary tumor bearing mice. *Int. J. Cancer* **12,** 568 (1973).
91. P. B. Blair, Natural and experimentally induced immunity in the murine mammary tumor infected mouse. In *Conference on Immunologic Control of virus associated tumors in man: Prospects and problems. (Cancer Res.,* In press.)
92. H. Fraenkel-Conrat and R. R. Wagner, *Comprehensive Virology,* Vol. 3, Reproduction, DNA animal viruses. Plenum Press, New York (1974).

93. R. Dulbecco, L. H. Hartwell and M. Vogt, Induction of cellular DNA synthesis by polyoma virus. *Proc. nat. Acad. Sci. Wash.* **53,** 403 (1965).
94. T. O. Fox and A. J. Levine, Relationships between virus induced cellular DNA synthesis and transformation by Simian virus 40. *J. Virol.* **7,** 473 (1971).
95. M. Takahashi, T. Ogino, K. Baba and M. Onakai, Synthesis of deoxyribonucleic acid in human and hamster kidney cells infected with human adenovirus type 5 and 12. *Virology* **37,** 513 (1969).
96. J. Y. Chou and R. G. Martin, DNA infectivity and the induction of DNA synthesis with thermosensitive mutants of SV40. *J. Virol.* **15,** 145 (1975).
97. J. Sambrook, H. Westphal, P. R. Srimivasan and R. Dulbecco, The integrated state of DNA in SV40 transformed cells. *Proc. nat. Acad. Sci. Wash.* **60,** 1288 (1968).
98. M. Botchan, B. Ozanne, B. Lugden, P. A. Sharp and J. Sambrook, Viral DNA in transformed cells. III. The amount of different regions of the SV40 genome present in a line of transformed mouse cells. *Proc. nat. Acad. Sci. Wash.* **71,** 4183 (1974).
99. L. D. Gelb, D. E. Kohne and M. A. Martin, Quantitation of SV40 sequences in African green monkey, mouse and virus transformed cell lines. *J. mol. Biol.* **57,** 129 (1971).
100. M. Green, Oncogenic viruses. *Ann. Rev. Biochem.* **39,** 701 (1970).
101. P. A. Sharp, V. Petterson and J. Sambrook, Viral DNA in transformed cells. I. A study of the sequences of adenovirus DNA in a line of transformed rat cells using specific fragments of the viral genome. *J. mol. Biol.* **86,** 709 (1974).
102. P. H. Gallimore, P. A. Sharp and J. Sambrook, Viral DNA in transformed cells. II. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. *J. mol. Biol.* **89,** 49 (1974).
103. F. L. Graham, A. J. Vandererb and H. L. Heynecker, Size and location of the transforming regions in human adenovirus type 5 DNA. *Nature (Lond.)* **251,** 687 (1974).
104. H. Renger and C. Basilico, Mutation causing temperature sensitive expression of cell transformation by a tumor virus. *Proc. nat. Acad. Sci. Wash.* **69,** 1009 (1972).
105. T. L. Benjamin, Physiological and genetic studies on polyoma virus. *Curr. Top. Microbiol. Immunol.* **59,** 107 (1972).
106. M. Fried, Cell transforming ability of a thermosensitive mutant of polyoma virus. *Proc. nat. Acad. Sci. Wash.* **53,** 486 (1965).
107. W. R. Eckhardt, R. Dulbecco and M. Burger, Temperature dependent surface changes in cells infected or transformed by a thermosensitive mutant of polyoma virus. *Proc. nat. Acad. Sci. Wash.* **68,** 283 (1971).
108. P. M. Biggs, G. de Thé and L. N. Payne, *Herpes viruses and oncogenesis.* Int. Agency for Research on Cancer, Publ., No. 2. Lyon, France (1972).
109. D. V. Ablashi and G. R. Pearson, *Herpesvirus saimiri*, a non-human primate model for herpesvirus associated neoplasia in man. *Cancer Res.* **34,** 1232 (1974).
110. F. Rapp and M. A. Jerkovsky, Persistent and latent infections. In *The Herpes Viruses.* (Edited by A. S. Kaplan), p. 271. Academic Press, New York (1973).
111. K. Nazerian, Marek's disease, a neoplastic disease caused by a Herpes virus. *Adv. Cancer Res.* **17,** 279 (1973).
112. P. C. Powell, L. N. Payne, J. A. Frazier and M. Rennie, T-lymphoblastoid cell lines from Marek's disease lymphomas. *Nature (Lond.)* **251,** 79 (1974).
113. J. W. Frankel and V. Groupe, Interactions between Marek's disease Herpes virus and avian leukosis virus in tissue culture. *Nature New Biol.* **234,** 125 (1971).
114. W. P. Peters, D. Kufe, J. Schlom, J. W. Frankel, C. O. Prickett, V. Groupe and S. Spiegelman, Biochemical and biological evidences for an interaction between Marek's disease Herpes virus and avian leukosis virus *in vivo. Proc. nat. Acad. Sci. Wash.* **70,** 3175 (1973).
115. F. Deinhardt, Herpesvirus Saimiri. In *The Herpes Viruses.* (Edited by A. S. Kaplan) p. 595, Academic Press, New York (1973).

116. G. KLEIN, G. PEARSON, A. RABSON, D. V. ABLASHI, L. FALKE, L. WOLFE, F. DEINHARDT and H. RABIN, Antibody reaction to *Herpesvirus saimiri* induced early and late (EA and LA) antigens in HVS infected squirrel, marmoset and owl monkeys. *Int. J. Cancer* **12,** 270 (1973).
117. H. RABIN, Studies on experimental lymphomas induced by *Herpesvirus saimiri* in non-human primates. *J. med. Primatology* **3,** 1 (1974).
118. R. LAUFS and H. STEINCKE, Vaccination of non-human primates against malignant lymphoma. *Nature (Lond.)* **253,** 71 (1975).
119. R. H. NEUBAUER, W. C. WALLEN and H. RABIN, Stimulation of *Herpesvirus saimiri* expression in the absence of evidence of type C virus activation in a marmoset lymphoid cell line. *J. Virol.* **14,** 745 (1974).

Europ. J. Cancer Vol. 12, pp. 341–351. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Treatment of Pain in Patients with Advanced Cancer

J. GYBELS*†, H. ADRIAENSEN‡ and P. COSYNS§

**Department of Neurology and Neurosurgery, ‡Department of Anesthesiology and §Department of Psychiatry, University of Leuven, B–3000 Leuven, Belgium*

1. INTRODUCTION

ALTHOUGH exact figures on the epidemiology of cancer pain are not available, some data are illustrative for the extent of the problem.

Beattie [1] calculates that in the United States one out of six patients is likely to develop a cancer during his lifetime. From observations published by J. Aitken-Swan [2] painful conditions arise in about 50% of all cancer patients. The incidence in terminal cancer hospitals is rated at 75% by R. Twycross [3]. In a survey of Cartwright and his colleagues [4] on the experiences of patients during their last year of life, the author states that pain was their main complaint that led them to the doctor. About 87% of them found some relief by their treatment. On 115 patients referred in 1974 to our pain clinic, subserving a multidisciplinary university hospital, 39% were cancer patients.

If it is difficult to provide valuable figures on the number of cancer patients with intractable pain, it is impossible to estimate the amount of their suffering. The treatment of the pain, even with complete relief, resolves only part of their problems, since their emotional stress is still present. Therefore, paintherapy for the incurable cancer patient is necessarily linked with the broader approach of terminal care [3].

The therapeutic modalities to face the pain problem in oncology, are rather modest. Once causal treatment is no longer able to cure the patient, the main goal of further therapy is the quality of life and the comfort of the patient. For palliative pain treatment, the analgesic drugs are still the most universal approach. In a number of specific situations neurosurgery, nerve blocks, psychotherapy or other forms of treatment have their indications, and may be of particular value [5]. The coordination and optimal utilisation of all different forms of pain treatment become possible through a progressive evolution of the former nerve block units towards the broader approach of the multidisciplinary pain clinic [6].

†Address reprint requests to: J. GYBELS, Department of Neurology and Neurosurgery, A.Z. Sint Rafael, Kapucijnenvoer 35, B–3000 LEUVEN, BELGIUM.

2. ANALGESIC DRUGS

The most universal approach in paintherapy is the use of analgesic drugs. They are easy to handle for the doctor, comfortable for the patient, and efficient in a large variety of painful conditions.

In search of the drug of choice, Admiraal and colleagues [7] described the properties of the ideal analgesic as follows:

—a strong analgesic potency.
—a long duration of action; at least long enough to assure a night without pain after a single dose administration.
—low toxicity, few side effects, no addiction potential.
—peroral activity; this eliminates the person who gives the injection, and prevents the kick after parenteral administration.

Although this perfect analgesic compound may not be available, the aims of a adequate treatment are quite well summarized. An attempt should be made to guide analgesic therapy in the direction of these ideal standards.

The analgesic drugs have been classified for many years in non-narcotic and narcotic drugs. In the meantime there have been synthetized

some morphinomimetic compounds with an intermediate analgesic potency, and low abuse liability. Drugs in this group are D-propoxyphene, pentazocine and tilidine. They should be classified between the minor antiphlogistic agents and the potent narcotics.

The rational use of analgesics should give just enough drug to achieve pain relief as completely as possible. Therefore a progression from weaker to stronger drugs is advised, taking maximal benefit of each compound, postponing as long as possible the exhaustion of the full number of available painkillers. For this purpose analgesic medication is best given in a preventive way on a 24-hr schedule [3].

A first trial with acetyl salicylic acid is worthwhile. This drug is a good analgesic, if given in high dose on regular intervals (1 g, 6 or 4 hourly). Potentiation of the therapy is possible with paracetamol and codeine.

A second step consists in the prescription of synthetic morphinomimetics with low addictive properties (D-propoxyphene-pentazocine-tilidine). As they are centrally active, they can be used in combination with acetyl salicylic acid, the effect of which is situated at the peripheral level [8]. For some of them analgesic activity shows a ceiling effect, so that further increase in dosage is without any benefit to the patient and will only enhance the undesired side effects.

When analgesia is insufficient with the former compounds one should not hesitate to replace them by narcotic drugs for the treatment of cancer pain. As Houde states "no patient should be made to suffer severe pain when the means are at hand to allay it" [9]. For the administration of orally active narcotic drugs methadone is widely used in the United States. More recently bezitramide has been introduced in Europe as a potent long acting pain killer [7]. In the United Kingdom good analgesic results have been obtained by Twycross, using morphine or diamorphine and cocaine by mouth as an elixir (Brompton coctail) [3]. This narcotic medication is often combined with an anti-emetic agent. The combination of narcotics with amphetamine-like drugs or tricyclic antidepressants should potentiate their analgesic activity [10].

In a last attempt to procure adequate pain relief in cancer patients the chronic parenteral use of analgesic drugs is mentioned. The disadvantages of chronic parenteral medication with analgesics are manifold and their oral administration, where possible, should be the treatment of choice. The patient who needs someone to give him his parenteral injection at regular intervals is made totally dependent. If he is not in the hospital, the risk of irregular administration is not imaginary. Periods of good relief and euphoria (the kick) are followed by periods of agony and pain, if the injection comes late. This could induce the development of drug dependency and tolerance; the dose could increase rapidly; the analgesic potency of the product might be exhausted in a short time.

3. NERVE BLOCKS

Nerve blocks are designed to interrupt the afferent sensory input to the central nervous system. This can be realised by the injection of local anaesthetic agents (diagnostic and prognostic blocks) or by neurolytic agents (therapeutic blocks). The injection can be done at different levels on the traject of the somatic or autonomic nerve fibres to the spinal cord (Fig. 1).

As the effect of a nerve block will be confined to the area supplied by the blocked sensible pathways, the indication for these procedures is one of localized pain.

Although nerve blocks have now been practized for about half a century, amazing progress

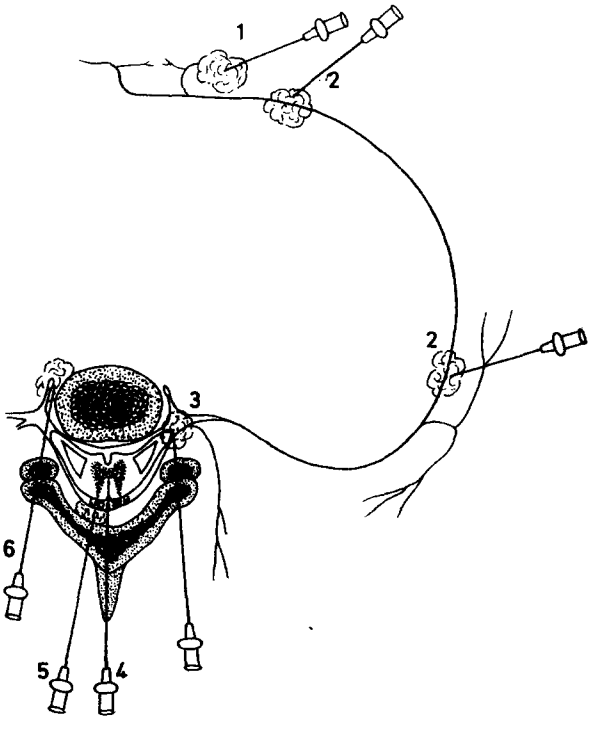


Fig. 1. Schematic representation of the sites of action of nerve blocks (with kind permission of H. A. Baar and H. U. Gerbershagen) [11]. (1) local infiltration; (2) nerve block; (3) paravertebral somatic root block; (4) spinal block; (5) peridural block; (6) paravertebral sympathetic ganglion block.

has not been made in this field. Improvements in the technique of injection with control of the position of the needle by radiologic image amplification or nerve stimulation; substitution of alcohol by other neurolytic agents such as phenol or chlorocresol; control of the extent of the block through evaluation of sympathetic activity by measuring skin conductivity (psychogalvanic reflex), temperature (thermometry—thermography), or bloodflow (impedance flow measurement) are modifications which have more or less recently been introduced to make this form of treatment more accurate in its application [12, 13].

Further publication of the results and complications with different types of blocks for cancer pain, has led to their more selective use. Amongst a variety of procedures, especially alcoholization of cranial nerves, intrathecal phenol and alcoholization of the coeliac ganglion have proven to be of great value in the treatment of cancer pain. The injection of iced saline or hypertonic saline and the barbotage of spinal fluid are still practised. There is much controversy on the validity of these procedures.

Pain relief by nerve blocks is not always complete, and lasts from several weeks to several months [14]. For those patients with only partial improvement, the combination of analgesic drugs with nerve blocks can make drug therapy, that often was unsatisfactory before, possible. If the use of nerve blocks is not always the most successful modality of pain treatment, an accurate injection, in experienced hands, for a good indication, can be very rewarding for the individual patient.

4. NEUROSURGERY IN THE TREATMENT OF CANCER PAIN

Generally speaking, the most favourable candidates for a neurosurgical procedure developed to relieve pain, are patients with malignancies which are beyond radiotherapeutic, surgical or chemical control. This is probably so because the patient's life expectancy is limited and at least part of the pain is due to activation of nociceptors, lying in the territory of the cancer process.

Although neurosurgical therapy for pain is often considered as a last resort, it is the authors' opinion that it should not be delayed unnecessarily until tolerance to narcotics develops. This is particularly so since new techniques have been developed in the last decade, which make old operations safer and easier, and new ideas about the neural mechanisms of pain have appeared, from which an entirely new form of pain control has evolved: electrical stimulation of some neuronal systems, which may be effective in pain control without destruction of neural tissue.

4.1 *Lesion techniques*

Lesion techniques are based on classical and more modern notions of the pathways in the nervous system which transmit the information from nociceptors to the higher levels of the central nervous system. In Fig. 2 the better known pathways are represented schematically. It is important to observe here that there is a lateral and a medial afferent system subserving pain. The lateral pathway which has been described for a long time is composed of the neospinothalamic tract, and projects largely

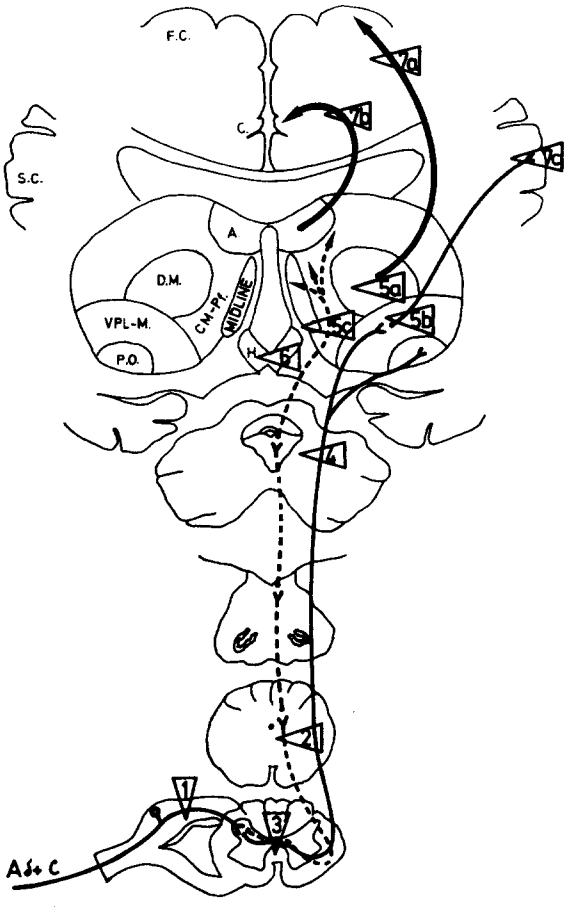


Fig. 2. ——: Tractus neo-spinothalamicus – – –: Tractus paleo-spinothalamicus and spino-reticulo-thalamicus →: Thalamic radiations to frontal cortex and cingulum. P.O.: posterior region. VPL–M: N. ventralis posterior lateralis and medialis. D.M.: N. dorsomedialis. CM–Pf.: centrum medianum-parafascicular complex. Midline: midline nuclei. A.: anterior nuclear group. H.: hypothalamus. F.C.: frontal cortex. C.: cingulum. S.C.: somato-sensory cortex. The nuclei relate to the neurosurgical procedures, indicated in Fig. 3.

Procedure	Frequency of indication
1. Dorsal rhizotomy	++
2. Antero-lateral cordotomy	+++++
3. Commissural myelotomy	+
4. Midbrain spinothalamic tractotomy	+
5. Thalamotomy :	++
a) Dorsomedial thalamotomy	
b) Ventroposterior thalamotomy (VPL, Vc p c)	
c) Medial thalamotomy (CM-Pf, intralaminar, limitans)	
d) Ventroposterior + medial thalamotomy (5b + 5c)	
e) Pulvinarectomy	
6. Hypothalamotomy	?
7. Cerebral operations for relief of pain.	+
a) Frontal leucotomy	+
b) Cingulotomy	+
c) Postcentral gyrectomy.	0

Fig. 3.

contralateraly in a somatotopically organized manner to the somato-sensory cortex; it is suggested that this pathway conditions the "epicritic" quality of pain. It is only in recent years that knowledge has accumulated concerning the medial pathways; this pathway, composed of paleo-spinothalamic and spino-reticulothalamic fibers projects diffusely and bilaterally to many subcortical and cortical non-specific areas; it is suggested that this medial system conditions the "protopathic" quality of pain. In Fig. 3, a list is given of the different neurosurgical techniques, developed to interrupt these pathways at a certain level, depending on the type of pain and on the area of the body in which the pain is felt.

It cannot be the purpose of this short review to describe the rationale, indications and dangers of each of these interventions, and we will limit our discussion to these aspects which are of particular interest to the oncologist. However, the interested reader can find much information and many references in e.g., White and Sweet's monography [15].

4.1.1. *Dorsal rhizotomy.* Since dorsal rhizotomy results in a restricted area of sensory loss involving all sensory modalities, it is rarely indicated in cancer pain. Rhizotomy of cranial nerves is far more successful than spinal dorsal rhizotomy, but it is also a major surgical procedure. To achieve sufficient de-afferentiation, often several cranial nerve roots must be sectioned.

4.1.2. *Antero-lateral cordotomy.* Section of the antero-lateral quadrant of the spinal cord is one of the oldest neurosurgical operations for pain relief. This operation can provide a selective loss of pain and temperature sensation in a widespread region of the body without an impairment of other sensory modalities. A percutaneous technique, introduced about a decade ago [16], in which an electrode is introduced into the antero-lateral quadrant of the spinal cord, has many advantages: no incision is necessary, no general anesthesia is required, pain pathways can be identified in the conscious cooperative patient by electrophysiological techniques, and lesions can be placed in sufficient precise manner. It follows that the indication for cordotomy can now be extended to those patients who are too ill for an open procedure, and that complications such as death, paralysis and sphincter impairment can be reduced.

Midline pain requires bilateral cordotomy for adequate relief. In that case, there remains a high risk of bladder dysfunction.

A rather important, but avoidable complication, seen with high cordotomies, is related to respiration: when this complication arises, the patient breathes normally when awake but becomes apneic when asleep. The complication is due to the interruption of an ascending system mediated through the upper cervical cord in the white matter immediately adjacent to the anterior horn and which contributes to the control of ventilation. This complication can, according to Rosomoff [17] be predicted by testing the patient for his response to breathing carbondioxide.

4.1.3. *Thalamotomy.* Since the introduction of the stereotactic technique in man in 1947, which permits to have a safe access to deep

cerebral structures with a fair accuracy, targets have been selected in the thalamus to interrupt the lateral specific pathways, the medial, multisynaptic, non-specific pathways, the thalamic projection to the frontal cortex (which destruction does not abolish pain, but changes the attitude towards pain), the thalamic projections to other associative cortical areas, possibly concerned with pain experience, or a combination of any of these systems. Although very great hope has been placed on those thalamic targets, experience has not lived up to those expectations.

In spite of this, stereotactic thalamotomy has a place in cancer patients with short life expectancy for which other procedures, such as a cordotomy, cannot be employed, and this because of the area involved. Pain involving multiple cranial and cervical nerves in cases of tumor of the head and neck is one of the best examples. The operative risk is minimal and the procedure can relieve the patient until death. As far as the most effective area at the thalamic level in relieving pain is concerned, this question cannot be answered precisely today. It seems that the lesion should be sufficiently large to include the specific neospinothalamic (VPL-M, Vcpc) and non-specific paleo-spinothalamic (CM-Pf-intralaminar, limitans) systems, and perhaps the DM nucleus and the pulvinar as well. Sometimes bilateral lesions may be necessary, but then unwanted side effects of the operation may become an important problem.

4.1.4. *Hypothalamotomy.* Although only a few neurosurgeons and only recently [18], have reported on favorable results in cancer pain after medial posterior hypothalamotomy, and obviously this intervention at the moment should be restricted to a very few neurosurgical centers, we refer to this intervention here because those favorable results may give a clue for the understanding of the surprisingly favorable results reported after chemical hypophysectomy by Moricca [19], and this as well in non-hormone-dependent as in hormone-dependent neoplastic disease. This author injects through needles placed in the sella tursica by way of the nose 0·6–2 ml ethanol.

According to Moricca pituitary adenolysis constitutes a non-traumatic and easily performed technique: it does not require special equipment, is absolutely inexpensive, and can be repeated without any technical limitations and with insignificant clinical contra-indications; it controls with a single block diffuse, unbearable cancer pain. Up to 1973, he treated 687 patients with advanced malignancy, performing 1593 pituitary chemolysis. The treatment was carried out mostly in patients with visceral and bone associated metastases. Most of these patients were in very poor condition; they all suffered from diffuse and severe cancer pain. In 605 cases pain relief was complete, immediate and long-lasting. These results, if they are true, are very impressive indeed. They need of course confirmation and proper evaluation by others, but at a first glance, would point to chemical hypophysectomy as an unique method for relieving "unbearable" cancer pain.

At this point, one may ask what could be the mechanisms through which chemical hypophysectomy produces its antalgic actions, and particularly so in the case of the non-hormone-dependent cancer disease. A possible explanation is the observation made by Miles and Lipton [20], who showed that fluids injected into the pituitary gland, propagate through the pituitary stalk and into the region of the hypothalamus; consequently pain relief in chemical hypophysectomy might be due to chemical hypothalamotomy.

4.1.5. *Cerebral operations.* Since a long time, frontal leucotomy and cingulotomy have been employed as a last resort to attenuate the psychic and emotional experience of pain. Well known and rarely used nowadays, they need not be discussed here. Gyrectomy of the somaesthesic cortical area is not effective and has been abandoned.

4.2. *Stimulation techniques*

The publication in 1965 of the gate theory of pain perception by Melzack and Wall [21] and the discovery, mainly by Mayer *et al.* [22] of a medial brainstem system with the specific and apparently endogenous property of pain inhibition has led to a renewed interest in the use of electrical stimulation of neural systems for pain relief. Although today not many clinical data are available of this form of treatment in cancer pain, the theoretical implications and the possible clinical applications are of such importance that they should be briefly discussed here. Important information on this topic can be found in a recent volume of *Advances in Neurology* devoted to pain [23].

4.2.1. *The gate control theory.* The gate control theory can be briefly summarized as follows (Fig. 4). Stimulation of the skin evokes nerve impulses that are transmitted to three spinal cord systems: the cells of the substantia gelatinosa in the dorsal horn, the dorsal column fibers

that project toward the brain and the first central transmission (T) cells in the dorsal horn. Wall and Melzack propose: (1) that the substantia gelatinosa functions as a gate control system that modulates transmission from peripheral fibers to T cells, (2) that the afferent patterns in the dorsal column act, in part at least, as a central control trigger which activates selective brain processes that influence the modulating properties of the gate control system, and (3) that the T cells activate neural mechanisms which compromise the action system responsible for pain response and pain perception. Phenomena related to pain would be determined by interactions amongst the three systems.

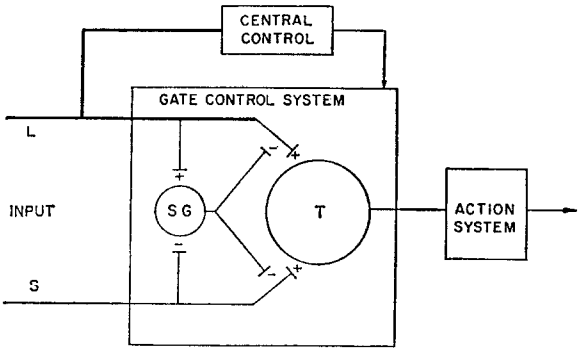


Fig. 4. SG: substantia gelatinosa. L: larger fibers of peripheral nerve. S: small fibers of peripheral nerve. T: transmission neurons in dorsal horn.

It should be stressed that many recent experimental data do not lend support to the gate control theory. However, it seems clear that nociceptive input, which is conducted to the central nervous system via small fibers [24], can be inhibited, and the gate control theory has stimulated new avenues for pain control. One of those is that, according to the gate control theory, and as is shown in the schema of Fig. 4, selective activation of myelinated fibers will decrease pain. At the present time myelinated fibers have been chiefly stimulated with an electrode applied to the skin, to a peripheral nerve, or to the posterior surface of the spinal cord. It is this last technique which has been mostly used in patients with cancer pain, which we will describe as an example.

4.2.2. *Dorsal column stimulation.* Schematically dorsal column stimulation is performed by implanting electrodes on the dorsal column. These electrodes are connected to a miniature radiofrequency receiver which is implanted under the skin, usually in the thoracic region under the clavicula. Electrical stimulation is supplied by a miniature battery-powered radio transmitter which produces a square wave of variable frequency, voltage and pulse width which is coupled to the receiver by the use of a flexible antenna. The patient chooses himself these parameters with which he experiences the best pain relief.

Dorsal column stimulation has been employed in several thousand patients with all sorts of pains. Initial results appeared quite promising with over 80% of patients describing pain relief. With time this percentage has steadily fallen. It now appears that in the most carefully selected series a success rate of about 50% is to be expected [25].

As far as cancer pain is concerned, no precise data are as yet available in the literature. Long, who discussed the usefulness of dorsal column stimulation in cancer pain at a recent symposium of cancer pain, organized by Bonica at the occasion of the first world congress of the International Association for the Study of Pain, summarized the situation as follows: "Dorsal column stimulation does not appear to be more satisfactory for the patient with cancer than the patient with chronic benign pain. It will be of real value in carefully selected patients but still remains a tool that should be limited to those people who are really interested in this modality of pain relief. Now that new types of stimulators, particularly percutaneous devices, are available, prospects may be better, because with the ease of application of percutaneous introduced electrodes, a success rate of 50% is much more acceptable than if the same success rate occurs only with a major surgical procedure."

4.2.3. *Brain stimulation.* One of the most exciting developments in recent years in pain physiology has been the discovery [22] that electrical stimulation of certain brainstem structures, and more particularly the serotonergic dorsal raphe nuclei, lying in the periaqueductal grey, can produce a profound analgesia. It was then found that the brain regions most sensitive to intracerebral microinjections of morphine are the same structures as those which when stimulated produce analgesia. It now seems clear that a neural substrate exists within the medial brainstem which serves normally to inhibit pain; and that this system, when activated electrically, possibly acts through the release of an endogenous, morphine-like substance.

It is to be expected that in the coming years pain reduction will be pursued by electrical stimulation of strategic areas of the nervous system.

5. PSYCHIATRIC ASPECTS IN THE TREATMENT OF CANCER PAIN

Physicians see pain mainly as one of the most reliable indicators of physical illness. For patients pain means a threat to the integrity of the body experienced as an object of concern to the self. The threat may not be apparent to the physician and the pain will then be classed as "psychogenic" [26].

In cancer pain there is obviously tissue damage and a real organic basis for the complaints. Therefore discussions about "pain prone" personality types and pain as a consequence of hostility or an expression of guilt for overt hostility are not immediately relevant for the problem we are dealing with.

There are two main reasons for a psychiatric approach in cancer pain problems.

1. It has become quite clear that in organic disease pain complaints are not solely a function of noxious peripheral input [27]. There is indeed a clear-cut relationship between pain and depression. An otherwise moderate pain experience can become unbearable when it occurs in a depressed patient, even if the depressive syndrome is reactive to the present illness and its familial, occupational or recreational consequences. Depressive syndromes, or even more frequently masked depressions, are common features of chronic pain states, whereas in the more acute states anxiety seems to be a major factor influencing pain [28].

Pain with its threat to the integrity of the body provokes anxiety, a specific unpleasant state of tension which in turn enhances the pain experience. Facing a fatal illness and death in the long run are additional sources of anxiety which steadily enhances the pain distress. Some patients tend to defend themselves by denial but the effect on pain experience can remain the same.

The communication of pain distress to the environment (family, physicians, nurses . . .) seems also to be dependent of some personality variables. The presence of symptoms is related to the neuroticism personality factor of the Eysenk Personality Inventory, whereas the extraversion dimension of personality determines the freedom with which those symptoms are communicated [29]. Extraverts have a tendency to exaggerate the painfulness of a situation whereas introverts minimise it. Clearly a lack of complaints does not necessarily mean absence of pain.

2. If in acute pain states the role of the psychiatrist is not evident, one can observe that nearly all patients with chronic pain show some psychological change, pointing to a possible intervention of the psychiatrist. Chronic pain is clearly associated with an increase of the neuroticism score. The "neurotic triad" on the MMPI (Hypochondriasis, Depression and Hysteria) is high in chronic pain patients, whereas it remains within normal limits in acute pain patients [28].

Moreover chronic pain is closely involved in human relationships and the response it evokes is a complex process termed "Painmanship" by Szasz [30], "Treatment Transaction" by Bond [31] or "Pain Games" by Sternbach [28].

Pain initiates complex behavioral changes in the environment such as receiving analgesics, attention for pain, rest, time out from stress or financial compensations. Those consequences can act as reinforcers and increase then the rate of pain behavior. After a while pain becomes a manipulative complaint between the patient who identifies himself as a painful suffering person and the doctor as being able to relieve the patient's suffering. The recent breakthrough in learning principles and behavior therapy in psychiatry provides a new useful theoretical and therapeutic model for understanding and managing those complex situations.

These considerations give some clues to the actual psychiatric pain control treatments. It would be wrong to expect from the psychiatrist a magic problem solving intervention or a simple push-button pain-control technique. His intervention must be a part of a comprehensive treatment program in which the above reported techniques will also find their place.

Most acute pain situations can be managed without a psychiatrist. Problems arise mainly when pain becomes a chronic feature in a progressive and debilitating disease ending up with death.

The first important aim is to make explicit for each particular patient a realistic and concrete goal of treatment. The patient always has certain expectations of the doctor and the doctor likewise has some expectations of the patient. They must be confronted until a consensus is fallen on a realistic goal. If pain relief appears not feasible, the therapist should aim at reducing it to bearable levels or help the patient to learn to live with his pain.

Which treatment methods are suitable to achieve these goals?

We can group them tentatively in three major categories: (1) Psychopharmacological and Psychotherapeutic management of the emotional distress; (2) Relaxation, Biofeedback and Hypnotic techniques to provide sympto-

matic pain relief; (3) Behavior modification methods to improve the pain behavior of patients with chronic pain strongly involving their environment.

(1) The first treatment methods aim at coping with the emotional repercussion of the pain distress and the whole severe somatic condition.

Anxiety and depression are common features enhancing the pain distress in cancer patients. Usually affects seem rather appropriate in proportion to the physical disability and discomfort. But many cases are characterised by masked depression or extensive denial of anxiety or illness.

Psychotropic drugs must be used in such cases and by reducing anxiety or depressive mood they also reduce the pain. There is furthermore increasing evidence that tricyclic antidepressants and phenothiazines have some analgesic effect independently of the changes they induce in mood and thinking.

Emergency and short-term psychotherapy are other useful tools to manage emotional distress enhancing pain. The restricted goal of brief psychotherapy is limited to the removal or amelioration of specific symptoms and of course, in cancer patients, it does not attempt the reconstitution of personality. The response of the patients to the illness can be clearly seen in relation to their pre-existing various personalities, but two major responses are likely to be found in nearly all the patients; an increased secondary narcissism with changes in the body image, and increased dependency needs, passivity and eventually anger stemming from the disappointments and frustrations.

Bellak [32] gives some practical guidelines:

—The therapist must assume a realistic attitude toward the seriousness of the patient's illness and pain, he must not minimise the realistic fears or the real extent of damage, nor the disabling effect of the pain or illness.

—Reassurance, support and addressing oneself to the lowered self-esteem is then generally in order.

—Clarification of the distortions of patients self-image, body-image or organ-image must be given.

—The specific meaning of the pain, the illness and the death to the patient must be identified and dealt with.

—Improvement of interpersonal relations may be achieved through removing or mitigating conflictual elements between the patient and his surrounding.

(2) A second series of techniques uses cognitive activities such as attention and suggestion to provide more specifically symptomatic relief, i.e., the temporary reduction or abolition of pain experience.

Progressive relaxation is a way of achieving deep muscular and mental relaxation. This method trains progressively the patient to focus intensively and selectively his attention on some specific bodily characteristics or states such as muscular tension, muscular relaxation, warmth, breathing and so on. The aim is to shift the attention of the patient from his pain and to focus it on a new induced body state incompatible with pain. There are two main methods to achieve such a goal, namely the method of Jacobson and the method of Schultz.

Biofeedback techniques are useful to accelerate the learning of the method or to enhance their effectiveness in some patients. Muscle or electromyogram feedback seems particularly effective, probably more than the so called α-training the physiological background of which seems more doubtful.

Suggestion and autosuggestion can also be utilized in combination with relaxation to act more specifically on pain and strengthen the pain relieving effect. Hypnotic methods rest mainly on that particular aspect. There is an enormous and conflicting literature on hypnosis, but therapists who are familiar with these techniques steadily report their beneficial effect on painstates. Orne [33] found hypnosis quite effective in the treatment of the severe chronic pain associated with terminal cancer. But he insists that it should be incorporated in an appropriate overall treatment program to meet the patient's psychological needs, i.e. breaking down the feeling of helplessness and allowing the patient and those around him to focus on positive improvements in functioning rather than the inevitable physical decline.

(3) The third group of therapeutic methods aims at modifying pain behavior which will theoretically be followed by changes in pain experience. This approach is useful mainly in more chronic pain patients when the pain complaint behavior has become a new way of life strongly involving the environment of the patient.

Fordyce [34] views pain as a learned behavior and as an operant, i.e., a behavior the frequency of which can be influenced by the manipulation of its consequences. Let us take the S–O–R–C model (stimulus–organism–pain response–consequences). The previously mentioned somatic or psychiatric methods tended to influence the pain response by manipulating antecedent variables such as the sensory input

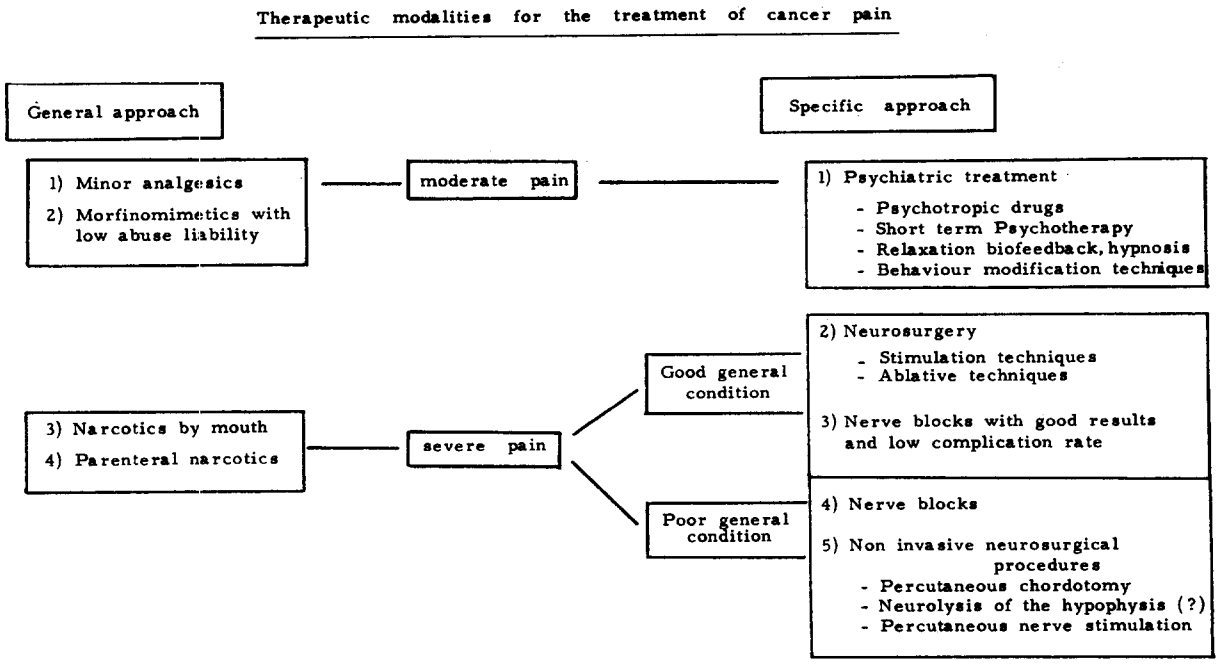


Fig. 5.

or organismic variables. But it is a matter of fact that pain has many consequences for the patient; some of them can act as reinforcers such as getting attention, rest or analgesics, while others are the avoidance of many painful activities or situations in which pain can be anticipated (avoidance learning).

Operant conditioning is the method by which behavior is changed or influenced through manipulation of consequences. It needs a throughout behavioral analysis of the pain mainly to disclose the pain controlling variables. The target behaviors are clearly defined and the followed operant procedure to achieve the goals is established with the patient who must agree with it. Many therapists establish written "treatment contracts" for this purpose.

This method is a very pragmatic and efficient way to deal with pain on a ward but can also be taught to the family of the patient and continued at home.

Pain behaviors are no longer reinforced, each progress is noticed and complimented by the nursing staff and patients are systematically engaged to develop pain incompatible behaviors. Reinforcers such as attention or rest are no longer contingent upon the pain behavior but do occur contingent upon the alternative pain incompatible behavior one is trying to increase. Analgesics are administered following a fixed time scheme (or schedule) so that they can no longer serve as a contingent reinforcer to pain behavior.

6. CONCLUSION

All of the methods described for the treatment of pain have their shortcomings. The best results are seen when a combination of them is used in the frame of a multidisciplinary pain clinic. The universal approach with analgesic drugs, as used (and misused) by every doctor, can often be corrected and improved by specialists with a larger pharmacological experience. At the same time, the screening for more specialistic intervention is easily realised by the multidisciplinary team, in which the oncologist and family physician are included. In Fig. 5 an attempt is made to summarize the possible modalities of treatment. Nevertheless this is an abstraction of the clinical situation, and it cannot be overemphasized that the treatment of pain has to be individual for each patient.

Acknowledgement—The authors are indebted to Mrs. M. Feytons-Heeren for technical assistance.

REFERENCES

1. E. J. Beattie, Philosophy of cancer care. *Surg. Clin. N. Amer.* **49,** 213 (1969).
2. J. Aitken-Swan, Nursing the late cancer patient at home. The family's impressions. *Practitioner* **183,** 64 (1959).

3. R. G. Twycross, Principles and practice of the relief of pain in terminal cancer. *From the Postgraduate Centres* **115** (1972).
4. A. Cartwright, L. Hockey, and A. B. M. Anderson, *Life Before Death.* Routledge & Kegan Paul, London (1973).
5. T. M. Murphy, Cancer pain. *Postgraduate Medicine* **53,** 187 (1973).
6. J. J. Bonica, Organisation and function of a Pain Clinic. In *Advances in Neurology.* (Edited by J. J. Bonica) Vol. 4, p. 433. Raven Press, New York (1974).
7. P. V. Admiraal, H. Knape, C. Zegveld, Experience with bezitramide and droperidol in the treatment of severe pain. *Brit. J. Anesth.* **44,** 1191 (1972).
8. A. Sunshine, Recent advances in non-narcotic analgesics. In *Advances in Neurology.* (Edited by J. J. Bonica). Vol. 4, p. 513. Raven Press, New York (1974).
9. R. W. Houde, The use and misuse of narcotics in the treatment of chronic pain. In *Advances in Neurology.* (Edited by J. J. Bonica). Vol. 4, p. 527. Raven Press, New York (1974).
10. W. H. Forrest, L. R. Brown, D. L. Mahler, J. Katz, P. Schroff, R. Defalque and B. Brown, The evaluation of morphine and dexamphetamine combination for analgesia. *Clin. Pharmacol. Therapy* **14,** 132 (1973).
11. H. A. Baar and H. V. Gerbershagen, *Schmerz, Schmerzkrankheit, Schmerzklinik,* Springer-Verlag, Berlin (1974).
12. M. Mehta, Intractable pain. In *Major Problems in Anaesthesia.* (Edited by W. Mushin), Vol. 2, p. 161. Saunders, London (1973).
13. W. H. Dam and J. J. Vive Larsen, Peripheral nerve blocks in relief of intractable pain. In *The relief of intractable pain.* (Edited by M. Swerdlow). Excerpta Medica, Amsterdam (1973).
14. J. Katz, Current role of neurolytic agents. In *Advances in Neurology.* (Edited by J. J. Bonica), Vol. 4, p. 471. Raven Press, New York (1974).
15. J. C. White and W. H. Sweet, *Pain and the Neurosurgeon.* Charles C. Thomas, Springfield (1969).
16. S. Mullan, P. V. Harper, J. Hekmatpanah, H. Torres and G. Dobben, Percutaneous interruption of spinal-pain tracts by means of a strontium-90 needle. *J. Neurosurg.* **20,** 931 (1963).
17. H. L. Rosomoff, A. J. Krieger and A. S. Kuperman, Effects of percutaneous cervical cordotomy on pulmonary function. *J. Neurosurg.* **31,** 620 (1969).
18. D. Fairman, Stereotactic hypothalamotomy for the alleviation of pain in malignant tumors. *J. Surg. Oncol.* **5,** 79 (1973).
19. G. Moricca, Chemical hypophysectomy for cancer pain. In *Advances in Neurology.* (Edited by J. J. Bonica), Vol. 4, p. 707. Raven Press, New York (1974).
20. J. Miles and S. Lipton, The mode of action by which pituitary alcohol injection relieves pain. *First World Congress on Pain.* Abstracts (Edited by J. J. Bonica *et al.*) Florence (1975).
21. R. Melzack and P. D. Wall, Pain mechanisms: A new theory. *Science* **150,** 971 (1965).
22. D. J. Mayer, T. L. Wolfle, H. Akil, B. Carder and J. C. Liebeskind, Analgesia from electrical stimulation in the brainstem of the rat. *Science* **174,** 1351 (1971).
23. J. J. Bonica, *Advances in Neurology: Pain.* Vol. 4. Raven Press, New York (1974).
24. J. Van Hees and J. Gybels, Pain related to single afferent C fibers from human skin. *Brain Res.* **48,** 397 (1972).
25. D. M. Long and N. Hagfors, Electrical stimulation in the nervous system: the current status of electrical stimulation of the nervous system for relief of pain. *Pain* **1,** 109 (1975).
26. T. S. Szasz, *Pain and Pleasure,* Basic Books, New York (1957).
27. R. Melzack, *The Puzzle of Pain,* Penguin Books (1973).
28. R. Sternbach, *Pain Patients: Traits and Treatment,* Academic Press, New York (1974).
29. M. R. Bond and I. B. Pearson, Psychological aspects of pain in woman with advanced cancer of the cervix. *J. psychosom. Res.* 13, 13 (1969).
30. T. S. Szasz, The psychology of persistent pain. In *Pain.* (Edited by Soulairac *et al.*) p. 93, Academic Press, New York (1968).

31. M. R. Bond and I. Pilowski, Subjective assessment of pain and its relationship to the administration of analgesics in patients with advanced cancer. *J. psychosom. Res.* **10,** 203 (1966).
32. L. Bellak and L. Small, *Emergency Psychotherapy and Brief Psychotherapy*, Grune & Stratton, New York (1965).
33. M. T. Orne, Pain suppression by hypnosis and related phenomena. In *Advances in Neurology*. (Edited by J. J. Bonica) Vol. 4, p. 563. Raven Press, New York (1974).
34. W. E. Fordyce, Pain viewed as learned behavior. In *Advances in Neurology*. (Edited by J. J. Bonica) Vol. 4, p. 415. Raven Press, New York (1974).

Europ. J. Cancer Vol. 12, pp. 353–356. Pergamon Press 1976. Printed in Great Britain

General Papers

Continuous Low-Dose Cyclophosphamide (NSC-26271) Therapy in Advanced Ovarian Cancer**

COSTANTINO MANGIONI*, GIORGIO BOLIS*, NICOLA NATALE*, and LUCIANO MORASCA†‡

**I Clinica Ostetrica e Ginecologica dell'Università di Milano, Italy*
†Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea, 62-20157 Milano, Italy

Abstract—*A low dose, continuous treatment by cyclophosphamide (100 mg/day p.o.) has been given to 141 patients with documented Müllerian ovarian cancer. From this total 134 have been evaluated for drug related toxicity. Response and survival have been studied in 72 patients in stages III, IV or in recurrence, all with at least one measurable tumor mass. Results demonstrate that this regimen of administration induces remission and prolongation of median survival with a very low level of toxicity and minimal discomfort to the patient.*

INTRODUCTION

OVARIAN cancer responds to alkylating agents in 45–75% of the patients [1–3], when the most favorable schedule of treatment is given. Other agents such as antibiotics, antimetabolites, antimitotics, are less effective [2] with the only exception being adriamycin, in which there is a good response in 55% of the treated patients [4]. Among alkylating agents melphalan is effective in 47% of the patients [5] and cyclophosphamide when given at a low dosage schedule is active in 49% of the patients [1]. Recently high doses of cyclophosphamide given intermittently have been used in an attempt to improve the patient response [6]. To improve survival and possibly the quality of life of patients affected by ovarian cancer we started, in 1968, a continuous low dose cyclophosphamide regimen. This paper reports the data obtained from patients with measurable tumor masses, undergoing this continuous cyclophosphamide therapy.

Accepted 2 January 1976.

**This work was supported by a grant from Mr. Mario Ratto.

‡To whom reprint requests should be addressed.

METHODS

Patients

From a total of 141 treated patients data on response and survival have been calculated on 72 patients. Reasons for excluding the other 69 were: 21 patients in stages IA, IB and IIA were receiving precautional therapy; 16 patients treated for less than two months for which therapy was considered insufficient; 25 patients with non measurable tumor in stages IC, IIB and IIC; 7 patients that withdrew from the study. All of the other patients were continuously followed after diagnosis.

Drug related toxicity has been evaluated in the total population of 134 follow-up patients.

Patients age at time of diagnosis was in the range of 32–75 years with mean age of 55·23 and median of 56 years. Diagnosis, confirmed histologically, was of Müllerian ovarian cancer of serous, mucinous or undifferentiated type (see Table 1). No patient had received prior chemotherapy; twelve had prior radiotherapy.

Staging

Patients had previously undergone a laparotomy which provided histological diagnosis and staging. Chest, bone and bowel X-rays were carried out routinely as were urography

Table 1. *Patients evaluated*

Stage	Typing			Total	%
	Serous	Mucinous	Undifferentiated		
III	33	0	11	44	61·2
IV	4	0	2	6	8·3
Recurrence	16	2	4	22	30·5
Totals	53	2	17	72	
%	73·6	2·7	23·7		100

Table 2. *Surgical treatment*

Stage	Total hysterectomy and bilateral salpingo-oophorectomy	Partial removal	Laparotomy	Total
III	9	9	26	44
IV	1	2	3	6
Recurrence	16	5	1	22
Total	26	16	30	72

and lymphography. Classification was performed following the recommendations of F.I.G.O. (Table 1).

Surgery

During laparotomy all the tumor masses which could be safely removed, were; when possible, a total hysterectomy with bilateral salpingo-oophorectomy (THBSO) was performed.

Out of the 72 patients evaluated, 30 had only exploratory laparotomy, 16 a partial removal of tumoral masses and 26 the THBSO (see Table 2).

Drug administration

Beginning the tenth postoperative day all patients received cyclophosphamide 100 mg/day p.o. (CTX). All the patients received at least 4 gm of CTX. In no case did treatment have to be terminated due to toxicity, it was however, suspended for two weeks whenever white-blood cell count fell below 2000/mm^3 or platelet count dropped below 100,000/mm^3.

The mean administered dosage was 20·8 g in a range from 4–64 g (median 17 g).

Evaluation of results

Tumor volume was evaluated by measuring two perpendicular diameters of at least one mass by clinical examinations. X-rays and ultrasound scanning were performed 1–2 times per month. Complete response is defined as the disappearance of every clinically measurable mass for at least two months. Partial response defines a more than 50% reduction in at least one of the masses for at least two months. Objective improvement defines a reduction of 25:50%. The other patients with progressive or unchanged tumor are listed as "Non Responders". Complete blood-cell count was performed every two weeks during the first three months and thereafter once a month. Blood urea nitrogen, alkaline phosphatase, SGPT, SGOT, electrolytes, serum creatinine, as well as EKG were checked once a month.

RESULTS

Clinical efficacy

Table 3 summarizes the response to the treatment of the 72 reported patients.

Approximately 15% had a complete disappearance of clinical tumor and approximately 50% responded with objective improvement or with a partial response. Thirty-three per cent of the patients had no response to the treatment.

According to histological typing the number of patients with a complete response seems to be percentually higher among serous carcinomas than among undifferentiated cancer. The difference however does not reach a significant level within the 0·05 confidence limits from 9·44 to 31·97% in serous carcinoma and from 0·15 to 28·69% in the undifferentiated. Response lasted from 2 to 47 months in the overall responding group (66·6%) and the

Table 3. Response to low dose cyclophosphamide (actual number and percentage of response)

	Histological typing						Total	
	Serous		Mucinous		Undifferentiated			
Level of response	No.	%	No.	%	No.	%	No.	%
non responders	18	34	1	—	5	29·5	24	33·3
objective improvement	13	24·6	—	—	5	29·5	18	25
partial response	12	22·7	1	—	6	35·3	19	26·39
complete response	10	18·9	—	—	1	6·9	11	15·28

duration seemed to be in some way related to the level of response (see Table 4). Objective improvements had a median duration of 5 months that went to 8 and 17 months respectively for partial response and complete response.

The median survival appears also to be increased in the population of responders in a way related to the level of response obtained (see Table 5). Particularly in the undifferentiated type of carcinoma the difference between the median survival of responders and non responders appears to be elevated. Patients still alive at the end of the study have not been included in Table 5. Among the 16 patients excluded 7 could be associated to TNO group A_1 (survival from 9 to 60 months); and 7 to group A_5 (3 to 47 months).

Toxicity

The side effects induced by this low dose treatment were mainly related to the gastrointestinal tract, bone marrow and skin adnexa.

A certain degree of nausea occurred in most of the patients and in a few cases was prolonged. Vomiting was, however, very rare. Bone marrow depression was very low.

Considering the 134 patients evaluated for toxicity during this low dose continuous regimen, in 19 cases a mild leucopenia or thrombocytopenia was observed, in 14 cases counts of 2000 WBC or 80,000 thrombocytes were re-

Table 4. Length of response (months)

	Histological typing							
	Serous		Mucinous		Undifferentiated		Total	
Level of response	Median	Range	Median	Range	Median	Range	Median	Range
Objective improvement	5	2 ÷ 8	—	—	4	2 ÷ 20	5	2 ÷ 20
Partial response	9	5 ÷ 31		6*	5	3 ÷ 30	8	3 ÷ 30
Complete response	17	12 ÷ 24	—	—	—	47*†	17	12 ÷ 47

*Single patient
†Still responding

*Table 5. Length of survival (months)**

	Histological typing							
	Serous		Mucinous		Undifferentiated		Total	
Level of response	Median	Range	Median	Range	Median	Range	Median	Range
Non responders	9	3 ÷ 33	—	5	5	4 ÷ 10	8	3 ÷ 33
Objective improvement	10	8 ÷ 18	—	—	11	8 ÷ 24	10	8 ÷ 24
Partial response	16	12 ÷ 43	—	17	14	12 ÷ 41	16	12 ÷ 43
Complete response	22	21 ÷ 72	—	—	—		22	21 ÷ 72

*Calculated on 58 deceased patients at the end of protocol December 1974; 16 patients still alive at this date have not been considered; their survival ranged from 3 to 60 months.

corded and in only 4 cases counts went below these figures.

In two patients red blood cells were slightly affected. Alopecia was the most frequently observed side effect. It appeared in about 50% of the patients, but, despite the continuation of therapy, hair regrew completely within a few months.

One patient complained of mild dysuria which disappeared after cessation of the treatment and did not recur after resuming the drug.

In one case pulmonary fibrosis occurred after 33 g of CTX without clinical symptoms except for positive X-rays.

No other side effect was noted.

DISCUSSION

These data demonstrate that the low dose cyclophosphamide regimen induces remission and prolongation of median survival with a very low toxicity.

Comparing the responses obtained with data from the literature the 49% of responses ($> 25\%$ decrease in tumor mass) obtained with oral administration of doses ranging from 50 to 150 mg/day, and by intravenous administration of 200 mg/day [1], can be compared with the figure of 67% that we obtained adding the three levels of response together. If we include in the group of "non responders" the 16 patients excluded for insufficient therapy, the percent of response after low dose cyclophosphamide regimen reaches its lowest value of 55%. It is however highly questionable whether terminal patients should be included in the evaluation of drugs requiring an active metabolization [7, 8].

As far as cyclophosphamide is concerned the high dose intermittent treatment, besides its very toxic implications, did not produce any real improvement in survival that ranged from 8 to 40 months [6].

Melphalan produced a 47% response, of which 20% are complete, with a very low toxicity [5]. These figures also, are not far from the responses produced by the low dose cyclophosphamide regimen.

To conclude it is certainly significant that cyclophosphamide can be used in ovarian cancer at low dose without loss of efficacy, with a very low level of toxicity and minimal discomfort for the patient.

REFERENCES

1. R. E. Beck and D. A. Boyes, Treatment of 126 cases of advanced ovarian carcinoma with cyclophosphamide. *Canad. med. Ass. J.* **98,** 539 (1968).
2. C. M. Bagley Jr, R. C. Young, G. P. Canellos and V. T. De Vita, Treatment of ovarian carcinoma: possibilities for progress. *New Engl. J. Med.* **287,** 856 (1972).
3. R. C. Young, S. P. Hubbard and V. T. De Vita, The chemotherapy of ovarian carcinoma. *Cancer Treat. Rev.* **1,** 99 (1974).
4. G. De Palo, M. De Lena, L. Luciani, F. Di Re, and G. Bonadonna, Controlled Study with Phenylanaline Mustard (L-Pam) versus Adriamycin (ADM) in Stage IV Ovarian Cancer. In *Abstracts, American European Conference on the Ovary*, Montreux, Switzerland. Dec. 9–13, p. 15 (1975).
5. J. P. Smith, and F. Rutledge, Chemotherapy in the treatment of cancer of the ovary. *Amer. J. Obstet. Gynec.* **107,** 691 (1970).
6. C. D. Buckner, R. Briggs, R. A. Clift, A. Fefer, D. D. Funk, H. Glucksberg, P. E. Neiman, R. Storb and E. D. Thomas, Intermittent high-dose cyclophosphamide (NSC-26271) treatment of stage III ovarian carcinoma. *Cancer Chemother. Rep.* part 1, **58,** 697 (1974).
7. J. Y. Jao, W. J. Jusko and J. L. Cohen, Phenobarbital effects on cyclophosphamide pharmacokinetics in man. *Cancer Res.* **32,** 2761 (1972).
8. C. M. Bagley, F. W. Bostick Jr and V. T. De Vita, Clinical pharmacology of cyclophosphamide. *Cancer Res.* **33,** 226 (1973).

Europ. J. Cancer Vol. 12, pp. 357–362. Pergamon Press 1976. Printed in Great Britain

Changes in the Deoxyadenylate Regions in 5 Cases of Chronic Lymphocytic Leukemic DNA*

RONALD W. PERO†, TOMAS BRYNGELSSON†, ADAM DEUTSCH† and ÅKE NORDÉN‡
†*Department of Biochemistry, Nucleic Acids Research Group, University of Lund, Box* 740, 220 07 *Lund, Sweden*
‡*Haematology Laboratory, University Hospital, Lund, Sweden*

Abstract—*The (dA) levels in the DNAs from normal human tissues and from the lymphocytes of five CLL patients were estimated by formation of ribonuclease A-resistant poly(U)-DNA complexes. Alterations in the (dA) levels of all five CLL DNAs were observed when comparison was made to the normal (dA) pattern. The type of (dA) alterations varied among the different CLL patients, but the variation correlated to the WBC levels of the patients at the time of study. A specificity of neoplastic-associated (dA) alterations for slow repeated + unique DNA sequences and for main band DNA was demonstrated with two of the CLL DNAs.*

INTRODUCTION

BIOCHEMICAL evidence for the neoplastic nature of chronic lymphocytic leukemia (CLL) has become rather substantial [1]. Some reports have been suggestive of changes in the DNA template of CLL lymphocytes. Neiman and Henry [2] have shown by DNA–RNA competitive hybridization techniques that there are rapidly labelled RNA species (HnRNA) present in CLL that are either in low concentration or absent in normal lymphocyte RNA. Sawada *et al.* [3] have determined that CLL chromatins had more transcribable repetitive DNA sequences than did normal chromatin. The work of Corneo *et al.* [4] using CsCl density gradient centrifugation and melting temperature profiles also indicated a heterogeneity of base composition of DNA from leukemic leucocytes when compared to normal leucocytes.

In our laboratory, we have investigated the DNA that may serve as template for the specialized oligoriboadenylic acid (oligo A) [5] regions found in CLL HnRNA. These specialized HnRNA regions are thought to be important in the processing of genetic information [6]. Our aim was accomplished by annealing DNA to polyuridylic acid (poly U) which formed DNA-poly(U) complexes with the deoxyadenylate (dA) rich sequences in the DNA.

It has now been well established in the chinese hamster [7], mouse [8], rat [9], duck [10] and human [11] that eukaryotic DNA indeed does contain small (dA) rich stretches which form ribonuclease A-resistant complexes with poly(U). These observations have provided the necessary basis for determining whether alterations in the (dA) regions of eukaryotic DNA can reflect a concurrent neoplastic transformation. We have found that the (dA) regions in DNA from both 7,12-dimethylbenz-(a)anthracene (DMBA) induced and Rous sarcoma virus induced rat tumors were changed considerably from the (dA) regions in normal rat DNA [9]. Likewise, similar alterations were found when human breast tumor DNAs were compared to normal human DNAs [12]. Since the (dA) regions in DNA appeared to be involved in neoplastic transformation in at least two unrelated biological systems, then the (dA) regions of several CLL DNAs were studied in an effort to verify this trend. We can now report that CLL DNA has alterations in the (dA) sequences when compared to normal human DNA and in much the same way as was previously observed with other neoplastic DNAs.

Accepted 11 December 1975.

*The present work has been supported by grants from the Swedish Natural Sciences Research Council and the Swedish Cancer Society.

MATERIAL AND METHODS

Normal tissue material

Lymphocytes, breast and placental tissues from apparently healthy individuals served as the normal tissue controls. They were collected and the DNA extracted as previously described [11,12].

The CLL patients

Five individuals having typical clinical symptoms of CLL were selected on the basis of their varied white blood cell (WBC) counts and ribonuclease–ribonuclease inhibitor patterns. It has been shown that CLL patients with high WBC counts differ from those with low WBC counts in that they have excess free inhibitor and no free alkaline ribonuclease present [13]. In accordance with these observations, a group of CLL cases were selected which represented the range of heterogeneity of this disease. A description of the five patients used in this study are summarized in Table 1. The high WBC patients with CLL were represented by case N.M., the intermediate WBC patients by cases A. H. and J. R., and the low WBC patients by cases H. A. and G. L. In all the CLL cases, the lymphocytes were isolated [14] and the DNA extracted [11] as already described.

Determination of the (dA) regions in DNA

In a previous study [11] we have described in detail the quantitative estimation of (dA) regions in human DNA. Our results indicated the formation of a 2 poly(U)–DNA complex with the (dA) stretches in the DNA. After mild ribonuclease A treatment (5 μg/ml at 25°C for 30 min) the more mismatched (dA) rich sequences in DNA were estimated. Exhaustive ribonuclease A treatment (5 μg/ml at 25°C for 6 hr), or the conditions which begin to digest pure poly(dA)–poly(U) triplexes, were used to estimate (dA) "pure" sequences in DNA. The annealing conditions which were used to form ribonuclease A-resistant poly(U)-DNA hybrids were carried out in 2*x*SSC (SSC = 0·15 M NaCl–0·015 M sodium citrate) at 25°C for 18 hr with approximately 10–20 μg DNA immobilized on nitrocellulose filters.

Other essential experimental details concerning the materials used, the DNA preparation and quantitative determination, the CsCl gradient preparation, the fractionation of human DNA into repeated classes using hydroxylapatite (HTP) chromatography, and the factors affecting poly(U) hybridization on nitrocellulose filters have also been described in our previous study [11]. The *t*-test was applied for statistical comparison ($P < 0{\cdot}05$) of normal and CLL DNAs.

RESULTS

As it was previously observed for rat [9] and human [11,12] DNAs, the level of (dA) regions in the normal DNA samples was not dependent on the specific tissue being analyzed (Fig. 1 and 2). This fact has allowed a quantitative comparison of the normal level of (dA) regions in the somatic cells of a human to those that may occur in certain cell types of the human following neoplastic transformation. In Fig. 1 and 2, just such a comparison has been made between normal and CLL DNAs. It can be seen that all 5 cases of CLL had significant disturbances in either the (dA) rich or (dA) "pure" regions o their lymphocyte DNAs when compared to the level in normal DNA. The pattern of these changes among the CLL DNAs was quite different for the (dA) rich sequences than was observed for the (dA) "pure" sequences, but taken together these changes did reflect a distinct correlation to the differences in WBC counts of the CLL patients studied (Table 1,

Table 1. *Description of the CLL patients in this study*

Description	Case N.M.	Case A.H.	Case J.R.	Case H.A.*	Case G.L.
Age in years	65	60	58	75	84
Sex	M	F	F	M	M
Date Studied	Feb. 1973	Feb. 1974	Sept. 1972	Sept. 1974	Jan. 1974
Treatment					
Prednisolone	+	+	–	–	–
Chlorambucil	–	+	–	–	–
Blood transfusion	–	1971	–	–	–
Splenectomy	–	–	–	–	–
WBC × 1000 per μl.	1000	214	91	65	25

*In 1942 Case H.A. was diagnosed as CLL and given arsenic. From 1942–1970 the WBC counts remained between 11,000 and 27,000 per μl blood, but in 1974 they had risen to 65,000.

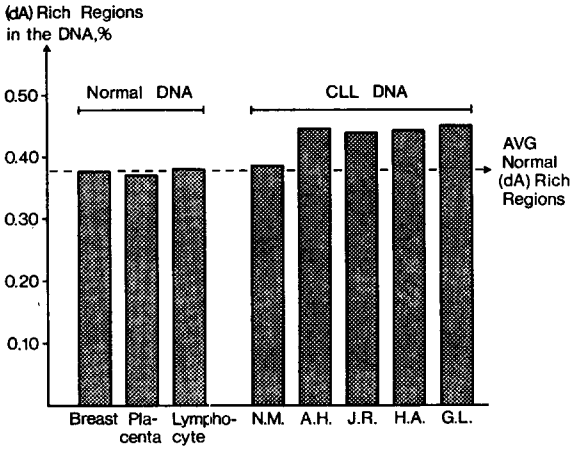


Fig. 1. The level of (dA) rich sequences in the native DNA from normal human tissues and from the lymphocytes of five cases of CLL. The CLL patients are displayed from left to right according to decreasing WBC levels (see Table 1). Only case N.M. DNA was not significantly different ($P < 0{\cdot}05$) from normal DNA when the (dA) levels were compared. Ten replicate determinations for the (dA) levels of each DNA sample were carried out.

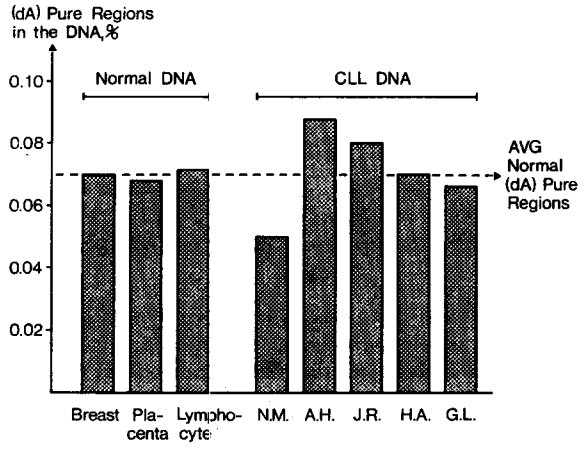


Fig. 2. The level of (dA) "pure" sequences in the native DNA from normal human tissues and from the lymphocytes of five cases of CLL. The CLL patients are displayed from left to right according to decreasing WBC levels (see Table 1). Cases N.M., A.H. and J.R. DNAs gave $P < 0{\cdot}05$ when compared to the % (dA) "pure" regions in normal DNA. Ten replicate determinations for the (dA) levels of each DNA sample were carried out.

Fig. 1 and 2). A high WBC level, represented by case N. M., had no significant differences in the (dA) rich sequences of its DNA but the (dA) "pure" sequences were reduced significantly when compared to the normal levels. The intermediate WBC levels of cases A. H. and J. R. had significant increases in both the (dA) rich and (dA) "pure" sequences of their DNAs, while the lymphocyte DNAs of the low WBC patients (cases H. A. and G. L.) were increased significantly in only the (dA) rich sequences and the (dA) "pure" sequences were at a normal level. These results support the contention that CLL is a heterogeneous disease with either several causes or several prolonged stages of malignant evolution.

An important criterion for understanding the role of (dA) regions in CLL was to determine if the observed (dA) alterations were specifically associated with certain nucleotide sequences within the DNA molecules. In a previous study [12] we separated neoplastic DNAs into adenine + thymidine (A+T) rich and guanine + cytosine (G+C) rich fractions by thermal elution chromatography on HTP. However, little specificity was found as the (dA) alterations in the native neoplastic DNAs were distributed into both the A+T rich and G+C rich fractions. Therefore, in an effort to see specificity in neoplastic-associated (dA) disturbances we have applied different fractionation procedures to the analysis of CLL DNAs; namely separation of DNA into different classes of repeated sequences and fractionation according to base composition by centrifugation to equilibrium in CsCl gradients. For this purpose, the DNAs from cases N. M. and G. L. were used, since these two cases represented the opposite extremes in the type of changes in their (dA) regions and also their WBC levels.

Case N. M. DNA was compared to normal DNA following fractionation into repeated classes. This was accomplished [11] by shearing the DNAs to small fragments of approximately $4{\cdot}4 \times 10^5$, denaturing them to single strands by heat treatment, and then reassociating them to double stranded complexes for prescribed time intervals or reassociation rates (C_0t values). The DNA sequences which have many copies per genome reassociate rapidly and have a small C_0t value of 0·05 while the opposite is true for the unique sequences ($C_0t > 50$).

The native DNA from case N. M. differed from normal native DNA in that it had a reduced level of (dA) "pure" sequences (Fig. 1 and 2). As can be seen in Fig. 3, when four classes of repeated DNA sequences were compared to the corresponding classes in normal DNA, the reduction in (dA) "pure" sequences was found to be confined to the slow repeated (C_0t 1–50) and unique (C_0t 50– ∞) DNA fractions. No differences were observed in the fast (C_0t 0–0·05) and intermediate (C_0t 0·05–1) repeated sequence classes between normal and case N. M. DNAs, thus indicating a specificity for neoplastic-associated (dA) changes in certain DNA fractions.

Case G. L. native DNA differed from normal

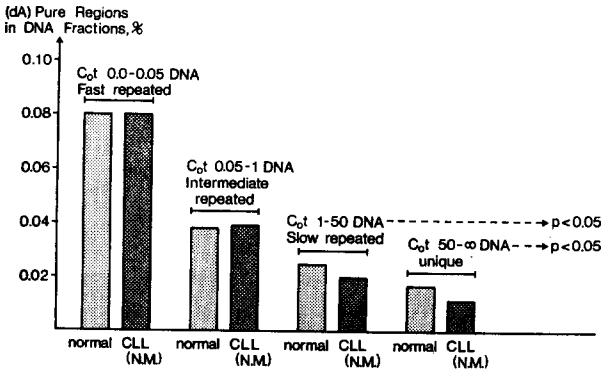


Fig. 3. Distribution of the neoplastic-associated (dA) alterations found in case N.M. DNA following fractionation into classes of repeated DNA sequences. Normal and case N.M. DNAs were sheared by sonication and separated into repeated sequence classes following denaturation-reassociation kinetics and using hydroxylapatite chromatography [11]. *Unfractioned case N.M. DNA had a reduced level of (dA) "pure" regions when compared to the normal DNA levels (see Fig. 2). Twenty-five replicate determinations for the (dA) levels in each repeated DNA class were carried out.*

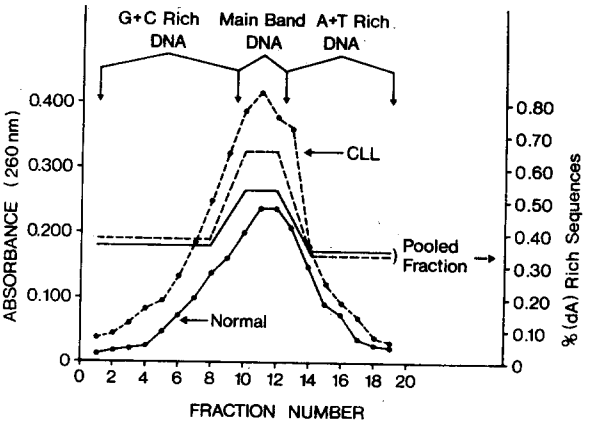


Fig. 4. Distribution of the neoplastic-associated (dA) alterations found in case G.L. DNA following fractionation into G+C rich, main band and A+T rich fractions using CsCl density gradients. A total of 700 μg case G.L. DNA and 400 μg normal DNA were centrifuged to equilibrium in CsCl solution (1·710 g/cm³) at 44,000 rev/min for 72 hr at 15 °C in a Ti 60 rotor. Fifteen drop fractions were collected and the absorbance at 260 nm recorded for the normal (●——●) and case G.L. (●- - -●) DNA fractions. Fractions 1–9, 10–12 and 13–19 were pooled, dialyzed against 2xSSC and the pooled fractions annealed to poly(U). The % (dA) rich sequences for the pooled fractions of normal (——) and case G.L. (- - -) DNAs was determined for replicate samples of each fraction. Unfractionated case G.L. DNA had an increased level of (dA) rich sequences when compared to normal DNA levels (see Fig. 1).

native DNA only in regard to the (dA) rich sequences which were increased over normal levels (Fig. 1 and 2). The distribution of this increased (dA) rich level was further studied by centrifuging both normal and case G. L. DNAs to equilibrium in CsCl gradients, dripping the gradients into approximately 20 fractions containing DNA, and then hybridizing poly(U) to the DNA fractions [11]. The results of this comparative study are presented in Fig. 4. The CsCl gradient fractions were pooled into G+C rich, main band and A+T rich fractions as indicated in Fig. 4 before they were annealed to poly(U). The increased level of (dA) rich regions originally noted in the case G. L. native DNA was now shown to be associated only with the main band DNA. Again, a specificity of neoplastic-associated (dA) alterations was observed.

The main band fractions isolated from normal and case G. L. DNAs (Fig. 4) were further studied by subfractionation into fast repeated (C_0t 0–1) DNA and slow repeated + unique DNA. The results from poly(U) hybridization experiments to these subfractions are presented in Table 2. Both fast repeated and slow repeated + unique main band DNA subfractions contained significant changes in the (dA) levels when compared to the similar subfractions of normal main band DNA. However, the slow repeated + unique subfraction was approximately 85% of the unfractionated main band DNA, and since there was actually a reduced level of (dA) rich sequences in the fast repeated case N. M. main band DNA, then the increased level of (dA)

Table 2. Distribution of neoplastic-associated (dA) alterations in case G.L. main band DNA following further subfractionation into classes of repeated DNA sequences

The main band DNAs used in this experiment were the same as indicated in Fig. 4. The normal main band DNA was 32·9 % of the native normal DNA and case G.L. main band DNA was 32·2 % of the native case G.L. DNA. The main band DNAs were further subfractionated into fast repeated (C_0t 0–1) and slow repeated + unique (C_0t 1–∞) DNA sequences as already described [11], and then the subfractions hybridized to poly(U). Main band case G.L. DNA originally had an increased level of (dA) rich sequences when compared to normal main band DNA (see Fig. 4).

Main band subfraction	Percentage of total main band	Percentage (dA) rich sequences
C_0t 0–1 DNA (fast repeated)		
normal	13·4	0·89
case G.L.	15·6	0·71*
C_0t 1–∞DNA (slow repeated + unique)		
normal	86·6	0·43
case G.L.	84·4	0·56*

*$P < 0·05$ when compared to normal main band subfractions.

rich sequences originally observed in unfractionated native case G. L. DNA, must have been principally associated with the slow repeated + unique subfraction of the main band DNA. These results are in agreement with those found for case N. M. DNA which was fractionated directly into repeated sequence classes and the (dA) levels measured; i.e., that the neoplastic-associated (dA) changes were localized into the slow repeated and unique DNA fractions (Fig. 3).

DISCUSSION

Among the five CLL patients studied, there was an evolution of neoplastic-associated (dA) changes from disturbances primarily in the (dA) rich sequences to disturbances primarily in the (dA) "pure" sequences (Fig. 1 and 2). This pattern of (dA) changes was parallelled by an increase in the WBC levels of the patients at the time of study (Table 1). We have observed a similar correlation of (dA) changes to histologic dedifferentiation using human breast tumor DNAs [12]. Hansen [15] has reported that high WBC levels in CLL patients statistically reflect a higher mortality rate than low WBC patients with CLL. Therefore, high WBC levels would suggest the more malignant condition as does an increased histologic dedifferentiation. It is of interest to note that the more malignant condition in both cases had the same (dA) changes which were reduced levels of (dA) "pure" sequences. Additional support for this general observation was also shown in a previous rat sarcoma study [9] when transplanted DMBA rat tumor DNA had even a greater reduction in the (dA) "pure" sequences than did primary DMBA tumor DNA.

A specificity of slow repeated and unique DNA sequences for neoplastic-associated (dA) alterations was demonstrated for both case N. M. and G. L. DNAs (Fig. 3 and 4, Table 2). This was true even though the two CLL patients differed greatly in their WBC levels and their type of neoplastic-associated (dA) changes. Since it has been shown [16–21] that most mRNA is transcribed from slow repeated or unique sequences in the DNA, then perhaps the (dA) changes observed in these DNA fractions reflect the differences in RNA transcription which have previously been described between normal and CLL lymphocytes [2,3,5]. In addition, Tapiero *et al.* [22] have reported that the main band DNA in chick has a higher proportion of slow repeated and unique DNA than does G + C rich or A + T rich DNA. Case G. L. DNA also had its (dA) alterations confined to the main band DNA which further implicates a close association of neoplastic-associated (dA) alterations to potential mRNA transcription sites.

The size range of (dA) regions in mammalian DNA has been shown to be rather heterogeneous varying from 25–150 nucleotides long [7]. These are very small sequences when considering the size of the transcribed regions in DNA which may be estimated from the molecular weight of HnRNA to be 5000–40000 nucleotides long [6]. The fact that (dA) regions in DNA are transcribed has been established by the presence of oligo(U) stretches in HnRNA [6, 23]. The (dA) sequences apparently play little role in the translation of genetic information since no complementary oligo(U) sequences have, as yet, been detected in mRNA [6]. In a sense, then, the biologic importance of the (dA) changes in CLL DNAs probably is intimately connected with the other nucleotide sequences that the (dA) regions are interspersed with, since the (dA) sequences which are transcribed are not translated into protein along with the genetic information in mRNA.

REFERENCES

1. S. Seeber and C. G. Schmidt, Makromolekulare Besonderheiten des Lymphocyten der Chronisch-lymphatischen Leukämie (CLL). *Klin. Wschr.* **52,** 1093 (1974).
2. P. E. Neiman and P. H. Henry, Ribonucleic acid-deoxyribonucleic acid hybridization and hybridization-competition studies of the rapidly labelled ribonucleic acid from normal and chronic lymphocytic leukemic lymphocytes. *Biochemistry* **8,** 275 (1968).
3. H. Sawada, V. H. Gilmore and G. F. Saunders. Transcription from chromatins of human lymphocytic leukemic cells and normal lymphocytes. *Cancer Res.* **33,** 428 (1973).
4. G. Corneo, P. Bianchi, E. Ginelli and E. Polli, Heterogeneity of base composition of DNA extracted from human leukemic leucocytes. *Europ. J. Cancer* **2,** 307 (1966).

5. P. E. Månsson, L. Holmquist, A. Deutsch and Å. Nordén, Heterogeneous nuclear RNA from lymphocytes of chronic lymphocytic leukemia: Adenylate-rich and double-stranded regions. *Scand. J. Haematol.* **14,** 42 (1975).
6. G. R. Molloy, W. L. Thomas and J. E. Darnell, Occurrences of uridylate-rich oligonucleotide regions in heterogeneous nuclear RNA of Hela cells. *Proc. nat. Acad. Sci. Wash.* **69,** 3684 (1972).
7. A. Shenkin and R. N. Burdon, Deoxydenylate-rich and deoxyguanylate-rich regions in mammalian DNA. *J. Mol. Biol.* **85,** 19 (1974).
8. A. Shenkin and R. H. Burdon, Deoxydenylate-rich sequences in mammalian DNA. *FEBS Lttr.* **22,** 157 (1972).
9. R. W. Pero, T. Bryngelsson, F. Mitelman and G. Levan, Changes in the deoxyadenylate regions of rat DNA in sarcomas induced by 7,12-dimethylbenz(a)anthracene and Rous sarcoma virus. *Hereditas* **80,** 153 (1975).
10. J. O. Bishop, M. Rosbash and D. Evans, Polynucleotide sequences in eukaryotic DNA and RNA that form ribonuclease-resistant complexes with polyuridylic acid. *J. mol. Biol.* **85,** 75 (1974).
11. R. W. Pero, T. Bryngelsson, C. Bryngelsson, A. Deutsch, Å. Nordén and A. Norgren. Hybridization of polyuridylic acid to human DNA immobilized onto nitrocellulose filters. *Nucleic Acids Res.* **2,** (7) 1163 (1975).
12. R. W. Pero, T. Bryngelsson, A. Norgren and A. Deutsch, Alterations in the deoxyadenylate regions of the DNA from four human breast tumours. *Europ. J. Cancer* **11,** 861 (1975).
13. P. E. Månsson, A. Deutsch and Å. Nordén, Ribonuclease–ribonuclease inhibitor patterns in lymphocytes of chronic lymphocytic leukemia. *Scand. J. Haemat.* **12,** 284 (1974).
14. A. Bøyum, Separation of leucocytes from blood and bone marrow. *Scand. J. clin. lab. Invest.* **21,** 97 (1968).
15. M. M. Hansen, Chronic lymphocytic leukaemia. *Scand. J. Haemat. Suppl.* **18,** 1 (1973).
16. R. B. Goldberg, G. A. Galau, R. J. Britten and E. H. Davidson, Non-repetitive DNA sequence representation in sea urchin embryo RNA. *Proc. nat. Acad. Sci. Wash.* **70,** 3516 (1973).
17. J. O. Bishop and M. Rosbash, Reiteration frequency of duck haemoglobin genes. *Nature New Biol.* **241,** 204 (1973).
18. Y. Suzuki, L. P. Gage and D. D. Brown, The genes for silk fibroin in *Bombyx mori*. *J. mol. Biol.* **70,** 637 (1972).
19. R. A. Firtel, A. Jacobson and H. F. Lodish, Isolation and hybridization kinetics of messenger RNA from *Dictyostelium discoideum*. *Nature New Biol.* **239,** 225, (1972).
20. D. Sullivan, R. Palacios, J. Staunezer, J. M. Taylor, A. J. Faras, M. L. Kiley, N. M. Summers, J. M. Bishop and R. T. Schimke, Synthesis of a deoxyribonucleic acid sequence complementary to ovalbumin messenger ribonucleic acid and quantification of ovalbumin genes. *J. Biol. Chem.* **248,** 7530 (1973).
21. W. H. Klein, W. Murphy, G. Attardi, R. J. Britten and E. H. Davidson, Distribution of repetitive and nonrepetitive sequence transcripts in Hela mRNA. *Proc. nat. Acad. Sci. Wash.* **71,** 1785 (1974).
22. H. Tapiero, M. N. Monier, D. Shaool and J. Harel, Distribution of repetitious sequences in chick nuclear DNA. *Nucleic Acids Res.* **1** (2), 309 (1974).
23. R. H. Burdon and A. Shenkin, Uridylate-rich sequences in rapidly labelled RNA of mammalian cells. *FEBS Lttr.* **24,** 11 (1972).

Europ. J. Cancer Vol. 12, pp. 363–370. Pergamon Press 1976. Printed in Great Britain

The Effects of Dexamethasone on Mouse Skin Initiation and Aryl Hydrocarbon Hydroxylase*

SARA THOMPSON and THOMAS J. SLAGA

Fred Hutchinson Cancer Research Center, 1102 *Columbia Street, Seattle, Washington* 98104, *U.S.A.*

Abstract—*Mouse epidermis contains a NADPH dependent aryl hydrocarbon hydroxylase (AHH), which is inducible by polycyclic hydrocarbons. The effects of dexamethasone, an anti-inflammatory steroid, on skin carcinogenesis and on mouse epidermal AHH were investigated. Dexamethasone was found to reduce tumor initiation by both 3-methylcholanthrene and 7,12-dimethylbenz(a)anthracene. Dexamethasone applied topically to mice at a dose level of 25 or 75 μg slightly stimulated the AHH activity in mouse epidermis with peaks of 150 or 180% of controls, respectively, at 2 hr and a return to control level by 4 hr. This was followed by a weak inhibition between 4 and 8 hr after treatment with either dose of dexamethasone. Dexamethasone did not inhibit benz(a)anthracene-induced AHH activity in vitro when added to the assay, even at concentrations greater than equimolar amounts to benzo(a)pyrene. However, if dexamethasone or fluocinolone acetonide were applied topically with 3-methycholanthrene (MC), they inhibited the MC induction of AHH by at least 50% between 4 and 18 hr after simultaneous treatment. The more potent anti-inflammatory steroids, fluocinolone acetonide and fluclorolone acetonide, had a greater inhibitory effect on MC induction of AHH after simultaneous treatment. The mouse skin tumor promoters, croton oil and 12-0-tetradecanoylphorbol-13-acetate, had only a weak stimulatory effect on the AHH activity. The mechanism by which the anti-inflammatory steroids inhibit tumor initiation may be related to their ability to inhibit the metabolism of polycyclic hydrocarbons to their ultimate carcinogenic form(s).*

INTRODUCTION

IT HAS been shown by Belman and Troll [1] that a series of steroidal anti-inflammatory agents inhibited, in a dose dependent manner, tumor promotion by croton oil in mouse skin (dexamethasone > Schering No. 11572 > prednesolone > hydrocortisone > cortisone) which correlated with their anti-inflammatory activities in mouse skin. They also showed that dexamethasone inhibited croton oil induced hyperplasia. Nakai [2] also showed that MC-induced sarcomas in mice was inhibited by steroids in the order dexamethasone > triamcinolone > methyl prednesolone > hydrocortisone > cortisone and that this correlated with their anti-inflammatory potencies. We later showed that dexamethasone could completely suppress tumor promotion by 12-0-tetradecanoylphorbol-13-acetate (TPA) for 6 months with a judicious choice of doses [3]. Recently, dexamethasone was found to reduce tumor initiation and complete carcinogenesis by MC-[4]. It also inhibited mouse epidermal DNA synthesis [5] and binding of radioactive phorbol esters to a cytosol protein and chromatin [6]. Dexamethasone decreased TPA-induced hyperplasia and also reduced several protein fractions of epidermal cytosol as revealed by polyacrylamide gels which were greatly enhanced after TPA treatment [6].

If a common mechanism exists for the inhibitory effect of dexamethasone on tumor initiation, promotion and complete carcinogenesis, it may be related to an alteration in the metabolism of the carcinogen and tumor promoter by the mixed-function oxidase system. In order to test this possibility, the effects of dexamethasone on epidermal aryl hydrocarbon hydroxylase (AHH) were investigated.

Accepted 22 October 1975.

*This work was supported in part by grant CA-13155 from the National Cancer Institute of NIH.

MATERIAL AND METHODS

Animals

Female Charles River CD-1 mice were purchased from Charles River Mouse Farms, North Wilmington, Mass. Mice 7–9 weeks old were carefully shaved with surgical clippers 2 days before treatment and only those mice in the resting phase of the hair cycle were used in biochemical and tumor experiments. Groups of 30 mice were used in the tumor experiments. The incidence of both papillomas and carcinomas were recorded weekly and at random papillomas and carcinomas were removed for histological verification.

Chemicals

Benz(a)anthracene (BA), benz(a)pyrene (BP) and 7,8-benzoflavone (7,8-BF) were obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. 3-Methycholanthrene (MC) was purchased from J. T. Baker, Philipsburg, New Jersey, and dexamethasone and 7,12-dimethylbenz(a)anthracene (DMBA) from Sigma Chemical Company, St. Louis, Missouri. Croton oil was obtained from S. B. Penick and Company, New York, New York. 12-0-Tetradecanoylphorbol-13-acetate (TPA) was prepared as previously described [7] and purified by preparative thin layer chromatography. 3-Hydroxybenzo(a)pyrene was a generous gift from Dr. H. V. Gelboin, National Cancer Institute, Bethesda, Maryland. The anti-inflammatory steroids, fluocinolone acetonide and fluclorolone acetonide* were generously supplied by Syntex Corporation, Palo Alto, California. All compounds were applied topically in acetone. In experiments where both a carcinogen and dexamethasone were given together, we applied them simultaneously in acetone. Similar results were obtained if dexamethasone was given 5 min before the carcinogen.

AHH enzyme assay

A modification of an assay described by Bowden *et al.* was used [8]. Three mice were used for each enzyme assay. Mice were killed between 8:00 and 10:00 a.m. by cervical dislocation, and Nudit cream (generously supplied by Helena Rubinstein, Inc.) was applied to the shaved area of the back. After 5 min the Nudit cream was thoroughly washed off under cold running water and the skins were removed and placed on ice. The whole skin was placed dermis side down on a cold glass plate and the epidermis was scraped off with fifteen strokes of a razor blade and placed in 1 ml of a 0·25M sucrose–0·05M Tris buffer pH 7·5. The epidermal material from three mice was homogenized with a Polytron PT10 homogenizer for 45 sec at setting "6". This epidermal homogenate was the source of enzyme for the majority of AHH assays.

The assay was performed in semi-darkness in a total volume of 1 ml which contained 50 μmoles Tris–HCl buffer pH 7·5, 0·5 mg of NADPH, 3 μmoles $MgCl_2$, 0·2 ml skin homogenate (containing 2–4 mg protein) and 100 nmoles of BP (added in 40 μl of acetone just prior to starting the incubation). The reaction mixture was incubated in the air at 37°C for 30 min. The reaction was stopped by adding one ml of acetone and placing the reaction flask on ice. Next, 3·25 ml of *n*-hexane was added to the flask. The flasks were returned to the 37°C incubator for 10 min with vigorous shaking and then a 2·5 ml aliquot of the organic phase was extracted with 2·5 ml of 1·0N NaOH by vigorous vortexing for 1 min. The concentration of the extracted phenolic derivatives in the alkaline phase was determined spectrophotofluorometrically with excitation at 396 nm and emission at 522 nm in a Turner spectrofluorometer. The spectrofluorometer was calibrated with an authentic sample of 3-hydroxybenzo(a)pyrene. The enzyme units are defined as the pmoles equivalent to the fluorescence of 3-hydroxybenzo(a)pyrene formed in 30 min of incubation. Enzyme activity was determined in duplicate and compared with a blank control flask to which acetone had been added to incubation. The protein content of the homogenates was determined by the Lowry method using bovine serum albumin as a standard.

The specific activity is expressed as pmoles of 3-hydroxybenzo(a)pyrene formed in 30 min of incubation per mg of protein. In most cases, the specific activities are expressed in the charts as a percentage of the acetone control groups and each time point tested represents an average of two to five groups containing three mice each. The average percentage standard deviation for the control groups for each experiment is plotted on each chart to indicate the extent of variation in the data.

*Fluocinolone acetonide, 6α, 9α-difluoro-11β, 16α, 17, 21-tetrahydroxypregnan-1,4-diene-3, 20-dione, cyclic 16,17-acetal; fluclorolone acetonide, 6α-fluoro-9α, 11β-dichloro-16α, 17α, 21-triol-pregnan-1,4-diene-3, 20-dione, cyclic 16,17-acetal.

RESULTS

Isolated epidermis has 4–5 times the AHH activity as the remaining dermis and about twice that of whole skin.* It was found that 15 strokes of a razor blade, if preceded by Nudit cream depilatory agent, was sufficient to remove all the epidermal layer without much dermal contamination. Additional scraping only added dermal material to the preparation. Without Nudit cream, no consistent separation of the epidermis from the dermis could be obtained.

Figure 1 shows the effect of a single topical application of dexamethasone on tumor initiation by MC. Dexamethasone caused at least a 50% reduction in both the percentage of animals with tumors and the number of papillomas per mouse. In the control mice, initiated and promoted only, 43% of the surviving mice developed carcinomas after 44 weeks, whereas only one out of 30 mice developed a carcinoma in the dexamethasone treated mice after 44 weeks. Figure 2 shows a similar inhibitory effect when a single topical application of dexamethasone was given with DMBA initiation.

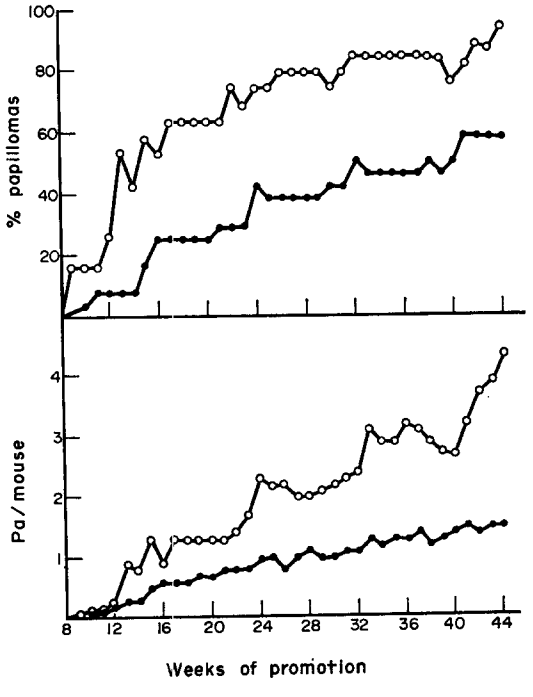


Fig. 1. Inhibition of MC tumor initiation by 75 μg of dexamethasone. Each group consisted of 30 mice. All animals were initiated with 13·4 μg MC and promoted with 10 μg TPA twice weekly. Dexamethasone was given once simultaneously with MC initiation. Control (MC) ○——○; dexamethasone + MC ●——●.

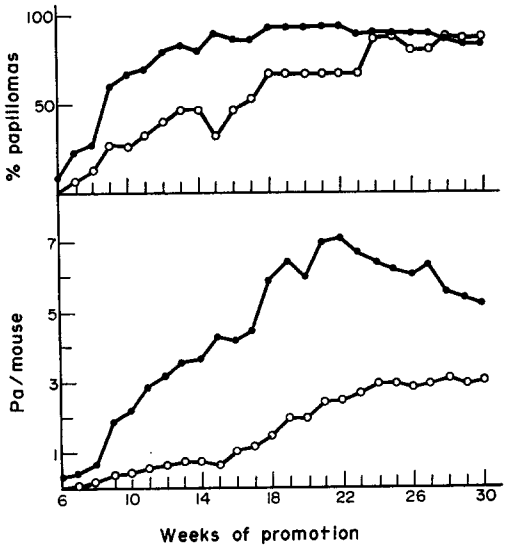


Fig. 2. Inhibition of DMBA tumor initiation by 75 μg of dexamethasone. Each group consisted of 30 mice. All animals were initiated with 2·5 μg DMBA and promoted with 10 μg TPA twice weekly. Dexamethasone was given once simultaneously with DMBA initiation. Control (DMBA) ●——●; dexamethasone + DMBA ○——○.

Since dexamethasone is so effective in inhibiting polycyclic hydrocarbon carcinogenesis, as well as phorbol ester promotion in mouse skin, it was decided to study the effect of dexamethasone on AHH activity. Figure 3 shows that dexamethasone, when applied topically to mice, slightly stimulated the AHH

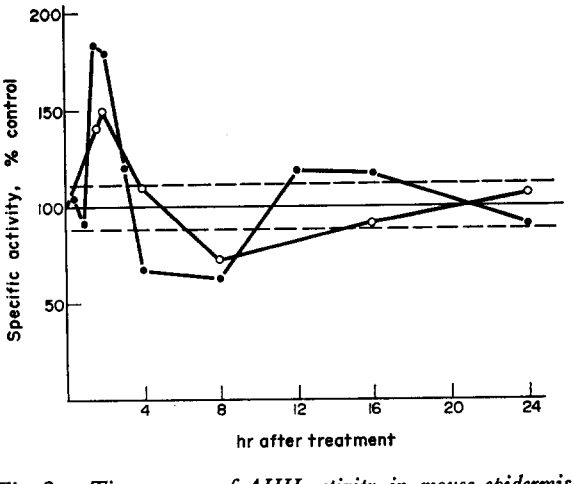


Fig. 3. Time course of AHH activity in mouse epidermis after a single topical application of either 25 (○) or 75 μg (●) of dexamethasone. The epidermis was isolated by 15 strokes of a razor blade. The specific activity is defined as pmoles of 3-OH-BP/mg protein/30 min. The specific activity is expressed as a percentage of the acetone control groups and each time point tested represents an average of 2–5 groups containing 3 mice each. The average percentage standard deviation for the control groups (dashed line) is plotted on the figure to indicate the extent of variation in the data.

*S. Thompson and T. J. Slaga: Mouse Epidermal Aryl Hydrocarbon Hydroxylase. Submitted to J. Investigative Dermatology.

specific activity in mouse epidermis with a peak of 180% of controls at 2 hr for the higher dose and then returning to control level by 4 hr. A similar response was observed when dexamethasone was applied topically at a dose of 25 μg. The *in vitro* effect of various concentrations of dexamethasone and fluocinolone acetonide on BA-induced mouse epidermal AHH activity was also determined. Dexamethasone did not inhibit the *in vitro* AHH activity when added directly to the incubation tubes even at concentrations greater than equimolar amounts to the substrate, BP (Table 1).

Table 1. In vitro *effect of dexamethasone, fluocinolone acetonide and 7,8-BF on AHH activity from mouse epidermis. The mice were pretreated topically with* 0·5 mg *of BA* 17 *hr before being sacrificed.**

In vitro addition†	BA-Induced Concentration	(SA % of Controls)
Dexamethasone	$2{\cdot}55 \times 10^{-6}$	93
	$2{\cdot}55 \times 10^{-5}$	99
	$6{\cdot}37 \times 10^{-5}$	105
	$1{\cdot}27 \times 10^{-4}$	96
	$2{\cdot}55 \times 10^{-4}$	90
Fluocinolone	10^{-4}	74
acetonide	2×10^{-4}	56
7,8-BF	10^{-4}	8
	2×10^{-4}	4

*The enzyme source for the assays was epidermal homogenate and the substrate concentration was 10^{-4} M benzo(a)pyrene (BP). The average SA for the BA-treated mice was 356 pmoles of 3-OH-BP formed/mg of protein.

†The compounds in acetone were added directly to the incubation tubes at the concentrations as indicated. Each value represents an average of at least 2 experiments with duplicate determinations for each experiment.

Similar *in vitro* results were obtained with dexamethasone when mouse epidermal AHH was not induced or was induced by MC. Fluocinolone acetonide had a mild inhibitory effect on the *in vitro* AHH activity.

However, 7,8-BF is a very effective inhibitor of AHH activity (96% inhibition) when added to the incubation mixture as was also reported by others [8, 9]. Table 2 depicts the *in vitro* effect of various concentrations of steroids without anti-inflammatory ability on BA-induced mouse epidermal AHH activity. All of the steroids tested inhibited to various degrees the *in vitro* AHH activity when added directly to the incubation tubes. Estradiol inhibited the *in vitro* AHH activity to a comparable level as shown for 7,8-BF in Table 1.

Table 2. In vitro *effect of a series of steroids on AHH activity from mouse epidermis. The mice were pretreated topically with* 0·5 *mg of BA* 17 *hr before being sacrificed.**

In vitro addition†	Concentration (M)	BA-induced (SA % of Control)
estrone	10^{-4}	8·9
	2×10^{-4}	11·9
estradiol	10^{-5}	8·8
	10^{-4}	6·0
	2×10^{-4}	4·8
estriol	10^{-4}	19·1
	2×10^{-4}	12·1
testosterone	10^{-5}	60·0
	10^{-4}	48·2
	2×10^{-4}	25·0
progesterone	10^{-4}	52·1
	2×10^{-4}	30·0

*The enzyme source for the assays was epidermal homogenate and the substrate concentration was 10^{-4} M benzo(a)pyrene (BP). The average SA for the BA-treated mice was 356 pmoles of 3-OH-BP formed/mg of protein.

†The steroids in acetone were added directly to the incubation tubes.

Figure 4 reveals the interesting results that

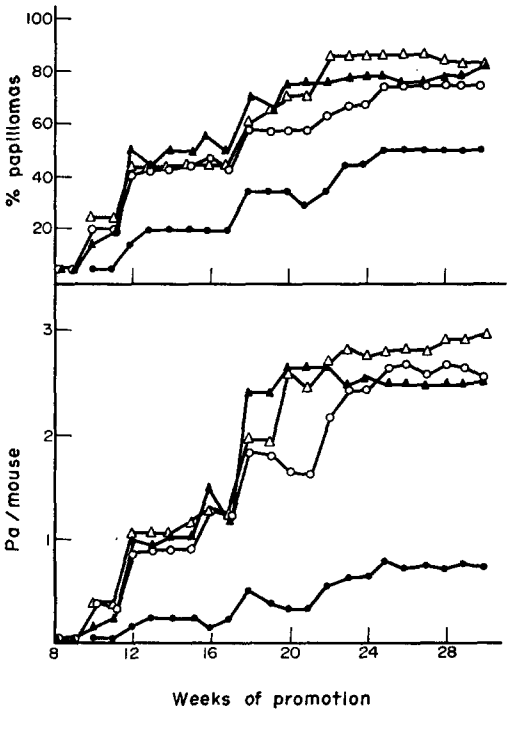


Fig. 4. The effect of simultaneous treatment of 20 μg *estradiol,* 20 μg *testosterone, or* 2·7 μg *7,8-BF on DMBA tumor initiation. Each group consisted of* 30 *mice and all animals were initiated with* 2·56 μg *of DMBA and promoted with* 10 μg *of TPA twice weekly. Control (DMBA)* ○——○; *DMBA* + 7,8-*BF* ●——●; *DMBA* + *estradiol* △——△; *and DMBA* + *testosterone* ▲——▲.

when either estradiol or testosterone were given simultaneously with DMBA they did not have any effect on DMBA tumor initiation, whereas, 7,8-BF at a concentration less than that used for the steroids was a potent inhibitor of DMBA tumor initiation.

Figure 5 depicts the inducibility of epidermal AHH by 200 nmoles of MC applied topically

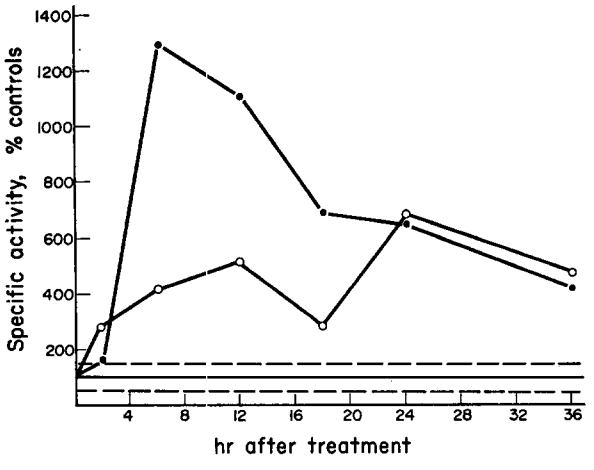


Fig. 5. Time course of AHH activity in mouse epidermis after a single topical application of either 200 nmoles of MC (●) or 200 nmoles of MC plus 75 μg of dexamethasone (○). See Fig. 3 for details. Each time point tested represents an average of 3–5 groups containing 3 mice each. The average percentage standard deviation for the control groups (dashed line) is plotted on the figure to indicate the extent of variation in the data.

and the effect of simultaneous dexamethasone treatment on AHH induction by MC. By 8 hr after MC treatment, there was about a 13-fold increase in AHH activity and greater than a 4-fold increase 36 hr after MC. Simultaneous treatment of 75 μg of dexamethasone *in vivo* inhibited the MC induction of AHH by more than 50% for the first 18 hr after treatment. Dexamethasone at a dose level of 75 μg had a much greater inhibitory effect on MC induction of AHH by 50 nmoles which was the same dose used in the tumor initiation experiments. Similar results were obtained when dexamethasone was given simultaneously with 200 nmoles DMBA *in vivo*. However, DMBA had less of an inducing effect on epidermal AHH than MC. We found that 10 μg of fluocinolone acetonide also inhibited MC-induced AHH activity, but for a longer period than 75 μg of dexamethasone (Table 3). Fluclorolone acetonide and fluocinolone acetonide at dose levels of 10 and 1 μg, respectively, also had an inhibitory action on MC-induced epidermal AHH activity, but less than that of 10 μg of fluocinolone acetonide (Table 3). Recently, fluocinolone acetonide was found to be at least 10 times more active in inhibiting TPA-induced hyperplasia and TPA tumor promotion than dexamethasone.*

The possibility was entertained that croton oil and tumor promoting phorbol esters may have an inducing effect on AHH activity and that dexamethasone may have a counteracting effect on the induction. Figure 6 reveals that croton oil and TPA had a minor effect on AHH activity in mouse epidermis when compared to that of polycyclic hydrocarbons. Both compounds gave a reproducible but small increase in epidermal AHH specific activity 12 and 24 hr after treatment. Because of the minor

*The inducibility of epidermal AHH by 200 nmoles of MC appears different when comparing the results in Fig. 5 and Table 3 because of the way we expressed the data. In Fig. 5, the AHH specific activity is expressed as a percentage of the acetone controls, whereas, in Table 3 it is not.

Table 3. The effect of steroidal anti-inflammatory agents on the induction of aryl hydrocarbon hydroxylase by 200 nmoles of 3-methylcholanthrene (MC) in mouse epidermis

Time after Treatment (hr)	SA after MC only*	Compounds given simultaneously with MC (Specific Activity expressed as percent of MC treated mice.)		
		10 μg F.A.	1 μg F.A.	10 μg F.C.
2	44	206		
6	126	83	115	82
12	349	39	75	79
18	274	71	79	55
24	353	50	112	115
36	253	58	73	

*The specific activity is expressed as pmoles of 3—OH—BP formed/mg of epidermal protein/30 min. The average specific activity of un-induced epidermis is 40·8.

inducing effect of croton oil and TPA on epidermal AHH activity, the effect of dexamethasone on the induction would be without meaning.

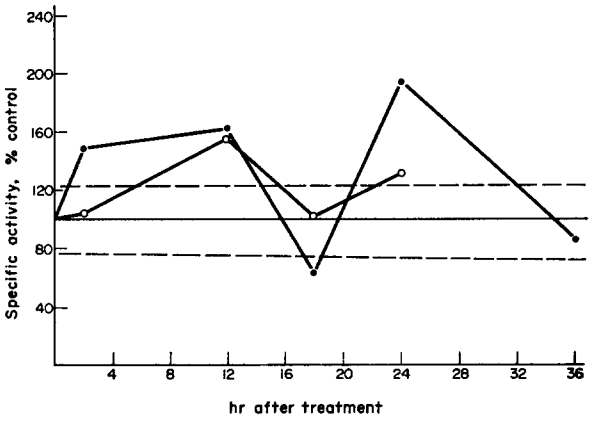


Fig. 6. Time course of AHH activity in mouse epidermis after a single topical application of either 1 mg of croton oil (●) or 5 μg of TPA (○). See legend to Fig. 3 for details. Each time point tested represents an average of 2 groups containing 3 mice each. The average percentage standard deviation for the control groups (dashed line) is plotted on the figure to indicate the extent of variation in the data.

DISCUSSION

Our results show that dexamethasone, a potent inhibitor of chemical carcinogenesis in mouse skin, has only a slight inducing effect on epidermal AHH activity, which occurs at 2 hr. Most polycyclic hydrocarbon carcinogens induce a several fold increase in AHH activity which lasts for at least 24 hr [8, 10]. Dexamethasone did not inhibit the *in vitro* AHH activity when added directly to the incubation tubes even at concentrations greater than equimolar amounts to the substrate, BP. Briggs and Briggs [11] also showed that several synthetic steroids did not inhibit the epidermal AHH activity when added to the *in vitro* assay at concentrations equimolar to BP. Hydrocortisone, cortisone and dexamethasone were reported to have no effect on the AHH activity in hamster fetal cell culture [12]. However, Briggs and Briggs [11] reported that certain synthetic corticosteroids applied topically in ointment twice daily for 7 days induced AHH activity in mouse epidermis comparable to that of MC. There are several differences in experimental design that could account for the lack of agreement in results. First, they applied their steroids in ointment, whereas we applied ours in acetone. Second, they applied the steroids twice daily for 7 days with a total dose ranging from approximately 2 to 35 mg, which may have had a systemic effect, whereas, we applied dexamethasone once at a dose of 25 or 75 μg. Finally, we used female mice in our experiments and they used male mice.

Several investigators have shown that steroidal anti-inflammatory agents can reduce the tumor response to mouse skin promoters [1, 13, 14]. With a judicious choice of doses, Scribner and Slaga [3] reported a complete suppression of TPA tumor promotion by dexamethasone for 6 months. Another report by the above authors showed that dexamethasone reduced tumor initiation and complete carcinogenesis by MC [4]. In this report, both DMBA and MC tumor initiation were inhibited by dexamethasone. Cortisone also was reported to reduce mouse skin tumors by the complete carcinogens, BP [13], MC [15] and DMBA [16]. A recent study showed that certain non-steroidal anti-inflammatory agents were partially effective in decreasing the promoting ability of TPA [4]. However, there was no correlation between their effects on tumorigenesis and on inflammation-hyperplasia [5, 17].

The slight induction of epidermal AHH by dexamethasone *in vivo* and the lack of inhibition by dexamethasone when added directly to the *in vitro* AHH assay was at first discouraging because we hypothesized that the inhibitory effect of dexamethasone on polycyclic hydrocarbon carcinogenesis was through its effect on the induction of AHH in mouse epidermis. However, we found that dexamethasone if applied simultaneously with MC inhibits the MC induction of AHH by approximately 61% at 12 hr after treatment (Fig. 4). Similar results were obtained with two new anti-inflammatory steroids (Table 3). It is possible that simultaneous dexamethasone treatment inhibits the metabolism of MC to an ultimate carcinogenic form; such a mechanism would explain its action on polycyclic hydrocarbon initiation and complete carcinogenesis. In a preliminary experiment, we found that microsome dependent covalent binding of MC to DNA *in vitro* was decreased when epidermal microsomes were isolated from mice pretreated with dexamethasone and MC 12 hr before sacrificing as compared to MC pretreated mice.

We considered the possibility that croton oil and tumor promoting phorbol esters may have an inducing effect on AHH activity and that dexamethasone may have an inhibitory effect on the induction. Figure 5 reveals that the tumor promoting agents, croton oil and TPA, had a minor but reproducible stimulatory effect on AHH activity in mouse epidermis. Since croton oil and TPA had such a slight

effect on AHH activity, the effect of dexamethasone on the induction of AHH by tumor promoters would be without meaning. The possibility still exists that esterase activity could be affected by dexamethasone which could explain the inhibition of tumor promotion and the binding of tumor promoters to a cytosol receptor protein and chromatin by simultaneous dexamethasone treatment [3, 6]. Kreibich *et al.* [18, 19] reported that tumor promoting phorbol esters are poorly metabolized by mouse skin and Hela cells, but are metabolized to a considerable extent in cultures of L-cells where phorbol esters do not block DNA synthesis or stimulate choline incorporation into phospholipids which showed an excellent correlation with the promoting ability of a series of phorbol esters [20, 21].

The mechanism by which dexamethasone and other glucocorticoids inhibit mouse skin tumor promotion and complete carcinogenesis may be related to their ability to inhibit DNA synthesis which consequently counteracts the induction of hyperplasia induced by tumor promoters and complete carcinogens [5]. We recently found that dexamethasone also inhibits TPA-induced increase in DNA synthesis. In preliminary experiments, we have found that fluocinolone acetonide is at least 10 times more active than dexamethasone in inhibiting DNA synthesis, counteracting TPA-induced increase in DNA synthesis and epidermal hyperplasia and in inhibiting the induction of epidermal AHH by MC. Also, our results indicate that there is excellent correlation between the promoting ability of a series of phorbol esters and their ability to induce epidermal hyperplasia. The correlation completely fails when other compounds are compared outside the phorbol ester series [22]. Furthermore, the anti-inflammatory ability of a series of steroids was correlated with their ability to inhibit mouse skin tumor promotion by TPA and their ability to counteract TPA-induced hyperplasia (fluocinolone acetonide > fluocinonide ⩾ dexamethasone ⩾ cortisol) [23]. Once again, the correlation fails when comparing non-steroidal anti-inflammatory agents [24]; Belman and Troll [1] also showed that a series of steroidal anti-inflammatory agents inhibited tumor promotion by croton oil in mouse skin. They also showed that the steroids they tested were truly anti-inflammatory to mouse skin, which we also showed for the steroids we tested using their criteria of inflammation [1, 24].

The effect of dexamethasone on tumor initiation by polycyclic hydrocarbons [4] is another point at issue. Low initiating doses of polycyclic hydrocarbons have been shown to inhibit DNA synthesis without subsequent inflammation or hyperplasia [5]. As stated earlier, we have found that dexamethasone and other powerful anti-inflammatory steroids if applied simultaneously with MC, inhibit the MC induction of AHH. The major effect of the anti-inflammatory steroids on tumor initiation may be related to their inhibiting the metabolism of MC to an ultimate carcinogenic form, which we are currently investigating.

REFERENCES

1. S. Belman and W. Troll, The inhibition of croton oil-promoted mouse skin tumorigenesis by steroid hormones. *Cancer Res.* **32,** 450 (1972).
2. T. Nakai, Influences of small doses of various corticosteroids on the incidence of chemically induced subcutaneous sarcomas in mice. *Cancer Res.* **21,** 221 (1961).
3. J. D. Scribner and T. J. Slaga, Multiple effects of dexamethasone on protein synthesis and hyperplasia caused by a tumor promoter. *Cancer Res.* **33,** 542 (1973).
4. T. J. Slaga and J. D. Scribner, Inhibition of tumor initiation and promotion by anti-inflammatory agents. *J. nat. Cancer Inst.* **51,** 1723 (1973).
5. T. J. Slaga, S. Thompson and E. A. Smuckler, Prolonged inhibition of mouse epidermal DNA synthesis by dexamethasone. *J. nat. Cancer Inst.* **54,** 931 (1975).
6. T. J. Slaga, J. D. Scribner, J. M. Rice, S. B. Das and S. Thompson, Inhibition by dexamethasone of intracellular binding of phorbol esters to mouse skin. *J. nat. Cancer Inst.* **52,** 1611 (1974).
7. W. M. Baird and R. K. Boutwell, Tumor-promoting activity of phorbol and four diesters of phorbol in mouse skin. *Cancer Res.* **31,** 1074 (1971).
8. G. T. Bowden, T. J. Slaga, B. G. Shapas and R. K. Boutwell, The role of aryl hydrocarbon hydroxylase in skin tumor initiation by 7,12-dimethylbenz(a)anthracene and 1,2,5,6-dibenzanthracene, using DNA binding and thymidine-^{3}H incorporation into DNA as criteria. *Cancer Res.* **34,** 2634 (1974).

9. H. V. Gelboin, F. J. Wiebel and L. Diamond, Dimethylbenzanthracene tumorigenesis and aryl hydrocarbon hydroxylase in mouse skin. Inhibition by 7,8-benzoflavone. *Science* **170,** 169 (1970).
10. N. Kenoshita and H. V. Gelboin, The role of aryl hydrocarbon hydroxylase in 7,12-dimethylbenz(a)anthracene in skin tumorigenesis: on the mechanism of 7,8-benzoflavone inhibition of tumorigenesis. *Cancer Res.* **32,** 1329 (1972).
11. M. H. Briggs and M. Briggs, Induction by topical corticosteroids of skin enzymes metabolizing carcinogenic hydrocarbons. *Brit. J. Dermatol* **88,** 75 (1973).
12. D. W. Nebert and H. V. Gelboin, Substrate-induced microsomal aryl hydroxylase in mammalian cell culture II. Cellular response during enzyme induction. *J. biol. Chem.* **243,** 6250 (1968).
13. R. K. Boutwell, Some biological aspects of skin carcinogenesis. *Progr. expt. Tumor Res.* **4,** 207 (1964).
14. N. Trainin, Adrenal imbalances in mouse skin carcinogenesis. *Cancer Res.* **23,** 415 (1963).
15. R. Baserga and P. Shubik, The action of cortisone on transplanted and induced tumors in mice. *Cancer Res.* **14,** 12 (1954).
16. R. N. Ghadially and H. N. Green, The effect of cortisone on chemical carcinogenesis in the mouse skin. *Brit. J. Cancer* **8,** 291 (1954).
17. J. D. Scribner and T. J. Slaga, Influence of non-steroid anti-inflammatory agents on protein synthesis and hyperplasia caused by a tumor promoter. *J. nat. Cancer Inst.* **52,** 1865 (1974).
18. G. Kreibich, I. Witte and E. Hecker, On the biochemical mechanism of tumorigenesis in mouse skin IV. Methods for determination of fate and distribution of phorbol ester TPA. *Z. Krebsforsch.* **76,** 113 (1971).
19. G. Kreibich, R. Süss and V. Kinzel, On the biochemical mechanism of tumorigenesis in mouse skin V. Studies of the metabolism of tumor promoting phorbol derivatives *in vivo* and *in vitro*. *Z. Krebsforsch* **81,** 135 (1974).
20. R. Süss, V. Kinsel and G. Kreibich, Cocarcinogenic croton oil factor A. Stimulates lipid synthesis in cell cultures. *Experientia (Basel)* **27,** 46 (1971).
21. R. Süss, G. Kreibich and V. Kinsel, Phorbol esters as a tool in cell research. *Europ. J. Cancer* **8,** 299 (1972).
22. T. J. Slaga, G. T. Bowden and R. K. Boutwell, Acetic acid, a potent stimulator of mouse epidermal macromolecular synthesis and hyperplasia but with weak tumor-promoting ability. *J. nat. Cancer Inst.* (In press).
23. T. J. Slaga, S. Thompson and J. A. Schwarz, unpublished results.
24. T. J. Slaga, Inflammation, hyperplasia and tumor promotion, to be submitted for publication in *J. invest. Dermatol.*
25. T. J. Slaga, G. T. Bowden, B. G. Shapas and R. K. Boutwell, Macromolecular synthesis following a single application of polycyclic hydrocarbons used as initiators of mouse skin tumorigenesis. *Cancer Res.* **34,** 771 (1974).

Europ. J. Cancer Vol. 12, pp. 371–379. Pergamon Press 1976. Printed in Great Britain

The Effects of Adriamycin and Daunomycin on Antitumoral Immune Effector Mechanisms in an Allogeneic System*

A. MANTOVANI†, A. VECCHI, A. TAGLIABUE and F. SPREAFICO‡

Istituto di Ricerche Farmacologiche "Mario Negri"
Via Eritrea, 62-20157 *Milano, Italy*

Abstract—*C3H mice pretreated with adriamycin (AM) 24 hr before transplantation with the allogeneic L1210 leukemia had a significantly longer survival than mice given an equitoxic dose of its analog daunomycin (DM). The effect of the two drugs on cellular and humoral immune effector mechanisms was thus investigated. The longer surviving AM-pretreated tumor allografted mice were found to have higher levels of cell-mediated cytotoxicity (CMC) in the peritoneal cavity, e.g., at the site where tumor growth was actually occurring. On the contrary when spleen CMC was investigated, either no differences were seen or DM-hosts had higher cytotoxic activity. DM was also found to cause a faster impairment than AM of the spleen capacity to mediate antibody-dependent cellular cytotoxicity (ADCC). As regards humoral responses, serum complement-dependent and arming activity were absent in both test groups, whereas higher levels of potentiating activity were found in AM pretreated hosts.*

The possible significance of the lower impairment of antitumoral immune effector mechanisms in giving AM its superior antineoplastic effectiveness is discussed.

INTRODUCTION

ADRIAMYCIN (AM), an agent currently regarded of great value in the treatment of a remarkably wide range of human neoplasms [1–5], has repeatedly been shown to possess a superior antitumoral activity in experimental and clinical conditions in comparison to its structural analog daunomycin (DM) [1–8]. Recently, evidence has been obtained strongly suggesting that a different interaction with the host's immune reactivity represents one important factor in giving AM its greater therapeutical efficacy over DM.

In fact the higher antineoplastic activity of AM is no longer detectable in previously immunosuppressed hosts [7] or when non-antigenic tumor variants are investigated [9]. In previous studies [10,11] we have also shown that at equitoxic doses AM is significantly less suppressive than DM of allogeneic skin and tumor rejection in mice. The complex effects of the two drugs on humoral antibody production to T lymphocyte-dependent and independent antigens have been described [11].

In order to gain an insight into the mechanisms sustaining the lower depression by AM of tumor allograft resistance, a comparative analysis of the effects of the two antibiotics on the cellular and humoral immune effector mechanisms operative in this condition was undertaken.

Since these mechanisms are in many respects similar to those detectable in the case of syngeneic tumors [12], the system employed could also be expected to be informative of the real *in vivo* conditions in which these agents are used.

Accepted 22 December 1975.

*Supported by NCI Contract No. C-72-3242.

†Recipient of a fellowship of the Anna Villa Rusconi Foundation.

‡To whom correspondence should be addressed at: Dept. Tumor Immunology and Chemotherapy—Istituto di Ricerche Farmacologiche "Mario Negri"—Via Eritrea, 62—20157 Milano, Italy.

MATERIAL AND METHODS

Animals. Female C3H mice (23 ± 1 g body weight) obtained from Charles River, Italy were used for this study.

Drugs. AM and DM, obtained from Farmitalia, Milan, Italy, were freshly dissolved in saline and injected i.v. 24 hr before tumor transplantation in the dose of 15 mg/kg. In this mouse strain, the acute LD_{50} dose is equal for both drugs [11].

Tumor. The L1210 leukemia was maintained by weekly i.p. passages in syngeneic DBA/2 (*H-2d*) hosts. After removal of contaminating erythrocytes by osmotic lysis and washings in 199 medium, 10^7 cells were transplanted i.p. into incompatible C3H ($H-2^k$) mice; cell viability was assessed microscopically by dye exclusion and only preparations showing over 95% viability were employed. In another series of experiments, the mice were transplanted on 3 successive days with 10^7 x-irradiated L1210 cells, beginning 1 day after drug treatment. The x-ray dose was 10,000 r delivered by a Philips RT 250 x-ray apparatus operating at 250 kV, 8 mA at a rate of 780 r/min.

Sera, splenocytes and peritoneal exudate cells (PECs). Serum was obtained from the retro-orbital plexus of 5–10 mice per group, heat-inactivated at 56°C for 30 min, and stored at −20°C until used. Spleens were gently teased in chilled 199 medium, washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI-FCS; Biocult Scotland). For the obtainment of PECs, the peritoneal cavity of the C3H recipients was washed by repeated flushing with 199; after 2 × washing, the cells were resuspended in RPMI-FCS.

When donors transplanted with live tumor cells were used, to separate PECs from the leukemia cells an agglutination procedure was employed. After osmotic shocking of the erythrocytes, 5 ml of ascites cell suspension containing $20–40.10^6$ cells per ml of 199 were incubated for 45 min at 37°C with 0·8 ml of an anti-L1210 serum prepared in C3H mice by the i.p. injection of 10^7 leukemia cells at 15 days intervals and killing the mice 10 days after the 3rd booster. At the end of the incubation the upper 2 ml were removed, and cells washed × 3. Cell preparations obtained in this way contained 1–2 leukemic cells per normal lymphoid element and this ratio was not significantly different in the various experimental groups in any given experiment.

Cell-mediated cytotoxicity (CMC) assay. A 4 hr ^{51}Chromium release assay was used, test conditions being as previously detailed [13,14] except for the use of only 10^4 target cells per sample. In each experiment, 5 mice per group were used with at least 2 replicates per sample. Specific cytotoxicity was expressed as the difference in percent isotope release in the presence of test and normal lymphoid cells. Routinely a range of attacker to target cell (A:T) ratios (20:1 to 200:1) was employed and a semilog plot of the specific cytotoxicity values versus the number of effector cells per sample was obtained. The number of cells giving 33% specific cytotoxicity was arbitrarily defined as 1 LU-33. Since in the first days after tumor transplantation splenocyte specific cytotoxicity did not reach the 33% value, the lower LU-10 limit was employed, this value being always within the linear part of the cytotoxicity versus effector cell number plot. This approach thus allowed a quantitative estimate of the total cytotoxic capacity of a given organ [12,15].

Complement-dependent serum cytotoxicity. A two step ^{51}Cr release assay was used [13]; technical conditions were as previously described [16].

Antibody-dependent cellular cytotoxicity (ADCC) assay. Serum arming activity [17] was assessed by mixing 0·1 ml of test serum with 5.10^4 labelled target cells in 1 ml RPMI-FCS followed by the addition of normal C3H splenocytes at an A:T ratio of 100:1. For the measurement of serum potentiating [17] activity, the same conditions were employed except for the use of immune C3H splenocytes, i.e., cells obtained from control, non drug-treated donors 7 days after transplantation of 10^7 L1210 leukemia cells. Percentage of arming and potentiation were expressed as the difference in isotope release between test and normal sera [14], the latter having been collected from sex and age-matched controls.

To investigate the effects of drug treatment on ADCC-mediating cells, splenocytes (5.10^6 in 0·1 ml) from individual organs were added to tubes containing 5.10^4 labelled L1210 cells in 1 ml RPMI-FCS followed by 0·1 ml of a 1:10 dilution of an ADCC-positive reference serum. Specifically, serum collected from C3H mice 45 days after challenge with 10^7 L1210 cells was employed. An index of the total spleen capacity to mediate ADCC was calculated from the formula: % specific cytotoxicity × total number of spleen cells ($\times 10^6$)/100, where the percentage of specific cytotoxicity in this case is the difference in isotope

release in the presence of ADCC-positive and normal C3H serum.

Statistical analysis. Data presented are mean ± S.E. of 5 mice per experimental group and are representative of at least 3 experiments; statistical significance was assessed by Duncan's new multiple range test.

RESULTS

When normal C3H mice are transplanted i.p. with 10^7 allogeneic L1210 leukemia cells, 100% of the animals are capable of surviving this tumor challenge. In these conditions the number of leukemia cells in the peritoneal cavity grows to a peak of approximately 200.10^6 cells on day 7–8 rapidly declining thereafter so that on days 10–11 the mice are leukemia-free. If the animals are injected with 15 mg/kg i.v. of AM or DM 24 hr before tumor transplantation, progressive tumor takes are observed in all recipients. However, leukemia growth is faster and death occurs significantly earlier in the DM-pretreated mice than in those given an equal dose of AM, survival being 8·0 ± 0·5 and 13·7 ± 1·2 days, respectively ($p < 0.05$). That this finding is not attributable to a greater residual chemotherapeutic activity of AM has been discussed elsewhere [11].

CMC. The effects of AM and DM in these experimental conditions on CMC were first examined in the spleen (Table 1). In tumor-rejecting controls, a progressive increase in CMC was observed from day 3 to day 10 as evidenced by the rising levels of specific isotope release at a given A:T ratio and by the appearance of a significant cytotoxicity at gradually lower A:T ratios. A drop in total spleen cytocydal capacity was then observed on day 12. In drug-treated mice a qualitatively similar pattern could be recognized, however the spleen cytotoxicity capacity was markedly lower than in controls as indicated by the LU values. With regard to the effects of the two analogs, no differences were found between AM and DM on days 3 and 5 whereas on day 7, i.e., on the day preceding death of the DM-injected mice, these animals actually showed a significantly higher cytotoxic capacity than their longer living AM-counterparts. At later times, the cytocydal activity of AM-splenocytes was not different from that of an equal number of control cells, although total spleen CMC capacity of the drug treated hosts was 40–50% lower than in control mice.

Since spleen CMC did not appear to correlate with the *in vivo* results, attention was turned to the evaluation of the direct cytotoxic activity of PEC's, i.e., at the site where tumor growth was actually occurring. The pre-

Table 1. *Spleen CMC in AM and DM pretreated tumor allografted mice*

Day	Experimental group	No. of cells (× 10^6)/spleen	% specific cytotoxicity 20:1[a]	100:1[a]	200:1[a]	No. of LU mouse
	Control	121·5	—	7·1 ± 0·4	14·3 ± 1·9	25 ± 5[b]
3	AM	48·5	—	—	9·0 ± 2·9	—
	DM	25·3	—	—	8·2 ± 1·5	—
	Control	130·0	4·0 ± 0·7	10·2 ± 1·9	19·5 ± 2·6	130 ± 15[b]
5	AM	38·9	—	6·5 ± 1·1	17·3 ± 2·6	8 ± 2[b]
	DM	40·2	—	4·4 ± 0·5	16·1 ± 1·2	7 ± 2[b]
	Control	155·3	5·4 ± 1·0	12·1 ± 0·4	30·1 ± 2·5	777 ± 40[b]
7	AM	40·2	—	8·9 ± 0·6	19·9 ± 0·8	76 ± 25[b]
	DM	38·3	4·8 ± 1·8	13·2 ± 0·6**	24·6 ± 0·6**	188 ± 20*[b]
	Control	151·1	15·5 ± 2·1	45·5 ± 2·4	30·8 ± 2·8	189 ± 15[d]
10	AM	63·0	17·1 ± 0·6	53·5 ± 2·3	36·2 ± 0·6	90 ± 14[d]
	DM	NT[c]	NT[c]	NT[c]	NT[c]	NT[c]
	Control	160·2	5·0 ± 1·5	36·5 ± 2·6	36·6 ± 0·9	168 ± 33[d]
12	AM	82·0	7·0 ± 0·4	30·4 ± 1·5	38·7 ± 0·9	67 ± 9[d]
	DM	NT[c]	NT[c]	NT[c]	NT[c]	NT[c]

[a]A:T ratio

[b]LU-10

[c]Not tested since mean survival in this group was 8·0 ± 0·5 days

[d]LU-33.

**$p < 0.01$ versus AM

*$p < 0.05$ versus AM

treatment with AM or DM of tumor allografted C3H mice resulted in a decrease in specific cytotoxicity in respect to controls. In addition the cytotoxic activity of PEC's collected from AM-pretreated mice was significantly higher than that displayed by equal number of cells recovered from DM-preinjected hosts. The specific cytotoxicity found on day 5 were in fact 24·2 ± 1·9; 12·5 ± 1·8 and 4·9 ± 2·1% for the control, AM and DM groups, respectively. On day 7, the values were 29·1 ± 1·6, 20·0 ± 2·2 and 8·9 ± 2·5%, respectively, the difference between the AM and DM groups being $p < 0·01$ in both cases. These results were obtained using 10^6 PEC effector cells, containing 1·25 contaminating tumor cell per normal lymphoid element, and 10^4 target leukemia cells, the A:T ratio thus being 0·8. Total cytotoxic capacity per animal could not be estimated in these conditions since the inability to completely remove contaminating leukemic cells from the PEC preparations, precluded an exact plotting of specific cytotoxicity values versus the number of effector cells. The method for preparation of PEC's from these tumor-bearing animals could in addition have resulted in the loss of potentially cytocydal elements. Moreover, the different tumor loads existing in the two drug-treated groups could have influenced CMC expression. Therefore, mice allografted with x-irradiated L1210 cells were also investigated.

It was observed (Table 2) that the pretreatment with AM or DM resulted in a decrease of the total number of recoverable PEC's evident already 3 days after allografting and persisting until day 13, when observation was discontinued. No statistically significant differences between the two drugs were found. As regards CMC, results obtained in these conditions were consistent with those found in mice given a live tumor challenge. In fact, the specific cytotoxicity of AM-PEC's was not significantly different from that displayed by equal numbers of control cells when tested in the 25:1–100:1 A:T range. However, as indicated by the LU-33 values, a lower total number of cytotoxically active PEC's was present in AM-treated mice than in controls. When cells from animals given an equitoxic dose of DM were assayed, it was found that their cytotoxic activity was significantly lower than that of AM-PEC's; in the days 3–7 period, the number of LU-33 was in fact approximately 1/3 of that estimated for AM-treated animals. The reduction caused by these drugs in the number of cytotoxic PEC's was long lasting since on day 13 the LU-33 values were still halved in respect to controls, with no differences at this time between AM and DM.

Serum cytolytic, arming and potentiating activities. In control mice, anti-L1210 complement dependent cytolytic antibodies were first detected 6 days after the transplantation of 10^7 leukemia cells, the 1:4 titer increasing progressively thereafter to a value of 1:64 on day 13. In drug-pretreated animals no significant activity could ever be detected in our conditions. The same sera were also assayed for the presence of

Table 2. Peritoneal CMC in Am and DM pretreated mice

Day	Experimental group	No. of PEC (× 10^6)/ mouse	% specific cytotoxicity 25:1[a]	50:1[a]	100:1[a]	No. of LU-33/ mouse
	Control	12·6	25·8 ± 1·1	33·2 ± 2·7	33·3 ± 1·8	25·2 ± 1·4
3	AM	7·5	24·3 ± 2·2	35·6 ± 2·0	44·8 ± 1·9	15·9 ± 1·9
	DM	5·2	20·0 ± 0·6	24·3 ± 1·6**	31·6 ± 2·6**	5·2 ± 2·1**
	Control	14·8	28·7 ± 2·1	31·1 ± 1·5	40·0 ± 3·0	35·9 ± 2·7
5	AM	10·3	24·4 ± 2·9	39·5 ± 1·0	39·9 ± 3·1	25·3 ± 2·3
	DM	8·7	19·2 ± 1·8	28·4 ± 2·9**	36·1 ± 2·2	9·7 ± 2·5**
	Control	15·6	34·5 ± 1·6	43·6 ± 1·3	54·8 ± 1·9	44·6 ± 2·2
7	AM	6·4	38·5 ± 2·6	52·8 ± 1·4	56·8 ± 3·4	32·0 ± 2·4
	DM	4·5	23·3 ± 1·5**	41·7 ± 1·8**	53·3 ± 1·7	11·8 ± 2·8**
	Control	12·5	19·5 ± 0·8	24·3 ± 2·2	38·7 ± 2·0	19·1 ± 1·1
13	AM	6·1	18·6 ± 2·9	30·8 ± 1·3	39·0 ± 1·5	10·4 ± 1·4
	DM	6·4	13·3 ± 0·6	20·1 ± 1·6**	35·9 ± 2·4	8·3 ± 1·6

C_3H mice were treated with AM or DM on day −1 and immunized with 10^7 x-irradiated L1210 leukemia cells i.p. on day 0, +1 and +2.

[a]A:T ratio

**$p < 0·01$ versus AM

Table 3. Cytolytic antibody levels and serum arming activity in AM and DM pretreated, tumor allografted mice

Day after tumor allograft	Serum	% Arming	Cytolytic antibody titer
	Control	—	—
3	AM	—	—
	DM	—	—
	Control	0·6 ± 0·5	1:2
6	AM	—	—
	DM	—	—
	Control	2·3 ± 1·1	1:4
7	AM	0·7 ± 0·5	—
	DM	1·5 ± 1·2	—
	Control	6·5 ± 0·5**	1:8
8	AM	1·4 ± 0·9	—
	DM	NT[a]	NT[a]

Arming values presented here were obtained employing a final serum dilution of 1:15 and an A:T ratio of 100:1.

[a] Not tested since mean survival in this group was 8·0 ± 0·5 days.

**$p < 0·01$ versus normal C3H serum.

arming and potentiating activities. As shown in Table 3, which gives representative results obtained with 1:15 serum dilutions, no significant arming capacity was found in drug-injected animals and tests performed with a range of serum dilutions (from 1:1 to 1:120) were also consistently negative. On the other hand, control mice possessed a significant and gradually rising serum arming activity from day 8 onwards.

For a first assessment of potentiating activity, the sera were tested at a final dilution of 1:15 and with the A:T ratio of 20:1. As judged from the fact that the level of CMC displayed by reference immune splenocytes was not modified, no potentiating activity was present in the sera from control or treated mice 3 and 5 days after tumor allograft (Table 4). A significant activity was detectable in controls and AM-treated hosts on day 6, whereas DM-sera became positive only on day 7 in 2 experiments and never showed activity in 2 other experiments. In addition, the potentiating activity displayed by the day 7 positive DM-sera was in these testing conditions, significantly lower than that of AM-sera, the latter being as active as control materials during the time period examined. Preliminary experiments had shown that in the absence of lymphoid attacker cells, these sera did not significantly modify isotope release from background levels, thus the observed changes in CMC do not appear to be attributable to non specific toxic effects. Moreover, the same sera were unable in the same conditions to affect the cytotoxic capacity of C3H or Balb/c splenocytes immune against the EL-4 lymphoma.

In view of the fact that the A:T ratio employed [14] and the serum concentration used in the test [18–20] can be critical variables in detecting synergistic or antagonistic effects of humoral factors on *in vitro* CMC, in successive experiments the potentiating activity of different sera obtained on day 6 from control and treated mice was evaluated employing a range of A:T ratios (20:1 to 100:1) as well as titered using the 20:1 A:T ratio.

Table 4. Serum potentiating activity in AM and DM pretreated tumor allografted mice.

Day	Serum	% specific cytotoxicity	% potentiation
	Normal	8·2 ± 1·6	—
	Control	8·9 ± 0·4	—
3	AM	7·9 ± 0·6	—
	DM	8·5 ± 0·5	—
	Normal	9·5 ± 0·4	—
	Control	8·9 ± 1·4	—
5	AM	9·8 ± 0·6	—
	DM	9·2 ± 1·8	—
	Normal	7·8 ± 1·5	—
	Control	15·2 ± 0·5	7·4
6	AM	14.8 ± 0·7	7
	DM	8·1 ± 1·8**	—
	Normal	7·8 ± 1·5	—
	Control	21·9 ± 1·1	14·1
7	AM	24·3 ± 1·3	16·5
	DM	13·1 ± 0·5**	5·3

Potentiation was assessed employing a final dilution of 1:15 and an A:T ratio of 20:1.

**$p < 0·01$ versus AM.

Table 5 shows that sera obtained on day 6 from DM-mice were negative for potentiating activity not only at low but also at higher A:T ratios. At variance, with control and AM-sera an increase in cytotoxic activity of a similar degree was observed passing from a 20:1 to a 40:1 A:T ratio, whereas no significant modifications in CMC were detected at a 100:1 A:T ratio. Although the potentiating activity displayed the AM-sera employed for these tests was lower than seen with other preparations, the differences in specific cytotoxicity observed were still statistically significant ($p < 0·01$) in respect to DM-sera. Since the highest potentiation had been found with these sera employing a 20:1 A:T ratio, this ratio

Table 5. *Serum potentiating activity of AM and DM sera at different A : T ratios*

A:T	Serum	% specific cytotoxicity	Potentiation
	Normal	32·2 ± 0·7	—
	Control	39·1 ± 1·0	6·9
20:1	AM	39·8 ± 1·8	7·6
	DM	27·2 ± 2·4**	—
	Normal	46·7 ± 0·9	—
	Control	56·4 ± 0·7	9·7
40:1	AM	55·9 ± 0·7	9·2
	DM	49·1 ± 2·1**	—
	Normal	52·3 ± 1	—
	Control	51·1 ± 0·9	—
100:1	AM	51·8 ± 0·5	—
	DM	53·2 ± 1·1	—

Sera were collected on day 6 after tumor allograft and tested for potentiating activity at a final dilution of 1:15.

** $p < 0.01$ versus AM.

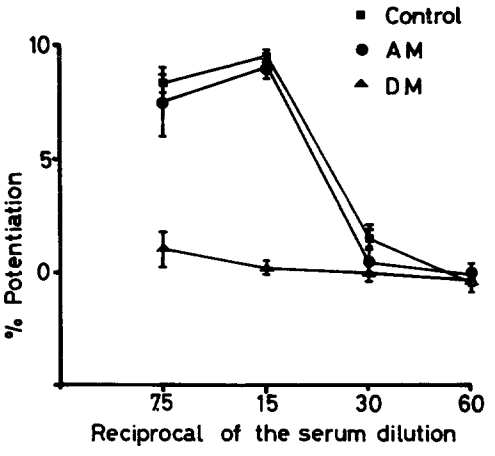


Fig. 1. *Potentiating antibody titers in control AM and DM pretreated, tumor allografted mice. Serum were collected* 6 *days after tumor allografting; the A : T ratio was* 20:1.

was used for investigating whether changes in serum concentration could influence the detection of potentiating capacity. As presented in Fig. 1, the negative DM-sera were devoid of activity at all dilutions tested and with the day 6 control and AM-sera used, activity was no longer detectable at a 1:30 dilution.

Spleen ADCC-mediating activity. Lastly, the effects of AM and DM on the functional activity of spleen ADCC-mediating cells were assessed. Spleen cell preparations obtained from non-tumor allografted C3H mice 2 days after the injection of 15 mg/kg AM or DM and exposed to a reference ADCC positive serum displayed an increased cytocydal activity with respect to control splenocyte preparations (Table 6),

Table 6. *ADCC-mediating activity in AM and DM pretreated, normal or tumor allografted mice*

Day after treatment	Exp. group	No. of cells (× 10^6)/spleen	% specific cytotoxicity	ADCC index
	Control	133	4·2 ± 0·5	5·6
2	AM	69	7·2 ± 0·7*	5·0
	DM	32	8·1 ± 0·5*	2·8
	Control	128	4·8 ± 0·6	6·1
6	AM	50	4·9 ± 0·8	2·5
	DM	43	5·1 ± 0·9	2·5
	Control	125	4·6 ± 1·4	5·7
9	AM	73	4·9 ± 0·9	3·6
	DM	82	4·8 ± 1·0	3·9
	Control	120	5·1 ± 1·0	6·1
2[a]	AM	75	8·5 ± 0·8*	6·4
	DM	30	9·4 ± 1·1*	2·8
	Control	131	9·2 ± 0·4	12·1
6[a]	AM	39	9·5 ± 0·7	3·7
	DM	40	8·9 ± 0·3	3·6
	Control	155	15·0 ± 0·3	23·3
8[a]	AM	38	13·2 ± 1·6	5·0
	DM	42	14·5 ± 0·9	5·5

Serum collected from C3H mice 45 days after transplantation of 10^7 L1210 leukemia cells was employed as ADCC positive serum at a dilution of 1:10. Specific cytotoxicity was calculated subtracting the isotope release with normal serum from samples containing the ADCC-positive serum.

[a]tumor allograft 24 hr after treatment

*$p < 0.05$ versus control spleen cells

the increase being of similar degree in both test groups. However, since spleen cellularity in drug-treated animals was lower than in controls and significantly more so in the DM group, the total spleen ADCC index was decreased only for the latter animals whereas remaining at control level in AM-injected mice. On day 6 after drug administration, no differences in splenocyte ADCC effector capacity were detected between control and test groups but the index was lower in treated mice than in controls, with no differences between AM and DM groups since cell depletion at this time was practically equal in these animals. On day 9, in parallel to the tendency to recover of spleen cellularity, a raise in the index value was observed. The findings obtained in tumor allografted animals were practically superimposable to those described for normal animals.

DISCUSSION

These studies confirm previous data [10,11, 21] showing that AM and DM differ in their capacity to affect immune responsiveness and, specifically, that AM is significantly less depressive than DM of those immune effector mechanisms currently regarded as the main determinants of host resistance against neoplasms.

Since CMC is generally considered to play a major role in the rejection of allogeneic neoplasma [12], it was not surprising to observe that in the AM-treated animals having a slower tumor progression, a higher level of CMC was detectable in the peritoneal cavity, e.g. at the actual site of tumor-host interaction. That this higher CMC is the result of a higher number of cytotoxically active PEC's is shown by the findings obtained when irradiated leukemia cells were used. The latter conditions not only allowed a quantitative assessment of the response in neater methodological conditions but also excluded the possible influences on CMC expression of different tumor loads between the two test groups. No immediate explanation is available for the lack of correlation between the data *in vivo*, those obtained with PEC's and the CMC results in the spleen. In fact the longer living AM-pretreated hosts appeared to contain significantly lower numbers of directly cytotoxic cells in the spleen than their DM-counterparts at a time (day 7) when the cellular depletion induced by the agents in this organ had attained similar levels. A migration of immune cells from the spleen to peripheral sites has been described in mice [22]; it could thus be hypothesized that in AM-animals this process occurred to a greater extent thus leading to a higher number of effector cells in the peritoneal cavity. Although no attempts were made to characterize the cellular composition of PEC's, it has been found that peritoneal cytotoxicity is largely dependent on lymphoid elements [23]. In addition, preliminary experiments have shown only a minor decrease in the cytotoxic activity of PEC preparations from which phagocytic-adherent elements had previously been removed. Whatever the underlying mechanism(s), the spleen results stress the importance of investigations of immune reactivity at the site(s) where tumor growth is actually occurring, for a more representative appraisal of real *in vivo* conditions from *in vitro* data.

Whereas no explanatory clues were provided by the measurement of complement dependent cytolytic antibodies which were absent in both test groups, a further immune parameter positively correlated with the relatively greater allograft resistance of AM-mice was their higher serum potentiating activity in comparison to DM-treated hosts. Since serum ADCC activity has been shown to segregate with IgG molecules [24–26], the observation of a lower potentiating capacity in DM-animals parallels our previous finding that this agent is more depressant than AM of secondary IgG responses [11].

As indicated by the ADCC index, both AM and DM reduced the total number of ADCC-mediating cells in the spleen, AM being however less destructive than DM since the latter induced a significantly faster and more prolonged cell depletion in this organ, as described in greater detail elsewhere [27]. The relative increase over controls in the *in vitro* spleen ADCC-mediating capacity seen in the first days after drug injection in both allografted and normal animals, indicates that at least some of the ADCC-effector cells have a lower sensitivity to these drugs than other splenocyte subpopulations. It appears thus, that both the cellular and humoral arms of ADCC were less severely impaired by AM than by DM and could therefore have activ ely contributed to the relatively greater tumor allograft resistance of AM treated hosts. A role for ADCC also in the rejection of syngeneic tumors has recently been advanced on the basis of serum transfer experiments [28,29]. Our findings showing that in the animals with the faster tumor growth, lower levels of potentiating factors and lower ADCC indexes were present, are in favour of the participation of

this mechanism also in mediating the resistance to tumor allografts.

The mechanism(s) responsible for the lower impairment of allograft resistance by AM in comparison to DM are still a matter of speculation. One possible hypothesis could be based on the pharmacokinetic differences between the two drugs and especially in the slower accumulation of AM in the spleen [7]. The lower cell depletion induced in this organ by AM [27] would thus permit the recruitment of a higher number of immune effector cells. On the basis of the differential sensitivity to AM and DM of the response to a T-independent antigen [11], a relative selectivity of DM for T cells, e.g., for the cell type which is considered crucial for allograft rejection [12] could have been suggested. However, evidence discussed in an accompanying report is difficult to reconcile with this hypothesis.

The reasons for the higher antitumoral effectiveness of AM over DM in clinical and experimental conditions have been the object of debate [7,11,30]. The possibility originally suggested by Schwartz and Grindey [7], that differences in immunodepressive activity between the two analogs could be, if not the exclusive, an important factor in this phenomenon seems substantiated by our data. In view of the fact that the reactivities evoked by syngeneic and allogeneic neoplasms can be regarded as similar in many respects, the lower impairment of antitumoral immune effector mechanisms could indeed be an element of great importance in giving AM its better therapeutic activity. In this regard it is noteworthy to add that we have confirmed in other syngeneic murine tumor models that the chemotherapeutic advantage of AM over DM is reduced or lost in previously immunosuppressed hosts (Filippeschi and Spreafico, in preparation).

REFERENCES

1. S. Monfardini, G. Bonadonna, S. Di Pietro, A. Guindani, F. Fossati-Bellani and M. De Lena, Sperimentazione clinica preliminare con Adriamicina in pazienti affetti di leucemia e tumori solidi in fase avanzata. *Tumori* **55,** 197 (1969).
2. G. Bonadonna, S. Monfardini, M. De Lena, F. Fossati-Bellani and G. Beretta, Phase I and preliminary phase II evaluation of Adriamycin (NSC 123127). *Cancer Res.* **30,** 2572 (1970).
3. J. J. Wang, E. Cortes, L. F. Sinks and J. F. Holland, Therapeutic effect and toxicity of Adriamycin in patients with neoplastic disease. *Cancer (Philad.)* **28,** 837 (1971).
4. E. Middleman, J. Luce and E. Frei III, Clinical trials with Adriamycin. *Cancer (Philad.)* **28,** 844 (1971).
5. R. H. Blum and S. K. Carter, Adriamycin. A new anticancer drug with significant clinical activity. *Ann. intern. Med.* **80,** 249 (1974).
6. J. S. Sandberg, F. L. Howsden, A. Di Marco and A. Goldin, Comparison of antileukemic effect in mice of adriamycin (NSC-123127) with daunomycin (NSC-82151). *Cancer Chemother. Rep.* **54,** 1 (1970).
7. H. S. Schwartz and G. B. Grindey, Adriamycin and Daunorubicin: a comparison of antitumor activities and tissue uptake in mice following immunosuppression. *Cancer Res.* **33,** 1837 (1973).
8. A. M. Casazza, A. Di Marco and G. Di Cuonzo, Interference of daunomycin and adriamycin on the growth and regression of Murine Sarcoma Virus (Moloney) tumors in mice. *Cancer Res.* **31,** 1971 (1971).
9. F. Guiliani, A. M. Casazza and A. Di Marco, Virologic and immunologic properties and response to Daunomycin and Adriamycin of a non-regressing mouse tumor derived from MSV-induced sarcoma. *Biomed. Express* **21,** 435 (1974).
10. A. Vecchi, A. Mantovani, C. Barale and F. Spreafico, Differential activity on humoral and cell mediated immunity of Adriamycin and Daunomycin. In *AbstractsXI International Cancer Congress*, Florence Oct. 20–26, V.3, p. 381, Casa Editrice Ambrosiana, Milan (1974).
11. A. Vecchi, A. Mantovani, A. Tagliabue and F. Spreafico, A characterization of the immunosuppressive activity of adriamycin and daunomycin on humoral antibody production and tumor allograft rejection. *Cancer Res.* In press (1975).

12. J.-C. Cerottini and K. T. Brunner, Cell-mediated cytotoxicity, allograft rejection and tumor immunity. *Advanc. Immunol.* **18,** 67 (1974).
13. A. Mantovani and F. Spreafico, Allogenic tumor enhancement by Levamisole, a new immunostimulatory compound: studies on cell-mediated immunity and humoral antibody response. *Europ. J. Cancer* **11,** 537 (1975).
14. A. Mantovani, A. Vecchi and F. Spreafico, The attacker: target cell ratio and serum effect on *in vitro* cell-mediated immunity. *J. nat. Cancer Inst.* **55,** 483 (1975).
15. J.-C. Cerottini and K. T. Brunner, *In vitro* assay of target cell lysis by sensitized lymphocytes. In *In Vitro Methods in Cell-Mediated Immunity,* (Edited by B. R. Bloom and P. R. Glade), p. 369, Academic Press, New York (1971).
16. A. Cohen and M. Schlesinger, Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation* **10,** 130 (1970).
17. I. Hellström, K. E. Hellström and G. A. Warner, Increase of lymphocyte-mediated tumor-cell destruction by certain patient sera. *Int. J. Cancer* **12,** 348 (1973).
18. R. Kiessling and E. Klein, Cytotoxic potential of mouse spleen cells on H-2 antibody-treated target cells. *J. exp. Med.* **137,** 527 (1973).
19. H. M. Skurzak, E. Klein, T. O. Yoshida and E. W. Lamon, Synergistic and antagonistic effect of different antibody concentrations on *in vitro* lymphocyte cytotoxicity in the Moloney Sarcoma Virus System. *J. exp. Med.* **135,** 997 (1972).
20. S. Britton and J. Forman, Some characteristics of the antibody-induced cell-mediated cytotoxicity against leukemia cells. *Transplantation* **17,** 180 (1974).
21. A. M. Casazza, F. Guiliani, A. M. Isetta, M. Soldati and A. Di Marco, Immunodepressive activity of antitumor drug: comparison between daunomycin and adriamycin. In *XI International Cancer Congress,* Florence Oct. 20–26, V.4 p. 753, Casa Editrice Ambrosiana, Milan (1974).
22. J. Sprent, Circulating T and B lymphocytes in the mouse. I. Migratory properties. *Cell. Immunol.* **7,** 10 (1973).
23. G. Berke, K. A. Sullivan and B. Amos, Rejection of ascites tumor allografts. I. Isolation and *in vitro* reactivity of peritoneal lymphoid effector cells from BALB/c mice immune to EL4 leukosis. *J. exp. Med.* **135,** 1334 (1972).
24. I. C. M. MacLennan, G. Loewi and A. Howard, A human serum immunoglobulin with specificity for certain homologous target cells which induces target cell damage by normal human lymphocytes. *Immunology* **17,** 897 (1969).
25. G. Möller and S. E. Svehag, Specificity of lymphocyte-mediated cytotoxicity induced by *in vitro* antibody-coated target cells. *Cell. Immunol.* **4,** 1 (1972).
26. P. Perlmann and H. Perlmann, Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. *Cell. Immunol.* **1,** 300 (1970).
27. A. Mantovani, A. Tagliabue, A. Vecchi and F. Spreafico, Effects of Adriamycin and Daunomycin on spleen cell populations in normal and tumor allografted mice. *Eur. J. Cancer* **12,** 381 (1976).
28. P. Hersey, New look at antiserum therapy of leukaemia. *Nature New Biol.* **244,** 22 (1973).
29. J. Zighelboim, B. Bonavida and J. L. Fahey, Antibody-mediated *in vivo* suppression of EL4 leukemia in a syngeneic host. *J. nat. Cancer Inst.* **52,** 879 (1974).
30. W. D. Meriwether and N. R. Bachur, Inhibition of DNA and RNA metabolism by Daunorubicin and Adriamycin in L1210 mouse leukemia. *Cancer Res.* **32,** 1137 (1972).

Europ. J. Cancer Vol. 12, pp. 381–387. Pergamon Press 1976. Printed in Great Britain

Effects of Adriamycin and Daunomycin on Spleen Cell Populations in Normal and Tumor Allografted Mice

A. MANTOVANI*, A. TAGLIABUE, A. VECCHI and F. SPREAFICO
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62-20157 Milano, Italy

Abstract—*In the attempt to elucidate the complex differential interaction of adriamycin (AM) and daunomycin (DM) with immune reactivity, the effects of these agents on spleen cell subpopulations were investigated in normal and tumor-allografted drug-pretreated mice. In terms of total organ cellularity, DM induced a faster and longer lasting cell-depletion than AM, although the nadir values were similar for both drugs. The percentages of theta positive and EA-rosette forming cells (EA-RFCs) were not modified by the two drugs in normal animals, whereas the increase in percent EA-RFCs observed in control tumor allografted mice was partially inhibited by both drugs. Macrophages were relatively spared by these agents as evidenced by an increase in their percent value.*

INTRODUCTION

AMONG the more recent cancer chemotherapeutic agents, adriamycin (AM) has become the object of much interest in view of its high activity on a remarkably wide range of human tumors. It has also been observed that AM possesses a higher antineoplastic effectiveness than its analog Daunomycin (DM) in both experimental and clinical conditions [1–4] but the reasons for this superiority of AM are still a matter of discussion [3, 5]. In previous studies evidence has been presented supporting the contention, originally advanced by Schwartz and Grindey [3], that differences in immunosuppressive activity between the two drugs could be an important factor in giving AM its higher antitumoral capacity. In fact, although the two analogs are equitoxic in terms of acute LD_{50} and of their effects on the bone marrow, AM was clearly less inhibitory than DM of tumor allograft resistance [6, 7] in a system in which the same immune effector mechanisms known to be operative in the response to syngeneic neoplasms are at play. Similarly, AM is less active than DM in prolonging skin allograft survival [8, 9]; both these reactivities are believed to be sustained by T lymphocytes, i.e., the elements credited with playing a major role in the response to syngeneic neoplasms [10,11]. From an analysis of the complex effects of the two analogs on the immune response to foreign erythrocytes and to a T-independent antigen such as pneumococcal polysaccharide S-III [6] the hypothesis was advanced that DM possessed in comparison to AM a relative selectivity for T cells. An investigation of the changes in the spleen lymphoid subpopulations after administration of these agents to normal and tumor-allografted mice was therefore of interest in the attempt to elucidate the cellular basis for the complex differential interaction of AM and DM with immune responsiveness.

Accepted 22 December 1975.

This work was supported by Contract NIH-NCI-C-72-3242.

*A. Mantovani was supported by a Fellowship of the Anna Villa Rusconi Foundation.

MATERIAL AND METHODS

Animals

Female C3H mice (23 ± 1 g body weight) obtained from Charles River, Italy were used throughout. In each experiment the animals were carefully matched for age and body weight.

Drugs

AM and DM were obtained from Farmitalia

Milan; they were freshly dissolved in saline and injected intravenously (i.v.) at the dose of 15 mg/kg.

Tumor

The L1210 leukemia was maintained by weekly intraperitoneal passages in syngeneic DBA/2 (H-2^d) mice. After removal of red cells from the ascites by osmotic lysis, 10^7 viable leukemia cells were injected intraperitoneally (i.p.) into istoincompatible C3H (H-2^k) mice 24 hr after drug treatment. After this inoculum, the survival time of DM and AM pretreated mice was 8·2 ± 1·2 and 13 ± 1·5 days respectively ($P < 0{\cdot}05$) whereas all controls were capable of rejecting this leukemia inoculum [6, 7].

Macrophages

The percentage of spleen macrophages was evaluated by a modification of Evans' procedure [12]. Individual spleens were gently teased in chilled RPMI 1640 medium (Biocult; England) supplemented with 10% fetal calf serum and repeatedly dispersed with a Pasteur pipette. Splenocytes preparations (3×10^6/ml) were then incubated in a hemocytometer for 60 min at 37°C in a humidified atmosphere of 95% air–5% CO_2 in the presence of carbon particles [13]. At the end of incubation the hemocytometer was immersed in phosphate-buffered (pH 7·4) saline and the floating coverslips lifted off; this step resulted in removal of non-adhering cells and uningested particles. The number of adherent cells which had ingested carbon was then determined microscopically.

Theta-bearing cells

Anti-theta C3H serum was prepared in AKR/J mice as described by Reif and Allen [14]; 50 μl of splenocyte suspension (3×10^6 cells/ml) were incubated (45 min at 37°C) with equivalent volumes of 1:8 diluted anti-theta serum and agarose-absorbed guinea pig serum [15]. Cell viability was checked microscopically by dye exclusion and at least 200 cells per tube (3 tubes/spleen) were examined. Per cent T cells was calculated according to the formula

$$\frac{T-C}{100-C} \times 100,$$

where T and C are the per cent mortality in test and control samples containing complement alone respectively [16]. In these conditions, the anti-theta serum was capable of killing > 95% of C3H thymocytes while showing < 5% cytotoxicity on bone marrow cells.

Fc receptor bearing cells

The percentage of membrane Fc receptor bearing splenocytes was assessed as described by Kedar *et al.* [17]. Sheep erythrocytes (E) were exposed to subagglutinating concentrations of rabbit anti-E serum in order to obtain sensitized E (EA); 0·3 ml of splenocyte suspension prepared as described above were incubated (5 min at 37°C) with 4×10^7/0·3 ml EA and then centrifuged for 5 min at 100 *g* at room temperature. After 2 hr at 4°C the pellets were gently resuspended, nucleated cells stained with crystal violet (1 mg/ml in RPMI 1640) and cells which had at least 3 erythrocytes firmly bound at their periphery scored as EA-RFC's. At least 200 cells/tube were scored.

Statistical analysis

Results presented are representative data of at least 3 experiments performed with 5–8 animals per group. Statistical significance was evaluated by means of Duncan's new multiple range test.

RESULTS

Spleen cellularity

As shown in Fig. 1, the injection of 15 mg/kg i.v. of AM or DM induced marked and long-lasting decreases in the total spleen cell number in both normal and tumor-allografted mice; however, clear differences in the kinetics of this effect were observable between the two drugs. Considering first normal animals, DM induced in fact a significantly more rapid fall in the number of nucleated spleen cells than seen after an equitoxic dose of AM. In fact splenocytic decrease had practically already reached its nadir one day after DM administration, the values observed until day 6 being not significantly lower than those observed at 24 hr. With AM, the decline was slower, reaching its peak only on day 5–6, at levels which were in the same range as those found with DM. A rise in spleen cellularity was seen after day 5–6 in both groups, but the return towards normal values after this drug dose was quite long since cellularity was still approximately 75% of control range 20 days after treatment. In tumor allografted controls (Fig. 1 B) a progressive increase in spleen cell number was found already 4 days after tumor transplantation and reached a plateau on the

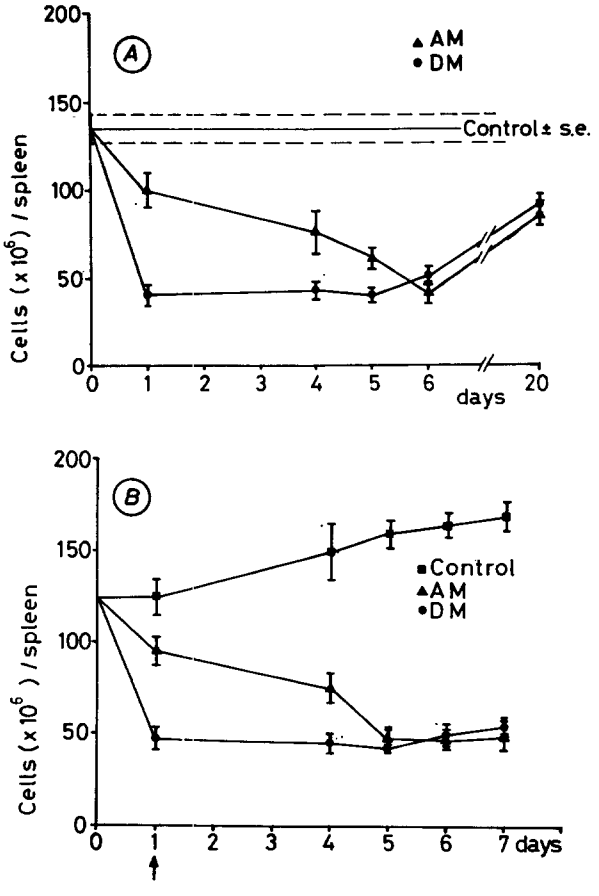


Fig. 1. Effect of AM and DM on spleen cell number in normal (A) *and tumor allografted mice* (B). *Arrow indicates the day of tumor allografting.*

10th–12th day to levels approximately 60% higher than normal. In the drug-pretreated, tumor-transplanted animals, a reduction was on the contrary observed and the cellular depletion induced by the two drugs was similar to that occurring in normal mice both in terms of the kinetics of the effect as well as with regard to the magnitudes of peak splenocyte decreases. Again in fact DM caused a much more rapid fall in cellularity with a reduction to 42% of controls already at 24 hr followed by a plateau until day 7; with AM the lowest cell number was seen 5 days after drug injection at levels not significantly different from those found in DM-pretreated animals and similar to those of non tumor-transplanted mice.

Macrophages

The changes in the percentage of phagocytic-adherent cells in the spleen of normal animals after administration of AM or DM are presented in Table 1 together with their calculated total numbers.

One day after treatment, both AM and DM-injected mice showed a similar and significant ($P < 0{\cdot}01$) increase in macrophage percentage which persisted throughout the 7 days observation period. In absolute numbers however, phagocytes were reduced in DM-treated hosts already on day 1, whereas a significant decrease was detectable after AM treatment only on day 6. In tumor-allografted control mice (Table 2), the percentage value of spleen macrophages was stable, thus in view of the increase in cellularity in these spleens, phagocyte number rose progressively and was approximately 50% higher than baseline 7 days after L1210 transplant. In drug-pretreated animals, the changes detected were essentially similar to those already described for non tumor transplanted mice; thus on the day of tumor grafting, DM-injected hosts had 50% less macrophages than either control or AM-treated mice, the latter reaching the same value only on day 6.

EA-RFC's

In normal animals, injection of these antibiotics did not cause significant changes in the percent values of Fc receptor-bearing cells as identified by their capacity to form EA-rosettes. The total number of these cells was, however, reduced by the drugs, DM causing a faster decline than AM, although the lowest values observed were practically in the same range for both agents. Following tumor-allograft a gradual increase in the percent of EA-RFC's occurred in controls so that 5 days later a 63·4% level was attained from a starting value of 44·7%. A slow decline thereafter ensued although also after complete disappearance of tumor cells from the peritoneal cavity which took place on day 10 in these conditions, the 55 ± 0·9% value observed was still significantly higher than in untreated controls. Total number of EA-RFC's obviously also increased in these mice having almost doubled on day 6. Pretreatment with AM or DM only partially inhibited this per cent increase, which however was still statistically significant in respect to normal controls; the changes in the absolute number of these elements reflected the different kinetics of cell depletion induced by the two drugs.

T cells

In non-allografted mice, neither agent induced appreciable modifications in the percent T cells, whereas in terms of the total size of this population, a reduction to 1/3–1/4 of control values was seen with both AM and DM. The duration of the depletion in this subpopulation found with DM-treatment was longer than seen with AM as previously observed also for

Table 1. Percentage and total numbers (in parenthesis, × 10^6) of spleen macrophages, EA-RFC's and T-cells in control and drug-treated C3H mice

Day after treatment*	% Macrophages			% EA-RFC's			% T-cells		
	C	AM	DM	C	AM	DM	C	AM	DM
1	8·9 ± 0·8	12·7 ± 1‡	14·3 ± 0·6‡	42·2 ± 2	41·2 ± 2·3	40·3 ± 3·2	22·7 ± 1·7	25·5 ± 2·3	25·7 ± 3·1
	(10·9 ± 0·9)	(12 ± 1)	(6·7 ± 1·2)	(52·7 ± 3·5)	(40·4 ± 6·4)	(19 ± 3)	(28·4 ± 3)	(25 ± 1·5)	(12·3 ± 2·5)
3	9·3 ± 0·5	13·6 ± 1‡	15·5 ± 0·9‡	45·4 ± 0·5	43·1 ± 2·1	44·6 ± 1·9	24·1 ± 2	22·0 ± 1·4	23·5 ± 1·1
	(11·6 ± 1·2)	(9·2 ± 1·5)	(5·3 ± 1·1)	(60·8 ± 5·4)	(29·3 ± 3·7)	(15·2 ± 2·9)	(32·3 ± 2·4)	(15·0 ± 2·2)	(8·0 ± 1·4)
4	10·8 ± 0·5	18·7 ± 2‡	19·1 ± 2‡	43·2 ± 2·5	48·0 ± 2·4	43·6 ± 0·7	21·4 ± 1·8	19·0 ± 2·0	21·2 ± 1·6
	(13·1 ± 2·1)	(12·7 ± 0·6)	(6·1 ± 1·9)	(53·1 ± 4·5)	(30·7 ± 3·9)	(13·0 ± 2·5)	(23·3 ± 3·0)	(12·3 ± 0·5)	(6·2 ± 1·4)
6	9·4 ± 0·6	13·0 ± 1·1†	12·9 ± 0·8†	45·5 ± 1·9	43·2 ± 3·1	42·0 ± 2·2	23·7 ± 1·5	22·0 ± 1·6	24·2 ± 0·9
	(12·2 ± 1·3)	(6·8 ± 1·5)	(7·0 ± 1·4)	(59·1 ± 8·5)	(22·5 ± 4·5)	(22·7 ± 4·5)	(30·8 ± 2·1)	(11·4 ± 3·2)	(13·1 ± 1·7)

*AM and DM (15 mg/kg, i.v.) were injected on day 0.
†$P < 0.05$ versus controls.
‡$P < 0.01$ versus controls.

Table 2. Percentage and total numbers (in parenthesis, × 10^6) of spleen macrophages, EA-RFC's and T-cells in drug-treated and control tumor allografted C3H mice

Day after treatment*	% Macrophages			% EA-RFC's			% T-cells		
	C	AM	DM	C	AM	DM	C	AM	DM
1	8·7 ± 0·8	12·5 ± 1·0‡	14·3 ± 0·7‡	44·7 ± 2·0	43·5 ± 2·7	43·4 ± 3·2	22·7 ± 1·7	26·9 ± 2·3	27·6 ± 3
	(11·1 ± 1·2)	(12·2 ± 0·7)	(6·7 ± 1·0)	(55·9 ± 7·4)	(42·6 ± 3·4)	(20·8 ± 3·4)	(28·4 ± 1·6)	(26·4 ± 2·2)	(13·2 ± 1)
4	9·8 ± 0·4	15·2 ± 0·7‡	13·7 ± 1·7‡	49·5 ± 1·7	46·0 ± 2·2	49·0 ± 0·6	24·8 ± 2·4	24·8 ± 0·9	27·0 ± 0·1
	(14·8 ± 1·6)	(10·9 ± 1·3)	(5·5 ± 1·7)	(74·7 ± 2·6)	(37·9 ± 8·0)	(19·6 ± 0·3)	(37·4 ± 3·6)	(19·7 ± 0·4)	(10·8 ± 1)
6	9·7 ± 0·1	14·7 ± 0·1‡	13·3 ± 0·9†	63·4 ± 1·7	50·2 ± 1·6‡	53·3 ± 1·4‡	16·9 ± 1·6	18·5 ± 1·6	17·3 ± 0·6
	(15·0 ± 1·2)	(5·6 ± 1·4)	(5·2 ± 1·6)	(98·3 ± 2·6)	(19·1 ± 2·6)	(23·0 ± 3·9)	(27·0 ± 2·6)	(7·0 ± 1·6)	(10·7 ± 2)
7	10·4 ± 1·0	14·6 ± 0·9†	16·2 ± 1·0‡	61·8 ± 2·4	51·7 ± 1·9†	53·8 ± 2·5†	18·0 ± 1·4	18·5 ± 1·2	20·2 ± 0·6
	(17·3 ± 1·7)	(5·4 ± 1·9)	(6·1 ± 1·5)	(97·9 ± 4·0)	(19·1 ± 1·7)	(22·9 ± 2·4)	(29·9 ± 2·3)	(6·8 ± 2·4)	(10·1 ± 1)

*AM and DM (15 mg/kg, i.v.) were injected on day 0 and 10^7 L1210 cells transplanted on day 1.
†$P < 0.05$ versus controls.
‡$P < 0.01$ versus controls.

EA-RFC's. In the tumor-transplanted controls a clear drop in T cells percentage was seen on day 6 and 7 after allografting, i.e., coincident with the rise in per cent EA-RFC's; this decline was transient since baseline levels were reached again on day 10. Accordingly, a significant raise in total T cells was present in the spleen of these tumor-rejecting animals only on days 8–10, e.g. at the time when in these conditions tumor cell numbers in the peritoneal cavity were rapidly declining [18]. In drug-pretreated hosts the changes in T cell percentage were practically superimposable to those of controls over the time-period investigated, total T cell numbers were reduced by both agents with the same temporal differences between DM and AM already described for EA-RFC's.

DISCUSSION

These results show that following the administration of AM and DM a marked reduction in spleen cellularity is observable together with changes in the relative proportions of the various cellular subpopulations present in the organ. In addition, it has been shown that the two analogs differed significantly in the kinetics of their effect and, consequently, in the total magnitude and duration of the cell depletion.

In fact, although the peak cell decrease was comparable with both agents, nadir depletion was reached already 24 hr after DM-injection and only on the 5th–6th day with an equitoxic dose of AM in both normal and allografted hosts. Since, as indicated by the data of normal mice, the kinetics of recovery towards normal values was similar for the two agents, DM caused a greater splenocyte depletion than its analog. In this differential effect of the two drugs on spleen cell counts it seems reasonable to attribute a major role to their different rates of entry in this organ. It has been shown in fact that DM accumulates in the spleen at significantly faster rates and reaches higher peaks than seen with AM, which is on the contrary more persisting [3, 19, Martini and Donelli, in preparation]. The differences in splenocyte depletion observed between these compounds can also be an important factor in explaining the higher immunosuppressive capacity of DM over AM in this tumor-allograft system and the changes in immune effector mechanisms operative in this condition [7]. It can be reasoned in fact that at the time of tumor allografting, a lower number of potentially responding cells were present in DM-treated mice than in their AM-preinjected counterparts thus permitting a higher degree of sensitization to the alloantigens.

This mechanism could thus be at the basis of the higher cytotoxic activity of peritoneal cells in AM than in DM-treated animals, and of the higher levels of potentiating activity in the serum of the longer living AM-hosts [7]. That the lower capacity of AM to interfere with antitumoral immune defences is probably one of the factors contributing to the higher antineoplastic effectiveness of this drug over DM has previously been discussed [3, 6, 7].

On the basis of the differential effect of AM and DM on the mouse primary and secondary responsiveness to sheep erythrocyte and the insensitivity of a T-independent stimulus to interferences by DM (6, 7), the hypothesis could in principle have been entertained that DM possessed a relative selectivity for T cells. Although no direct measurement of B cells was obtained in this study since also other cell types [17] can form EA-rosettes, the present findings are difficult to reconcile with such a hypothesis.

The observation that the percentage of T cells and EA-RFC's were essentially similar in unstimulated animals treated with either drug and, in addition, not significantly different from controls, indicates in fact that spleen depletion did not occur at the preferential expense of either of these subpopulations. An alternative explanation suggesting that the differential immunosuppressive activity of the two agents results from the interplay of the different rates of drug entry and persistence in the spleen and the different durations of the inductive periods for primary and secondary responsiveness seems, on the other hand, compatible with the complex of our data.

Spleen macrophages showed a relatively higher resistance to AM and DM in comparison to EA-RFC's and T lymphocytes as indicated by the increase in their percentage which persisted throughout the observation period in both normal and tumor-allografted mice. It is possible that the low turnover [20] of the phagocytic subpopulation plays a role in its comparatively lower destruction by these cytotoxic agents. Whatever the reasons, since macrophages have been credited with an important role in tumor resistance [21, 22], the relative sparing of these cells could contribute to the remarkable antineoplastic activity of these antibiotics.

REFERENCES

1. J. S. Sandberg, F. L. Howsden, A. Di Marco and A. Goldin, Comparison of antileukemic effect in mice of adriamycin (NSC-123127) with daunomycin (NSC-82151). *Cancer Chemother. Rep.* **54,** 1 (1970).
2. G. Bonnadonna, S. Monfardini, M. De Lena, F. Fossati-Bellani and G. Beretta, Phase I and preliminary phase II evaluation of Adriamycin (NSC 123127). *Cancer Res.* **30,** 2572 (1970).
3. H. S. Schwartz and G. B. Grindey, Adriamycin and Daunorubicin: a comparison of antitumor activities and tissue uptake in mice following immunosuppression. *Cancer Res.* **33,** 1837 (1973).
4. R. H. Blum and S. K. Carter, Adriamycin. A new anticancer drug with significant clinical activity. *Ann. intern. Med.* **80,** 249 (1974).
5. W. D. Meriwether and N. R. Bachur, Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. *Cancer Res.* **32,** 1137 (1972).
6. A. Vecchi, A. Mantovani, A. Tagliabue and F. Spreafico, A characterization of the immunosuppressive activity of Adriamycin and Daunomycin on humoral antibody production and tumor allograft rejection. *Cancer Res.* In press (1975).
7. A. Mantovani, A. Vecchi, A. Tagliabue and F. Spreafico, The effect of adriamycin and daunomycin on cellular and humoral effector mechanism in an allogeneic tumor model. Submitted for publication to *Europ. J. Cancer* **12,** 371 (1976).
8. A. M. Casazza, F. Giuliani, A. M. Isetta, M. Soldati and A. Di Marco, Immunodepressive activity of antitumor drug: Comparison between Daunomycin and Adriamycin. In *XI Int. Cancer Cong., Florence* Oct. 20–26, (Edited by Casa Editrice Ambrosiana, Milan) V. 4, p. 753 (1974).
9. A. Vecchi, A. Mantovani, C. Barale and F. Spreafico, Differential activity on humoral and cell mediated immunity of adriamycin and daunomycin. In *Abstracts XI Int. Cancer Cong., Florence,* Oct. 20–26 (Edited by Casa Editrice Ambrosiana Milan) V. 3, 0. 381 (1974).
10. J.-C. Cerottini and K. T. Brunner, Cell-mediated cytotoxicity, allograft rejection and tumor immunity. *Advanc. Immunol.* **18,** 67 (1974).
11. D. Collavo, A. Colombatti, L. Chieco-Bianchi and A. J. S. Davies, T lymphocyte requirement for MSV tumour prevention or regression. *Nature (Lond.)* **249,** 169 (1974).
12. R. Evans, Macrophages in syngeneic animal tumours. *Transplantation* **14,** 468 (1972).
13. F. de Halleux, H. S. Taper and C. Deckers, A simple procedure for identification of macrophages in peritoneal exudates. *Brit. J. exp. Path.* **54,** 352 (1973).
14. A. E. Reif and J. M. V. Allen, The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. exp. Med.* **120,** 413 (1964).
15. A. Cohen and M. Schlesinger, Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation* **10,** 130 (1970).
16. L. W. Poulter and J. L. Turk, Proportional increase in the θ carrying lymphocytes in peripheral lymphoid tissue following treatment with cyclophosphamide. *Nature New Biol.* **238,** 17 (1972).
17. E. Kedar, M. Ortiz de Landazuri and B. Bonavida, Cellular immunoadsorbents: a simplified technique for separation of lymphoid cell populations. *J. Immunol.* **112,** 1231 (1974).
18. A. Mantovani and F. Spreafico, On the nature of blocking factors and their lymphoid target cells in an allogeneic tumor system. *Europ. J. Cancer* **11,** 451–455 (1975).
19. D. W. Yesair, E. Schwartzbach, D. Schuck, E. P. Denine and M. A. Asbell, Comparative pharmacokinetics of Daunomycin and Adriamycin in several animal species. *Cancer Res.* **32,** 1177 (1972).
20. W. Zschiesche, Alkylating anticancer agents and phagocytosis. *J. Reticuloendothel. Soc.* **12,** 16 (1972).

21. R. EVANS, Macrophages and the tumour bearing host. *Brit. J. Cancer* **28,** suppl. I, 19 (1973).
22. S. A. ECCLES and P. ALEXANDER, Macrophage content of tumours in relation to metastatic spread and host immune reaction. *Nature* (*Lond.*) **250,** 667 (1974).

Europ. J. Cancer Vol. 12, pp. 389–393. Pergamon Press 1976. Printed in Great Britain

Cross-Reactions among Mouse Tumors of Different Etiology as Detected by Macrophage Electrophoretic Mobility (MEM) Test

LUISE PASTERNAK*, H. L. JENSSEN†, H. KÖHLER† and G. PASTERNAK*

**Central Institute of Cancer Research, Academy of Sciences of the Ger. Dem. Rep., Department of Experimental and Clinical Immunology, 1115 Berlin-Buch, Ger. Dem. Rep.*

and †Institute of Physiological Chemistry, University of Rostock, Research Department of Immunology, 25 Rostock, Ger. Dem. Rep.

Abstract—*The macrophage electrophoretic mobility (MEM) test was applied to experimental mouse tumors. In this experiment mice bearing transplanted tumors of chemical, physical or leukemic virus origin are sensitized against rabbit encephalitogenic protein (REP) and antigen extracts prepared from these tumors. On the basis of the extensive cross-reactions observed, the antigen(s) involved are considered different from the tumor-associated transplantation antigens (TATAs).*

INTRODUCTION

THE MACROPHAGE electrophoretic mobility (MEM) test is a sensitive method for the detection of antigen-sensitized lymphocytes [1]. In the presence of specific antigen a factor is released from the lymphocytes which reduces the electrophoretic mobility of macrophages even if of heterologous origin. The test is increasingly used in oncology for immunodiagnostic purposes. Tumor patients are supposed to be sensitized against tumor associated antigens. It was found by Field and Caspary [2] that patients with tumors are sensitized against a basic encephalitogenic protein the appearance of which is apparently connected with tumor formation. Basic proteins isolated from tumor tissue were likewise reactive in the MEM test [3].

Cross-reactions observed in this test among human tumors of different histology and organ origin indicate that sensitization occurs against antigens other than those detectable by the cellular cytotoxicity test. Cross-reactions by the cytotoxic test are restricted to tumors having the same organ origin [4].

In our experiments the MEM test has been applied to mice sensitized against tumors which reveal distinct tumor-associated transplantation antigens, TATAs [5–7]. Of particular interest was the reactivity of lymphocytes from tumor-bearing animals with the encephalitogenic proteins from human or rabbit origin and with antigen extracts from mouse tumors.

Accepted 18 December 1975.

MATERIAL AND METHODS

(a) *Antigens*

Antigens in the MEM test were encephalitogenic proteins from man and rabbits, supernatant from homogenized mouse tumors, and a potassium chloride extract from u.v. light-induced mouse sarcoma UV 14306. Human encephalitogenic protein (HEP) was prepared from human brain according to Dau and Peterson [8]. After extraction with chloroform/methanol (2:1, v/v) and repeated washings at pH 5·0–5·5 the protein was precipitated with ammonium sulfate. The protein was dissolved in 0·01 N HCl. Finally, the protein concentration was adjusted to 100 μg/ml.

The same technique was used for the preparation of rabbit encephalitogenic protein (REP).

Supernatant from homogenate was obtained after 10 min centrifugation at about 1000 × *g* of a 1:10 (w/v) in Eagle's medium diluted tumor homogenate. For homogenization of

tumors an ultraturrax (Janke & Kunkel KG, Staufen, Breisgau, FRG) homogenizer was used.

Soluble tumor antigens were isolated by the potassium chloride method according to Blasecki and Tevethia [9]. In brief, tumor cells at a concentration of 3×10^7 cells/ml were suspended in 3 M KCl in 0·07 M phosphate-buffered saline, pH 7·2. After 24 hr of extraction at 4°C the suspension was centrifuged for 1 hr at 100,000 × ***g***. Dialysis against 0·1 NaCl was followed by concentration using collodium bags (Sartorius GmbH,. Göttingen, FRG) and new dialysis against 0·15 M NaCl. The material was then stored at −20°C. Before the tests the optimal concentration was estimated by diluting the antigens.

(b) Donors of lymph node cells

Donors of lymph node cells were inbred mice of the XVII/Bln and AKR/Bln strains.

The experimental animals had i.m. transplanted tumors of different etiology. The size of the tumors varied between 10 and 20 mm dia. Tumors in XVII mice were two u.v. light-induced sarcomas (UV 14306, UV 15264), a methylcholanthrene-induced sarcoma (MC 11504), a nitrosomethyl-urea-induced leukemia (NMU 3), and a Graffi virus-induced leukemia (Gr 101). An AKR mouse with spontaneous leukemia was included in the tests. Lymph node cells were also obtained from XVII mice immunized repeatedly with X-ray killed tumor cells of UV 14306 or Gr 101.

(c) Preparation of mouse lymph node cells

After extirpation the peripheral lymph nodes were put into Eagle's medium, minced and pressed through a metal net. Aggregated cells and connective tissue were eliminated by filtration of the suspension through gauze. After 2 washings with cool medium the cell concentration derived was adjusted by means of a Thoma chamber. The number of dead cells, according to the trypan blue exclusion test, was less than 5%.

(d) Preparation of guinea pig macrophages

Macrophages were derived from normal guinea pigs 6–10 days after injection of 20 ml sterile liquid paraffin into the peritoneal cavity. Eagle's medium was used to wash off the cells from the peritoneal cavity. After 2 washings *in vitro* the cell concentration desired was adjusted. Only cells which had phagocytized paraffin-oil were evaluated in the tests.

(e) Macrophage electrophoretic mobility (MEM) test

Incubation of lymphocytes with antigens was carried out according to the split method of Pritchard *et al.* [10].

In the first step of incubation 1·0 ml of antigen was added to a given number of lymphocytes in 2·0 ml Eagle's medium.

In the case of HEP and REP the protein concentration was 100 μg/ml. When using the other antigens an optimal non-toxic concentration was adjusted by titration. The antigen-lymphocytes mixture was kept at room temperature (23°C) for 90 min. In the second step the cell-free supernatant of this mixture was again incubated at 23°C with 10^7 indicator macrophages for 90 min. Macrophages were then measured in the cell electrophoretic test. Controls were (1) macrophages after incubation with supernatant from mouse lymphocytes without any antigen, (2) macrophages in the presence of antigen, and (3) macrophages without antigen. The calculated mean value of macrophage mobility of controls (1) and (2) did not exceed 1% as compared with that of control (3).

Macrophage mobility was measured in an electrical field (about 6–10 V/cm) using a cell electrophoretic apparatus (Zytopherometer, Zeiss, Oberkochen, FRG).

The mean value of macrophage mobility in each single experiment was calculated from 40 distinct measurements. Only macrophages were used which had a diameter of about 16 μm and which contained paraffin oil. Reduction of macrophage mobility indicates a positive reaction and is expressed in per cent on the basis of control (3). A reduction $\geqq 5\%$ was arbitrarily considered positive. Details of the technique are given in previous papers [11,12].

RESULTS

Tumor-bearing mice are apparently sensitized against an antigen which is present in the rabbit encephalitogenic protein (REP). After incubation of lymphocytes from these animals with REP a factor is released which reduces the mobility of guinea pig macrophages in the MEM test up to about 20% (Table 1).

Normal lymphocytes do not react. The sensitizing antigen is not restricted to a particular tumor type. Leukemias as well as sarcomas of different etiology induce the same pattern of reactivity.

Table 1. *Slowing of macrophages in per cent induced by REP antigen–lymphocyte–macrophage interaction*

Lymphocyte donor	Number of lymphocytes × 10^6	Macrophage mobility time in sec.	Mobility reduction in per cent
1. UV 14306	0	2·94 ± 0·05	—
tumor-bearing	0·5	3·16 ± 0·08	7·5
XVII mouse	1·0	3·18 ± 0·12	8·2
	2·0	3·46 ± 0·10	17·7
2. PVC 11677	0	2·94 ± 0·05	—
tumor-bearing	0·5	2·96 ± 0·08	0·8
CBA mouse	1·0	3·09 ± 0·11	5·3
	2·0	3·21 ± 0·08	9·2
3. NMU 3	0	2·88 ± 0·12	—
leukemia-bearing	0·5	3·14 ± 0·09	9·1
XVII mouse	1·0	3·28 ± 0·10	13·8
	2·0	3·37 ± 0·07	16·8
4. AKR mouse	0	2·94 ± 0·05	—
with spontaneous	0·5	3·01 ± 0·11	2·5
leukemia	1·0	3·15 ± 0·08	7·3
	2·0	3·44 ± 0·06*	19·4
5. Normal	0	2·88 ± 0·09	—
XVII mouse	0·5	n.t.†	—
	1·6	2·90 ± 0·11	0·6
	2·0	2·91 ± 0·11	0·9

*Same macrophages as in experiment 3.
†n.t. = not tested.

Table 2 confirms the reactivity of REP with lymphocytes from tumor-bearing or tumor-immunized mice and it shows that the human encephalitogenic protein (HEP) is much less effective. In addition, there is cross-reaction among Graffi leukemia Gr 101 and UV-induced sarcoma UV 14306, i.e. the antigen extract from Gr 101 reacts with lymphocytes of mice sensitized against UV 14306 and vice versa.

Table 2. *Slowing of macrophages in per cent induced by antigen–lymphocyte–macrophage interaction. Demonstration of cross-reaction between a UV sarcoma and a viral leukemia in mice*

	Antigen							
Lymphocyte donor	HEP		REP		Gr101 supernatant		UV 14306 supernatant	
UV 14306 tumour-bearing XVII mouse	6·6*	(0·9)†	24·9	(1·8)	14·9	(1·8)	18·7	(2·4)
UV 14306 immunized XVII mouse	2·5	(0·9)	9·9	(1·8)	15·7	(1·8)	14·5	(1·8)
Gr 101 leukemia-bearing XVII mouse	0	(1·5)	16·5	(3·0)	5·9	(3·0)	8·5	(3·0)
Gr 101 leukemia-immunized and boostered XVII mouse	6·6	(1·2)	12·4	(2·4)	19·8	(2·4)	9·5	(2·4)
Gr 101 leukemia-immunized XVII mouse	n.t.‡		13·2	(1·5)	17·4	(3·0)	0	(3·0)

*Macrophage slowing in per cent.
†Number of lymphocytes used for incubation with antigen × 10^6.
‡n.t. = not tested.

Table 3. Slowing of macrophages in per cent induced by antigen–lymphocyte–macrophage interaction. Reactivity of UV 14306 KCl extract with lymphocytes of mice bearing sarcomas of different etiology

Lymphocyte donor	Number of lymphocytes × 10^6	Macrophage mobility time in sec.	Mobility reduction in per cent
UV 14306	0	2·86 ± 0·07	—
tumor-bearing	0·5	3·07 ± 0·06	7·4
XVII mouse	1·0	3·21 ± 0·05	12·2
	2·0	3·22 ± 0·03	12·6
UV 15264	0	2·86 ± 0·07	—
tumor-bearing	0·5	3·04 ± 0·06	6·3
X II mouse	1·0	3·08 ± 0·05	7·7
	2·0	3·14 ± 0·06	9·8
MC 11504	0	2·86 ± 0·07	—
tumor-bearing	0·5	2·93 ± 0·07	2·5
XVII mouse	1·0	3·04 ± 0·06	6·3
	2·0	3·09 ± 0·06	8·0

Cross-reactions among mouse sarcomas induced by chemical or physical carcinogens are also evident from experiments with potassium chloride extract of sarcoma UV 14306 (Table 3). This antigen induced the release of macrophage slowing factor from lymphocytes of mice bearing UV 14306, UV 15264 or MC 11504 sarcomas. The reactivity seemed to be slightly higher in the homologous system with UV 14306 extract.

DISCUSSION

Except HEP, all the other antigen preparations used reacted with lymphocytes from tumor-bearing or tumor-immunized mice in that they induced release of the macrophage slowing factor as demonstrated by the MEM test. The strong reactivity with REP is a surprising fact since this protein is of heterologous origin. Apparently tumor-bearing mice are sensitized against an antigen which is closely related to REP. It remains to be seen whether encephalitogenic proteins of rodents generally share common antigenic determinants. In contrast, the reactivity of HEP is negligible.

Antigen extracts prepared from the mouse tumors behaved like REP. Thus, potassium chloride extract from UV 14306 or supernatants from tumor homogenate reacted with the sensitized lymphocytes, independently of the tumor type of lymphocyte donor. Whether the cross-reacting antigen in the tumors is different from REP is not yet known. With high probability the antigen measured by the MEM test is unrelated to the TATAs. Sarcomas induced by chemical or physical carcinogens do not cross-react in the transplantation test and they do not share common antigens with viral tumors such as Graffi or AKR Gross leukemia [5,13]. However, viral antigens as a cause of cross-reactivity among murine tumors cannot completely be ruled out since chemically induced sarcomas were shown to possess C-type viral antigens [14]. Even UV 14306 has the gs-1 antigen of murine leukemia viruses (unpublished results).

On the basis of the cross-reactivity the antigens measured by the MEM test apparently are not responsible for tumor-specific transplantation resistance. There is another fact against the identity of TATA and the MEM antigen. Although NMU 3 [7] is only slightly immunogenic in the transplantation test the MEM test is as high as in the strongly immunogenic UV sarcomas. The same is true for AKR mice with spontaneous leukemia.

The nature of the antigen involved in sensitization is still an open question. It cannot be ruled out that individual tumor antigens participate in the MEM reaction since the reduction of macrophage mobility seems to be higher in the homologous tumor antigen-lymphocyte combination, i.e., UV 14306 extract apparently produces more slowing factor from lymphocytes of UV 14306-bearing mice than of mice bearing other tumors. In order to understand the reaction, it seems necessary, however, to apply the MEM test to defined antigens.

Acknowledgement—We thank Dr. J. Günther, Research Department of Immunology at the Institute of Physiological Chemistry, University of Rostock, for kind supply of HEP and REP.

REFERENCES

1. E. J. FIELD, Delayed hypersensitivity studies: some applications of cell electrophoresis. *J. roy. Coll. Phycns Lond.* **6,** 316 (1972).
2. E. J. FIELD and E. A. CASPARY, Lymphocyte sensitisation: an *in vitro* test for cancer? *Lancet* **ii,** 1337 (1970).
3. J. P. DICKINSON, E. A. CASPARY and E. J. FIELD, A common tumor specific antigen. I. Restriction *in vivo* to malignant neoplastic tissue. *Brit. J. Cancer* **27,** 99 (1973).
4. I. HELLSTRÖM, K. E. HELLSTRÖM, H. O. SJÖGREN and G. A. WARNER, Demonstration of cell-mediated immunity to human neoplasms of various histological types. *Int. J. Cancer* **7,** 1 (1971).
5. A. GRAFFI, K.-H. HORN and G. PASTERNAK, Antigenic properties of tumors induced by different chemical and physical carcinogens. *UICC Monograph Series, Munksgaard, Copenhagen* **2,** 204 (1967).
6. G. PASTERNAK, Differences in the antigenic behavior of Graffi and Gross virus-induced leukemias in mice. *UICC Monograph Series, Munksgaard, Copenhagen* **2,** 86 (1967).
7. G. PASTERNAK and L. PASTERNAK, Virus-coded antigens in chemically induced leukemia. *Boll. Ist. sieroter. Milan.* **50,** 192 (1971).
8. P. C. DAU and R. D. A. PETERSON, Transformation of rodent lymphoid cells by an encephalitogen of human origin. *Int. Arch. Allergy appl. Immunol.* **35,** 353 (1969).
9. J. W. BLASECKI and S. S. TEVETHIA, *In vitro* assay of cellular immunity to tumor-specific antigen(s) of virus-induced tumors by macrophage migration inhibition. *J. Immunol.* **110,** 590 (1973).
10. J. A. V. PRITCHARD, J. L. MOORE, W. H. SUTHERLAND and C. A. F. JOSLIN, Evaluation and development of the macrophage electrophoretic mobility (MEM) test for malignant disease. *Brit. J. Cancer* **27,** Suppl. I, 229 (1973).
11. H. MEYER-RIENECKER, H. L. JENSSEN, H. KÖHLER und J. GÜNTHER, Zur Bedeutung des Makrophagen-Elektrophorese-Mobilitätstestes für die Diagnostik der Geschwülste des Zentralnervensystems. *Dtsch. med. Wschr.* **100,** 538 (1975).
12. E. J. FIELD, B. K. SHENTON, H. MEYER-RIENECKER, H. L. JENSSEN, H. KÖHLER, J. GÜNTHER und H. FRIEMEL, Die Makrophagen-Elektrophorese-Mobilitäts-(MEM) Methode: ihre Anwendung in der klinischen Immunologie. *Dtsch. Gesundh.-Wes.* **30,** 377 (1975).
13. L. PASTERNAK und G. PASTERNAK, Immunologische Charakterisierung von künstlich mit dem Graffi-Leukämievirus infizierten Sarkomen. I. Immunologischer Nachweis von Virus und virusinduzierten Transplantationsantigenen. *Arch. Geschwulstforsch.* **31,** 243 (1968).
14. R. J. HUEBNER and R. V. GILDEN, Inherited RNA viral genomes (virogenes and oncogenes) in the etiology of cancer. In *RNA Viruses and Host Genome in Oncogenesis.* (Edited by P. EMMELOT and P. BENTVELZEN) p. 197. North-Holland, Amsterdam (1972).

Europ. J. Cancer Vol. 12, pp. 395–403. Pergamon Press 1976. Printed in Great Britain

Multiple Agent Chemotherapy for Advanced Histiocytic Lymphoma

STEVAN ADLER, KATSUTARO SHIMAOKA, TIN HAN and YOSHIAKI TSUKADA

Departments of Medicine B and Pathology, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York, 14263, *U.S.A.*

Abstract—*Seventy-eight patients with stage III and IV histiocytic lymphoma were randomized for treatment with cyclophosphamide (CYP), vincristine (VCR), and prednisone (PRD) (COP) or COP plus BCNU and HN_2. The study was done in three phases: the first compared COP with COP plus BCNU and HN_2 at a "low" dose, the second compared the same agents using a 50% increase in the dose of the alkylating agents ("high dose") and the third compared the second combination with the addition of 10 weekly doses of bleomycin. Upon entering remission, the patients were randomized to receive daily CYP p.o. or daily CYP p.o. plus reinforcing doses of PRD and VCR every two months.*

The response rates were not significantly different for each treatment group; the overall remission rate was 50%. The median duration of remission was 4 months. Twenty-one patients were observed during maintenance chemotherapy. Neither regimen showed an advantage over the other. The overall median survival was 6 months, but patients achieving CR had a median survival of 14 months. The addition of BCNU, HN_2 or bleomycin did not improve the response rate for COP.

PREVIOUS studies have demonstrated the value of cyclophosphamide (CYP) plus vincristine (VCR) [1] and CYP, VCR and prednisone (PRD) ("COP" regimen) [2,3] in the treatment of advanced histiocytic lymphoma, yielding a response rate of about 40% for the three drug combination. Therapy with single agents such as mechlorethamine (nitrogen mustard, HN_2) and bis-1,3-(2-chloroethyl)-1-nitrosourea (BCNU) produced a response rate of 18 and 28% respectively [4,5]. Recent attention has been focused on the antibiotic agent bleomycin (BLEO) and response rates of between 36 and 45% have been reported [6,7]. Induction of remission with combination chemotherapy (COP) has been shown to improve survival [4]. The duration of remission has been considerably shorter than that for lymphocytic lymphosarcoma or Hodgkin's disease. The employment of maintenance chemotherapy did not initially show any advantage over no maintenance in prolonging remission [2,3]; however, the study of Hayes and Glidewell indicates an improvement of remission duration using oral CYP maintenance [8].

Since the alkylating agents commonly used in the treatment of neoplasia presumably act by different mechanisms, it is conceivable that the addition of multiple agents given on an intermittent schedule could improve the remission rate in this disease [9,10]. A therapeutic protocol utilizing the combination of CYP, VCR and PRD with the addition of BCNU, HN_2 and BLEO was designed to evaluate the performance of these drugs in inducing and maintaining remissions in patients with advanced histiocytic lymphoma. The results of this evaluation form the basis of this report.

METHODS

All patients with growing, histologically proven histiocytic lymphoma in stages III and IV, regardless of prior therapy, were selected for entrance into the study. The presence of one or more measurable tumors was required. Adequate marrow function as manifested by peripheral WBC counts of 4000/mm^3 and platelet counts of 100,000/mm^3 was desirable; lower counts did not prevent entry into the study, as provisions for dose modification of

Accepted 18 December 1975.

Reprint requests to: Dr. Katsutaro Shimaoka, Department of Medicine B, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14263.

marrow depressant drugs were made. Adequate renal and pulmonary function must have been present and the patients were not to have received any form of radiation or chemotherapy within two weeks of entry. Patients previously treated with one or more agents used in this study were not excluded.

Pre-study work-up included a complete physical examination, complete blood count, bone marrow aspiration and determination of the serum bilirubin, urea nitrogen, uric acid, alkaline phosphatase and glutamic-oxaloacetic transaminase. Appropriate roentgenographic and isotopic studies were performed when indicated. Blood counts and serum chemistry determinations were done weekly and tumor masses were measured twice weekly or at every clinic visit.

Patients were staged according to the Ann Arbor criteria [11] and in addition were classified as to physical condition at entrance according to the following definitions:

Good: Ambulatory, normal nutritional status, normal blood counts.
Fair: Limited ambulation, moderate malnutrition, mild depressions of blood counts.
Poor: Bedridden, cachectic, marked hematological abnormalities.

Remission status was determined according to the following definitions:

Complete remission (CR): complete disappearance of all objective and subjective evidence of disease.
Partial remission (PR): a regression of at least 50% in the product of two perpendicular measurements of all mass lesions in comparison with pre-treatment measurements and disappearance of all symptoms of disease.
No response (NR): no change or regression of less than 50% in measurable lesions within 6 cycles of therapy or progression of disease of less than 25% of the initial size prior to completion of the induction phase; complete or partial remission lasting less than 30 days.
Relapse: the appearance of new tumor after partial or complete remission or growth of stable tumors by more than 25% of their size after remission.

The duration of remission was calculated from the date the patient was considered in remission to the date of relapse, death or date of last determination of status.

Patients were randomized to receive one of two possible arms (designated A and B) of the induction phase. To ensure equal distribution of previously treated patients into both arms, the randomization was stratified for prior and no prior chemotherapy. The induction phase was designed to terminate 30 days from the time of complete response in patients showing complete tumor regression. Those showing only partial response continued therapy for a total of 6 cycles. Upon entering remission and completing the induction phase, the patients were then randomized to one of two possible maintenance schedules. The study was divided into three sequential parts, termed Phases I, II and III.

THERAPY

Figure 1 summarizes the schedule and sequence of chemotherapy in this study. Each phase is described below:

Phase 1

Arm A consisted of CYP 600 mg/m^2 i.v. weekly for the first two weeks of each 4 week period plus daily oral PRD 30 mg/m^2 for 14 days during the same interval. VCR, 1·4 mg/m^2 i.v., was given concurrently with CYP during months 1, 3 and 5 of the induction period. Arm B contained the same three drugs except that BCNU 60 mg/m^2 i.v. was substi-

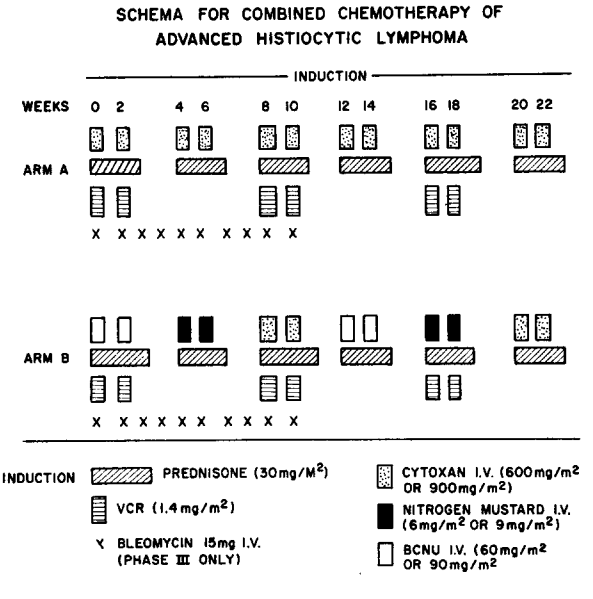


Fig. 1. Schema for administration of chemotherapy. Arm A indicates COP therapy for a period of 22 weeks. Arm B shows the alternating schedule for the multiple agent regimen. Only patients showing partial remission received the full course of therapy. The doses of the agents used are shown beneath the figure.

tuted for CYP during months 1 and 4 and HN_2, 6 mg/m^2 i.v. was substituted during months 2 and 5.

Phase II

Arms A and B were identical to those of Phase I, except that the doses of CYP, BCNU and HN_2 were increased by 50% (i.e., 900, 90 and 9 mg/m^2, respectively). The doses of VCR and PRD remained the same.

Phase III

Arms A and B were identical to those of Phase II and BLEO, 15 mg i.v. weekly, was added to each arm during the first 10 weeks of treatment.

Each phase ran for approximately one year until patient accrual was sufficient to permit evaluation of response to therapy and degree of toxicity. Upon termination of one phase, all new patients were entered into the subsequent one in operation.

Maintenance

Those patients considered in CR or PR were randomized to one of two schedules of maintenance chemotherapy.

Schedule I

CYP, 150 mg p.o. in divided daily doses, was given along with reinforcement therapy consisting of VCR, 1·4 mg/m^2 i.v. (one dose) plus PRD, 30 mg/m^2 p.o. for 14 days, repeated at 6-week intervals.

Schedule 2

CYP, 150 mg p.o. in divided daily doses, was given with no other therapy.

The drugs and doses for this treatment remained the same throughout all three phases of the study. Maintenance therapy was given for an indefinite period until relapse or excessive toxicity intervened.

Dose modification

In order to accommodate patients with depression of bone marrow function, dose modification of alkylating agents was made according to the following:

WBC/mm^3	Platelets/mm^3	% Dose given
> 4000	> 100,000	100
3000–4000	75,000–100,000	75
2000–3000	50,000– 75,000	50
< 2000	< 50,000	0

Vincristine was discontinued in the presence of symptomatic muscular weakness or a decrease of 50% in peripheral nerve conduction velocity. Conduction studies were not carried out routinely, but were done if clinically indicated. Persistent, severe paresthesias and constipation resistant to therapy also resulted in discontinuation of the drug. In the presence of obstructive jaundice, only 25% of the prescribed dose was given. Fifty % of the dose was given for those with hepatocellular jaundice [22].

Prednisone dosage was modified at the discretion of the investigators for any of the known corticosteroid-induced side effects.

Statistical methods

Statistical analysis was carried out using the chi square method with the Yates continuity correction and the exact binomial probability test. Survival curves were calculated according to the method of Kaplan and Meier [12] and analyzed by the method described by Breslow [13].

RESULTS

Between March 1970 and June 1973, a total of 91 patients was admitted to this study. Seven patients were disqualified after review of pathological material failed to confirm the diagnosis of histiocytic lymphoma. Six others were disqualified because of lack of measurable disease in one and insufficient duration of follow-up in the rest, leaving 78 evaluable patients. The age, sex, stage, condition and history of prior therapy is summarized in Table 1.

Induction

Phase I. Twenty-four patients (13 on Arm A and 11 on Arm B) entered this phase. The overall response rate (CR+PR) was 50% (Table 2). There was no difference between the response rates of Arms A and B. One cycle of chemotherapy was sufficient to achieve a complete response in 3 patients and one required six cycles of induction in order to enter complete remission. Eight patients entered partial remission with one cycle of chemotherapy, but 5 relapsed before completing six cycles. The median duration of remission for each arm was 3 months.

The hematologic toxicity is summarized in Table 3. White cell and platelet counts were equally affected in both arms. One-third of the patients had WBC depressions to less than

Table 1. Characteristics of patients with advanced histiocytic lymphoma undergoing chemotherapy

Phase		Age			Sex		Stage		Condition			Prior therapy			
		< 41	41–65	> 65	M	F	III	IV	Good	Fair	Poor	None	CT	RT	RT+CT*
Arm A	I	4	8	1	9	4	2	11	5	6	2	6	2	2	3
	II	1	6	5	5	7	4	8	3	6	3	5	0	4	3
	III	1	10	2	7	6	0	13	4	6	3	7	1	4	1
Arm B	I	2	6	3	9	2	3	8	6	4	1	5	1	2	3
	II	0	9	4	6	7	1	12	4	7	2	6	1	2	4
	III	2	12	2	7	9	2	14	7	5	4	5	2	7	2

*CT = chemotherapy, RT = radiation therapy.

Table 2. Response to multiple agent chemotherapy of patients with advanced histiocytic lymphoma

Phase	Arm	No. patients	CR (%)	PR (%)	NR (%)*
I	A	13	3 (23)	4 (31)	6 (46)
	B	11	1 (9)	4 (36)	6 (55)
II	A	12	4 (33)	5 (42)	3 (25)
	B	13	3 (23)	4 (31)	6 (46)
III	A	13	5 (39)	2 (15)	6 (46)
	B	16	4 (25)	2 (13)	10 (62)
Totals		78	20 (26)	21 (27)	37 (47)

*CR = complete remission; PR = partial remission; NR = no response.

Table 3. Summary of hematologic toxicity according to regimen

	Number of patients					
	Phase I		Phase II		Phase III	
	A	B	A	B	A	B
Decrease in Hb* (g/100 ml)						
0	4	2	4	4	1	5
< 2	3	4	5	2	6	4
2–4	5	4	3	6	6	4
> 4	1	1	0	1	0	3
Nadir of WBC† (per mm³)						
> 4000	9	7	4	7	5	11
3000–4000	0	2	0	2	1	1
2000–3000	3	1	5	1	2	0
< 2000	1	1	3	3	5	4
Nadir of platelets (per mm³)						
> 100,000	12	9	9	6	11	8
75,000–100,000	0	0	0	2	0	1
50,000–75,000	0	1	3	2	1	1
< 50,000	1	1	0	3	1	6

*Hb = hemoglobin.
†WBC = white blood count.

4000/mm³, whereas only 3 (10%) had platelet count depressions to less than 100,000/mm³. Decreases in hemoglobin of more than 2 g/100 ml were seen in 46% of the patients.

Non-hematologic toxicity consisted of nausea and/or vomiting in 10 of 24 patients and neurotoxicity, as evidenced by loss of deep tendon reflexes and/or paresthesias, occurred in 11 of the 24 cases. There were no complications of PRD administration.

The necessity for dose modification of the alkylating agents was examined among those patients entering CR or PR. In Arm A, the average dose of CYP received was 95% of the total calculated dose. In Arm B, 90% of the calculated dose of CYP, 88% of the calculated dose of HN_2 and 96% of the calculated dose of BCNU, were received.

Of the patients entering this phase, 21 died of their disease, one of unrelated causes, one is still alive and one has been lost to follow-up. The median duration of survival from entrance to the study to the the date of analysis is 5 months for both arms.

Phase II. Twenty-five patients (12 on Arm A and 13 on Arm B) entered this phase. The overall response rate was 64% (Table 2). The difference in response between the two arms is not significant. One cycle of chemotherapy was sufficient to achieve a complete response in 5 patients and one required six cycles of induction in order to enter complete remission. Eight patients achieved a partial response with one cycle, but 4 relapsed before completing six cycles. The median duration of response was 7 months for Arm A and 4 months for Arm B.

Table 3 shows depressions of WBC to under 4000/mm³ in 56% of the patients and depressions of platelet counts to under 100,000/mm³ in 40%. Decreases in hemoglobin of more than 2 g/100 ml were seen in 40% of the patients.

Gastrointestinal toxicity occurred in 6 of 25 patients and neurotoxicity in 12 of the 25. PRD was modified or discontinued in 3 patients: two developed diabetes mellitus (with cushingoid facies in one case) and one developed hypertension.

Dose modification of alkylating agents resulted in an average dose of 75% of the calculated total dose of CYP in Arm A. In Arm B the average doses received were 73% for CYP, 68% for HN_2 and 96% for BCNU.

Nineteen of the patients admitted to this study have died of their disease. Three patients from Arm B are still alive and in remission. Three patients from Arm A are still living, but only one is in remission. The median duration of survival from entrance to the study is 7 months for Arm A and 5 months for Arm B.

Phase III. Twenty-nine patients (13 on Arm A and 16 on Arm B) entered this phase. The overall response rate was 45% (Table 2). Both arms gave similar responses. Four patients achieved a complete remission with one cycle of therapy, while 5 others required more than one cycle of treatment to enter complete remission; 3 patients relapsed before completing induction. Five patients achieved a partial response with one cycle; 3 relapsed before completing 6 cycles. The median duration of response for Arm A was 6 months and for Arm B, 5 months.

White cell depression to less than 4000/mm^3 appeared in 45% and platelet count depression to less than 100,000/mm^3 in 35% of the patients in both arms. Hemoglobin losses of over 2 g/100 ml occurred in 45% of patients (Table 3).

Gastrointestinal toxicity appeared in 15 of 29 patients and neurotoxicity in 10. Two patients developed upper gastrointestinal hemorrhage from peptic ulcer disease associated with PRD administration. Toxicity related to BLEO therapy occurred in 10 patients (32%); three developed dermatologic lesions, 6 interstitial pneumonitis and/or pulmonary fibrosis and one developed excessive febrile reactions requiring discontinuation of the drug.

Dose modification of alkylating agents resulted in an average dose of 87% of the calculated dose of CYP in Arm A. In Arm B, average doses received were 69% for CYP, 64% for HN_2 and 98% for BCNU.

Nineteen of the patients in this group have died of their disease, 3 of other causes and one has been lost to follow-up. Two patients from Arm A are alive and in remission. Four from Arm B are still living, but only 2 remain in remission. The median duration of survival from entrance to the study is 6 months for Arm A and 4 months for Arm B.

Maintenance

The same maintenance schedules were used throughout all phases of the study. Of the 78 patients entered, 44 went into remission, but only 22 were randomized for maintenance chemotherapy. One patient was disqualified for failure to take his drugs. Of the 22 patients who were not entered into maintenance, 16 relapsed before completing induction, 2 were not entered due to physician error, one died of a pulmonary abscess, one failed to return for treatment, one developed a relapse of previous mycosis fungoides and new therapy was given, and one case involved a patient with a chronic psychosis who was unable to take oral medication reliably.

The patients were analyzed together without regard to type of induction therapy, although more patients from Phases II and III were able to enter maintenance. Table 4 summarizes the characteristics of the patients and response to therapy for both groups.

Table 4. Characteristics of patients entering maintenance chemotherapy for advanced histiocytic lymphoma

	Schedule 1	Schedule 2
Number of patients	10	11
Male/Female	5/5	4/7
Median age (yr)	54	59
Remission status (CR/PR)	6/4	8/3
Condition:		
Good	6	5
Fair	3	5
Poor	1	1
Stage:		
III	4	2
IV	6	9
Induction:		
Phase I	4	1
Phase II	3	5
Phase III	3	5

The patients receiving drugs on Schedule I tolerated the agents quite well. Seven patients had mild hematologic toxicity (decreases in Hb. of less than 2 g/100 ml, depression of WBC to not more than 3000/mm^3 and depression of platelet counts to not more than 75,000/mm^3), while 2 showed depressions exceeding these values. There were no cases of neurotoxicity ascribable to the administration of VCR and the oral PRD was well tolerated. The median duration of remission is 5 months. There have been 7 relapses during therapy.

The median survival from entrance to the protocol to date of analysis is 14 months (range 8–32 months).

The patients receiving therapy on Schedule 2 showed mild toxicity, as above, in 6 and more severe depressions in 5 cases. Eight patients required dose reductions of about 50% of the calculated dose of CYP. The median duration of remission is 4 months. There have been 7 relapses. The median survival from entrance to the protocol is 12 months (range 6–35 months).

Currently, 3 patients are alive and in remission on Schedule 1. Seven patients on Schedule 2 are still alive, but only four are in remission.

ANALYSIS

In order to evaluate the addition of BCNU, HN_2 and BLEO and the effect of increasing the doses of alkylating agents on remission rate, it is necessary to show that the populations treated are comparable, since the trials were sequential rather than concomitant.

The composition of the patient population, with regard to those factors which most influence the prognosis of lymphoma, was examined for homogeneity.

Figure 2 shows the response to therapy (expressed as the mean CR + PR rate) for the prognostic factors of age, sex, stage, physical condition and history of prior therapy. Of these factors, only stage at the time of treatment had a statistically significant effect ($P < 0{\cdot}05$ for stage III vs stage IV). Women showed an advantage over men and the factor of prior therapy suggested that previously untreated patients responded better. However, the differences failed to reach significance by the exact binomial probability test.

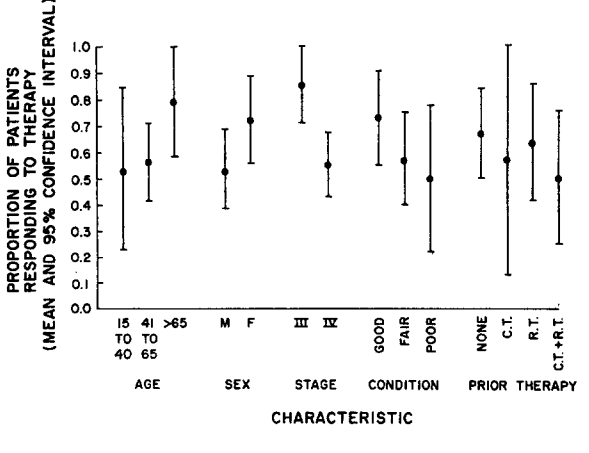


Fig. 2. Response to chemotherapy for the prognostic factors which most influence lymphoma. Only stage at time of treatment appeared to influence response (P = 0·013).

The distribution of these prognostic factors among the three phases was not entirely homogeneous. Patients in the age category of 41–65 years were distributed equally in all phases, but there was a cluster of those under 40 years in Phase I which was significant ($P < 0{\cdot}05$). Women predominated in Phases II and III, but the difference in proportion, in comparison to Phase I, was not significant.

Patients classified as being in good, fair or poor condition were present in equal proportion in all phases. Patients who had received no prior treatment were equally represented in all phases. Phase I contained more patients who had received prior chemotherapy and Phase III contained more patients who had received prior radiation therapy. These differences in proportion were not significant.

The distribution of histologic types was examined for the entire population under study. Sixty-six cases showed a diffuse pattern, while 12 showed the nodular pattern. The distribution of types in each phase was not significantly different: Phase I–21 diffuse, 3 nodular; Phase II–21 diffuse, 4 nodular; Phase III–24 diffuse, 5 nodular.

It is recognized that larger numbers of patients in each phase might have made certain prognostic factors more important in determining response to therapy. However, the differences in patient composition were not statistically significant and comparison of one phase with another is permissible.

Response to chemotherapy was compared using the chi square test. The results of Phase I vs Phase II, Phase I vs Phase III and Phase I vs Phases II plus III showed no significant difference. Similarly, the duration of response and survival were not different in each of the phases and there was no advantage in the addition of BCNU, HN_2 and BLEO to the combination of CYP, VCR and PRD. The response to chemotherapy was examined according to histological type. The diffuse type was in the majority in each phase and response was distributed equally. However, the nodular type was present in only a few patients in each group and so was looked at as a separate population. Among the group of 12 patients there were 5 CR, 5 PR and 2 NR. The median survival for this group was 4·5 months, which is not significantly different from that of the entire study population. The depression of platelet counts to below 100,000/mm^3 was significantly increased when Phase I and Phases II and III were compared ($P < 0{\cdot}025$). Arm B showed

significantly more depression of platelet counts, both mild ($< 100{,}000/mm^3$) and severe ($< 50{,}000/mm^3$), over Arm A ($P < 0{\cdot}05$). This would probably indicate the effect of BCNU on platelets in Arm B. On the other hand, depression of leukocytes was not significant except for severe leukopenia ($< 2000/mm^3$) between Phases I and III ($P < 0{\cdot}05$). Mortality associated with hematologic depression was limited to three patients: two died of sepsis and one from a disseminated intravascular coagulopathy and anemia. Gastrointestinal and neurologic toxicity occurred in equal proportion in all phases.

The role of BLEO in the production of interstitial pneumonia and pulmonary fibrosis was investigated by one of us (Y.T.). Slides from lung specimens obtained at post mortem were reviewed without knowledge of patient identity or form of therapy in 35 cases, including those of 5 patients previously identified as having clinical evidence compatible with BLEO toxicity. Four of 25 patients who had not received BLEO were identified as having minimal pulmonary fibrosis or interstitial pneumonia. Two of these patients had received radiation therapy to the chest; one through a mantle port and the other, a single dose of 200 rads to the lung on the day before he expired. Five of 10 patients who had received BLEO showed pulmonary fibrosis or interstitial pneumonia; 3 showed marked fibrosis and 2, minimal fibrosis with interstitial pneumonia. One of the latter two patients had received prior radiation therapy through a mantle port to the chest. The incidence of pulmonary toxicity was not significantly different between BLEO and non-BLEO groups ($P > 0{\cdot}05$). However, severe pulmonary toxicity was only observed in the group to which BLEO was given ($P < 0{\cdot}05$). The mean dose of BLEO received by those patients manifesting pulmonary toxicity was 120 mg while that of those patients not manifesting such toxicity was 70 mg. The incidence of broncho-pneumonia, pulmonary lymphoma and chronic lung disease was the same in the patients treated with BLEO and those who were not.

DISCUSSION

Gamble *et al.* introduced the combination of CYP, VCR and PRD for advanced non-Hodgkin's lymphoma in 1967 [14]. Hoogstraten *et al.* presented a detailed evaluation of this combination, showing superiority over CYP alone [2]. The response rates for histiocytic lymphoma were 53% for "low" dose and 85% for "high" dose combinations. Luce *et al.* expanded their earlier observations, reporting a 39% response rate [3]. The doses used were comparable with the "low" dose regimen used by Hoogstraten's group. The introduction of maintenance chemotherapy did not alter the duration of remission in either study.

Attempts to improve these results have included the alteration of drugs, sequence of administration and substitution of alkylating agents and the addition of antimetabolites to the "COP" regimen. Lowenbraun *et al.* used a combination of HN_2, VCR, PRD and procarbazine ("MOPP") and obtained remissions in 5 of 8 patients; the median duration of response was 20 months [15]. Schein *et al.* treated 10 patients with MOPP and all responded [16]. Fourteen others were treated with a similar regimen replacing HN_2 with CYP ("C-MOPP") and 13 responded. The median duration of remission for 9 patients responding completely to either therapy was 25 months. More recently, Stein *et al.* reported a randomized trial of chemotherapy for non-Hodgkin's lymphoma [17]. Four patients with histiocytic lymphoma received COP plus procarbazine and 2 responded; 6 others received VCR and PRD and 3 responded.

Host and Abrahamsen found a 57% response rate using CYP, PRD, procarbazine and vinblastine [18]. Levitt *et al.* utilized CYP in sequential combination with methotrexate and cytosine arabinoside and achieved responses in all of 15 treated patients [19]. Remissions lasted a median of 10 months.

Previous studies in multiple myeloma [9,10] have indicated that multiple alkylating agent regimens are advantageous in inducing remissions. We, therefore, substituted HN_2 and BCNU for CYP in the COP sequence in both low and high doses [20]. BCNU was chosen because previous experience indicated that it might be active in histiocytic lymphoma [5]. BLEO has been used in the treatment of Hodgkin's disease [21] and non-Hodgkin's lymphoma [6] and has been called an effective drug with little in the way of myelosuppressive activity. We incorporated this drug in order to test its efficacy and safety in the treatment of histiocytic lymphoma.

In our study, the response to COP containing CYP in "low" and "high" dose combination was not significantly different. However, there appeared to be a trend in that a greater number of CR and PR patients appeared in Phases II and III, indicating that a larger patient population might have shown a statisti-

cally significant result. The doses chosen for CYP were comparable with the low dose combination of Hoogstraten *et al.* [2] and with those of Luce *et al.* [3]. Our regimen differed in that we did not alter the doses of VCR and PRD and did not taper the dose of PRD.

The addition of BCNU and HN_2 to the COP regimen did not improve the rate of remission or significantly prolong survival. In contrast to the suggestion that response may improve by increasing the doses of alkylating agents, the addition of BCNU and HN_2 did not produce a trend to greater response. Toxicity induced by increasing the doses and adding BCNU, HN_2 and BLEO in Phase III, Arm B was significantly greater than that in Arm A of Phase I, especially for platelets. The necessity for dose modification of alkylating agents among responding patients was significantly greater after the doses were increased by 50% (Phase I vs Phase II, $P < 0{\cdot}001$).

Bleomycin failed to improve remission or survival rate. Pulmonary fibrosis and interstitial pneumonia appeared as an identifiable hazard to therapy with this drug, although their incidence did not reach statistical significance in this study.

Duration of remission in each of the arms of the study was not significantly different, with a median of 4 months for the entire group. By comparison, Hoogstraten's patients had a median remission of about 2 months [2] and Luce's a period of 6 months [3].

Survival was not significantly different in any of the arms. The median survival for the entire group was about 6 months. The induction of remission was correlated with prolonged survival and a median of 14 months was found for those entering complete remission. The survival of patients entering PR was not found to be significantly different from those not responding. The survival of those entering CR was significantly better than that of those not responding or in PR (Fig. 3). The poor survival of patients who entered partial remission is explainable by the fact that 73% relapsed before completing the induction phase. It appears that unless a patient with advanced histiocytic lymphoma enters CR with chemotherapy, the treatment will not influence his survival.

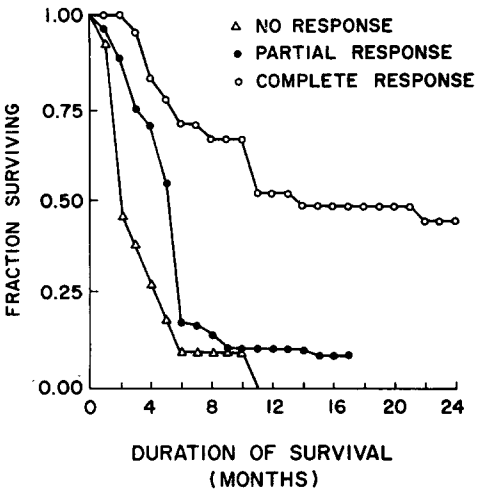


Fig. 3. Life table plot of survival of patients with advanced histiocytic lymphoma. Improved survival is seen among those patients showing complete response to chemotherapy (P = 0·01 *for CR vs PR and* P = 0·001 *for CR vs NR*).

Both maintenance chemotherapy schedules were well tolerated and relatively easy to administer. The duration of maintained remission was the same for each group, as was the duration of survival. The lack of patients in an unmaintained remission status prevents us from determining whether or not such chemotherapy prolongs either remission or survival.

In summary, chemotherapy for stage III and IV histiocytic lymphoma with COP was shown to be useful in inducing remissions. The escalation of doses of alkylating agents to higher levels may produce an improved response rate. The substitution of BCNU and HN_2 for CYP and the addition of BLEO to the COP sequence did not improve response. Survival was prolonged in those patients entering complete remission independent of the agents used. Maintenance chemotherapy with daily oral CYP with or without reinforcement with VCR and PRD, was well tolerated. Neither schedule has an advantage in terms of remission duration or survival.

Acknowledgment—The authors wish to thank Mrs. Oki Jung and Mr. Jack Tidings for their invaluable assistance in the statistical evaluation of this study and Dr. Leon Stutzman for his helpful criticism of this manuscript.

REFERENCES

1. P. P. Carbone, C. Spurr, M. Schneiderman, J. Scotto, J. F. Holland and B. Shnider, Management of patients with malignant lymphoma: a comparative study with cyclophosphamide and vinca alkaloids. *Cancer Res.* **28,** 811 (1968).
2. B. Hoogstraten, A. H. Owens, R. E. Lenhard, O. J. Glidewell, L. A. Leone, K. B. Olson, J. B. Harley, S. R. Townsend, S. P. Miller and C. L. Spurr, Combination chemotherapy in lymphosarcoma and reticulum cell sarcoma. *Blood* **33,** 370 (1969).
3. J. K. Luce, J. F. Gamble, H. E. Wilson, R. W. Monto, B. L. Isaacs, R. L. Palmer, C. A. Coltman, Jr., J. S. Hewlett, E. A. Gehan and E. Frei, III, Combined cyclophosphamide, vincristine and prednisone therapy of malignant lymphoma. *Cancer (Philad.)* **28,** 306 (1971).
4. P. P. Carbone, Non-Hodgkin's lymphoma: recent observations on natural history and intensive treatment. *Cancer (Philad.)* **30,** 1511 (1972).
5. H. E. Lessner, BCNU (1,3-bis(B-chloroethyl)-1-nitrosourea: effects on advanced Hodgkin's disease and other neoplasia. *Cancer (Philad.)* **22,** 451 (1968).
6. European Organization for Research on the Treatment of Cancer, Cooperative Group for Leukemia and Reticulocytoses. Bleomycin in reticuloses. *Brit. med. J.* **1,** 285 (1972).
7. R. H. Blum, S. K. Carter and K. Agre, A clinical review of bleomycin—a new antineoplastic agent. *Cancer (Philad.)* **31,** 903 (1973).
8. D. M. Hayes and O. Glidewell, Intensive combination chemotherapy for lymphosarcoma and reticulum cell sarcoma. *Blood* **38,** 803 (1971).
9. J. B. Harley, S. V. Ramanan, I. Kim, P. V. Thiagarajan, J. H. Chen, R. Gomez, D. Koppel, F. Hyde, S. Gustke and J. Kroll, The cyclic use of multiple alkylating agents in multiple myeloma. *W. Va. med. J.* **68,** 1 (1972).
10. B. J. Lee, G. Sahakian, B. D. Clarkson and I. H. Krakoff, Combination chemotherapy of multiple myeloma with alkeran, cytoxan, vincristine, prednisone and BCNU. *Cancer (Philad.)* **33,** 533 (1974).
11. P. P. Carbone, H. S. Kaplan, K. Musshoff, D. W. Smithers and M. Tubiana, Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res.* **31,** 1860 (1971).
12. E. L. Kaplan and P. Meier, Non-parametric estimation from incomplete observations. *Amer. Stat. Assoc.* **453,** 457 (1958).
13. N. Breslow, A generalized Kruskal–Wallis test for comparing K samples subjected to unequal patterns of censorship. *Biometrika* **57,** 579 (1970).
14. J. F. Gamble, J. K. Luce, E. Frei, III, H. E. Wilson, R. L. Palmer, B. L. Isaacs and R. W. Monto, Combination (COP) chemotherapy of malignant lymphoma in adults. *Abst. Amer. Soc. clin. Oncol.* (1967).
15. S. Lowenbraun, V. T. DeVita and A. A. Serpick, Combination chemotherapy with nitrogen mustard, vincristine, procarbazine and prednisone in lymphosarcoma and reticulum cell sarcoma. *Cancer (Philad.)* **25,** 1018 (1970).
16. P. S. Schein, B. A. Chabner, G. P. Canellos, R. C. Young, C. Berard and V. T. DeVita, Potential for prolonged disease-free survival following combination chemotherapy of non-Hodgkin's lymphoma. *Blood* **43,** 181 (1974).
17. R. S. Stein, E. M. Moran, R. K. Desser, J. B. Miller, H. M. Golomb and J. E. Ultmann, Combination chemotherapy of lymphomas other than Hodgkin's disease. *Ann. int. Med.* **81,** 601 (1974).
18. H. Host and A. F. Abrahamsen, Combination chemotherapy with cyclophosphamide, vinblastine, procarbazine and prednisone in the treatment of malignant lymphomas. *Scand. J. Haemat.* **10,** 170 (1973).
19. M. Levitt, J. C. Marsh, R. C. DeConti, M. S. Mitchell, R. T. Skeel, L. R. Farber and J. R. Bertino, Combination sequential chemotherapy in advanced reticulum cell sarcoma. *Cancer (Philad.)* **29,** 630 (1972).
20. S. Adler, K. Shimaoka and T. Han, Multiple agent chemotherapy for advanced reticulum cell sarcoma. *Abstr. Amer. Soc. clin. Oncol.* (1974).
21. A. Yagoda, B. Mukherji, C. Young, E. Etcubanas, C. Lamonte, J. R. Smith, C. T. C. Tan and I. H. Krakoff, Bleomycin, an antitumor antibiotic. *Ann. int. Med.* **77,** 861 (1972).
22. I. S. Johnson, J. G. Armstrong, M. Gorman and J. P. Burnett, Jr., The vinca alkaloids: a new class of oncolytic agents. *Cancer Res.* **23,** 1390 (1963).

Europ. J. Cancer Vol. 12, pp. 405–409. Pergamon Press 1976. Printed in Great Britain

Plasma Dehydroepiandrosterone Concentration in Normal Women and in Patients with Benign and Malignant Breast Disease

B. S. THOMAS, PAMELA KIRBY, ELIZABETH K. SYMES and D. Y. WANG
Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX

Abstract—*Studies on plasma dehydroepiandrosterone (DHEA) concentrations employing a new gas chromatographic analytical method with electron capture detection were performed on women with breast cancer and benign breast disease. Significantly lower values were found in both early and advanced disease when compared with normal women. Likewise, a similar observation was made in younger women (age range 20–39 years) with benign breast disease. No significant correlation was found between plasma DHEA and plasma DHEA sulphate concentrations, nor between plasma DHEA and urinary 11-deoxy-17-oxosteroids. This indicates that measurement of plasma DHEA may be a useful independent parameter in the study of breast cancer.*

INTRODUCTION

IT IS now well established that there is a strong association between the amounts of urinary androgen metabolites and the aetiology and clinical course of breast cancer. Subnormal amounts of urinary androgen metabolites are excreted by women with a high risk of developing breast cancer, and similar abnormalities have been observed in patients with a poor prognosis following mastectomy and endocrine ablation [1–7]. In spite of these data, relatively few studies have been undertaken to establish the biochemical and physiological reasons involved. A logical approach to these problems would be to establish to what extent the excretion of the urinary androgen metabolites relates to changes in the peripheral blood levels of their precursors. So far dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) have been extensively studied [8]. For instance, low urinary androgen metabolite excretion was found to be associated with subnormal levels of plasma DS and AS. Reports from two sources show no difference in plasma testosterone values between normal women and women with breast cancer [9, 10]. However, the role of other unconjugated androgens such as androstenedione and dehydroepiandrosterone (DHEA) has not been fully examined. Although the biological activity of DHEA is only 10% of that of testosterone [11], nevertheless its much higher production rate in the normal woman, of approximately 7 mg/day compared to 0·2–0·3 mg of testosterone, makes it a major source of androgen [12].

Whether the urinary androgen metabolite abnormalities found in breast cancer are due to a low production rate of DS, an almost inert androgen, or whether concomitant changes occur in the more potent androgens, is unknown. Therefore, this laboratory is presently engaged in measuring the plasma concentrations of DHEA, androstenedione and testosterone in women with breast cancer. This paper reports the studies on DHEA in which results have been compared between normal women and those with benign or malignant breast disease. The opportunity has also been taken to compare the results of plasma DHEA levels with those of plasma DS and urinary 11-deoxy-17-oxosteroids concentrations.

MATERIAL AND METHODS

The patients studied were 35 women with benign breast disease (age range 21–88),

Accepted 8 December 1975.

65 women with early breast cancer, comprising 32 before mastectomy (age range 36–73) and 33 post-mastectomy (age range 36–65) and 35 women with advanced breast cancer (age range 28–73). Diagnosis was confirmed by histology and the benign breast disease group included fibroadenosis, duct ectasia, lobular hyperplasia, fibroadenoma and adenosis. The normal women described in this study were 45 volunteers living in the island of Guernsey (age range 20–75).

Blood was taken from all subjects by venepuncture and the heparinised plasma stored at −20°C. The blood was taken from hospital patients between 8.0 a.m.–12.00 a.m. The specimens from the early breast cancer cases were taken either 24 hr before operation or 10 days following mastectomy. Blood from the normal control group was collected between 4.0 p.m.–10.0 p.m.

The plasma DHEA was assayed by gas–liquid chromatography with electron capture detection, using the method of Symes and Thomas [13]. Urines were collected over 24 hr and analysed for 11-deoxy-17-oxosteroids [14].

RESULTS

The regression lines of plasma DHEA concentration on age for the various groups studied are shown in Fig. 1–3. Only in normal women is the level of plasma DHEA significantly correlated with age ($P < 0{\cdot}001$). However, all the regression lines are shown in the figures since they represent the best-fit line for the data.

(a) *Benign disease*

The regression lines for patients with benign breast disease and normal women (Fig. 1) were analysed by comparing age-adjusted means. This analysis showed no significant difference between the elevations of the two lines. However, a comparison of the DHEA levels in the age group of 20–39 years using the Student t test showed that normal women have a significantly higher concentration of DHEA in their blood than young women with benign breast disease ($t = 2{\cdot}59$; $P < 0{\cdot}02$).

(b) *Early breast cancer*

The plasma DHEA concentration for normal women and patients with early breast cancer are shown in Fig. 2. For simplicity, the regression line shown in Fig. 2 represents that for the combined pre- and post-mastectomy patients; however, the elevations of the regression lines for either pre-operative or post-

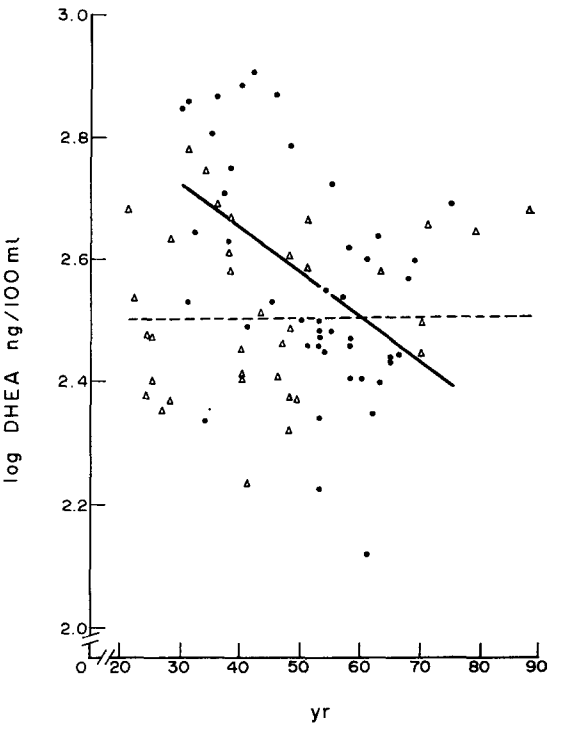


Fig. 1. ● = *Normal controls (regression line* —— y = −0·007x+2·937); △ = *Benign breast disease (regression line* – – – y = 0·001x+2·461); *where* y *is log DHEA;* x *is age.*

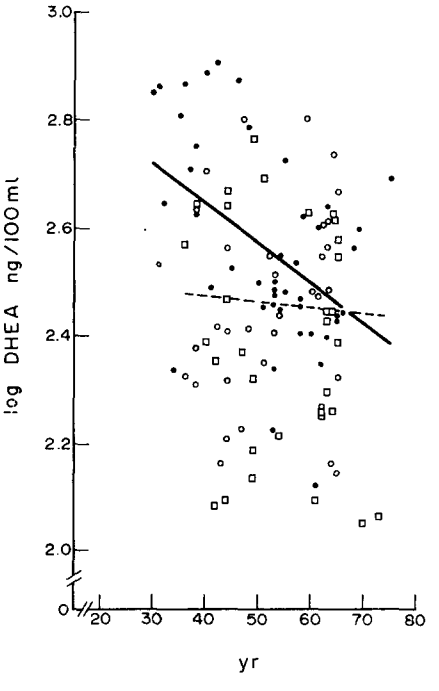


Fig. 2. ● = *Normal controls;* □ = *Early breast cancer; pre-mastectomy;* ○ = *Early breast cancer; post-mastectomy (pre- and post-mastectomy regression line* – – – y = −0·004x+2·426); *where* y *is log DHEA;* x *is age.*

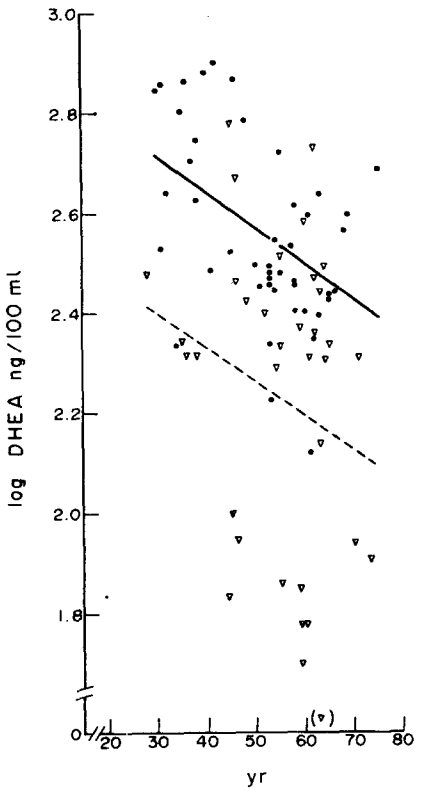


Fig. 3. ● = *Normal controls;* ▽ = *Advanced breast cancer (regression line* $---y = -0.007x + 2.618$) *where* y *is log DHEA;* x *is age;* (▽) *represents* 11 *ng/100 ml plasma.*

operative DHEA levels are significantly lower than that for normal women ($F = 11.5$; $P < 0.001$ and $F = 6.4$; $P < 0.025$, respectively). No consistent change in DHEA concentration was observed as a result of mastectomy.

(c) *Advanced breast cancer*

The DHEA levels in women with advanced breast cancer are very much reduced compared with those found in normal women (Fig. 3). The differences between the elevations of the normal regression line and that for patients with advanced breast cancer is highly significant ($F = 26.7$; $P < 0.001$).

(d) *Relationship between plasma DHEA and DS levels*

In most of the subjects studied plasma DS concentration had also been measured in addition to DHEA. A direct comparison of the plasma levels of these compounds showed no significant correlation in any of the groups of women studied except in women with early breast cancer after mastectomy, the correlation just reaching formal significance ($r = 0.37$; $P < 0.05$. See Table 1).

Table 1. Correlation of plasma DHEA and DS levels

Clinical status	*r*	*N*
Normal controls	−0·01	29
Benign breast disease	0·23	23
Early breast cancer (pre-operative)	0·28	29
Early breast cancer (post-operative)	0·37*	33
Advanced breast cancer	−0·12	27

*$P < 0.05$

Similarly, no significant correlation was found between plasma DHEA concentration and the amount of urinary 11-deoxy-17-oxosteroids excreted in 24 hr except in women with early breast cancer after mastectomy ($r = 0.39$; $P < 0.05$. See Table 2).

Table 2. Correlation of plasma DHEA level an the amount of 11-deoxy-17-oxosteroid excreted

Clinical status	*r*	*N*
Early breast cancer (pre-operative)	0·03	19
Early breast cancer (postsoperative)	−0·39*	24
Advanced breast cancer	0·04	14

*$P < 0.05$

DISCUSSION

These results add more evidence to the generalization that androgen production is subnormal in women with breast cancer. Although it has not yet been established whether the measurements of plasma DHEA have any prognostic value in the course of the disease, it is evident from this study that the level of DHEA is a sensitive indicator in discriminating between normal women and patients with early breast cancer, particularly in patients below the age of 60. A similar difference was also found for subjects with advanced breast cancer or young women with benign breast disease. Since the latter group have an appreciably increased risk of developing breast cancer it is probable that women with an enhanced risk of breast cancer are characterized by a subnormal concentration of plasma DHEA [15].

The pattern of results of DHEA concentration are similar to those reported for urinary androgen metabolites and plasma DS; the fact

that there is no correlation between the levels of DHEA and DS, although there is between the amounts of plasma DS and urinary androgen metabolites, indicates that the measurement of this steroid might be very useful since DHEA appears to be an independent parameter. This finding is reinforced by other workers who have shown that plasma concentrations of DS and DHEA do not necessarily follow the same pattern in puberty and hypertension [16, 17]. A further illustration of this phenomenon is the significant difference found for plasma DHEA between normal women and patients with early breast cancer before mastectomy, which was not found in the study on plasma DS. It will have been noted that the plasma from the control group was obtained in the afternoon and evening, whereas that from the patients was obtained in the morning. A diurnal variation in DHEA levels has been reported; the morning values are higher than evening ones [18]. Thus the differences between women with breast disease and the controls found in the present studies may be greater than those reported here.

It is not known whether androgenicity *per se* is important in the association between the risk of breast cancer and the amount of urinary androgen metabolite excreted. Although DHEA is a very much weaker androgen than testosterone, if one considers the ten-fold higher blood concentration of DHEA [19] and the lower binding of DHEA, to plasma proteins [20], it is conceivable that DHEA may provide a strong androgenic stimulus in women in some target organs. Differences in plasma DHEA concentration between normal women and patients with breast cancer might indicate an appreciable difference in androgen status between these two groups.

Acknowledgements—The authors wish to thank Mr. J. L. Hayward, F.R.C.S., of Guy's Hospital, and Mr. Niall O'Higgins, F.R.C.S., late of the Hammersmith Hospital, for their co-operation. Also Dr. R. D. Bulbrook of the Imperial Cancer Research Fund for his helpful advice.

REFERENCES

1. R. D. Bulbrook, F. C. Greenwood and J. L. Hayward, Selection of breast cancer patients for adrenalectomy or hypophysectomy by determination of urinary 17-hydroxycorticosteroids and aetiocholanolone. *Lancet* **i,** 1154 (1974).
2. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, Abnormal excretion of urinary steroids by women with early breast cancer. *Lancet* **ii,** 1238 (1962).
3. R. D. Bulbrook, J. L. Hayward and B. S. Thomas, The relation between the urinary 17-hydroxycorticosteroids and 11-deoxy-17-oxosteroids and the fate of patients after mastectomy. *Lancet* **i,** 945 (1964).
4. P. Juret, M. Hayem and A. Fleisler, A propos de 150 implantations d'yttrium radio-actif intra-hypophysaires dans le traitement du cancer du sein à un stade avancé. *J. Chir. (Paris)* **87,** 409 (1964).
5. J. Marmorston, Urinary hormone metabolite levels in patients with cancer of the breast, prostate and lung. *Ann. N.Y. Acad. Sci.* **125,** 959 (1966).
6. H. Miller, J. A. Durant, A. G. Jacobs and Jacqueline F. Allison, Alternative discriminating function for determining hormone dependency of breast cancer. *Brit. med. J.* **1,** 147 (1967).
7. S. Kumaoka, N. Sakauchi, O. Abe, M. Kusama and O. Takatano, Urinary 17-ketosteroid excretion of women with advanced breast cancer. *J. clin. Endocr.* **28,** 667 (1968).
8. D. Y. Wang, R. D. Bulbrook, Marenka Herian and J. L. Hayward, Studies on the sulphate esters of dehydroepiandrosterone and androsterone in the blood of women with breast cancer. *Europ. J. Cancer* **10,** 477 (1974).
9. D. Y. Wang, J. L. Hayward and R. D. Bulbrook, Testosterone levels in the plasma of normal women and patients with benign breast disease or with breast cancer. *Europ. J. Cancer* **2,** 373 (1966).
10. M. Horn and G. S. Gordon, Plasma testosterone in advanced breast cancer. *Oncology* **30,** 147 (1974).
11. W. R. Eberlien, J. A. Winter and R. L. Rosenfield, The Androgens. In *Hormones and Blood,* (Edited by C. H. Gray and A. L. Bacharach.) Vol. 2, p. 189. Academic Press, New York and London (1967).
12. R. Horton and J. F. Tait, *In vivo* conversion of dehydroepiandrosterone to plasma androstenedione and testosterone in man. *J. Clin. Endocr.* **27,** 79 (1967).

13. ELIZABETH K. SYMES and B. S. THOMAS, A method for the measurement of plasma dehydroepiandrosterone by gas–liquid chromatography with electron capture detection. *J. Chromatogr.* **116,** 163 (1976).
14. B. S. THOMAS, R. D. BULBROOK, J. A. DURANT, H. MILLER and D. M. ROSS, A rapid method for the estimation of urine 11-deoxy-17-oxosteroids and its use in the management of breast cancer. *Clin. Biochem.* **2,** 311 (1969).
15. M. J. BRENNAN, R. D. BULBROOK, N. DESHPANDE, D. Y. WANG and J. L. HAYWARD, Urinary and plasma androgens in benign breast disease: Possible relation to breast cancer. *Lancet* **i,** 1076 (1973).
16. B. R. HOPPER and S. S. C. YEN, Circulating concentrations of dehydroepiandrosterone and dehydroepiandrosterone sulphate during puberty. *J. clin. Endocr.* **40,** 458 (1975).
17. H. SEKIHARA, N. OSHAWA and K. KOSAKA, Serum dehydroepiandrosterone sulphate and dehydroepiandrosterone levels in essential hypertension. *J. clin. Endocr.* **40,** 156 (1975).
18. B. B. SAXENA and H. M. GANDY, Use of radioisotopes for measurement of follicle stimulating hormone and luteinizing hormones, testosterone, androstenedione. dehydroepiandrosterone, pregnenolone, cortisol and sulphates in plasma. In *In Vitro Procedures with Radioisotopes in Medicine*, p. 613. International Atomic Agency, Vienna (1970).
19. H. M. GANDY and R. E. PETERSON, Measurement of testosterone and 17-ketosteroids in plasma by the double isotope dilution technique. *J. clin. Endocr.* **28,** 949 (1968).
20. D. Y. WANG and R. D. BULBROOK, The binding of steroids to plasma proteins in normal women and those with breast cancer. *Europ. J. Cancer* **5,** 247 (1969).

Europ. J. Cancer Vol. 12, pp. 411–412. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *May, 1976*

Human Cancer

E. A. Grimm, H. K. B. Silver, J. A. Roth, D. O. Chee and D. L. Morton: Detection of tumor-associated antigen in human melanoma cell-line supernatants.

C. Sundström and K. Nilsson: Establishment and characterization of a human histiocytic lymphoma cell line (U-937).

T. Svenberg: Carcinoembryonic antigen-like substances of human bile. Isolation and partial characterization.

P. Phillips, J. K. Steward and S. Kumar: Tumour angiogenesis factor (TAF) in human and animal tumours.

P. A. Trumper, M. A. Epstein and B. C. Giovanella: Activation *in vitro* by BUDR of a productive EB virus infection in the epithelial cells of nasopharyngeal carcinoma.

M. J. Embleton, J. C. Wagner, M. M. F. Wagner, J. S. P. Jones, G. Sheers, P. D. Oldham and R. W. Baldwin: Assessment of cell-mediated immunity to malignant mesothelioma by microcytotoxicity tests.

A. B. Miller, J. Lindsay an G. B. Hill: Mortality from cancer of the uterus in Canada and its relationship to screening for cancer of the cervix.

Yu. S. Tatarinov, D. M. Falaleeva and V. V. Kalashnikov: Human pregnancy-specific $beta_1$-globulin and its relation to chorioepithelioma.

Experimental Cancer

E. Yefenof, I. P. Witz and E. Klein: Interaction of antibody and cell surface localized antigen.

E. Okazaki, T. Aoki and S. J. de Kozlowski: AKR leukemia-specific surface antigens acquired by malignant transformation: Their common and individual specificities.

M. F. A. Woodruff, A. Ghaffar and V. L. Whitehead: Modification of the effect of *C. parvum* on macrophage activity and tumour growth by X-irradiation.

R. J. Perper, A. L. Oronsky and M. Sanda: The effect of BCG on extravascular mononuclear cell accumulation *in vivo*.

I. Gresser, C. Maury and M. Tovey: Interferon and murine leukemia. VII. Therapeutic effect of interferon preparations after diagnosis of lymphoma in AKR mice.

L. Manuelidis and E. E. Manuelidis: Localization of mouse satellite DNA on chromosomes of experimentally induced glioblastomas; noncentromeric label in one glioblastoma producing C-type particles.

P. B. Brandchaft, T. Aoki and T. N. Silverman: Immunogenic and immunosuppressive components in soluble preparations from Moloney murine leukamie virus-induced tumor cells.

N. D. Kon, J. T. Forbes and P. A. Klein: Ability of delayed-type hypersensitivity reactions to distinguish tumor associated antigens and histocompatibility antigens in soluble extracts from murine fibrosarcomas.

J. K. Reddy, M. S. Rao and M. V. Jago: Rapid development of hyperplastic nodules and cirrhosis in the liver of rats treated concurrently with thioacetamide and the pyrrolizidine alkaloid lasiocarpine.

British Journal of Cancer *May, 1976*

J. L. Foster, P. J. Conroy, A. J. Searle and R. L. Wilson: Metronidazole (flagyl): Characterization as a cytotoxic drug specific for hypoxic tumour cells.

M. F. A. Woodruff, A. Ghaffar, N. Dunbar and V. L. Whitehead: The effect of *C. parvum* on immunization with irradiated tumour cells.

R. R. Joseph, D. Belpomme and G. Mathé: Increase in 'null' cells in acute lymphocytic leukemia in remission on long-term immunotherapy.

B. W. Hancock, L. Bruce and J. Richmond: Neutrophil function in lymphoreticular malignancy.

F. M. Taylor, C. B. Freeman and R. Harris: The response of remission lymphocytes to autochthonous leukaemic myeloblasts.

Tessa E. Sadler and Peter Alexander: Trapping and destruction of blood-borne syngeneic leukaemia cells in lung, liver and spleen of normal and leukaemic rats.

Taisei Nomura: Comparison of tumour susceptibility among various organs of foetus young, and adult ICR/Jcl mice concerning their reproductive activities.

A. L. Latner and G. A. Turner: Effect of aprotinin on immunological resistance in tumour-bearing animals.

Lea Cercek and B. Cercek: Changes in the structuredness of cytoplasmic matrix (SCM) in human lymphocytes induced by PHA and CaBP as measured in single cells.

G. M. MacNab, J. B. Urbanowicz, E. W. Geddes and M. C. Kew: Hepatitis-B surface antigen and antibody in Bantu patients with primary hepatocellular cancer.

Fali S. Mehta, J. J. Pindborg, R. B. Bhonsle and P. N. Sinor: Incidence of oral leukoplakias among 20,358 Indian villagers in a 7-year period.

C. L. M. Olweny, A. Kaddumukasa, I. Atine, R. Owor, I. Magrath and J. L. Ziegler: Childhood kaposi's sarcoma: Clinical features and therapy.

Brief Communications

H. D. Preisler: Bromodeoxyuridine inhibition of post-DMSO induced erythroleukemic cell differentiation *in vitro*.

J. R. W. Masters, K. Sangster, I. I. Smith and A. P. M. Forrest: Human breast carcinomata in organ culture: The effect of hormones.

Europ. J. Cancer Vol. 12, pp. 413–418. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Specific Activities of Immunoglobulins Produced in Monoclonal Gammapathy—Maladies of Derepression

JAN G. WALDENSTRÖM
Emeritus Professor of Medicine, University of Lund, Malmö, Sweden

INTRODUCTION

THERE has been much discussion regarding the nature of the immunoglobulins that are produced in patients suffering from multiple myeloma or macroglobulinemia. For many years it was assumed that these proteins were abnormal as they were the products of pathological tumour cells. This led to the introduction of the term paraprotein indicating that these molecules were different from normal globulins. Already at an early date the present author pointed out the fact that we have no proofs regarding the correctness of this assumption. To me it always seemed much more probable that these immunoglobulins were normal antibodies only formed in excess. I have always avoided the term paraprotein for this reason. In principle this discussion is the same as the present debate regarding *ectopic* polypeptide hormone *production by cancer cells*, even if these latter paraneoplastic products are much more foreign to the tissue from which the tumour originates than the immunoglobulins, that are really *topic* products of *plasma cells* or lymphocytes. Is the ACTH or ADH produced by some cancer cells identical to the normal hormone or not?

We started to collect as many data as possible regarding antibody activity of myeloma globulins already in the fifties. Together with Winblad and others, we published a paper entitled "The occurrence of serological 'antibody' reagins or similar γ-globulins in conditions with monoclonal hypergammaglobulinemia such as myeloma, macroglobulinemia, etc." in which a battery of different immunological tests was performed on each serum with an M-component. It was remarked, however, that a false positive Wasserman reagin, a false Bunnel or a false Widal reaction against a number of bacteria was never seen [1].

In a Harvey lecture in 1961 I discussed these questions and pointed out that we had seen a number of myeloma sera with abnormally high titers of certain immunoglobulin activities (antistreptolysin, antistaphylolysin etc.). Other authors published observations regarding high cold agglutinin activity or increased titers of rheumatoid factor in sera from patients with macroglobulinemia. It has now been amply confirmed, that the monoclonal increase in plasma cells associated with benign conditions as well as with the malignant processes may be connected with a large number of serological abnormalities. It has also been proved that this monoclonal product, the M-component, is responsible for the activity. It is evident, however, that the affinity of this antibody for the antigen may be either high or low.

In a series of lectures held in Nashville, Tennessee in 1965 and published later [2], I have discussed at some length the problem of what I called the Weil–Felix phenomenon giving a number of examples of "non-specific specificity". The Weil–Felix reaction against Proteus X in carriers of Rickettsia, the occurrence of false positive Wasserman, cold agglutinins, hemolysins or other antibodies not directly connected with the antigen in certain infectious diseases such as measles, rubella, mononucleosis etc. are examples of this. My idea was that the same antibody molecule may contain sites for several antigens and that e.g.,

some measles antibody molecules may show such non-specific activity beside the specific effect on measles antigens. This idea has been confirmed by later experimental work showing that immunization against a certain well-defined antigen may give an antibody response that is also active against some other antigen when tested against a large battery.

We know that the normal antibody response develops in a standard sequence. The first phase is usually characterized by increase in macromolecular antibodies (macroglobulins, IgM) with a sedimentation constant of 19–20 S. The next response occurs as an increase in 7 S components with a molecular weight of 150,000 and belonging to the immunological type IgG and to a certain extent IgA. In multiple myeloma these latter two classes are represented. Macroglobulinemia is characterized by an increase in IgM. Each of these molecules contains one pair of connected heavy chains and one pair of light chains bound to the heavy chains. The light chains are often excreted in the urine in the form of Bence Jones proteins. These light chains may belong to two immunological types, kappa or lambda, and it is characteristic for the neoplastic "clonal" diseases of immunocytes (lymphocytes or plasma cells) that only one type of immunoglobin is produced in large amounts, e.g., IgG lambda or IgM kappa etc.

It has been shown that this clone of cells proliferating as a neoplasm only produces one individual immunoglobin and the proliferation is therefore called monoclonal. Because of this limited proliferation the term *monoclonal gammapathy* has been introduced embracing both progressive malignant myeloma with plasma cell proliferation and progressive macroglobulinemia, which is a lymphocytic disease, as well as corresponding benign neoplasms [3]. Thus recent investigations have shown, that similar increases in one single immunoglobulin of myeloma type is observed also in persons, who do not show any signs of malignant progression. It seems probable that this should be regarded as a benign plasmocytoma. This non progressive level of cell proliferation and immunoglobulin production may in rare instances change into a malignant form. We have seen more than 200 patients with constant levels of the M-component both as an IgC, IgA or IgM persisting for many years. From the point of view of prognosis it is quite important to remember that patients with such benign conditions do not need any treatment and should only be observed closely at regular intervals in order to detect if their plasma cell disease becomes malignant. The non-progressive condition has been called *benign monoclonal gammapathy* (BMG). It is quite common. Axelsson and Hällén [4] found the condition in 1% of healthy persons (7000 examined) above the age of 25. Above 70 it is present in 3% [5, 6].

Extensive research regarding the antibody nature of all such monoclonal globulins have been performed in many centers and many interesting data have been collected. It was already mentioned that antibodies against the two common infectious agents: streptococci and staphylococci were observed early in this research. In addition to our first cases we have personally seen no less than 10 cases of the first kind and 10 of the second but the number published in the literature has become rather extensive and there is no question about the fact that *antistreptolysin* and *antistaphylolysin* are the two most common monoclonal antibodies that are found in myeloma. For biological reasons this seems quite interesting as a large group in the normal population must have been challenged with a corresponding antigen at some stage in their life. This could mean that the monoclonal gammapathy only appears in persons who have had such a previous "exercise" of the corresponding clone of cells. Several authors have tried to find out if antibodies against antigens that could not have induced an earlier antibody response may also occur as a monoclonal disease. There are some indications that this may be the case but reliable proof is naturally difficult to obtain.

Zettervall has very closely investigated the immunoglobulin molecule in some myeloma patients with antistreptolysin activity of the IgG M-component [7]. He could find that the activity was located in the Fab part of the molecule which contains the normal antigen binding site. He was also able to separate light chains from heavy chains with loss of activity, whereas reconstitution of the molecule led to restoration of activity. Attempts at molecular "hybridization" with addition of light chains from a serologically inactive myeloma globulin to the heavy chains from a globulin with such activity were unsuccessful.

There is thus no question about the fact that some immunoglobulins produced by neoplastic plasma cells have a true antibody activity. Expressed as titer per weight it must be much lower than the activity of antibodies produced by immunization and the great question still remains whether the myeloma antibodies have a lower affinity indicating that they are different from normal antibodies in some way or other.

The affinity of these two antibodies as found in different myeloma cases seems to vary considerably.

Also in the IgM series we may find a marked increase in one special antibody activity. This is often an antibody occurring after challenge with an infectious agent that is common in our climate. Such *cold agglutinins* are often produced in high titers during infections with mycoplasma or Epstein–Barr virus, but these antibodies are always polyclonal. When such antibody activity is found in a patient with clinical macroglobulinemia, the molecule is always monoclonal and the titers may be excessively high (dilution: one in several millions). Interestingly enough all these macroglobulins have the kappa type of light chain with very few exceptions. This is remarkable as we do not have any indication that the kappa structure is necessary for the activity of cold agglutinin. The action of this agglutinin is directed against an antigen on the red cell surface that is called I. Practically, all adult individuals with very rare exceptions have this antigen on their red cell surface but their fetal blood contained red cells with the antigen i. Cold agglutinin directed against i are only found in polyclonal form, e.g., after the infections that have been mentioned. ABO blood group antibodies were never found in high titers.

It is of course easy to use the word cross-reactivity to explain such a phenomenon as antibody against mycoplasma and antibody against "blood group" I. This term implies that similar structures are present as antigens in both conditions. It is also possible that the same antibody molecule has affinity for several antigens and that there is a very strong affinity for one antigen and a weaker for another. This possibility was already mentioned in the introduction to this review as the Weil–Felix phenomenon [2].

There is a special clinical syndrome connected with the presence of cold agglutinins in high titer: the clinical disease macroglobulinemia presenting as a lymphoma with lymph gland swelling and splenomegaly, bleeding tendency, hyperviscosity etc. may be present or absent. The bone marrow may be full of lymphocytes, lymphocytoid plasma cells and tissue mast cells *or normal*. Most cases with the cold agglutinin syndrome may have a high titer of the antibody without very marked increase in IgM but a monoclonal fraction is always present. We have followed a number of patients with the cold agglutinin syndrome for many years, even decades, without any marked increase in macroglobulin level or cold agglutinin titer. In that case they resemble benign monoclonal gammapathy. The clinical picture includes also a hemolytic anemia. Sometimes the patients suffer from acrocyanosis. In several instances we have seen patients with a cold agglutinin that was also a cryoglobulin and in such patients it may be hard to decide the rôle of cold agglutinated red cells as compared to precipitated cryoglobulin in the obstruction of circulation, e.g., in the fingers. Acrocyanosis may be caused by both processes.

One of the most enigmatic problems in internal medicine is the influence of cold on health. Without any question cold is regarded by the layman as one of the most common causes of disease but the number of conditions with a well-defined mechanism accounted for by cold is very small. Cold agglutinin is one instance, another is precipitation of cryoglobulins in the skin with development of intravascular precipitates that may cause impaired circulation, e.g., acrocyanosis in the cold. Some patients also develop cold purpura or necrosis in the skin that is probably explained by ischemia from protein precipitation or agglutination in small vessels. The mechanism of cold urticaria is more complicated even if it seems proven that precipitation of cryoglobulin, possibly as needle-like crystals, may have something to do with histamine liberation from mast cells in the skin [8].

Cryoglobulins are usually monoclonal macroglobulins and the severe clinical syndrome that we see in many patients with this complication are sometimes explained by *hyperviscosity*. The reaction of macroglobulins on cooling is variable. Some sera with a high macroglobulin content have a high viscosity that increases moderately on cooling. In other sera the macroglobulin content is not very high, the viscosity at 37° is only moderately increased but cooling may increase the viscosity excessively leading to precipitation at different temperatures. In the latter type of case acrocyanosis is quite common and this must be differentiated from agglutination of red cells in the cold. We do not yet know which is the most common explanation of the hyperviscosity syndrome. In some cases there is an interaggregation of macroglobulin or in rare instances of IgG molecules. This is of course not to be regarded as a true immunological reaction but rather as a physicochemical phenomenon. In a few patients with monoclonal macroglobulinemia without the clinical symptoms of this disease we may see complex formation between the macroglobulin and all sorts of IgG molecules. These sera

usually show very high titers of what we call rheumatoid factor activity and it is important to remember that this immunological activity may be directed both against polyclonal rabbit gammaglobulins (Waaler–Rose test) or against human gammaglobulins, e.g. in a latex test. Such immunoglobulin complexes may be clinically very important. They are probably the basis for a condition that was described in the 1940s under the name of purpura hyperglobulinemica and may also cause renal tubular defects in such patients. This is an interesting example of an antibody against antibodies, a sort of immunological "cannibalism".

A very rare result of such interactions is the occurrence of excessive hyperviscosity with a very special clinical syndrome that may be much improved by repeated plasmapheresis. The whole problem of immune complex disease is intimately connected with the relation between different immunoglobin molecules. Not only antigen antibody bindings but also complex formation may be responsible for enigmatic clinical pictures. In some instances of immune complex disease it seems probable that the monoclonal IgM acts as rheumatoid factor and complexes with normal IgG. Binding of complement may be an important part of this process and kidney damage is probably the most serious complication.

The anticomplementary effects of many myeloma sera have been known for more than 30 years. Some of the anticomplementary titers may be extremely high. We found one IgG protein with an activity persisting at a dilution of over 1/100,000. Cooper screened 200 myeloma sera and found 4 with anticomplementary activity after heating to 56°. This has also been found by other authors. The anticomplementary activity has not been analysed in detail. It seems most probable that the heating disturbs the tertiary structure of the molecule and the binding occurs on the Fc fragment.

A large variety of other symptoms both in myeloma and in macroglobulinemia may possibly be connected with the production of antibody molecules. One of the most striking examples is the formation of *amyloid* by precipitation of light chains in such a way that the amyloid structure appears. It has been proved that amyloid is not characterized by a special chemical composition but rather by the formation of polypeptide aggregates in a special spatial arrangement as so-called non parallel, β-pleated sheets. The most common is probably caused by light chains, preferably of the lambda type, and this is the amyloid seen in diseases of immunocytes. There are, however, may other polypeptides that may form substances with amyloid characteristics. It seems probable that parts of such polypeptide hormones as calcitonin in medullary thyroid carcinoma and insulin in the pancreas may be amyloid constituents. The clinical results of amyloid formation in multiple myeloma and macroglobulinemia may be quite varied. Characteristically the heart may be infiltrated with amyloid resulting in impaired function. Not uncommonly nerves are infiltrated with amyloid and this may give severe disturbances with the picture of a polyneuropathy.

Postural hypotension has also been described from amyloid infiltration of the sympathetic system. In these cases the mechanism of disturbed function is comparatively easy to understand but other conditions may appear as very mysterious.

All these observations must be kept in mind when we talk about the connection of antibody activity and neurological disease. The number of neurological pictures connected with the production of monoclonal immunoglobulins in myeloma and macroglobulinemia is quite large [9]. It is not certain, however, that this is caused by antibody activity of the M-component and as a matter of fact we hardly know of any such globulin with a defined and well-established action against some component in nervous tissue. The same also was true regarding a number of conditions appearing in myeloma patients and disappearing during effective treatment of the primary disease. Such observations make us believe that the primary cause of the illness is not the neoplastic disease but the activity of the abnormal globulins, in producing the symptoms is improved.

We have seen patients, who have a curious combination of severe polyneuropathy resembling the *Guillain–Barré* syndrome and *myeloma* with *osteosclerosis*. A number of such cases have been published [10]. This condition has recently been found in a number of Japanese patients and their syndrome is also characterized by gynecomastia and hirsutism. It is hard to explain if this is in some way caused by the activity of the antibody or if the whole picture is a disease by itself that is not really to be regarded as a variant of myeloma. The M-component is always very small, the patients usually young and the prognosis surprisingly good.

In other myeloma cases, the patients have

signs of polyneuritis chiefly characterized by paresthesiae and in many instances also combined with deafness. This is specially typical for macroglobulinemia and has been called the *Bing–Fog–Neel syndrome*. Most authors regard it as caused by infiltration of the nerves by the proliferating lymphocytes and this is probably true even if it is difficult to dispose clinically the possibility of amyloid or antibody action.

The systematic search for antibody activity in myeloma globulins has led to the detection of several interesting myeloma antibodies. One of the most surprising connections has been the finding of wide-spread xanthomata together with myeloma in a series of patients. We described a patient with probable IgA myeloma and very marked hyperlipidemia but without xanthoma in 1951. The connection with xanthoma has been explained by a possible antibody activity of myeloma globulins against lipoproteins (Beaumont).

During the last year, a group in Switzerland has observed such myeloma globulins with an activity against apolipoproteins. As might be expected this was accompanied by low cholesterol values. A similar situation is at present under investigation in Malmö. It is possible that antilipoprotein activity is not very rare among myeloma globulins [11].

Ozer and Chaplin found a macroglobulin of monoclonal type with the capacity to agglutinate human cells that have been preserved for some time at 4°C or 37°C only, the antigen is not known [12]. It is interesting that we never found any increased activity in the ordinary blood group agglutinins (AB O) although they were tested on a large number of sera. This may be an argument in favour of the fact that monoclonal proliferation occurs, when the clone has been challenged with the corresponding antigen. Killander found a patient [13] who had a positive Wasserman but a negative Kline and TPI test. The reagin was a macroglobulin M-component. Lecithin was probably the antigen. It seems probable that several cases with antithromboplastin activity causing coagulation disorders may belong to the same group even if this is much more common in the polyclonal type of gammaglobulin increase. Castaldi *et al.* published investigations on a monoclonal macroglobulin with activity against antihemophilia globulin (Factor VIII) [14].

A patient with myeloma and hypercalcemia who did not show any obvious clinical signs of hypercalcemia was recently observed in Malmö. The monoclonal IgG has been investigated in some detail by Lindgärde *et al.* [15] and it was found that calcium ions were bound specifically to the immunoglobulin. It could be shown that the binding was localized to the Fab fragment and that the light chains were active in calcium binding. These findings are compatible with the assumption that calcium is bound in the antibody combining site.

In 1972, Westerhausen *et al.* published interesting data regarding a patient, who obviously produced an IgG that formed a complex with transferrin [16]. The serum iron level was excessively high (750) and signs of pigment cirrhosis were present. Transferrin was obviously bound to the Fab part of the monoclonal IgG_1 Kappa molecule probably in relation. Nothing is mentioned regarding signs indicating presence of a gammapathy. This is so far a unique "autoantibody".

Potter, who has made very important experiments with mice developing experimentally induced myeloma, has written an excellent review regarding antibody activity of myeloma proteins.

The connection between appearance of monoclonal globulins and a number of different clinical conditions is not well established. It has been known for a long time that the two dermatological conditions pyoderma gangranosum [17] and lichen myxoedematosus [18] are connected with increase in IgG, in the last disease and chiefly IgA in the second. It is not clear if these patients have real myeloma or if they may have developed a monoclonal antibody that is active against some constituent of the skin. This would then be an autoimmune disease caused by the proliferation of one plasma cell clone.

In general oncology it is common to find carcinoma patients who suffer from the production of specific polypeptides with hormone activity. These paraneoplastic phenomena may probably be compared with formation of immunoglobulins by special clones of plasma cells or lymphocytes. It must be remembered, however, that the normal function of these latter cells is to synthetize immunoglobulin. The product is therefore topic but the *derepression* is active only in one clone of cells. In many carcinomas the paraneoplastic product is completely ectopic, whereas it is clear that *templates for the formation of one specific polypeptide* are *activated* in such cases. The parallel is therefore near at hand. I would regard all these conditions, both topical and ectopic, as caused by *derepression of one polypeptide-forming system* with continued production without regulation in the absence of normal stimulation [10].

REFERENCES

1. J. G. Waldenström, S. Winblad, J. Hällen and S. Ljungman, The occurrence of serological "antibody" reagins or similar γ-globulins in conditions with monoclonal hypergammaglobulinemia, such as myeloma, macroglobulinemia etc. *Acta med. scand.* **176,** 619 (1964).
2. J. G. Waldenström, Monoclonal and polyclonal hypergammaglobulinemia: Clinical and biological significance. Cambridge Univ. Press (1968).
3. J. G. Waldenström, The occurrence of benign, essential monoclonal (M type), non-macro-molecular hyperglobulinemia and its differential diagnosis. IV. Studies in the gammapathies. *Acta med. scand.* **176,** 345 (1964).
4. U. Axelsson and J. Hällen, A population study on monoclonal gammapathy. Follow-up after 5½ years on 64 subjects detected by electrophoresis on 6995 sera. *Acta med. scand.* **191,** 111 (1972).
5. J. Hällen, Discrete gammaglobulin (M-) components in serum. *Acta med. scand.* Suppl. 462 (1966).
6. C. Derycke, J. M. Fine and G. A. Boffa, Dysglobulinemies "essentielles" chez les sujets âgés. *Nouv. Rev. Hématol.* **5,** 729 (1965).
7. O. Zettervall, Antibody activity in monoclonal immunoglobulin G. *Acta med. scand.* Suppl. 492 (1968).
8. J. J. Costanzi and C. A. Coltman Jr., Kappa chain cold precipitable immunoglobulin G (IgG) associated with cold urticaria. *Clin. exp. Immunol.* **2,** 167 (1967).
9. J. Bichel, J. Bing and N. Harboe, Another case of hyperglobulinemia and affection of the central nervous system. *Acta med. scand.* **138,** 1 (1950).
10. J. G. Waldenström, Maladies of derepression. *Schweiz. med. Wschr.* **100,** 2197 (1970).
11. W. Riesen, G. Noseda and R. Bütler, Anti-lipoprotein activity of human monoclonal immunoglobulins. *Vox Sang. (Basel)* **22,** 420 (1972).
12. F. L. Ozer and H. Chaplin, Agglutination of stored erythrocytes by a human serum; characterization of the serum factor and erythrocyte changes. *J. clin. Invest.* **42,** 1735 (1963).
13. A. Killander, J. Killander, L. Philipson and R. Wilten, A monoclonal gamma-macroglobulin complexing with lecithin. In: Nobel Symposium 3. Gamma Globulins (Edited by J. Killander), Interscience, New York, 359 (1967).
14. P. A. Castaldi and R. Penny, A macroglobulin with inhibitory activity against coagulation factor VIII. *Blood* **35,** 370 (1970).
15. F. Lindgärde and O. Zettervall, Hypercalcemia and normal ionized serum calcium in a case of myelomatosis. *Ann. intern. Med.* **78,** 396 (1973).
16. M. Westerhausen, B. Kickhöfen, P. Werner and H. J. Germann, Transferrin-Autoimmunsyndrom. Ein neues pathogenetisches Prinzip. *Verhandl. Deutsch. Gesellschaft für innere Medizin.* Verlag J. F. Bergmann, München. p. 901 (1972).
17. S. Jablonska, A. Stachow and H. Dabrowska, Rapports entre la pyodermite gangreneuse et le myélome. *Ann. Derm. Syph.* **94,** 121 (1967).
18. K. James, H. Fudenberg, W. L. Epstein and J. Shuster, Studies on a unique diagnostic serum globulin in papular mucinosis (lichen myxedematosus). *Clin. exp. Immunol.* **2,** 153 (1967).

Europ. J. Cancer Vol. 12, pp. 419–424. Pergamon Press 1976. Printed in Great Britain

Effect of Tamoxifen (ICI 46,474) on Initiation and Growth of DMBA*-Induced Rat Mammary Carcinomata†

V. C. JORDAN‡

Department of Pharmacology, School of Medicine, Leeds LL2 9NL, England
Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, 01545, U.S.A.

Abstract—*Tamoxifen (5 mg/day × 2) administered s.c. at the time of DMBA administration reduced the number of mammary tumour found 120 days later to < 10% of controls. A second dose regimen of tamoxifen 30 days after DMBA inhibited tumour-genesis up to 120 days. Tamoxifen (5 mg/day × 2) administered to rats with established mammary tumours produced anti-tumour effects for three weeks. Treatment tumours were significantly ($P < 0.01$) smaller than controls. The binding of [^3H]oestradiol-17β in vivo was significantly lower in uteri ($P < 0.01$) vaginae ($P < 0.001$) and mammary tumours ($P < 0.01$) of treated animals compared with controls. Tamoxifen (5 mg/day × 2) inhibited oestrogen-stimulated elevations in plasma prolactin ($P < 0.01$) and uterine wet weights ($P < 0.001$) in ovariectomized rats. Inhibition of oestrogen action at the tumour level probably plays a more fundamental role in the anti-tumour effects of tamoxifen than reductions in the circulating prolactin levels.*

INTRODUCTION

THE SUCCESSFUL induction of rat mammary tumours with DMBA requires the presence of oestrogenic hormones and prolactin [1, 2]. Surgical techniques which lower circulating levels of oestrogen and prolactin e.g. ovariectomy and hypophysectomy, produce regression of established mammary tumours [3] therefore oestrogens and prolactin appear to be necessary for continued tumour growth.

Daily administrations of the non-steroidal anti-oestrogen tamoxifen (ICI 46,474) [4] inhibits the growth of DMBA-induced rat mammary carcinomata [5–7]. Single administrations of large doses of tamoxifen produce prolonged anti-oestrogenic effects in laboratory animals [8, 9] and the present study describes the utilization of this technique to inhibit the initiation and growth of DMBA-induced rat mammary carcinomata.

Accepted 7 January 1975.

*DMBA. 7,12-dimethylbenz(a)anthracene.

†This study was supported by grants from ICI United States, ICI Ltd. and VCJ was supported at WFEB by NCI contract No. 1-CB-43967.

‡Present address and reprint requests: Department of Pharmacology, School of Medicine, Leeds LS2 9NL, England.

MATERIAL AND METHODS

DMBA and oestradiol-17β were obtained from Sigma Chemicals. Tamoxifen (*trans*-1-(p-β-dimethylaminoethoxyphenyl) 1,2 diphenyl but-1-ene) was a gift from ICI Ltd. All subcutaneous injections in peanut oil were prepared from stock solutions in absolute ethanol. The ethanol was evaporated under N_2 to produce the required concentration in 0·1 ml peanut oil. DMBA was dissolved in peanut oil by warming and the solution was allowed to cool before administration. DMBA (20 mg in 2 ml peanut oil) was administered by gavage to 50-day-old female Sprague Dawley rats as previously described [10]. Throughout the study, statistical comparisons of means were made by Student's *t*-test.

(a) *Inhibition of tumour initiation*

After the administration of DMBA (day 1), rats were weighed and divided into four groups each of seven rats: A, B, C & D. Group A received 5 mg tamoxifen subcutaneously on days 1 and 2; Group B received 5 mg tamoxifen subcutaneously on days 1 and 2 and days 30 and 31; Group C was ovariectomized under ether anaesthesia (day 1) and given peanut oil subcutaneously (day 1 and 2); and Group D was used as control and given peanut oil

subcutaneously (days 1 and 2). At 80, 100 and 120 days after DMBA, animals were weighed and examined for tumours. Tumours were measured with calipers and tumour area was calculated using the formula $\pi \times$ length/2 $\times$ width/2. Vaginal smears were taken for one week starting 120 days after DMBA and then tumour samples were taken for histological examination.

(b) *Inhibition of tumour growth*

Animals were obtained from a pool of rats treated with DMBA. One hundred days after DMBA, 5 rats with 14 established mammary tumours were biopsied and tumour samples were taken for histological examination. Tumours were measured and each rat received a subcutaneous injection of 5 mg tamoxifen on the two consecutive days after biopsy. Similarly 6 rats with 9 tumours were biopsied and received vehicle alone. Treatment and control groups were weighed and tumours were measured at weekly intervals for three weeks.

After the final measurement, animals were ovariectomized under ether anaesthesia. One treatment and one control rat did not recover from the effects of anaesthesia and died during the night. Twenty-four hours after ovariectomy each rat received a subcutaneous injection of 30 μCi 6,7 [^{3}H]oestradiol-17β (49·3 Ci/mmol. New England Nuclear Corporation) in 0·1 ml peanut oil. Four hours later rats were killed and uteri, vaginae and samples of heart and tumour tissue were dissected out, dried overnight in a 100°C oven and weighed on a torsion balance. Tissues were combusted in a Packard Tricarb Tissue Oxidiser and samples were automatically collected in 10 ml tritium scintillator (6 g Butyl-PBD [2(4′-tert-butylphenyl)-5(4″-biphenylyl)-1, 3, 4-oxadiazole], 135 ml tolene, 720 ml dioxan, 100 g naphthalene, 45 ml absolute methanol). Vials were counted for 10 min in a Packard Tricarb Liquid Scintillation Spectrometer (model 3320). Counts per minute were converted to disintegrations per minute (dis/min) by the external standard channels ratio method and results were expressed as dis/min per mg dry weight tissue.

Inhibition of oestrogen-stimulated plasma prolactin levels

Female Sprague–Dawley rats (180–200 g Charles River Breeding Laboratories) were ovariectomized under ether anaesthesia and used one week later. Animals were divided into five Groups (1–5) each of 7 rats. All administrations were made subcutaneously. Groups 1 and 2 received 5 mg tamoxifen on days 1 and 2 of the experiment. Group 3 received 1 mg tamoxifen on days 1 and 2. Groups 4 and 5 received peanut oil. Groups 1, 3 and 4 received daily injections of 5 μg oestradiol-17β for 8 days of the experiment. Groups 2 and 5 received peanut oil. On day 8 animals were treated and placed in a noise-free environment. Animals were killed between 20.00 and 22.00 in a screened area of the laboratory by stunning and decapitation. Blood was collected in ice-cold heparinized tubes and plasma was obtained by centrifugation at 1800 $\times$ ***g*** for 10 min (4°C). Plasma was frozen immediately and stored at -10°C until radioimmunoassay. Uteri were dissected out and weighed wet on a torsion balance.

Radioimmunoassay of prolactin

All samples were determined in one assay. The method used was that described by Odell, Rayford and Ross [11]. Rat prolactin and antibody were a gift from the National Institute for Arthritis, Metabolic and Digestive Diseases (NIAMDD). Results were expressed as ng. NIAMDD-rat prolactin-RPL standard per ml rat plasma. Assay sensitivity was 0·25 ng/ml.

RESULTS

(a) *Inhibition of tumour initiation*

At 80 days after DMBA no tumours were detectable in tamoxifen treated or ovariectomized groups, whereas 7 tumours (range 0·25–1·53 cm^2) were present in the controls. At 100 days after DMBA 1 tumour (0·91 cm^2) was present in the animals treated only with tamoxifen at the time of DMBA administration. No other tumours appeared in this group at 120 days. No tumours were present at 120 days in the ovariectomized group or the group treated with two courses of tamoxifen (Group B). Control rats had a total of 14 tumours (range 0·31–5·37 cm^2) at 120 days after DMBA although one rat did not develop tumours. All tumours were identified histologically as adenocarcinomata.

At the end of the experiment all animals in the control and tamoxifen treated groups were found to have oestrous cycles. Ovariectomized rats had leukocytic smears.

At 120 days after DMBA ovariectomized rats (384 $\pm$ 13 g) were significantly heavier ($P < 0{\cdot}001$) than controls (293 $\pm$ 9 g). The

group treated with tamoxifen at the time of DMBA (272 ± 7 g) were not significantly different ($P > 0.05$) than controls but Group B (260 ± 5 g) which received a subsequent injection of tamoxifen were significantly lighter ($P < 0.01$) than controls. At the time of DMBA administration, rats were within the range 156–217 g before randomization into groups.

(b) *Inhibition of tumour growth*

The administration of tamoxifen (10 mg) to rats with established DMBA-induced mammary carcinomata, resulted in a significant ($P < 0.01$) reduction in tumour area at 3 weeks when compared with controls (Table 1). Rats treated with tamoxifen experienced a stabilization in body weight whereas control animals continued to gain weight.

Control tumours (8) grew steadily throughout the 3 week observation period although one tumour underwent spontaneous regression. In the tamoxifen treated group (14 tumours), three types of tumour response were observed (Fig. 1). A continued growth was seen in 3 tumours although the growth rate was less than that of controls. Stasis was observed in 3 tumours, but the majority of tumours (8) underwent a steady regression.

Throughout the experimental period, oestrous cycles occurred in the control rats, whereas the tamoxifen treated group went into a prolonged dioestrus.

Three weeks after the administration of tamoxifen, treatment and control rats were ovariectomized, and the following day each rat was injected with 30 μCi [^{3}H]oestradiol-17β. At 4 hr after administration, the levels of

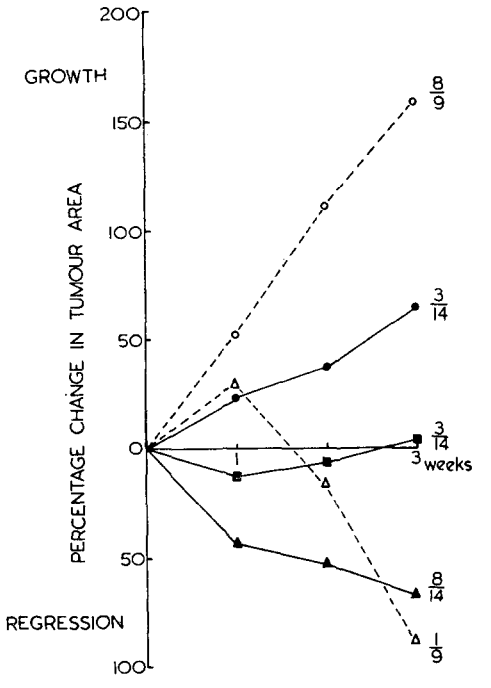


Fig. 1. Graph of the mean percentage growth or regression of DMBA-induced rat mammary tumours from rats treated with tamoxifen (5 mg/day × 2) (●——● ■——■ ▲——▲) on the first two days of the experiment or control rats receiving peanut oil (○——○ △——△). Tumour areas were calculated each week and the results were expressed as a percentage of the tumour area at the start of the experiment. Number of tumours in each category are shown on the right.

radioactivity in uterus ($P < 0.01$), vagina ($P < 0.001$) and mammary tumours ($P < 0.01$) of tamoxifen treated rats was significantly lower than controls. (Fig. 2). The level of radioactivity in heart tissue was unchanged. There was no significant difference between the levels of radioactivity in the tumours of treated rats and that in the heart tissue.

Table 1. The effect of tamoxifen (5 mg/day × 2) on the growth of DMBA-induced rat mammary tumours. Tumours were measured weekly for 3 weeks after the administration of tamoxifen. Statistical analysis was by Student's t-test between related treatment and control groups.

		Time (weeks)			
		0		3	
	Number of tumours	Wt (g)	Tumour area†	Wt (g)	Tumour Area
Control (6 rats)	9	305 ± 5	2·19 ± 0·45	316 ± 4	4·93 ± 1·12
Tamoxifen treated (5 rats)	14	283 ± 9	1·7 ± 0·19	284 ± 7*	1·38 ± 0·28*

†Mean tumour area ± S.E.M. (cm^2).
*$P < 0.01$.
Other values $P > 0.05$.

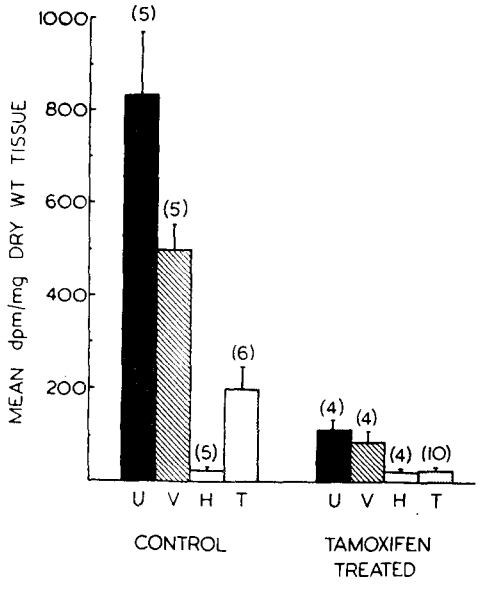


Fig. 2. Effect of tamoxifen therapy (5 mg/day × 2) on the levels of radioactivity (dis/min per mg dry wt ± S.E.M.) in uterus (U), vagina (V), heart (H) and DMBA-induced mammary tumour (T). Three weeks after tamoxifen, animals were ovariectomized and each rat was given 30 μCi 6,7[^{3}H]-oestradiol-17β. Animals were sacrificed 4 hr later and tissue levels of radioactivity was obtained by combustion. Number of samples in each group is shown in parenthesis.

(c) *Inhibition of oestrogen-stimulated plasma prolactin levels*

The administration of a total dose of 2 or 10 mg tamoxifen to ovariectomized rats at the start of a 8 day course of oestradiol treatment, significantly ($P < 0{\cdot}001$) reduced uterine wet weight increases but only 10 mg tamoxifen significantly reduced ($P < 0{\cdot}01$) oestradiol-stimulated rises in plasma prolactin levels (Fig. 3). The administration of tamoxifen (10 mg) stimulated significant rises in uterine wet weight ($P < 0{\cdot}001$) and plasma prolactin levels ($P < 0{\cdot}001$) when compared with ovariectomized controls. A combination of oestradiol and tamoxifen (10 mg) did not reduce the uterine wet weights and plasma prolactin levels to those of tamoxifen alone. A statistical comparison of tamoxifen (10 mg) with and without oestradiol, for uterine wet weights and plasma prolactin levels gave values of $P < 0{\cdot}05$ and $P < 0{\cdot}001$ respectively.

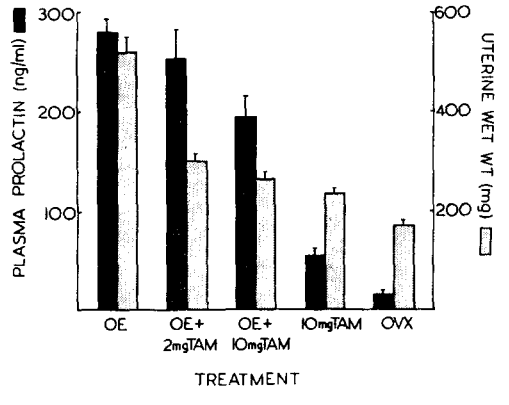


Fig. 3. Effect of tamoxifen (TAM. 5 mg/day × 2 or 1 mg/day × 2) on oestradiol-17β (OE. 5 μg/day × 8)—stimulated increases in uterine wet weights and plasma prolactin. (7 ovariectomized [OVX] rats/group).

DISCUSSION

The daily administration of anti-oestrogens [12] or oestrogen withdrawal [13, 14] have been reported to impair or abolish the induction of mammary tumours with polycyclic hydrocarbons.

A two day course of large doses of tamoxifen can produce long term anti-oestrogenic effects in rats [8] and mice [8, 9] and in the present study, the administration of a dose regimen which has been found to produce long term anti-oestrogenic effects, was successful in inhibiting the induction of mammary tumours by DMBA. This result is consistent with the results obtained in a preliminary study. [15].

An interesting feature of the data was the significant weight gain in ovariectomized rats, compared with the rats treated twice with the anti-oestrogen tamoxifen. Neither group produced tumours. Further studies are planned to determine the mechanism by which tamoxifen regulates rat body weight whereas oestrogen withdrawal results in increased body weight.

DMBA-induced tumours grow in response to oestrogens [16] or prolactin [17] but since oestrogens can stimulate prolactin production (Fig. 3) [18] anti-oestrogens may either impair the direct effects of oestrogens at the tumour level [19, 20] or block oestrogen-stimulated prolactin release.

In previous studies the non-steroidal anti-oestrogens tamoxifen, MER 25 [21] and nafoxidine [1] have been shown to only partially reduce oestrogen stimulated prolactin levels. Similarly in the present study, a dose of tamoxifen which produced prolonged tumour regression, in the majority of tumours (Fig. 1), was unable to reduce oestrogen-stimulated prolactin levels to those of the ovariectomized rat (Fig. 3).

By contrast, whilst tumour regression was in progress and three weeks after tamoxifen administration, the binding of [^{3}H]oestradiol in DMBA-induced tumours was reduced to that of non-target tissue (heart) (Fig. 2). The

daily administration of tamoxifen reduces oestrogen binding in regressing mammary tumours [6] and similarly in the present study, a short course (2 days) of large doses of tamoxifen has been found to be effective for an extended period. This prolonged anti-tumour effect demonstrates that daily administration may be unnecessary.

In the United Kingdom, tamoxifen is used for the treatment of breast cancer [22, 23]. Assuming that the mechanism of action of tamoxifen is the same in human and rat mammary cancer then it may be possible to reduce the frequency of administration by raising the dose. Tamoxifen has been reported to have a biological half-life of several days in female patients [24].

DMBA-induced tumours, like those in the human [22, 23], do not all respond to tamoxifen. This is illustrated in Fig. 1 and we have previously found [24, 26] that the heterogeneous tumour response to tamoxifen is related to the oestrogen binding capacities of the tumours i.e. tumours with low levels of oestrogen receptors have been found to remain static or continue to grow, but as the concentration of oestrogen receptors in the tumours increase there is an increase in tumour regression.

In the present experiments tamoxifen inhibited the initiation and growth of DMBA-tumours. The blockade of oestrogen binding in the tumours may be of fundamental importance since tamoxifen produced only partial inhibition of oestrogen-stimulated prolactin release. It has been reported that tamoxifen may have a direct action at the ovarian level [27], thus reducing circulating oestrogen levels. This would facilitate competitive blockade of oestrogen receptors at the tumour level but may not significantly affect the release of prolactin [7].

Acknowledgements—I would like to thank Carol Robison (WFEB) for undertaking the prolactin assay, Drs. Pamela and David Tarin for histology and pathological identification of tumours and Philip Rylett for preparing the figures.

REFERENCES

1. J. C. Heuson, C. Waelbroek, C. Legros, G. Gallez, C. Robyn and N. L'Hermite. Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-Br-α ergocrytine (CB154), an inhibitor of prolactin secretion, and by nafoxidine (U-11, 100A), an estrogen antagonist. *Gynecol. Invest.* **2,** 130 (1971/72).
2. P. Chan and L. A. Cohen, Effect of dietary fat, antiestrogen and antiprolactin on the development of mammary tumours in rats. *J. nat. Cancer Inst.* **52,** 25 (1974).
3. A. Sterental, M. Dominguez, C. Weissman and O. H. Pearson, Pituitary role in the estrogen dependency of experimental mammary cancer. *Cancer Res.* **23,** 481 (1963).
4. M. J. K. Harper and A. L. Walpole, A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J. Reprod. Fertil.* **13,** 101 (1967).
5. R. I. Nicholson and M. P. Golder, The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Europ. J. Cancer* **11,** 571 (1975).
6. V. C. Jordan and L. J. Dowse, Tamoxifen as an anti-tumour agent I. Effect of oestrogen binding. *J. Endocr* **68,** 297 (1976).
7. V. C. Jordan and S. Koerner, Tamoxifen as an anti-tumour agent II. Role of oestradiol and prolactin. *J. Endocr* **68,** 305 (1976).
8. C. W. Emmens, Compounds exhibiting prolonged anti-oestrogenic and anti-fertility activity in rats and mice. *J. Reprod. Fertil.* **26,** 175 (1971).
9. V. C. Jordan, Prolonged anti-oestrogenic activity of ICI 46, 474 in the ovariectomized mouse. *J. Reprod. Fertil.* **42,** 251 (1975).
10. C. Huggins, L. C. Grand and P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* **198,** 204 (1961).
11. W. D. Odell, P. L. Rayford and G. T. Ross, Simplified, partially automated method for radioimmunoassay of human thyroid-stimulating hormone, grow, luteinizing and follicle stimulating hormones. *J. lab. & clin. Med.* **70,** 973 (1967).
12. L. Terenius, Effect on anti-oestrogens on initiation of mammary cancer in the female rat. *Europ. J. Cancer* **7,** 65 (1971).

13. C. Huggins, G. Brizarelli and H. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumours. *J. exp. Med.* **109,** 25 (1959).
14. T. L. Dao, The role of the ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. *Cancer Res.* **22,** 973 (1962).
15. V. C. Jordan, Anti-tumour activity of the anti-estrogen ICI, 46, 474 (Tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J. Steroid Biochem.* **5,** 354 (Abstr.) (1974).
16. D. Sinha, D. Cooper and T. L. Dao, The nature of estrogen and prolactin effects on mammary tumorigenesis. *Cancer Res.* **33,** 411 (1973).
17. B. S. Leung, G. H. Sasuki and J. S. Leung, Estrogen–prolactin dependency in 7,12 dimethylbenz(a)anthracene-induced tumour. *Cancer Res.* **35,** 621 (1975).
18. C. L. Chen and J. Meites, Effect of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. *Endocrinology* **86,** 503 (1970).
19. C. Lee and R. Oyasu, Direct action of estradiol on rat mammary tumours. *J. nat. Cancer Inst.* **52,** 283 (1974).
20. L. Y. Arbogast and E. R. De Sombre, Estrogen-dependent *in vitro* stimulation of RNA synthesis in hormone dependent mammary tumours of the rat. *J. nat. Cancer Inst.* **54,** 483 (1975).
21. V. C. Jordan, S. Koerner and C. Robison, Inhibition of oestrogen stimulated prolactin release by anti-oestrogens. *J. Endocr.* **65,** 151 (1975).
22. M. P. Cole, C. T. A. Jones and I. D. H. Todd, A new anti-oestrogenic agent in late breast cancer. *Brit. J. Cancer* **25,** 270 (1971).
23. H. W. C. Ward, Antioestrogenic therapy for breast cancer: a trial of tamoxifen at two dose levels. *Brit. med. J.* **1,** 13 (1973).
24. J. M. Fromson, S. Pearson and S. Bromah, The metabolism of tamoxifen (ICI 46,474). Part II: in female patients. *Xenobiotica* **3,** 711 (1973).
25. V. C. Jordan and T. Jaspan, Oestrogen binding as a predictive test for DMBA-induced tumour response to tamoxifen therapy. *Proc. 9th Int. Cong. of Chemotherapy* **7,** (1975) (in press).
26. V. C. Jordan and T. Jaspan, Tamoxifen as an anti-tumour agent. Oestrogen binding as a predictive test for tumour response. *J. Endocr.* **68,** 453 (1976).
27. J. Watson, F. B. Anderson, M. Alam, J. E. O'Grady and P. J. Heald, Plasma hormones and pituitary luteinizing hormone in the rat during the early stages of pregnancy and after post-coital treatment with tamoxifen (ICI 46,474). *J. Endocr.* **65,** 7 (1975).

Europ. J. Cancer Vol. 12, pp. 425–432. Pergamon Press 1976. Printed in Great Britain

Burkitt's Lymphoma in Ibadan: Response to Various Doses of Cyclophosphamide and Long-Term Survivors

JAMES I. DURODOLA

Department of Surgery (Oncology Unit), University of Ibadan, Ibadan, Nigeria

Abstract—*The results of therapy with various doses and dose-schedules of cyclophosphamide of* 146 *patients with Burkitt's lymphoma are reported. Sixty-five patients were in stage I,* 36 *in stage II,* 41 *in stage III, and* 4 *in stage IV. Various doses in mg/kg used were* 5, 10, 15, 20, 30, 40, 50, 60, 100, *and* 130*; and administered either as i.v. infusion, or single i.v. injection or multiple (chronic) i.v. injection-schedule. In spite of the difficulty of follow-up of the patients thereby making an accurate summary of the long-term survivors impossible, evidence was presented of many patients surviving up to* 5 *years and longer. The study shows that:* 1. *complete and durable remissions could be achieved with cyclophosphamide;* 2. *long-term survivors are independent of the dosage or dose-schedules of the drug even in patients with stage III disease;* 3. *patients in stage IV disease did very poorly even with intensive chemotherapy;* 4. *patients can tolerate very high doses of cyclophosphamide.*

INTRODUCTION

MANY patients with Burkitt's lymphoma have rapid tumor regression following administration of cyclophosphamide, and in some the tumor regression was complete and sustained, producing long-term survivors. Burkitt and others [1–4] have suggested that host defence participate in tumor regression and that intensive chemotherapy might suppress possible immunity against the tumor. Ziegler and others [5, 6] on the other hand thought that because Burkitt's lymphoma cells are very susceptible to cytotoxic agents, intensive chemotherapy with cyclophosphamide might prevent tumor relapse and prolong remission.

Oettgen *et al.* [7] compared the results of chronic daily low doses of cyclophosphamide at 2·5 to 10·00 mg/kg p.o. or i.v. with aggressive single large dose at 30–40 mg/kg i.v. and felt that the aggressive treatment was preferable. However, a later report [8] suggested that their 17 patients treated with those doses of cyclophosphamide had died without producing long-term survivors.

Accepted 19 January 1976.

Ngu [9] giving no figures, observed that 90% of those completing the first course of treatment (cyclophosphamide) showed complete tumor regression and over 40% of treated cases have survived for five years and more, several over ten years.

Ziegler *et al.* [5] and Nkrumah and Perkins [6] who used only large doses of cyclophosphamide reported the follow-up of their patients for only 2 or 3 years; they compared the effects of a single large dose with multiple large doses of cyclophosphamide at 40 mg/kg i.v.

Considering the fact that the cases cited above are either too few or/and the period of follow-up of the survivors too short, no important conclusion could be made as to the advantages of large doses over small doses of cyclophosphamide in producing remission and long-term survivors.

The present report consists of the study of a total of 146 Burkitt's lymphoma patients who received various doses and dose-schedules of cyclophosphamide either as primary or secondary agent, and at least 20 of who have been followed up for 5 years or more.

Because cyclophosphamide used in our series ranged from very small doses to extremely large doses with various dose-schedules, and the number of patients treated was large, fairly definite conclusions about effects of doses and dose-schedules can now be made.

MATERIAL AND METHODS

Patients

A total of 146 unselected patients who had been treated by the Oncology Unit of Department of Surgery were reviewed. They were mostly children (except 3 adults) in whom diagnosis of Burkitt's lymphoma was made clinically (10), radiologically (9), or histopathologically/cytologically (127). There were 111 males (76%) and 35 females (24%), giving roughly a sex ratio of 3 to 1. The peak incidence is at age 8 years (Fig. 1).

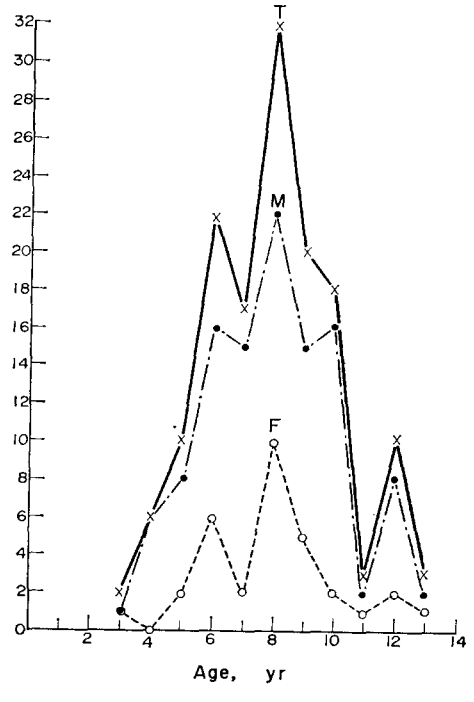


Fig. 1. *Burkitt's lymphoma: age and sex distribution of* 146 *patients treated with cyclophosphamide.* (*T* = *total number*, *M* = *males*, *F* = *females*)

Facial bones (105 = 72%), mainly maxilla and mandible, were affected most commonly either alone (79) or in combination with other parts of the body (26). The next common site of involvement was the abdomen (42 = 29%), mainly the peritoneum, ovary, spleen, kidneys, and liver.

Investigations and staging

Routine clinical investigations were done in majority of the cases, which included white blood cells count, differential, platelets; hemoglobin determination (pack cell volume = pcv), serum electrolytes, blood urea nitrogen (bun). Routine roentgenography was also done. Special clinical investigations were done only when absolutely necessary; these included liver function tests, lumbar puncture for CSF, bone marrow aspirate, intravenous pyelography, and abdominal lymphangiography. In most cases clinical staging was done retrospectively by the author and was based on the sites of involvement recorded when the patient was first seen. They were classified according to the four clinical stages (commonly used) outlined in Table 1. In the absence of routine lumbar puncture, bone marrow aspiration, or/and laparotomy, clinical staging is not infallible.

Table 1. *Clinical staging of Burkitt's lymphoma*

Stage	Extent of disease
I	Single tumor mass*
II	Two or more separate tumor masses*
III	Intra-thoracic, intra-abdominal, paraspinal, or osseus tumor (excluding facial bones)
IV	Central nervous system (malignant cells in the CSF) or bone marrow involvement

*Usually facial involvement

Of the 146 patients, 65 (44·5%) were in stage I, 36 (24·7%) in stage II, 41 (28·1%) in stage III, and 4 (2·7%) in stage IV (see Fig. 2). With recurrences, however, the staging pattern changed as many who were in the early stages moved to advanced stages.

Treatment

One-hundred and twenty-seven patients received cyclophosphamide as primary agent while 19 received the drug as secondary agent. Those 19 patients had received the following drugs as primary agents: leukeran (4), melphalan (3), cytosine arabinoside (3), methotrexate (2), immune serum (2), i.v. nitrogen mustard (1), orthomelphalan (1), interferon (1), vinblastine (1), and colcemid (1).

The number of patients who received various doses of cyclophosphamide is shown in Table 2. In many of these cases the initial dosages were increased or decreased depending on the state of the bone marrow and evidence of response to the drug.

Various dose-schedules of administration of

Table 2. Various doses of cyclophosphamide and the long-term survivors

Dosage (mg/kg)	No. of patients	Survival (years)												Recorded deaths
		½	1	2	3	4	5	6	7	8	9	10	11	
5	3	1	—	—	—	—	—	—	—	—	—	—	—	1
10	66	23	14	10	8	7	7	6	5	4	3	2	1	8
15	29	11	7	6	5	5	4	4	4	2	1	—	—	4
20	23	9	7	4	3	2	2	2	1	1	1	1	1	4
30	8	6	4	4	4	4	3	2	2	2	1	—	—	1
40	10	6	4	3	—	—	—	—	—	—	—	—	—	0
50	1	1	—	—	—	—	—	—	—	—	—	—	—	0
60	2	—	—	—	—	—	—	—	—	—	—	—	—	0
100	2	1	1	1	1	1	—	—	—	—	—	—	—	0
130	1	—	—	—	—	—	—	—	—	—	—	—	—	0
Total	146	58	37	28	21	19	16	14	12	9	6	3	2	17

the drug was also used, the larger doses, e.g. 130 mg/kg were given as i.v. infusion in 500 ml of normal saline over 15–24 hr, while 60 mg/kg and 50 mg/kg were given as single i.v. injections. About half of the patients who received 40 mg/kg had single i.v. injection-schedule while the other half received multiple doses and some received further 10 mg/kg maintenance therapy. Most of the lower doses were given on the multiple-injection-schedules with maintenance therapy.

RESULTS

Inadequate follow-up of 43 of our patients has precluded accurate assessment of the long-term survivors. Survival analyses were restricted to 103 of the 146 patients for whom partial or complete follow-up information was available. Of the 103 evaluable patients, 54 deaths were recorded, 23 died in the hospital (post-mortem was done in 12 of them); and 31 died at home at various times, the cause of death was unknown. The report of 2 of the 12 post-mortems gave cause of death as pneumothorax and bronchopneumonia respectively; in the other 10, death was due to generalized involvement of body organs, particularly the kidneys.

Further breakdown of the mortality showed: thirty-nine deaths occurred within 6 months of diagnosis of their disease, 10 others died before one year and 5 more died before two years. None of our patients who survived two years has had any recurrence of the tumor, and no deaths as result of the tumor had occurred among them. Most of the patients who died early (4–6 weeks of diagnosis of their disease) were moribund when first seen, and most of those who died at home were among those who were removed from the hospital by the parents or relatives because they thought that all hopes of cure were gone.

Of the 43 defaulting patients (who did not complete a course of treatment or come back for adequate check-up or follow-up), 14 defaulted in the first month, 12 in the second, 5 in the third, 4 in the fourth, and the rest within 12 months from diagnosis of their disease.

Table 2 presents the overall follow-up and long-term survivors of the 103 evaluable patients from the total of 146 patients treated with various doses of cyclophosphamide, and who survived up to 6 months. Table 3 shows the doses and dose-schedules used for a selected number of long-term survivors (28) from Table 2. Since Tables 2 and 3 were prepared 6 months ago, 4 more patients surviving 5 years or longer have been traced, making the number of those surviving 5 years or longer 20 instead of the 16 recorded in Table 2. Table 4 shows the doses and dose-schedules used in treating 29 patients described as short-term survivors. Table 5 presents the clinical stages of 128 evaluable patients (with adequate data on doses and stages of the disease) who received various doses of cyclophosphamide.

In Table 3 the clinical stages of the 28 patients showing good response (= complete regression of their tumor) are as follows: stage I = 15 (53·7%), stage II = 7 (25·00%), stage III = 6 (21·43%). These patients have had no recurrences of their tumor and 20 of them are now known definitely to have survived 5 years or longer. The number of patients in similar clinical stages but showing poor/no response (= no regression of their tumor) is shown in Table 4 as short-term survivors.

Table 3. Daily doses and dose-schedules of cyclophosphamide and long-term survivors

Sex	Age	Stage	Daily doses and schedules of administration	Last visit	Survivals (years)
M	8	II	10 mg/kg × chronic†	1975	11
M	8	II	10 mg/kg × chronic	1975	10
M	10	I	10 mg/kg × chronic	1975	9¼
M	9	III	10 mg/kg × chronic	1973	9
M	7	I	10 mg/kg × chronic	1975	8⅔
F	11	I	10 mg/kg × chronic	1975	7¼
M	8	I	10 mg/kg × chronic	1973	6⅓
F	7	II	10 mg/kg × chronic	1971	5½
M	8	III	10 mg/kg × 10*	1972	3½
F	3	III	10 mg/kg × 2	1970	2
M	8	II	10 mg/kg × 7	1975	2
F	8	III	15 mg/kg × 7, 10 mg/kg × 7	1975	8¼
M	8	I	15 mg/kg × chronic	1975	7½
M	4	III	15 mg/kg × chronic	1975	7½
M	5	I	15 mg/kg × 7, 10 mg/kg × 5	1972	4
M	5	I	15 mg/kg × 14	1975	2
M	12	I	20 mg/kg × chronic	1975	11½
M	11	I	20 mg/kg × chronic	1968	6
M	6	I	20 mg/kg × 1, 10 mg/kg × chronic	1973	3½
M	7	I	20 mg/kg × chronic	1966	2
F	10	I	30 mg/kg × 5, 10 mg/kg × 5	1975	9¼
M	4	I	30 mg/kg × chronic	1975	8½
M	4	II	30 mg/kg × chronic	1971	5
M	6	II	30 mg/kg × 1, 10 mg/kg × chronic	1974	4
M	9	III	40 mg/kg × 2	1970	2½
M	10	I	40 mg/kg × 2, 10 mg/kg × chronic	1968	2
M	7	II	40 mg/kg × 1, 10 mg/kg × chronic	1975	2
M	6	I	100 mg/kg × 1 infusion in 15 hours	1971	4½

*Number of doses
†Chronic = more than 10 doses; in many cases doses were increased, or decreased, or changed to p.o.

Figure 3 shows the observed survival rate percentage for 2 years of 103 evaluable patients for whom adequate data were available. Figure 4 presents the stages and observed survival rate % of 64 patients who survived the follow-up of up to 6 months. Figure 5 shows three dose-schedules and observed survival rate % of 65 patients with evaluable data for doses and survival beyond 6 months. The number of patients who received doses larger than 40 mg/kg is too few to include in this figure.

Of the 146 patients treated with various doses of cyclophosphamide (Table 2), 48 were recorded as having complete regression of their tumors, 38 partial regression, and 60 no regression. Of the 86 patients thus showing

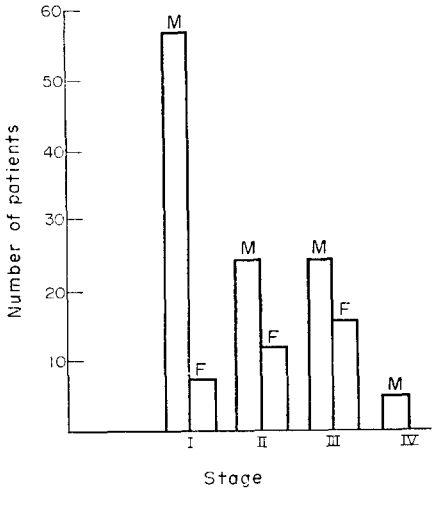


Fig. 2. Clinical staging of Burkitt's lymphoma (146 patients). M = males, F = females.

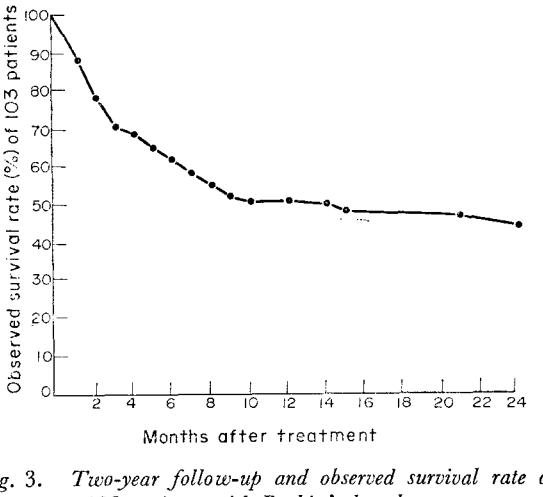


Fig. 3. Two-year follow-up and observed survival rate of 103 patients with Burkitt's lymphoma.

Table 4. Daily doses and dose-schedules of cyclophosphamide and short-term survivors

Sex	Age	Stage	Daily doses and schedules of administration	Survivals (months)
M	10	III	5 mg/kg × 5*, 30 mg/kg × 3	2
M	10	III	10 mg/kg × chronic†	4
F	8	I	10 mg/kg × chronic	3
M	4	II	10 mg/kg × 7, 5 mg/kg × 10	2
M	9	I	10 mg/kg × 10	2
M	10	I	10 mg/kg × chronic	2
M	11	> III	10 mg/kg × chronic	2
M	6	I	10 mg/kg × chronic	1
F	8	I	10 mg/kg × 7, 20 mg/kg × 7	2
M	9	I	10 mg/kg × 28	1
M	12	III	15 mg/kg × 14, 40 mg/kg × 1	4
M	8	III	15 mg/kg × 6, 20 mg/kg × 30	2
F	6	III	15 mg/kg × chronic	2
M	3	II	15 mg/kg × chronic	2
M	8	I	15 mg/kg × chronic	2
F	9	III	15 mg/kg × chronic	2
M	5	II	20 mg/kg × 7, 30 mg/kg × 5	1
M	5	II	20 mg/kg × chronic	4
M	9	I	20 mg/kg × chronic	3
F	12	I	20 mg/kg × chronic	3
F	9	I	20 mg/kg × chronic	2
F	8	II	30 mg/kg × 21	4
M	8	I	40 mg/kg × 5, 30 mg/kg × 5	2
M	5	I	40 mg/kg × 5	1
M	7	I	40 mg/kg × 1	1
M	6	I	60 mg/kg × 1	2
M	6	I	60 mg/kg × 1	1
M	12	III	100 mg/kg × 1 in 24 hours in saline	1
M	6	III	130 mg/kg × 1 in 24 hours in saline	1

*Number of doses
†Chronic = more than 10 doses; in many cases doses were increased, or decreased, or changed to p.o.

Table 5. Clinical stages of patients who received various doses of cyclophosphamide

Dose schedule	Total No. of patients	Number of patients by clinical stages I	II	III	IV
10 mg/kg × 2 to 10*	13	7	5	1	0
10 mg/kg × chronic†	43	20	10	13	0
15 mg/kg × 2 to 10	7	2	2	3	0
15 mg/kg × chronic	19	4	5	9	1
20 mg/kg × 2 to 10	7	3	0	3	1
20 mg/kg × chronic	14	7	3	3	1
30 mg/kg × 2 to 10	6	3	3	0	0
30 mg/kg × chronic	2	1	1	0	0
40 mg/kg × 2 to 8	10	4	2	4	0
50 mg/kg × 2 to 4	1	0	1	0	0
60 mg/kg × 2 to 4	3	2	0	1	0
100 mg/kg × 1	2	1	0	1	0
130 mg/kg × 1	1	0	0	1	0
	128	54	32	39	3

*Number of doses
†Chronic = more than 10 doses; in many cases doses were increased, or decreased, or changed to p.o.

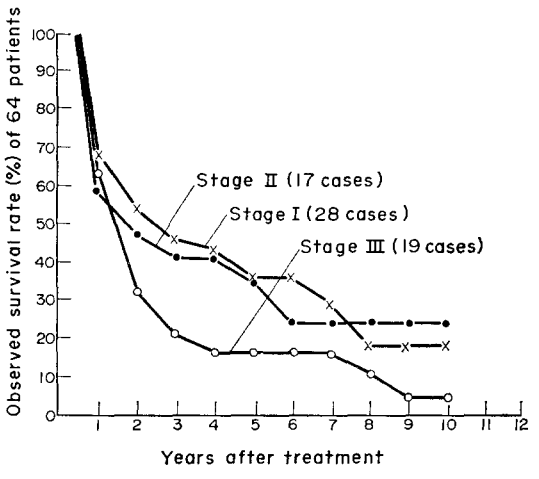


Fig. 4. Follow-up and observed survival rate of 64 patients with Burkitt's lymphoma who survived more than 6 months.

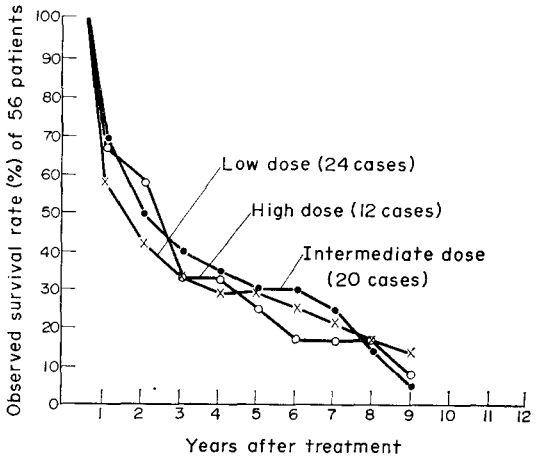


Fig. 5. Follow-up and observed survival rate of 56 patients with Burkitt's lymphoma treated with low-dose (5 and 10 mg/kg), intermediate-dose (15 and 20 mg/kg), and high-dose (30 and 40 mg/kg) cyclophosphamide.

response to cyclophosphamide, 58 were followed up for 6–10 months. Of these, 11 were known to have died before the end of the first year follow-up period while 10 were lost to follow-up although they were quite well when last seen; that is, 37 of the 58 patients were known to be alive and in good health at the end of one year follow-up period. Twenty-eight of the 37 followed for 1 year were known to be very well and no recurrences of their tumors at the end of 2 years, 5 were known to have died while 4 did not complete the 2-year follow-up period but they were in good health when last seen. Twenty-one were followed for 3 years and were in good health with no recurrences, 7 were lost to follow-up. Nineteen completed the 4-year follow-up period with no recurrences, 2 were lost to follow-up. Sixteen were well when seen at 5-year follow-up period with no recurrences, 3 were lost to follow-up. There were no known deaths in these patients after the 2-year follow-up period. Other survivors have now begun to surface as a result of efforts to trace them.

DISCUSSION

Analyses by clinical stage are of limited usefulness because, in the absence of routine lumbar puncture for CSF and bone marrow aspiration or routine gastro-intestinal X-ray studies and laparotomy, stage IV and stage III respectively could not be excluded. With the facilities available to us in form of equipment, personnel, and hospital beds for the patients, most of these special investigations could not be carried out routinely.

The problems of follow-up of patients are familiar to any one who has worked in any of the developing countries. A patient who has responded to treatment and is in good health sees no need to go back to the hospital for follow-up or a check-up and he does not want to be reminded of his past ill-health. Also there is constant movement of people from rural areas and villages to the cities that permanent addresses are not possible in some cases. Because of this difficulty of follow-up, an accurate data for long-term survivors of our patients in this series is impossible at this time. Since Table 2 was prepared about 6 months ago, 4 more patients surviving 5 years or longer have been traced, and they are quite well and no recurrences of their tumors. That makes the number of those surviving 5 years or longer 20 instead of the 16 recorded then. Efforts by the Medical Social Services Department of this hospital are continuing to trace many of the patients who are lost to follow-up.

It must also be pointed out that in Table 2 the fact that 28 patients are recorded as surviving for 2 years while only 3 for 10 years does not mean that the rest of the patients died, it means that only a very few of the surviving patients have been followed for as long as 10 years. For this review, only 2 patients are recorded for 1962 and 5 for 1963.

Figure 3 represents the true pattern of survivals in this series because in our experience and that of Levine *et al.* [10] patients surviving 2 years rarely relapse. There is some correlation of survival with stage as depicted in Fig. 4 where survivals in stages I and II are better than in stage III; however, there seems to be no advantage of high and intermediate dosages over low dose in producing long-term survivors as shown in Fig. 5.

We find it inconceivable that the parents

who showed so much concern and sorrow when they brought their children with the initial occurrence of the tumor, and so much joy when they left the hospital with their "cured" and healthy children, will fail to bring them back if they have recurrences of their tumors after such dramatic response to our treatment and "cure". Therefore we believe that many more of our patients are alive today and tumor-free than our records show.

Data have been presented that of all the 146 patients treated with various doses of cyclophosphamide, 60 showed no tumor regression, 38 showed partial tumor regression, while 48 showed complete tumor regression. A further breakdown of the 86 patients showing tumor response is summarized below in tabular form.

Pattern of tumor response to cyclophosphamide and relapse

	Stage			
	I	II	III	IV
Complete tumor regression	24	12	11	1
Relapse	4	2	3	0
Response to re-treatment	3	1	3	
Partial tumor regression	14	13	11	
Relapse (massive)	7	3	8	
Response to re-treatment	1	0	1	

Almost all relapses after partial tumor regression were massive recurrences and failed to respond to re-treatment with cyclophosphamide or other chemotherapeutic agents, while only 2 of the 9 relapses from complete regression group failed to respond to cyclophosphamide, the 2 developed CNS involvement.

The possibility that sex-related factor influences resistance of young female patients to the disease process cannot be completely ruled out as indicated by a lower incidence (Figs. 1 and 2). If such sex-related factor exists, it needs to be explored, particularly because every center in Africa which had reported Burkitt's lymphoma had given a higher incidence for the male patients.

The unpredictability of response of Burkitt's lymphoma patients to chemotherapeutic agents is still very puzzling. Why there should be such a difference in survivals of almost identical patients, disease conditions, and treatment (Tables 3 and 4) still challenges our knowledge of Burkitt's lymphoma and its response to treatment.

The role of immune system in the initial response of tumor cells to the drug and in the maintenance of tumor regression has been the subject of speculation and intensive investigations for over a decade. Evidence of both cell-mediated and humoral anti-tumor immune mechanisms has been demonstrated by Klein in 1971 [11]. However, similar experience of unpredictability as regards response of Burkitt's lymphoma cells to cyclophosphamide has been observed in the *in vitro* experiment (H. Strander, 1974, Personal Communication) where some cell lines respond to extremely small doses of the drug while some other cell lines fail to respond to even extremely large doses of the same drug. It seems that host defence is not the only factor operating in the response of Burkitt's lymphoma because the cells studied *in vitro* cannot be said to be enjoying the protection of the host defence, they are maintained in a new medium and growing independent of the host (the patient).

CONCLUSION

This review permits several important conclusions regarding the chemotherapy of Burkitt's lymphoma. First, that complete and durable remissions could be achieved with cyclophosphamide and that the long-term survival is independent of the dosage or dose-schedule of the drug even in patients with stage III disease. Second, it confirmed earlier observations of several investigators that patients in stage IV disease do very poorly even with intensive chemotherapy. Third, that patients can tolerate very high dose of cyclophosphamide, as high as 130 mg/kg (as seen in our series). Fourth, we still do not have a precise understanding of the mechanism by which Burkitt's lymphoma cells respond to cyclophosphamide and it is impossible at present to predict how a Burkitt's lymphoma patient will respond to the drug. Fifth, clearly the optimal management of Burkitt's lymphoma patients is not yet established, but further controlled trials in the areas where the tumor is endemic will assist in clarifying this important therapeutic problem.

REFERENCES

1. D. Burkitt, Long-term remissions following one- and two-dose chemotherapy for African lymphoma. *Cancer (Philad.)* **20,** 756 (1967).
2. P. Clifford, Further studies in the treatment of Burkitt's lymphoma. *E. Afr. med. J.* **43,** 179 (1966).

3. D. Burkitt, M. S. R. Hutt and D. H. Wright, African lymphoma—Preliminary observations on response to therapy. *Cancer (Philad.)* **18,** 399 (1965).
4. R. H. Morrow, M. C. Pike and A. Kisuule, Survival of Burkitt's lymphoma patients in Mulago Hospital, Uganda. *Brit. med. J.* **4,** 323 (1967).
5. J. L. Ziegler, L. Fass, S. K. Kyalwazi, R. H. Morrow and P. P. Carbone, Treatment of Burkitt's tumor with cyclophosphamide. *Cancer (Philad.)* **26,** 474 (1970).
6. F. K. Nkrumah and I. V. Perkins, Burkitt's lymphoma in Ghana: Clinical features and response to chemotherapy. *Int. J. Cancer* **11,** 19 (1973).
7. H. F. Oettgen, P. Clifford and D. Burkitt, Malignant lymphoma involving the jaw: Treatment with alkylating agents and actinomycin D. *Cancer Chemother. Rep.* **28,** 25 (1963).
8. P. Clifford, S. Singh, J. Stjernsward and G. Klein, Long-term survival of patients with Burkitt's lymphoma: An assessment of treatment and other factors which may relate to survival. *Cancer Res.* **27,** 2578 (1967).
9. V. A. Ngu, Chemotherapy of Burkitt's tumor at the University of Ibadan, Nigeria. *J. Amer. med. Ass.* **222,** 1166 (1972).
10. P. H. Levine, B. R. Cho, R. R. Connelly, C. W. Berard, G. T. O'Conor, R. F. Dorfman, J. M. Easton and V. T. DeVita, The American Burkitt Lymphoma Registry: A Progress Report. *Ann. intern. Med.* **83,** 31 (1975).
11. G. Klein, Immunological studies on a human tumor—dilemmas of the experimentalist. *Israel J. med. Sci.* **7,** 111 (1971).

Europ. J. Cancer Vol. 12, pp. 433–439. Pergamon Press 1976. Printed in Great Britain

Diagnostic Tests of Bovine Leukemia

Comparison between an Hematological Test and the Serological Diagnosis*

M. MAMMERICKX†, D. PORTETELLE‡§, R. KETTMANN‡¶, J. GHYSDAEL‡‖, A. BURNY‡ and D. DEKEGEL#

†*Département de Virologie, Institut National de Recherche Vétérinaire, 99 Groeselenberg, 1180 Bruxelles, Belgium*

‡*Faculté d'Agronomie, 5800 Gembloux, Belgium,* and *Département de Biologie Moléculaire, Université Libre de Bruxelles, 67 rue des Chevaux, 1640 Rhode-Saint-Genèse, Belgium*

#*Laboratoire de Microscopie Electronique, Institut Pasteur du Brabant, 22 rue du Remorqueur, 1140 Bruxelles, Belgium*

Abstract—*The classical hematological test (lymphocytes counts) was applied to 5 herds of cattle where the enzootic form of bovine leukemia was diagnosed. In the same five herds, a search for antibodies directed against bovine leukemia virus (BLV) p 24 was carried out using an agar gel immunodiffusion (AGID) technique.*

Among the 245 animals screened, 30 reacted positively to both tests, 11 were positive by hematological test only and 15 were positive by serological test only. From these results we would advise to eliminate from so heavily infected herds, all the animals showing abnormal blood pictures and all these reacting positively to the immunological test.

INTRODUCTION

ENZOOTIC bovine leukemia is a lymphoproliferative disease. Epidemiological and experimental observations lead many investigators to consider a virus (or viruses) as probable etiological agent(s) of the disease. A major progress towards identification of a viral agent was the observation [1] that leukemic lymphocytes kept in survival cultures release "C-type" particles. This observation was rapidly confirmed by several groups [2–4]. These particles, now called bovine leukemia virus (BLV) contain a high molecular weight RNA–reverse transcriptase complex. ^{3}H-C DNA synthesized *in vitro* in the disrupted particles hybridizes only to a very limited extent to normal bovine DNA, but anneals to a maximum extent to bovine tumor DNA [5]. These biochemical results also point to enzootic bovine leukemia being an infectious disease caused by a viral agent, BLV.

If this is so, one expects the infected animal to rise antibodies against BLV or some BLV components. It is now known that leukemic animals carry antibodies directed against an ether sensitive antigen and an ether resistant antigen both present in homogenates of cultured leukemic lymphocytes [6]. In the present study we screened animals of five herds (total = 245 animals) for the presence of antibodies directed against the ether resistant antigen, a viral protein with a molecular weight: 24.000. The results obtained by the agar gel immunodiffusion test (AGID test) were compared to these provided by hematological examination of all the animals. Hematological tests, interpreted here according to the key of Göttingen, detect cases of persistent lymphocytosis. It might be useful to notice here that persistent lymphocytosis, whilst frequently considered as a pretumoral situation is not necessarily the rule [7, 8]. In that respect, recent results from

Accepted 16 January 1976.

*Part of this work was supported by a Research contract with the Caisse Générale d'Epargne et de Retraite (Fonds Cancérologique).

§Assistant of the "Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite", Belgium.

¶Aspirant of the "Fonds National de la Recherche Scientifique", Belgium.

‖Fellow of the "Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture", Belgium.

our laboratory showed that BLV sequences are present in the DNA of lymphocytes from persistent lymphocytosis cases. It therefore seems legitimate to consider persistent lymphocytosis as one possible clinical aspect of bovine leukemia. As the sanitary policy in several Western Europe countries (Denmark, German Federal Republic, Belgium) is presently based only on hematological figures, it seemed to us of basic interest to compare the hematological test, detecting a clinical symptom of the disease, and the serological test, detecting antibodies directed against a viral product.

MATERIAL AND METHODS

Animals

We recently detected, in Belgium, five herds with high incidence of bovine leukemia (high risk herds). Hematological as well as serological tests were performed on the 245 animals of these herds.

Hematological tests

Total leucocytes counts were run in an electronic Coulter Counter (model ZF) carefully calibrated for bovine cells [9]. The leucocytes formula was established on smears stained with the May–Grünwald–Giemsa solution. Animals were classified as normal, suspicious or leukemic according to the key of Göttingen [10].

Serological tests

Agar gel immunodiffusion (AGID) was run as described in the literature [11–17] with slight modifications. The antigen was prepared from a short-term culture of leukemic lymphocytes derived from cow No. 950. This is the best virus producer of our experimental herd. After 4 days of culture, cells were harvested by centrifugation, resuspended in two volumes of water, frozen and thawed three times and homogenized at full speed with an ultraturrax homogenizer (Janke and Kunkel KG). The isotonicity of the aqueous suspension was then restored by addition of 10 × TNE (TNE = 0.01 M Tris–HCl, pH 8.3, 0.15 M NaCl, 0.001 M EDTA). The suspension was then adjusted to 0.5% NP-40 (non-ionic detergent; Shell Company) and clarified by a 20 minutes centrifugation at 10.000 ***g*** and 2°C. The supernatant was stored at −50°C and used as antigen in all the experiments described.

It was obviously of crucial importance to clearly monitor the viral origin of the antigen being tested. This stems from five lines of evidence (Portetelle *et al.*, in preparation):

1. The antigen appears with time during short term cultures of leukemic lymphocytes as virus particles do.

2. The antigen is concentrated in the 1155 density region of sucrose gradients after equilibrium centrifugation of the virus pellet. This is also the density where BLV equilibrates [5]. No virus (by SD test and EM) and no antigen (by AGID test) can be detected when normal lymphocytes cultures are processed for virus purification.

3. The positive sera (cows No. 907, 15, 929, . . .) keep 100% of their potency after adsorption by normal bovine cells extracts; they lose all their potency when adsorbed against cultured leukemic lymphocytes extracts.

4. Contamination by Bovine syncytia virus (BSV) is improbable for this virus was never found in our experimental herd and moreover, our endogenous reverse transcriptase reactions never worked in the presence of Mn^+ cations.

5. After banding of BLV in sucrose gradients and its solubilization by NP 40 or Triton X-100 (Rohm & Haas, Cy), the viral antigen was purified through DEAE-cellulose, phosphocellulose and Sephadex G200 columns. The fractions of the G200 profile reacting positively with the antibody were then analyzed by SDS polyacrylamide gel electrophoresis and showed one major band corresponding to a protein of molecular weight: 24000.

All these controls and observations make us quite confident that we are dealing with a true BLV antigen.

Immunodiffusion tests were performed in Petri dishes with 1% Noble agar (Difco) in TNE containing 3% polyethylene glycol 6000 (Fluka AG). The central well was filled once with antigen. In all plates, test sera were alternated with our best known positive serum from cow No. 929. Plates were read after 4 days incubation in a humidified chamber at room temperature.

RESULTS

All immunodiffusion tests were run with the same antigen preparation from cultured lymphocytes from cow No. 950. In these tests, unknown sera alternated with our most potent reference serum. When the level of serum precipitin to bovine viral specific antigen was high enough, a precipitation line appeared in continuity with the precipitation line of the reference serum. Even weakly positive sera could be detected with the present experimental design. The lines of precipitate became obvious

Table 1. *Total numbers and percentages of animals per age groups; comparative hematological and serological diagnosis*

Age groups	Total numbers of animals per group and their percentage in the total population		Total numbers and percentages of animals diagnosed as leukemic by the hematological test		Total numbers and percentages of animals diagnosed as antibody carriers by the the serological test	
	n	%	n	%	n	%
0–1	39	15·9	0	0·0	1	2·2
1–2	58	23·7	8	19·5	6	13·3
2–3	34	13·9	8	19·5	8	17·8
3–4	31	12·7	9	22·5	9	20·0
4–5	18	7·3	2	4·9	3	6·7
5–6	29	11·8	7	17·1	7	15·6
6–7	16	6·5	4	9·8	6	13·3
7–8	4	1·6	1	2·4	1	2·2
+8	16	6·5	2	4·9	4	8·9
Total	245	100	41	100	45	100

on both sides when fusing with the corresponding band of the reference sera (presence of hooks on the control line).

The 245 animals were divided into 9 age groups as follows: from 0 to 1 year of age, from 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8 and over 8 years of age. Total numbers of animals per age group, the percentage they represent in the population tested and the diagnostic results encountered by the two methods in comparison are listed in Table 1. Among a total number of 245 animals, 41 are positive according to the hematological test while 45 are positive by the serological test.

Considered simultaneously (Table 2) both tests agreed on 219 (189+30) animals over 245, they disagreed on 26 (11+15) animals.

Detailed results are reported in Tables 3, 4 and 5. All those animals for which both tests gave similar or opposite results are listed respectively in these tables.

In table 6 are compared hematological and serological data according to the age groups of animals tested.

DISCUSSION

In this study, persistent lymphocytosis was diagnosed in about 20% of the animals tested.

About the same percentage of animals was diagnosed as carriers of antibodies directed against BLV p24 when their sera were examined in an AGID test. The reactors belonged to the 5 herds. Except for the first age groups (0 to 1 year of age) both methods detected reactors among all age groups of the population (Table 1). Thirty animals reacted positively to both methods. Among these were found almost all (21 out of 23) animals diagnosed as frankly leukemic according to the key of Göttingen and all the tumor carriers (3 out of 3). In our view, they constitute typical cases of enzootic leukemia and, except for the young age groups

Table 2. *Total numbers and percentages of animals in each age group considering the various results obtained from both tests, hematology and serology (For total number of animals in each age group see table* 1)

Age	Hematology — Serology —		Hematology + Serology +		Hematology + Serology —		Hematology — Serology +	
	n	%	n	%	n	%	n	%
0–1	38	20·1	0	0·0	0	0·0	1	6·7
1–2	47	24·9	3	10·0	5	45·5	3	20·0
2–3	24	12·7	6	20·0	2	18·2	2	13·3
3–4	20	10·6	7	23·3	2	18·2	2	13·3
4–5	14	7·4	1	3·3	1	9·1	2	13·3
5–6	21	11·1	6	20·0	1	9·1	1	6·7
6–7	10	5·3	4	13·3	0	0·0	2	13·3
7–8	3	1·6	1	3·3	0	0·0	0	0·0
+8	12	6·3	2	6·7	0	0·0	2	13·3
Total	189	100	30	100	11	100	15	100

Table 3. Hematological picture of all the animals who reacted positively to the serological test. The eventual presence of tumors is reported in the last column

Herd	Age groups	Hematology		Tumor
		Absolute number of lymphocytes	Key of Göttingen*	
A	1–2	20·916	L	—
A	1–2	17·154	L	—
B	1–2	10·022	S	—
A	2–3	20·160	L	—
A	2–3	9·522	S	—
A	2–3	24·260	L	—
A	2–3	9·280	S	—
A	2–3	7·737	S	—
A	2–3	21·829	L	—
A	3–4	11·659	L	—
A	3–4	10·032	L	—
A	3–4	13·090	L	—
A	3–4	16·830	L	—
A	3–4	6·932	S	—
A	3–4	6·548	S	—
A	3–4	6·725	S	—
B	4–5	10·254	L	—
B	5–6	11·233	L	+
C	5–6	12·642	L	—
C	5–6	12·608	L	—
D	5–6	14·051	L	—
E	5–6	13·452	L	—
E	5–6	6·600	S	+
A	6–7	6·413	S	—
A	6–7	12·528	L	—
D	6–7	22·102	L	—
D	6–7	22·585	L	—
E	7–8	12·665	L	+
D	+8	11·355	L	—
E	+8	14·378	L	—

*L = leukemic; N = normal; S = suspicious.

Table 4. Hematological picture of animals diagnosed as leukemic by hematological test. No tumor and no positive serological reaction were detected.

Herd	Age groups	Hematology	
		Absolute number of lymphocytes	Key of Göttingen
A	1–2	9·390	S
A	1–2	12·873	L
A	1–2	10·279	S
A	1–2	10·267	S
B	1–2	11·366	S
A	2–3	7·672	S
A	2–3	15·397	L
B	3–4	7·083	S
B	4–5	7·700	S
B	5–6	7·144	S

(0 to 2 years of age), they appear in all the age groups at about the same percentages as those observed in the total population (see Tables 1 and 2).

The 11 animals exhibiting a positive reaction in the hematological test and a negative one in the serological test (Table 4), were almost all borderline cases (suspicious according to the key of Göttingen). This type of data was not observed in all the age groups with percentages comparable to those observed in the total population (see Tables 1 and 2). Perhaps, except for two animals (L) we are dealing with normals which statistically must appear out of the normal range; they were mainly young animals, a class of age where the physiological variations of the lymphocytes count are frequent [9]. The two positive cases (L) probably have a too low level of antibodies to be detected by AGID. We are therefore tempted to conclude

Table 5. *Hematological picture of animals considered as normal according to the key of Göttingen. They showed a positive serological reaction and no tumor was detected*

Herd	Age groups	Hematology: Absolute number of lymphocytes
B	0–1	7·513
A	1–2	6·342
A	1–2	6·767
E	1–2	3·572
A	2–3	7·319
D	2–3	6·362
A	3–4	6·177
C	3–4	6·033
E	4–5	5·115
E	4–5	6·208
A	5–6	2·777
B	6–7	4·697
E	6–7	3·735
E	+8	3·696
E	+8	5·280

that 9 out of these 11 animals constitute the margin of error of the hematological test. Great carefulness must however be exercised for, it might quite well happen that hematological disorders precede any serological reaction. The negative outcome of serological tests should be reassessed in the future by radioimmunoassay.

The second interesting group contained 15 animals (Table 5). They reacted as normals in the hematological test but appeared as antibody carriers in the serological test. Except in the young age groups, the percentage of such animals in the other age groups was about the same as in the total population. The percentage of such cases in our study was 33% (15 animals out of 45). This is close to the 30% observed by Olson *et al.* in their study [14]. The discrepancy between hematological and serological tests could be explained by any of the three following possibilities:

1. These cases represent normoleucocythemic leukemia (aleukemic leukemia) ignored, of course, by the hematological test. In this case, AGID could not be considered as an early diagnostic method of bovine leukemia (neither hematology nor serology detect cases among animals from 0 to 1 year of age) but as a specific method able to detect a new series of animals ignored so far by hematology.

2. These cases represent animals recently infected by BLV. Here AGID could be considered as a technique of choice for early diagnosis of bovine leukemia, if we however, admit that an old animal can still get contaminated by the virus. It does not seem to be the case as the percentage of leukemia in the various age groups (Table 6) increases from 0 to 3 years of age and gets stabilized thereafter [14, 18]. The conclusion seems then to be that infection occurs in young age and not later.

3. These cases represent animals efficiently fighting BLV infection (immune reaction without disease). The actual infection could have happened a long time before. These animals could be either virus-free (cured animals with antibodies but no antigens), or virus-carriers (animals with both antigens and antibodies). They might never develop tumors.

From the above detailed discussion, it is expected that this group of animals, negative by hematology and positive by serology, will expand with the sensitivity of the method used to detect antibody carriers. It is therefore urgently required to purify extensively BLV

Table 6. *Comparison between hematological and serological data according to the age group of the animals tested*

Age groups	Hematological test +: Total number of positive/ Total number in the group	Hematological test +: Percentage in the group	Serological test +: Total number of positive/ Total number in the group	Serological test +: Percentage in the group
0–1	0/39	0·0	1/39	2·6
1–2	8/58	13·8	6/58	10·3
2–3	8/34	23·5	8/34	23·5
3–4	9/31	29·0	9/31	29·0
4–5	2/18	11·1	3/18	16·7
5–6	7/29	24·1	7/29	24·1
6–7	4/16	25·0	6/16	37·5
7–8	1/4	25·0	1/4	25·0
+8	2/16	12·5	4/16	25·0
Total	41/245	16·7	45/245	18·4

p24 and raise potent monospecific antibodies against it. Highly specific radioimmunoassays would indeed allow investigators not only to detect any antibody carrier but also putative virus carriers devoid of immunological reactions.

In conclusion, we consider AGID as a useful tool in the hands of those who, today, have to decide about sanitary policy measures. As the evidence for BLV being the infectious agent of bovine leukemia gets reinforced every day, it is mandatory to design sensitive and precise tools for detection of BLV infection. As on the other hand, some herds remain BLV-free even in Western Europe, where the disease spreads, we are strongly encouraged to believe that efficient detection of BLV and elimination of virus carriers is the key way towards eradication of enzootic bovine leukemia from our herds.

Acknowledgements—We warmly thank P. Ridremont, J. Severs and G. Vandendaele for their skillful technical assistance.

REFERENCES

1. J. M. Miller, L. D. Miller, C. Olson and K. G. Gilette, Virus-like particles in phytohemagglutininstimulated lymphocyte cultures with reference to bovine lymphosarcoma. *J. nat. Cancer Inst.* **43,** 1297 (1969).
2. S. K. Dutta, V. L. Larson, D. K. Sorensen, V. Perman, A. F. Weber, R. F. Hammer and R. E. Shope, Isolation of C-type virus particles from leukemic and lymphocytotic cattle. *Bibl. Haemat.* **36,** 548 (1970).
3. J. F. Ferrer, N. D. Stock and P. S. Lin, Detection of replicating C-type viruses in continuous cell cultures established from cows with leukemia: effect of the culture medium. *J. nat. Cancer Inst.* **47,** 613 (1971).
4. T. G. Kawakami, A. L. Moore, G. M. Theilen and R. J. Munn, Comparisons of virus-like particles from leukotic cattle to feline leukosis virus. *Bibl. Haemat.* **36,** 471 (1970).
5. R. Kettmann, D. Portetelle, M. Mammerickx, M. Galoux, D. Dekegel, J. Ghysdael, Y. Cleuter, A. Burny and H. Chantrenne. Bovine leukemia virus: an exogenous RNA oncogenic virus? *Modern Trends in Human Leukemia II* (Edited by R. Neth, R. C. Gallo, S. Spiegelman) J. F. Lehmans, München, (In press 1975).
6. M. Onuma, C. Olson, L. E. Baumgartener and L. D. Pearson, An ether-sensitive antigen associated with bovine leukemia virus infection. *J. nat. Cancer Inst.* **55,** 1155 (1975).
7. M. Mammerickx and D. Dekegel, Studies on relationship between persistent lymphocytosis, infection with C-type particles and presence of *Trypanosoma theileri* associated with bovine enzootic leukosis. *Zbl. Vet.-Med. B.* **22,** 411 (1975).
8. J. F. Ferrer, D. A. Abt, D. M. Bhatt and R. R. Marshak, Studies on the relationship between infection with bovine C-type virus, leukemia, and persistent lymphocytosis in cattle. *Cancer Res.* **34,** 893 (1974).
9. M. Mammerickx and H. van Lierde, Les paramètres hématologiques normaux chez les bovidés des races bleue de Moyenne Belgique, rouge de Flandre Occidentale et pie-rouge de Flandre Orientale. *Ann. de Gembloux* **80,** 201 (1974).
10. A. Tolle, Zur Beurteilung quantitativer hämatologischer Befunde im Rahmen der Leukose-Diagnostik beim Rind. *Zbl. Vet.-Med. B.* **12,** 281 (1965).
11. J. F. Ferrer, Antigenic comparison of bovine type C virus with murine and feline leukemia viruses. *Cancer Res.* **32,** 1871 (1972).
12. J. M. Miller and C. Olson, Brief communication: Precipitating antibody to an internal antigen of the C-type virus associated with bovine lymphosarcoma. *J. nat. Cancer Inst.* **49,** 1459 (1972).
13. J. M. Miller and M. J. van der Maaten, A complement-fixation test for the bovine leukemia (C-type) virus. *J. nat. Cancer Inst.* **53,** 1699 (1974).
14. C. Olson, H. E. Hoss, J. M. Miller and L. E. Baumgartener, Evidence of bovine C-type (leukemia) virus in dairy cattle. *J. Amer. vet. Assoc.* **163,** 355 (1973).

15. J. PAULSEN, R. RUDOLPH and J. M. MILLER, Antibodies to common ovine and bovine C-type virus specific antigen in serum from sheep with spontaneous leukosis and from inoculated animals. *Med. Microbiol. Immunol.* **159,** 105 (1974).
16. A. A. RESSANG, M. MASTENBROEK, J. QUAK, L. J. L. D. VAN GRIENSVEN, J. CALAFAT, J. HILGERS, P. C. HAGEMAN, T. SOUISSI and S. SWEN, Studies on bovine leukemia. I. Establishment of type C virus producing cell lines. *Zbl. Vet.-Med. B.* **21,** 602 (1974).
17. O. C. STRAUB, E. WEILAND and B. FRENZEL, Ergebnisse von hamatologischen und serologischen Untersuchungen bei natürlichen und experimentellen Rinderleukose-Ubertragungsversuchen. *Dtsch. tierärztl. Wschr.* **81,** 549 (1974).
18. M. MAMMERICKX, La race, l'âge et le pourcentage d'infection des bovidés leucémiques dans les foyers découverts en Belgique. *Ann. Méd. vét.* **116,** 475 (1972).

Europ. J. Cancer Vol. 12, pp. 441–445. Pergamon Press 1976. Printed in Great Britain

Catechols in Primary and Metastatic Human Malignant Melanoma Cells in Monolayer Culture*

Ch. AUBERT†‡, C. LAGRANGE†‡, H. RORSMAN§ and E. ROSENGREN¶

†*Laboratoire de Biologie—Endocrinologie, Institut Gustave Roussy*, 94800 *Villejuif*

§*Department of Dermatology, University of Lund*, *S*-221-85 *Lund, Sweden*

¶*Department of Pharmacology, University of Lund S*-221-85 *Lund, Sweden*

Abstract—*Dopa and 5-S-cysteinyldopa were determined in permanent cell lines obtained from two primary and two metastatic human malignant melanoma and also in one clone of one of the primary melanoma cell lines. The cell lines differed considerably with regard to catechol content and secretion into the media. The clone also differed from the original cell line. Results in the exponential growth phase differed from those in the plateau phase and change of medium affected catechol content.*

Determination of catechols in cultured melanoma cells can be used for characterization of cell lines that cannot be separated by optical or ultrastructural morphological methods.

INTRODUCTION

NUMEROUS biochemical studies have been performed on animal melanotic tumors [1–7] and cell lines derived from these tumors [8–10]. For obvious reasons, fewer biochemical investigations have been carried out on human malignant primary or metastatic melanomas [11–13].

However, recently cultures of established cell lines of human malignant melanomas made possible the biochemical investigation of these cells and their specific metabolic functions. Melanin synthesis have been studied in particular [14–15].

It has been shown that 5-S-cysteinyldopa, which plays a role in pheomelanin production, could be detected in the serum and in the urine of patients with melanoma metastases [16–18].

We wanted to study the production of 5-S-cysteinyldopa in subcultured malignant melanocytes and in their culture medium under various experimental conditions.

Accepted 15 January 1975.

*This investigation was supported by grant A.T.P. 23 from the Institut National de la Santé et de la Recherche Médicale, Paris, France, the Swedish Cancer Society and the Swedish Medical Research Council.

‡Present address: U. 119 I.N.S.E.R.M., 27, Bd Lei Roure—13009 Marseille, France.

MATERIAL AND METHODS

For this purpose we used permanent cell lines obtained in our laboratory from primary (IGR 3, IGR 22) [19] and metastatic lymph node (IGR 1, IGR 2) [20] human malignant melanomas. We also investigated a clone, IGR 36, derived from the 5th subculture of the IGR 22 line, immediately after spontaneous redifferentiation of this line [19].

Some clinical, pathological and cytological data are given in Table 1 and Table 2. The determination of 3,4-dihydroxyphenylalanine (dopa) and 5-S-cysteinyldopa were performed on the subcultures given in Table 2. The methods of cell culture [19] and determination of dopa and 5-S-cysteinyldopa have been described previously [21].

The fibroblasts (IGR 20) were obtained from normal skin of the patient with superficially spreading melanoma giving the IGR 22 line.

All cultures were initiated by inoculation of $2 \cdot 10^5$ cells, suspended in 6 ml of minimal essential medium (M.E.M.) and 1 ml of foetal bovine serum into Falcon Plastic T-30 flasks.

For each cell line, cells and culture media were collected from three Falcon flasks. The medium was removed from the three Falcon flasks, then the cells were harvested by trypsinization (0·2% trypsin in Dulbecco's solution), centrifuged and washed with fresh

Table 1. *Clinical and pathological characteristics of the patients with melanoma*

Patients	Age	Hair colour	Freckles	Primary tumor	Level of invasion*	Origin of cultured cells	Established cell lines
J.M. ♂	42	blond	yes	nodular	V	metastasis lymph node	IGR 1
C.B. ♀	55	dark	no	superficial spreading	IV	metastasis lymph node	IGR 2
R.N. ♂	60	dark	no	nodular	IV	primary cutaneous	IGR 3
A.E. ♀	26	blond	yes	superficial spreading	IV	primary cutaneous	IGR 22
A.E. ♀	26	blond	yes			clone of IGR 22	IGR 36
A.E. ♀	26	blond	yes			fibroblasts from normal skin	IGR 20

*From the classification of Clark *et al.* (1969).

Table 2. *Cytological characteristics of the cell lines*

Cell lines	Morphology	Type of melanosomes	Number of subcultures
IGR 1	dendritic	fibrillar	155 (14)
IGR 2	dendritic	fibrillar	148 (96)
IGR 3	epithelioid	granular	96 (40)
IGR 22	dendritic	fibrillar	64 (7)
IGR 36	dendritic	fibrillar	40 (8)
IGR 20	fibroblastic	—	24 (12)

( ) No. of the subcultures chosen for the experimentations.

culture medium. One third of the total number of cells was resuspended in 6·6 ml of new medium and used for extraction of catechols. One third (6·6 ml) of the original nutritive mixture of the three Falcon flasks was also tested for catechols. To preserve the catechols, 0·2 ml glacial acetic acid and 0·2 ml of 2% sodium metabisulfite were added.

RESULTS

First, we compared the dopa and 5-S-cysteinyldopa content in the cells and in the medium during the exponential growth and plateau phases. For the exponential growth phase, seven determinations were performed and the average value was chosen; for the plateau phase, three determinations were made and the average value used. In order to study the importance of frequency of culture media changes, we compared the 5-S-cysteinyldopa levels in the cells and in the medium at seven days of culture after trypsinization, at the end of the exponential growth phase. Medium change were made every day and not at all during the seven days.

Dopa determinations

Dopa *in the cells* was detected only in one cell strain (IGR 2). The dopa level was below 10 ng/1 . 10^6 cells and dopa was only detectable at the end of the exponential growth phase and in the plateau growth phase. Dopa was not detected *in the culture medium* in any of the 24 hr collections during the exponential growth phase. However, the medium that was not renewed between 48 and 168 hr contained small amounts of dopa in IGR 1 and IGR 2. During the plateau phase of cell growth dopa was formed in significant amounts in IGR 2. The cells of IGR 1 and IGR 36 formed small amounts of dopa (Table 3).

Table 3. *Dopa in cell culture medium during the exponential growth phase and during the plateau phase* (*ng*/1.10^6 *cells*)

Cell lines	Exponential growth phase	Plateau phase
IGR 1	9	8
IGR 2	9*	29*
IGR 3	0	0
IGR 22	0	0
IGR 36	0	3
IGR 20	0	0

*Dopa was also detectable in the cells.

5-S-cysteinyldopa determinations

All melanocytes except IGR 3 contained and secreted 5-S-cysteinyldopa, but in different amounts. 5-S-cysteinyldopa could not be de-

Table 4. *5-S-Cysteinyldopa in cultured melanoma cells and in the medium in exponential and in plateau phases (ng/1.10⁶ cells)*

Cell lines	Cells		Medium	
	Exponential growth phase	Plateau phase	Exponential growth phase	Plateau phase
IGR 1	31	121	81	64
IGR 2	83	257	123	73
IGR 3	0	0	69	0
IGR 22	64	37	150	77
IGR 36	58	44	99	265
IGR 20	0	0	0	0

tected in the cells of IGR 3 strain in any phase and secretion into the medium was only found in the exponential growth phase. Fibroblasts (IGR 20) did not contain nor secrete any 5-S-cysteinyldopa (Table 4).

Influence of the phase

In the cells, for IGR 1 and IGR 2, the amount of 5-S-cysteinyldopa increased considerably during the plateau phase when compared with the exponential growth phase. In contrast the cells of IGR 22 and IGR 36 showed about the same concentrations of 5-S-cysteinyldopa in the plateau phase when compared with the exponential growth phase. *In the medium*, the concentration of this metabolite decreased for IGR 1, IGR 2 and IGR 22 at the plateau phase and increased considerably with IGR 36 at the same period (Table 4). We found a certain amount of 5-S-cysteinyldopa in the medium of IGR 3 during the exponential growth phase but not during the plateau phase, but the catechol was never detectable in the cells (Table 4).

Influence of the renewal culture medium

Change of medium every day increased the 5-S-cysteinyldopa content in the cells of IGR 22 and IGR 36, but there was no significant modification in the medium (Table 5). In contrast, for IGR 1 and IGR 2 the renewal of the culture medium inhibited the formation of 5-S-cysteinyldopa in the cells and its secretion into the medium (Table 5). In IGR 3, when the medium was not renewed, we observed the excretion of a small amount of 5-S-cysteinyldopa into the medium, whereas none was detectable in the cells.

DISCUSSION

All the melanocytic cultures have 5-S-cysteinyldopa either in the cells or in their culture medium, or in both. Detectable amounts of dopa were found in the IGR 2 cells, but dopa could not be demonstrated in the cells of the other lines. Dopa was secreted into the medium of IGR 2 and small amounts were also detected in the media of IGR 1 and IGR 36.

During the exponential growth phase, the intracellular concentrations of 5-S-cysteinyldopa could be lower or higher than in the plateau phase depending on the cell strains. The level of 5-S-cysteinyldopa *in the cells* increased during the plateau phase for the metastatic cell lines but its secretion into the

Table 5. *5-S-Cysteinyldopa in cultured melanoma cells and in medium after 7 days of culture (ng/1.10⁶ cells)*

Cell lines	Cells		Medium	
	Medium renewed every 24 hr	Medium not renewed	Medium renewed every 24 hr	Medium not renewed
IGR 1	21	80	22	130
IGR 2	110	188	43	132
IGR 3	0	0	0	15
IGR 22	145	21	68	62
IGR 36	100	70	57	31
IGR 20	0	0	0	0

culture medium remained identical or decreased. It seems possible that a substance with catechol-metabolic inhibitory functions may be produced in the cells during the plateau phase. In contrast, for the primary cell lines the content of 5-S-cysteinyldopa *in the cells* during the plateau phase showed small changes, but 5-S-cysteinyldopa secretion decreased to zero in IGR 3 and somewhat in IGR 22. The IGR 36 clone showed increased secretion.

The renewal of the culture medium influenced production and secretion of 5-S-cysteinyldopa of the cell lines differently. The formation of 5-S-cysteinyldopa was stimulated by the renewal of the culture medium in the primary cell line IGR 22 and to a lesser degree in the IGR 36 clone. The secretion of 5-S-cysteinyldopa into the medium was very slightly modified for both cell lines. In contrast, for the metastatic cell lines the changes of the culture medium inhibited the formation of 5-S-cysteinyldopa in the cells and its excretion into the medium. We suggest that the renewal of the culture medium which stimulated the multiplication of the cells [22] inhibited the synthesis of the 5-S-cysteinyldopa.

It is obvious that our cell lines, metastatic and primary ones, differed considerably with regard to dopa and 5-S-cysteinyldopa levels. In IGR 3, cells did not contain 5-S-cysteinyldopa and they also secreted very small amounts of this metabolite. IGR 3 cells possessed granular melanosomes in contrast to the other cell lines which had fibrillar ones (Table 2). It is possible that the formation of 5-S-cysteinyldopa could be related to the presence of fibrillar melanosomes; this is also suggested by studies on the heterotransplantation in the "nude" mouse [23]. Such differences may be highly significant in the analysis of tumor metabolism *in vivo*. A line from primary melanoma (IGR 22) and its clone (IGR 36) which had no morphological differences either at the optical or ultrastructural level showed pronounced biochemical differences (Table 4).

Most patients with melanoma metastasis show pathologically increased urinary excretion of 5-S-cysteinyldopa, but normal values have been observed in some cases [17]. The quantitative relations between different clones or between the primary tumor and the metastasis may certainly be of importance for the clinical catechol findings.

The difficulties of diagnosing a tumor, containing cells like IGR 3, by determination of 5-S-cysteinyldopa in the urine are of course evident.

Acknowledgements—We thank Mrs. Georgette Mascaro and Miss Françoise Rougé who provided technical assistance and Mrs. A. Lipcey for the English text.

REFERENCES

1. M. Seiji, M. Birbeck and T. B. Fitzpatrick, Subcellular localization of melanin biosynthesis. *Ann. N.Y. Acad. Sci.* **100,** 497 (1963).
2. H. Takahashi and T. B. Fitzpatrick, Large amounts of deoxyphenylalanine hydrolysate of melanosomes from Harding-Passey mouse melanoma. *Nature, (Lond.)* **209,** 888 (1966).
3. S. Pomerantz, The trypsin hydroxylase activity of mammalian tyrosinase. *J. biol. Chem.* **242,** 161 (1966).
4. J. B. Burnett, H. Seiler and I. V. Brown, Separation and characterization of multiple forms of tyrosinase from mouse melanoma. *Cancer Res.* **27,** 1, 880 (1967).
5. C. Aubert, C. Bohoun and E. Comoy, Excrétion urinaire de la 3,4-dihydroxyphénylalanine (D.O.P.A.) et de la dopamine chez les hamsters porteurs de mélanomes induits ou greffés. *C.R. Acad. Sci. (Paris)* **268,** 2850 (1969).
6. M. Seiji, H. Itakura and K. Miyazaki, The membrane system and melanin formation in melanocytes. In *Biology of Normal and Abnormal Melanocytes* (Edited by T. Kawamura, T. B. Fitzpatrick and M. Seiji) Tokyo Press, Vol. 22 (1971).
7. M. R. Okun, L. M. Edelstein, N. Or, G. Hamada, G. Blumental, B. Donnellan and J. Burnett, Oxydation of tyrosine and dopa to melanin by mammalian peroxidase: the possible role of peroxidase in melanin synthesis and catecholamine synthesis *in vivo*. In *Pigmentation, Its Genesis and Biologic Control* (Edited by V. Riley) Vol. 45, p. 571, Appleton-Century-Crofts, New York (1972).

8. C. Claunch, A. Vikawa, T. T. Tchen and F. Hu, Biochemical studies in certain "pigmented" and "non pigmented" strains of melanoma cells. In *Advances in Biology of Skin* (Edited by W. Montagna and F. Hu) Pergamon Press, Oxford, Vol. 8, p. 479 (1967).
9. F. Hu, Melanin production in mammalian cell cultures. In *Biology of Normal and Abnormal Melanocytes* (Edited by T. Kawamura, T. B. Fitzpatrick and M. Seiji) Tokyo Press, p. 303 (1971).
10. A. Oikawa, M. Nohara and M. Nakayasu, Melanin synthesis in cultures of melanoma cells. In *Biology of Normal and Abnormal Melanocytes* (Edited by T. Kawamura, T. B. Fitzpatrick and M. Seiji) Tokyo Press, p. 209 (1971).
11. C. Bohuon, E. Comoy, R. Jouannet and C. Aubert, Mélanomes humains: élimination de la DOPA, des catécholamines, de l'acide vanylmandélique (VMA) et de l'acide homonanilique (HVA). *Enzym. biol. clin.* **10,** 458 (1969).
12. M. L. Voorhess, Urinary excretion of dopa and metabolites by patients with melanoma. *Cancer (Philad.)* **26,** 146 (1970).
13. A. N. Siakotos, V. Patel and A. Cantaboni, The isolation and chemical composition of premelanosomes and melanosomes: human and mouse melanosomes. *Biochem. Med.* **7,** 14 (1973).
14. M. Romsdahl and T. C. Hsu, Establishment and characterization of human malignant melanoma cell lines. Presented at the *Seventh Int. Pigment Cell Conf.*, Seattle, Washington, Sept. 2–6 (1969).
15. G. G. Maul and M. M. Romsdahl, Ultrastructural comparison of two human malignant melanoma cell lines. *Cancer Res.* **30,** 2782 (1970).
16. A. Bjorklund, B. Falck, S. Jacobson, H. Rorsman, A. M. Rosengren and E. Rosengren, Cysteinyldopa in human malignant melanoma. *Acta derm.-venereol. (Stock.)* **52,** 357 (1972).
17. C. Agrup, P. Agrup, T. Andersson, B. Falck, J. A. Hansson, S. Jacobson, H. Rorsman, A. M. Rosengren and E. Rosengren, Urinary excretion of 5-S-cysteinyldopa in patients with primary melanoma or melanoma metastasis. *Acta derm.-venereol. (Stock.)* **55,** 337 (1975).
18. C. L. Vogel, Dh. Dhru, H. Rorsman, A. M. Rosengren and E. Rosengren, Dopa and 5-S-cysteinyldopa in malignant melanoma in Uganda Africans. *Acta derm.-venereol. (Stock.)* **54,** 19 (1974).
19. C. Aubert, Cultures de mélanomes malins primitifs humains—Evolution des mélanocytes malins *in vitro*. *Ann. Dermat. Syphil. (Paris)* **102,** 59 (1975).
20. C. Aubert, E. Chirieceanu, Cultures de mélanomes humains. Caractérisation. *Rev. Inst. Pasteur (Lyon)* **6,** 265 (1973).
21. H. Rorsman, A. M. Rosengren and E. Rosengren, A sensitive method for determination of 5-S-cysteinyldopa. *Acta derm.-venereol. (Stock.)* **53,** 248 (1973).
22. G. M. Hahn and J. B. Little, Plateau-phase cultures of mammalian cells. An *in vitro* model for human cancer. In *Current Topics in Radiation Research Quarterly* (Edited by North Holland) Vol. 8, 39 (1972).
23. C. Aubert, E. Chirieceanu, C. Foa, H. Rorsman, E. Rosengren and F. Rouge, Ultrastructural and biochemical changes in cultured human malignant melanoma cells after heterotransplantation in "nude" mice. *Cancer Res.* (In press).

Europ. J. Cancer Vol. 12, pp. 447–453. Pergamon Press 1976. Printed in Great Britain

Adenylate Cyclase and Protein Phosphokinase Activities in Human Thyroid. Comparison of Normal Glands, Hyperfunctional Nodules and Carcinomas*

G. SAND†, A. JORTAY‡, R. POCHET† and J. E. DUMONT†

†*Institut de Recherche Interdisciplinaire, Faculté de Médecine, Université de Bruxelles and Biology Department*§, *Euratom, and* ‡*Service de Chirurgie de l'Institut Bordet, B-1000 Bruxelles, Belgium*

Abstract—*Basal and stimulated adenylate cyclase and protein phosphokinase activities were measured in normal and diseased human thyroids. Seven specimens of thyroid carcinomas (2 papillary, 2 mixed papillo-follicular or trabecular, 1 follicular and 2 medullary), and 6 hyperfunctional autonomous nodules were obtained at thyroidectomy. Nine specimens of normal tissue, taken from the opposite lobe of the nodular thyroids and from entirely normal glands, constituted the control group.*

Biochemical observations were the following: adenylate cyclase: basal activity and stimulation by TSH were rather similar in all cases. Hyperfunctional nodules were characterized by a significant increase of stimulation, as compared to the normal, by sodium fluoride (13·0 vs 7·8 fold) and prostaglandin E_1 *(2·6 vs 1·3 fold). Adrenalin had no significant effect. Protein phosphokinase: basal activity was significantly higher in carcinomas by a factor of up to 4-fold. Activation by cyclic AMP was similar in normal and pathological homogenates.*

These data show that adenylate cyclase activity is not unequivocally related to the degree of malignancy in human thyroid tumors and that protein phosphokinase activity is increased in such tissues.

The data are discussed within the framework of current hypotheses derived from studies of transformed cells in vitro.

INTRODUCTION

THE CYCLIC AMP (adenosine 3′,5′-cyclic monophosphate) system has been shown to be one of the major intracellular systems for relaying a hormone message from the plasma membrane to the nucleus. It has been implicated in cell differentiation and growth in *in vitro* preparations [1]. In fibroblasts in culture there is an inverse relation between growth and intracellular levels of cyclic AMP [2]. Moreover an essential event in transformation appears to be a depression in adenylate cyclase activity resulting in a decrease of cyclic AMP concentrations in transformed cells [1]. However, in apparent contradiction with this observation, cyclic AMP levels in rapidly growing animal tumors are high [1] and tropic hormones, such as thyrotropin (TSH), raise cyclic AMP levels and activate growth in their target tissues [3].

The aim of the present work was to compare the activity of two key enzymes of the cyclic AMP system: adenylate cyclase and cyclic AMP-dependent protein phosphokinase in normal and cancerous human thyroid tissue, and in autonomous hyperfunctioning nodules.

MATERIAL AND METHODS

Seven specimens of thyroid carcinomas were obtained from patients at thyroidectomy. There

Accepted 15 January 1976.

*This work was supported by a grant from the "Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite", Belgium.

§Publication No. BIO 1200.

were 2 papillary, 2 mixed papillo-follicular or trabecular, 1 follicular and 2 medullary carcinomas. The patients ranged in age from 7 to 66; 5 were female and 2 were male. None of these patients had received previous neck irradiation. ^{131}I iodide uptakes were within normal limits in all patients and thyroid scans uniformly demonstrated low or absence of activity over tumor sites.

Six hyperfunctional nodules characterized by elevated ^{131}I iodide uptake (50% at the 6th hr) and marked hyperactivity at scan were excised. In 3 cases, the T_3 suppression test and TSH stimulation of the surrounding tissue confirmed the diagnosis (see Table 2) [4, 5]. All patients were female and ranged in age from 33 to 40.

Nine specimens of normal tissue taken from the opposite lobe of the nodular thyroids and from entirely normal glands constituted the control group.

For normal thyroids and hyperfunctional nodules, the vascular supply was interrupted 3 min or less before the specimen was excised. This time interval was considerably longer for carcinomas and ranged from 10 to 30 min.

The excised tissue was immediately placed in normal saline solution at 0°C, transported to the laboratory, and frozen in liquid nitrogen until the time of biochemical measurements. In 1 experiment with normal thyroid, the tissue was cut in 6 samples, 3 were assayed freshly and the 3 others were frozen.

Before freezing, a portion was taken for histologic examination in standard hematoxylin and eosin sections; 15 different fields distributed in the 3 most representative sections of each specimen were observed.

The structural pattern of thyroid tissue was defined as either homogeneous or heterogeneous after comparison of nodules and carcinomas with normal thyroids. Criteria used were the regularity of follicle size, presence of colloid cysts, amount of connective tissue and

Table 1. Histologic and biochemical results obtained on thyroid carcinomas. DNA and protein content are expressed in mg/g wet tissue, adenylate cyclase activity in picomoles cyclic AMP formed/10 min/μg DNA, and protein phosphokinase activity in picomoles of phosphate incorporated into protein/min/mg DNA. Effectors added: TSH: 10 mU/ml; sodium fluoride (NaF): $1 \cdot 10^{-2}$ M; adrenalin: $5 \cdot 10^{-4}$ M; prostaglandin E_1: 5μg/ml; cyclic AMP: $2 \cdot 10^{-6}$ M

Patient	Carcinoma	Structural aspect	Cells / Colloid	DNA	Proteins	Adenylate cyclase: Basal	+ TSH	+ NaF	+ Adren.	+ PGE_1	Protein phosphokinase: Basal	+ cAMP
M.V.	Papillary	Homog.	High	2·8	134	8·9	11·0	60·0	14·6	—	916	1,125
M.S.	Papillary	Homog.	Variable	1·6	71	2·4	4·5	16·7	2·1	4·0	446	1,325
W.S.	Mixed	Homog.	High	0·9	68	1·9	2·1	—	—	—	934	1,540
A.R.	Mixed	Homog.	High	3·5	152	1·4	2·8	12·5	1·7	3·3	481	494
G.S.	Follicular	Heterog.	High	2·7	172	—	1·6	22·5	2·5	5·0	2,120	3,750
J.M.	Medullary	Heterog.	Low	0·6	40	1·0	1·2	5·8	1·1	1·2	1,015	1,355
S.V.	Medullary	Heterog.	Variable	1·6	64	—	—	—	—	—	1,250	1,510

Table 2. Clinical definition and histological results of hyperfunctional nodules. $PB^{127}I$ is expressed in μg%, ^{131}I uptake in % of tracer dose and $PB^{131}I$ in mg/L plasma. T_3 suppression: 0 = no suppression; — not done

Patient	$PB^{127}I$	^{131}I Uptake 6th hr	^{131}I Uptake 24th hr	$PB^{131}I$	T_3 Suppression	TSH activation of surrounding tissue	Pathological findings	Structural aspect	Population cells/colloid
J.C.	5·5	28·9	42·5	0·30	—	—	Cylindrical epithelium	Heterog.	Variable
M.F.	11·7	63·8	60·3	0·35	—	—	Cylindrical epithelium	Heterog.	Low
J.W.	5·1	53·9	78·8	0·49	0	+	Colloid Goiter	Homog.	Average
C.E.	9·5	—	70·0	0·48	—	—	(Flattened epithelium)	Homog.	Low
M.I.	6·5	—	69·0	0·14	—	+	(Flattened epithelium)	Homog.	Low
M.D.	7·7	69·6	76·5	1·54	0	+	Colloid cysts	Heterog.	Variable

fibrosis or degenerative changes disrupting the basic follicular organization.

Cell density was roughly evaluated using similar criteria applied to normal and pathological tissues. The cell population was expressed as low or average (typical of normal thyroid) or as high when colloid material was almost totally replaced by cell proliferation (mainly in carcinomas), or as variable if cell population varied greatly from one area to another.

From 3 days to 2 months after excision, the frozen glands were thawed, rapidly trimmed free of fat and surrounding connective tissue, sliced with a Stadie Riggs microtome, chopped with a MacIlwain slicer, and homogenized with a motor driven teflon glass homogenizer by 4 strokes at a low speed in ice-cold medium (0·25 M sucrose, 0·05 M tris–HCl buffer, pH 7·4). The preparation was filtered through a teflon sieve and used for enzymatic assays (homogenate).

Protein content was assayed by the method of Lowry *et al.* [6] with bovine serum albumin as standard, and DNA by the method of Burton [7].

Adenylate cyclase was measured as previously described [8]. Briefly, aliquots of the homogenate were incubated 10 min at 30°C in a medium (200μl) containing 50 mM tris–HCl buffer, pH 7·4, 2 mM cyclic AMP, 5 mM $MgCl_2$, 3 mM ATP, 5 μCi α^{32}P–ATP (Radiochemical Centre, Amersham, U.K.), 0·1% cristalline bovine serum albumin, 10 mM creatine phosphate and 60 μg cristalline rabbit muscle creatine kinase. At the end of the incubation, the test tubes were transferred to an ice bath and 100 μl of an ice-cold solution containing 20 mM cyclic AMP, 50 mM ATP and ^{3}H-cyclic AMP (20,000 counts/min) were added. Cyclic AMP was then isolated by elution on dry neutral alumina columns [9]. The eluate was diluted in Bray's solution and ^{3}H and ^{32}P radioactivities were counted in a liquid scintillation counter.

Protein phosphokinase activity was assayed in a total volume of 1 ml: the complete reaction mixture contained enzyme plus 0·1 M potassium phosphate buffer pH 6·5, 1 mM EDTA, 10 mM magnesium acetate, 10 mM sodium fluoride, 2 mM theophylline, 2 mg of a mixture of histones (from calf thymus type II, Sigma), 0·2 mM γ-^{32}P-ATP (15–20 dis. min^{-1}/p mole) and, when added, 2 μM cyclic AMP. Assays were carried out at 30°C. After 4 min preincubation, the reaction was initiated by the addition of γ-^{32}P-ATP and stopped after 30 sec, 1 min, 2 min and 4 min by the addition of 1 ml of 40% trichloroacetic acid (TCA). The TCA precipitable material was washed two times by dispersion in cold NaOH 1N and reprecipitation in TCA 20%. The final TCA precipitate was digested in 1 ml soluene-100 (Packard, Warenville, Ill., U.S.A.) and counted in toluene-omnifluor 4% (NEN, Boston, Mass., U.S.A.) with a Nuclear Chicago Mark II liquid scintillation counter (efficiency of 80–90% for ^{32}P). Enzyme activity was linear with time up to 4 min and up to a protein concentration of 1 mg.

Cyclic AMP, ATP, phosphocreatine, creatine kinase and histones were purchased from Sigma (Saint Louis, Mo., U.S.A.), and adrenalin from Parke-Davis. Other reagents were of analytical grade (Merck, Darmstadt, F.R.D.). Bovine thyrotropin was obtained from Armour and from the N.I.H. Prostaglandin E_1 was a generous gift of Dr. J. Pike, Upjohn Co., Kalamazoo, Michigan, U.S.A. γ-^{32}P-ATP was prepared according to the method of Glynn and Chappell [10] modified by Walsh *et al.* [11], or was obtained from the Radiochemical Centre (Amersham, Great Britain). ^{3}H labelled cyclic AMP was obtained from New England Nuclear.

RESULTS

The clinical definition of the cases and the histologic study of carcinomas and nodules are respectively presented in the left part of Table 1 and in Table 2. Among the 7 thyroid carcinomas, the histological aspect is considered as homogeneous in 4 cases and heterogeneous in 3 cases. Among the latter, it is not surprising to see the two medullary carcinomas which are known to present varying structure. The cell population is high to variable in 6 cases and is low in only one. Such findings are consistent with the reduced colloid space partially replaced by cell proliferation and stroma [12], as in experimental models [13]. Except in one case, a good correlation was obtained in double blind between the estimation of the cells: colloid ratio by histologic examination and biochemical DNA measurement.

In the 6 hyperfunctional nodules, 3 were classified as heterogeneous and 3 as homogeneous. The cell density was more variable than in the carcinomas and ranged from average to low values. This fact is explained by the similarity between nodular and normal thyroid tissue, but also by marked structural changes induced by colloid deposits and cysts frequently encountered in goitrous disease. Here also a good correlation has been obtained between

histologic examination and biochemical DNA measurement.

The absolute values of DNA and protein content, and basal and stimulated adenylate cyclase and protein phosphokinase activities in carcinomas, hyperfunctional nodules and normal thyroid glands are presented in Tables 1, 3 and 4. As in other studies of this type [14], biochemical levels are highly variable from one case to another. However, it can be observed that the protein: DNA ratio is similar in all types of glands. With regard to the absolute levels of protein phosphokinase activities, activity is significantly higher in several carcinomas, by a factor of up to 4-fold. Indeed, basal protein kinase specific activity (expressed in picomoles of phosphate incorporated into protein/min/mg DNA) in carcinomas is similar to cyclic AMP-stimulated protein kinase activity in normal glands. Activation by cyclic AMP was similar in carcinomas, nodules, and normal glands homogenates; however in one case of papillary carcinoma, the activation factor reached a relatively high value. Adenylate cyclase basal activity was rather similar in normal, nodular and cancerous tissue. The stimulation factor by TSH was also similar. Freezing did not seem to affect TSH stimulation (Table 5). The effect of adrenalin was irregularly obtained and not statistically significant in the three series. A significant effect of prostaglandin E_1 was only observed on the nodular adenylate cyclase. In this tissue the stimulation by sodium fluoride was also markedly enhanced compared to normal tissue.

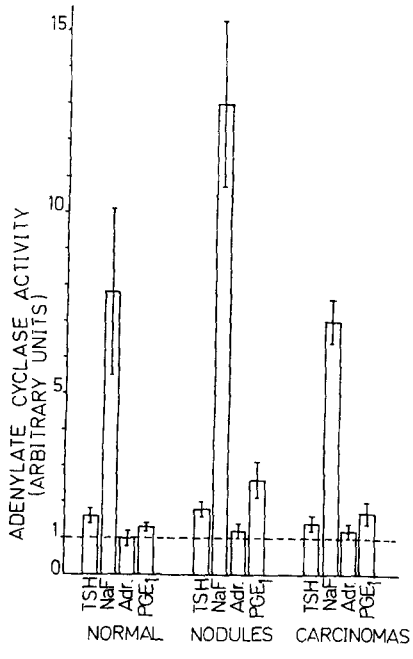


Fig. 1. Adenylate cyclase activity in normal thyroid tissue, autonomous nodules and carcinomas. Mean value ± SEM. Arbitrary units are pmoles of cyclic AMP formed/10 min./μg DNA at 30°C with basal activity taken equal to 1.

DISCUSSION

Studies of fibroblasts and transformed cell lines in culture have led to the general conclusion that there is an inverse relation between intracellular cAMP levels and growth, and that the characteristics of transformed cells are secondary to a decrease in the cell concentration of this nucleotide [1, 2].

However two facts are not easily reconciliable with this generalization: (1) tropic hormones enhance the accumulation of cAMP in their target tissues and promote growth; (2) cAMP levels in rapidly growing animal tumors are often high.

It was therefore of interest to measure the activity of enzymes of the cAMP system in human tumors. Previous studies on adenylate cyclase in medullary carcinomas [15] and in two functioning human thyroid adenomas [16] did not demonstrate a clear cut relation between adenylate cyclase activity and its stimulation by TSH and the degree of malignancy. However, medullary carcinomas cannot be considered strictly as thyroid tumors, because this cell type is not implicated in thyroid hormone synthesis.

These results have been confirmed in this study. There is no clear cut difference in adenylate cyclase activity and its activation by effectors such as TSH, sodium fluoride or prostaglandin between normal and carcinomatous tissue. Thus, so far as it can be evaluated on the limited samples of human tumors, these data do not support the hypothesis of an inverse relation between adenylate cyclase activity and malignancy.

In contrast, in the hyperfunctional tissue of the autonomous nodules, the activation of adenylate cyclase by prostaglandin E_1 and sodium fluoride was increased by a factor of 2. In some nodules adenylate cyclase has been shown to be more sensitive to TSH which could explain their growth and hyperfunctioning [16]. The present data suggest that increased sensitivity to other effectors might also play a role in the pathogenesis of these lesions.

The low or absent adenylate cyclase activation in some normal and cancerous tissues does not fit in well with the previously observed stimulation of several TSH-dependent metabolisms in human normal thyroid tissue [17].

Table 3. *Biochemical results obtained on hyperfunctional nodules. Units: see legend of Table* 1

Patient	DNA	Prot.	Adenylate cyclase					Protein phosphokinase	
			Basal	+TSH	+NaF	+Adr.	+PGE_1	Basal	+cAMP
J.C.	1·9	94	1·3	2·3	20·4	1·3	4·8	138	304
M.F.	0·9	86	3·1	5·4	49·0	3·9	10·0	619	826
J.W.	1·5	57	0·5	1·2	4·0	0·6	0·8	63	109
C.E.	1·4	74	2·4	5·0	44·0	4·2	8·6	686	752
M.I.	1·3	33	2·7	3·5	20·0	2·4	3·6	1,505	1,500
M.D.	3·1	86	—	—	—	—	—	645	710

Table 4. *Biochemical results obtained on normal glands. Units: see legend of Table* 1

Patient	DNA	Prot.	Adenylate cyclase					Protein phosphokinase	
			Basal	+TSH	+NaF	+Adr.	+PGE_1	Basal	+cAMP
J.M.	1·0	291	1·0	2·7	6·4	1	1·5	480	880
W.S.	0·8	88	2·1	3·2	—	—	—	—	974
J.C.	1·8	119	1·0	1·3	6·0	1·2	1·2	142	216
J.W.	1·8	79	2·8	3·5	7·8	4·4	3·5	246	292
M.S.	1·4	57	2·1	5·6	13·4	1·9	3·1	454	696
A.M.	2·7	73	1·1	1·4	23·0	0·7	2·0	386	454
M.I.	1·6	55	5·9	5·9	41·0	5·1	7·2	596	942
R.V.	0·9	38	3·3	2·9	17·1	2·8	5·8	400	474
M.D.	2·4	139	—	—	—	—	—	292	750

Table 5. *Comparison of adenylate cyclase and protein phosphokinase activities in fresh and frozen tissue. Units: see legend of Table* 1

Adenylate cyclase Activity	Fresh tissue sample				Frozen tissue sample			
	1	2	3	Mean Value	1	2	3	Mean Value
Basal	1·6	1·6	1·5	1·6	1·7	2·1	1·6	1·8
+TSH	2·5	3·2	2·0	2·6	3·3	2·8	2·1	2·7
TSH/Basal	1·6	2·0	1·3	1·6	1·9	1·3	1·3	1·5
+NaF	5·7	7·9	3·4	5·7	7·3	6·9	7·1	7·1
NaF/Basal	3·6	4·9	2·3	3·6	4·3	3·3	4·4	3·9
Protein kinase activity								
Basal		170				178		
+ cyclic AMP		284				281		

However sensitivity of adenylate cyclase to effectors is notoriously fragile and in this case could have been lost because of the freezing of the tissue. However, a comparison of adenylate cyclase and protein phosphokinase activity in fresh and frozen normal thyroid do not show significant differences.

If adenylate cyclase activity seems not to be related to the degree of malignancy, the situation is not the same for protein phosphokinase activity. Indeed, although absolute enzymatic levels were variable from case to case, basal and cAMP-stimulated activity were higher in carcinomas than in normal tissue by a factor of up to 4-fold. Nevertheless, the activation by cAMP was the same in normal

and pathologic glands. Granner [18] has also observed 3-fold higher basal protein phosphokinase activity in a cell line established from a rat hepatoma (HTC) than in normal liver, but in the crude extracts, cAMP was unable to activate the enzyme.

Thus protein phosphokinase and probably cyclic AMP-dependent protein phosphokinase were found in greater amounts in several types of human thyroid carcinomas. However, one cannot exclude at the present stage of work the possibility of modifications of the enzyme(s) which would increase the affinity for protein substrates or the inactivation of the thermostable protein phosphokinase inhibitor [19], if present in the thyroid.

In conclusion, whereas from extrapolation of *in vitro* work on transformed cells one would have expected to find decreased levels of adenylate cyclase and decreased sensitivity to effectors in human thyroid carcinomas, no such change has been observed. However, the activity of protein phosphokinase, the enzyme of which cyclic AMP is an effector, has been found to be markedly increased. It would be therefore of interest to investigate the end result of the modulation of adenylate cyclase and protein phosphokinase activities, i.e. protein phosphorylation in normal and cancerous human tissue.

Acknowledgements—This work was supported by a grant of the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite (Belgium). The authors wish to thank Dr. P. Dor for his collaboration in providing the thyroid tissue specimens, Dr. E. Schell-Frederick for help in revising the text and Ms. D. Legrand for the careful typing of the manuscript.

REFERENCES

1. W. L. Ryan and M. L. Heidrick, Role of cyclic nucleotides in Cancer. In *Advances in Cyclic Nucleotide Research.* (Edited by P. Greengard and G. A. Robison), Vol. 4, p. 81, Raven Press, New York (1974).
2. J. Otten, G. S. Johnson and I. Pastan, Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. *Biochem. biophys. Res. Comm.* **44,** 1192 (1971).
3. J. E. Dumont, The action of thyrotropin on thyroid metabolism. In *Vitamins and Hormones.* Vol. 29, p. 287 Academic Press, New York (1971).
4. S. C. Werner and M. Spooner, New and simple test for hyperthyroidism employing L-triiodothyronine and the twenty-four hour ^{131}I uptake method. *Bull. N.Y. Acad. Press* **31,** 137 (1955).
5. J. M. Miller and J. I. Hamburger, The thyroid scintigram. I. The hot nodule. *Radiobiology* **84,** 66 (1965).
6. O. H. Lowry, N. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
7. K. Burton, Determination of DNA concentration with diphenylamine. In *Methods in Enzymology.* (Edited by L. Grossman and K. Moldave), Vol. 12b, p. 163 Academic Press, New York (1968).
8. R. Pochet, J. M. Boeynaems and J. E. Dumont, Stimulation by thyrotropin of horse thyroid plasma membranes adenylate cyclase: evidence of cooperativity. *Biochem. biophys. Res. Comm.* **58,** 446 (1974).
9. J. Ramachandran, A new simple method for separation of adenosine 3'-5'-cyclic monophosphate from other nucleotides and its use in the assay of adenyl cyclase. *Analyt. Biochem.* **43,** 227 (1971).
10. I. M. Glynn and J. B. Chappell, A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. *Biochem. J.* **90,** 147 (1964).
11. D. A. Walsh, J. P. Perkins, Ch. O. Brostrom, E. S. Ho and E. G. Krebs, Catalysis of the phosphorylase kinase activation reaction. *J. biol. Chem.* **246,** 1968 (1971).
12. W. O. Russell, M. L. Ibanez, R. L. Clark, C. S. Hill Jr and E. C. White, Follicular (organoid) carcinoma of the thyroid gland. Report of 84 cases. In *Thyroid Cancer. UICC Monograph.* (Edited by Ch. E. Hedinger). Vol. 12, p. 14 Springer-Verlag, Berlin (1969).
13. J. Matovinovic, R. D. Hilbert, W. F. Armstrong and R. G. Helgensen, Thyroid tumor and thyroid transplant tumor in iodine-deficient rat. In *Current Topics in Thyroid Research.* (Edited by Cassano-Andreoli). p. 920 Academic Press, New York (1965).

14. J. E. DUMONT, Carbohydrate metabolism in the thyroid gland. *J. clin. Endocr.* **20,** 1246 (1960).
15. F. DE RUBERTIS, K. YAMASHITA, A. DEKKER, P. R. LARSEN and J. B. FIELD, Effects of thyroid-stimulating hormone on adenyl cyclase activity and intermediary metabolism of "cold" thyroid nodules and normal human thyroid tissue. *J. clin. Invest.* **51,** 1109 (1972).
16. P. R. LARSEN, K. YAMASHITA, A. DEKKER and J. B. FIELD, Biochemical observations in functioning human thyroid adenomas. *J. clin. Endocr.* **36,** 1009 (1973).
17. J. OTTEN and J. E. DUMONT, Glucose metabolism in normal human thyroid tissue *in vitro*. *Europ. J. clin. Invest.* **2,** 213 (1972).
18. D. K. GRANNER, Protein kinase: altered regulation in a hepatoma cell line deficient in adenosine 3′,5′-cyclic monophosphate-binding protein. *Biochem. biophys. Res. Comm.* **46,** 1516 (1972).
19. D. A. WALSH, CH. D. ASHBY, C. GONZALEZ, D. CALKINS, E. H. FISCHER and E. G. KREBS, Purification and characterization of a protein inhibitor of adenosine 3′-5′-monophosphate-dependent protein kinases. *J. biol. Chem.* **246,** 1977 (1971).

Europ. J. Cancer Vol. 12, pp. 455–463. Pergamon Press 1976. Printed in Great Britain

Influence on Metastatic Spread of Whole-Body or Local Tumor Hyperthermia*

A. YERUSHALMI
Radiation Unit, The Weizmann Institute of Science, Rehovot, Israel

Abstract—*This paper reports on an experimental model for quantitative examination of the effects of whole-body and local hyperthermia on the advanced or delayed appearance of metastases in the lungs of mice inoculated with the Lewis lung carcinoma tumor.*

Tumor-bearing animals were submitted to whole-body heating with hot air at temperatures of 45°C *and* 50°C. *Local hyperthermia hot-air temperatures were* 45°C, 50°C *and* 55°C. *In comparison to untreated tumor-bearing animals, the appearance of metastases was advanced in whole-body heated animals, whereas there was a delay in metastasis appearance in animals which received local hyperthermic treatment.*

INTRODUCTION

THERE exists substantial experimental evidence to indicate that hyperthermia (temperatures above 40°C) has selective inhibitory and destructive effects on cancer cells [1–5]. Regression and destruction of tumors in experimental animals and in humans has been achieved by hyperthermia accompanying infectious diseases, by administration of pyrogens, and by physical heating [4–11]. Studies on mammals and mammalian cells have shown clearly that radiation sensitivity is modified by elevated temperatures [12–17]. Hyperthermia was achieved by whole-body [10,18–22] or local heating [14,17,23–28]. Only a few reports dealt with the optimal heating conditions, or compared the consequences of whole-body vs local hyperthermia [14,29–31]. In one of these, the VX-2 carcinoma in the rabbit limb was treated by local and whole-body hyperthermia [29], and local heating was found to be more effective than whole-body hyperthermia. However, heating to a degree insufficient to produce cure stimulates the spread and growth of metastases and may allow repair of sublethal damage to the tumor cells to take place [31, 32], and the cell's sensitivity to hyperthermia may be dependent on their position in the cell cycle [33].

In the present study, an experimental system was developed for the quantitative examination of the results of whole body versus local hyperthermia on the advanced or delayed appearance of metastases in the lungs of mice which had been inoculated with the highly malignant, rapidly growing Lewis lung carcinoma. I report on the observation that metastasis appearance is advanced in whole-body heated animals in comparison to untreated tumor bearing animals, while there is a delay in metastasis appearance in locally heated animals. This finding is of particular interest with respect to the application of whole-body hyperthermia in humans with metastatic tumors.

Accepted 7 January 1976.

*This work was supported by a grant from the Gulton Foundation, Englewood, New Jersey, U.S.A.

MATERIAL AND METHODS

Animals used were 3–6-months old inbred C3H/eB/BLF1 mice of both sexes. The animals were housed 10 per cage and provided with a standard laboratory diet and tap water ad libitum. The tumor used throughout these experiments was the 3LL Lewis carcinoma. This tumor spontaneously metastasizes to the lungs [34]. The tumor was maintained by serial transplantation of tumor cells i.m. in C3H/eB/BLF1 mice. Tumor inocula were prepared from 3–5 tumors. Tumors were aseptically removed, minced with scissors, washed twice in cold phosphate buffered saline, and then trypsinized for 30 min in 0·25% trypsin solution to produce a disaggregated cell suspension. The suspension was filtered through a fine stainless steel mesh. Tumor cells were then centrifuged, washed

twice in Dulbecco's modified Eagle's medium, and viable cells estimated by exclusion of trypan blue counted in a hemocytometer; $0{\cdot}5 \times 10^6$ viable tumor cells in 0·5 ml were injected i.m. into the left hind leg. In preliminary experiments, and in the control groups of each experiment, the tumor take rate was higher than 95%.

Tumor diameter measurement

Measurements of the size of the i.m. tumor diameters were taken daily from the first day of tumor inoculation. The external greatest diameter at the tumor site was measured with calipers (60 inoculated animals). Figure 1 shows

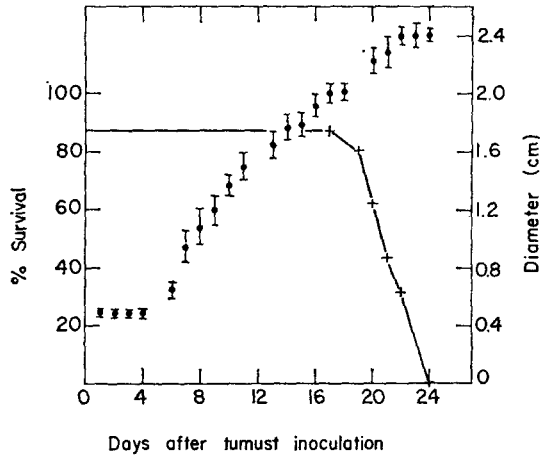


Fig. 1. Tumor diameter growth and mortality of untreated tumor-bearing animals. ●*—Tumor-bearing leg diameter in cm. Bars indicate standard deviation.* +*—Percentage of survival of tumor-bearing animals.* 100 % = 60 *animals in each curve.*

the exponential increase curve of the diameter of the tumor-bearing leg. The time it took for the tumor-bearing leg to reach 1 cm leg diameter after inoculation of $0{\cdot}5 \times 10^6$ cells is in agreement with the experiments of others [35]. The tumor-bearing leg diameter increased according to $D = D_0 (1 - e^{-at})$, where D is the tumor-bearing leg diameter on days 6–22; D_0 is the leg diameter on the day of inoculation ($D_0 = 0{\cdot}485$ cm); a was found to be $-2{\cdot}74 \times 10^{-3} \pm 1{\cdot}9 \times 10^{-4}$.

Heating system

The heating system has been described elsewhere [17]. In short, it consisted of: (a) a hot air blower; (b) a heat-insulated cylinder (dia 10·5 cm, length 24 cm); and (c) a temperature control unit. For local heating, the tumor-carrying limbs of 4 mice were inserted into the four holes (forming a Maltese cross) drilled in the upper part of the cylinder. For whole-body heating, 4 mice at a time were inserted into the cylinder. Hot air was blown through a pipe connecting the blower with the cylinder.

Temperature measurements

The hot air atmosphere was achieved by passing the air through an attenuator to reduce turbulence. Homogeneity was achieved and confirmed by a system of valves and thermocouples installed for temperature stability and control. Simultaneous temperature measurements were made in the cylinder, in the tumor (to specify intratumor temperature) and in the ear of the mouse (to record skin temperature). The temperature measurements were taken by copper–constantan thermocouples 1·8 mm thick. A thermocouple was inserted into the tumor through a channel made with a 2-mm dia trocar. Skin temperature was measured by inserting a thermocouple deep into the ear. Preliminary experiments showed that intra-ear temperature measurements were reliable indicators of skin temperature. The leads from the installed thermocouples were connected to an electronic control unit which included a continuous digital temperature read-out and X–Y recorder.

Experimental schedule

The tumor cell suspension was inoculated ($0{\cdot}5 \times 10^6$ viable cells) into the hind left limb of stock C3H/eB/BLF1 mice. At different days after tumor transplantation animals were randomly selected for the experiment. The experiments consisted of the following groups: (a) a control group, inoculated only; (b) whole body heated animals (heating temperature 45°C, 50°C); (c) locally heated animals (heating temperatures 45°C, 50°C and 55°C); (d) stock animals treated at the same experimental conditions as in (b) and (c).

Preliminary experiments in untreated tumor-bearing animals showed that no visible distant blood-borne metastases can be detected before the 12th day following implantation, and the lung nodules are clinically occult. From the 12th day after inoculation, 3 or 4 animals of each group were randomly selected daily and killed by ether. The chest of these animals was opened, the lungs observed under a low power dissecting microscope, and the number of lung metastases was counted. Lung metastases appeared as pink round nodules 0·2–3·0 mm in diameter protruding on the surface of the lungs; they had well defined borders.

RESULTS

The tumors became palpable 7–8 days after inoculation and increased in volume until 22 days. Animals died 23 ± 1·4 days following inoculation, with a primary tumor diameter of approximately 2·4 cm (Fig. 1). There was no life span prolongation, no restraint of tumor, no slow down in tumor growth in both locally and whole-body heated animals. Both groups tolerated well the heating temperatures and none died as a result of hyperthermia. The mean survival time for locally heated and whole-body heated tumor-bearing animals and of control untreated tumor-bearing animals was 23 ± 1·4 days. Fig. 2 and 3 illustrate the temperature build-up in whole-body and locally heated tumor-bearing animals 10 days after inoculation, when the tumor-bearing leg diameter was 1·37 ± 0·06 cm. In all the experiments, heating time was 30 min.

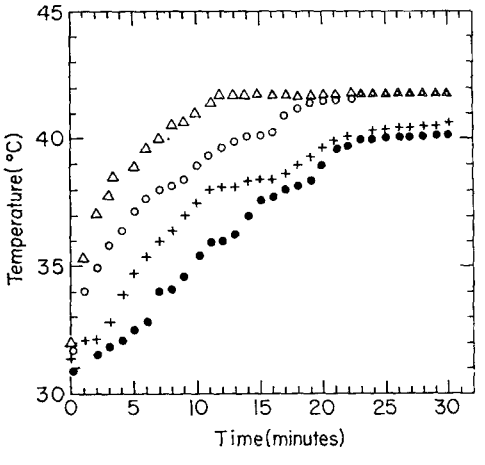


Fig. 2. Temperature build-up in whole body heated tumor-bearing animals. Hot air temperature 45°C: +—body temperature; ●—intratumor temperature. Hot air temperature 50°C: △—body temperature; ○—intratumor temperature.

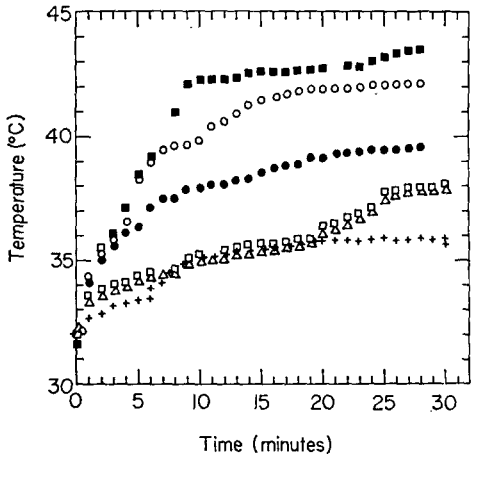


Fig. 3. Temperature build-up in locally heated tumor-bearing animals. Hot air temperature 45°C: +—body temperature; ●—intratumor temperature. Hot air temperature 50°C: △—body temperature; ○—intratumor temperature. Hot air temperature 55°C; □—body temperature; ■—intratumor temperature.

Whole-body heated animals (Fig. 2) achieved body temperatures of 40·5°C when heating at 45°C was applied. At the same time, the temperature of the tumor-bearing leg rose up to 40·2°C. Heating at 50°C raised body and tumor-bearing leg temperatures to 41·9°C.

When tumor-bearing leg was locally heated at 45°C, body temperature reached 35·8°C, limb temperature reached 39·5°C. At 50°C heating, body temperature rose to 37·7°C and tumor-bearing limb temperature to 42·2°C. At 55°C local heating, body temperature reached 38·1°C and the temperature in the tumor-bearing limb reached 43·5°C.

At whole-body heating at 45°C, the temperature of the tumor-bearing limb rose to 40·2°C as compared to 42·2°C when 50°C hot air was applied locally. When whole body heating at 50°C was applied, the temperature of the tumor-bearing limb rose to 41·9 as compared to 43·5 in mice receiving 55°C hot air locally on the tumor-bearing limb.

While the temperature in the locally heated mice was higher in the tumor area than in whole-body heated animals, the tumor temperatures of the whole-body heated mice were equal to or lower than those of the whole-body heated mice. These comparisons are of importance in examining the results and comparing the effects of whole-body versus local hyperthermia.

The effect of whole-body and local hyperthermia on the first day of metastasis appearance is summarized in Table 1. In 45°C hot air temperature heated tumor-bearing animals, metastases appeared on the same day in both tumor-bearing controls and locally heated animals, while in the whole body heated animals metastases appeared one day earlier in one group, and on the same days as in the controls in 3 groups. In 50°C hot air temperature heating in locally heated animals metastasis appearance was delayed by 2 days in one group as compared to unheated controls, while in the remaining three groups metastases appeared on the same day as in the controls. However, metastasis appearance was accelerated in all the six groups which were whole-body heated, as compared to their unheated tumor-bearing controls. Locally heated tumor-bearing animals at hot-air temperatures of 55°C demonstrated late metastasis appearance in locally heated animals. While in whole-body heated animals at 50°C heating applied 10 days after tumor

Table 1. First day of metastasis appearance in whole-body and locally heated animals

Heat treatment at No. of days after tumor inoculation	First day of metastasis appearance			
	Unheated controls	Locally heated	Controls	Whole body heated
Hot air temperature 45°C				
1			14	14
3	14	14	14	13
6	14	14	14	14
8			15	15
10	14	14	14	14
Hot air temperature 50°C				
1			15	13
2	16	16	16	15
3			15	14
5	15	17	16	15
8	14	14	14	13
10	15	15	15	13
Hot air temperature 55°C				
3	14	16		
5	14	15		
6	14	15		
8	15	15		

Table 2. Daily number of metastases in whole-body and locally heated animals at 45°C heating temperature

Hot air temperature 45°C									
	Local heating				Whole body heating				
Days after tumor inoculation	Unheated control	Heated 3 days after tumor inoculation	Heated 6 days after tumor inoculation	Heated 10 days after tumor inoculation	Unheated control	Heated 1 day after tumor inoculation	Heated 6 days after tumor inoculation	Unheated controls	Heated 10 days after tumor inoculation
12	0/3	0/3	0/3	0/3	0/4	0/4	0/4	0/3	0/3
13	0/3	0/3	0/3	0/3	0/4	0/4	0/4	0/3	0/3
14	1/3	2/3	1/3	3/3	2/4	2/4	1/4	1/3	2/3
15	3/3	2/3	2/3	2/3	12/4	40/4	3/4	3/3	3/3
16	3/3	1/3	2/2	1/3	10/4	41/4	23/4	21/3	11/3
17					14/4	40/4	23/4		
18	21/3	3/3	2/3	4/3	19/4	44/4	26/4	40/3	46/3
19	20/3	3/3	3/3	2/3	21/4			21/3	21/3
20	24/3	3/3	5/3	4/3	21/4	40/4	24/4	24/3	22/3
21	3/3	14/4	16/4	18/4	22/4	23/4	25/4	7/3	5/3
22	21/3	24/4	25/4	26/4	28/4	21/4	40/4	2/2	24/3
23					29/4	40/4	40/4		
24					20/4	10/4	42/4		

inoculation, metastases appeared earlier than in the controls, local heating at 55°C did not accelerate metastases appearance as compared to unheated controls. Furthermore, in the groups locally heated up to 6 days after inoculation, metastasis appearance was delayed as compared to the unheated tumor-bearing controls.

Tables 2, 3 and 4 summarize the results of daily metastases counting of locally and whole-body heated animals at 45°C, 50°C and 55°C hot air temperature. From day 12 after inoculation, animals were daily randomly selected and lung metastases were counted as described in methods.

As compared to control unheated tumor-bearing animals, the daily counted number of metastases in locally heated animals is in general less than those of the controls at the three heating temperatures. On the other hand,

Table 3. Daily number of metastases in whole-body and locally heated animals at 50°C heating temperature

Hot air temperature 50°C												
	Local Heating						Whole Body Heating					
Days after tumor inoculation	Unheated controls	Heated 2 days after tumor inoculation	Unheated controls	Heated 8 days after tumor inoculation	Unheated controls	Heated 10 days after tumor inoculation	Unheated controls	Heated 1 day after tumor inoculation	Unheated controls	Heated 8 days after tumor inoculation	Unheated control	Heated 10 days after tumor inoculation
12	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
13	0/4	0/4	0/4	0/4	0/4	0/4	0/4	3/4	0/4	1/4	0/4	2/4
14	0/4	0/4	7/4	13/4	0/4	0/4	0/4	2/4	1/4	2/4	0/4	7/4
15	0/4	0/4	16/4	1/4	13/4	7/4	8/4	2/4	4/4	20/4	3/4	20/4
16	7/4	1/4	8/4	3/4	11/4	20/4	12/4	60/4	21/4	20/4	7/4	36/4
17	9/4	5/4	23/4	3/3	30/4	31/4	8/4	39/4	11/4	22/4	8/4	26/4
18	26/4	21/4			46/4	13/4	42/4	22/4	23/3	27/4	60/4	72/4
19			20/4	8/4								
20	34/4	7/4	62/4	7/4			41/4	24/4	23/4	44/4	41/4	42/4
21	11/4	11/3	42/4	41/4			31/4	32/4	21/4	45/4	31/4	55/4
22			24/4	17/4			30/4		24/4	38/4	30/4	46/4
23									23/4	45/4		

Table 4. Daily number of metastases in locally heated animals at 50°C heating temperature

	Hot air temperature 55°C			
	Local heating			
Days after tumor inoculation	Unheated control	Heated 3 days after tumor inoculation	Unheated controls	Heated 8 days after tumor inoculation
12	0/4	0/4	0/4	0/4
13	0/4	0/4	0/4	0/4
14	1/4	0/4	0/4	0/4
15	1/4	0/4	2/4	5/4
16	3/4	1/4		
17	20/4	20/4	15/4	16/4
18	28/4	22/4	26/4	19/4
19	40/4	23/4	23/4	18/4
20	33/4	25/4	31/4	26/4
21	60/4	8/4	26/4	19/4

Table 5. Daily number of metastases in animals locally heated twice

	Local heating					
					Hot air temperature 55°	
Days after tumor inoculation	Unheated control	Hot air temperature 45°C heated at days 4 & 6 after tumor inoculation	Hot air temperature 50°C heated at days 4 & 6 after tumor inoculation	Unheated controls	Heated at days 6 & 8 after tumor inoculation	Heated at days 9 & 11 after tumor inoculation
12	0/4	0/4	0/4	0/4	0/4	0/4
13	0/4	0/4	0/4	3/4	6/4	0/4
14	4/4	4/4	0/4	4/4	3/4	3/4
15	5/4	1/4	2/4	6/4	1/4	1/4
16	11/4	3/4	1/4	8/4	16/4	10/4
17	13/4	3/4	5/4	19/4	15/4	13/4
18	26/4	4/4		23/4	23/4	9/4
19	29/4	4/4	11/4			
20	34/4	20/4	12/4	32/4	18/4	8/4
21	15/4	9/4	8/4	11/4	9/4	16/4

the daily counted number of metastases in whole-body heated animals is generally higher than in the unheated tumor-bearing controls.

The advantage of local heating over whole-body heating is clearly demonstrated in Table 5. In this set of experiments, tumor-bearing animals were locally heated twice, at intervals of 2 days. Here again, local heating at 45°C hot air temperatures at days 4 and 6 after tumor inoculation had not caused earlier metastasis appearance. Two local heating treatments at 50°C hot air temperature on days 4 and 6 after tumor inoculation resulted in 1 day delay of metastases as compared to its unheated tumor-bearing controls.

Finally, two treatments of local heating at 55°C hot air temperature at 2-day intervals show a delay of metastasis appearance in one group, while in the second group the first day of metastasis appearance was the same as in the unheated tumor-bearing controls. In no case was there any acceleration in metastasis appearance in locally heated animals, while whole-body heating resulted in earlier appearance of lung metastases.

DISCUSSION

There is little available data on the relative effectiveness of local hyperthermia compared with whole-body heating [14, 29–31]. Local hyperthermia (immersion in water bath) was found to be more effective than whole-body heating (radiating heating of glow lamps) in

the treatment of the VX-2 carcinoma in the limb of the rabbit [29]. In the present investigation, local and whole-body hyperthermia were produced with the same heat source, namely, hot air, so that heat was applied under the same physical conditions.

Whole-body heating is used and is claimed to be a means of destroying metastatic as well as primary cancer cells [21]. Moreover, whole-body hyperthermia is used clinically in cancer therapy [6]. However, it is also reported that in some cases, whole-body heated patients seemed to develop a less heat sensitive tumor [19].

This communication represents the results of a large series of experiments with groups of animals inoculated with the 3LL Lewis lung carcinoma, conducted for the purpose of devising an appropriate controlled model for the study of the influence of whole-body vs local hyperthermia on the appearance of metastases of a malignant tumor. Early in the course of the disease, when the primary tumor is comparatively small, the host is in good condition and can tolerate the heating treatment. As with human tumors, this transplantable tumor has not been observed to regress spontaneously. The animals developed pulmonary metastases in a known period. Also, the survival times were sufficient to permit a distinct separation between results from controls and from heat-treated animals. The parameters studied were: (1) The delay or acceleration of metastasis appearance in local versus whole-body heated tumor-bearing mice, and (2) the daily counted average number of metastases in the two groups. The biological model used lends itself to an *in vivo* quantitative analysis of the effectiveness of local and whole-body heat treatment in metastatic tumors.

The results indicate that in the present model, local hyperthermia applied once or twice does not accelerate metastasis appearance, whole-body heating results in earlier appearance, of lung metastases. Moreover, in comparison with controls, in locally heated animals the average number of lung metastases was in general smaller, while a higher daily average number of lung metastases was observed in whole-body heated animals. The results indicate that in whole-body heating, body temperature was higher or equal to the intratumor temperature. However, at local heating, body temperature was lower than intratumor temperature. These data and the facts mentioned earlier suggest that in hyperthermia treatment a primary tumor–body temperature gradient must exist in order to avoid stimulation of metastasis appearance. Thus, the elevating of whole-body temperature above that of the tumor and artificial elevated temperatures may have altered a metabolic or biological border in animals bearing metastatic tumors, leading to (a) stimulation of premature activity in 3LL cells present in metastatic sites at the time of heating; (b) changes in the mobilities of the cells of the primary tumor; (c) alterations in properties of the tissues concerned in metastatic distribution of blood-mediated metastasis or (d) a possible depression in the immune defence mechanism(s) of the host at artificially lowered primary tumor–body temperature gradient, leading to a more rapid spread of metastases. Whether or not the early appearance of metastases as a result of whole-body heating is general or applies only to this particular tumor remains to be tested. Nevertheless, the results of this investigation provide evidence in favor of the local heating as an adjuvant to therapy and should be considered in the planning of clinical studies.

Acknowledgement—I wish to thank Mr. S. Milchan for his competent and careful technical assistance.

REFERENCES

1. B. Mondovi, A. F. Agro, G. Rotilio, R. Storm, G. Moricca and A. Rossi Fanelli, The biochemical mechanism of selective heat sensitivity of cancer cells. II. Studies on nucleic acids and protein synthesis. *Europ. J. Cancer* **5,** 137 (1969).
2. B. Mondovi, R. Storm, G. Rotilio, A. F. Agro, R. Cavaliere and A. Rossi Fanelli, The biochemical mechanism of selective heat sensitivity of cancer cells. I. Studies on cellular respiration. *Europ. J. Cancer* **5,** 129 (1969).
3. B. C. Giovanella, A. C. Morgan, J. S. Stehlin Jr. and L. J. Williams, Selective lethal effect of supranormal temperatures on mouse sarcoma cells. *Cancer Res.* **33,** 2568 (1973).
4. D. S. Muckle and J. A. Dickson, The selective inhibitory effect of hyperthermia on the metabolism and growth of malignant cells. *Brit. J. Cancer* **25,** 771 (1971).

5. R. Cavaliere, E. C. Ciocatto, B. C. Giovanella, C. Heidelberger, R. O. Johnson, M. Margottini, B. Mondovi, G. Morica and A. Rossi Fanelli, Selective heat sensitivity of cancer cells. *Cancer (Philad.)* **20,** 1351 (1967).
6. J. Isseles, Immunotherapy in progressive metastatic cancer. *Clin. Trials J.* **3,** 357 (1970).
7. H. C. Nauts, G. A. Fowler and F. H. Bogatko, A review of the influence of bacterial infections and of bacterial products (Coley's toxin) on malignant tumors in man. *Acta med. scand.* suppl. **276,** 145, 1 (1953).
8. K. Overgaard and J. Overgaard, Investigation on the possibility of a thermic tumor therapy, I: Short-wave treatment of a transplanted isologous mouse mammary carcinoma. *Europ. J. Cancer* **8,** 65 (1972).
9. O. S. Selawry, J. D. Carlson and G. E. Moore, Tumor response to ionizing rays at elevated temperatures. *Amer. J. Roentgenol.* **80,** 833 (1958).
10. S. L. Warren, Preliminary study of effect of artificial fever upon hopeless tumor cases. *Amer. J. Roentgenol.* **33,** 75 (1935).
11. N. Westermark, Effect of heat upon rat tumors. *Skand. Arch. Physiol.* **52,** 257 (1927).
12. J. A. Belli and F. J. Bonte, Influence of temperature on the radiation response of mammalian cells in tissue culture. *Radiat. Res.* **18,** 272 (1963).
13. E. Ben-Hur, M. M. Elkind and B. V. Bronk, Thermally enhanced radioresponse of cultured Chinese hamster cells: Inhibition of repair of sublethal damage and enhancement of lethal damage. *Radiat. Res.* **58,** 38 (1974).
14. G. Crile Jr., The effect of heat and radiation on cancers implanted on the feet of mice. *Cancer Res.* **23,** 372 (1963).
15. J. E. Robinson, M. J. Wizenberg and W. A. McCready, Radiation and hyperthermal response of normal tissue *in situ*. *Radiology* **113,** 195 (1974).
16. J. E. Robinson, M. J. Wizenberg and W. A. McCready, In: Abstracts of Contributed papers IV International Congress of Biophysics, Moscow, August 7–14, Section I–IV, p. 289 (1972).
17. A. Yerushalmi and I. Har-Kedar, Enhancement of radiation effects by heating of the tumor. *Israel J. med. Sci.* **10,** 772 (1974).
18. R. T. Pettigrew, J. M. Galt, C. M. Ludgate, D. B. Horn and A. N. Smith, Circulatory and biochemical effects of whole body hyperthermia, *Brit. J. Surg.* **61,** 727 (1974).
19. R. T. Pettigrew, J. M. Galt, C. M. Ludgate and A. N. Smith, Clinical effects of whole-body hyperthermia in advanced malignancy. *Brit. med. J.* **4,** 679 (1974).
20. M. A. Henderson and R. T. Pettigrew, Induction of controlled hyperthermia in treatment of cancer. *The Lancet* **i,** 1275 (1971).
21. M. Von-Ardenne, *Theoretische und experimentelle Grundlagen der Krebs-Mehrschrift-Therapie.* 2nd ed. VEB Verlag Volk und Gesundheit Berlin (1971).
22. R. Kirsch and D. Schmidt, In: *Aktuelle probleme aus dem Gebeit der Cancerologie,* pp. 53–70, Springer Verlag, Heidelberg (1966).
23. A. T. K. Cockett, M. Kasmin, R. Nakamura, A. Fingerhurt and J. J. Atein, Enhancement of regional bladder megavoltage irradiation in bladder cancer using local bladder hyperthermia. *J. Urol.* **97,** 1034 (1967).
24. W. W. Shingleton, F. A. Bryan, W. L. O'Quinn and L. C. Krueger, Selective heating and cooling of tissue in cancer chemotherapy. *Ann. Surg.* **156,** 408 (1962).
25. K. Woeber, The effects of ultrasound in the treatment of cancer. *The Ultrasonic Energy* Edited by E. Kellys, pp. 138–149. Chicago Univ. Press (1965).
26. D. B. Cater, I. A. Silver and D. A. Watkinson, Combined therapy with 220 kV roentgen and 10 cm microwave heating in rat hepatoma. *Acta Radiol.* **2,** 321 (1964).
27. E. W. Hahn, A. A. Alfieri and J. H. Kim, Increased cures using fractionated exposure of X irradiation and hyperthermia in the local treatment of the Ridgeway osteogenic sarcoma in mice. *Radiology* **113,** 199 (1974).
28. A. Yerushalmi, Cure of a solid tumor by simultaneous administration of microwaves and X-irradiation. *Rad. Res.* **64,** 602 (1975).
29. J. A. Dickson and D. S. Muckle, Total body hyperthermia versus primary tumor hyperthermia in the treatment of the rabbit VX2 carcinoma. *Cancer Res.* **32,** 1916 (1972).

30. D. S. Muckle and J. A. Dickson, Hyperthermia (42°C) as an adjuvant to radiotherapy and chemotherapy in the treatment of the allogeneic VX2 carcinoma in the rabbit. *Brit. J. Cancer* **27,** 307 (1973).
31. J. A. Dickson and M. A. Ellis, Stimulation of tumor cell dissemination by elevated temperature (42°C) in rats with transplanted Yoshida tumors. *Nature (Lond.)* **248,** 354 (1974).
32. R. J. Palzer and C. Heidelberger, Studies on the quantitative biology of hyperthermic killing of HeLa cells. *Cancer Res.* **33,** 415 (1973).
33. R. J. Palzer and C. Heidelberger, Influence of drugs and synchrony on the hyperthermic killing of HeLa cells. *Cancer Res.* **33,** 422 (1973).
34. K. Sugiura and C. C. Stock, Studies in a tumor spectrum. III. The effect of phosphoramides on the growth of a variety of mouse and rat tumors. *Cancer Res.* **15,** 38 (1955).
35. G. Gordon Steel and K. Adams, Stem cell survival and tumor control in the Lewis lung carcinoma. *Cancer Res.* **35,** 1530 (1975).

Europ. J. Cancer Vol. 12, pp. 465–473. Pergamon Press 1976. Printed in Great Britain

Skeletal Muscle Metabolism in Patients with Malignant Tumor*

KENT LUNDHOLM, ANN-CHRISTIN BYLUND, JAN HOLM and TORE SCHERSTÉN

Surgical Metabolic Research Laboratory, Department of Surgery, Sahlgrenska sjukhuset, University of Göteborg, Göteborg, Sweden

Abstract—*Skeletal muscle biopsies were taken from 43 cancer patients and 55 matched, presumably metabolically healthy controls for determinations of enzyme activities, capacity of glucose assimilation and metabolism and the incorporation rate of leucine into proteins.*

In muscle tissue from cancer patients the activities of the lysosomal enzymes, cathepsin-D and β-glucuronidase, were increased. Acid phosphatase activity was the same as in muscle tissue from the controls. The activities of hexokinase, phosphofructokinase, lactate dehydrogenase, and cytochrome-c-oxidase were significantly decreased. This decrease was accompanied by a decreased incorporation rate of glucose-carbon into glycogen, lactate and CO_2. The incorporation rate of leucine into proteins was significantly decreased in cancer patients and the fractional degradation rate of proteins was increased. Amino acids caused the same degree of stimulation on the leucine incorporation rate into proteins as in the controls. The RNA concentration was unchanged in muscles from the cancer patients.

The results suggest that malignant tumors provoke specific alterations of the skeletal muscle metabolism of the host. The rate of protein biosynthesis seems to be decreased together with an increased degradation rate. The capacity for glucose assimilation and metabolism is decreased indicating a disturbed energy regulation in the muscles of cancer patients.

INTRODUCTION

PATIENTS with malignant tumor frequently present a rapid loss of body-weight, wasting of tissues, and apparently disturbed metabolic regulatory functions. The progressive catabolism and tumor cachexia cannot always be fully explained by a secondary nutritional deprivation caused by increased loss of proteins or by low caloric intake. Some other factors are probably of importance.

We have earlier reported an increased catabolic enzyme activity in liver and muscle tissue from patients with malignant tumor suggesting a changed metabolism in tissues, which were not invaded by tumor growth [1–3]. Similar observations in tumor-bearing animals have also been reported [4]. These findings indicate a tumorhost relationship of the metabolism on the molecular level.

Hydrolytic enzymes have been reported to be increased concomitantly with increased incorporation rate of amino acids into muscle proteins in experimental animals with dystrophic muscles [5, 6]. These findings imply that changes in hydrolytic enzyme activities may be of functional importance, particularly in relation to protein metabolism.

The present studies were aimed to shed further light on the relationships between lysosomal enzyme activities, the incorporation rate of leucine into proteins and the degradation rate of proteins in skeletal muscles of cancer patients. An additional aim was to investigate whether other metabolic changes of relevance for cellular energy production occur in skeletal muscles of cancer patients.

Accepted 30 December 1975.

*This work was supported by the Swedish Cancer Society (project No. 93), the Swedish Medical Research Council (project No. 536) and the Assar Gabrielsson Foundation.

MATERIAL AND METHODS

Reagents and isotopes

L-[U-^{14}C] leucine 250 mCi/mmol, puromycinhydrochloride, Sigma Chem. Corp.,

USA; D-[U-^{14}C] glucose 1–5 mCi/mmol, New England Nuclear Chemicals (West Germany). The reagents and enzymes for the enzyme assays were all from Sigma Chem. Comp. A sterile amino acid solution consisting of 19 L-amino acids with a concentration equivalent to 100 times that of human plasma was kindly supplied by AB Astra, Södertälje, Sweden.

Clinical material

The study material comprised 43 patients, 19 men aged 62 ± 3 years, and 24 women aged 63 ± 2 years. The patients were all admitted to our hospital because of diagnosed or suspected malignant tumor. In Table 1 is listed the diagnoses and other pertinent data on the patients. Normal serum levels for liver tests are defined as follows: serum bilirubin < 1·2 mg/100ml, alkaline phosphatase < 260 U, aspartate amino transferase (SASAT) < 16 U, alanine amino transferase (SALAT) < 16 U. The histological grading of the tumor cells was performed by a pathologist and was given as: high, low and anaplastic grade of differentiation. Besides the laboratory tests, the following examinations of the patients were performed prior to operation: radiography of the lungs and the bone system and, in most patients, aortography. In 13 patients anemia and a rapid weight loss the past 1–2 months were recorded. Five of these patients were in a more advanced catabolic state. However, none of them had apparent loss of body fluids and the daily caloric intake was not apparently decreased.

All patients were explored surgically, and "curative" or "palliative" resection was performed in all of them. The degree of tumor spread was judged from preoperative examinations and the findings during operation.

The control material was obtained from 55 patients admitted to the hospital for operation of uncomplicated gall-bladder disease or peptic ulcer. The mean age in the control group, which comprised 28 men and 27 women, was 56 ± 2 years (SEM). These patients revealed no signs of malignancy, and they were in caloric balance as judged from weight records. They had normal liver function according to the definition above, and none had any other disease of known importance for the study.

Muscle biopsy

In the cancer patients as well as in the controls biopsies were taken from the rectus abdominal muscle at time of the operation. The patients were fasted for 12 hr before operation and were premedicated one hour before initiation of anesthesia with diazepam and atropine. Anesthesia consisted of hexobarbital (Evipan^{R}) or penthanyl (Lepthanal^{R}), oxygen and nitrous oxide. The biopsies were taken as the first procedure during the operation and without unnecessarily touching the muscle.

Experimental procedures

The biopsy specimens were prepared and incubated as described previously [7, 8]. Muscle fibres were teased away from the specimen and collected in incubation vessels.

For determination of the incorporation rate of glucose-carbon into various metabolites, 100 mg tissue was incubated in a medium consisting of Krebs Ringer phosphate buffer (KRP), glucose 8·25 mmol/l and [^{14}C]-glucose (10^6 dis/min); pH 7·4.

For determination of the incorporation rate of leucine into muscle fibre proteins, 200 mg tissue was incubated for 4 hr in a medium consisting of Krebs Ringer bicarbonate buffer (KRB), 19 L-amino acids at a concentration corresponding to 10 times the human plasma level and [^{14}C]-leucine (10^6 dis/min); pH 7·4. After incubation the fibres were homogenized. Protein preparation, counting of radioactivity, and protein determination were performed as described earlier [8].

For determination of the fractional degradation rate of proteins and the degree of reutilization of leucine from degraded proteins three duplicate fibre specimens were radioactively prelabelled by incubation for 2 hr in KRB buffer with all amino acids at human plasma level and [^{14}C]-leucine. The specimens were then washed for 15 min in 12 ml KRB buffer at 37°C, pH 7·4 to decrease the tissue soluble radioactivity. One duplicate specimen was analysed directly for remaining total tissue soluble radioactivity and protein radioactivity to be used as references for the calculations. The two other duplicate specimens were incubated for further two hours in an unlabelled medium consisting of KRB buffer and all amino acids at plasma level. In one of these duplicates puromycin was added to the medium (125 μg/ml). The soluble radioactivity in the incubation medium and the total tissue soluble radioactivity were determined as described previously [9]. The sum of this radioactivity was given per unit protein and incubation time (dis/min × hr^{-1} × mg $prot.^{-1}$). The

Table 1. *Data of patients with malignant tumor*

Diagnosis	Number	Liver serum tests		Grade of differentiation			No spread	Tumor spread	
		Normal	Not normal	High	Low	Undiff.		Regional lymph nodes	Generalized
Oesophageal carcinoma	3	2	1	1	1	1		3	
Gastric carcinoma	11	8	3	3	7	1	2	8	1
Gastric leiomyoblastoma	1	1			1		1		
Pancreatic carcinoma	6		6	1	5			4	2
Choledochal carcinoma	1		1		1		1		
Gall-bladder carcinoma	1	1		1				1	
Colon carcinoma	4	3	1	2	2		2	2	
Rectal carcinoma	3	3		2	1		2	1	
Renal carcinoma	4	4		3	1		3	1	
Liver melanocarcinoma	1		1	1					1
Liposarcoma	1		1		1			1	
Malignant cyst adenoma of the ovary	1		1		1				1
Undiff. carcinoma of uncertain origin	3		3			3			3
Hepatic carcinoma	3		3	2	1		1	1	1

soluble radioactivity in the medium plus the total tissue soluble radioactivity minus the total tissue soluble radioactivity of the reference specimens constituted the released radioactivity from proteins. This value thus gave the degradation rate of prelabelled proteins (dis/min × hr^{-1}).

The degree of reutilized leucine from proteolysis was calculated from the released radioactivity in the incubations with and without puromycin. The validity of this technique for determination of the degradation rate is supported by our previous finding that puromycin *per se* does not influence the rate of leucine release from proteins or the oxidation and rate of metabolism of leucine [9]. The fractional degradation rate (V% × hr^{-1}) could be calculated from the ratio between the absolute degradation rate and the radioactivity of the proteins in the reference specimen.

Determination of RNA

RNA was determined according to Munro & Fleck [10], slightly modified for muscle tissue. Immediately after excision the muscle tissue was frozen in liquid nitrogen and homogenized in ice-chilled saline (1:20 w/v). The extraction of RNA was performed with 0·3 M potassium hydroxide during incubation at 37°C for 60 min. The RNA was determined as the absorbance of 260 nm in a spectrophotometer, Beckman DU2. Every sample was also read at 232 nm to check for nonspecific absorbance.

Counting of radioactivity

The radioactivity was counted in a Packard-Tri-Carb (3320) liquid scintillation spectrometer. Correction for quenching was performed by the external standard method. Protein samples were counted in toluene, PPO-POPOP and aqueous solutions in Insta-GEL^{R} (Packard Instrument Comp., USA).

Enzyme assay methods

The muscle specimens were rinsed, cut with scissors and homogenized in an all-glass homogenizer, immersed in redistilled ice-water. For measurement of phosphofructokinase (EC 2.7.1.11), the homogenate was prepared in 10 vol. (w/v) of tris–HCl buffer (50 umol/l), pH 8·2, containing EDTA (1 mmol/l) and magnesium sulphate $MgSO_4$ (5 mmol/l). The homogenates for assay of all other nonlysosomal enzymes were prepared in 10 vol. of disodium/monopotassium phosphate buffer (100 mmol/l), pH 7·2, containing EDTA (2 mmol/l). The homogenates were centrifuged at 130 × 10^3 × *g* for 30 min and the supernatant fraction was used for the enzyme assays, except for cytochrome-c-oxidase (EC 1.9.3.1), which activities were analysed in the homogenate fraction. For determination of 3-OH-acyl-CoA dehydrogenase (EC 1.1.1.35) Triton-X 100 was added to the homogenate one hour before centrifugation at 130 × 10^3 × *g*. Spectrophotometric assays were performed in a Beckman model DU2 spectrophotometer at 25°C and 340 nm. Glycogen phosphorylase (EC 2.4.1.1), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27) and 3-OH-acyl-CoA dehydrogenase activities were determined according to Bass *et al.* [11]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was determined according to Noltman *et al.* [12]. Phosphofructokinase activity was determined according to Mansour *et al.* [13] as modified by Opie *et al.* [14]. Cytochrome-c-oxidase activity was estimated polarographically as oxygen consumption, with a Clark-oxygen electrode according to the method described by Tottmar *et al.* [15].

The tissue specimens for determination of lysosomal enzyme activities were homogenized in the presence of Triton-X 100 as described earlier [16]. Cathepsin-D (EC 3.4.4.23) was determined according to Anson [17]. Acid phosphatase (EC 3.1.3.2) was determined according to Vaughan *et al.* [18] and beta-glucoronidase (EC 3.2.1.31) according to Greenberg [19].

Statistical methods

In the comparison between different groups, the significance was checked with t-test. Linear regression was calculated by the method of least squares, and standard procedures were used to calculate correlation coefficients. T-test was supplied to test the significance of the regression coefficients.

RESULTS

Enzyme activities

The patients with malignant tumor had significantly increased cathepsin-D and β-glucuronidase activities in muscle tissue (Table 2). Acid phosphatase activity and the soluble protein concentration were the same in skeletal muscle tissue from cancer patients and controls.

Table 2. Cathepsin-D-, β-glucuronidase-, acid phosphatase activity and soluble protein concentrations in muscle tissue from patients with malignant tumor and controls. Mean ± SEM

	Cathepsin-D (μmol tyrosin × min^{-1} × g prot^{-1})	β-glucuronidase (μmol × hr^{-1} × g prot^{-1})	Acid phosphatase (μmol × hr^{-1} × g prot^{-1})	Soluble protein (g/l)
Cancer	3·54 ± 0·25 [36]	11·63 ± 0·99 [13]	34·4 ± 3·2 [15]	3·81 ± 0·08 [36]
Controls	2·40 ± 0·17 [55]	7·19 ± 0·35 [15]	31·4 ± 2·6 [22]	3·93 ± 0·09 [55]
	$P < 0·0005$	$P < 0·0005$	n.s.	n.s.

The results in Table 3 show that the cancer patients had significantly lower activities of hexokinase ($P < 0·0125$), phosphofructokinase ($P < 0·0025$), lactate dehydrogenase ($P < 0·0005$) and cytochrome-c-oxidase ($P < 0·025$). A trend to lower activities of glycogen phosphorylase ($P < 0·15$) and glucose-6-phosphate dehydrogenase ($P < 0·25$) was also found. 3-OH-acyl-CoA dehydrogenase was almost the same in the both groups.

Incorporation rate of leucine into proteins and glucose-carbon into various metabolites

The incorporation rate of leucine into muscle proteins was significantly decreased and the fractional degradation rate of previously labelled proteins was significantly increased in the cancer patients (Table 4). The degree of reutilization of leucine from proteolysed radioactive proteins was not significantly different in the cancer patients as compared with controls.

The RNA concentration in muscle tissue was the same in cancer patients and controls (Table 5). In Table 5 is also shown that the degree of stimulation by amino acids on the incorporation rate of leucine into skeletal muscle proteins was the same in cancer patients and controls.

The incorporation rate of glucose-carbon into muscle glycogen, lipids, lactate and CO_2 is shown in Table 6. In the cancer patients the incorporation rate of glucose-carbon into glycogen, lactate and CO_2 was significantly decreased ($P < 0·0025$, $P < 0·0125$, $P < 0·05$ respectively).

Relationships between enzyme activities, protein degradation rate and incorporation rate of leucine into muscle proteins

The cathepsin-D and β-glucuronidase activities in muscle tissue of cancer patients were significantly correlated to each other ($r = 0·74$, $P < 0·0005$; Fig. 1). This correlation was not found in the control group. As illustrated in Fig. 2 the slopes of the regression lines between the relationship between the cathepsin-D activity and the incorporation rate of leucine into muscle proteins in cancer patients and controls were significantly different ($P < 0·005$).

Table 3. Different glycolytic and mitochondrial enzymes in skeletal muscle tissue from cancer patients and controls. For enzyme determination see "Experimental procedures". Mean ± SEM. Number of patients within the brackets

	Glycogen phosphorylase	Hexokinase	Glucose-6-phosphate dehydrogenase	Phosphofructokinase
	(μmol NADPH × min^{-1} × g^{-1} wet weight)		(μmol NADH × min^{-1} × g^{-1} wet weight)	
Cancer	3·92 ± 0·52 [13]	1·79 ± 0·13 [13]	0·095 ± 0·016 [8]	28·6 ± 2·3 [13]
Controls	4·83 ± 0·62 [12]	2·31 ± 0·16 [12]	0·147 ± 0·069 [12]	42·3 ± 3·6 [12]
	$P < 0·15$	$P < 0·0125$	$P < 0·25$	$P < 0·0025$

	Lactate dehydrogenase	3-OH-acyl-CoA dehydrogenase	Cytochrome-c-oxidose
	(μmol NADH × min^{-1} × g^{-1} wet weight)		(μmol O_2 × min^{-1} × g^{-1} wet weight)
Cancer	74·6 ± 9·0 [13]	3·24 ± 0·32 [13]	1·48 ± 0·17 [13]
Controls	188·0 ± 16·0 [13]	3·17 ± 0·32 [15]	2·01 ± 0·18 [19]
	$P < 0·0005$	n.s.	$P < 0·025$

Table 4. Incorporation rate of leucine into skeletal muscle proteins, fractional degradation rate of proteins and the degree of reutilization of leucine from degraded proteins, in muscle tissue from cancer patients and controls in vitro. *Mean ± SEM*

	Incorporation rate of leucine (μmol × hr^{-1} × g prot^{-1})	Fractional degradation rate of radioactive protein (V% × hr^{-1})	Degree of reutilized radioactivity from degraded proteins (%)
Cancer	0·197 ± 0·016 [20]	33·1 ± 5·0 [18]	10·4 ± 2·5 [13]
Controls	0·230 ± 0·012 [40]	21·4 ± 2·8 [16]	8·6 ± 1·8 [14]
	$P < 0·05$	$P < 0·025$	n.s.

Table 5. Total RNA content of muscle tissue and the degree of stimulation on the incorporation rate of leucine into muscle proteins by increased amino acid concentration in the medium (See "Material and Methods"). Mean ± SEM

	RNA content of muscle tissue ($\Delta\lambda$ 260 absorbance × 0·25 wet weight^{-1})	The degree of stimulation on the incorporation rate of leucine into muscle proteins by all amino acids at 10 times plasma level compared to that of plasma level (%)
Cancer	167 ± 9 [12]	74·4 ± 1·8 [15]
Controls	174 ± 12 [14]	73·3 ± 2·4 [17]
	n.s.	n.s.

Table 6. The incorporation rate of glucose-carbon into various metabolites of skeletal muscle tissue. The muscle fibres were incubated in KRP buffer (pH 7·4) with glucose 8·25 mmol/l and D-^{14}C-glucose. See "Experimental procedures". Mean ± SEM

	Glycogen	Lipids	Lactate	CO_2
	(μmol × hr^{-1} × g^{-1} wet weight)			
Cancer	0·066 ± 0·015 [11]	0·034 ± 0·010 [11]	4·66 ± 0·25 [11]	0·231 ± 0·034 [11]
Controls	0·166 ± 0·024 [16]	0·031 ± 0·004 [16]	6·12 ± 0·54 [15]	0·313 ± 0·020 [15]
	$P < 0·0025$	n.s.	$P < 0·0125$	$P < 0·05$

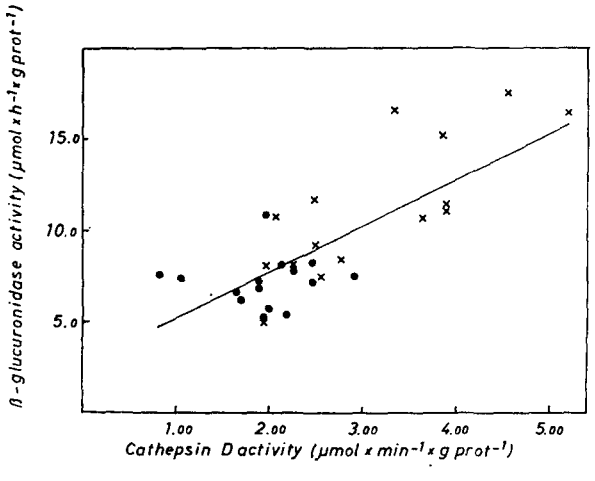


Fig. 1. The relationship between cathepsin-D and β-glucuronidase activities in skeletal muscle tissue from cancer patients (x) and in controls. (●). In the cancer patients these activities were significantly correlated (r = 0·74; P< 0·0005) *and the observations fitted the equation* Y = 2·66X+ 2·59. *No correlation was found in the controls.*

The proteolytic capacity as represented by the cathepsin-D activity in muscle tissue and the degradation rate of muscle proteins were significantly correlated (Fig. 3) ($r = 0·59$, $P < 0·025$).

DISCUSSION

The influence of malignant tumors on the host tissues is incompletely known. Recently we reported increased lysosomal enzyme activities in liver and muscle tissue in patients with malignant tumor [1–3]. These increased enzyme activities could be a sign of an unspecific effect on the protein metabolism. However, the present results showing increased cathepsin-D and β-glucuronidase activities concomitantly with decreased activities of glycolytic and oxidative enzymes in skeletal muscles from

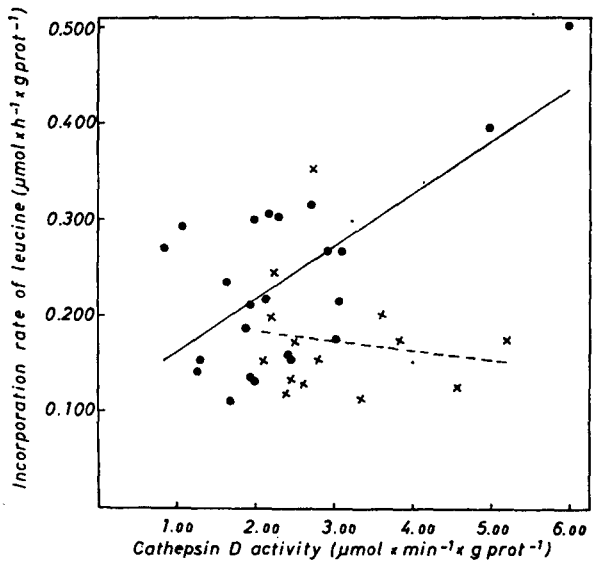


Fig. 2. The relationship between cathepsin-D activity and the incorporation rate of leucine into muscle proteins in cancer patients (x) and in controls (●). The observations in cancer patients fitted the equation Y = 0·01X + 0·20 (r = −0·13) *and those in controls the equation* Y = 0·06X + 0·10 (r = 0·69). *The slopes of the regression lines were significantly different* (P < 0·005).

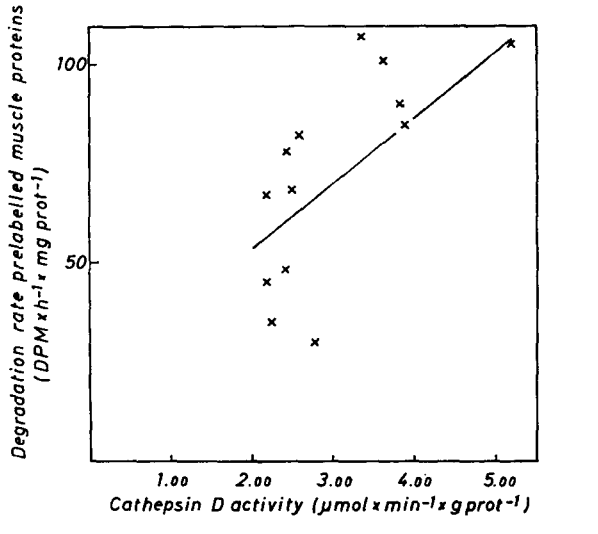


Fig. 3. The relationship between cathepsin-D activity and the proteolytic rate of skeletal muscles in cancer patients. The observations fitted the equation Y = 17·6X + 17·6 (r = 0·59; P < 0·025).

cancer patients point to the possibility of more specific effects provoked by the malignant tumor. The finding of increased cathepsin-D and β-glucuronidase activity but unchanged activity of acid phosphatase suggests a differential effect also on lysosomal enzymes. The significant correlation between cathepsin-D activity and β-glucuronidase activity, which was found in the cancer patients but not in the controls, may be taken as an indication for a common induction mechanism for these enzymes.

In experimental animals and humans with dystrophic muscles it has been shown that there is an increased muscle-cathepsin activity associated with increased incorporation rate of amino acids into muscle proteins [4, 6]. Increased amino acid incorporation rate into skeletal muscle proteins was recently also found in immobilized patients [20]. Our cancer patients showed another pattern. They had increased muscle cathepsin-D activity but decreased incorporation rate of leucine into proteins and maintained RNA concentration in the muscles. In this respect the pattern also differed from that in debilitation because of starvation [21, 22]. The decreased leucine incorporation rate into proteins suggests an impaired protein biosynthetic capacity in muscle tissues in malignancy. The mechanism for this impairment of protein biosynthesis in the cancer patients cannot be explained by the results. However, the fact that it was possible to stimulate the incorporation rate of leucine by amino acids to the same degree in cancer patients as in the controls argues against a defect of initiation, elongation and termination of peptides.

The participation of lysosomal enzymes in the intracellular breakdown of macromolecules is well documented, but their role in the regulation of the balance between anabolic and catabolic reactions is not known. The patophysiological significance of the increased activity of hydrolytic enzymes in muscle tissue in our cancer patients cannot be decided. However, the concomitant increase of cathepsin-D activity and fractional degradation rate of muscle proteins in cancer patients provides at least circumstantial evidence for a contributory role of these enzymes in the development of cachexia in cancer disease. This was further strengthened by the finding of a significant positive correlation between cathepsin-D activity in muscle tissue and degradation rate of proteins (Fig. 3).

Although the present material of cancer patients was heterogenous no clear cut correlation was found between the degree of debilitation and the metabolic parameters in the muscles. This may support the assumption that the metabolic changes in the muscles are preceding phenomenons to the clinical cachexia. On the other hand in one patient with liposarcoma we had the opportunity to repeat the determinations of enzyme activities and the incorporation rate of leucine into muscle proteins within 12 weeks after the primary

operation. At the second exploration it was found that the tumor was wide-spread and far beyond surgical cure. The cathepsin-D activity in muscle tissue had increased threefold and the incorporation rate of leucine had decreased to about one third during this time. The patient was extremely cachectic and died within two weeks. The observation in this patient together with the other results support the hypothesis that cancer cachexia is characterized, among other things, by a decreased protein biosynthetic capacity as well as by an increased protein catabolism in skeletal muscles. The significantly changed relationship between muscle-cathepsin-D activity and the incorporation rate of leucine into muscle proteins (Fig. 2) illustrates this disturbed balance between anabolism and catabolism.

The decreased activities of hexokinase, phosphofructokinase (the rate limiting step in glycolysis), lactate dehydrogenase and cytochrome-c-oxidase, as well as the decreased incorporation rate of glucose-carbon into glycogen, lactate and CO_2 in skeletal muscles from cancer patients, indicate an impairment of the ability to utilize glucose and also a disturbed energy regulation in the muscles of cancer patients [23, 24]. These results support also the hypothesis that the increased lactate concentration in plasma from tumor-bearing animals [25] is dependent on an increased glycolysis in the tumor tissue [26]. Recently there has been shown a positive correlation between the *in vitro* capacity of glucose uptake and utilization in human skeletal muscle tissue and insulin sensitivity of the patient [27, 28]. In view of that it seems reasonable to assume a decreased insulin sensitivity in cancer patients. Actually, we have preliminary results supporting this hypothesis.

Acknowledgements—The skilful technical assistance of Mrs. Barbro Jilderos, Mrs. Ingela Jonson, Mrs. Margareta Jonsson and Miss Ingela Ostrand is gratefully acknowledged.

REFERENCES

1. T. Scherstén, L. Wahlqvist and L.-G. Johansson, Lysosomal enzyme activity in liver tissue from patients with renal carcinoma. *Cancer (Philad.)* **23,** 608 (1969).
2. T. Scherstén, L. Wahlqvist and Barbro Jilderos, Lysosomal enzyme activity in liver tissue, kidney tissue, and tumor tissue from patients with renal carcinoma. *Cancer (Philad.)* **27,** 278 (1972).
3. T. Scherstén and K. Lundholm, Lyosomal enzyme activity in muscle tissue from patients with malignant tumor. *Cancer* **30,** 1246 (1972).
4. R. J. Shamberger, M. Hozumi and H. P. Morris, Lyosomsal and non-lysosomal enzyme activities of Morris hepatomas. *Cancer Res.* **31,** 1632 (1971).
5. G. Monckton and T. Nihei, A correlation of histology and amino acid incorporation studies in Duchenne muscular dystrophy. *Neurology* **19,** 415 (1969).
6. G. E. Nichoalds, J. F. Diehl and C. D. Fitch, Vitamin E deficiency and metabolism of amino acids in skeletal muscle. *Amer. J. Physiol.* **213,** 3, 759 (1967).
7. J. Holm and T. Scherstén, *In vitro* metabolism of glucose by human skeletal muscle. Method and normal values. *Scand. J. Clin. Lab. Invest.* **29,** 99 (1972).
8. K. Lundholm, A-C. Bylund, J. Holm, S. Smeds and T. Scherstén, Metabolic studies in human skeletal muscle tissue. Evaluation of an *in vitro* preparation intended for clinical metabolic studies. *Europ. surg. Res.* **7,** 65 (1975).
9. K. Lundholm and T. Scherstén, *In vitro* determination of protein synthesis and degradation rate in human skeletal muscle tissue. *Europ. J. Biochem.* **60,** 181 (1975).
10. H. N. Munro and A. A. Fleck, Determination of nucleic acids. In *Methods of Biochemical Analyses.* (Edited by D. Glick) Vol. 14, p. 113. Interscience Publ. John Wiley, N.Y., (1966).
11. A. Bass, D. Brdiczka, P. Eyer, S. Haufer and D. Pette, Metabolic differentiation of distinct muscle types of the level of enzymatic organization. *Europ. J. Biochem.* **10,** 198 (1969).
12. E. A. Noltman, C. J. Gobler and S. A. Kuby, Glucose-6-phosphate dehydrogenase. 1. Isolation of the crystalline enzyme from yeast. *J. biol. Chem.* **236,** 1225 (1961).
13. T. E. Mansour, Studies on heart phosphofructokinase. *J. biol. Chem.* **240,** 2166 (1965).

14. L. H. Opie and E. A. Newsholme, The activities of fructose 1,6-diphosphatase, phosphofructokinase and phosphorynolpyruvate-carboxykinase in white muscle and red muscle. *Biochem. J.* **103,** 391 (1967).
15. S. O. C. Tottmar, H. Pettersson and K. H. Kiessling, The Subcellular distribution and properties of aldehyde dehydrogenase in rat liver. *Biochem. J.* **135,** 577 (1973).
16. K. Lundholm and T. Scherstén, Leucine incorporation into proteins and cathepsin-D activity in human skeletal muscles. *Exp. Geront.* **10,** 155 (1975).
17. M. L. Anson, The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. gen. Physiol.* **22,** 79 (1938).
18. A. Vaughan, G. G. Guilbault and D. Hackney, Fluorometric methods for analysis of acid and alkaline phosphatase. *Anal. Chem.* **43,** 721 (1971).
19. L. J. Greenberg, The use of 2-naphtyl β-D-glucuronide as a substrate for the fluorometric analysis of β-glucuronidase. *Anal. Biochem.* **14,** 265 (1966).
20. A-C. Bylund, J. Holm, K. Lundholm and T. Scherstén, Skeletal muscle metabolism in tetanus. *Acta Chir. Scand.* In press (1976).
21. V. R. Young, S. C. Stothers and G. Vilaire, Synthesis and degradation of mixed proteins, and composition changes in skeletal muscle of malnourished and refed rats. *J. Nutr.* **101,** 1379 (1971).
22. V. R. Young, *Mammalian Protein Metabolism* (Edited by H. N. Munro) Vol. IV, p. 621. Academic Press, New York and London (1970).
23. C. Waterhouse, How tumors affect host metabolism. *Ann. N.Y. Acad. Sci.* **230,** 86 (1974).
24. C. Waterhouse and J. H. Kemperman, Carbohydrate metabolism in subjects with cancer. *Cancer Res.* **31,** 1273 (1971).
24. C. F. Cori and G. T. Cori, The carbohydrate metabolism of tumors—II. Changes in the sugar, lactic acid, and CO_2-combining power of the blood passing through a tumor. *J. biol. Chem.* **65,** 397 (1925).
26. J. Gold, Proposed treatment of cancer by inhibition of gluconeogenesis. *Oncology* **22,** 185 (1968).
27. J. Holm, A-G. Dahllöf, P. Björntorp and T. Scherstén, Glucose tolerance, plasma insulin and lipids in intermittent claudication with reference to muscle metabolism. *Metabolism* **22,** 1395 (1973).
28. T. Scherstén, P. Björntorp and J. Holm, Glucose metabolism and insulin sensitivity in patients with intermittent claudication. Effects of physical conditioning and arterial reconstruction. Abstract, *Europ. J. clin. Invest.* **4,** 370 (1974).

Europ. J. Cancer Vol. 12, 475–479. Pergamon Press 1976. Printed in Great Britain

Increase in Cell Surface Antigen Expression by Leukemic Cells Following *In Vitro* Treatment with Papain*

A. COUDERT†, J. F. DORE†§, R. HUCHET†, C. GUIBOUT†¶ and C. SANTANA†

†*Institut de Cancérologie et d'Immunogénétique (INSERM)*, 14–16, *avenue Paul-Vaillant Couturier*, 94800-*Villejuif, France, and Centre Léon-Bérard*, 28, *rue Laënnec*, 69008-*Lyon, France*

Abstract—*A variety of enzymes have been used to increase the immunogenicity of tumor cells in syngeneic hosts, including neuraminidase, trypsin and papain. The results of the investigation presented here suggest that, under defined conditions,* in vitro *treatment by papain of living cells from a Gross virus induced leukemia results in a quantitative modification of the antigens expressed at the cell surface without any detectable qualitative modification. Cells from the W/Fu C58(NT)D leukemia (leukemia induced in W/Fu rats by the Gross agent) were submitted to a moderate* in vitro *treatment by papain. Antisera were raised in syngeneic W/Fu rats immunized with W/Fu C58 (NT)D cells, either untreated (G-typing serum) or papain treated. The cytotoxic activity of both of these types of antisera was assessed on W/Fu C58 (NT)D cells, untreated or papain treated. Target cells treated with papain showed an increased susceptibility to lysis by antibody in the presence of complement. Cross-absorption studies showed that the activity towards papain-treated target cells could be absorbed out with untreated W/Fu C58(NT)D cells and was not affected by absorption with papain-treated normal lymphocytes.*

Hence papain treatment appears to induce an increase in antigen expression at the cell surface, rather than the unmasking of "cryptantigens", and this could be of interest in producing antibodies against weak antigens.

INTRODUCTION

DURING the past few years several attempts have been made to increase tumor antigenicity by means of enzymatic treatment. Neuraminidase was the principal agent used to remove sialic acid which is considered the major masking component of tumor specific antigens at the cell surface [1–5]. Although the action of neuraminidase was found to be dependent upon its origin [6] and upon the nature of the link between sialic acid and the glycocalyx, it has been reported that such a treatment could significantly increase tumor antigenicity [7,10]. It has been shown with chemically-induced tumors, that treated tumor cells given to an animal prior to non treated cells prevented lethal tumor growth [11]. On the other hand, unsuccessful attempts have been reported [12]. Recently it was reported that trypsin treatment increases Gross virus induced antigen sites in rat lymphoma cells [13] and that papain treatment of murine leukemia cells increased tumor cell antigenicity when measured by quantitative absorption of specific antisera [14]. All previous work has demonstrated that enzymatic treatment of tumor cells may result in an increase of their immunogenicity or antigenicity. But it was not shown whether the new antigenic sites were similar to those existing before the treatment.

In this report, an analysis of the antigens expressed by Gross virus-induced rat leukemia cells treated with papain was made, using absorption of specific antisera. The evidence indicates that papain treatment does not unmask cryptantigens but increases the expression of preexisting antigenic sites.

Accepted 29 December 1975.

*Supported by INSERM, grant 74.5.014.2.

§Maitre de Recherche (INSERM) to whom correspondence may be addressed.

¶Fellow of the Lady Tata Memorial Trust.

MATERIAL AND METHODS

Animals

W/Fu rats, originally provided by Dr. J. C. Leclerc, (Hôpital Cochin, Paris), were bred at the "Institut de Cancérologie et d'Immunogénétique".

Tumor cells

W/Fu C58(NT)D leukemia, a Gross virus-induced leukemia of W/Fu rats [15], kindly provided by Dr. R. B. Herberman (National Cancer Institute, Bethesda) was used. The leukemia was serially transplanted as an ascites by intraperitoneal inoculation of $2 \cdot 10^7$ viable cells into weanling W/Fu rats. Cells to be used for immunization or tests were harvested from the animals 7 days after transplantation and were washed three times in 0·15 M NaCl before use.

Rat spleen cells

Normal spleen cells were obtained from W/Fu rats by mincing the spleen in Hank's Balanced Salt Solution (BSS). The cell suspension was filtered through a gauze and washed three times in Hank's BSS before use.

Papain treatment

Papain solution was prepared extemporaneously by dissolving crude papain (750 I.E.U./g, Laboratoires Trouette–Perret, 93-Aubervilliers, France) in 0·15 M NaCl to give a final concentration of 0·1 mg/ml. Ten million viable cells were added to each ml of papain solution and the mixture (0·1 mg papain/10^7 cells/ml) was incubated for 10 min at room temperature. The cells were then washed 3 times in 0·15 M NaCl.

Antisera

Antisera to C58 (NT)D leukemia cells were raised in syngeneic W/Fu adult rats by repeated subcutaneous injections of viable leukemia cells untreated or papain treated. Two antisera were produced:

(a) S27: W/Fu rats were given 150×10^6 C58(NT)D cells subcutaneously, followed one month later by 8 weekly injections of the same number of cells.

(b) Sp: a second antiserum was obtained with papain treated cells using the same protocol.

Usually, small tumors were produced, but regressed spontaneously.

Antiserum to SRBC

An antiserum to sheep red blood cells (SRBC) was obtained in W/Fu adult rats by 4 i.p. injections of 1 ml of a suspension of SRBC at a concentration of 15%. The rats were bled 7 days after the last injection.

Cytotoxicty tests

Trypan Blue exclusion tests were performed in 3 ml plastic test tubes. Five-hundredths/ml aliquots of antiserum or control serum dilution, cell suspension (4.10^6/ml) and rabbit complement (diluted 1:10, as determined by previous titration) were incubated at 37°C for 45 min. Hank's BSS was used as diluent. The tubes were then transferred into an ice-bath and 0·05 ml Trypan Blue solution was added to each tube. Trypan Blue solution was extemporaneously prepared by mixing 4 vol of a 0·2% Trypan Blue aqueous solution with 1 vol 4·25% NaCl. The percentage of dead cells in each tube was counted under the microscope. Untreated and papain-treated C58(NT)D cells were used as target cells. A cytotoxicity index (A–B/100–B was calculated where A is the percentage of killed cells in the test, and B the percentage of dead cells in the control.

Absorption experiments

C58(NT)D leukemia cells and normal W/Fu rat spleen cells were prepared as described above. One-tenth/ml aliquots of antiserum dilution were incubated for 45 min at room temperature with increasing number of untreated or papain-treated cells ($1–50.10^6$ cells), and then centrifuged. The residual cytotoxic activity of the supernatant was tested on both untreated and papain-treated C58(NT)D leukemia cells.

RESULTS

Cytotoxic activity of antisera

The titration curves obtained with S27 and Sp on both untreated and papain-treated C58(NT)D target cells are shown on Fig. 1.

Antiserum Sp, raised against papain-treated C58(NT)D leukemia cells was found to have a greater cytotoxic activity than antiserum S27, raised against untreated C58(NT)D cells.

The cytotoxicity activity of both S27 and Sp was found to be greater when assayed against papain-treated target cells.

Cross-absorption of antisera

In order to determine whether different antigenic specificities were revealed by the papain-treatment of the leukemia cells used for immunization, the two sera were absorbed

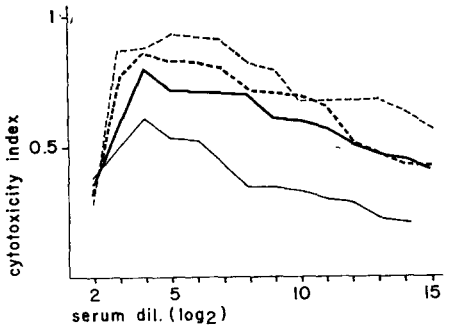


Fig. 1. *Cytotoxic activity of S27 and Sp antisera. S27, raised in syngeneic W/Fu rats with C58(NT)D leukemia cells, was titrated against untreated (——) or papain-treated C58(NT)D (——) cells. Sp, raised in a similar manner with papain-treated C58(NT)D cells, was titrated against untreated (- - -) or papain treated (- - -) C58(NT)D leukemia cells.*

in vitro with increasing numbers (1, 2, 5, 10, 30 and 50.10^6) of untreated and papain-treated C58(NT)D leukemia cells, and their residual cytotoxic activity was determined using untreated and papain-treated target cells.

Antiserum S27 was diluted 1:50 prior to absorption, as determined from the titration curve shown in Fig. 1. It can be seen from Fig. 2 drawn from a typical experiment, that

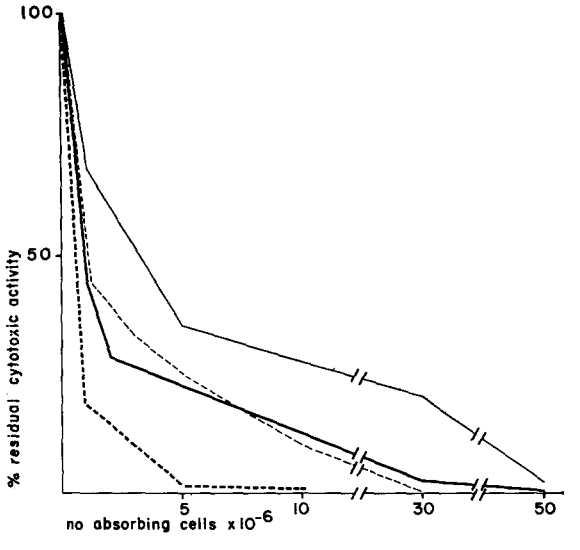


Fig. 2. In vitro *absorption of S27 antiserum, S27 was diluted* 1:50 *and absorbed with C58(NT)D leukemia cells either untreated (solid lines) or papain-treated (dotted lines).*

Percentage of residual cytotoxic activity was determined against C58(NT)D leukemia cells, untreated (thick lines) or papain-treated (thin lines).

the cytotoxic activity of S27 was more rapidly absorbed out with papain-treated leukemia cells than with untreated cells. Furthermore, the use of papain-treated target cells tended to delay the absorption of S27 antiserum, thus increasing the number of untreated C58(NT)D cells needed for the complete absorption of 0·1 ml antiserum.

Antiserum Sp was diluted 1:500 prior to absorption, and absorption studies, shown in Fig. 3, were quite similar to those given above

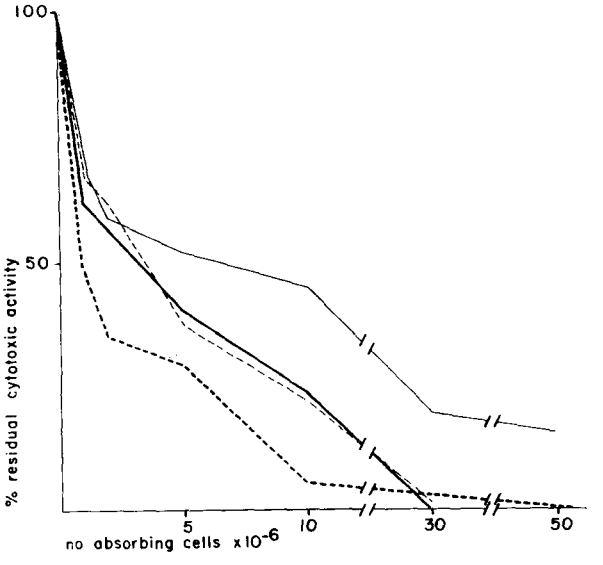


Fig. 3. In vitro *absorption of Sp antiserum. Sp was diluted* 1:500 *and absorbed with C58(NT)D leukemia cells either untreated (solid lines) or papain-treated (dotted lines), and the percentage of residual cytotoxic activity was determined on both untreated (thick lines) and papain-treated C58(NT)D leukemia cells (thin lines).*

for antiserum S27. It is worthy to notice that, although the number of untreated C58(NT)D cells required for the absorption of the cytotoxic activity of 0·1 ml antiserum tested against papain-treated target cells, was high, the cytotoxic activity of an antiserum raised against papain-treated leukemia cells and assayed with papain treated target cells could be absorbed out using untreated leukemia cells.

Specificity controls

The specificity of the S27 and Sp antisera was tested on normal W/Fu rat spleen cells, treated or not with papain. None of the two antisera was cytotoxic for normal rat spleen cells, even when the target cells had previously been treated with papain. (Table 1).

Table 1. *Cytotoxicity index of S27 and Sp antisera on normal W/Fu rat spleen cells*

Antisera	Target cells	
	Untreated	Papain treated
S27 (1/2)	0·01*	< 0·01
Sp (1/2)	0·01	0·01

*Cytotoxicity index.

The specificity of the absorption studies was checked in the following manner. A W/Fu hyperimmune serum anti sheep red cells was absorbed as described above with untreated or papain-treated C58(NT)D leukemia cells, and the hemagglutination titers of the unabsorbed serum were determined. No modification of the titer was observed following absorption with leukemia cells, even when those cells were papain-treated. (Table 2).

Table 2. Absorption of A W/Fu serum anti-SRBC with C58 (NT) D cells untreated or papain treated

	Hemagglutination titer Absorbed with	
Unabsorbed	Untreated C58(NT)D cells	Papain-treated C58(NT)D cells
1/640	1/640	1/640

DISCUSSION

Several reports have clearly shown that *in vitro* treatment of cells from leukemias [2,7,10, 14] or solid tumors [8,12] with neuraminidase or Trypsine [13] increases their antigenicity or immunogenicity. Such an effect is not restricted to tumor antigens since it has also been reported with transplantation antigens [9]. In a preliminary report, we showed that a mild *in vitro* treatment with papain of cells from a murine leukemia induced by the Gross agent was able to increase the expression of Gross virus-induced cell surface antigens [14]. However, *in vivo* immunotherapy experiments based on the use of enzyme-treated leukemia cells failed to give positive results [12]. Therefore, the present work was undertaken to provide a further insight in the mode of action of papain treatment on the expression of tumor associated cell surface antigens.

The data presented here tend to indicate that a mild papain treatment of rat leukemia cells increases the expression of cell surface antigens.

The cytotoxic activity of syngeneic antibodies to rat leukemia cells is increased when determined in an *in vitro* cytotoxic antibody test using papain treated target cells. Such an effect could be attributed either to a non specific increase in the susceptibility of the cells to complement-mediated lysis, to a redistribution of antigenic determinants at the cell surface thus allowing more complement to be activated, or to an increase in the number of antigenic sites exposed at the cell surface.

It is rather unlikely that a non specific increase in susceptibility to lysis of the target cells may occur, since papain treatment, at the dosage and incubation time used, has virtually no effect on cell viability [14] and since the cytotoxic activities shown in Fig. 1 are expressed in an index form taking in account cell death in the controls. The data obtained in the absorption experiments support the view that a greater number of antigenic determinants are expressed at the surface of papain-treated cells: the cytotoxic activity against untreated cells of both the antisera may be absorbed out with a smaller number of leukemia cells when papain treated cells are used for absorption. Such an effect can be considered as a specific one, since papain-treated leukemia cells are unable to affect the activity of an unrelated antibody. So, the evidence greatly favours the view that papain-treatment increases the number of antigenic sites at the cell surface. However, it is not known whether such an increased antigenicity is due to an unmasking of hidden sites or to a rearrangement of sites at the cell surface increasing the multivalency of the antigen [13]. Furthermore, the additional antigenic determinants revealed by papain treatment appear to be identical to those present at the cell surface before the treatment, since the activity against papain-treated target cells of an antiserum raised with papain-treated leukemia cells could be absorbed out with untreated leukemia cells.

Antisera raised in syngeneic rats with papain-treated C58(NT)D cells were usually found to have a higher cytotoxic activity than antisera raised in a similar manner with untreated C58(NT)D leukemia cells. Whether this increase in the immunogenicity of the tumor cells relies upon an increase in the antigenic expression is not known, and it is not unlikely that the enzyme may strengthen the tumoral antigen, considered as a hapten, by unmasking carrier determinants.

From the above discussed evidence, it can be concluded that papain treatment by leukemia cells results in a quantitative modification of the tumor antigens expressed at the cell surface, without any detectable qualitative modification and that the immunogenicity of the tumor cell is increased. This may prove to be useful in detecting weak tumor antigens or in raising potent antisera specific for tumor-associated cell surface antigens.

Acknowledgements—We wish to thank Dr. R. Motta for helpful discussion.

REFERENCES

1. J. F. Codington, B. H. Sandford and R. W. Jeanloz, Glycoprotein coat of the TA3 cell. I. Removal of carbohydrate and protein material from viable cells. *J. nat. Cancer Inst.* **45,** 637 (1970).
2. G. A. Currie, Masking of antigens in the Landschütz ascites tumor. *Lancet* **ii,** 1336 (1967).
3. G. A. Currie and K. D. Bagshawe, The role of sialic acid in antigenic expression: further studies of the Landschütz ascites tumor. *Brit. J. Cancer* **22,** 843 (1968).
4. W. P. van Beck, L. A. Smets and P. Emmelot, Increased sialic acid density in surface glycoprotein of transformed and malignant cells. A general phenomenon? *Cancer Res.* **33,** 2913 (1973).
5. L. Weiss, The topography of cell surface sialic acids and their possible relationship to specific cell interaction. *Behring Inst. Mitt.* **55,** 185 (1974).
6. R. Drzeniek, Substrate specificity of neuraminidases. *Histochem. J.* **5,** 271 (1973).
7. J. G. Bekesi, J. P. Roboz, L. Walter and J. F. Holland, Stimulation of specific immunity against cancer by neuraminidase treated tumor cell. *Behring Inst. Mitt.* **55,** 309 (1974).
8. R. L. Simmons, A. Rios, G. Lundgren, P. K. Ray, C. F. McKhann and G. R. Haywood, Immunospecific regression of methylcholanthrene fibrosarcoma with the use of neuraminidase. *Surgery* **70,** 38 (1971).
9. B. H. Sandford, An alteration in tumor histocompatibility induced by neuraminidase. *Transplantation* **5,** 1273 (1967).
10. J. G. Bekesi, G. St. Arneault, L. Walter and J. F. Holland, Immunogenicity of leukemia L1210 cells after neuraminidase treatment. *J. nat. Cancer Inst.* **49,** 107 (1972).
11. G. A. Currie and K. D. Bagshawe, Tumor specific immunogenicity of methylcholanthrene-induced sarcoma cells after incubation in neuraminidase. *Brit. J. Cancer* **23,** 141 (1969).
12. J. F. Dore, M. J. Hadjiyannakis, A. Coudert, C. Guibout, L. Marholev and K. Imai, Use of enzyme-treated cells in immunotherapy of leukemia. *Lancet* **i,** 600 (1973).
13. P. B. Brandchaft and C. W. Boone, Increase in Gross (G) antigen sites on the surface of AkR virus-induced rat lymphoma cells after treatment with trypsin. *J. Immunol.* **113,** 94 (1974).
14. A. Coudert, E. Ajuria, J. F. Dore, M. J. Hadjiyannakis and L. Marholev, Augmentation de l'antigénicité de cellules tumorales traitées par la papaïne. *C. R. Acad. Sci. (Paris)* **274,** 2833 (1972).
15. G. Geering, L. J. Old and E. A. Boyse, Antigens of murine leukemias induced by naturally occuring murine leukemia virus. Their relations to the antigens of Gross virus and other murine leukemia viruses. *J. exp. Med.* **124,** 753 (1966).

Europ. J. Cancer Vol. 12, pp. 481–491. Pergamon Press 1976. Printed in Great Britain

Comparison of Some Response to Hyperthermia by Normal Human Diploid Cells and Neoplastic Cells from the Same Origin*

KENNETH R. KASE† and GEORGE M. HAHN
Department of Radiology, Stanford University School of Medicine, Stanford, CA 94305, *U.S.A.*

Abstract—*Human diploid fibroblasts (WI-38) and SV-40-transformed WI-38-VA13/2RA cells were used to investigate the differences in survival and population kinetics between normal and neoplastic cells exposed to hyperthermia at 43°C. Survival is measured by colony-forming ability and by population growth following exposure using a mathematical growth model. The WI-38 cells are shown to be more resistant to heat killing than the 2RA cells at low population densities, but the difference in sensitivity disappears at very high population densities where both cell systems are very resistant. The differences in sensitivity to hyperthermic killing at different population densities may result from multiple "targets" for heat damage but we show that it may also be explained by differences in cell cycle distributions at the time of heating. Population kinetics studies indicate that both cell systems suffer a mitotic delay following exposure with the cells apparently stopped in G2 or mitosis. For equal exposures the 2RA cells incur a longer delay than do the WI-38 cells, and it is possible that this difference might be exploited in cancer therapy.*

INTRODUCTION

FOR OVER 100 years clinicians have reported cancer cures or tumor regressions following whole-body hyperthermia or localized tumor heating. More recently a number of *in vivo* animal studies have indicated that tumors can be cured by subjecting them to supranormal temperatures while normal tissues seem to be unaffected [1–9]. Several *in vitro* studies have been reported which lead one to believe that cultures of malignant cells (or cells of malignant origin) are more sensitive to heat than are non-malignant (or normal) cells [10–17]. However, there are reported studies which indicate there is little or no difference between so-called normal and malignant cells [18,19], and other work shows the opposite response [20–22]. However, in almost all cases the normal and neoplastic cells compared have originated from different sources. Few investigators have actually measured survival, particularly with human cells.

Recently three investigations involving normal and neoplastic cells of the same origin have given evidence that neoplastic cells may be more susceptible to hyperthermic killing. Only one of these investigations involved measurement of cell survival by colony formation and it suffered from low plating efficiencies [10]. Another used dye exclusion [15] as a measure of viability, but dye exclusion has been shown to be a poor indicator of survival after heating [23]. The third study used a measurement of cell loss rather than growth following hyperthermia [11] and this measurement may simply reflect different rates of detachment of heat-inactivated cells. Thus, in each of these studies there are shortcomings which leave open the question of the relative sensitivities of normal and transformed cells.

This investigation was undertaken to try to circumvent some of these shortcomings. Survival of normal and transformed human

Accepted 1 December 1975.
*This investigation was supported by USPHS Grants CA-05008-17, CA-04542, and CA-10372.
†Present address: Department of Radiation Therapy, Harvard Medical School, 50 Binney Street, Boston, MA 02115, U.S.A.

cells has been determined two ways: by colony formation and by analysis of cell population growth following hyperthermic exposure. Cell kinetics after heat treatment were investigated by observing population DNA distributions and by measuring parameters of cell growth. We show that at low cell densities the neoplastic cells are more sensitive than normal cells, but this difference vanishes at a high population density. This differential response may result from differences in the population age distributions at the time of the exposure. We also demonstrate that the cell kinetics following hyperthermic exposure differ between the normal and transformed cells. This last finding may be useful when designing combined treatments of hyperthermia with drugs or radiation.

MATERIAL AND METHODS

Cells

The normal cells used were human fibroblasts (WI-38) derived from embryonic lung [24, 25] obtained from Dr. L. Hayflick (Stanford University) in their 14th to 18th doubling and used by us until they reached their 35th to 40th doubling. These cells undergo about fifty population doublings before they cease proliferating. As reported by Saksela and Moorhead [25], WI-38 cultures remain 97–99% diploid until the 40th passage when aneuploidy begins to appear.

The neoplastic cells were WI-38 fibroblasts which had been transformed by SV-40 virus [26] and were received in their 199th passage from Dr. A. Girardi (Wistar Institute). This is an established cell line designated WI-38-VA13/2RA and will be referred to here as 2RA. Girardi *et al.* [26] report that cultures of 2RA cells are dominated by a tetraploid cell population. In addition, their appearance is more epithelial than fibroblastic.

Cells were grown in Eagles MEM supplemented with 15% fetal calf serum, and were subcultured once weekly by detaching with 0·2% trypsin. WI-38 cells were split 1:4 and 2RA cells, 1:5. Both cell strains were tested for tumorgenicity as described previously [27].

Method of heating

Cells were grown in 60 mm dia plastic Petri dishes and placed in a holder which allowed the cells and growth medium to be immersed in a plastic water tank at 43°C. The tank was covered and the entire assembly was placed inside an incubator.

The 95% air, 5% CO_2 gas supply was bubbled through the heated water of the bath. Two telethermometer probes (Yellow Springs Instrument Company) with a measurement precision of $\pm 0{\cdot}05$°C were inserted in the water for monitoring and control. The temperature controller was a Versatherm proportional controller (Cole-Parmer Company) capable of regulating the temperature to $\pm 0{\cdot}05$°C. Temperature control was achieved by controlling the incubator heater based on the water bath temperature.

Temperature variations during an experiment were kept within $\pm 0{\cdot}2$°C of the desired temperature and these lasted less than 5 min. In general the cells were exposed to a temperature within $\pm 0{\cdot}1$°C of the desired temperature for at least 90% of the exposure time. Temperature of the medium in the dish was monitored and found to agree precisely with the water temperature.

Cell survival

Cell survival experiments were done under four different conditions which resulted in different age distributions, levels of cell-to-cell contact and availability of nutrient supply.

A. Actively proliferating 2 to 3 day old cultures of 10^5 to 5×10^5 cells per dish;

B. Plateau-phase 8 to 10 day old cultures of 2 to 5×10^6 cells per dish which had no medium change since plating ("unfed");

C. Plateau-phase cultures as in B which were supplied with fresh medium 24 hr prior to heating and again immediately prior to heating ("stimulated unfed");

D. Very dense 10 to 12 day old cultures of 1 to $3{\cdot}5 \times 10^7$ cells per dish which had daily medium exchanges including one immediately prior to heating ("dense, fed").

To study survival by colony formation following exposure to 43°C replicate dishes were heated for each data point and the cells subcultured immediately following their removal from the high temperature environment. Cells were detached with 1 ml trypsin (either 0·2 or 0·05%) following two trypsin rinses. Cell concentration was determined by counting on a Coulter Counter (Coulter Instrument Company), and the appropriate dilutions made for plating to obtain 20–50 clones per 60 mm dish or 60–100 clones per 100 mm dish. Four or five dishes were plated per point. All visible colonies containing 50 or more cells were scored.

Cell survival was also determined by measuring the growth of the cell population following exposure to 43°C. In one group of experiments the cells were subcultured after removal from

the high temperature as described above and plated for growth. The number of heat-treated cells plated varied depending on the length of exposure, but radiation-killed cells (3000–4000 rad) of the same type were added as required to make the plated cell density equal on all dishes. This was done to equalize the growth rate of the viable cells. Cells were plated at a cell number (less than 2×10^3 viable cells) which was expected to result in 9 to 12 days of exponential cell growth with no medium change.

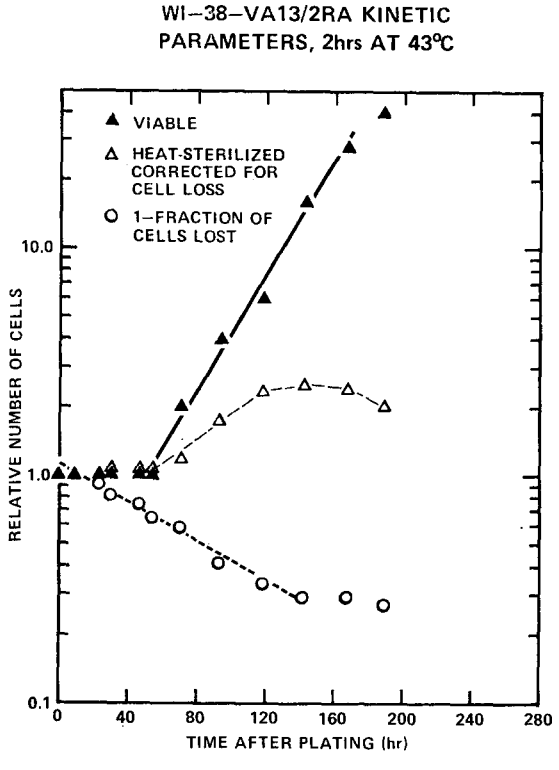


Fig. 1. Graph showing the growth and loss of viable and heat-sterilized WI-38-VA13/2RA cells following 2 hr at 43°C. Graphs such as this were used to determine growth rates, mitotic delay, mean number of doublings of heat-sterilized cells and cell loss rate.

In a second group of experiments cells were plated at 5×10^4 cells/dish. This resulted in sustained exponential growth for 8–10 days with medium exchanges beginning on a daily basis when the cell number exceeded 5×10^5 cells/dish. On the second day following plating the exponentially growing cells were exposed to 43°C for various lengths of time. At least 6 dishes were exposed at each time point.

Growth of the cells in both groups following the exposure was followed for up to two weeks following heating. Growth curves were analyzed to provide an estimate of the number of viable cells present at the beginning of the growth period which could then be used to calculate a surviving fraction and compared with the results of colony formation experiments. (Viable cells are those which are capable of producing colonies of 50 cells or more.)

Kinetic parameters

Several parameters of cell kinetics following exposure to supranormal temperatures are needed for the mathematical model for cell growth. These are: division delay following hyperthermia (τ), growth rate of viable (λ_{gc}) and heat-sterilized (λ_{gd}) cells, the number of divisions which heat-sterilized cells undergo before they stop proliferating (m), and the rate at which heat-sterilized cells are lost from the culture (λ_d). To measure these parameters experiments were done in which individual cells were observed and their behavior following hyperthermia was recorded.

Cells were plated at a density of 800–1000 cells/dish on 60 mm Petri dishes with a 2 × 2 mm grid imprinted on the bottom. Five to six hr following plating each dish was scanned. Single cells were selected and their position recorded using the X–Y coordinates on the vernier stage adjustment. Fifty to 100 cells were observed at each dose point.

Following heating at 43°C for 1–2 hr, the cells were returned to 37°C. Periodically thereafter the dishes were removed from the 37°C incubator, the previously selected cells scored and the dishes returned to 37°C. The time required for scoring was typically 15–30 min/dish during which time the dish was at room temperature. Unheated controls were observed in the same way.

Each of the cells selected for observation could be classified as either viable or heat-sterilized. The ratio of viable cells at a given time to the viable cells immediately following exposure was plotted against time after heating to determine τ and λ_{gc}. To determine λ_{gd} and m for heat-sterilized cells the following equations were used:

$$R_t = N_{dt}/(N_{do} - N_{dl}),$$

where N_{dt} = number of heat-sterilized cells present at time t,

N_{do} = number of heat-sterilized cells present immediately following heating,

N_{dl} = number of heat-sterilized cells present immediately following heating which have disappeared by time t.

The ratio R_t was plotted against time. Graphs such as that in Fig. 1 were produced.

The quantities λ_{gc} and λ_{gd} are the slopes of the growth curves and τ for viable and heat-sterilized cells is found from the intercepts of the growth curves with the abscissa. The value of m was found from the number of population doublings which occurred before the heat-sterilized cell curve peaked and began to fall. A graph of the loss of heat-sterilized cells gave estimates of λ_d and n (the extrapolation number of the cell loss curve).

Cell cycle analysis

Cell cycle analysis was performed using a Cytofluorograph (Biophysics, Inc.). Cells were fixed in ethanol, treated for 30 min with 0·1% ribonuclease (Ribonuclease-A, Sigma Corp.) and stained with ethidium bromide. The resulting DNA distributions were analyzed to determine the fraction of cells in each of the three portions of the cell cycle: G1, S and G2-M.

RESULTS

Cell survival measured by colony-forming ability

All survival experiments were performed twice, some as many as seven times. Colony-formation efficiency appeared to be dependent upon the serum used in the medium and ranged from 5 to 35%. Since substantially the same survival results were obtained over the whole range of colony formation efficiencies, it appears that no selective elimination of cells was occurring at the lower efficiencies.

Survival of WI-38 and 2RA cells in exponentially growing cultures (condition A above) and plateau-phase cultures (conditions B & C above) are reported elsewhere [27] and shown in Figs. 2 and 3.

To determine if the 2RA cells would continue to increase in resistance to hyperthermic killing as they were grown to more and more crowded conditions, experiments were performed using cultures grown as described in D above. At 3·5 × 10^7 cells/dish the 2RA cultures still had not reached a growth plateau, whereas the WI-38 cells reached a growth plateau at 1 to 1·5 × 10^7 cells/dish. A minimal quantity of medium (2 ml) was present during heat exposure to avoid problems of the cells becoming hypoxic. Results are shown in Fig. 4.

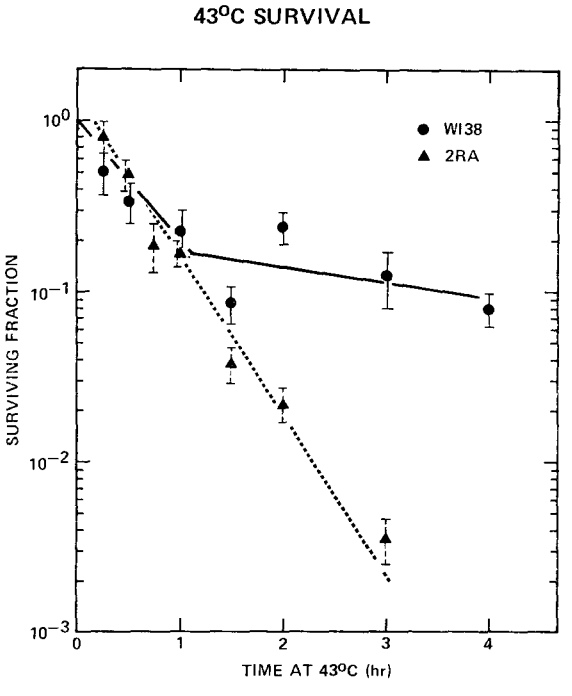


Fig. 2. Survival by colony formation of exponentially growing WI-38 and WI-38-VA13/2RA cells following exposure to 43°C. Error bars represent 1 S.E. from the mean. Lines were fit by eye.

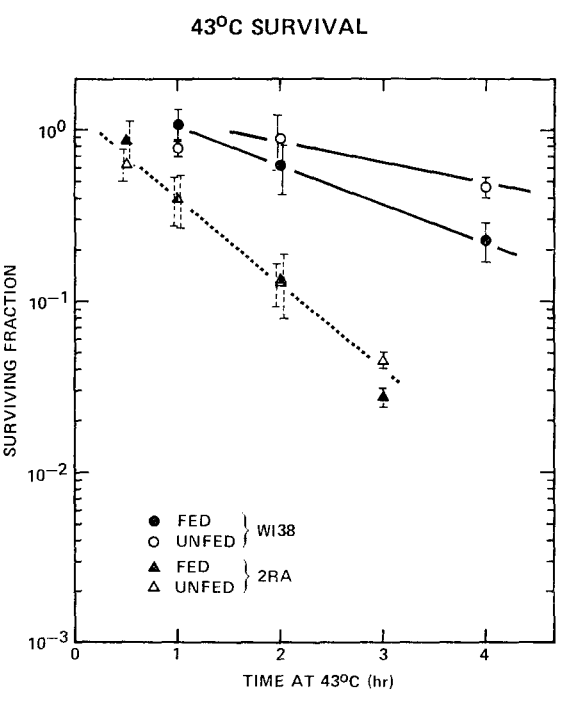


Fig. 3. Survival by colony formation of unfed and stimulated ("fed") plateau-phase WI-38 and WI-38-VA13/2RA cells following exposure to 43°C. Error bars represent 1 S.E. from the mean. Lines were fit by eye.

Cell survival by growth analysis

Several experiments were done in which the growth of the cell population was followed after exposure to 43°C for various times. Growth data were analyzed according to a model similar to one recently published by

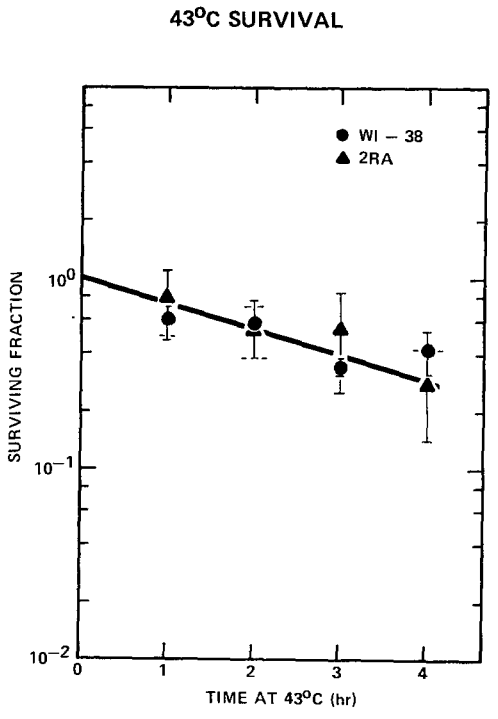


Fig. 4. Survival by colony formation of fed, densely growing cultures of WI-38 and WI-38-VA13/2RA cells. Error bars represent 1 S.E. from the mean. Lines were fit by eye.

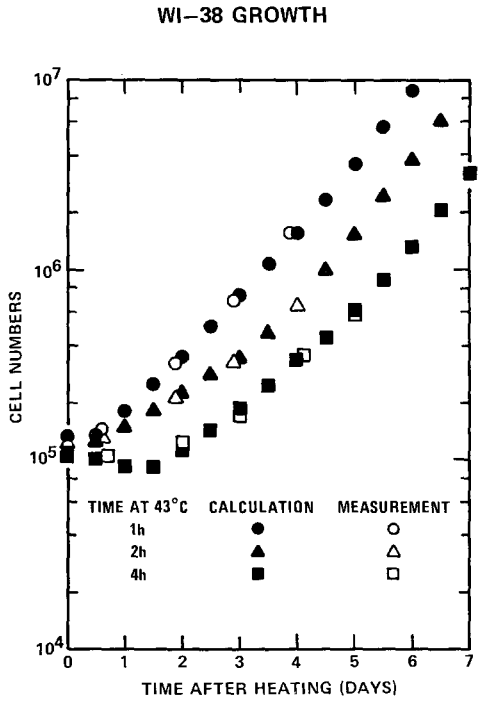


Fig. 5. Comparison of WI-38 measured and calculated growth curves following exposures of 1, 2 and 4 hr at 43°C.

Okumura and Uchiyama [28]. The equations used to model cell growth are given below [29]:

$$0 \leqq t \leqq \tau\colon N = N_0 + (fN_p - N_0)(1 - (1 - \exp(-\lambda_{dt}))^n)$$

$$\tau \leqq t \leqq T'\colon N = N_0 \exp(\lambda_{gc}(t-\tau)) + (fN_p - N_0) \exp(\lambda_{gd}(t-\tau))(1 - (1 - \exp(-\lambda_{dt}))^n)$$

$$T' < t\colon N = N_0 \exp(\lambda_{gc}(t-\tau)) + (fN_p - N_0) \exp(\lambda_{gd}(T'-\tau))(1 - (1 - \exp(-\lambda_{dt}))^n)$$

where:

f = Fraction of cells planted which attach to the dish (if the cells are not subcultured following the insult, $f = 1$);
N_p = Total number of cells planted in dish: or total number of cells present at the time of insult;
N_0 = Number of viable cells present immediately following the insult (survivors);
N_d = Number of heat-sterilized cells present immediately following the insult;
$N_d = fN_p - N_0$;
T' = Time during which heat-sterilized cells proliferate;
$T' = m(\ln 2\ .\ )/\lambda_{gd}$.

Examples of measured growth curves and points calculated from the growth model are shown in Fig. 5. The survival curve obtained for exponentially growing WI-38 cells is shown in Fig. 6 which also shows the mean surviving fraction values found in the colony formation experiments reported above. Survival curves obtained for exponentially growing 2RA cells and plateau-phase WI-38 cells show substantially the same agreement between the two methods of determining survival.

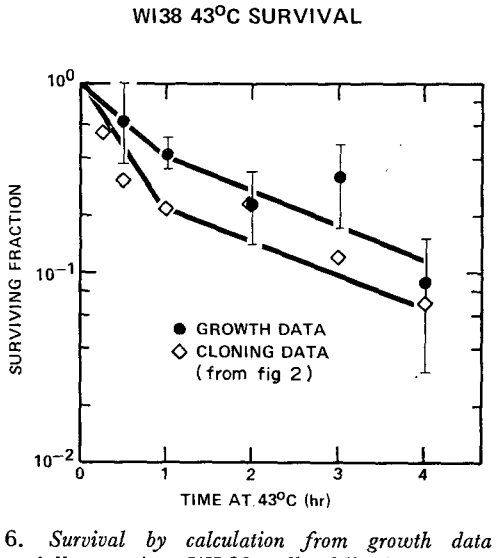


Fig. 6. Survival by calculation from growth data for exponentially growing WI-38 cells following exposure to 43°C. Error bars represent 1 S.E. from the mean. Lines were fit by eye. Comparison is made with survival data from colony formation.

Table 1. *WI-38 kinetic parameters. Values of doubling times, mitotic delay, half-time for loss of heat-sterilized cells, mean number of doublings of heat-sterilized cells and extrapolation number of cell loss curve for WI-38 cells exposed to 43°C for 0, 1 and 2 hr*

Time at 43°C (hr)	T_{dv}[a] (hr)	T_{dhs}[b] (hr)	τ_v[c] (hr)	τ_{hs}[d] (hr)	$T\frac{1}{2}$[e] (hr)	m[f]	n[g]
0	23 (20–25)	45 (32–62)	16 (10–23)	24 (20–30)	351 (123–640)	2·15 (1·81–2·46)	1·04 (1·02–1·05)
1	28 (24–33)	51 (41–62)	16 (13–19)	20 (15–25)	210 (172–248)	2·20 (1·70–2·70)	1·07 (1·05–1·10)
2	22 (22–22)	50 (30–71)	26 (21–31)	33 (28–37)	130 (72–196)	1·92 (1·58–2·17)	1·17 (1·10–1·25)

Symbols used in table:
[a] Doubling time of viable cells.
[b] Doubling time of heat-sterilized cells.
[c] Division delay time of viable cells.
[d] Division delay time of heat-sterilized cells.
[e] Half-time of heat-sterilized cell loss.
[f] Number of doublings of heat-sterilized cells.
[g] Extrapolation number of heat-sterilized cell loss curve.
Note: Mitotic delays at 0 hr exposure are due to the subculturing process.

Table 2. *WI-38-VA13/2RA kinetic parameters. Values of doubling times, mitotic delay, half-time for loss of heat-sterilized cells, mean number of doublings of heat-sterilized cells and extrapolation number of cell loss curve for WI-38-VA13/2RA cells exposed to 43°C for 0, 1 and 2 hr*

Time at 43°C (hr)	T_{dv} (hr)	T_{dhs} (hr)	τ_v (hr)	τ_{hs} (hr)	$T\frac{1}{2}$ (hr)	m	n
0	35	50	14	17	232	1·81	1·10
1	33	140	27	23	156	0·85	1·03
2	25	56	50	50	71	1·32	1·12

Kinetic parameters

Results of experiments to obtain values for the kinetic parameters used in the growth model are shown in Figs. 1 and 7. Values of the parameters measured are tabulated in Tables 1 and 2. Mean values are given with the range of values measured in parentheses if more than one experiment was done.

A qualitative observation made during these experiments was that heat-sterilized cells appearing to be mitotic at one observation either had disappeared completely 24 hr later or degenerated over a period of days without dividing. On the other hand, there were many cells which never appeared mitotic, never divided, and did not grow, but remained attached for many days. Still others grew into giant cells.

DISCUSSION

Cell survival

Exponentially growing normal (WI-38) cells exhibit a biphasic survival with time at 43°C while the neoplastic cells (2RA) exhibit simple exponential survival with a small shoulder. Normal cells in exponential growth may undergo an adaptive process under hyperthermic exposure or there may be a sub-population, amounting to 10–20%, which is very resistant to killing by hyperthermia. The adaptive process or the resistant sub-population of normal cells allows the WI-38 cultures to survive much longer treatments than the 2RA cultures are able to survive.

A quantity, D_0 can be defined for hyperthermic killing which measures the time at 43°C required to reduce the surviving fraction to e^{-1} (37%) of its original value on the exponential portion of the survival curve. Similarly, an extrapolation number, n, can be defined as the intercept of the exponential portion of the survival curve extrapolated to the ordinate. The mean values of D_0 and n for exponentially growing cultures are tabulated in Table 3.

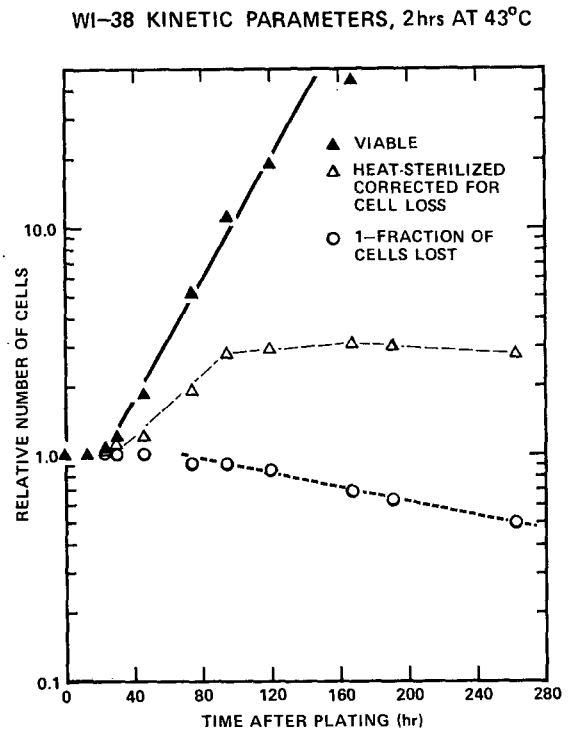


Fig. 7. Graph showing the growth and loss of viable and heat-sterilized WI-38 cells following 2 hr at 43°C.

In the unfed plateau condition WI-38 cells are significantly more resistant to killing at 43°C than are the 2RA cells (Table 4). However, there is no indication of a biphasic survival curve for the WI-38 cells. Confluent monolayers of both WI-38 and 2RA cells are much more resistant to hyperthermic killing than are exponentially growing cultures. Also, it is apparent that stimulated WI-38 cultures are less resistant than unfed cultures. This differential does not appear in the 2RA cultures. DNA distributions of the cultures may provide an explanation and this will be discussed later.

The finding that exponentially growing cultures are more sensitive than are the confluent monolayers is exactly opposite from the results of Hahn [30] and Schulman and Hall [31] using Chinese hamster cells exposed to 43°C. Another set of experiments comparing exponentially growing cultures of mouse 3T3 cells and Hela cells with the same cells in a "confluent phase" was reported by Kim *et al.* [12]. For exposures at 42°C they report that the cells in "confluent phase" are more resistant to killing than are cells growing exponentially.

Plateau-phase WI-38 cells are at least as resistant to killing at 43°C as the resistant portion of the exponentially growing cultures. The D_0 for 2RA cells increased more than a factor of two from that of the exponentially growing cultures so that they are more resistant than the sensitive portion of the WI-38 exponentially growing cultures.

An interesting result is observed in dense, fed cultures. Survival of WI-38 cells is practically identical with their survival in the unfed plateau state. However, survival of 2RA cells increases to the level of the WI-38. Under these conditions of growth there appears to be no difference in survival at 43°C between WI-38 and 2RA cells (Table 4).

The D_0 measured for the resistant portion of the WI-38 exponentially growing population and the D_0 of the densely growing cultures (135–200 min) corresponds well with the D_0 of 150 min measured by Schrek [15] for normal human lymphocytes ,but is significantly less than the D_0 of 260 min for the initial portion of the survival curve of human melanocytes reported by Stehlin, *et al.* [17]. These are about the only direct comparisons which can be made with other reported data on normal cells.

The D_0 measured for 2RA cells in exponential growth, 23 min, compares well with 30 min measured by Palzer and Heidelberger [32] for exponentially growing HeLa cells at 43°C. Also the surviving fractions are comparable over the exposure times measured. This indicates some similarity in the response of two human neoplastic cell lines to hyperthermia. Stehlin *et al.* [17] report a much longer D_0 of about 180 min for human malignant melanoma

Table 3. Mean values for D_0 and n for exponentially growing cultures of WI-38 and WI-38-VA13/2RA cells exposed to 43°C. Numbers in parentheses indicate the range of values obtained in the several experiments. For WI-38 cells two values of D_0 are given, one for the sensitive portion and one for the resistant portion of the population

	D_0		n
	Sensitive	Resistant	
WI-38	41 m (31–54 m)	137 m (125–156 m)	1·1 (1·0–1·5)
WI-38-VA13/2RA	23 m (18–34 m)	—	2·6 (1·0–5·0)

Table 4. Mean values for D_0 and n for unfed plateau phase and dense, fed cultures of WI-38 and WI-38-VA13/2RA cells exposed to 43°C. Numbers in parentheses indicate the range of values obtained in the several experiments

	D_0	n
WI-38 stimulated	120 m (114–126 m)	1·7 (1·4–2·4)
Unfed	176 m (144–228 m)	1·8 (1·4–2·1)
Dense fed	194 m (156–354 m)	1·0 (1·0–1·0)
2RA stimulated	58 m (44– 71 m)	1·2 (1·0–1·4)
Unfed	62 m (54– 70 m)	1·1 (1·0–1·2)
Dense fed	194 m (186–226 m)	1·0 (1·0–1·1)

cells heated to 43°C but there is no indication in their paper of the conditions of growth or the method of determining survival.

Figure 6 shows that survival parameters as determined from growth measurements agree quite well both qualitatively and quantitatively with those determined in colony formation experiments. The survival calculated from growth data is generally somewhat higher than the survival measured by colony formation, but the shapes of the curves are practically identical. It is possible that the greater number of cells present on the dish in the growth experiments compared with the low number plated for colony formation promoted greater cell survival. D_0 values determined from the growth experiments are compared with D_0 values determined in the colony formation experiments in Table 5, and one can see that the values agree quite well except for the unfed plateau cells. Since the two methods (growth and colony formation) yield similar results, they reinforce the belief that each method provides reasonably accurate survival information.

Relationship of culture density to survival

It is apparent that the culture density significantly affects cell survival. For WI-38 cells it is possible that differences in age distribution account for this finding. Figure 8 shows that there are significant differences in the DNA distributions depending on cell density and nutrient supply. Table 6 gives a typical breakdown of the distribution of cells through the cell cycle for each condition.

Table 5. Comparison of mean values of D_0 obtained from survival experiments analyzed by growth and colony formation

	Growth	Colony formation
WI-38 exponential	65 m	41 m
	146 m	139 m
unfed	296 m	176 m
2 RA exponential	34 m	23 m

Other authors [32–35] have found a cell cycle dependence of survival in cells exposed to supranormal temperatures. These reports indicate that cells in G1 are much more resistant to hyperthermic killing than are cells in S or G2-M. Thus the net survival could depend on the fraction of cells in G1 at the time of the exposure.

A similar argument does not hold for the 2RA cells. As shown in Fig. 9, there is little, if any, difference in the DNA distributions of 2RA cells in exponential growth or plateau-phase. Only in the dense, fed cultures is there a shift to a population containing mostly G1 cells. An analysis of typical distributions is given in Table 7.

Table 6. Distribution of cells through the cell cycle for WI-38 cells in various proliferative states. Fraction of WI-38 cells in each portion of the cell cycle for cultures in exponential growth, plateau-phase (unfed and stimulated to proliferate) and dense, fed cultures

	Exponential	Plateau		Dense
Stage	Growth	Unfed	Stimulated	Fed
G1	0·46	0·83	0·64	0·68
S	0·27	0·05	0·11	0·12
G2-M	0·27	0·12	0·25	0·20

One can make the argument that the closeness of the DNA distributions is the reason no difference in survival is measured between stimulated and unfed plateau 2RA cells. But the difference in survival between unfed plateau and exponentially growing cells cannot be accounted for in this way. It is also clear that the total difference in response between the normal and neoplastic cells cannot be accounted for simply by differences in DNA distributions.

Kinetics

From the kinetic data it is apparent that viable and heat-sterilized cells grow at different rates. The ratio of doubling times (or growth rate constants) is about 2 for both WI-38 and 2RA cells and not affected by hyperthermic exposure.

Heat-induced division delay is about the same for both viable and heat-sterilized cells and is a function of the amount of cell killing caused by the hyperthermic exposure. Other investigators have reported mitotic delays

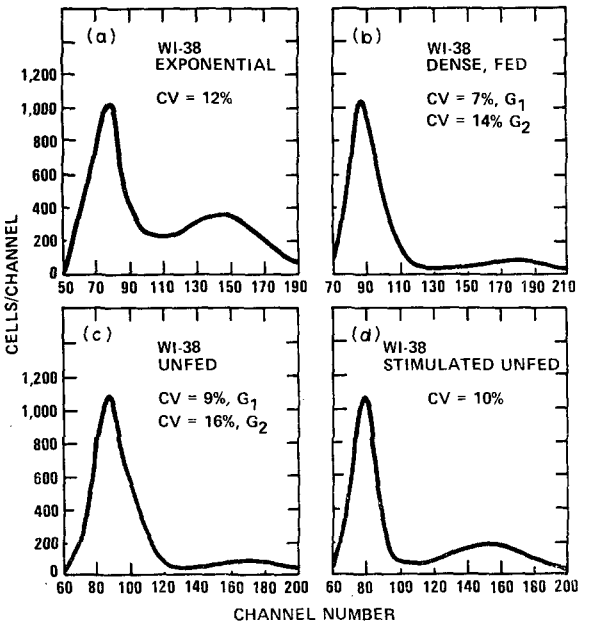


Fig. 8. DNA distributions for WI-38 cells in (a) exponential growth, (b) dense, fed culture, (c) unfed plateau-phase and (d) stimulated unfed cells. The abscissa represents fluorescent intensity, the ordinate is the number of cells. Peaks in channels 80–90 represent cells in G1; peaks in channels 150–180 represent cells in G2-M. C.V. is coefficient of variation.

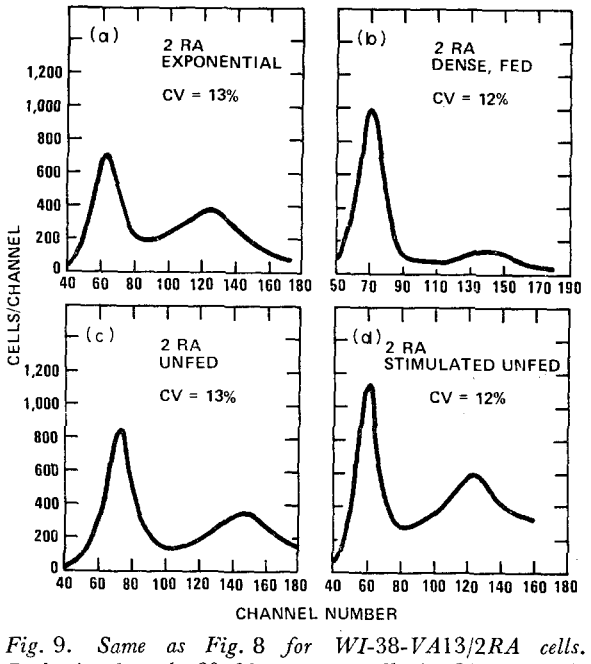


Fig. 9. Same as Fig. 8 for WI-38-VA13/2RA cells. Peaks in channels 60–80 represent cells in G1; peaks in channels 120–150 represent cells in G2-M.

following hyperthermia, but at temperatures other than 43°C. The number of doublings undergone by heat-sterilized cells is somewhat different for the two cell strains, but in both cases is relatively independent of cell killing or duration of hyperthermic exposure.

Rate of loss of heat-sterilized cells appears to be very dependent on the amount of cell killing caused by the hyperthermic exposure. The extrapolation number of the cell loss curve is the same for both cell strains and is independent of cell killing or duration of hyperthermic exposure.

Experimental results reported previously [27] and those discussed above show that, although the proliferation kinetics of WI-38 and 2RA cells following exposure to 43°C are qualitatively similar, the time scales differ somewhat. Both cell systems experience a mitotic delay following hyperthermia, but the delay appears to be longer for 2RA cells than for WI-38 cells for equal exposure times.

CONCLUSIONS

Variation in sensitivity of WI-38 cells to hyperthermic killing seems to be more related to cell cycle distribution than to the degree of cell-to-cell contact since stimulated plateau cultures are more sensitive than are unfed plateau cells at the same density. Comparisons with results of experiments using non-human mammalian cells show very little agreement. WI-38 cells appear much more resistant to killing at 43°C and the resistance increases with cell density while other mammalian cells exhibit a decreased resistance with increasing cell density. Thus, data from experiments using non-human mammalian cells may not be applicable to human cells.

Table 7. Distribution of cells through the cell cycle for WI-38-VA13/2RA cells in various proliferative states. Fraction of WI-38-VA13/2RA cells in each portion of the cell cycle for cultures in exponential growth, plateau-phase (unfed and stimulated to proliferate) and dense, fed cultures

	Exponential	Plateau		Dense
Stage	Growth	Unfed	Stimulated	Fed
G1	0·39	0·43	0·40	0·68
S	0·21	0·22	0·24	0·12
G2-M	0·40	0·35	0·36	0·20

Data reported here indicate that the process of transformation in human fibroblasts from a normal to a neoplastic condition involves changes in the cell which make it more sensitive to killing by supranormal temperatures. The increased sensitivity of the transformed cells persists until the cells are grown into a very dense culture. This could imply that damage to the plasma membrane is the critical lethal event for 2RA cells at low densities and becomes less important as the cells grow into

close contact. Judging from heat-sterilized cell loss rates and number of doublings by heat-sterilized cells, it appears that 2RA cells are more easily detached from the dish. 2RA cells are also detached much more readily with trypsin than are WI-38 cells. This could reflect membrane differences between the two cell strains which might make the 2RA cells more susceptible to damage than the WI-38 cells. Because the variations in sensitivity of the normal cells appear to be primarily related to cell cycle distribution while variations in sensitivity of the neoplastic cells appear primarily related to cell contact, the primary target for lethality at 43°C may be different for the two cell systems. However, other explanations are possible.

Baserga *et al.* [36] have demonstrated that WI-38 cells more readily enter the non-proliferative G0 state than do 2RA cells. They show that upon stimulation of unfed plateau cultures of WI-38 cells a period of 3 hr elapses before the cells demonstrate a reentry into the cell cycle in G1 while 2RA cells appear to begin functioning immediately as G1 cells. The increased resistance of WI-38 cells could be explained if a significant fraction of cells are in G0, even in exponentially growing cultures. The three hr period prior to entry into G1 following stimulation by subculturing could afford a period for recovery from heat-induced damage which the 2RA cells do not have. The increase in resistance of 2RA cells as they become more crowded could then be explained by assuming that more of these cells enter the G0 state as the culture becomes more dense.

The results of these experiments have interesting applications for hyperthermic cancer therapy. In solid tumors where cells are closely packed, hyperthermic killing as demonstrated in the experiments on plateau-phase cultures may give clear selective killing of neoplastic cells. However, the disappearance of the differential in cell killing in dense, fed cultures is disturbing and needs further investigation. Nutrient supply appears to have much less effect than cell cycle distribution or cell density. Consequently tumor cells might be expected to be more readily destroyed than normal tissue cells for identical exposure conditions regardless of their nutrient supply.

Based on the results of cell kinetics studies, judiciously timed fractionated treatments might take advantage of the possibility that surviving normal cells may be primarily in the resistant G1 (or G0) phase while a large fraction of the neoplastic cells may be blocked in the more sensitive G2-M phase of the cycle. The same effects may also offer advantages for combined hyperthermic and chemotherapeutic treatment using drugs which act primarily on cells in certain phases of the cell cycle.

Acknowledgements—We thank Drs. P. Hanawalt and D. Clayton for their helpful criticisms; Louise Gordon and Mike Hatfield for their assistance in the experiments; Drs. L. Hayflick and A. Girardi for supplying the cells; and Drs. H. S. Kaplan and A. Epstein for the nude mice and assistance in the tumorgenicity test.

REFERENCES

1. F. M. Allen, Biological modifications of effects of roentgen rays. *J. Roentgenol.* **73,** 836 (1955).
2. D. B. Cater, I. A. Silver and D. A. Watkinson, Combined therapy with 220 KV roentgen and 10 cm microwave heating. *Acta Radiol. Ther. Phys. Biol.* **2,** 321 (1964).
3. G. Crile, Jr., Selective destruction of cancers after exposure to heat. *Ann. Surg.* **156,** 404 (1962).
4. G. Crile, Jr., The effects of heat and radiation on cancers implanted in the feet of mice. *Cancer Res.* **23,** 372 (1963).
5. J. A. Dickson and M. Suzangar, *In vitro–in vivo* studies on the susceptibility of the solid Yoshida sarcoma to drugs and hyperthermia (42°C). *Cancer Res.* **34,** 1263 (1974).
6. K. Overgaard and J. Overgaard, Investigations on the possibility of a thermic tumor therapy—I. *Europ. J. Cancer* **8,** 65 (1972).
7. G. L. Rohdenburg and F. Prime, Effect of combined radiation and heat on neoplasms. *Arch. Surg.* **2,** 116 (1921).
8. N. Westermark, The effect of heat on rat tumors. *Skand. Arch. Physiol.* **52,** 257 (1927).
9. A. Yerushalmi and I. Har-Kedar, Enhancement of radiation effects by heating of the tumor. *Israel J. Med. Sci.* **10,** 772 (1974).
10. T. T. Chen and C. Heidelberger, Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons *in vitro*. *Int. J. Cancer* **4,** 166 (1969).

11. B. C. Giovanella, A. C. Morgan, J. S. Stehlin and L. J. Williams, Selective lethal effect of supranormal temperatures on mouse sarcoma cells. *Cancer Res.* **33,** 2568 (1973).
12. J. H. Kim, S. H. Kim and E. Hahn, Thermal enhancement of the radiosensitivity using cultured normal and neoplastic cells. *Amer. J. Roentgenol. Radiat. Ther. Nucl. Med.* **121,** 860 (1974).
13. E. M. Levine and E. B. Robbins, Differential temperature sensitivity of normal and cancer cells in culture. *J. Cell. Physiol.* **76,** 373 (1970).
14. R. A. Lambert, Demonstration of the greater sensitivity to heat of sarcoma cells as compared with actively proliferating connective tissue cells. *J. Amer. Med. Assoc.* **59,** 2147 (1912).
15. R. Schrek, Sensitivity of normal and leukemic lymphocytes and leukemic myeloblasts to heat. *J. Nat. Cancer Inst.* **37,** 649 (1966).
16. R. Cavaliere, E. C. Ciocatto, B. C. Giovanella, C. Heidelberger, R. O. Johnson, M. Margottini, B. Mondovi, G. Moricca and A. Rossi-Fanelli, Selective heat sensitivity of cancer cells. *Cancer* **20,** 1351 (1967).
17. J. S. Stehlin, Jr, B. C. Giovanella, P. D. de Ipolyi, L. R. Muenz and R. F. Anderson, Results of hyperthermic perfusion for melanoma of the extremities. *Surg., Gynec. Obstet.* **140,** 338 (1975).
18. H. B. Friedgood, On the thermal death point of sarcoma and normal mononuclear cells. *Arch. Exp. Zellforsch.* **7,** 243 (1928).
19. R. Love, R. Z. Soriano and R. J. Walsh, Effect of hyperthermia on normal and neoplastic cells *in vitro*. *Cancer Res.* **30,** 1525 (1970).
20. N. Auersperg, Differential heat sensitivity of cells in tissue culture. *Nature (Lond.)* **209,** 415 (1966).
21. L. Ossovski and L. Sachs, Temperature sensitivity of polyoma virus, induction of cellular DNA synthesis and multiplication of transformed cells at high temperature. *Proc. Nat. Acad. Sci. U.S.A.* **58,** 1938 (1967).
22. Z. F. Ch. Kachani and A. B. Sabin, Reproductive capacity and viability at higher temperatures of various transformed hamster cell lines. *J. Natl. Cancer Inst.* **43,** 469 (1969).
23. M. Harris, Criteria of viability in heat-treated cells. *Exp. Cell. Res.* **56,** 382 (1968).
24. L. Hayflick, The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37,** 614 (1965).
25. E. Saksela and P. S. Moorhead, Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proc. Natl. Acad. Sci. U.S.A.* **50,** 390 (1963).
26. A. J. Girardi, D. Weinstein and P. S. Moorhead, SV-40 transformation of human diploid cells. *Ann. Med. Exp. Fenn.* **44,** 242 (1966).
27. K. Kase and G. M. Hahn, Differential heat response of normal and transformed human cells in tissue culture. *Nature (Lond.)* **255,** 228 (1975).
28. Y. Okumura and Y. Uchiyama, A model of growth kinetics of irradiated cultured cells. *Int. J. Radiat. Biol.* **26,** 321 (1974).
29. K. R. Kase, Comparison of the response to hyperthermia by human normal diploid cells and neoplastic cells from the same origin. Ph.D. Thesis, Stanford University (1975).
30. G. M. Hahn, Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment. *Cancer Res.* **34,** 3117 (1974).
31. N. Schulman and E. J. Hall, Hyperthermia: Its effect on proliferative and plateau phase cell cultures. *Radiology* **113,** 207 (1974).
32. R. J. Palzer and C. Heidelberger, Studies on the quantitative biology of hyperthermic killing of HeLa cells. *Cancer Res.* **33,** 415 (1973).
33. R. J. Palzer and C. Heidelberger, Influence of drugs and synchrony on the hyperthermic killing of HeLa cells. *Cancer Res.* **33,** 422 (1973).
34. W. C. Dewey, A. Westra, H. H. Miller and H. Nagasawa, Heat-induced lethality and chromosomal damage in synchronized Chinese hamster cells treated with 5-bromodeoxyuridine. *Int. J. Radiat. Biol.* **20,** 505 (1971).
35. A. Westra and W. C. Dewey, Variation in sensitivity to heat shock during the cell cycle of Chinese hamster cells *in vitro*. *Int. J. Radiat. Biol.* **19,** 467 (1971).
36. R. Baserga, M. Costlow and G. Rovera, Changes in membrane function and chromatin template activity in diploid and transformed cells in culture. *Fed. Proc.* **32,** 2115 (1973).

Europ. J. Cancer Vol. 12, pp. 493–496. Pergamon Press 1976. Printed in Great Britain

Effects of Progesterone on Rat Uterine Adenocarcinoma Cells *In Vitro*: An Electron Microscopic Study

SOUEI SEKIYA,* NAOHIDE TAKAYAMA,† and HIROYOSHI TAKAMIZAWA*

**Department of Obstetrics and Gynecology, Chiba University School of Medicine, 1-8-1 Inohana, Chiba 280, Japan, †Department of Pediatrics, Tokyo University Branch Hospital, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo, Japan*

Abstract—*Ultrastructural features of cloned uterine adenocarinoma cells derived from a rat (HTP/C1 cells) were compared with those of their variant cell line which was adapted to grow in a culture medium containing 8 μg/ml progesterone* in vitro *(HTP/C1/P8 cells). Poorly formed cell contacts were prominent in the monolayer of HTP/C1 cells, whereas the intercellular spaces of HTP/C1/P8 cells were narrow and the cell-cell connection was tight. However, no significant difference of acid mucopolysaccharride on the cell surface observed between the both cell lines. Other ultrastructural changes in the HTP/C1/P8 cells were an increased number of organelles such as mitochondria, Golgi complex and rough endoplasmic reticulum with coexistence of relatively abundant lipid droplets in cytoplasm. These ultrastructural changes induced by the direct application of progesterone* in vitro *coincide with the* in vivo *results described previously in human tumours and confirm that progesterone is involved in the regulation of cellular differentiation*

INTRODUCTION

CHANGES in endometrial adenocarcinoma after the administration of large doses of progestational agents have been reported [1–11]. They were the induction of secretory and/or acanthomatous changes in the carcinoma tissues and conversion of the cells to a benign morphology.

We reported previously that progesterone directly increased morphological differentiation of uterine adenocarcinoma cells of rat *in vitro* [12]. The present study describes further confirmation by electron microscopy of the direct effects of progesterone on these cells *in vitro*.

MATERIAL AND METHODS

Cells

Transplantable cloned cell line HTP/C1, derived from a uterine adenocarcinoma induced by an intra-uterine administration of 7,12-dimethylbenz(a)anthracene into Sprague–Dawley rat [13, 14], and its variant line HTP/C1/P8, which adapted to grow in the culture medium containing 8 μg/ml progesterone [12, 15], were used. The HTP/C1/P8 cells were grown for more than 6 months in the culture medium with progesterone and sub-cultured 17 times or more. These cells were grown at 37°C in a humified 5% CO_2 and 95% air, and cultivated in 60-mm dia Petri dishes (Falcon Plastics, Oxnard, Calif.). The growth medium consisted of Eagle's minimum essential medium supplemented with 10% calf serum and antibiotics as described previously [14].

Electron microscopic procedure

When the culture was at a subconfluent state 5 ml of the culture fluid was discarded and the monolayer of HTP/C1 or HTP/C1/P8 cells fixed with 3% glutaraldehyde in phosphate buffered saline (PBS) solution for 24 hr at 4°C. The monolayer was then washed with 7% sucrose in PBS and post fixed with 1% osmium tetroxide for 60 min at 4°C. The cells in monolayer were scraped off with a rubber policeman and centrifuged at 1000 rev/min for 5 min to obtain a cell pellet. The pellet was mixed with 4% melted agar on a slide glass kept at 50°C and left for 1–2 min to allow the agar infiltrate into the pellet. The slide was

Accepted 24 October 1975.

then placed on an ice to solidify the agar which was cut into smaller pieces. These pieces were dehydrated in a graded ethanol series and embedded in Luft's Epon 812. Then sections were made using a Porter Blum MT-1 ultramicrotome, double-stained with uranylacetate followed by Reynold's lead acetate and examined with a Hitachi 11-B electron microscope. Ruthenium red staining was performed following the method described by Martinez-Palomo and Brailvsky [16].

For light microscopic observation semi-thin 1 μm sections were stained with 10% Giemsa solution in PBS on a hot plate at 70°C.

RESULTS

Although marked variations in the cell size were observed in both HTP/C1 and HTP/C1/P8 cells, HTP/C1 and HTP/C1/P8 cells could be distinguished by light microscopy. The former cells were square in shape and formed poor cell–cell connections, whereas the latter were roundish and formed close cell contacts.

By electron microscopy, loose contacts between HTP/C1 cells in monolayer was clearly illustrated (Fig. 1). Although HTP/C1 cells were considered to be epithelial in origin because of the existence of microvilli on the cell surface, the appearance of their junctional apparati was far from those of the typical epithelial cells; in as much as the intercellular spaces were dilated, the contact surface region between cells was reduced, and typical tight junctions and desmosomes were very sparse (Fig. 2 and 3). The cytoplasm of HTP/C1 cells was darker than that of HTP/C1/P8 cells, presumably because abundant free ribosomes in cytoplasma of HTP/C1 cells. Some of the nuclei of HTP/C1 cells exhibited many infoldings which resulted in an irregular appearance of the nuclei. Microvilli were found not only on the free cell surfaces but also they projected into the wide intercellular spaces (Fig. 1–3).

In contrast to HTP/C1 cells, HTP/C1/P8 cells were tightly connected each other and desmosomes were well developed, but tight and intermediate junctions were not clearly demonstrated (Fig. 6). The cytoplasm of HTP/C1/P8 cells was characterized by abundant mitochondria, rough endoplasmic reticulum and well-developed Golgi complex. Lipid droplets were frequently observed in the cytoplasm (Fig. 4 and 5).

In view of the correlation between the malignancy of cell and the amount of acid mucopolysaccharide on the cell surface was reported in some virus-transformed cells *in vitro* [16, 17] the staining of the surface of the two cell lines by ruthenium red was studied. This electron dense coats on the cell surface were observed in both cell lines, but there was no significant difference in the thickness of the electron dense material (Fig. 7 and 8).

DISCUSSION

Descriptions transmission and scanning electron microscopy of the endometrial carcinoma have been published by several authors [18–22], but there are only a few accounts of the effect of progestational agents on the cell ultrastructure [23–26].

Hayakawa *et al.* [23, 24] first reported that the changes occurred in the well differentiated adenocarcinoma cells following the treatment with large doses of dimethisterone resembled to those observed in the normal, non-malignant endometrial glandular cells obtaining during the luteal phase. Similar observations were reported by Akashi [25] and by Richart and Ferenczy [26]. These ultrastructural changes of endometrial carcinoma cells by progestational agents were confirmed by the alterations of organelles such as an increased number and size of mitochondria, large aggregations of glycogen in the subapical area, an increase of lysosomes and the appearance of dense nuclear material.

However, it may be considered that the carcinoma tissues are composed of cancer cells that are at different stages of differentiation or cell cycle *in vivo*. Therefore, the effects of the drugs on these carcinoma cells may vary from area to area, as suggested by Hayakawa [24]. The use of cloned cell lines in tissue culture may be an important method to avoid this difficulty. The HTP/C1 cells in the present study produced moderately undifferentiated adenocarcinomata when inoculated subcutaneously into isologous newborn rats [12–14]. In contrast to HTP/C1 cells the tumours developed after the inoculation of HTP/C1/P8 cells were differentiated adenocarcinomata [12].

These early observations and the present results indicate that fairly high dose of progesterone acts directly on uterine adenocarcinoma cells growing as undifferentiated *in vitro* and convert them into more benign looking cells as judged on morphological appearances.

Acknowledgement—The authors thank Prof. Yutaka Shimada, Department of Anatomy, Chiba University School of Medicine, for his helpful suggestions.

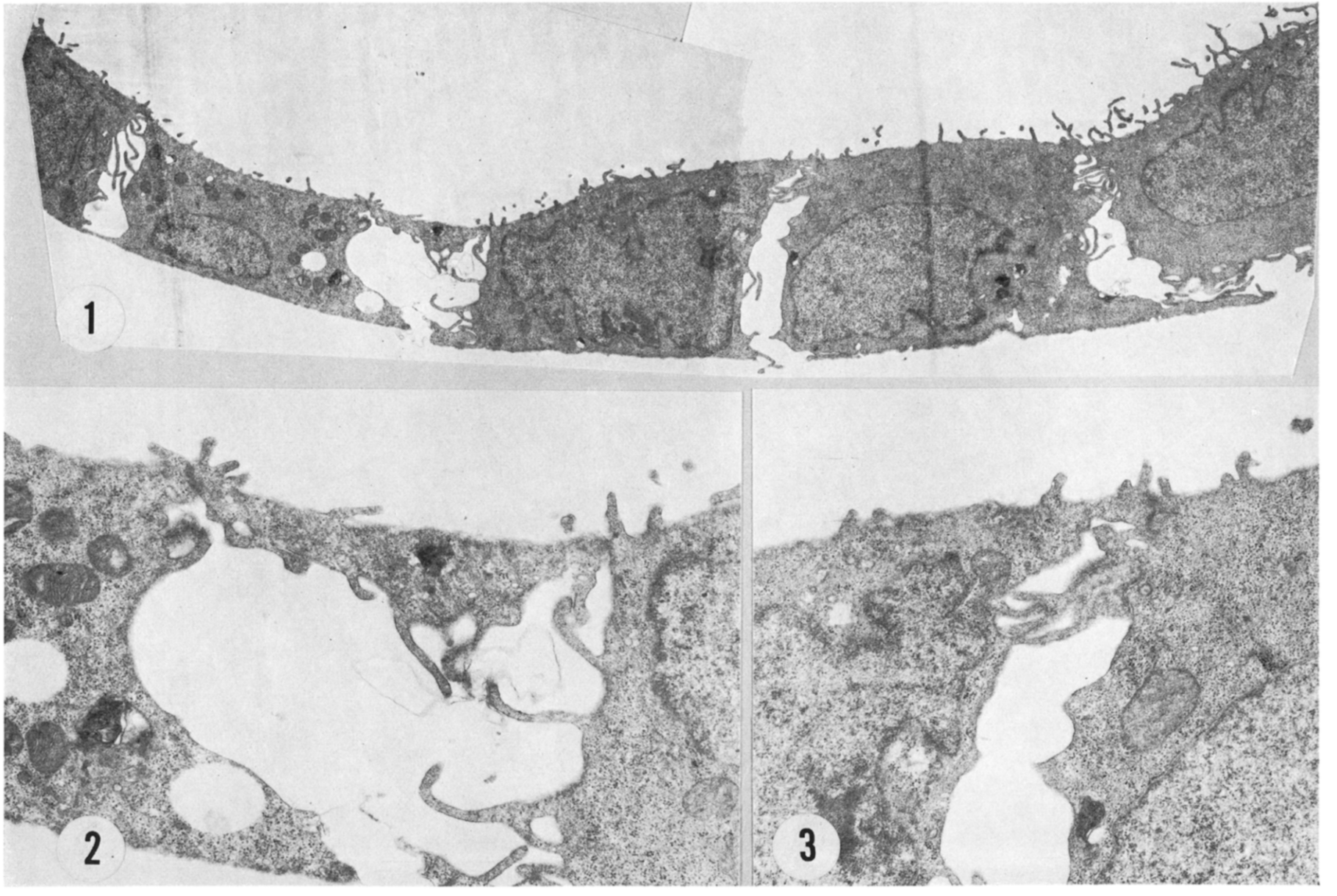
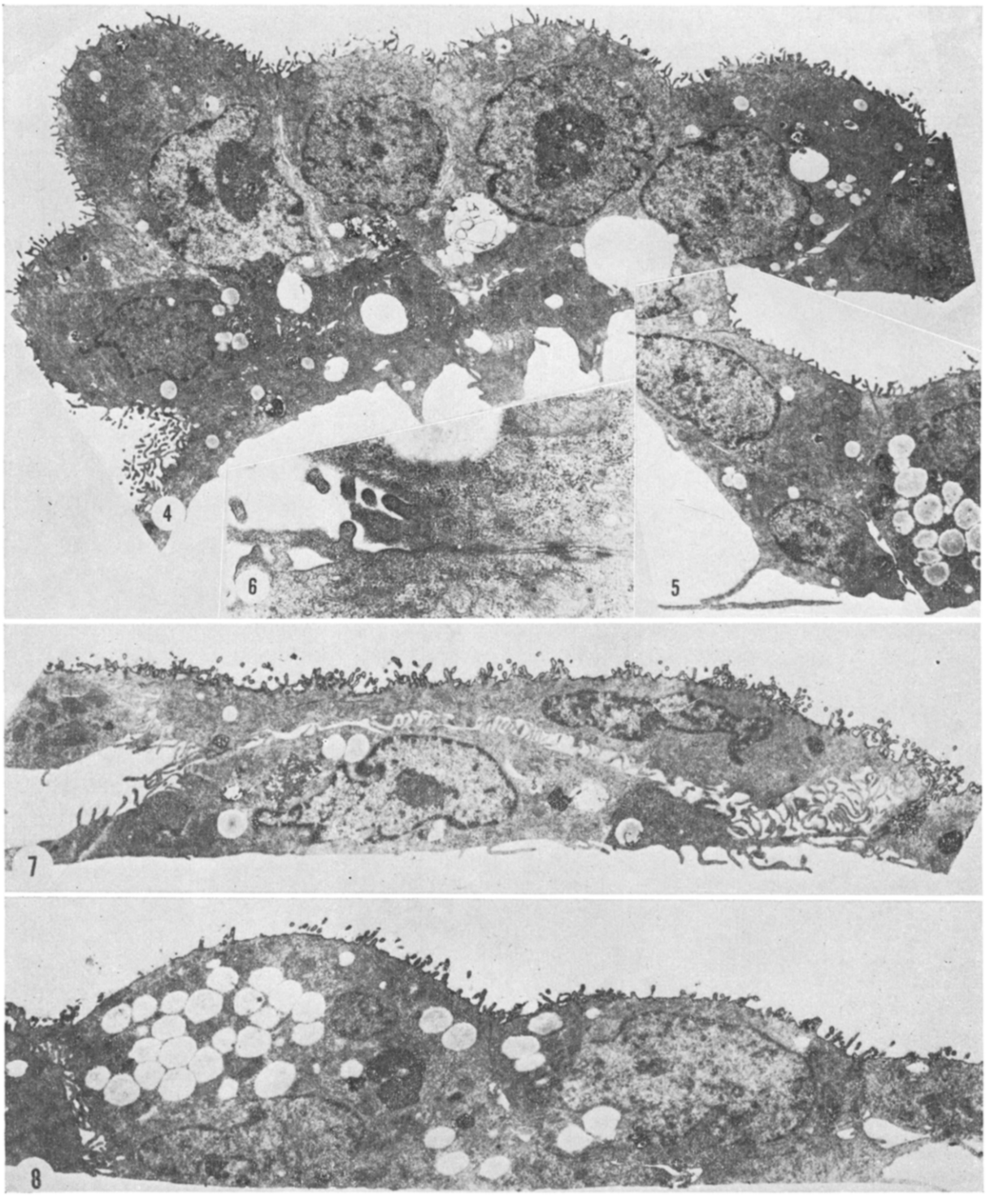


Fig. 1. *A part of the monolayer of HTP/C1 cells. Poorly formed cell contacts characterized by incomplete intercellular junctional complex are prominent.* (× 6000)

Figs. 2 *and* 3. *Intercellular junction of HTP/C1 cells. Neither intermediate junctions nor desmosomes were observed.* (× 20000)

(to face page 494).



Figs. 4 *and* 5. *Parts of HTP/C1/P8 monolayer are characterized by narrow intercellular spaces, abundant mitochondria and rough endoplasmic reticula. Lipid droplets are also seen.* (× 10000)

Fig. 6. *Intercellular junction of HTP/C1/P8 cells. A desmosome is clearly present.* (× 40000)

Fig. 7. *The monolayer of HTP/C1 cells stained with ruthenium red. Only free cell surface is stained.* (× 10000)

Fig. 8. *Ruthenium red-stained HTP/C1/P8 monolayer.* (× 10000)

REFERENCES

1. R. W. KISTNER, Histological effects of progestins on hyperplasia and carcinoma in situ of endometrium. *Cancer (Philad.)* **12,** 1106 (1959).
2. R. W. KISTNER, C. T. GRIFFITHS and J. CRAIG, Use of progestational agents in the management of endometrial cancer. *Cancer (Philad.)* **18,** 1563 (1965).
3. W. B. WENTZ, Effect of a progestational agent on endometrial hyperplasia and endometrial cancer. *Obstet. Gynecol.* **24,** 370 (1964).
4. A. VERGA and E. HENRIKSEN, Histologic observations on the effect of 17-alpha-hydroxyprogesterone-17-*n*-caproate on endometrial carcinoma. *Obstet. Gynecol.* **26,** 656 (1965).
5. D. G. ANDERSON, Management of advanced endometrial adenocarcinoma with medroxyprogesterone acetate. *Am. J. Obstet. Gynecol.* **92,** 87 (1965).
6. P. BERGSJÖ, Progesterone and progestational compounds in the treatment of advanced endometrial carcinoma. *Acta endocr. (Kbh)* **49,** 412 (1965).
7. J. B. BONTE, A. DROCHMANS and P. IDE, 6-Alpha-methyl-17 alpha-hydroxy-progesterone acetate as a chemotherapeutic agents in adenocarcinoma of the uterus. *Acta obstet. gynec. scand.* **45,** 121 (1966).
8. C. N. HUDSON, M. CHIR and A. G. STANSFELD, The histological changes produced in untreated adenocarcinoma of the endometrium by a progestogen. *J. Obstet. Gynaec. Brit. Cwlth.* **74,** 442 (1967).
9. J. HUSTIN, Endometrial carcinoma and synthetic progestogens results of intrauterine treatment. *J. Obstet. Gynaec. Brit. Cwlth.* **77,** 915 (1970).
10. N. MOE, Short-term progestogen treatment of endometrial carcinoma. *Acta obstet. gynecol. scand.* **51,** 55 (1972).
11. A. H. JOHN, J. S. CORNES, W. D. JACKSON and P. BYE, Effect of systemically administered progesterone on histopathology of endometrial carcinoma. *J. Obstet. Gynaec. Brit. Cwlth.* **81,** 786 (1974).
12. S. SEKIYA, M. KAMIYAMA and H. TAKAMIZAWA, Cellular differentiation of rat uterine adenocarcinoma cells by progesterone *in vitro*. *Cancer Res.* **35,** 1713 (1975).
13. S. SEKIYA, H. TAKAMIZAWA, F. WANG, T. TAKANE and T. KUWATA, *In vivo* and *in vitro* studies on uterine adenocarcinoma of rat induced by 7,12-dimethylbenz(a)anthracene. *Am. J. Obstet. Gynecol.* **113,** 691 (1972).
14. S. SEKIYA, Y. KIKUCHI and H. TAKAMIZAWA, High- and low-tumorigenic culture lines of rat uterine adenocarcinoma and their isozyme patterns of lactate dehydrogenase and hexokinase. *Cancer Res.* **33,** 3324 (1973).
15. S. SEKIYA, Y. KIKUCHI and H. TAKAMIZAWA, The effects of hormones and chemotherapeutic agents on rat uterine adenocarcinoma cells in tissue culture. *Am. J. Obstet. Gynecol.* **119,** 675 (1974).
16. A. MARTINEZ-PALOMO and C. BRAILVSKY, Surface layer in tumor cells transformed by adeno-12 and SV_{40} viruses. *Virology* **34,** 379 (1968).
17. H. R. MORGAN, Ultrastructure of the surfaces of cells infected with avian leukosis-sarcoma viruses. *J. Virol.* **2,** 1133 (1968).
18. O. NILSSON, Electron microscopy of the human endometrial carcinoma. *Cancer Res.* **22,** 492 (1962).
19. O. NILSSON, H. L. KOTTMEIER and K. G. TILLENGER, Some differences in the ultrastructure of normal and cancerous human uterine epithelium. *Acta obstet. gynecol. scand.* **42,** 73 (1963).
20. C. GOMPEL, Ultrastructure of endometrial carcinoma. Review of fourteen cases. *Cancer (Philad.)* **28,** 745 (1971).
21. T. V. THRASHER and R. M. RICHERT, An ultrastructural comparison of endometrial adenocarcinoma and normal endometrium. *Cancer (Philad.)* **29,** 1713 (1972).
22. A. J. WHITE and H. J. BUCHSBAUM, Scanning electron microscopy of the human endometrium. II. Hyperplasia and adenocarcinoma. *Gynecol. Oncol.* **2,** 1 (1974).
23. K. HAYAKAWA, Y. OKUDAIRA, K. NASU, H. IIJMA and I. TAKI, Effect of gestagen on uterine adenocarcinoma of the endometrium in the human, with special reference to electron microscope study. *J. Jap. obstet. gynec. Soc.* **20,** 1528 (1968). (In Japanese).

24. K. HAYAKAWA, Effects of dimethisterone on endometrial adenocarcinoma: electron microscopic study. *Acta obstet. gynec. Jap.* **16,** 285 (1969).
25. E. AKASHI, Effects of gestagen on human endometrial adenocarcinoma—electronmicroscopic study. *J. Jap. obstet. gynec. Soc.* **25,** 851 (1973). (In Japanese).
26. R. M. RICHART and A. FERENCZY, Endometrial morphologic response to hormonal environment. *Gynecol. Oncol.* **2,** 180 (1974).

Europ. J. Cancer Vol. 12, pp. 497–498. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *June, 1976*

Human Cancer

G. Klein, G. Clements, J. Zeuthen and A. Westman: Somatic cell hybrids between human lymphoma lines. II. Spontaneous and induced patterns of the Epstein–Barr virus (EBV) cycle.

E. Yefenof, G. Klein, M. Jondal and M. B. A. Oldstone: Surface markers on human B and T lymphocytes. IX. Two-colour immunofluorescence studies on the association between EBV receptors and complement receptors on the surface of lymphoid cell lines.

C. O'Toole, S. Nayak, Z. Price, W. H. Gilbert and J. Waisman: A cell-line (SCaBER) derived from squamous cell carcinoma of the human urinary bladder.

J. L. Ziegler, M. Anderson, G. Klein and W. Henle: Detection of Epstein–Barr virus DNA in American Burkitt's lymphoma.

Experimental Cancer

T. Tanino, M. Saito and K. Egawa: The cell line-specific cell surface components of hypotetraploid axcitic mammary carcinoma cells which induce humoral reactions in the syngeneic host.

T. Tanino and K. Egawa: Humoral reactions by the syngeneic host against incomplete saccharide moieties on tumor cell surface.

K. Egawa and T. Tanino: The induction of immunity against tumor cell transplantation with a complex of tumor cell surface substance and a serum component found in the tumor-regressor animals.

E. Podmaniczky, B. Szende, K. Lapis and G. Ferenc: Particular cell surface changes observed in MC–29 virus infected chicken-embryo fibroblast (CEC) cultures.

M. Börzsönyi, A. Pintér, A. Surján and I. Farkas: Transplacental induction of lymphomas in Swiss mice by carbendazim and sodium nitrite.

I. Hellström and K. E. Hellström: Specific sensitization of lymphocytes to tumor antigens by co-cultivation with peritoneal cells exposed to such antigens.

C. Biddle: Stimulation of transplanted 3-methylcholanthrene-induced sarcomas in mice by specific immune and by normal serum.

D. J. Koropatnick and H. F. Stich: DNA fragmentation in mouse gastric epithelial cells by precarcinogens, ultimate carcinogens and nitrosation products: An indicator for the determination of organotropy and metabolic activation.

A. D. Altstein, V. M. Zhdanov, T. N. Omelchenko, S. G. Dzagurov, G. G. Miller and J. Zavada: Phenotypic mixing of vesicular stomatitis virus and D-type oncornavirus.

L. Falk, F. Deinhardt, L. Wolfe, D. Johnson, J. Hilgers and G. de-Thé: Epstein–Barr virus: Experimental infection of *Callithrix jacchus* marmosets.

Y. Ichikawa, M. Maeda and M. Horiuchi: *In vitro* differentiation of Rauscher-virus-induced myeloid leukemia cells.

E. S. Ievleva, N. V. Engelhardt and G. I. Abelev: Specific antigen of murine erythroblasts.

L. A. Radov, J. S. Haskill and J. H. Korn: Host immune potentiation of drug responses to a murine mammary adenocarcinoma.

British Journal of Cancer *June, 1976*

L. P. Shah, R. C. Rees and R. W. Baldwin: Tumour rejection responses in rats sensitized to rat embryonic tissue. I. Rejection of tumour cells implanted subcutaneously and detection of cytoxic lymphoid cells in embryo-sensitized rats.

M. J. Embleton: Serial measurement of tumour-directed cell-mediated cytotoxicity and serum blocking factor correlated with tumour growth, in rats treated with cells of an aminoazo-dye-induced hepatoma and BCG.

G. A. Currie: Serum lysozyme as a market of host resistance. II. Studies in patients with malignant melanoma, hypernephroma or breast carcinoma.

V. Geddes-Dwyer and D. A. Cameron: Influence of exogenous transfer ribonucleic acid on the growth of transplantable 32_p-induced osteosarcomas.

G. Pizza, D. Viza, C. Boucheix and F. Corrado: *In vitro* production of a transfer factor specific for transitional cell carcinoma of the bladder.

C. E. Searle and E. L. Jones: The carcinogenic action of *n*-ethyl-*n*-nitrosourea administered neonatally to mice of the A, C57Bl, DBAf and IF strains.

Muranyi-Kovacs, G. Rudali and J. Imbert: Bioassay of 2,4,5-trichlorophenoxyacetic acid for carcinogenecity in mice.

Harvey D. Preisler, Sigurdur Bjornsson, Mayumi Mori and Gary H. Lyman: Inducers of Friend leukemic cell differentiation *in vitro*—effects of *in vivo* administration.

U. Møller and J. Bojsen: Circadian variations in $32p$ uptake of DMBA-induced mammary tumors and Walker 256 carcinosarcomas in rats.

S. M. Shalet, C. G. Beardwell, P. H. Morris Jones and D. Pearson: Ovarian failure following abdominal irradiation in childhood.

Brief Communications

M. G. Brattain, T. G. Pretlow, J. M. Pittman and Anne Weiler: Variability in the binding of concanavalin A by human colon and colonic tumour I.

R. Saffhill, T. M. Dexter, S. Muldal and N. G. Testa: Terminal deoxynucleotidyl transferase in a case of PH^1 positive infant chronic myelogenous leukemia.

Letter to the Editor

R. Schoental: Alkylating carcinogens: Their acute (alkylating) versus mutagenic and carcinogenic (cross linking) action.

Europ. J. Cancer Vol. 12, pp. 499–510. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Oncogenic Viruses: Implications for Human Diseases (Part II)

J. M. PREVOST
Service de Médecine et Laboratoire d'Investigation Clinique, Institut Jules Bordet, 1000 *Bruxelles, Belgium*

PUTATIVE HUMAN CANCER VIRUSES

AT THE present time, the relationship between viruses and cancer is largely circumstantial and based on indirect evidence, i.e., the "footprint" of viral activity, as outlined in the preceding sections. Most of this evidence was obtained through studies and analogies with animal models and through the development of more sophisticated methodologies, particularly at the molecular level. We shall review here briefly the relationships between some particular viruses and human tumors.

Among DNA viruses, the Epstein–Barr virus (EBV), a member of the Herpes family, is perhaps one of the very first candidates on the list of candidate human tumor viruses. The EBV is recognized as the causal agent of a nonmalignant, self-limited proliferation of B-lymphocytes, infectious mononucleosis with Paul Bunnel heterophile antibodies and is strongly implicated as causal factor in Burkitt's lymphoma and nasopharyngeal carcinoma [5,120,121]. Seroepidemiological surveys have shown the widespread distribution of virus infection as well as specific associations with these diseases. Later, longitudinal serologic studies demonstrated that specific EBV-associated antigen-antibody systems (see Table 4), early and membrane antigens, were directly related to the evolution of the malignancy and were useful prognostic indexes for treatments and follow-up of patients [122]. Tumor cells harbor the virus genome but complete virus is usually seen only after long term culture of biopsies, along with immunological markers such as the early (EA) and viral capsid antigens (VCA); other viral markers, however, such as the membrane antigen complex, the EBV nuclear antigen (EBNA) [123] and by molecular hybridization, the DNA of the virus, often in multiple copies, part of which might be integrated in the cell genome [124–126], are readily demonstrable in the tumor cell itself, as well as in virogenic and nonproducer cell lines. One of the remarkable properties of the EB virus is the transforming activity [120, 127–129] for cord blood leukocytes, exhibited by some isolates, but not by others, and paradoxically more often by virus obtained from the saliva of patients with infectious mononucleosis, healthy chronic bearers, or excreters than from other sources (Burkitt cell lines, for example). Furthermore, inoculation of virus preparations to some monkey species (primates, New-World species) have produced lymphomas similar to the human malignancy, with B-lymphocytes proliferations and corresponding serologic pictures [130–131]. *In vitro* studies in the EB virus system have been hampered by the lack of fully permissive cells in which EBV replicates with a large yield of infectious progeny. The replication is restricted to lymphoid cells, and production of complete virus is incompatible with lymphoid cell proliferations in virogenic cell lines [5]. Multiple antigen-antibody systems (Table 4) have been recognized using various methods but three of them deserve a particular mention: (i) The so-called EBNA [123] is present in every cell harboring the viral genome and might be equivalent to the T-antigen of papova- and adenoviruses; (ii) in parallel to the EBNA, the LYDMA or lymphocytotoxicity dependent membrane antigen, recently described, is detected by lymphocytotoxicity tests on the membrane of cells bearing the EBV DNA; and, (iii) the early antigen complex (EA) [5,138],

Table 4. Epstein–Barr virus and related antigen–antibody systems. Relationships with human diseases

Antigen*	Source of antigen	Source of antibodies (Relations with tumors)	Methods†	References
VCA	Producer cell lines (Fixed smears)	Past EBV Infection (High levels in tumor patients)	IF (d or id)	[132]
MA	Producer cell lines (Living cells) Tumor Cells +	Past EBV Infection (Pattern related to disease)	—IF (d or blocking) —Immunoelectron-microscopy —RIA	[133–135] [136] [137]
EA (D or R)	Non producer cell lines, infected with EBV (Fixed smears)	(D) IM or NPC patients (R) BL patients (Pattern related to disease)	IF (id)	[138]
S (or soluble)	Non producer cell lines, cell extracts or supernatant Tumor cells +	Past EBV infection (Pattern related to disease)	CF	[139,140]
EBNA	Non producer cell lines (Fixed smears) Tumor cells + (Related to the presence of EBV genome in cells)	Past EBV infection	IF (ACIF)	[123]
N	Virus concentrate from Producer Lines	Past EBV infection (Related to MA specificities)	Virus Neutralization	[141–142]
LYDMA	Non producer lines (live cells) (EBV genome containing cells)	Lymphocytes from EBV infected subjects	Lymphocytotoxicity	[143,144]

Abbreviations—

*VCA, viral capsid antigen; MA, membrane antigens; EA, early antigens; S, soluble antigen; EBNA, Epstein-Barr nuclear antigen; N, neutralizing antigen; LYDMA, lymphocyte dependent membrane antigen.

†IF, immunofluorescence, (d) direct or (id) indirect; ACIF, anticomplementary IF; RIA, radioimmunoassay; CF, complement fixation tests; EBV, Epstein-Barr virus; IM, infectious mononucleosis; NPC, nasopharyngeal carcinoma; BL, Burkitt's lymphoma.

an early product of the viral genome whose appearance is incompatible with cell survival. Generally, EA is spontaneously present only in a very small percentage of transformed cells and rarely in tumor cells except after explantation in tissue culture. This fact appears paradoxical in relation to the *in vivo* immunological picture (high antibody titers, with a pattern clearly related to the evolution of the tumor) in Burkitt's lymphomas and anaplastic nasopharyngeal carcinomas [122,145], but might perhaps be an indicator of the tumor cell burden, a proportion of which spontaneously produce EA and then die and expell, in the extracellular medium, antigens immediately recognized by the immunocompetent cells.

In the nasopharyngeal carcinoma (NPC) story, the situation was far less clear despite strong serologic association [146] very similar to the Burkitt's lymphoma. However, recent reports associate the virus genome with the epithelial malignant cell: the *in situ* hybridization technique on cytological preparations [147] and the transplantation of NPC tumors in homozygous Nude mice [148], procedures allowing the cytological identification of the genome bearing cells or the elimination of lymphoid cell infiltrates with concomittant enrichment in epithelial cells. The EBNA was particularly useful in this context. This does not constitute a proof of causal relationship, however, and reminds us of the particular dual tropism of other members of the herpesvirus group for epithelial and lymphoid cells.

EBV is also associated, by serological analysis (high anti-EBV, VCA titers), with other lymphomatous proliferations, notably Hodgkin's disease [149, 150], chronic lymphocytic leukemias [151] and nonmalignant diseases characterized by autoimmunity and abnormal lymphocyte functions, such as Sarcoidosis [152] and Systemic Lupus Erythematosus [153].

These observations may be explainable, however, by indirect effects of the disease on virus bearing lymphocytes or on the immunological containment mechanisms for the herpesvirus carrier state, established earlier after primary infections. No EBV DNA is present in tumor cells in these cases [154].

These facts are impressive indeed, but obscured by two other findings. First, the so-called non-African Burkitt's tumor is very similar histologically to the African lymphoma but on immunological, serological and biochemical grounds, completely lacks relationships with EBV in most cases [155, 156]. Secondly, Kufe and other members of Spiegelman's group demonstrated the presence of a particulate fraction containing reverse transcriptase and high molecular weight RNA, characteristic of leukoviruses, in Burkitt's lymphoma biopsies and NPCarcinomas but not from specimens of infectious mononucleosis or leukocytes from normal patients; they also found newly acquired DNA sequences, related to the Rauscher-murine leukemia virus in the same cells [157]. Questions arise immediately as to which is the primary cause or the cofactor, whether both act in cooperation or whether one or both is nonessential. These questions are still unresolved but they emphasize the need for distinguishing between virogenic and transforming functions, i.e., the incompatibility of some viral functions leading to virus production and cell death versus cellular proliferation and the problems of establishing correlations between clinical, pathological pictures and viroimmunologic parameters in attempting to assess the role of viruses in cancer.

The problems outlined in the EBV case are much more acute for herpes simplex viruses and their oncogenic potential in humans. Their lytic interaction with cells *in vitro*, and sometimes *in vivo* (immunologic disabilities), contrasts with their propensity to induce long lasting recurrent or latent infections [110, 158]. The association between herpes simplex and human cancer was first based on observations of tumors occasionally arising at sites of recurrent herpetic lesions and documented extensively thereafter by serologic correlations. Table 5 summarizes the arguments relating Herpes simplex to some form of human tumors, for Herpes simplex hominis type 2 and cervical carcinoma particularly. Altogether these findings cannot exclude either a causal role of the virus in squamous cancer or an "innocent bystander", a passenger state. Two observations are particularly important in this context: (i) the model of Herpes simplex transformation of hamster cells, developed by Rapp and coworkers, after reducing the lytic potential of the virus by u.v. irradiation or photodynamic inactivation [163] and (ii) the possibility of analysis of putative transforming functions by the demonstration of infectious properties of the viral DNA [170] or fragment thereof and

Table 5. *Summary of the arguments relating Herpes simplex virus type* 2 *and cervical carcinoma*

I.	SEROEPIDEMIOLOGY: [159, 160]
	—Sexual transmission of HSV type 2
	—Comparative epidemiology of HSV infection and cervical cancer
	—Herpes infection precedes for the development of premalignant and preinvasive lesions of cervix
	—Excess viral antibody in cancer patients
II.	VIROLOGICAL ARGUMENTS:
	—HSV, ubiquitous agent, propensy to latent and recurrent infections [110]
	—Recurrent infections at the site of cancer lesion
	—Virus recovered from atypical or cancer cells [161]
	—Viral or virus induced (specific nonstructural) antigens in tumor cells [162]
	—*In vitro* transformation by HSV (hamster, mouse, rat, human cells [163–165]
	—chromosomal aberrations [166]
III.	IMMUNOLOGICAL ARGUMENTS:
	—Virus specific antigens in cancer cells [162]
	—Prevalence of antiviral antibodies higher in cases of invasive cervical carcinoma, *in situ* and atypia than in control groups (Seroneutralization) [159, 160]
	—Nonvirionic (or nonstructural) viral antibodies: prevalent in cancer patients—relationship to cancer evolution and prognosis [162, 167, 168]
IV.	HERPES VIRUS GENETIC INFORMATION IN CANCER CELLS:
	—Virus structural and nonstructural antigens in cancer cells and premalignant lesions [162]
	—Detection of nucleic acids (fragment HSV-2 DNA) in one case of invasive cervical cancer [154, 169]
V.	ANALOGIES WITH ANIMAL MODELS:
	—Hamster model for HSV transformation [163]
	—Other oncogenic herpes viruses: Marek's disease (MDHV), *Herpesvirus saimiri* and Lucke frog carcinoma (Adenocarcinoma of the kidney)
VI.	ONCOGENIC POTENTIAL OF OTHER HUMAN HERPES VIRUSES:
	—Epstein-Barr virus (EBV), Burkitt's lymphomas and nasopharyngeal carcinomas (NPC)
	—HSV Type 1 (?)

the transforming properties of conditional lethal mutants of Herpes simplex virus [165, 171]. These models have now been extended to transformation assays in other cells (mice, rat) [164, 165] and, in addition, have raised the question that interactions between herpes simplex and other viruses, like oncornavirus or endogenous integrated viruses, might permit the expression of the oncogenic potential of the DNA virus [164, 172]. Particularly relevant to the distinction between lytic and transforming properties of the virus has been the demonstration of early and nonvirionic (non-structural) antigens in cervical tumors which appear to be tumor specific [162]. The level of antibodies directed at these constituants has some prognostic significance, but these results are the subject of controversy due to the lack of reproducibility of some tests in some laboratories but not in others [162, 167, 168, 173]. At the molecular level, hybridization experiments with very sensitive methods are very frustrating at the present time; only one tumor was found to contain viral genetic information, corresponding to only one part of the viral genome (about 40%) [154, 169].

On epidemiological grounds, the relationships between squamous cervical cancer and HSV hominis, type 2 is far from clear. Careful controlled studies showed certainly that herpetic genital infections are among the covariables associated with cervical carcinoma and sexual activities [159, 160], but that other viruses have also the same epidemiologic patterns, such as the condyloma acuminata virus, a member of the papovavirus group [154]. The question then arises; Are other DNA viruses linked to human tumors? Extensive studies have ruled out the role of human adenoviruses in human cancer [9]; on the other hand, the recent isolates of members of the papovavirus group from the urogenital tract [174] and from brain cultures [175] deserve particular attention, with regard to their biology in other systems [176].

RNA tumor viruses are known to cause sarcomas and leukemia-lymphomas in a variety of animals, in chickens, mice, cats, monkeys and probably guinea-pigs and bovines, and to cause a carcinoma, mammary cancer, in mice. The existence of such an etiological relationship between viruses and tumors in natural hosts does not prove that this situation must also prevail in man. Nevertheless, based on biochemical, biophysical and biological properties of C- and B-type RNA viruses in animal models, evidence is now accumulating that such viruses are indeed present in man and in some human tumors. These "putative human viruses" or their footprints are somewhat related to their animal counterparts.

The demonstration by Dmochowski and coworkers of virus particles of typical morphology in murine leukemia and later, in tissues of some patients with leukemia and lymphoma, was an important first step and raised the interest in the search for viruses in tumors in man [177]. Many years later, in 1970, Moore and his colleagues [178, 179], studying a particular population in India characterized by an extensive consanguinity and a high prevalence of breast cancer, extended clearly the initial observations of Dmoschowski [180] and Gross [181]: B-type particles, with morphology similar to the mouse agent, were present in the milk and in some tumors. Furthermore, the virus bore some antigenic relationship to the mouse mammary tumor virus (MMTV) as demonstrated by the presence of antibodies against MMTV in human sera [182]. In the American population, B-type particles were found by electron microscopy, in the milk of 5% of women with no familial history of breast cancer, compared to 60% of those with a positive history [178]. But these viruses, like C-type particles, were not confined to tumor cells and were found in tissues which never become neoplastic; they are present long before clinical symptoms developed. Type C particles have also been identified in embryos and placental structures of man and in a variety of mammals. They are present in secretions (saliva, milk) and urine, raising questions about their mode of transmission in the human genus [183–186].

The discovery of the enzyme reverse transcriptase provided, in fact, a powerful and much more sensitive molecular aid in approaching the problems of cancer etiology and in searching for viral agents in tumors. The properties and requirements of the enzyme were rapidly characterized (Table 2) and using these new kinds of probes, specific for RNA tumor viruses, Spiegelman and coworkers were able to demonstrate the presence of particulate material having all the characteristics of RNA tumor viruses in leukemia, lymphoma, sarcomas [187] and breast cancer [188]. These included the presence of reverse transcriptase, high molecular weight RNA with 70S sedimentation coefficient and particles having a buoyant density of 1·14–1·18 g/ml in sucrose. These tests are known now as the simultaneous detection test [189]. In addition, the reverse transcriptase allowed for the *in vitro* synthesis of radioactive DNA transcripts from the viral

RNA template, from murine leukemia viruses for example, and attempts were soon successful in detecting related sequences between this labelled probe and nucleic acids extracted from neoplastic cells (molecular hybridization). In the surveys of a variety of human tumors, for which there were suitable animal models, a pattern emerged in concordance with the predictions deducible from murine systems. Leukemia, lymphomas and sarcomas contained enough RNA sequences, in their polysomes, homologous to the genome of the murine leukemia–sarcoma complex (Rauscher leukemia virus) to form detectable, stable hybrids; human breast cancer contained sequences related to the MMTV; in the same condition, none of these tumors contained nucleic acid sequences detectably hybridizable to that of avian viruses.

Other tumors were studied along with these methods, but at this time without experimental animal background and many neoplasms were found to contain particles related to oncornaviruses, with the simultaneous detection test: brain tumors [190], pulmonary carcinomas, tumors of the digestive tract [191], etc. The sequences transcribed *in vitro* into a DNA probe were isolated, back-hybridized to the cell nucleic acids and found specific for each tumor localization; no related sequences were detected in corresponding normal tissues.

The question arises then as to whether viral related sequences are transmitted vertically (oncogene theory) or horizontally. In other words, are these sequences present in each cell or do the malignant cells contain virus related information which is not present in normal tissues? Two approaches have been useful in answering these questions. The labeled DNA probes synthesized from the particulate material obtained from malignant cells were purified by extensive hybridization and recycling with normal DNA and then hybridized to normal or tumoral cell DNA. Stable Crick–Watson pairs were not obtained with the normal DNA but only with nucleic acids extracted from tumors, indicating that a new set of genetic information was associated with the malignant state [192, 193]. However, these experiments did not rule out the possibility of transmission of this set of genes via the germ line. The second approach tries to overcome these critics through the study of monozygous twin pairs, one of which is leukemic [194]. A hybridization-recycling experiment as outlined above was performed. The leukemic twin had in his DNA particle related sequences not detectable in the leucocytes of his healthy sibling. The same situation held, at random, between leukemic and other nonleukemic patients. Furthermore, this newly acquired information is related to the genome of animal oncornaviruses.

Gallo and associates, on the other hand, were focusing their studies on the properties and distinguishing features of DNA polymerases in normal and leukemic lymphocytes [195, 196]. An enzyme, clearly distinct from normal cell components by its biochemical and biophysical properties (as outlined in Table 2) was observed in the postmitochondrial cytoplasmic particulate components in association with high molecular weight 70S RNA in cells from some patients with acute leukemia. In addition, by immunological criteria, this reverse transcriptase was related to primate C-type isolates, the Gibbon ape leukemia virus (GALV) and the woolly monkey sarcoma virus (WMSV or SSV-1) and also to murine leukemia virus, but less extensively [197–199].

The relationships between primate viral constituents and virus traces in human tumors (genetic sequences, enzyme) [200] suggest that other virus products such as polypeptides and glycoproteins should also be related on immunological grounds. Todaro and his group [201, 202], working in parallel to Gallo, Strand and August's group [203], used to test this prediction the very sensitive and quantitative radioimmunoassay technique for viral specific proteins, using polypeptides isolated from primate viruses (SSV-1) or others. So far, virus constituants antigenically related to polypeptides of SSV-1 virus have been identified in some cases of human leukemia [202] but the significance and potential of these observations is not understood, for Strand and August detect some viral components in normal tissues or in nonmalignant specimens, such as in S. L. E. [203]. Furthermore, Gilden and associates [204] reported that the screening of serum samples of patients with various tumors and controls, by radioimmunoassay for antibody detection against structural virus constituents, was uniformly negative; the antigen used in their report was the major polypeptide of one murine leukemia virus and was based on the fact that this antigen bears interspecies mammalian determinants. Another observation is relevant to these reports: Leukemia and Hodgkin's disease are sometimes associated with damages at the level of the renal glomerulus. This glomerulosclerosis or chronic membranous glomerulonephritis is caused by deposits of immune complexes, complement components and fibrin as in some animal

models [205, 206]. Studying renal eluates, Sutherland and collaborators showed that virus related constituants were present in the deposits, such as virus group-specific antigens, and related antibodies [207]. It must be constantly kept in mind, in connection to these reports, that experimental animal models have shown that virus polypeptides and glycoproteins are expressed in normal cells, in the cytoplasm and at the cell membrane as the result of the presence and expression of integrated genome of endogenous viruses, as outlined in the preceding sections.

Other approaches, followed by the same groups as well as others, were attempts to activate viruses or to isolate viruses from tumor cells. Various methods are at hand now, coupled with the very potent biochemical and immunological methods: derivation of cell lines from tumors [208], short term tissue culture [209], immunological activation of potential virus bearing lymphocytes [210, 211], chemical induction [212], functional complementation of defective viruses by extraneous leukemia or helper viruses or interactions with other nontumorigenic viruses. But immediately the question of origin and identification of the cells and the viral isolates comes to our mind; this can actually be ascertained by current technologies, as well as for the cell (chromosome patterns and markers, isoenzymes, transplantation antigens or HL-A typing ,etc.) [213] as the virus (Table 1 and 2). Misleading claims—and perhaps much more of human virus isolations were rapidly corrected along these lines. Other reports, such as the demonstration of characteristic virus particles in long term human cell lines [214, 215], await further assessment of their role in human diseases. *In vivo* activation, on the other hand, gave confusing results. McAllister and colleagues were able to transplant successfully rhabdomyosarcoma cells into very young kittens and isolate a virus from the transplanted tumor cells after tissue culture explantation [175]. The virus known as RD-114 was thought at first to be of human origin but as soon as nucleic acid hybridization and reverse transcriptase immune enzyme inhibition were performed it was obvious that in fact, an unknown virus of feline origin was picked up [20]. This holds true also for a similar attempt made by Todaro and others with the same RD cells injected in ALS treated NIH Swiss mice: the AT-124 was activated from mouse cells and was able to replicate readily in the human cells [75]. The isolate was characterized as a murine virus.

All these works are actually culminating in long term attempts to grow, in the laboratory, viruses or isolates from human tumors. MacGrath and coworkers reported first the continuous production of viruses from a mammary tumor derived cell line in tissue culture, by using culture media conditioned by the addition of hormones, insuline, corticosteroids and estrogens [6]. More recently, Gallo and Gallagher repeatedly isolated C-type viruses from one case of acute myelogenous leukemia again using conditioned media, from a non-characterized human fetal culture which was able to support the continuous growth and differentiation of white blood cellular elements. In these two cases, characterization of the isolates was made as far as the origin of the cells and the virus were concerned by methods outlined above. The isolate from the mammary tumor culture has typical B-type morphology and all characteristics of oncornaviruses; it also bears some antigenic determinants related to the MMTV [6, 216]. The leukemia isolate is a C-type particle and is related immunologically to primate oncornaviruses, particularly the woolly monkey sarcoma virus (SSV-1) and gibbon ape isolate (GALV) [7]. The question now is whether this "leukemia" virus is an endogenous or an exogenous form and to ascertain its origin. One very puzzling point is the fact that Gallagher and Gallo were unable to detect, in leukemic cells, DNA sequences related to the RNA genome of their isolate [217]. The results obtained by Mak and others are well in line with those of Gallo's group, when considering the relationships between putative human leukemia viruses and primate C-type isolates [218].

CONCLUSIONS

The last decade and particularly the past 5 years were characterized by a tremendous amount of new acquisitions and new knowledge in the wide field of tumor viruses, as well as in the biology of these agents using the new technology developed in these researches. We are still unable to answer the question "Are viruses etiologically implicated in human tumors, and if so, how"? The words of R. Weiss are particularly relevant to these questions: "Are viruses causing cancer or are viruses induced by cancer?" Nevertheless, a great deal of hope arises, with the recognition of consistent association between tumor and viruses, at three practical levels for patients, in addition to progress made in various forms of treatments: immunodiagnosis and immunotherapy,

immunoprophylaxis and therapy directed at causative agents. First, knowledge on the biology of agents closely related to tumors might permit us to devise specific early virus-related detection tests, particularly useful in high risk populations, as well as preventive measures associated with cancer screening. Viral components and virus products coded for by the viral genome in the cell (nonstructural components) are the targets for those assay; in other words, the recognition of transformation-related and virogenic functions is acutely needed in order to be able to use tumor specific virus-directed tests. In addition, research directed at the biology of the tumor and virus associated antigens, particularly at the immunological level, might resolve arguments concerning the etiology of the disease. Moreover, immunopreventive attitudes might be another important aim, perhaps on a more long term issue, for the assessment of these measures needs work on large population samples and long term follow-up before being able to see any significant effect on the incidence of a particular tumor. Experimental models are particularly useful to circumvent these problems, as long as these situations are comparable with each other. As proposed by M. A. Epstein [219], one particular tumor is particularly interesting in this context: the EBV and Burkitt's lymphoma in Africa, where a clear response as far as efficacy is concerned might be established in a decade or so by the application of some kind of vaccine in a very young high risk population. In case of success, arguments regarding the etiology of the disease might also be available. But these propositions, if certainly valid, are not the only ones to be studied in these cases, for the role of cofactors such as malaria might be equally important in the Burkitt's lymphoma context.

Finally, if we have better and better understanding of the molecular mechanisms underlying malignant transformation by viruses one might soon be able to devise appropriate therapeutic drugs or agents more specific for the malignant event and less and less toxic for normal cell components.

Acknowledgements—I am indebted to Dr. Gary R. Pearson, National Cancer Institute, Bethesda, Maryland, for his valuable suggestions and critical discussion. I also greatly appreciate the continuous support of Professor H. Tagnon, Dr. L. Thiry and Dr. J. Klastersky.

REFERENCES

120. G. Miller, The oncogenicity of Epstein–Barr virus. *J. inf. Dis.* **130,** 187 (1974).
121. M. A. Epstein and B. G. Achong, Various forms of Epstein–Barr virus infection in man: established facts and a general concept. *Lancet* **ii,** 836 (1973).
122. W. Henle and G. Henle, Epstein–Barr virus and human malignancies. *Cancer (Philad.)* **34,** 1368 (1974).
123. B. M. Reedman and G. Klein, Cellular localisation of an Epstein–Barr virus (EBV) associated complement fixing antigen in producer and non-producer lymphoblastoid cell lines. *Int. J. Cancer* **11,** 499 (1973).
124. M. Nonoyama and J. S. Pagano, Detection of Epstein–Barr viral genome in non-productive cells. *Nature New Biology* **233,** 103 (1971).
125. H. zur Hausen, H. Schulte-Holthausen, G. Henle, W. Henle, P. Clifford and L. Santeson, Epstein–Barr virus DNA in biopsies of Burkitt's Lymphoma and anaplastic carcinomas of the nasopharynx. *Nature (Lond.)* **288,** 1056 (1970).
126. A. Tanaka and M. Nonoyama, Latent DNA of Epstein–Barr virus: separation from high molecular weight cell DNA in a neutral glycerol gradient. *Proc. nat. Acad. Sci. Wash.* **71,** 4658 (1974).
127. R. S. Chang, Umbilical cord leukocytes transformed by lymphoid cell filtrates from healthy peoples. *Nature New Biology* **233,** 124 (1971).
128. R. S. Chang and H. D. Golden, Transformation of human leukocytes by throat washings from infectious mononucleosis patients. *Nature (Lond.)* **234,** 359 (1971).
129. G. Miller, J. C. Niederman and L. L. Andrews, Prolonged oropharyngeal excretion of Epstein–Barr virus after infectious mononucleosis. *New Engl. J. Med.* **289,** 229 (1973).
130. T. Shope, D. Dechairo and G. Miller, Malignant lymphoma in cotton top marmosets after inoculation with Epstein–Barr virus. *Proc. nat. Acad. Sci. Wash.* **70,** 2487 (1973).

131. F. Deinhardt, L. A. Falk and F. G. Wolfe. Transformation of non human primate lymphocytes by Epstein–Barr virus. *Cancer Res.* **34,** 1241 (1974).
132. G. Henle, W. Henle, P. Clifford, V. Diehl, G. W. Kafuko, B. Kirya, G. Klein, R. H. Morrow, G. M. Munube, P. Pike, P. M. Tukei and J. L. Ziegler, Antibodies to EBV in Burkitt's Lymphoma and control group. *J. nat. Cancer Inst.* **43,** 1147 (1969).
133. G. Klein, P. Clifford, E. Klein, R. T. Smith, J. Minowada, M. Kourilsky and J. H. Burchenal, Membrane fluorescence reaction of Burkitt's lymphoma cells from biopsy specimen and tissue culture. *J. nat. Cancer Inst.* **39,** 1027 (1967).
134. G. Goldstein, G. Klein, G. Pearson and P. Clifford, Direct membrane fluorescence reaction of Burkitt's lymphoma cells in culture. *Cancer Res.* **29,** 749 (1969).
135. G. Pearson, G. Klein, G. Henle, W. Henle and P. Clifford, Relation between EBV and cell membrane fluorescence in Burkitt's tumor cells—IV/ Differentiation between antibodies responsible for membrane and viral fluorescence. *J. exp. Med.* **129,** 707 (1969).
136. D. Silvestre, F. M. Kourilsky, G. Klein, Y. Yata, C. Neauport-Sautes, and J. P. Levy, Relationship between the EBV associated membrane antigen on Burkitt's lymphoma cells and the viral enveloppe, demonstrated by immunoferritin labelling. *Int. J. Cancer* **8,** 222 (1971).
137. J. F. Hewetson, B. Gotoshkar and G. Klein, Radioiodine labelled antibody test for the detection of membrane antigens associated to Epstein–Barr virus. *J. nat. Cancer Inst.* **48,** 87 (1972).
138. G. Henle, W. Henle and G. Klein, Demonstration of two distinct components in the early antigen complex of EB virus infected cells. *Int. J. Cancer* **8,** 272 (1971).
139. D. Armstrong, G. Henle and W. Henle, Complement fixation tests with lines derived from Burkitt's lymphoma and acute leukemia. *J. Bact.* **91,** 1257 (1966).
140. J. H. Pope, M. K. Hoene and E. J. Wetters, Significance of complement fixing antigens associated to herpes like virus and detected in Raji cell line. *Nature (Lond.)* **222,** 166 (1969).
141. G. Pearson, F. Dewey, G. Klein, G. Henle and W. Henle, Relation between neutralization of EB virus and antibodies to cell membrane antigens induced by the virus. *J. nat. Cancer Inst.* **45,** 989 (1970).
142. G. Rocchi and J. F. Hewetson, A practical and quantitative microtest for the detection of neutralizing antibodies against EB virus. *J. gen. Virol.* **18,** 385 (1973).
143. E. Svedmyr and M. Jondal, Cytotoxic effector cells specific for B-cell lines transformed by Epstein–Barr virus are present in patients with infectious mononucleosis. *Proc. nat. Acad. Sci. Wash.* **72,** 1622 (1975).
144. G. Klein, Epstein–Barr virus determined membrane antigens. In *International Workshop on Epstein–Barr virus* (Production concentration and purification of EBV and viral antigens), Int. Ag. Res. Cancer, Int. Techn. Rep., 75–003, 729 (1975).
145. W. Henle, G. Henle, P. Gunven, G. Klein, P. Clifford and S. Singh, Patterns of antibodies to Epstein–Barr virus induced early antigens in fatal cases of Burkitt's lymphoma and long term survivors. *J. nat. Cancer Inst.* **50,** 1163 (1973).
146. G. De The and A. Gresser, Nasopharyngeal carcinoma: recent studies and outlook for a viral etiology. *Cancer Res.* **34,** 1196 (1974).
147. H. Wolf, H. zur Hausen, G. Klein, Y. Becker, G. Henle and W. Henle, Attempts to detect virus specific DNA sequences in human tumors III/ Epstein–Barr virus DNA in non lymphoid nasopharyngeal carcinoma cells. *Med. Microbiol. Immunol.* **161,** 15 (1975).
148. G. Klein, B. C. Giovanella, T. Lindhal, P. J. Fialkow, S. Singh and J. S. Stehlin, Direct evidence for the presence of EBV DNA and nuclear antigen in malignant epithelial cells from patients with anaplastic carcinoma of the nasopharynx. *Proc. nat. Acad. Sci. Wash.* **71,** 4737 (1974).
149. B. Johannson, G. Klein, W. Henle and G. Henle, Epstein–Barr (EBV) associated antibody patterns in malignant lymphoma and leukemia. I/ Hodgkin's disease. *Int. J. Cancer* **6,** 450 (1970).

150. P. H. Levine, D. V. Ablashi, C. W. Berard, P. P. Carbone, D. E. Waggoner and L. Malan, Elevated antibody titers to Epstein–Barr virus in Hodgkin's disease. *Cancer (Philad.)* **27,** 416 (1970).
151. G. Miller, T. Shope, L. Heston, R. O'Brien, A. Schwartz and H. Pearson, Prospective study of EBV infection in acute leukemia of childhood. *J. Pediatrics* **80,** 932 (1972).
152. E. B. Byrne, A. S. Evans, D. W. Fouts and H. L. Israel. A seroepidemiological study of EB virus and other viral antigens in sarcoidosis. *Amer. J. Epid.* **97,** 355 (1973).
153. A. S. Evans, N. F. Rothfield and J. C. Niederman, Raised antibody titers to EB virus in Systemic Lupus Erythematosus. *Lancet* **i,** 167 (1971).
154. H. zur Hausen, DNA viruses in human cancer: biochemical approaches. In *Conference on Immunologic Control of Virus associated Tumors in man: prospects and problems. (Cancer Res.,* in press).
155. P. H. Levine and B. R. Cho, Burkitt's Lymphoma: clinical features of North American cases. *Cancer Res.* **34,** 1219 (1974).
156. J. S. Pagano, C. H. Huang and P. H. Levine, Absence of Epstein–Barr viral DNA in American Burkitt's lymphoma. *New Engl. J. Med.* **289,** 1395 (1973).
157. S. Spiegelman, D. Kufe, R. Hehlman and W. P. Peters, Evidence for RNA tumor viruses in human lymphomas including Burkitt's disease. *Cancer Res.* **33,** 1575 (1973).
158. A. J. Nahmias and B. Roizman, Infection with Herpes Simplex viruses type 1 and 2. *New Engl. J. Med.* **289,** 667 (1973).
159. I. D. Rotkin, A comparison review of key epidemiological studies in cervical carcinoma related to current searches for transmissible agents. *Cancer Res.* **33,** 1353 (1973).
160. A. J. Nahmias, Z. M. Naib and W. E. Josey, Epidemiological studies relating genital herpetic infections to cervical carcinoma. *Cancer Res.* **34,** 1111 (1974).
161. L. Aurelian, J. D. Strandberg, L. V. Melendez and L. A. Johnson, Herpes simplex virus type 2 isolated from cervical carcinoma cells grown in tissue culture. *Science* **174,** 704 (1971).
162. L. Aurelian, Virions and antigens of Herpes simplex virus type 2 in cervical carcinoma. *Cancer Res.* **33,** 1539 (1973).
163. F. Rapp and R. Duff, Transformation of hamster embryo fibroblasts by Herpes simplex viruses type 1 and 2. *Cancer Res.* **33,** 1527 (1973).
164. R. Duff and F. Rapp, Quantitative assay for transformation of 3T3 cells by Herpes simplex type 2. *J. Virol.* **15,** 490 (1975).
165. J. C. MacNab, Transformation of rat embryo cells by temperature sensitive mutants of Herpes simplex virus. *J. gen. Virol.* **24,** 143 (1974).
166. J. O'Neill and F. Rapp, Early events required for the induction of chromosome abnormalities in human cells by Herpes simplex virus. *Virology* **44,** 544 (1971).
167. A. C. Hollinshead, O. Lee, P. B. Chretien, J. L. Tarpley, W. E. Rawls and E. Adam, Antibodies to herpes virus non virion antigen in squamous carcinoma. *Science* **182,** 713 (1973).
168. A. B. Sabin and G. Tarro, Herpes simplex and herpes genitalis virus in etiology of some human cancers. *Proc. nat. Acad. Sci. Wash.* **70,** 3225 (1973).
169. B. Roizman and N. Frenkel, The transcription and state of Herpes simplex virus DNA in human cervical tumor: a comparison with productive infection in human cells. In *Possible Episomes in Eukaryotes* (Edited by L. G. Silvestri) p. 202, North Holland, Amsterdam (1973).
170. F. L. Graham, G. Veldhuisen and N. M. Wilkie, Infectious herpes virus DNA. *Nature New Biology* **245,** 265 (1973).
171. M. Takahashi and K. Yaminishi, Transformation of hamster embryo and human embryo cells by thermosensitive mutants of Herpes simplex virus type 2. *Virology* **61,** 306 (1974).
172. R. Garfinkle and B. R. MacAuslan, Transformation of cultured mammalian cells by viable herpes simplex virus subtype 1 and 2. *Proc. nat. Acad. Sci. Wash.* **71,** 220 (1974).
173. A. B. Sabin, Herpes simplex genitalis virus nonvirion antigen and their implication in certain human cancers, unconfirmed. *Proc. nat. Acad. Sci. Wash.* **71,** 3248 (1974).

174. S. D. Gardner, A. M. Field, D. V. Coleman and B. Hulme, New human papovavirus (BK) isolated from the urine after renal transplantation. *Lancet* **i**, 1253 (1971).
175. B. L. Padgett, D. L. Walker, G. M. Zurhein and R. J. Eckroade, Cultivation of papova-like virus from human brain with progressive multifocal leukoencephalopathy. *Lancet* **i**, 1257 (1971).
176. E. O. Major and G. di Mayorca, Malignant transformation of BHK-21 clone 13 cells by BK virus, a human papovavirus. *Proc. nat. Acad. Sci. Wash.* **70,** 3210 (1973).
177. L. Dmochowski and C. E. Gey, Studies on submicroscopic structures of leukemias of known or suspected viral origin, a review. *Blood* **13,** 1017 (1958).
178. D. H. Moore, J. Charney, B. Kramarsky, E. Y. Lasfargues, N. H. Sarkar, M. J. Brennan, J. H. Burrows, S. M. Sirsat, J. C. Paymaster and A. B. Vaidya, Search for human breast cancer virus. *Nature (Lond.)* **229,** 611 (1971).
179. N. H. Sarkar and D. H. Moore, On the possibility of a human breast cancer virus. *Nature (Lond.)* **236,** 103 (1972).
180. L. Dmochowski, G. Serman and B. Myers, Relationship of viruses to the origin of human breast cancer. *Texas Med. Rec. Ann.* **61,** 384 (1968).
181. L. Gross, H. S. McCarthy and A. E. Gessler, The significance of particles in human milk. *Ann. N.Y. Acad. Sci.* **54,** 1018 (1952).
182. J. Charney and D. H. Moore, Neutralization of Murine Mammary tumor virus (MMTV) by sera of women with breast cancer. *Nature (Lond.)* **229,** 627 (1971).
183. L. Dmochowski, Molecular mechanisms in viral neoplasia of animals and their implication in the origin of cancer in man. *Amer. J. clin. Pathol.* **60,** 3 (1973).
184. S. Chandra, T. Liszczak, W. Korol and E. M. Jensen, Type C particles in human tissues—I/Electron microscopy of embryonic tissues *in vivo* and *in vitro*. *Int. J. Cancer* **6,** 40 (1970).
185. S. S. Kalter, R. J. Helmke, R. L. Heberling, M. Panigel, A. K. Fowler, J. E. Strickland and A. Hellman, C-type particles in normal human placentas. *J. nat. Cancer Inst.* **50,** 1081 (1973).
186. D. E. Strickland, A. K. Fowler, P. P. Kind, A. Hellman, S. S. Kalter, R. L. Heberling and R. J. Helmke, Group specific gs antigen and RNA-dependent-DNA-polymerase activity in normal baboon placenta. *Proc. Soc. exp. Biol. (N.Y.)* **144,** 256 (1973).
187. R. Hehlman, W. Baxt, D. Kufe and S. Spiegelman, Molecular evidences for a viral etiology of human leukemias, lymphomas and sarcomas. *Amer. J. clin. Pathol.* **60,** 65 (1973).
188. J. Schlom and S. Spiegelman, Evidences for viral involvement in murine and human mammary adenocarcinomas. *Amer. J. clin. Pathol.* **60,** 44 (1973).
189. J. Schlom and S. Spiegelman, Simultaneous detection of reverse transcriptase and high molecular weight RNA unique to oncogenic viruses. *Science* **174,** 840 (1971).
190. W. Cuatico, J. R. Cho and S. Spiegelman, Particles with RNA of high molecular weight and RNA-directed-DNA-polymerase in human brain tumors. *Proc. nat. Acad. Sci. Wash.* **70,** 2789 (1973).
191. W. Cuatico, J. R. Cho and S. Spiegelman, Evidence of particles associated RNA-dependent-DNA-polymerase and high molecular weight RNA in human gastro-intestinal and lung malignancies. *Proc. nat. Acad. Sci. Wash.* **71,** 3304 (1974).
192. N. C. Goodman, R. M. Rupprecht, R. W. Sweet, R. Massey, F. Deinhardt and S. Spiegelman, Viral related DNA sequences before and after transformation by RNA tumor viruses. *Int. J. Cancer* **12,** 752 (1973).
193. R. W. Sweet, N. C. Goodman, J. R. Cho, R. M. Rupprecht, R. R. Redfield and S. Spiegelman, The presence of unique DNA sequences after viral induction in mice. *Proc. nat. Acad. Sci. Wash.* **71,** 1705 (1974).
194. W. Baxt, J. W. Yates, H. J. Wallace Jr, J. F. Holland and S. Spiegelman, Leukemia specific DNA sequences in leukocytes of the leukemic member of identical twins. *Proc. nat. Acad. Sci. Wash.* **70,** 2629 (1973).

195. R. C. Gallo, P. S. Sarin, A. M. Wu, M. G. Sarngadharan, M. Reitz, M. S. Robert, N. Miller, W. C. Saxinger and D. Gillespie, On the nature of the nucleic acids and RNA-dependent-DNA-polymerase from RNA tumor viruses and human cells. In *Possible Episomes in Eukaryotes.* (Edited by L. G. Silvestri) p. 14. North Holland, Amsterdam (1973).
196. B. J. Lewis, J. W. Abrell and R. C. Gallo, Human DNA-polymerase III (R-DNA polymerase). Distinction from DNA-polymerase I and Reverse transcriptase. *Science* **183,** 867 (1974).
197. R. C. Gallo, N. R. Miller, W. C. Saxinger and D. Gillespie, Primate RNA tumor virus like DNA synthesized endogenously by RNA-dependent-DNA-polymerase in virus like particles from fresh human acute leukemia blood cells. *Proc. nat. Acad. Sci. Wash.* **70,** 3219 (1973).
198. R. E. Gallagher, G. J. Todaro, R. G. Smith, D. M. Livingston and R. C. Gallo, Relationship between RNA-directed-DNA-polymerase (Reverse transcriptase) from human acute leukemia blood cells and primate type C viruses. *Proc. nat. Acad. Sci. Wash.* **71,** 1309 (1974).
199. R. C. Gallo, R. E. Gallagher, M. G. Sarngadharan, P. Sarin, M. Reitz, N. Miller and D. H. Gillespie, The evidence for involvement of type C RNA tumor viruses in human leukemia. *Cancer (Philad.)* **34,** 1398 (1974).
200. N. R. Miller, W. C. Saxinger, M. S. Reitz, R. E. Gallagher, A. M. Wu, R. C. Gallo and D. H. Gillespie, Systematic of RNA tumor viruses and virus like particles of human origin. *Proc. nat. Acad. Sci. Wash.* **71,** 3177 (1974).
201. C. J. Sherr and G. J. Todaro, Type C viral antigens in man—I/Antigens related to endogenous primate virus in human tumors. *Proc. nat. Acad. Sci. Wash.* **71,** 4703 (1974).
202. C. Sherr and G. J. Todaro, Primate type C virus p30 antigens in cells from humans with acute leukemia. *Science* **187,** 855 (1975).
203. M. Strand and J. T. August, Type C RNA virus gene expression in human tissues. *J. Virol.* **14,** 1584 (1974).
204. H. P. Charman, N. Kim, M. White and R. V. Gilden, Failure to detect in human sera, antibodies cross-reactive with group-specific antigens of murine leukemia virus. *J. nat. Cancer Inst.* **52,** 1409 (1974).
205. J. C. Sutherland, R. V. Markham, H. E. Ramsey and M. R. Mardiney Jr, Subclinical immune complex nephritis in patients with Hodgkin's disease. *Cancer Res.* **34,** 1179 (1974).
206. M. B. Oldstone, T. Aoki and F. J. Dixon, The antibody response of mice to murine leukemia virus in spontaneous infections: absence of classical immunological tolerance. *Proc. nat. Acad. Sci. Wash.* **69,** 134 (1972).
207. J. C. Sutherland and M. R. Mardiney Jr, Immune complex disease in the kidneys of lymphoma-leukemia patients: the presence of an oncornavirus related antigen. *J. nat. Cancer Inst.* **50,** 633 (1973).
208. G. J. Todaro, V. Zeve and S. A. Aaronson, Cell culture technique in the search for cancer virus in man. *In Vitro* **6,** 355 (1971).
209. T. W. Mak, J. Manaster, A. F. Howatson, E. A. McCullock and J. E. Till, Particles with characteristics of leukoviruses in culture of marrow cells from leukemic patients in remission and relapse. *Proc. nat. Acad. Sci. Wash.* **71,** 4336 (1974).
210. P. Lonai, A. Decleve and H. S. Kaplan, Spontaneous induction of endogenous murine leukemia virus related expression during short term *in vitro* incubation of mouse lymphocytes. *Proc. nat. Acad. Sci. Wash.* **71,** 2008 (1974).
211. M. S. Hirsch, S. M. Phillips, C. Solnick, P. H. Black, R. S. Schwartz and C. B. Carpenter, Activation of leukemia viruses by graft-versus-host and mixed lymphocytes reactions *in vitro. Proc. nat. Acad. Sci. Wash.* **69,** 1069 (1972).
212. S. E. Stewart, G. Kasnic, C. Draycott, W. Feller, A. Golden and E. Mitchell, Activation *in vitro* by 5-Iododeoxy-uridine of a latent virus resembling C-type virus in a human sarcoma cell line. *J. nat. Cancer Inst.* **48,** 273 (1972).
213. W. A. Nelson-Rees, Banded marker chromosomes as indicators of intraspecies cellular contamination. *Science* **184,** 1093 (1974).

214. V. M. Zdhanov, V. D. Soloviev, T. A. Betkemirov, K. V. Ilyin, A. F. Bikovsky, N. P. Mazurenko, I. S. Irlin and F. I. Yerhov, Isolation of oncornaviruses from continuous human cell culture. *Intervirology* **1,** 19 (1973).
215. H. Gelderblom, H. Bauer, H. Ogura, R. Wigand and A. B. Fisher, Detection of oncornavirus-like particles in Hela cells.—I/Fine structure and comparative morphological classification.—II/Immunological characterization of the virus.—III/Biochemical characterization of the virus. *Int. J. Cancer* **13,** 246 (1974).
216. C. M. McGrath, P. Furmanski, H. Soule, P. Grant, C. Longley and M. A. Rich, Immunological relationships between MuMTV, 734-B and a virus-like particle isolated from human milk. Abstr. 653, *Proc. Am. Assoc. Cancer Res.* (1975).
217. R. C. Gallo, Biochemical studies of type C viral molecules in leukemia. In *Conference on Immunologic control of virus associated tumors in man, problems and prospects* (*Cancer Res.*, in press).
218. T. W. Mak, S. Kurtz, J. Manaster and D. Heusma, Viral related information in oncorna-virus-like particles isolated from cultures of marrow cells from leukemic patients in relapse and remission. *Proc. nat. Acad. Sci. Wash.* **72,** 623 (1975).
219. M. A. Epstein, Approaches to immunological control of virus associated tumors in man. In *Conference on Immunologic control of virus associated tumors in man, problems and prospects* (*Cancer Res.*, in press).

Europ. J. Cancer Vol. 12, pp. 511–517. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Immunological Aspects of Acute Leukemia in Man

WILLY F. PIESSENS* and WILLIAM C. MOLONEY
Departments of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Massachusetts 02115, *U.S.A.*

Abstract—*During recent years the immunological aspects of acute leukemia in man have been the subject of an ever increasing number of studies. Our purpose is to review progress made in four specific areas of research: the relationship between immune competence and susceptibility to leukemia, the role of immune competence during overt leukemia and its modification by chemotherapy, the characterization of leukemia antigens, and the effect of active immunotherapy on acute leukemia in man.*

1. IMMUNE COMPETENCE AND SUSCEPTIBILITY TO LEUKEMIA

THE SUSCEPTIBILITY of inbred strains of mice to viral leukemias is linked to the major histocompatibility (H-2 locus). The same chromosomal region appears to contain genes controlling the immune responsiveness to various antigens, the susceptibility to leukemia and the major histocompatibility antigens [1, 2]. Whether a similar relationship exists in man between the incidence of leukemia and the histocompatibility (HL-A) type has been the subject of several studies.

HL-A antigens can be detected on leukemia cells by conventional tissue typing methods [3]. Whereas several studies failed to reveal any differences in the number and distribution of HL-A antigens on leukemic cells [3, 4], other studies suggest an increased incidence of certain HL-A antigens or haplotypes in leukemia, in comparison to their incidence in the normal population [5, 6]. In addition, anomalies in the HL-A typing of leukemic cells occur rather regularly [7]. These include the finding of extra HL-A antigens which should not be present on the basis of what can be predicted from genetic family studies, and additional antigens found on cells obtained during active disease but not during remission. The exact nature of these "new" antigens is not clear but similar anomalies of HL-A typing occur after exposure of normal lymphocytes to phytohemagglutinin [8] or proteolytic enzymes [9], indicating that this phenomenon is not specific for leukemia and may represent phenotypic rather than genotypic alterations. Thus, unequivocal evidence for a relationship between the histocompatibility type and the incidence of leukemia in man is not available at the present time.

Other evidence that immune competence correlates with the incidence of leukemia in man is also lacking. Despite some early reports to the contrary [10] it is clear that "immunoprophylaxis" with BCG vaccination at an early age neither increases nor decreases the incidence of acute leukemia in children [11, 12]. Primary immunodeficiency diseases and chronic immunosuppression to prevent the rejection of organ grafts are associated with an increased incidence of tumors. However, most of these are lymphomas and solid tumors rather than leukemias [13, 14]. There is an increased incidence of leukemias following exposure to high doses of radiation, but whether this is due to the leukemogenic effect of irradiation *per se* or to its immunosuppressive effects remains unknown [15].

*Cancer Research Scholar of the American Cancer Society, Massachusetts Division Inc.

Reprint requests to Willy F. Piessens, M.D., Peter Bent Brigham Hospital, 721 Huntington Ave., Boston, MA 02115, U.S.A.

2. IMMUNE COMPETENCE DURING OVERT LEUKEMIA

Studies on the immune competence of patients with overt leukemia and on the effects of chemotherapy on the immune responsiveness are important for several reasons.

Infections continue to be the leading cause of death in the leukemias; therefore, how the modification of the host's immune responses by chemotherapy alters the patient's resistance to infections should be of primary concern to everyone treating this disease.

Furthermore, as immunotherapy is rapidly becoming an acceptable treatment modality, it is important to determine whether the patient's immunity to his own leukemia has any bearing at all on long-term prognosis. The effects of immunosuppressive chemotherapy and of immunotherapeutic manipulations have to be examined with regard to these basic questions.

Most studies reveal a qualitatively normal but a quantitatively decreased general immune competence in patients with acute leukemia [16–20].

In addition, specific immunological reactions against leukemia-associated antigens can be detected in over half of all patients (see below).

It has been claimed that those patients with an intact immune system at the time of diagnosis are more likely to obtain a remission and thus have a better prognosis, presumably because leukemic cells remaining after a drug induced remission are further eliminated by the host's immune defense against "foreign" tumor cells [207]. However, a correlation between immune competence and prognosis can not always be demonstrated [18]. This could be explained by the variable effects of different drug regimens on the immune competence and on the leukemia itself.

It is obvious that if a chemotherapeutic regimen that would effectively eradicate leukemia were available, the effect of the host's immune competence would be largely irrelevant. On the other hand, even the strongest expression of cellular immunity may be without detectable effect on the prognosis following treatment with ineffective drugs that are also immunosuppressive. Thus, the prognostic significance of the patient's immune competence may depend in part on the nature of the chemotherapy, but this possibility has not been adequately studied.

An interesting finding in these studies is the demonstration that immunosuppression by chemotherapeutic drugs is to a large extent reversible, not only after cessation of treatment [21], but also with continued intermittent chemotherapy [22].

Long-term survivors of acute leukemia have high avidity antibody to leukemia-associated antigens in their serum, but this may result from prolonged exposure to low doses of antigen rather than being the cause of high intrinsic resistance to this disease [23]. Thus, the hypothesis that the immunological reactivity of leukemic patients towards their own tumor contributes to the overall prognosis in this disease is an attractive one, but many more studies are needed to settle this problem.

3. LEUKEMIA ANTIGENS

Attempts to demonstrate leukemia-associated antigens have been numerous, have employed a variety of immunological methods, and have met with encouraging albeit variable success [24].

Autoantibodies against leukemic cells have been demonstrated in the sera of untreated patients with several forms of leukemia. Doré obtained positive results in 12 of 51 patients and Yoshida in 15 of 30 patients tested [25, 26]. More recently, antibody dependent lymphocyte killing of myeloblasts has also been reported [27]. The failure to demonstrate autoantibodies in the majority of patients has resulted in numerous attempts to produce leukemia specific heteroantisera by immunizing a variety of animal species with human leukemic cells. The prevalence of positive results varies considerably, although surprisingly less than one might expect from the differences in the clinical material and the immunization procedures used. It is unlikely that all antisera detect the same antigen(s); nevertheless, some generalizations can be made. Following the appropriate absorptions with normal leukocytes, most antisera detect antigens more or less specific for either the myeloid or the lymphoid leukemias. In general, the same antigens appear to be shared by the acute and chronic forms of the same cell type, myeloid or lymphoid. Although most leukemias of a given cell type have common antigens, antigens unique for each individual leukemia can also be detected following the appropriate absorptions of the antisera [28, 29]. By analogy with the situation in murine leukemias, the finding of common leukemia antigens has been interpreted as evidence for a viral origin of human leukemia.

In contrast to the antisera mentioned above, the antibodies developed by Mann and Herbermann react with AML and ALL cells but not

with leukocytes from normal donors or from patients with CML, CLL or Hodgkin's disease [30]. Antisera with similar specificity were developed by Billing and Terasaki using either Raji cells or fresh lymphosarcoma cells as the source of the antigen, which appears to be a glycoprotein [31, 32]. In a long-term study of 10 patients with acute leukemia (8 ALL, 2 AML), reactivity of the antisera with peripheral blood cells correlated well with the amount of tumor in the marrow, as reactivity disappeared during remission [33]. Hence, the use of such antisera may be useful for immunodiagnosis of early relapse.

The major problems and pitfalls in the characterization of leukemia antigens with xenogeneic antisera have been summarized [24]. Whether the antigens that are good immunogens for animals play any role in the immune response of the cancer host to his own tumor remains to be determined.

Cellular immune reactions to leukemia antigens were first studied with the mixed lymphocyte culture test, in which remission lymphocytes are exposed *in vitro* to autologous blasts. Stimulation of remission lymphocytes by autologous blasts has been reported by several investigators, indicating the presence of "new" antigens on the leukemic cells [34–38]. In some studies the degree of stimulation is less when the culture medium is supplemented with autologous rather than allogeneic serum. This has been attributed to the presence of "blocking factors" in these sera; in view of recent animal studies this should be detrimental to the patient; however, the contrary seems to be true [38]. It is of interest that the cells of 7 of 8 patients with AML and serum inhibition in the MLC had IgG on their membrane detectable by direct immunofluorence. Perhaps the lymphocytes in this case were stimulated not by antigen(s) present on the blasts, but by antigen–antibody complexes, as noted in other systems [39].

A short-lived increase in the reactivity of lymphocytes to autologous blasts occurs following immunotherapy. Enhanced lymphocyte reactivity can also be observed as a rebound phenomenon after the cessation of chemotherapy [22, 37].

The mixed lymphocyte reaction probably represents an *in vitro* expression of delayed hypersensitivity to a new antigen present on the leukemic cells. However, other possible mechanisms for lymphocyte stimulation in mixed cell cultures, such as the formation of mitogenic factors by leukemic cells, have never been adequately studied. The antigens are certainly tumor-associated, but proof that they are true tumor specific antigens is still lacking. The issue has become more confusing by the demonstration that normal lymphocytes react in the MLC to autologous cultured lymphoblastoid cell lines or to mitogen induced autologous lymphoblasts [40, 41]. The hypothesis that during the process of transformation by mitogens or by long-term culture "hidden" (fetal?, viral?) antigenic components become exposed is an attractive one, but the relationship, if any, of these antigens to leukemia antigens is a matter of speculation. Because these cells clearly are not leukemic but nevertheless are capable of stimulating autologous lymphocytes in the MLC, caution should be used to attribute the lymphocyte stimulation by leukemic cells to the presence of leukemia specific antigens.

Lymphocytes from histocompatible, identical siblings also react in the MLC to blast cells from the leukemic sibling, but not to remission leukocytes [42, 43]. This again suggests the appearance of a new antigen on the leukemic cells, as cells from normal identical siblings do not interact in the MLC. However, equally convincing negative findings have been reported in four sets of identical siblings (2 ALL, 2 AML) [44].

4. IMMUNOTHERAPY

Since the turn of the century, numerous attempts have been made to immunize cancer patients with tumor cells or tumor extracts but until recently the results have been consistently disappointing. Based on the results from extensive studies with the murine leukemias (summarized in reference [45]) the first successful clinical trial of immunotherapy for ALL was reported by Mathé [46]. Twenty patients with ALL in complete remission induced by chemotherapy were treated with irradiated leukemia cells, BCG or both. Ten control patients were left untreated. All 10 controls relapsed within 130 days after the chemotherapy was stopped, but only 9 of the 20 patients given immunotherapy had relapsed by that time.

These preliminary studies have since been extended to include over 100 patients treated with a variety of experimental protocols, continuously modified to incorporate new concepts derived from results obtained in animal models or the preceding clinical trials [47]. The results continue to support the original conclusions that under well defined circumstances certain modalities of active

immunotherapy increase the duration of remissions in ALL. However, in trials of similar design by the Medical Research Council and by the Children's Cancer Study Group A, BCG alone did not prolong remissions in ALL [48, 49].

Trials of immunotherapy for AML have met with more consistent success. Powles *et al.* [50] have shown that the duration of remissions induced by chemotherapy can be significantly prolonged by treatment with weekly injections of irradiated allogeneic leukemia cells and BCG, in addition to monthly courses of maintenance chemotherapy. The median duration of remissions was 188 days with chemotherapy and 312 days with the combination of chemotherapy and immunotherapy. It is not clear whether the prolongation of remissions was due to the BCG or to the tumor cells.

In a pilot study by the same group, immunotherapy without maintenance chemotherapy appeared equally effective as the combination of both for the maintenance of remission of AML [51], but this was not corroborated in another study of similar design [52]. When relapses occurred, successful reinduction of remissions was easier in the group treated with immunotherapy alone than in those patients receiving maintenance chemotherapy as well; this was attributed to a greater bone marrow reserve in those patients not receiving chronic maintenance chemotherapy.

Gutterman *et al.* studied the effect of immunotherapy with BCG alone on the length of remission in AML maintained with intermittent chemotherapy [53]. Median duration of remission was 72+ weeks on the combined treatment as compared to 60 weeks on chemotherapy alone. Curiously, they could not demonstrate a beneficial effect on the length of remissions in patients with ALL treated with the same regimen. Even a less intensive course of immunotherapy (with BCG given twice a week for a total of 4 weeks) prior to the start of maintenance chemotherapy prolonged remissions in AML from 26 to 39.4 weeks [54].

Several interesting features emerge from these studies. In most trials the initial relapse rates for patients in the control and the immunotherapy groups are very similar. However, while the control group continues its downward trend, the relapse rate for patients on immunotherapy levels off. If the results from animal studies can be extrapolated to man, this would suggest that the tumor burden in those patients who relapse early was not decreased to a number of cells the immune response can cope with. At the present time no methods are available to differentiate *a priori* these unfortunate patients from those who will do well. Perhaps the use of immunodiagnostic methods such as the one described by Haltermann may eventually be a major step in this direction.

The morbidity associated with long-term immunotherapy with living tubercle bacilli appears to be acceptable but by no means negligible. The side effects of BCG as used for the treatment of malignant diseases have been recently summarized [55]. Fortunately, enhancement of tumor growth has so far not been a major problem in the human leukemias, although it regularly occurs in some experimental tumor systems [56, 57].

Finally, many different BCG vaccines are available. Most of them will protect against tuberculosis (for which they were developed in the first place), but there are considerable differences among the vaccines, notably in the mycobacterial strains used and the technical procedures employed to deliver the final product. The conflicting results from various trials have been attributed in part to the use of different vaccines and different routes of administration with variable effects on the immune response. This may be true to a certain extent as, for instance, dead mycobacteria favor antibody responses rather than cell mediated immunity which appears to be more important for resistance to tumors [58]. The ratio of living versus dead organisms varies considerably between different BCG vaccines and even from one lot to another of the same vaccine. The many uncertainties of using such a variable material as BCG cannot be overemphasized, especially as the desired immunoprophylactic antileukemic effect is merely a byproduct of the specific immunization (against tuberculosis). It is imperative that standardized extracts of tubercle bacilli with effects comparable to those of intact bacilli be tested in controlled, standardized, and preferably international clinical trials of immunotherapy for acute leukemia in man.

CONCLUSION

Although the studies on the immunological aspects of human acute leukemia have increased our understanding of the biology of this disease, there is no unequivocal evidence to date that immune reactions play a major role in the incidence, the morbidity and the prognosis of acute leukemia in adults. There is some evidence

that certain forms of immunotherapy can prolong remissions in AML in perhaps as many as one-half of the patients, but the importance of this finding is lessened by the fact that less than 50% of all adults with acute leukemia ever achieve a remission. Because immunotherapy is ineffective when large numbers of tumor cells are present, the immediate improvement of the outlook for adults with acute leukemia will result from improvements in chemotherapy and not from the addition of immunotherapy to our therapeutic arsenal. Nevertheless, further studies on immunity in human leukemia are urgently needed to answer the many unsolved questions discussed in this brief review.

REFERENCES

1. F. Lilly, E. A. Boyse and L. J. Old, Genetic basis of susceptibility to viral leukaemogenesis. *Lancet* **ii,** 207 (1964).
2. B. Benacerraf and M. E. Dorf, Genetic control of specific immune responses In: *Progress in Immunology II,* (Edited by L. Brent and J. Holborow), Vol. 2, p. 181 North Holland, Amsterdam (1974).
3. F. M. Kourilsky, J. Dausset and J. Bernard, A qualitative study of normal leukocyte antigens of human leukaemic lymphoblasts. *Cancer Res.* **28,** 372 (1968).
4. S. D. Lawler, P. T. Klouda, R. M. Hardisty and M. M. Till, Histocompatibility and acute lymphoblastic leukaemia. *Lancet* **i,** 699 (1971).
5. G. D. Pegrum, I. C. Balfour, C. A. Evans and V. L. Middleton, HL-A antigens on leukaemic cells. *Brit. J. Haemat.* **19,** 493 (1970).
6. R. L. Walford, S. Finkelstein, R. Neerhout, P. Konrad and E. Shanbrom, Acute childhood leukaemia in relation to the HL-A human transplantation genes. *Nature (Lond.)* **225,** 461 (1970).
7. G. D. Pegrum, Annotation: Leukaemia Antigens. *Brit. J. Haemat.* **24,** 1 (1973).
8. P. MacKintosh, D. A. Hardy and T. Aviet, Lymphocyte typing changes after short term culture. *Lancet* **i,** 1019 (1971).
9. A. Gibofsky and P. I. Terasaki, Trypsinization of lymphocytes for HL-A typing. *Transplantation* **13,** 192 (1972).
10. L. Davignon, P. Lemonde, P. Robillard and A. Frappier, BCG vaccination and leukaemia mortality. *Lancet* **ii,** 638 (1970).
11. L. J. Kinlen and M. C. Pike, BCG vaccination and leukaemia. Evidence of vital statistics. *Lancet* **ii,** 398 (1971).
12. G. W. Comstock, V. T. Livesay and R. G. Webster, Leukaemia and BCG. A controlled trial. *Lancet* **ii,** 1062 (1971).
13. R. A. Good, Relations between immunity and malignancy. *Proc. nat. Acad. Sci. (Wash.)* **69,** 1026 (1972).
14. I. Penn and T. E. Starzl, Malignant tumors arising *de novo* in immunosuppressed organ transplant recipients. *Transplantation* **14,** 407 (1972).
15. Editorial, Radiation, immunity and cancer. *Lancet* **ii,** 217 (1972).
16. J. M. Dupuy, F. M. Kourilsky, D. Fradelizzi, N. Feingold, C. L. Jacquillat, J. Bernard and J. Dausset, Depression of immunological reactivity of patients with acute leukaemia. *Cancer (Philad.)* **27,** 323 (1971).
17. M. E. Oren and R. B. Herberman, Delayed hypersensitivity reactions to membrane extracts of human tumor cells. *Clin. exp. Immunol.* **9,** 45 (1971).
18. W. H. Greene, S. C. Schimpff and P. H. Wiernik, Cell-mediated immunity in acute non-lymphocytic leukemia: Relationship to host factors, therapy and prognosis. *Blood* **43,** 1 (1974).
19. J. S. Walker, D. Davis, P. Davies, C. B. Freeman and R. Harris, Immunological studies in acute myeloid leukaemia: PHA responsiveness and serum inhibitory factors. *Brit. J. Cancer* **27,** 203 (1973).
20. E. M. Hersh, J. P. Whitecar, K. B. McCredie, G. P. Bodey and E. J. Freireich, Chemotherapy, immunocompetence, immunosuppression and prognosis in acute leukemia. *New Engl. J. Med.* **285,** 1211 (1971).
21. A. A. Green and L. Borella, Immunological rebound after cessation of long term chemotherapy in acute leukemia. II. *In vitro* response to phytohemagglutinin and antigens by peripheral blood and bone marrow lymphocytes. *Blood* **42,** 99 (1973).

22. E. M. Hersh, J. U. Gutterman, G. M. Mavligit, K. B. McCredie, M. A. Burgess, A. Matthews and E. J. Freireich, Serial studies on immunocompetence of patients undergoing chemotherapy for acute leukaemia. *J. clin. Invest.* **54,** 401 (1974).
23. D. G. Jose and J. H. Colebatch, High avidity antibody in long-term survivors of acute leukaemia. *Lancet* **ii,** 835 (1974).
24. R. Harris, Leukaemia antigens and immunity in man. *Nature (Lond.)* **241,** 95 (1973).
25. J. F. Doré, L. Marholev, H. Colas de la Noue, F. de Vassal, R. Motta, I. Hrsak, G. Seman and G. Mathé, New antigens on human leukaemic cells and antibody in the serum of leukaemic patients. *Lancet* **ii,** 1249 (1967).
26. T. O. Yoshida and K. Imai, Autoantibody to human leukaemic cell membrane as detected by immune adherence. *Eur. J. clin. biol. Res.* **15,** 61 (1970).
27. P. Hersey, I. C. M. MacLennan, A. C. Campbell, R. Harris and C. B. Freeman, Cytotoxicity against human leukaemic cells. I. Demonstration of antibody dependent lymphocyte killing of human allogeneic myeloblasts. *Clin. exp. Immunol.* **14,** 159 (1973).
28. R. S. Metzgar, T. Mohanakumar and D. S. Miller, Antigens specific for human lymphocytic and myeloid leukemia cells. Detection by nonhuman primate antiserums. *Science* **178,** 986 (1972).
29. M. A. Baker, K. Ramachandar and R. T. Taub, Specificity of heteroantisera to human acute leukemia associated antigens. *J. clin. Invest.* **54,** 1273 (1974).
30. D. L. Mann, G. N. Rogentine, R. Halterman and B. Leventhal, Detection of an antigen associated with acute leukemia. *Science* **174,** 1136 (1971).
31. R. Billing and P. I. Terasaki, Human leukemia antigen. I. Production and characterization of antisera. *J. nat. Cancer Inst.* **53,** 1635 (1974).
32. R. Billing and P. I. Terasaki, Human leukemia antigen. II. Purification. *J. nat. Cancer Inst.* **53,** 1639 (1974).
33. R. B. Halterman, B. G. Leventhal and D. L. Mann, An acute leukemia antigen. Correlation with clinical status. *New Engl. J. Med.* **287,** 1272 (1972).
34. D. C. Viza, O. Bernard-Degani, C. L. Bernard and R. Harris, Leukemia antigens. *Lancet* **ii,** 493 (1969).
35. W. H. Fridman and F. M. Kourilsky, Stimulation of lymphocytes by autologous leukaemic cells in acute leukaemia. *Nature (Lond.)* **224,** 227 (1969).
36. B. G. Leventhal, R. H. Halterman, E. B. Rosenberg and R. B. Herberman, Immune reactivity of leukemia patients to autologous blast cells. *Cancer Res.* **32,** 1820 (1972).
37. R. L. Powles, L. A. Balchin, G. Hamilton Fairley and P. Alexander, Recognition of leukaemia cells as foreign before and after autoimmunization. *Brit. med. J.* **1,** 486 (1971).
38. J. U. Gutterman, R. D. Rossen, W. T. Butler, K. B. McCredie, G. P. Bodey, E. J. Freireich and E. M. Hersh, Immunoglobulin on tumor cells and tumor induced lymphocyte blastogenesis in human acute leukemia. *New Engl. J. Med.* **288,** 169 (1973).
39. R. I. Handin, W. F. Piessens and W. C. Moloney, Stimulation of unimmunized lymphocytes by platelet-antibody complexes in idiopathic thrombocytopenic purpura. *New Engl. J. Med.* **289,** 714 (1973).
40. M. E. Weksler and G. Birnbaum, Lymphocyte transformation induced by autologous cells: Stimulation by cultured lymphoblast lines. *J. clin. Invest.* **51,** 3124 (1972).
41. S. C. Knight, D. A. Hardy and N. R. Ling, The interaction of normal lymphocytes and cells from lymphoid cell lines. *Immunology* **19,** 343 (1970).
42. M. L. Bach, F. H. Bach and P. Joo, Leukemia associated antigens in the mixed leukocyte culture test. *Science* **166,** 1520 (1969).
43. T. Han and J. Wang, Antigenic disparity between leukaemic lymphoblasts and normal lymphocytes in identical twins. *Clin. exp. Immunol.* **12,** 171 (1972).
44. R. H. Rudolph, E. Michelson and E. D. Thomas, Mixed leukocyte reactivity and leukemia. Study of identical siblings. *J. clin. Invest.* **49,** 2271 (1970).
45. G. Mathé, Active immunotherapy. *Adv. Cancer Res.* **14,** 1 (1971).
46. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat and F. de Vassal, Active immunotherapy for acute lymphoblastic leukaemia. *Lancet* **i,** 697 (1969).

47. G. Mathé, P. Pouillart, L. Schwarzenberg, R. Weiner, M. Hayat, J. L. Amiel, C. Rosenfeld, M. Schneider, A. Cattan and F. deVassal, Essais d'immunothérapie active dans la leucémie aiguë lymphoide. *Nouv. Presse méd.* **2,** 557 (1973).
48. Medical Research Council, Treatment of acute lymphoblastic leukaemia. *Brit. med. J.* **4,** 189 (1971).
49. Children's Cancer Study Group A, BCG in the treatment of acute lymphoblastic leukemia. *Proc. Amer. Assoc. Cancer Res.* **14,** 45 (1973) (abst.).
50. R. C. Powles, D. Crowther, L. J. T. Baterman, M. E. J. Beard, J. McElwain, J. Russell, T. A. Lister, J. M. A. Whitehouse, P. F. M. Wrigley, M. C. Pike, P. Alexander and G. Hamilton Fairley, Immunotherapy for acute myelogenous leukaemia. *Brit. J. Cancer* **28,** 365 (1973).
51. R. L. Powles, Immunotherapy for acute myelogenous leukaemia. *Brit. J. Cancer* **28,** Suppl. 1, 262 (1973).
52. C. B. Freeman, R. Harris, C. G. Geary, M. J. Leyland, J. E. MacIver and I. W. Delamore, Active immunotherapy used alone for maintenance of patients with acute myeloid leukaemia. *Brit. med. J.* **4,** 571 (1973).
53. J. U. Gutterman, E. M. Hersh, V. Rodriguez, K. B. McCredie, G. Mavligit, R. Reed, M. A. Burgess, T. Smith, E. Gehan, G. P. Bodey and E. J. Freireich, Chemotherapy of adult acute leukaemia. Prolongation of remission in myeloblastic leukemia with BCG. *Lancet* **ii,** 1405 (1974).
54. W. R. Vogler and Y. K. Chan, Prolonging remission in myeloblastic leukaemia by Tice strain bacillus Calmette-Guérin. *Lancet* **ii,** 128 (1974).
55. R. C. Bast, Jr., B. Zbar, T. Borsos and H. J. Rapp, BCG and cancer. *New Engl. J. med.* **290,** 1413, 1458 (1974).
56. W. F. Piessens, F. C. Lachapelle, N. Legros and J. C. Heuson, Facilitation of rat mammary tumor growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).
57. F. C. Sparks and J. Breeding, Tumor regression and enhancement resulting from immunotherapy with BCG and neuraminidase. *Cancer Res.* **34,** 3262 (1974).
58. G. B. Mackaness, D. J. Auclair and P. H. Lagrange, Immunopotentiation with BCG. I. Immune response to different strains and preparations. *J. nat. Cancer Inst.* **51,** 1655 (1973).

Europ. J. Cancer Vol. 12, pp. 519–526. Pergamon Press 1976. Printed in Great Britain

Fractionation of Ascites Tumour Cells at 1 *g*: Separation of Cells in Specific Stages of the Life Cycle

A. TULP and J. J. M. N. WELAGEN
Division of Biophysics, Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract—*Novikoff rat hepatoma ascites cells were separated by sedimentation at 1 g, and populations were obtained which were highly enriched with respect to cells in specific stages of the cell cycle. Sedimentation was carried out using linear Ficoll gradients in a newly developed sedimentation chamber. This procedure yielded populations which were enriched in G_1, S and G_2+M phase cells, as demonstrated by pulse cytophotometry. In addition, cells containing (sub)octaploid amounts of DNA were found. After fractionation, generally more than 94% of the cells were viable. Their protein content, lactate dehydrogenase activity and succinic dehydrogenase activity were measured.*

The same fractionation procedure was also found to be applicable to leukemia ascites cells. Labelling index, ^{3}H-thymidine incorporation and lactate dehydrogenase activity of the cells were determined. In general, viability was about 95%.

The newly devised sedimentation chamber allows separation of classes of cells in sufficient quantity for chemical determinations.

INTRODUCTION

SEVERAL methods exist for the separation of classes of cells. Recently the field has been reviewed extensively by Pretlow *et al.* [1]. Practically all methods make use of differences in physical properties of the cells concerned [2–8]. Apart from the mitotic selection method [9], the only physical method for separating cells in different stages of their life cycle in reasonable quantities [10] from homogeneous suspensions, is velocity sedimentation at 1 *g* [11] or by centrifugal force [12, 13]. However, one of the drawbacks of rate-zonal centrifugation in standard centrifugation tubes is, that serious losses up to 60–80% occur due to wall-sedimentation effects [14, 15].

Wall sedimentation effects are virtually absent at unit gravity. We have recently developed a sedimentation chamber [16, 17], in which suspensions of cells are layered on preformed density gradients and are then fractionated with the use of natural gravity. With our method, "streaming" [18–20] is reduced by adding a polymer of high intrinsic viscosity to the cell suspension. For a discussion, see Tulp and Bont [16], and Leif *et al.* [21].

We have now applied this procedure to the fractionation of rat and murine ascites tumour cells.

Accepted 5 February 1976.

MATERIAL AND METHODS

Cell preparation

(a) Novikoff tumour ascites cells: Male or female 3 months old Sprague–Dawley rats were inoculated i.p. with about 10^7 tumour cells. Cells were harvested after 6 days by injecting i.p. 20 ml phosphate-buffered saline-citrate, and immediately afterwards removing the ascites fluid by suction. Cells were spun down for 1 min at 600 *g* at 4°C, and washed four times with phosphate-buffered saline to remove red blood cells.

(b) Leukemia ascites cells: Male or female 8–12 weeks old GR mice were inoculated i.p. with 10^7 GRSL-2c leukemia cells. Cells were harvested after 7 days in medium 199 (no Ca or Mg added) and sedimented by centrifugation for 10 min at 300 *g* at 4°C. The cells were washed two times. For sedimentation, gradients

contained Earle's balanced salt solution (minus bicarbonate) plus 10 mM phosphate pH 7·5; the sediment that developed was removed by centrifugation.

Sedimentation and collection of cells

Sedimentation of cells was carried out in a cylindrical perspex sedimentation chamber (height 12 cm, diameter 12·7 cm) as described before [16]. When applying 10 ml cell suspension to the sedimentation chamber it was found necessary to add an overlay of 45 ml buffer in order to disperse the cell band uniformly over the total surface of the gradient. The contents of the chamber were fractionated via the cone (see also Peterson and Evans [22]) by upward displacement of the gradient by a cushion of 6% Ficoll (w/v) in 0·9 %NaCl. (Dense sucrose solution was found unsatisfactory since it disturbed the gradient.) The chamber was emptied at a velocity of 80 ml/min. Fractions were centrifuged for 10 min at 500 ***g***. Pellets were resuspended in 10–20 ml of the appropriate buffer to give counts up to 60,000/0·50 ml. Cells were counted in a Coulter Counter (model A, Coultronics, France) with a 100 μl orifice. Corrections were made for coincidences. In addition, fractions were inspected by microscopy for singularity of cells and viability.

Precursor incorporation

About 7×10^7 cells were added to 10 ml of warm medium containing 30 μCi of ^{3}H-labelled thymidine, followed by incubation at 37°C for 30 min. After washing two times with medium, cells were applied to the sedimentation chamber.

Autoradiography

Smears of cells on microscopic slides were fixed in methanol, washed with 50% ethanol and dried by air. Slides were washed twice with ice-cold 0·1 M perchloric acid and autoradiography was performed with Emulsion K5 (Ilford). Time of exposure was 14 days. After developing, cells were stained with hematoxylin-eosin. Two hundred cells were examined for labelling index.

Pulse cytophotometry

An ICP 11 impulse cytophotometer (PHYWE Aktiengesellschaft, Göttingen, Germany) was used as described by Dittrich and Göhde [23]. GRSL cells were stained with ethidium bromide after RNA digestion. After RNA digestion, Novikoff tumour cells were treated with pepsin and then with ethidium bromide [24]. The surfaces of the curves were measured by planimetry.

Assay of enzyme activity

Activities were measured at 30°C. Lactate dehydrogenase activity [25] was measured in 0·50–1·0 ml supernatant of fractions treated with 1·0% Triton X-100; 2 μg rotenone was added to the enzymatic mixture. Succinic dehydrogenase activity was measured in twice freezed-thawed fractions in the presence of 2 μg rotenone as described by Fleischer and Fleischer [26]. Fractions were obtained by centrifuging 7·5 ml volumes after Coulter counting and resuspending the cells in 2·5 ml volumes.

Other determinations

Protein was determined according to Lowry *et al.* [27]. Incorporated radioactivity was determined after quenching the reaction with 0·1 M perchloric acid essentially as described by Vernie *et al.* [28]. Viability was estimated by mixing cell suspensions with 0·5% trypan blue in Hanks-phosphate solution.

Chemicals

Ficoll (average molecular weight 400,000 and 70,000) was obtained from Pharmacia, Fine Chemical Co., Uppsala, Sweden. Cytochrome *c*, pepsin, NADH and pyruvate were obtained from Boehringer and Söhne, Mannheim, Germany. Ribonuclease, rotenone and ethidium bromide were products of Sigma Chemical Co., Milwaukee, Mo. (^{3}H-CH_3)-Thymidine was obtained from The Radio Chemical Centre, Amersham, England. Polyethylene oxide was a gift from Heybroek and Co., Handel Mij. N.V., Amsterdam, The Netherlands.

RESULTS AND DISCUSSION

Novikoff tumour cells

Sedimentation time was considerably reduced in gradients of Ficoll (average molecular weight 70,000) due to their lower intrinsic viscosity. However, after removal of the Ficoll (average mol. wt. 70,000), the cells showed a

substantial loss in viability. Much better results were obtained using Ficoll of average mol. wt. 400,000. Viability of the fractionated cells, as judged by the trypan blue exclusion test, was about 92% after sedimentation in Ficoll (400,000) gradients (Fig. 1). Viability of the slowest sedimenting cells was strongly depressed. Indeed, it is common experience in velocity sedimentation, that viability of the slowest moving cells is relatively low [11, 29–33], and this has been observed by us in the fractionation of rat-liver cells, Yoshida tumour

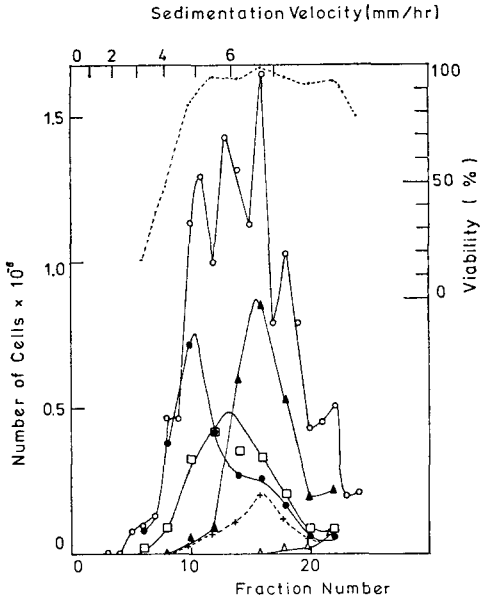


Fig. 1. *Separation of Novikoff ascites tumour cells by velocity sedimentation at unit gravity.* Ordinate (*left*): *Number of cells* $\times 10^{-6}$; (*right*): *Viability* %; Abscissa: *fraction number;* Abscissa (*top*): *sedimentation velocity* (*mm/hr*). 2×10^7 *Novikoff tumour cells suspended in* 50 *ml Hanks' medium* (*Ca, HCO_3^- and glucose were absent*), *containing* 10 *mM phosphate buffer pH* 7·5 *and* 0·05% *polyethylene oxide, were layered on a continuous linear* 1–4% (*w/v*) *Ficoll gradient. Sedimentation time was* 6 *hr* 40 *min.*

(●------●) *viability*
(○——○) *total number of cells*
(●——●) *G_1-phase cells*
(□——□) *S-phase cells*
(▲——▲) *G_2 + M-phase cells*
(+ - - - +) *S'-phase cells*
(△——△) *G_2 + M'-phase cells*

cells (unpublished observations) and murine ascites tumour cells (see below) as well. A faint pinkish band of erythrocytes (not shown in Fig. 1), corresponding with fractions 2 and 3 could be seen when the perspex sedimentation chamber was viewed from the side during the sedimentation process. The profile of Novikoff tumour cells after 6 hr sedimentation at unit gravity is shown in Fig. 1. Recoveries varied from 90%–95%. The shape of the curve of total cell number indicates the presence of cells in various phases of the life cycle. This is substantiated by the histograms of Fig. 2, where a selection of histograms of DNA analysis is given. Fraction 6 (B) consisted primarily of the (sub)diploid [34] G_1-phase cells. In fraction 16 (C) is shown how the phases of the life cycle were determined by graphical analysis; it is evident that the phase corresponding to the (sub)tetraploid amount of DNA became relatively more important. In fraction 24 (D) cells were present, which contained the (sub)octaploid amount of DNA (G_2+M'). For measurement of DNA content, the cells were subjected to pepsin treatment, and this yields bare nuclei. It therefore is unlikely that the detection of cells in the G_2+M' phase could be attributed to binary cell clumps. Moreover, these were absent when cells were inspected by microscopy after fractionation. Using pulse cytophotometry, Plagemann *et al.* [35] have demonstrated that Novikoff tumour cells with a (sub)octaploid amount of DNA are G_2+M phase cells of the tetraploid tumour cells. Furthermore they assumed that the cells designated S' were S-phase cells of the (sub)tetraploid cells. It should be emphasized that, although a considerable percentage of octaploid-like cells was found in fraction 24 (D), these cells were almost undetectable in the control (A). The histograms were used to construct the curves of Fig. 1. Although this simple planimetric determination is likely to introduce errors [36], we consider that it gave a fair approximation of the degree to which cells in different phases of their life cycle were separated. Figure 1 shows that the velocity of the modal fraction of the (sub)diploid cells was 5 mm/hr, and that the velocity of the modal fraction of the (sub)tetraploid cells was 6·4 mm/hr.

The distribution of specific activity of lactate dehydrogenase, a soluble cytoplasmic enzyme, followed an irregular pattern (Fig. 3). The irregular variation of the specific activity of this enzyme is consistent with data of Klevecz and Ruddle [37] and Hooper [38] for lactate dehydrogenase activity during the cell cycle of Chinese hamster cells. Smaller variations have been reported for neoplastic mast cells by Warmsley *et al.* [39], and by Martin *et al.* [40] for hepatoma cells (our data on leukemia cells are given below). Protein content was also estimated in the experiment illustrated in Fig. 3. Protein level per cell increased gradually when the fractions containing the faster sedimenting cells were assayed. Experiments in which the activity of a mitochondrial marker

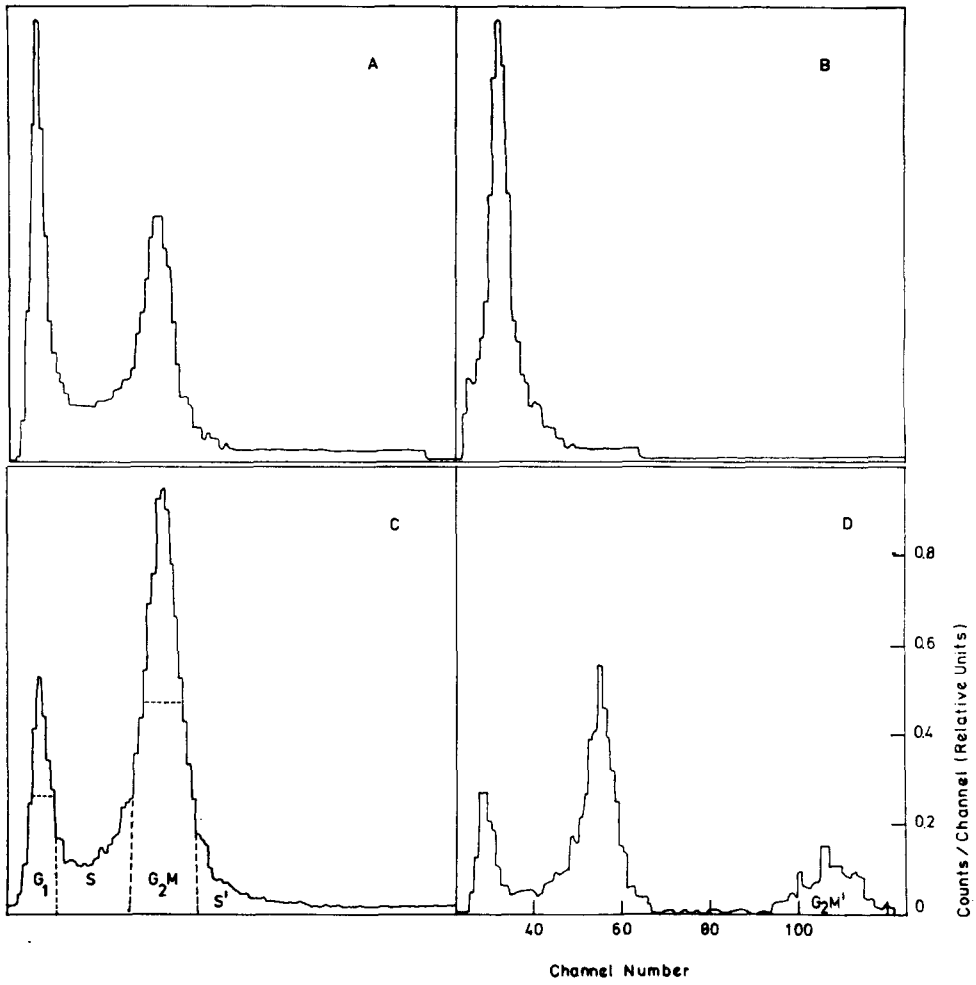


Fig. 2. Relative DNA content of Novikoff tumour cells as a function of fraction number. Ordinate: *Counts/channel (relative units);* Abscissa: *channel number (relative fluorescence intensity). The details of the experiment are described in the legend to Fig.* 1. *A = control; B = fraction* 8*; C = fraction* 16 *and D = fraction* 24.

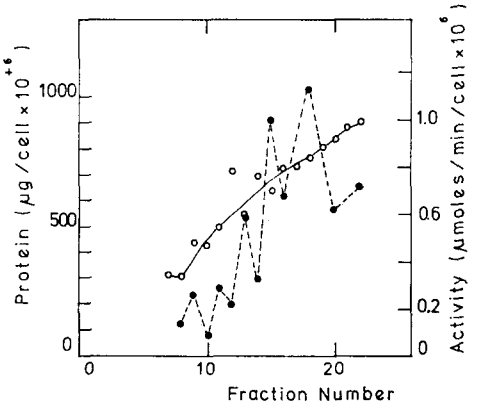


Fig. 3. Lactate dehydrogenase activities and protein contents of fractionated Novikoff cells. Ordinate *(left): protein (μg/cell* × 10^6), *(right): activity lactate dehydrogenase (μmoles/min/cell* × 10^6); Abscissa: *fraction number. For details see legend to Fig.* 1.

(○——○) *protein content*
(●- - -●) *lactate dehydrogenase activity*

enzyme, succinic dehydrogenase, was measured and the average cell viability amounted to 93%, revealed (not shown) that during the life cycle of Novikoff ascites cells the activity of succinic dehydrogenase increased gradually without the irregular variations in activity found with lactate dehydrogenase. Activity was 47 nmoles succinate/min/10^6 cells for fraction 9 (containing 75% G_1 and 25% S phase cells). At the plateau, activity was 95·3 nmoles/min/10^6 cells for fraction 23 (containing 29% G_1, 20·5% S, 37% G_2+M, 8·5% S′ and 7·5% G_2+M′). Since twice as many cells were loaded onto the gradient as in the experiment of Fig. 1, the latter separation was somewhat less good in the higher fractions.

Murine leukemia cells

Leukemia cells from a cell line (GRSL-2c) different from that used previously [16] were

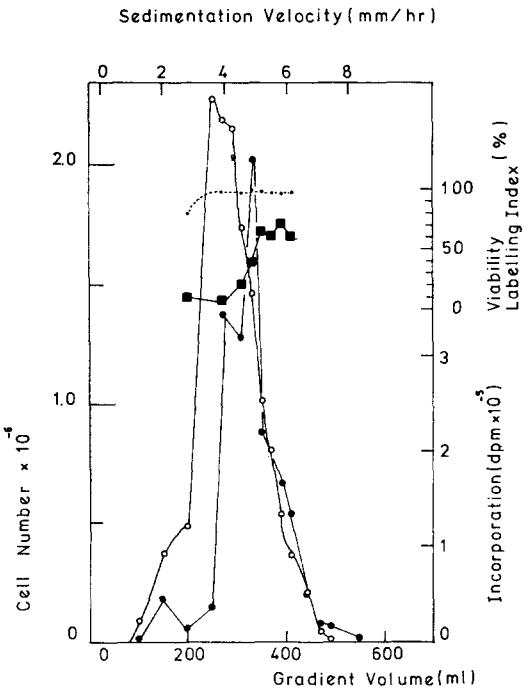


Fig. 4. *Separation of murine leukemia cells by velocity sedimentation at* 1*g and incorporation of* ^{3}*H-thymidine.* Ordinate *(left): cell number* $\times 10^{-6}$*; (right upper part): labelling index, viability* (%)*; (right lower part) incorporated* ^{3}*H-thymidine (dis/min* $\times 10^{-5}$*);* Abscissa: *gradient volume (ml);* Abscissa *(top): sedimentation velocity (mm/hr).*

GRSL cells were prelabelled with ^{3}*H-thymidine as described under "Material and Methods". About* 2×10^7 *leukemia cells in* 10 *ml medium containing* 10 *mM phosphate pH* 7·5, 0·4% *Ficoll and* 0·03% *polyethylene oxide were layered via the sieve onto a continuous linear* 1–4% *Ficoll gradient. An overlay of* 45 *ml medium* 199 *was then layered onto the gradient. All solutions contained* 2% *fetal calf serum. Sedimentation time was* 5 *hr* 15 *min.*

(●- - -●) *viability*
(■———■) *labelling index*
(○———○) *total cell number*
(●———●) *incorporated* ^{3}*H-thymidine*

applied to the gradient. Grace to the fact that the ascites fluid was less hemorrhagic with this cell line, the final cell suspension was free of erythrocytes. Lymphocytes and macrophages were found only occasionally in this preparation. In order to keep the pH constant during the sedimentation procedure, bicarbonate was replaced by 10 mM phosphate. The sedimentation profile of leukemia cells is depicted in Fig. 4. Recoveries varied between 84–96%. A broad peak was present in the 250 ml region and a definite shoulder was apparent in the 400 ml region. Viability after the fractionation procedure was generally 97%. The peak of the ^{3}H-thymidine incorporation curve was markedly shifted to the right in the direction of the more rapid sedimenting cells. Autoradiography revealed that the percentage of nuclei which contained grains, in the fraction at 200 ml gradient volume was 7·5%. After this the labelling index increased gradually to 70·5% in the fraction at 390 ml. Evidence that the proportion of S-phase cells increased towards the fractions which contained the more rapid sedimenting cells, was corroborated by the micro-fluorometric analysis.

Fig. 5 shows a series of representative DNA analyses of fractionated murine ascites tumour cells. The non-fractionated cell suspension (A) contained relatively low amounts of S-phase (21%) and G_2+M-phase (9%) cells. Note the striking difference between A and D. In the latter case, early and mid S-phase cells were virtually absent and the majority of cells consisted of late S and G_2+M-phase cells. Graph B shows that G_1-phase cells were predominant (96%) in the slower sedimenting cells. C shows a situation intermediate between

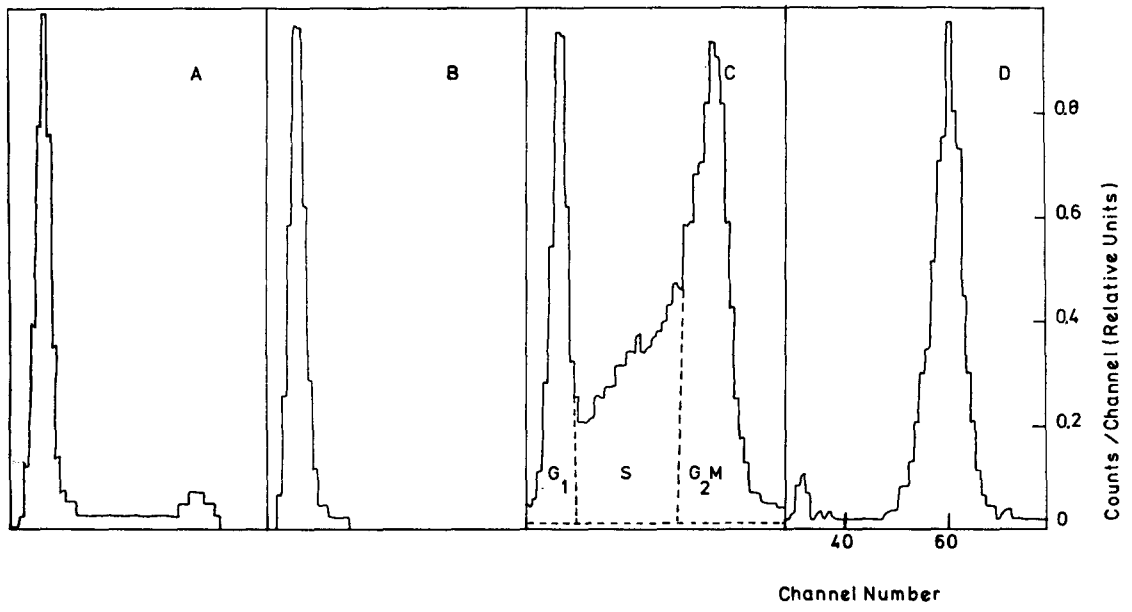


Fig. 5. *Relative DNA content of murine leukemia cells after separation at unit gravity.* Ordinate: *Counts/channel (relative units);* Abscissa: *channel number (relative fluorescence intensity). A = control; B = fraction at* 240 *ml; C = fraction at* 340 *ml and D = fraction at* 450 *ml. The details of the experiment are described in the legend to Fig.* 4.

Table 1. Activity of lactate dehydrogenase (LDH) of GRSL cells separated at unit gravity and percentage of enrichment of cell cycle components

Exp.	Fraction at gradient volume (ml)	Percentage			Number of cells × 10^{-6}			Activity of LDH (μmoles/min/10^6 cells)
		G_1	S	G_2+M	G_1	S	G_2+M	
1.	Control	70	21	9	11·6	3·50	1·50	—
	200	88	10	2	0·43	0·05	0·01	—
	220	94	6	0	2·12	0·14	0	—
	240	96	4	0	2·10	0·9	0	—
	260	93	7	0	2·00	0·15	0	—
	280	87	10	3	1·51	0·17	0·05	—
	300	68	19	13	1·00	0·28	0·19	—
	320	45	36	19	0·46	0·37	0·19	—
	340	21	36	43	0·17	0·29	0·35	—
	360	8	38	54	0·04	0·20	0·29	—
	380	10	31	59	0·004	0·09	0·26	—
	400	3	25	72	0·006	0·05	0·15	—
	450	3	21	76	0.003	0.02	0.06	—
	Sum				9·84	2·71	1·55	
	Recovery of cells in phase of cell cycle (%)				85	78	103	
	Total recovery					84%		
2.	Control	62	24	14	17·3	6·7	3·9	—
	200	100	0	0	0·62	0	0	0·08
	250	91	9	0	2·60	0·26	0	0·21
	270	89	11	0	3·40	0·42	0	0·29
	290	75	19	6	2·94	0·86	0·24	0·29
	315	75	22	3	2·82	0·83	0·11	0·33
	340	52	31	17	1·57	0·93	0·51	0·35
	360	41	31	28	0·83	0·63	0·57	0·37
	380	27	36	37	0·33	0·44	0·45	0·42
	400	19	32	49	0·19	0·32	0·48	—
	420	8	43	49	0·04	0·23	0·26	0·37
	440	11	22	67	0·03	0·07	0·21	0·46
	Sum				15·4	5·0	2·83	
	Recovery of cells in phase of cell cycle (%)				89	75	73	
	Total recovery					85%		

For details see caption to Fig. 5.

B and D. The low percentage of G_1-phase cells in Fig. 6D underlines that the "streaming" phenomenon in our standard procedure was strongly reduced.

Table 1 shows the percentages of the life-cycle components as determined by the simple planimetric method. From these data we calculated that peak G_1-phase cells sedimented at a velocity of 3·4 mm/hr, peak S-phase cells at 4·9 mm/hr and G_2+M-phase cells at 5·4 mm/hr.

In another experiment (Table 1.2) we found that the specific activity of lactate dehydrogenase increased gradually, and it was clear that variations in enzymatic activity were small in the life cycle of murine leukemia cells. Viability was about 95% in this particular experiment.

Since we used only one-tenth of each fraction for the determination of lactate dehydrogenase activity, it seems that cell loads of $2{\cdot}10^7$ ascites cells are generally sufficient for the measurement of the majority of constitutive enzymes.

Stokes' law forms the theoretical basis for separations at unit gravity. It can be calculated from the measurements of viscosity and density of Ficoll-solutions made by Pretlow and co-workers [41] that separation time at 4°C can be reduced almost two-fold at 25°C, provided cells remain viable.

As long as the cells remain viable during the sedimentation procedure, the relatively long separation of hours for cells of about 1,000 $m\mu^3$ at 4°C can be no objection, and the technical simplicity of the method is a major advantage as compared to the more complicated manipulation of rate-zonal centrifugation [13].

Acknowledgement—The authors gratefully acknowledge the interest and support of Professor P. Emmelot, Dr. M. Sluyser for reading the manuscript, and the technical assistance of Mr. A. Visser and Mr. D. Kamlag in maintaining the tumour cells.

REFERENCES

1. T. G. PRETLOW, E. A. WEIR and J. G. ZETTERGREN, Problems connected with the separation of different kinds of cells. In *International Review of Experimental Pathology*. (Edited by M. A. EPSTEIN) Vol. 14, p. 91. Academic Press, New York (1975).
2. D. GLICK, D. VON REDLICH, E. J. JUHOS and C. R. MCEWEN, Separation of mast cells by centrifugal elutriation. *Exp. Cell Res.* **65,** 23 (1971).
3. K. SHORTMAN, The separation of different cell classes from lymphoid organs. I. *Aust. J. exp. Biol. med. Sci.* **44,** 271 (1966).
4. K. SHORTMAN, The separation of different cell classes from lymphoid organs. II. *Aust. J. exp. Biol. med. Sci.* **46,** 375 (1968).
5. H. C. MEL, L. T. MITCHELL and B. THORELL, Continuous free-flow fractionation of cellular constituents in bone marrow. *Blood* **25,** 63 (1965).
6. H. WALTER, E. J. KROB, R. GARZA and G. S. ASCHER, Partition and counter current distribution of erythrocytes and leukocytes from different species. *Exp. Cell Res.* **55,** 57 (1969).
7. K. HANNIG and K. ZEILLER, Zur Auftrennung und Charakterisierung immunokompetenter Zellen mit Hilfe der trägerfreien Ablenkungselektrophorese. *Hoppe-Seylers Z. physiol. Chem.* **350,** 467 (1969).
8. C. W. BOONE, G. S. HARELL and H. E. BOND, The resolution of mixtures of viable mammalian cells into homogenous fractions by zonal centrifugation. *J. Cell Biol.* **36,** 369 (1968).
9. T. TERASIMA and L. J. TOLMACH, Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* **30,** 344 (1963).
10. M. J. FULWYLER, Electronic separation of biological cells by volume. *Science* **150,** 910 (1965).
11. H. R. MCDONALD and R. G. MILLER, Synchronization of mouse L-cells by a velocity sedimentation technique. *Biophys. J.* **10,** 834 (1970).
12. L. K. EVERSON, D. N. BUELL and G. ROGENTINE, Separation of human lymphoid cells into G_1, S and G_2 cell cycle populations by use of a velocity sedimentation technique. *J. exp. Med.* **137,** 343 (1973).
13. A. H. M. WARMSLEY and C. A. PASTERNAK, The use of conventional and zonal centrifugation to study the life cycle of mammalian cells. *Biochem. J.* **119,** 493 (1970).
14. J. K. V. WILLSON, D. E. LUBEROFF, A. PITTS and T. G. PRETLOW, A method for the separation of lymphocytes and plasma cells from the human palatine tonsil using sedimentation in an isokinetic gradient of Ficoll in tissue culture medium. *Immunology* **28,** 161 (1975).
15. M. J. STEWART, T. G. PRETLOW and R. HIRAMOTO, Separation of ascites myeloma cells, lymphocytes and macrophages by zonal centrifugation on an isokinetic gradient. *Amer. J. Pathol.* **68,** 163 (1972).
16. A. TULP and W. S. BONT, An improved method for the separation of cells by sedimentation at unit gravity. *Anal. Biochem.* **67,** 11 (1975).
17. A. TULP and W. S. BONT, *First Int. Symp. on Pulse-Cytophotometry*, p. 260 (Edited by C. A. M. HAANEN, H. F. P. HILLEN and J. M. C. WESSELS) European Press Medikon, Ghent (1975).
18. H. C. MEL, Stable-flow free boundary migration and fractionation of cell mixtures. *J. theor. Biol.* **6,** 181 (1964).
19. M. K. BRAKKE, Zone electrophoresis of dyes, proteins and viruses in density-gradient columns of sucrose solutions. *Arch. Biochem. Biophys.* **55,** 175 (1955).
20. N. G. ANDERSON, Brei fractionation. *Science* **121,** 775 (1955).
21. R. C. LEIF, W. C. KNEECE, R. L. WARTERS, H. GRINVALSKY and R. A. THOMAS, Density gradient system. III. Elimination of hydrodynamic, wall and swirling artifacts in preformed isopycnic gradient centrifugation. *Anal. Biochem.* **45,** 357 (1972).
22. E. A. PETERSON and W. H. EVANS, Separation of bone marrow cells by sedimentation at unit gravity. *Nature (Lond.)* **214,** 824 (1967).
23. W. DITTRICH and W. GÖHDE, Impulsefluorometrie bei Einzelzellen in Suspensionen. *Z. Naturf.* **246,** 360 (1969).
24. E. BERKHAN, DNS-Messung von Zellen aus Vaginalabstrichen. *Ärztl. Lab.* **18,** 77 (1972).

25. H. U. Bergmeyer, E. Bernt and B. Hess, *Methods of Enzymatic Analysis* (Edited by H. U. Bergmeyer) p. 736, Academic Press, New York (1963).
26. S. Fleischer and B. Fleischer, Assay of DPNH or succinate-cytochrome c reductase activity. In *Methods in Enzymology* (Edited by N. O. Kaplan) Vol. X, p. 427. Academic Press, New York (1967).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
28. L. N. Vernie, W. S. Bont and P. Emmelot, Ribosome monomers in rat liver following administration of dimethylnitrosamine. *Cancer Res.* **31,** 2189 (1971).
29. R. A. Phillips and R. G. Miller, Antibody-producing cells: analysis and purification by velocity sedimentation. *Cell Tissue Kinet.* **3,** 263 (1970).
30. T. G. Pretlow, D. E. Luberoff, L. J. Hamilton, P. C. Weinberger, W. A. Maddox and J. R. Durant, Pathogenesis of Hodgkin's disease: separation and culture of cells from Hodgkin's disease in a sterile isokinetic gradient of Ficoll in tissue culture medium. *Cancer (Philad.)* **13,** 1120 (1973).
31. S. R. Ayad, M. Fox and D. Winstanley, The use of Ficoll gradient centrifugation to produce synchronous mouse lymphoma cells. *Biochem. biophys. Res. Commun.* **37,** 551 (1969).
32. M. Omine and S. Perry, Use of cell separation at 1 *g* for cytokinetic studies in spontaneous AKR leukemia. *J. nat. Cancer Inst.* **48,** 697 (1972).
33. D. S. Barkley, L. L. Rakic, J. K. Chaffee and D. L. Wong, Cell separation by velocity sedimentation of postnatal mouse cerebellum. *J. Cell Physiol.* **81,** 271 (1973).
34. T. C. Hsu, The chromosomes of the Novikoff rat hepatoma. *Cytologia (Tokyo)* **24,** 62 (1959).
35. P. G. W. Plagemann, D. P. Richey, J. M. Zylka and J. Erbe, Cell cycle and growth stage-dependent changes in the transport of nucleosides, hypoxanthine, choline and deoxyglucose in cultured Novikoff rat hepatoma cells. *J. Cell Biol.* **64,** 29 (1975).
36. P. N. Dean and J. H. Jett, Mathematical analysis of DNA distributions derived from flow microfluorometry. *J. Cell Biol.* **60,** 523 (1974).
37. R. R. Klevecz and F. H. Ruddle, Cyclic changes in enzyme activity in synchronized mammalian cell cultures. *Science* **159,** 634 (1968).
38. A. W. Hooper, Thymidylate synthetase in mammalian cells *in vitro*. *J. Cell Biol.* **35,** 59A (1967).
39. A. M. H. Warmsley, B. Phillips and C. A. Pasternak, The use of zonal centrifugation to study membrane formation during the life cycle of mammalian cells. *Biochem. J.* **120,** 683 (1970).
40. D. Martin, G. M. Tomkins and D. Granner, Synthesis and induction of tyrosine amino transferase in synchronized hepatoma cells in culture. *Biochemistry* **62,** 248 (1969).
41. T. G. Pretlow, C. W. Boone, R. I. Shrager and G. H. Weiss, Rate zonal centrifugation in a Ficoll gradient. *Anal. Biochem.* **29,** 230 (1969).

Europ. J. Cancer Vol. 12, pp. 527–534. Pergamon Press 1976. Printed in Great Britain

Ultrastructural Analysis of Malignant Conversion *In Vitro* of Epithelial Cells Derived from Normal Adult Rat Liver*

S. KARASAKI†‡, A. SIMARD† and G. DE LAMIRANDE§‡
Institut du Cancer de Montréal, Centre Hospitalier Notre-Dame, and
†Département d'Anatomie et §Département de Biochimie, Université de Montréal, Montréal, Canada

Abstract—*A tumorigenic cell line of epithelial-type was established from a long-term culture of isolated liver cells from a normal adult Wistar rat. Electron microscopic examinations of foci of transformed epithelial cells in the primary culture revealed production of virus particles in association with membranes of the nuclear envelope, endoplasmic reticulum and cell surface. The epithelial cells were found in quiescent state and had intranuclear canalicular membranes. By trypsinization, the cells were initiated into a rapid replication and showed subcellular differentiation. Annulate lamellae and paired cisternae emerged in passage* 1. *Viral production ceased at this stage. Close apposition of adjacent cells became prominent in passage* 9, *when the cell line first revealed tumorigenicity upon transplantation into neonatal Wistar rats. Concomitantly, cytochemical tests gave a positive reaction of ATPase activity on the plasma membranes. Surface ATPase activity continued to show in the epithelial cells following passages* in vitro *as well as in tumor cells* in vivo. *The possible origin of epithelial cells* in vitro *from hepatocytes was suggested by the demonstration of peroxisomes and bile canalicular-like structures in the carcinoma cells.*

INTRODUCTION

ISOLATED hepatocytes from normal rat liver usually have a limited life span *in vitro* [1–4]. Under certain conditions, however, liver cells may continue to multiply [5–12] and eventually become malignant during years of long term cultivation [13–15]. Malignant conversion *in vitro* of cultured liver cells has been induced or accelerated by treatments with chemical carcinogens [8, 16–19] or oncorna viruses [20]. The detection of malignant conversion *in vitro* of epithelial cells is particularly difficult, since little alteration is induced in cell morphology at the critical step [21]. Although many studies were performed on hepatocarcinogenesis *in vivo* [22–25], there are few reports of subcellular analyses on *in vitro* transformation of liver cells [8, 17, 26]. Of these reports, Weinstein *et al.* [8, 26] have focused on the presence of oncorna virus particles in rat liver cell lines and their correlation with transformed cells. The sequential process of *in vitro* neoplastic transformation is very little understood at the subcellular level.

This paper reports the establishment and morphological characterization of a tumorigenic cell line of epithelial-type, derived from the liver of a normal adult rat. The studies of cells in the primary culture and subcultures involve fine structural and cytochemical observations with the objective of obtaining clues to the nature and sequence of subcellular events which may constitute cytodifferentiation toward malignant conversion *in vitro*. The carcinomas, produced by the implantation of cultured cells in rats, are ultrastructurally examined in special attention to cytochemical properties of differentiated hepatocytes.

Accepted 26 January 1976

*This work was supported by grants from the National Cancer Institute of Canada, le Ministère des Affaires sociales du Québec, La Fondation J. H. Biermans and Les Fondations J. Rhéaume.

‡Research Associate of the National Cancer Institute of Canada.

MATERIAL AND METHODS

Cell separation

Rats of the Wistar strain were obtained from

Canadian Breeders, Inc., St. Constant, Que., Canada. The cell line studied here was derived from the liver of a normal male weighing 200 g. The liver was dissociated on May 10, 1973. The cell separation technique employed was essentially the same as that described by Berry and Friend [3]. After perfusion through the hepatic vein with Ca^{2+}-deficient Hank's solution containing 0·05% hyaluronidase (Sigma) and 0·025% collagenase II (Sigma), liver cells were suspended in a mixture (1:1) of Ham's F12 culture medium (GIBCO) and of Hank's solution containing 0·5 M sucrose and passed through two successive sieves with 250 and 40 μ mesh. The enzymatic dissociation of an adult rat liver yielded 2·5 × 10^8 cells. The cell population consisted almost exclusively of hepatocytes (99·1%) as determined with Carnoy-fixed smears stained with hematoxylin-eosin.

Cell culture

The standard culture medium was Ham's F12 containing 100 units of penicillin G and 100 μg of streptomycin per ml and, supplemented with 5 or 10% fetal calf serum (GIBCO). Isolated hepatocytes were plated at 37°C in plastic T-flasks (25 cm^2, Falcon). Cellular concentration was 5 × 10^6 per 10 ml.

Within 6 hr of explanting in a plastic flask, a majority of spherical hepatocytes were packed in a layer of aggregates. They had an angular outline and were loosely attached to the plastic surface during the 1st week period. The medium was partially changed 2 or 3 times a week and were maintained in the same flasks from 4 months to more than 18 months in some cases. Regular determination of cell numbers in flasks showed little variation or a very slow decrease. Six months after explantation, 7 separate cultures showed signs of cell growth. On January 4, 1974, the first subculture was performed on one of the flasks. To dislodge the cells, a mechanical dispersion method was applied after 10 min of treatment with a 0·25% trypsin solution at 37°C. Further passages *in vitro* were continuously done at 5–8 days intervals with trypsinization.

Plating efficiency in earlier passages was less than 5% of the cells. In passage 4, cells grew well in monolayer (Fig. 1). In passage 6 or later, the cells were easily detachable by trypsinization. In passage 12, the logarithmic growth was readily resumed after a lag period of 18 to 24 hr. At this point, the plating efficiency was in excess of 20% and the population doubling time was less than 30 hr in the logarithmic phase (Fig. 1). The saturation of cell density resulted in the inhibition of growth (Fig. 5). More than 90 passages have been done over a period of 20 months.

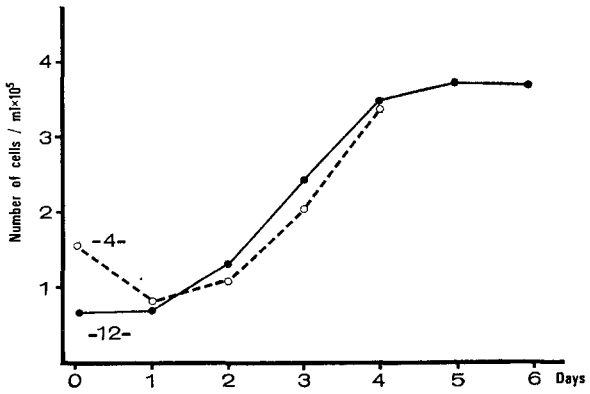


Fig. 1. Growth patterns of cultured liver cells at the 4th (– –○– –) and 12th (—●—) passages. Cells were placed in a series of T-5 flasks (75 cm^2) to each of which 15 ml of Ham's F12 medium plus 10% fetal bovine serum was added. Each day, the culture was trypsinized and the number of cells was measured in Coulter counter.

Tumorigenicity

Newborn Wistar rats (6–18 hr after birth) were used for tumorigenicity tests of the cultured cells. Cells were dissociated by either trypsinization or mechanical dispersion. A suspension of 10^6 to 2 × 10^6 cells in 0·05–0·1 ml of culture medium was injected subcutaneously or intraperitoneally. When transplanted either i.p. or s.c. into newborn rats with 2 × 10^6 cells from passage 6, no tumors were formed (Table 1). In the same batch of animals, positive results were obtained on s.c. transplantation of an established cell line, RLC [4, 27]. 2 × 10^6 cells of passage 9 could produce tumors on i.p. transplantation. Further passages developed tumors on s.c. transplantation of 1–2 × 10^6 cells with a latency period of 10–18 days (Table 1). Some of the tumors were explanted and grown *in vitro*.

A s.c. inoculation of 10^6 cells gave a solid tumor of 10 cm^3 volume after 4 weeks and killed the recipient. I.p. transplants developed into many solid tumors on the surface of peritoneum and the diaphragm. Metastasis into the eye was found in an i.p. transplant. Routine histologic examinations were done on Carnoy-fixed, paraffin-embedded and toluidine blue-stained tissues.

Electron microscopy

The cells in plastic culture flasks were fixed *in situ* for 30 min to 1 hr with 2·5% glutar-

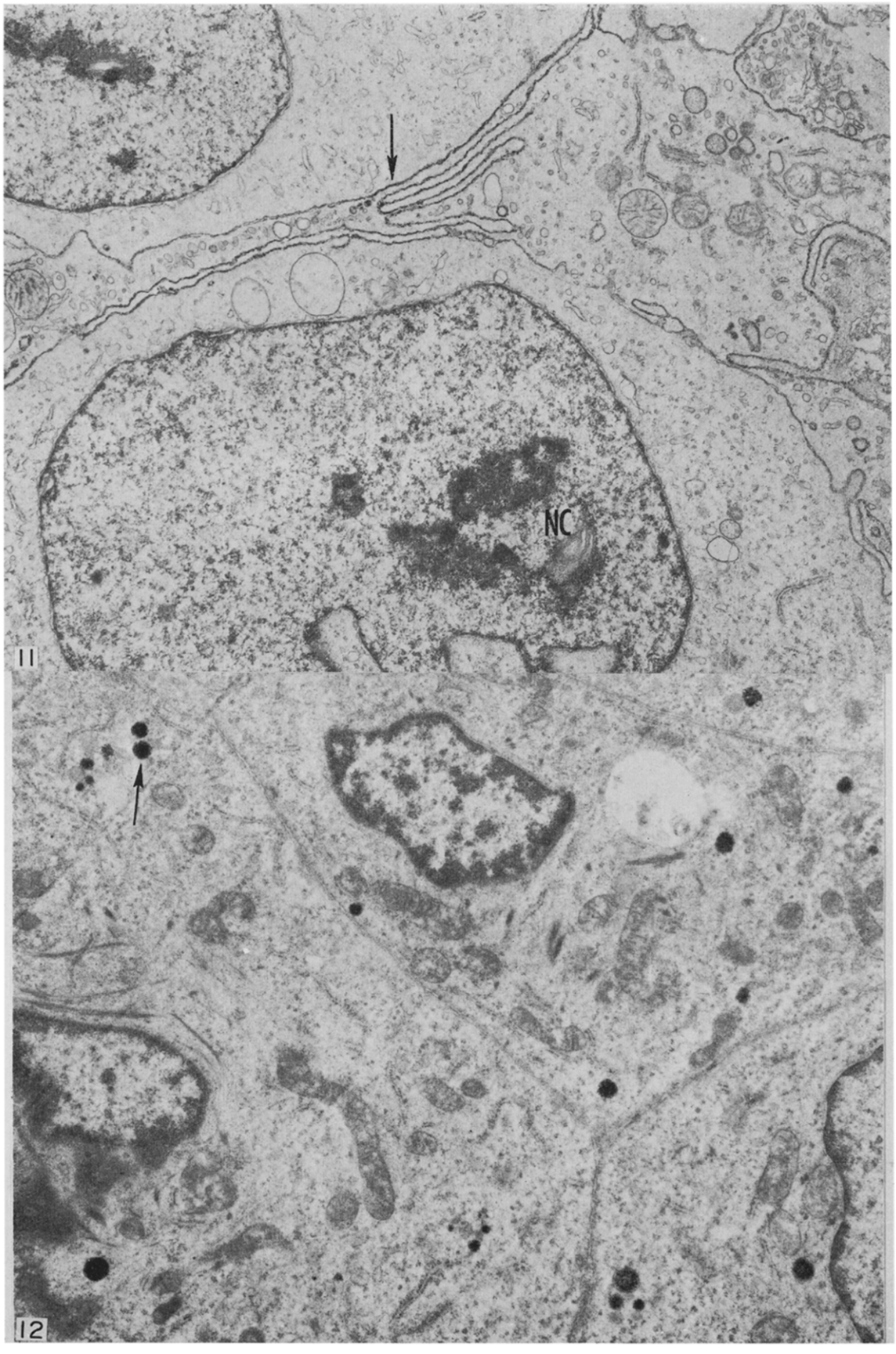


Fig. 11. *ATPase reaction in glutaraldehyde-fixed tumor section. A positive reaction on the pattern of lead phosphate precipitates follows the course of the plasma membranes (arrows). Intranuclear canaliculi (NC).* × 12,000.

Fig. 12. *Diaminobenzidine-H_2O_2 reaction in glutaraldehyde-fixed tumor section. Peroxidase activity is seen located in microbodies of carcinoma cells (arrow).* × 30,000.

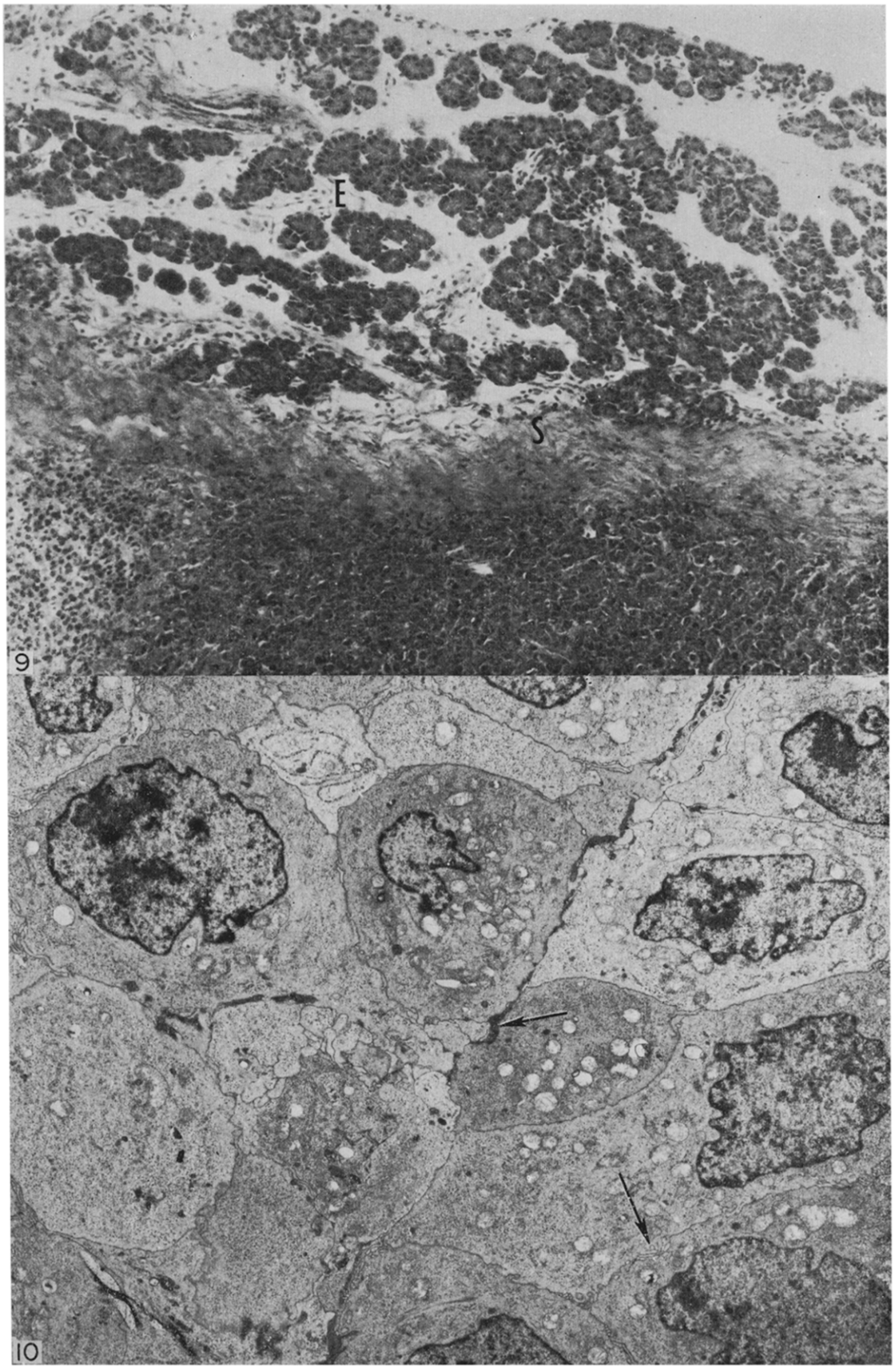


Fig. 9. *Photomicrograph of a solid tumor produced by i.p. transplantation of the cultured cells in passage* 9. *The peripheral portion of a solid tumor shows three parts with different histological patterns as well as variation in staining characteristics. Fibro-stromal tissues* (*S*) *and capillaries* (*E*) *of the host. Carnoy-toluidine blue.* × 225.

Fig. 10. *Electron micrograph of a part of carcinoma tissue. Bile canalicular-like surfaces* (*arrows*). *Opaque amorphous material resembling biliary products.* × 6,750.

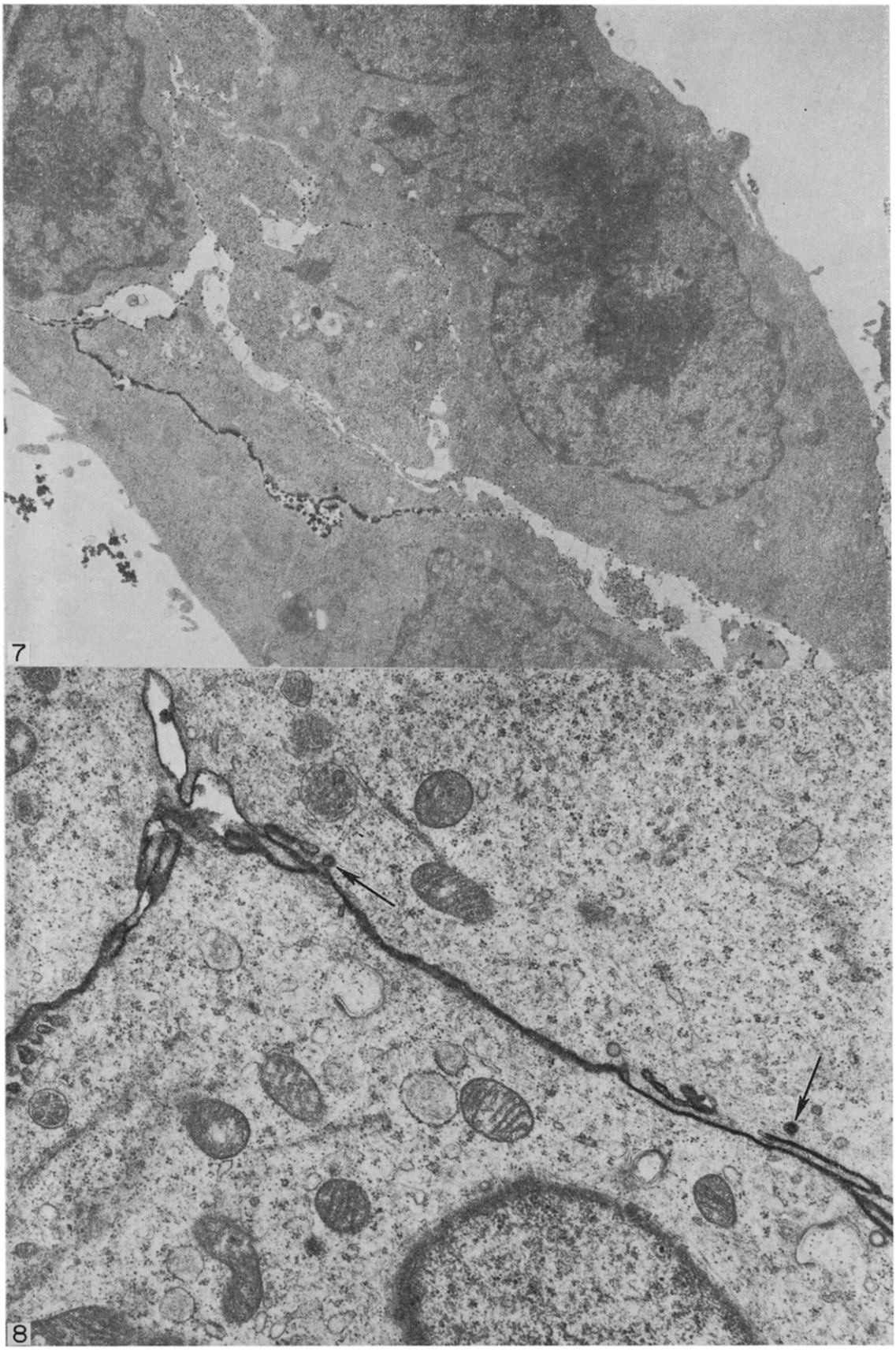


Fig. 7. ATPase reaction in epithelial cells from a preconfluent culture of passage 9. The surface membranes of adjacent cells were seen covered with bands of opaque reaction product. Regional differences in the surface deposits of the reaction product, ranging from few tiny particles to continuous bands. × 7,500.

Fig. 8. Ruthenium red staining of epithelial cells from a confluent of passage 20. In tangential section of 3 adjacent cells, the surface membranes are closely apposed and covered with an electron opaque layer of ruthenium red stained coat. Pinocytotic vesicles (arrows). × 15,000.

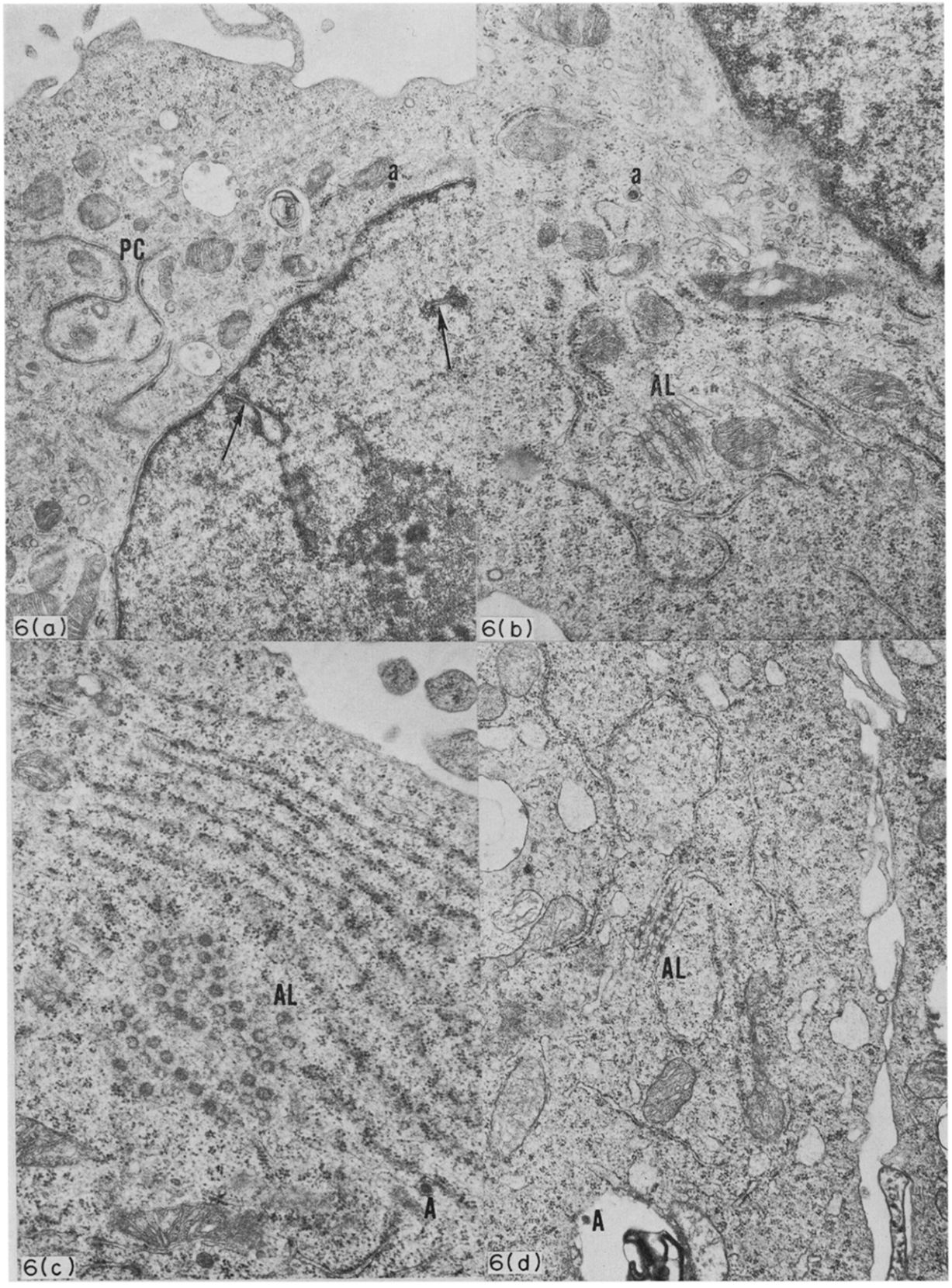


Fig. 6.(a)–(d). *Rat liver epithelial cells in passage* 1.

(a) *Paired cisternae (PC) in the cytoplasm. Small A particle within a smooth vesicle (a). Intranuclear canaliculi (arrows). A large nucleolus with prominent granular components. Uniformly distributed chromatin.* × 15,000.

(b) *Perpendicular section of annuli in cytoplasmic annulate lamellae (AL). Small A particle within a smooth vesicle (a).* × 52,500.

(c) *Tangential section of annuli in cytoplasmic annulate lamellae (AL). Parallel arrays of rough endoplasmic reticulum cisternae. Large A particles within a smooth vesicle (A).* × 30,000.

(d) *Cytoplasmic annulate lamellae (AL) in continuity with rough endoplasmic reticulum. Large A particle within a smooth vesicle (A).* × 22,500.

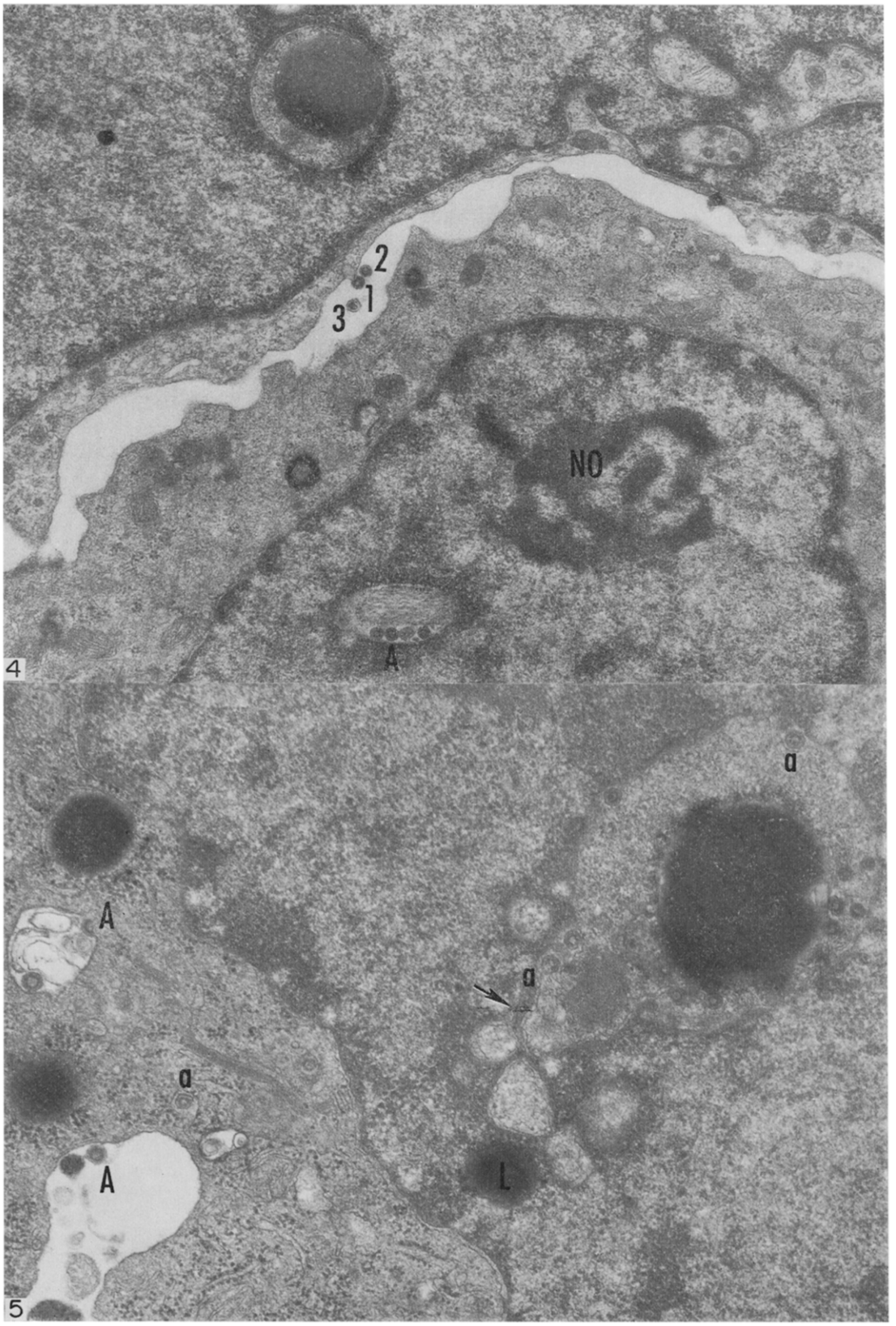


Fig. 4. *Liver epithelial cells in the primary culture (after 7 months).*
Intercellular and intracisternal virus particles. Budding (1), *immature* (2) *and mature* (3) *forms of C particles. Large A particles in the cisternae of nuclear envelope. The nucleolus (NO) show a decreased amount of granular components which are segregated from fibrillar components.* × 30,000.

Fig. 5. *Liver epithelial cells in the primary culture (after 7 months).*
Free and budding forms of intracisternal A particles. Small A particles (a) within the perinuclear cisternae and the vesicles of endoplasmic reticulum. Large A particles (A) within cytoplasmic vacuoles. Intranuclear membranes (arrow) and lipid (L). × 67,500.

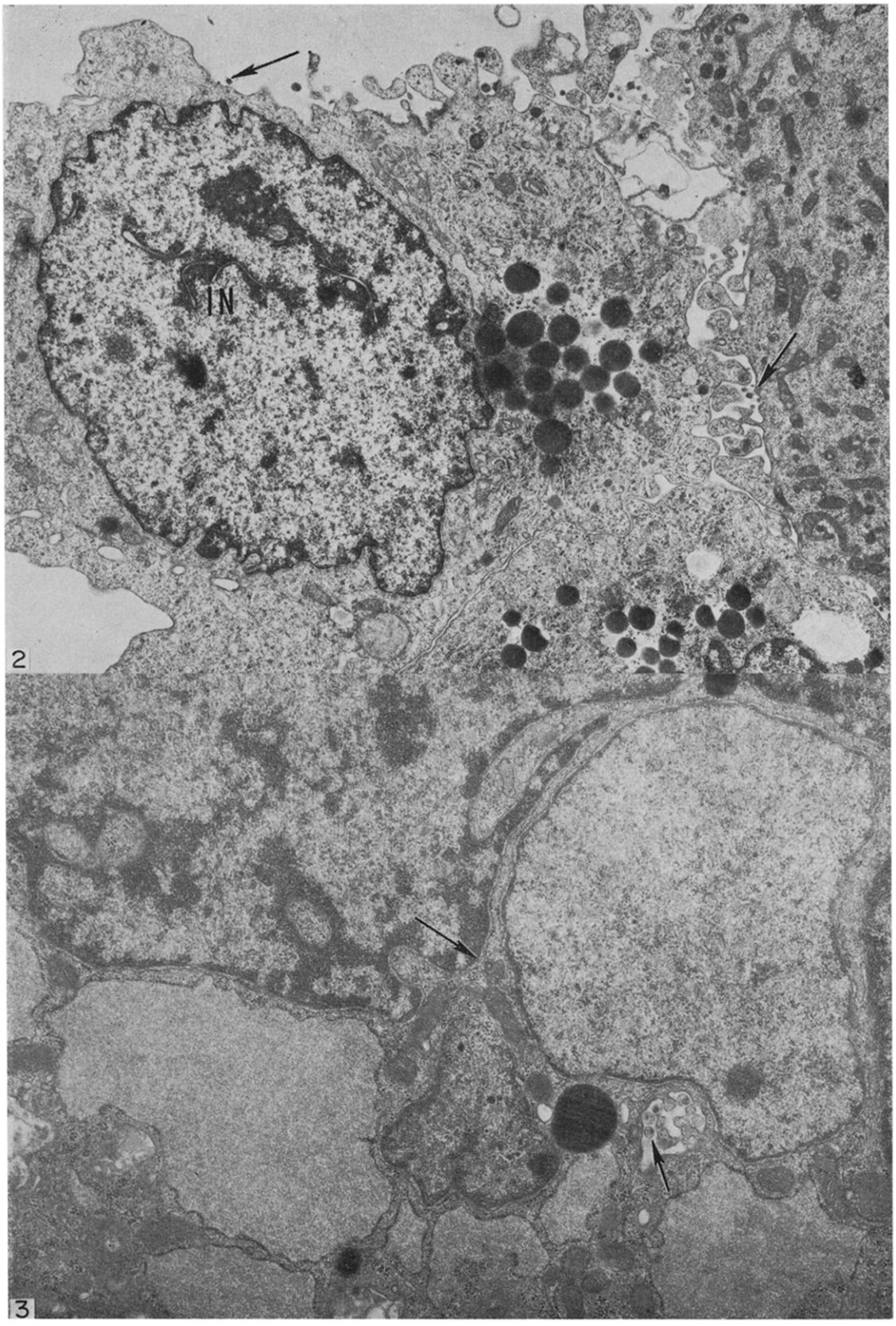


Fig. 2. *Electron micrographs of rat liver epithelial cells in the primary culture (after 7 months). Intranuclear canaliculi (IN). Virus particles (arrows). Undulated cell surface.* × 12,000.

Fig. 3. *Electron micrographs of rat liver epithelial cells in the primary culture (after 7 months). Large and small nuclei with different patterns of chromatin distribution. Virus particles (arrows). Endoplasmic reticulum with flattened sacs, the content of which are moderately dense.* × 22,500.

(to face page 528)

Table 1. *Tumorigenicity of cultured epithelioid cells from rat liver*

Days after subculture*	Passage number†	Tumor incidence‡ i.p.§	s.c.§	Average latency period (days)
44	—6—	0/3	0/2	—
55	—9—	2/3	0/4	—
65	—12—	—	1/3	15
80	—14—	2/2	2/5	18
98	—9—Cl—1—	—	5/6	12
118	—20—	2/4	3/4	13
157	—16—Cl—2—	—	2/2	14
389	—50—	—	3/4	10

*Plus 237 days in the primary culture of isolated hepatocytes.
†Passage number *in vitro*, except Cl (1 passage *in vivo*).
‡Number of animals with carcinoma/Number of newborns inoculated.
§Route of implantation of 1–2 × 10^6 cells per animal.

aldehyde (Ladd) in 0·1 M sodium cacodylate-HCl buffer (pH 7·1), washed for 1–4 hr in 7·5% sucrose solution, post-fixed for 1 hr with 1·4% OsO_4 in 0·1 M *s*-collidine buffer (pH 7·1), dehydrated in a graded ethanol–water series and embedded in Epon. Sections were prepared for both light and electron microscopy. Thin sections were stained with uranyl acetate and lead citrate, and were viewed in a JEM-7A electron microscope. Specimens of tumor tissues were similarly processed.

Cytochemistry

To detect cytochemical alterations of surface membrane of the cells during passages *in vitro* and *in vivo*, the method of Wachstein and Meisel [28] for ATPase demonstration was applied on glutaraldehyde-fixed cells *in situ* in plastic flasks or on sections of fixed tumors. The method of Novikoff and Goldfisher [27] was similarly applied for detection of peroxisomes [29]. Fixatives containing ruthenium red were also used to identify carbohydrate-containing structures on the cell surface [30].

RESULTS

The primary culture

During 6 months period the originally explanted hepatocytes remained attached over the surface of flask bottom. Each of 7 separate cultures from the single preparation ($< 2{\cdot}5 \times 10^8$ cells) contained 3–10 foci of colonies of epithelial cells and sparsely scattered stellate-type cells. The foci of epithelial cells grew very slowly, although mitoses were never detected.

The ultrastructural examinations were performed on the colonies of epithelial cells after 7 and 8 months in culture. It appeared that they had lost almost all of their original tissue characteristics at the subcellular level, but they were found to vary markedly from one another in fine structure. The cross section of epithelial cells *in situ* showed contacts of the plasma membrane to the plastic substratum and an accumulation of microfilaments and microtubules adjacent to the membrane, suggesting a strong adhesion of cells with the substratum. Cell-to-cell contacts were mostly focal at the crests of surface undulation (Fig. 2). Cells contained more than one nucleus, which demonstrated different patterns of chromatin distribution (Fig. 3). The nuclei were extremely deformed and lobulated in outline, frequently possessing deep cytoplasmic invaginations (Fig. 2–5). The nucleoplasm included canalicular membranes, of which structure appeared to be similar to that of the inner nuclear membrane (Fig. 2). In the cytoplasm, there were membranous profiles of endoplasmic reticulum scattered singly or in parallel arrays (Fig. 2–5). Numerous structures resembling oncorna virus particles were frequently seen (Fig. 2–5). Extracellular C particles [31] and two types of intracisternal A particles [32] were identified (Fig. 4 and 5). C particles measured 90–100 nm dia and were formed by a typical process of budding from the cell membrane into the intercellular space (Fig. 4). Immature C particles comprised a relatively electron-lucent core surrounded by an electron dense shell (Fig. 4). Mature C particles contained a central dense core surrounded by an irregularly contoured membrane (Fig. 4). Small and large types of A particles were present in the cisternae of nuclear envelope and the vesicles of endoplasmic reticulum (Fig. 5). Particle size ranged from 70–80 nm for the small type and

from 90–110 nm for the large type (Fig. 5). Both types of particles appeared to contain 2 concentric dense shells and an electron lucent center (Fig. 5). Budding forms of both types of particles were seen from the membranes of either endoplasmic reticulum or nuclear envelope (Fig. 5).

Subcultures

The cultures of passage 1 contained epithelial cells mixed with stellate-type cells. Mitoses were frequently seen. Following further subcultures, the cells became more homogeneous in size and shape.

Comparative studies at the ultrastructural level were performed and the subcellular features in epithelial cells in the primary culture as well as in subcultures are summarized in Table 2 with reference to the sequence of passage numbers. In passage 1, the majority of cells showed less irregularities of nuclear fine structure when compared to those of primary culture. Only a few exhibited intranuclear canalicular membranes (Fig. 6a). Paired cisternae (Fig. 6a) and annulate lamellae (Fig. 6b–d) were frequently found in passage 1. These cytoplasmic membranous structures morphologically resembling the nuclear membrane were often located adjacent to the nucleus (Fig. 6a). They were also found in continuity with the membranes of endoplasmic reticulum (Fig. 6d). Virus particles were seen within the vesicles of endoplasmic reticulum adjacent to annulate lamellae or paired cisternae (Fig. 6a–d) and rarely in association with the cell surface, but none within the perinuclear cisternae.

The fine structure of nuclei and cytoplasm did not appear to change remarkably with passage number. There were few specializations of cytoplasmic organelles, accompanying the confluency of cells. Rough endoplasmic reticulum cisternae were quite prominent in post-confluent cultures. Peroxisomes and glycogen particles were not detectable by cytochemical methods. Microfilaments adjacent to the plasma membranes were rare at the preconfluency and increased significantly at the confluency. A few virus particles were detectable by passage 6, while later their detection was seldom.

In passages 4 and 6, the junctions of close apposition between individual cells were mostly focal and the intracellular dilations were evident. Using the Wachstein–Meisel technique, ATPase reaction was not seen by passage 8 (Table 2). In passage 9, however, some colonies showed the reaction product at the outer surface of plasma membranes of epithelial cells (Fig. 7). The distribution of the reaction product varied among individual cells, such as complete absence from some and its restriction to certain regions on the surface of others (Fig. 7). The ATPase-positive cells were ultrastructurally indistinguishable from neighboring cells which showed no activity. All epithelial cells from passage 12 exhibited reaction products at random along the entire plasma membranes, although it was more abundant on the apposed surfaces than the

Table 2. Sequential alterations of cultured epithelial cells after in vitro *and* in vivo *passages*

Passage number*	Electron microscopic detection†				Cytochemical reaction†
	Virus particles	Intranuclear canalicular membranes	Cytoplasmic annulate lamellae	Surface close appositions	Surface ATPase activity
—0—	+	+	−/+	−/+	—
—1—	+/−	−/+	+	−/+	—
—4—	−/+	−/+	−/+	−/+	—
—6—	−/+	—	—	−/+	—
—8—	—	—	—	−/+	—
—9—	—	—	—	+	+/−
—12—	—	—	—	+	+
—14—	—	—	—	+	+
—9—Cl—1—	−/+	−/+	−/+	+	+
—20—	—	—	—	+	+
—16—Cl—2—	—	—	—	+	+
—50—	—	—	—	+/−	+

*Passage number *in vitro*, except Cl (1 passage *in vivo*).
† + : Frequent detection in thin sections of cells.
+/− : infrequent, −/+ : rare, —: seldom or never encountered.

apical one. In passage 12 or later, intracellular junctions of close apposition became prominent. The ruthenium red stained layer of the cell surface was similar in thickness and configuration at the different stages of passages (Fig. 8).

Carcinomas produced by transplantation in vivo

In either s.c. or i.p. transplantation, solid tumors were formed in close association with the surrounding fibrostromal tissues of the recipient animals. As seen in Fig. 9, tumor nodules had the appearance of malignant hepatomas or hepatocellular carcinomas at various degrees of histological differentiation. Tumor cells were irregularly packed or arranged in interlacing strands, but sometimes showed adenomatous formations. Trabecular patterns were often found with the host fibrostromal network and in association with capillary proliferation. In undifferentiated tumors, individual cells were various in size, and polygonal or occasionally fusiform in shape. Mitotic figures were observed more frequently than *in vitro*.

In thin sections tumor cells appeared related to each other by their parallel, often interdigitating cell surfaces (Fig. 10). In many areas, the interdigitation of cell membranes had the appearance of bile canaliculi. It was noted that tumor cells were often closely apposed with leukocytes and other host cells. Cytochemical tests for ATPase activity gave a positive reaction along the surface membranes of tumor cells (Fig. 11). Only solitary virus particles infrequently were seen within the intercellular spaces. The nuclei sometimes contained intranuclear canaliculi. In the cytoplasm, many peroxisomes [29] were identified by the cytochemical test (Fig. 12).

In vitro *cultivation of carcinomas*

Peripheral areas of the tumors produced by the implantation of cultured cells were explanted in the plastic dishes. After 1 day, many round cells migrated out of the explants. This process was followed by the outgrowth of flattened epithelial cells, which appeared to be morphologically identical to those observed in continuous subcultures (Table 2). One million cells were harvested and implanted into newborn rats after 2 and 3 weeks *in vitro*. In the implanted rats, carcinomas were produced after 2 weeks (Table 1).

DISCUSSION

Only a few reports have been published on the development of carcinomas upon *in vivo* transplantation of liver cells which were grown *in vitro* [14]. The present experiment has demonstrated the production in Wistar rats of hepatocellular carcinomas derived from normal adult liver cells in culture. A series of implantation experiments and morphologic examinations have suggested that the cultured cells acquired malignant properties, step by step. This may be true, not only for the growth pattern of cell populations but also for the subcellular differentiation of individual cells *in vitro* and *in vivo*.

The development of tumors resembling hepatocellular carcinomas *in vivo* supports the possibility that unspecific epithelial cells observed *in vitro* were originated from hepatocytes. The findings of peroxisomes and canalicular-like structures in the carcinomas are of particular significance, since these organelles are prominent in differentiated hepatocytes [33]. It seems probable that the induction of a differentiated state occurs *in vivo* rather than *in vitro*.

In the present investigation, the primary culture was initiated with a highly homogeneous population of hepatocytes which were isolated from normal adult rat liver. Regular examinations during several months in the period of explantation, showed little variation in cell numbers without detectable mitosis. An excessively long period before subcultures would be expected to be selective for a population of cells capable of growth under adverse conditions. It should be pointed out that the culture condition in the present experiment resembles those which Borek [13] found to result in "spontaneous transformation" of established liver epithelial cells. A sublethal damage of cells at the time of liver dissociation or explantation as well as a long residence in quiescent phases might be considered important effects toward "transformation" *in vitro*. The "transformed" colonies of epithelial cells found in the primary cultures, however, were practically in non-proliferative state.

At the first subculture, trypsinization could trigger the conversion of quiescent cells into continuously replicating ones. The mode of cell proliferation changes markedly from that of the parental cells in the course of relatively a few mitotic cycles, and they could soon divide typically every 30 hr or shorter at the logarithmic growth as seen in other established liver cell lines [5, 9, 13, 34]. With repeated passages of the cell line over a period of 2 months, the fast-proliferating cells could produce tumors on *in vivo* transplantation. The increase of proliferating pool due to an altered pattern

of cell cycle is a prerequisite for malignant conversion and, in this respect, the sequence of events during early passages *in vitro* also resembles that observed in premalignant lesions *in vivo* [22, 24, 35, 36].

The present study has demonstrated A and C virus particles in epithelial cells derived from normal rat liver. A close examination of the picture published by Gerschenson *et al.* [5] also shows a similar particle (but designated as "microbody") in cultured rat liver cells (RLC). Recently, Weinstein *et al.* [8] have succeeded in demonstrating endogenous A and C particles in 5 hepatoma cultures and in a spontaneously transformed liver cell line, but so far such particles were not detectable in the 5 independently isolated cell cultures from normal rat liver. The association of virus particles would not be considered a sign of malignancy, since our cell line rather ceased viral production after passages *in vitro* when it became tumorigenic.

Virus particles of hepatic cells have been found in association with the cell surface and the endoplasmic reticulum or its derivatives [19, 26, 37–39], but little attention has been paid to the presence of virus particles within the nuclear envelope. We found in the primary cultures two types of A particles which appeared to be budding from the nuclear membranes. Coincidentally, there were unusual formations of intranuclear canaliculi which might be derived by deep invaginations of only the inner nuclear membrane [40]. The development of intranuclear membranes in non-dividing cells is suggestive of a continued synthesis of the inner nuclear membrane under an interaction of viral ligaments at a period when nucleoplasm volume is stable. This type of intranuclear membranes were also demonstrated in various neoplastic cells which could produce A or C type virus particles [12, 37, 38, 41]. An interaction of viral ligaments might cause abnormal membrane formations.

When released from a mitotic block by subculture, the proliferating cells had transitory cytoplasmic structures, such as annulate lamellae and paired cisternae. These organelles have been suggested to arise from the nuclear envelope [42–44]. Their apparent structural relationship to endoplasmic reticulum has been also indicated in actively growing or proliferating cells in normal and malignant tissues [25, 38, 39, 42–44]. The emergence of these transitory organelles in the present cell line might result from viral interaction with the nuclear envelope, since it is frequently accompanied by virus particles in the endoplasmic vesicles. The sequential phenotypic expressions at the specific stages *in vitro* suggest a developmental role of oncorna virus in subcellular differentiation leading to a neoplastic state.

An essential prerequisite for the malignant conversion *in vitro* would be alterations which eventually influence the cell surface properties characteristic of neoplastic cells [13, 21, 23, 24, 30, 39, 45]. With the Wachstein–Meisel method for cytochemical demonstration of ATPase activity, a positive reaction at the surface membrane of cells was found concomitant with the acquisition of tumorigenicity in the cell population. An intense ATPase reaction was biochemically demonstrated at the surface of intact cells from all tumorigenic liver cell lines tested, including the present line [46]. Similar surface changes in phosphatase localizations have been reported in hepatocellular carcinomas during *in vivo* transplantation [39, 44] and in liver parenchyma during the administration of a carcinogenic azo dye [23, 24]. Since there was a close temporal correlation between the cytochemical pattern *in vitro* (Table 2) and tumorigenicity *in vivo* (Table 1), the cell surface alteration would be considered as a useful marker for a further study of malignant conversion of epithelial cells.

It was recognized during passages *in vitro* that epithelial cells decreased their adhesiveness toward the plastic substratum while intercellular junctions of close apposition became gradually prominent. The implantation of cultured cells into rats exhibited histological differentiation which might involve the process of preferential convergence of cells within the stromal tissue of a recipient. The differential adhesiveness as well as the increase of surface ATPase activity of the epithelial cells would facilitate for a growth advantage upon *in vivo* transplantation.

Acknowledgement—We thank Luciano Borsato for valuable technical help, and Bernard Szirth for assistance in photography. We also thank Andrée LeMyre, Aline Farner and Alec Tretiak who prepared cell cultures during the course of this work.

REFERENCES

1. R. W. Alexander and J. W. Grisham, Explant culture of rat liver. I. Method, morphology, and cytogenesis. *Lab. Invest.* **22,** 50 (1970).

2. D. M. Bissell, L. E. Hammaker and U. A. Meyer, Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I. Functional studies. *J. Cell Biol.* **59,** 722 (1973).
3. M. N. Berry and D. S. Friend, High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43,** 506 (1969).
4. G. de Lamirande and A. Simard, Étude biochimique d'hépatocytes en culture provenant de rats normaux adultes. *Rev. canad. Biol.* **34,** 111 (1975).
5. L. E. Gerschenson, T. Okigaki, M. Andersson, J. Molson and M. E. Davidson, Fine structural and growth characteristics of cultured rat liver cells. *Expt. Cell Res.* **71,** 49 (1972).
6. P. T. Iype, Cultures from adult rat liver cells. I. Establishment of monolayer cell-cultures from normal liver. *J. Cell Physiol.* **78,** 281 (1971).
7. T. Okigaki and S. G. Quan, Morphological inconsistency in liver cells in culture. *Proc. Japan Acad.* **50,** 517 (1974).
8. I. B. Weinstein, J. M. Orenstein, R. Gebert, M. E. Kaighn and U. C. Stadles, Growth and structural properties of epithelial cell culture established from normal rat liver and chemically induced hepatomas. *Cancer Res.* **35,** 253 (1975).
9. G. M. Williams and J. M. Gunn, Long-term cell culture of adult rat liver epithelial cells. *Exp. Cell Res.* **89,** 139 (1974).
10. G. M. Williams, K. Stromberg and R. Kroes, Cytochemical and ultrastructural alterations associated with confluent growth in cell cultures of epithelial-like cells from rat liver. *Lab. Invest.* **29,** 293 (1973).
11. G. M. Williams, E. K. Weisburger and J. H. Weisburger, Isolation and long-term culture of epithelial-like cells from rat liver. *Exp. Cell Res.* **69,** 106 (1971).
12. G. Yasuzumi and R. Sugihara, The fine structure of nuclei as revealed by electron microscopy. The fine structure of Ehrlich ascites tumor cell nuclei in preprophase. *Exp. Cell Res.* **37,** 207 (1965).
13. C. Borek, Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. *Proc. nat. Acad. Sci. (Wash.),* **69,** 956 (1972).
14. Y. Oshiro, L. E. Gerschenson and J. A. DiPaolo, Carcinomas from rat liver cells transformed spontaneously in culture. *Cancer Res.* **32,** 877 (1972).
15. J. Sato, M. Namba, K. Usui and D. Nagano, Carcinogenesis in tissue culture. VIII. Spontaneous malignant transformation of rat liver cells in long-term culture. *Jap. J. exp. Med.* **38,** 105 (1968).
16. H. Katsuta and T. Takaoka, Carcinogenesis in tissue culture. XIV. Malignant transformation of rat liver parenchymal cells treated with 4-nitroquinoline 1-oxide in tissue culture. *J. nat. Cancer Inst.* **49,** 1563 (1972).
17. K. Koshiba, M. Namba and T. Oda, Electron microscopic studies on cultured rat liver cells transformed by 4-nitroquinoline 1-oxide. *Gann* **61,** 233 (1970).
18. J. Sato, Malignant transformation in culture of rat liver cells treated with or without 3'-methyl-4-dimethylaminoazobenzene. In *Cancer Cells in Culture.* (Edited by H. Katsuta) p. 335. University of Tokyo Press, Tokyo (1968).
19. G. M. Williams, J. M. Elliott and J. H. Weisburger, Carcinoma after malignant conversion *in vitro* of epithelial-like cells from rat liver following exposure to chemical carcinogens. *Cancer Res.* **33,** 606 (1970).
20. C. Altaner and E. Hlavayova, Transformation of rat liver cells with chicken sarcoma virus B77 and murine sarcoma virus. *J. Virol.* **11,** 177 (1973).
21. K. K. Sanford, Biologic manifestation of oncogenesis *in vitro*: A critique. *J. nat. Cancer Inst.* **53,** 1481 (1974).
22. S. Karasaki, The fine structure of proliferating cells in preneoplastic rat livers during azo-dye carcinogenesis. *J. Cell Biol.* **40,** 322 (1969).
23. S. Karasaki, Subcellular localization of surface adenosine triphosphatase activity in preneoplastic liver parenchyma. *Cancer Res.* **32,** 1703 (1972).
24. S. Karasaki, Cell proliferation and subcellular localization of alkaline phosphatase activity in rat liver during azo dye carcinogenesis. *Cancer Res.* **35,** 482 (1975).

25. L. P. Merkow, S. M. Epstein, E. Farber, M. Pardo and B. Bartas, Cellular analysis of liver carcinogenesis. III. Comparison of the ultrastructure of hyperplastic liver nodules and hepatocellular carcinomas induced in rat liver by 2-fluorenyl acetamide. *J. nat. Cancer Inst.* **43,** 33 (1969).
26. I. B. Weinstein, R. Gebert, V. C. Stadler, J. M. Orenstein and R. Axel, Type C virus from cell cultures of chemically induced rat hepatomas. *Science* **178,** 1098 (1972).
27. A. B. Novikoff and S. Goldfisher, Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* **17,** 675 (1969).
28. M. Wachstein and H. Meisel, Histochemistry of hepatic phosphatase at physiologic pH. *Amer. J. clin. Pathol.* **27,** 13 (1957).
29. Z. Hruban, E. L. Vigil, A. Slevers and E. Hopkins, Microbodies—constituent organelles of animal cells. *Lab. Invest.* **27,** 184 (1972).
30. A. Martinez-Palomo, C. Braislovsky and W. Bernhard, Ultrastructural modifications of the cell surface and intercellular contacts. *Cancer Res.* **29,** 925 (1969).
31. W. Bernhard, The detection and study of tumor viruses with the electron microscope. *Cancer Res.* **20,** 712 (1960).
32. D. G. Chase and L. P. Piko, Expression of A- and C-type particles in early mouse embryo. *J. nat. Cancer Inst.* **51,** 1971 (1972).
33. C. Rouiller and A. M. Jezequiel, Electron microscopy of the liver. In *The Liver. Morphology, Biochemistry, Physiology.* I. (Edited by C. Rouiller) p. 195. Academic Press, New York (1963).
34. E. B. Thompson, G. M. Tomkins and J. F. Curran, Induction of tyrosine-α-keto glutarate transaminase by steroid hormones in a newly established tissue culture line. *Proc. nat. Acad. Sci. (Wash.)* **56,** 296 (1966).
35. W. Oehlert, Cellular proliferation in carcinogenesis. *Cell Tissue Kinet.* **6,** 325 (1973).
36. A. Simard, G. Cousineau and R. Daoust, Variations in the cell cycle during azo dye carcinogenesis. *J. nat. Cancer Inst.* 41, 1257 (1968).
37. S. Karasaki, Virus particles associated with the transplantable Novikoff hepatoma in the rat. *Cancer Res.* **29,** 1313 (1969).
38. J. Locker, P. J. Goldblatt and J. Leighton, Some ultrastructural features of Yoshida ascites hepatoma. *Cancer Res.* **28,** 2039 (1968).
39. A. B. Novikoff and L. Biempice, Cytochemical and electron microscopic examination of Morris 5123 and Reuber H-35 hepatomas after several years of transplantation. *Gann Monogr.* **1,** 65 (1966).
40. S. Karasaki, An electron microscope study of intranuclear canaliculi in Novikoff hepatoma cells. *Cancer Res.* **30,** 1736 (1970).
41. E. Bucciarelli, Intranuclear cisternae resembling structures of the Golgi complex. *J. Cell Biol.* **30,** 664 (1966).
42. H. Hanaoka and B. Friedman, Paired cisternae in human tumor cells. *J. Ultrastruct. Res.* **32,** 323 (1970).
43. R. O. Kelly, Ultrastructural comparisons of paired cisternae in leukemic and mesenchymal cells. *Anat. Rec.* **171,** 559 (1971).
44. R. G. Kessel, Annulate lamellae. *J. Ultrastruct. Res. Suppl.* **10,** 1 (1968).
45. E. Essner and M. D. Reuber, Localization of acid phosphatase activity in well-differentiated hepatocellular carcinoma 146. *J. nat. Cancer Inst.* **47,** 25 (1971).
46. S. Karasaki, Surface membrane ATPase activity in transformed rat liver epithelial cell lines. *J. Cell Biol.* **67,** 200a (1975).

Europ. J. Cancer Vol. 12, pp. 535–540. Pergamon Press 1976. Printed in Great Britain

Radiosensitivity of the Acute Leukaemic Infiltrate

K. ATKINSON, P. R. M. THOMAS, M. J. PECKHAM and T. J. McELWAIN
Leukaemia Unit and the Department of Radiotherapy, Institute of Cancer Research and Royal Marsden Hospital, Sutton, Surrey, U.K.

Abstract—*Local radiotherapy was given to* 23 *patients with acute leukaemia who had clinically apparent extramedullary leukaemic infiltrates which were unresponsive to systemic cytotoxic chemotherapy. A further patient with a localized granulocytic sarcoma also received local irradiation. Altogether* 28 *different sites in* 24 *patients were irradiated. Acute leukaemic infiltrates showed variable radioresponsiveness. They usually responded to doses of the order of* 2000–2500 *rads, but not invariably so. Although in some instances complete response was obtained with lower doses* (400–800 *rads), experience has shown that occasionally both acute lymphoblastic and acute myelogenous leukaemic infiltrates fail to regress completely after doses of more than* 2000 *rads. Response to irradiation was not influenced by the state of the bone marrow at the time of irradiation. Cytotoxic chemotherapy, effective for disease at other sites, and given concurrently with irradiation, similarly had no influence on the local radiation result. A tumour dose of* 2500 *rads is recommended in those patients where there is a reasonable prospect of long term overall control of the disease and where radiation is not regarded as a purely palliative procedure. The rhythm and fractionation and overall treatment time will depend upon the site and volume irradiated.*

INTRODUCTION

APART from the use of radiotherapy for the treatment of neuroleukaemia in acute lymphoblastic leukaemia (ALL) [1], little has been written about the therapeutic, as opposed to the prophylactic, application of this form of treatment in acute leukaemia. Aur *et al.* [1] showed that 2400 rads craniospinal irradiation was insufficient to eradicate central nervous system leukaemia once it had developed, although more recent results combining neuraxis irradiation with intrathecal methotrexate are promising [2]. Apart from involvement at this site, however, little has been written about the radiosensitivity of the acute leukaemic infiltrate, although it is generally considered to be relatively radiosensitive. D'Angio *et al.* [3] emphasized the value of local irradiation for symptomatic relief in acute leukaemia, but did not employ doses of more than 1400 rads. We therefore decided to look retrospectively at a group of patients who had received radiotherapy for extramedullary leukaemic infiltrates in an attempt to assess their radiosensitivity.

Accepted 23 January 1976.

Requests for reprints should be sent to Dr. K. Atkinson, Hutchinson Cancer Center, Seattle, WA 98104, USA.

MATERIAL AND METHODS

Twenty-three patients had acute leukaemia, 10 had acute lymphoblastic leukaemia (ALL), 11 had acute myeloblastic leukaemia (AML), one had acute undifferentiated leukaemia (AUL), and one had blast transformation of chronic myeloid leukaemia; one patient had a granulocytic sarcoma without evidence of overt acute leukaemia. Of the 10 patients with ALL, four had the variety associated with a large anterior mediastinal mass; four patients had two different sites irradiated. Clinical details are shown in Tables 1 and 2. All patients with central nervous system leukaemia also received intrathecal chemotherapy (cases 2, 3, 4, 9, 13, 21, 25, 28). For convenience those patients with ALL are designated the lymphoblastic group (Table 2), and all the other patients the myelogenous group (Table 1). The general management of these patients has been described elsewhere [4, 5].

RESULTS

Of the 24 patients in the group four patients had two different sites irradiated making a total of 28 sites irradiated altogether. The results are shown in Table 3. Overall, a complete response without subsequent local

Table 1. *Clinical details of patients in the myelogenous group*

Response	Site	Radiation details: Dose/Fractions/Days (rads)	N.S.D. (rets)	Follow-up time for site irradiated (months)	Time to local recurrence (months)	Overall survival
Complete response with no local recurrence (10)	1. Tonsil (AML)	880/ 6 / 6	220	1	—	2 months. Dead
	2. Cranium (CGLBT)	2052/ 6 /14	757	2	—	8 months. Dead
	3. (i) Cranium and (ii) Spine (AML)	(i) 2500/ 14 /20 (ii) 900/ 5 / 8	700 253	2	—	13 months. Alive and well.
	4. Cranium (AML)	2400/ 7 /16	887	3	—	3 months. Dead
	5. Kidneys (AML)	1400/ 14 /22	273	3	—	14 months. Alive without leukaemia
	6. Abdomen (AML)	1700/ 8 /12	528	6	—	13 months. Alive and well
	7. Lymph node (AUL)	2500/ 10 /17	819	12	—	17 months. Dead
	8. Sclera (AML)	2700/ 8 /15	988	13	—	26 months. Dead
	9. Cranium (AML)	2500/ 7 /17	945	16	—	32 months. Dead
	10. Sinuses (GS)	2916/ 17 /29	774	24	—	24 months. Dead
Complete response with subsequent local recurrence (3)	11. Skin (AML)	2000/ 5 / 7	879	2	2	8 months. Dead
	12. Abdomen (AML)	400/ 3 / 3	95	4	4	12 months. Dead
	13. Cranium and Spine (AML)	2200/ 12 /24	572	7	7	11 months. Dead
Partial response (3)	14. Breasts (AML)	2120/ 10 /29	553	3		6 months. Dead
	15. Skin	2500/ 12 /23	698	3		
	16. Bone (AML)	1800/ 6 /14	617	2		14 months. Alive
No response (1)	17. Spleen (AML)	500/ 8 /10	78	1		3 months. Dead

Key—CGLBT Chronic granulocytic leukaemia blast transformation.
GS Granulocytic sarcoma.

recurrence was obtained at 17 of the 28 sites (10 of 17 sites in the myelogenous group; seven of 11 sites in the lymphoblastic group). The duration of post-irradiation follow-up on the complete responses without local recurrence varied: 1, 2, 2, 3, 3, 6, 12, 13, 16 and 24 months in the myelogenous group; 1, 1, 4, 7, 9, 26 and 59 months in the lymphoblastic group. The radiation dose in this group of complete responses without local recurrence also varied but was 1700 rad or more, in 14 of the 17 sites. Only two received low doses, and it is interesting that one of these (case 24, 750 rad in six fractions over 14 days for ALL testicular infiltration) remains in complete local remission 59 months after irradiation.

Table 2. Clinical details of patients in the lymphoblastic group

Response	Site	Radiation details: Dose/Fractions/Days (rads)	N.S.D. (rets)	Follow-up time for site irradiated (months)	Time to local recurrence (months)	Overall survival
Complete response with no local recurrence (7)	18. Testicles (ALL/ mediastinal mass)	2200/ 11 /14	657	1	—	17 months. Dead
	19 Vertebrae (ALL)	2336/ 12 /19	666	1	—	65 months. Dead
	20. Retinae (ALL)	2500/ 12 /16	745	4	—	11 months. Dead
	21. (i) Cranium and (ALL) (ii) Spine	(i) 2400/ 14 /20 (ii) 980/ 5 / 5	665 289	7	—	36 months. Alive and well
	22. Mediastinum (ALL/ mediastinal mass)	1700/ 7 / 9	565	9	—	9 months. Dead
	23. Lymph node areas above diaphragm (ALL/ mediastinal mass)	2600/ 9 /19	873	26	—	26 months. Alive and well
	24. Testicle (ALL)	750/ 6 /14	157	59	—	60 months. Alive and well
Complete response wtth subsequent local recurrence (2)	25. Cranium (ALL)	2500/ 13 /17	727	2	2	9 months. Dead
	26. Lymph node areas above diaphragm (ALL/ mediastinal mass)	3500/ 13 /30	1292	24	24	25 months. Dead
Partial response (2)	27. Testicle (ALL)	2850/ 11 /60	741	2		45 months. Dead
	28. Cranium (ALL/ mediastinal mass)	2460/ 8 /21	795	24		26 months. Alive

Table 3. Results of local irradiation

	Myelogenous group	Lymphoblastic group	Total
No. patients	14	10	24
No. sites irradiated	17	11	28
Complete response without local recurrence	10	7	17
with local recurrence	3	2	5
Partial response	3	2	5
No response	1	0	1

A complete response, but with subsequent local recurrence at 2, 2, 4, 7 and 23 months after irradiation, was also obtained at five other sites, bringing the total of complete responses to 22 out of the 28 sites irradiated. Three patients were in the myelogenous group and two in the lymphoblastic group. The radiation dose was only less than 2000 rad in one of these patients (case 12): he received 400 rad for hepatosplenomegaly and para-aortic lymphadenopathy causing inferior vena caval obstruction: renal infiltration occurred four months later. Thus a total of 18 out of the 22 sites in which a complete response was obtained received 1700 rad or more. In order to compare

the various doses a scattergram of the nominal standard doses (NSD) calculated in rets (6) has been made (Fig. 1): 18 of the 22 complete responses received 500 rets or more.

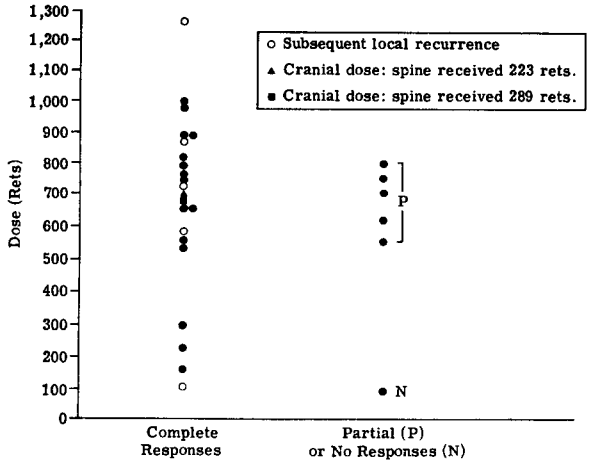


Fig. 1. Correlation of radiation response with radiation dose (rets).

This scattergram (Fig. 1) emphasizes the wide range of radiosensitivity of these infiltrates not only by the occasional complete responder given low dose irradiation, but by the small group of partial responders given relatively high dose irradiation; thus, a poor partial response was obtained in one patient with acute myeloblastic leukaemia who had both skin and breast deposits (case 14). These deposits were confirmed histologically before irradiation and a second biopsy after completion of irradiation, when tumour volume reduction was approximately 40%, still showed leukaemic infiltration. In the lymphoblastic group a boy with testicular involvement (case 27) showed only a poor response (approximately 40% tumour volume reduction) after radiotherapy using a split course: the initial therapy was given as 1350 rads in six fractions over 10 days, and this produced the 40% reduction in tumour volume. He was then given a five and a half week break to see if further reduction would occur. It did not, and he received a second course of 1500 rads in five fractions over eight days to no further effect. The NSD of the two courses combined was 741 rets.

The state of the bone marrow at the time of irradiation of the leukaemic infiltrate made no difference to the result of the irradiation (Table 4). Chemotherapy given at the time of irradiation also seemed to make little difference to the result of the irradiation (Table 5): in 16 cases where chemotherapy effective for disease at other sites was given concurrently with irradiation, 14 complete responses and two partial responses were obtained; in eight cases where chemotherapy which was ineffective for disease at other sites was given concurrently, seven complete responses and one partial response were obtained.

Table 4. Results of irradiation correlated with state of the bone marrow at the beginning of irradiation

	Result of irradiation		
	Complete response	Partial response	No response
*State of bone marrow at beginning of irradiation**			
Remission or hypoplastic without blast cells	11	3	—
Relapse	9	1	1

*No data in 3 cases.

Table 5. Results of irradiation correlated with chemotherapy given concurrently

	Complete response	Partial response	No response
Chemotherapy EFFECTIVE for disease at other sites given concurrently with local irradiation*	14	2	—
Chemotherapy INEFFECTIVE for disease at other sites given concurrently with local irradiation*	7	1	—

*Chemotherapy not given at time of irradiation of 4 sites.

The rate of tumour regression in the complete response group was often strikingly rapid. A patient with acute myeloblastic leukaemia (case 12) with massive intraabdominal disease causing inferior vena caval obstruction achieved complete clinical remission within five days of starting radiotherapy. A further patient with AML causing renal infiltration and uraemia (case 5) had a virtually normal blood urea again within eight days of starting irradiation (Fig. 2).

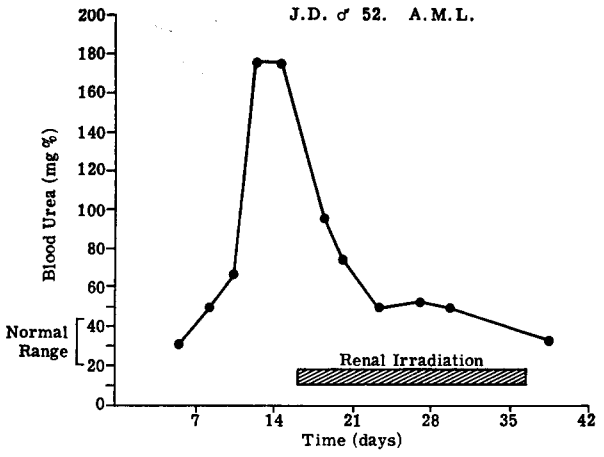


Fig. 2. Rate of response of uraemia to renal irradiation.

DISCUSSION

These results confirm what has generally been accepted by radiotherapists—that acute leukaemic infiltrates are usually more radiosensitive than most tumours. In addition, however, they emphasize the little known feature that these tumours, like some other tumours of the haemopoietic/lymphoproliferative system, particularly diffuse histiocytic lymphomas [7], have a wide range of radiosensitivity: occasional examples (e.g., case 24) are apparently eradicated completely by low dose therapy, whereas others (cases 14, 27) show only a partial response to relatively high dose therapy. Cases 24 and 27 make a particularly striking contrast since both had local irradiation for testicular infiltration by ALL with totally different results.

Established neuroleukaemia is notoriously difficult to eradicate [8]. Aur *et al.* [1] found 2400 rads craniospinal irradiation insufficient to produce eradication, although it temporarily relieved symptoms and cleared the cerebrospinal fluid of blast cells. More promising results have been reported by Willoughby [2], in patients who have never previously had craniospinal irradiation: a combination of intrathecal methotrexate to clear the cerebrospinal fluid of blast cells followed by 2400 rads cranial irradiation and 1000 rads spinal irradiation was used. We have now adopted this treatment policy (cases 3 and 21); consideration should perhaps be given to reducing the cranial dose to 1000 rads in very young children who have previously had 2400 rads cranial irradiation, in view of the disseminated necrotizing leuco-encephalopathy reported by Rubenstein *et al.* [9] as a complication seen in patients with neuroleukaemia and neurolymphoma treated by triple intrathecal chemotherapy and whole brain irradiation.

For extramedullary acute leukaemic infiltration, with the exception of the spine when the neuraxis is being treated for central nervous system disease, we would recommend, in view of the wide range of radiosensitivity, a tumour dose of at least 2500 rads if an attempt is being made to secure complete eradication of the leukaemic deposit within the irradiated volume. This policy would appear well worth-while if there is a reasonable prospect of systemic chemotherapy achieving and maintaining overall long term control of the disease. The type of fractionation and overall treatment time will depend upon the volume and site irradiated. Of course lower doses may be more appropriate if palliation is the objective of treatment.

Acknowledgements—We should like to thank Miss L. Pegus and the Medical Art Department for their assistance with the illustrations, and Miss S. Moore for her help with the manuscript.

REFERENCES

1. R. J. A. Aur, J. V. Simone, O. Hustu, M. S. Verzosa, A comparative study of central nervous system irradiation and intensive chemotherapy early in remission of childhood acute lymphocytic leukaemia. *Cancer (Philad.)* **29,** 381 (1972).
2. M. L. N. Willoughby, Treatment of overt meningeal leukaemia. *Lancet* **i,** 363 (1974).
3. G. J. D'Angio, A. E. Evans, A. Mitus, Roentgen therapy of certain complications of acute leukaemia in childhood. *Amer. J. Roentgenol.* **82,** 541 (1959).
4. K. Atkinson, D. G. Wells, H. McD. Clink, H. E. M. Kay, R. Powles, T. J. McElwain, Adult acute leukaemia. *Brit. J. Cancer* **30,** 272 (1974).
5. R. Powles, Immunotherapy for acute myelogenous leukaemia using irradiated and unirradiated leukaemia cells. *Cancer (Philad.)* **34,** 1558 (1974).
6. F. Ellis, Nominal standard dose and the ret. *Brit. J. Radiol.* **44,** 101 (1971).
7. M. J. Peckham, J. P. Guay, I. M. E. Hamlin, R. J. Lukes, Survival in localized nodal and extranodal non-Hodgkin's lymphomata. *Brit. J. Cancer* **31,** Suppl. II, 413 (1975).

8. M. J. Duttera, W. A. Bleyer, T. C. Pomeroy, C. M. Leventhal, B. Leventhal, Irradiation, methotrexate toxicity, and the treatment of meningeal leukaemia. *Lancet* **ii,** 703 (1973).
9. L. J. Rubinstein, M. M. Herman, T. F. Lond, J. R. Wilbur, Disseminated necrotizing leukoencephalopathy: a complication of treated nervous system leukaemia and lymphoma. *Cancer* (*Philad.*) **35,** 291 (1975).

Europ. J. Cancer Vol. 12, pp. 541–549. Pergamon Press 1976. Printed in Great Britain

The Value of Adriamycin in Overcoming Resistance to Methotrexate in Tissue Culture

BRIDGET T. HILL*†, L. A. PRICE‡ and J. H. GOLDIE§

** The Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX, U.K.*

‡ The Institute of Cancer Research, Royal Marsden Hospital, London SW3 6JJ, U.K. and

§St. Michael's Hospital and the University of Toronto, Canada

Abstract—*Adriamycin effectively reduces the colony-forming ability of L1210 and L5178Y cells in culture. Its cytotoxicity is significantly greater against cells resistant to methotrexate (MTX), whether this resistance is due to an elevated level of dihydrofolate reductase or to impaired MTX transport. In all cell lines the toxicity of adriamycin increases with dose and duration of exposure.*

Tritiated-adriamycin enters the cells by a process that is temperature dependent, and is reduced by the presence of various metabolic inhibitors. Drug uptake occurs against a concentration gradient. Most of the drug remains associated with the trichloracetic acid-soluble fraction (approx. 70%), and the majority of drug bound to macro-molecules is associated with the DNA. A higher drug concentration is achieved in the MTX-resistant L5178Y cells than in the MTX-sensitive L5178Y cells, and therefore the ability of a cell to take up adriamycin may be correlated with the drug's cytotoxicity.

These findings suggest that adriamycin may be a useful agent for treating neoplasms resistant to MTX or for use in combination with MTX against mixed tumour cell populations.

INTRODUCTION

ADRIAMYCIN has been shown to exert anti-tumour activity in a spectrum of animal tumour systems [1–4], and in patients with acute leukaemia [5, 6] and different kinds of solid tumours [5, 7]. More recently this agent is now being successfully used in combination therapy in clinical trials. Combinations of adriamycin and methotrexate (MTX) have been used in the treatment of Ewing's sarcoma [8], lung carcinoma [9], osteogenic sarcoma [8, 10], and certain gynaecological cancers [11]. Since resistance to MTX is a well established clinical problem, we wanted to determine the effectiveness of adriamycin against cells resistant to MTX. Such information might allow the selection of agents which did not show any reciprocal cross-resistance and might provide a rational basis for the designing of multiple drug combinations.

Accepted 19 February 1976

†To whom requests for reprints should be addressed.

In this report we present the results of the effects of adriamycin on MTX-sensitive and MTX-resistant lines of L5178Y lymphoblasts and L1210 cells in culture.

MATERIAL AND METHODS

Chemicals and reagents

Gifts of tritiated adriamycin and non-radioactive adriamycin were kindly provided for these studies by Pharmitalia U.K. Ltd. The radiochemical purity of adriamycin-^{3}H(G)-hydrochloride (specific activity 7·09 mCi/m-mole) was 99%, as checked by TLC and paper chromatography. Methotrexate was obatined from Lederle Laboratories. Other chemicals were purchased from Sigma Chemical Co. or British Drug Houses Ltd.

Cell cultures

L5178Y lymphoblasts were grown in suspension culture in Fischer's medium for leukaemic cells of mice (Gibco Bio-cult, Renfrewshire, Scotland) containing 10% foetal calf serum (Flow Laboratories Ltd.) as described previ-

ously [12, 13]. Two sublines were used: (i) an MTX-sensitive line, which is inhibited by continuous exposure to 10^{-8}M MTX and (ii) an MTX-resistant line, which can grow in 10^{-6}M MTX but is inhibited by drug concentrations of 5×10^{-6}M. Resistance in this cell line has been previously found to be related to impaired MTX transport [14–16]. These cells had a doubling time of approximately 36 hours during exponential growth phase.

L1210 cells were kindly donated by Dr. R. C. Jackson, the Chester Beatty Research Institute, Surrey, U.K. and were maintained in RPMI 1640 medium (Gibco Bio-cult, Renfrewshire, Scotland) containing 10% foetal calf serum. Under these conditions, when growing logarithmically these cells had a doubling time of approximately 12 hr. Two L1210 sublines were used: (i) an MTX-sensitive line with an LD_{50} (the concentration of drug which in 48 hr. reduced the number of cells to 50% of the untreated control) of $5{\cdot}9 \times 10^{-9}$M and (ii) an MTX-resistant line with an LD_{50} of $3{\cdot}4 \times 10^{-6}$M, which was maintained in the presence of 2×10^{-6}M MTX [17]. Resistance in this cell line was associated with an elevated level ($\times 37$) of dihydrofolate reductase (DHFR).

Cell viability assays were performed according to the method of Chu and Fischer [18] with the modifications described previously [12, 13]. Under these conditions the cloning efficiency for L5178Y cells was 60–75% and for L1210 cells was 55–70%. The data, expressed in survival curves, represents the mean value of colony counts of 5 replicate cultures expressed as a percentage of the control (non-treated) cultures, which were given the value of 100%.

Transport studies

L5178Y lymphoblasts and L1210 cells selected from cultures in logarithmic growth at a density of $2–3 \times 10^5$ cells/ml were counted and removed from suspension by centrifugation at 350 g at room temperature for 3 min. The cells were resuspended to a concentration of 5×10^5 cells/ml in Hank's balanced salt solution pH 7·4 (Gibco Bio-cult, Renfrewshire, Scotland) at 37°. Adriamycin was added to aliquots of these cell suspensions, resulting in a final drug concentration of 7×10^{-6}M (4·5 μg/ml and 0·05μ Ci/ml). At various times after drug addition aliquots of cell suspension were removed, chilled rapidly by immersion in ice and diluted 10 times with ice-cold Hank's balanced salt solution. The cells were then removed from suspension by centrifugation at 500 ***g*** for 2 min and washed once with ice-cold 0·85% isotonic saline. The resulting cell pellets were solubilized in 100 μl of 1N NaOH and mixed with 10 ml Aquasol (New England Nuclear Chemicals GmbH Research Products, West Germany). Radioactivity was estimated using a Packard Tricarb Liquid Scintillation Counter Model No. 3385 at 36% efficiency for ^{3}H.

The distribution of adriamycin-^{3}H(G)-hydrochloride between acid soluble and acid insoluble fractions was estimated under similar experimental conditions, following exposure of cells to 7×10^{-6}M adriamycin. The cell pellets resulting after centrifuging aliquots of cell suspension were extracted in 2×2 ml volumes of 10% trichloracetic acid (TCA) and left to stand at 4°C for 20 min before centrifugation to provide TCA-soluble and TCA-insoluble fractions. The pellets representing the TCA-insoluble fractions were dissolved finally in 1N NaOH. The radioactivity associated with aliquots of each cell extract was then measured by scintillation counting. Similarly, the binding of ^{3}H-adriamycin to DNA, RNA and protein was determined after extraction of 2 ml aliquots of cell suspension according to the Scott procedure [19]. DNA was estimated according to Burton [20], RNA by U.V. absorption [21] and protein by the method of Lowry *et al.* [22].

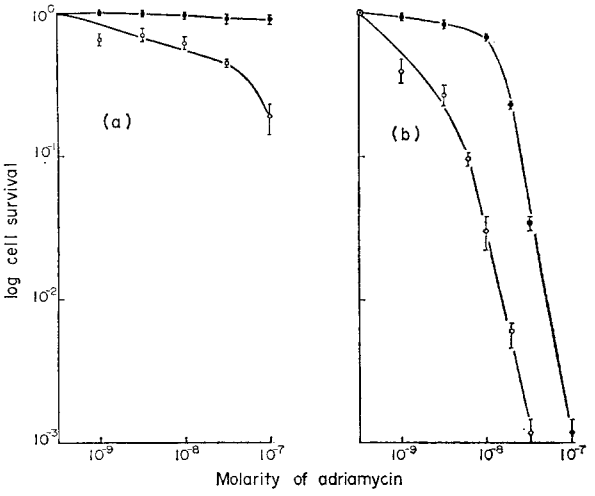


Fig. 1(a). *The effects of a* 6 *hr exposure to Adriamycin on the colony forming ability of MTX-sensitive and MTX-resistant L5178Y cells. At no point did the scatter exceed* 20%.

○—○ *MTX-resistant cells*
●—● *MTX-sensitive cells*

Fig. 1(b). *The effects of a* 24 hr *exposure to Adriamycin on the colony forming ability of MTX-sensitive and MTX-resistant L5178Y cells. At no point did the scatter exceed* 20%.

○—○ *MTX-resistant cells*
●—● *MTX-sensitive cells*

For measurement of drug uptake at 4°C, the cell suspensions were pre-cooled in ice for 15 min before the drug was added.

The rate of efflux of ^{3}H-adriamycin from the cells at 37°C was determined by incubating cells with the drug, using the conditions described above, for 30 min. The cells were then centrifuged at 350 *g* for 2 min at room temperature and resuspended to the same volume in fresh Hank's solution without drug at 37°C. Aliquots were removed at subsequent intervals as described above, in order to determine their radioactivity content.

RESULTS

Effects of adriamycin on cell viability

Figures 1(A) and 1(B) shows the effect of adriamycin on cell viability after the exposure of MTX-sensitive and MTX-resistant L5178Y cells for 6 and 24 hr respectively. The toxicity of adriamycin in both cell lines increases with both dose and duration of exposure. Adriamycin is significantly more effective in reducing the viability of cells resistant to MTX than of cells of the parent MTX-sensitive line. This difference is most pronounced at the higher drug doses (10^{-7}M) for the short time (6 hr). However, it is interesting to note that this short exposure (6 hr) to 10^{-7}M adriamycin has little effect on the viability of MTX-sensitive cells but can reduce survival of MTX-resistant cells by 20%, similarly a 24 hr exposure to 10^{-8}M adriamycin only reduces the viability of MTX-sensitive cells to 80% of controls whilst that of the MTX-resistant cells falls as low as 0·3%.

Adriamycin is also effective in reducing the colony-forming ability of L1210 cells in culture (see Fig. 2) following a 24-hr exposure.

The superiority of adriamycin over MTX itself is shown in Table 1 in terms of its ability to kill cells resistant to MTX either by virtue of a transport defect (L5178Y cells) or an elevated DHFR level (L1210 cells).

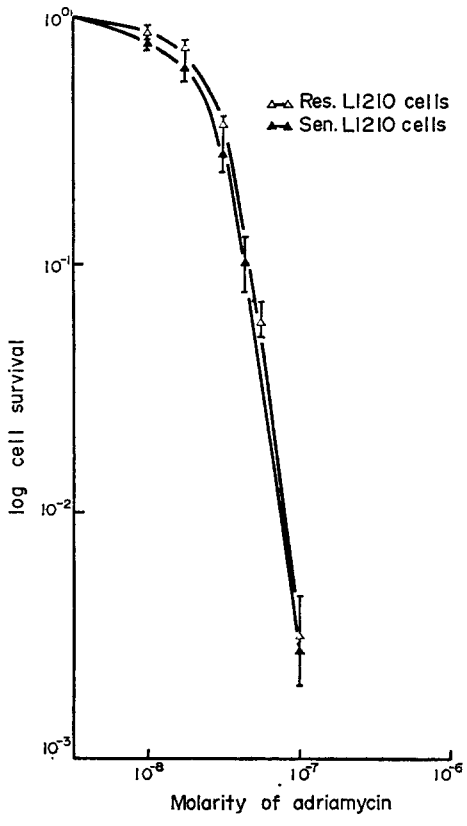


Fig. 2. The effects of a 24 hr *exposure to Adriamycin on the colony forming ability of MTX-sensitive and MTX-resistant L1210 cells. At no point did the scatter exceed* 20%.

△—△ *MTX-resistant cells*
▲—▲ *MTX-sensitive cells*

Table 1. *Drug concentrations (M) resulting in a* 1 *log cell kill following a* 24 *hr exposure in culture*

Drugs	MTX-sensitive cells L5178Y	MTX-sensitive cells L1210	MTX-resistant cells L5178Y	MTX-resistant cells L1210
MTX	8×10^{-7}	2×10^{-8}	$>10^{-4}$*	10^{-5}
Adriamycin	3×10^{-8}	6×10^{-8}	7×10^{-9}	6×10^{-8}

*10^{-4}M MTX produces approximately a 0·5 log cell kill under these experimental conditions.

Influx of adriamycin

The rates of uptake of ^{3}H-adriamycin by L5178Y and L1210 cells are shown in Fig. 3 and 4 respectively. The pattern of drug influx in all cell types is characterised by an early rapid association of the drug with the cells, followed by a period during which the quantity of drug within the cells increases linearly as a function of time until a "steady-state" concentration is achieved after approximately 150 min incubation. Both the early rate (between 1 and 60 min incubation) and the final extent of drug uptake is greater by the L5178Y cells than by the L1210 cells. In these experiments there appears to be a correlation between the extent of uptake of the drug and its ability to reduce cell viability. A higher drug concentration is achieved in the MTX-resistant L5178Y cells than in the sensitive cells, and the MTX-resistant line are also most readily killed by adriamycin. Whilst the pattern of drug uptake and the cytotoxicity of adriamycin are comparable in the two L1210 lines (see Table 2).

Further characteristics of the transport of adriamycin were examined using the L5178Y cells:

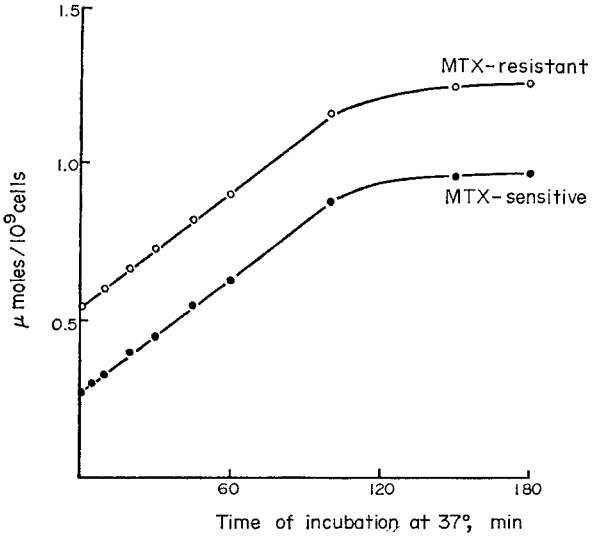


Fig. 3. The rates of uptake of ^{3}H-Adriamycin by MTX-sensitive and MTX-resistant L5178Y cells expressed in μmole/10^9 cells as a function of time (min) of incubation at 37° following exposure to 7×10^{-6}M drug. Each figure represents the mean of eight determinations. Overall scatter not in excess of 10%.

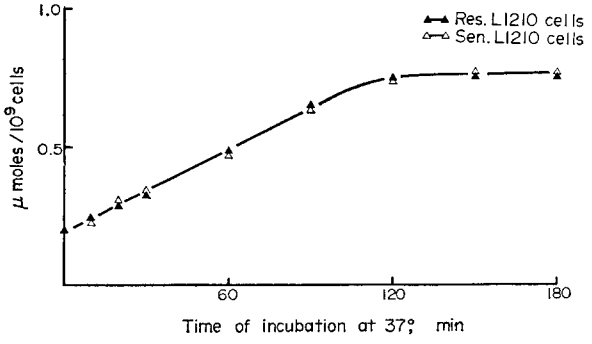


Fig. 4. The rates of uptake of ^{3}H-Adriamycin by MTX-sensitive and MTX-resistant L1210 cells expressed in μmole/10^9 cells as a function of time (min) of incubation at 37° following exposure to 7×10^{-6}M drug. Each figure represents the mean of eight determinations. Overall scatter not in excess of 10%.

△—△ *MTX-resistant cells*
▲—▲ *MTX-sensitive cells*

(i) *Intracellular drug concentrations.* In these L5178Y cells the intracellular drug concentrations achieved at the plateau levels of the graphs (see Fig. 3) after 150 min incubation at 37° in the presence of 7×10^{-6}M drug are 1·57 mM and 2·5 mM for the MTX-sensitive and MTX-resistant cells respectively. These values were calculated using measurements of the intracellular free space determined as described previously [23]. The intracellular water content was 6·1 and $5{\cdot}0 \times 10^{-10}$ml/cell for MTX-sensitive and -resistant cells respectively. These results indicate that adriamycin, under these experimental conditions, was accumulated against a concentration gradient by these cells.

Table 2. A comparison of the cytotoxic effects of adriamycin and the "steady-state" intracellular drug levels achieved in a series of cultured cell lines

Cell line	% viability after exposure to 10^{-8}M drug*	"Steady-state" level†
MTX-resistant L5178Y cells	3	1·30
MTX-sensitive L5178Y cells	60	0·98
MTX-sensitive L1210 cells	79	0·75
MTX-resistant L1210 cells	86	0·75

*The cells were exposed at 37° for 24 hr prior to assessing colony-forming ability. Results are taken from Fig. 1 and 2 and expressed as % of control values.

†"Steady-state" levels achieved after 150 min incubation at 37°. Results taken from Figs. 3 and 4 and expressed in μmoles/10^9 cells.

(ii) *Effect of temperature on drug uptake.* Drug uptake was also shown to be temperature dependent (see Fig. 5). After a 60-min incubation, uptake of adriamycin was depressed at the lower temperature to approximately 41% and 55% of those achieved at 37°C in MTX-sensitive and MTX-resistant cells respectively. Comparable results (not shown) were obtained with the L1210 cells, with ^{3}H-adriamycin uptake being depressed at 4° to 36% and 51% of the values at 37°C in MTX-sensitive and MTX-resistant cells respectively. However, even at this lower temperature (4°C) there is still a considerable quantity of ^{3}H-adriamycin which associated with the cells and which increases linearly over the first 10 min of incubation and then levels off. This rapid associating fraction of ^{3}H-drug, which is seen at both temperatures, may represent non-specific binding to the cell surface.

(iii) *Effect of various metabolic inhibitors on drug uptake.* The metabolic inhibitors, dissolved in phosphate buffered saline and the pH adjusted to 7·4 where necessary, were used at concentrations established previously as inhibitory to the active transport of ^{3}H-lysine into these cells. The inhibitors were added to the cell suspensions at the same time as the ^{3}H-adriamycin and the cells were incubated for 30 min at

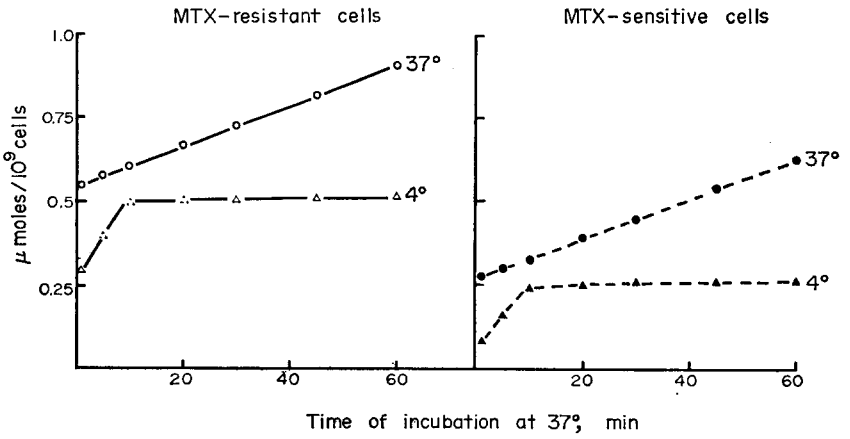


Fig. 5. A comparison of the rates of uptake of ^{3}H-Adriamycin by MTX-sensitive and MTX-resistant L5178Y cells expressed in μmole/10^9 cells as a function of time (min) of incubation at 37° or 4° following exposure to 7×70^{-6}M drug. Each figure represents the mean of eight determinations. Overall scatter not in excess of 10 %.

Table 3. Effects of various metabolic inhibitors on the uptake of ^{3}H-adriamycin at 37°C

Chemical	Concentration (mM)	Values expressed as % of control: L5178Y cells MTX-sensitive	L5178Y cells MTX-resistant	L1210 cells MTX-sensitive	L1210 cells MTX-resistant
NaF	10	79	61	81	61
NaCN	0·1	48	56	51	57
2,4–DNP	0·5	46	65	48	65

Each value represent the mean of five estimations. The overall scatter did not exceed 10%.

37°C. As shown in Table 3 uptake was markedly reduced in the presence of 2,4-dinitrophenol (2,4-DNP—an inhibitor of oxidative phosphorylation) and sodium cyanide (a general respiratory poison) and less so by sodium fluoride (an inhibitor of glycolysis). These results would be consistent with earlier observations that adriamycin crosses the cell membranes by an energy dependent process [24, 25].

(iv) *Kinetics of drug uptake.* For these studies requiring estimations of the initial velocity of drug uptake, the uptake velocity was determined from the difference between drug associated with the cells after 2 vs 10 min incubation at 37°C, when the rate of influx (see Fig. 3) approximated to linearity. Uptake of adriamycin by L5178Y lymphoblasts approximated Michaelis-Menten kinetics for drug concentrations from $1–40 \times 10^{-6}$M. The kinetic parameters are described in Table 4. The K_m's are comparable for all the cell lines, whilst the V_{max} values are slightly higher for the L1210 cells compared with the L5178Y cells. No significant differences in these kinetic parameters were noted between MTX-sensitive and MTX-resistant cells.

(v) *Efflux of adriamycin.* L5178Y lymphoblasts were preloaded with adriamycin to an approximately equivalent concentration of 0·5 μmoles of drug per 10^9 cells by incubation for 30 min at 37°C with ^{3}H-Adriamycin (7×10^{-6}M for MTX-sensitive cells and 5×10^{-6}M for MTX-resistant cells). The cells were then centrifuged, washed and resuspended in drug-free medium at 37°C. Aliquots of cell suspension were removed at the times shown in Fig. 6, which illustrates the rate and extent of efflux of adriamycin from these two cell types. There is a rapid efflux of adriamycin from both MTX-sensitive and MTX-resistant cells. The final amount of drug associated with the cells is comparable in both cell types.

*Table 4. Kinetic data for the uptake of Adriamycin by L5178Y and L1210 cells**

Cell line	K_m†	V_{max}‡
MTX-resistant L5178Y	1·06 ± 6%	0·36 ± 2·3%
MTX-sensitive L5178Y	1·08 ± 8%	0·38 ± 3·6%
MTX-resistant L1210	1·10 ± 5%	0·45 ± 3·8%
MTX-sensitive L1210	1·08 ± 4%	0·46 ± 4·4%

*The data were derived by calculation of enzyme Michaelis–Menten parameters with standard errors by the statistical method of Wilkinson [26].

†K_m is expressed as mM concentration.

‡V_{max} is expressed in μmoles per min per 10^9 cells.

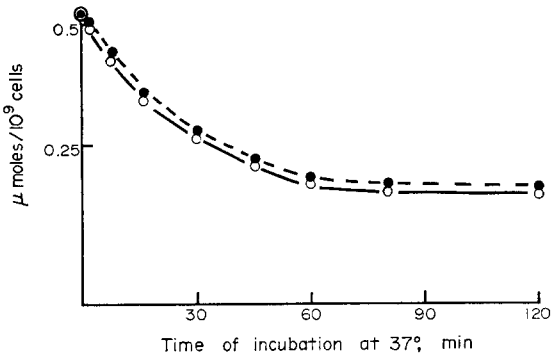


Fig. 6. The rate of efflux of ^{3}H-Adriamycin from "pre-loaded" MTX-sensitive and MTX-resistant L5178Y cells expressed in μmole/10^9 cells as a function of time of re-incubation in drug-free medium at 37°. Each figure represents the mean of eight determinations. Overall scatter not in excess of 15%.

○—○ *MTX-resistant cells*
●—● *MTX-sensitive cells*

(vi) *Intracellular distribution of adriamycin.* Figure 7 shows that in both cell lines the majority of the intracellular drug remains associated with the TCA-soluble fraction. Approximately 25–30% of the total drug taken by the two cell lines after a 60-min incubation is associated with the TCA-insoluble fraction, indicating that this proportion may be bound to cell macromolecules. The extent of binding of ^{3}H-adriamycin to DNA, RNA and protein increased with time as shown in Table 5. The majority of the tritiated drug is associated with DNA and the least with cellular protein. Significantly more ^{3}H-adriamycin is bound to DNA in the MTX-resistant cells than the

Table 5. Extent of binding of ^{3}H-adriamycin-derived radioactivity to cell macromolecules extracted from MTX-sensitive and -resistant L5178Y cells after exposure to 7×10^{-6}M adriamycin

Cell line	Time of incubation (min)	μmoles/mg DNA	RNA	Protein
MTX-sensitive cells	10	3·12	1·00	0·33
	60	6·95	1·63	0·37
	120	13·40	3·50	0·40
MTX-resistant cells	10	3·75	1·08	0·35
	60	10·50	2·75	0·40
	120	17·50	3·90	0·45

DNA, RNA and protein were extracted by the Scott procedure [19] and assayed as described in the Material and Methods section. Each value represents the mean of 4 determinations. Overall scatter did not exceed 10%.

In these calculations it is assumed that all the tritium was associated with Adriamycin.

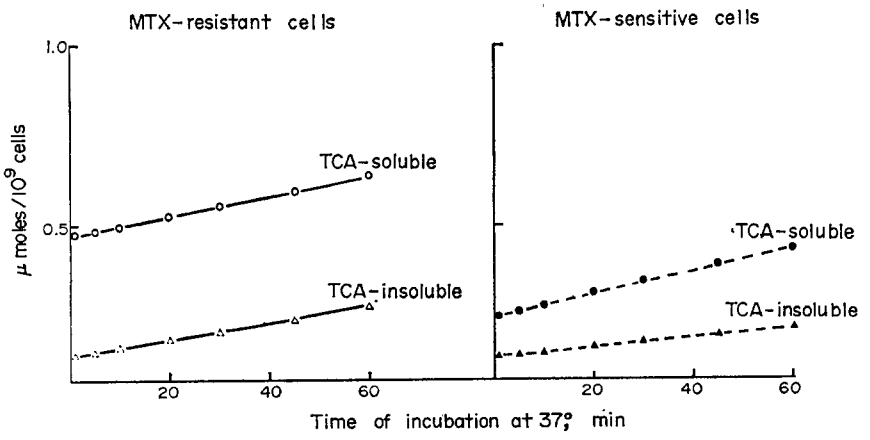


Fig. 7. Distribution of ^{3}H-Adriamycin between TCA-soluble and TCA-insoluble extracts following exposure of L5178Y cells to 7×10^{-6}M drug. Each figure represents the mean of 6 *determinations. Overall scatter not in excess of* 10%.

MTX-sensitive cells, whilst the extent of binding to RNA and protein is comparable in the two cell lines. This increased binding to DNA in the MTX-resistant cells probably reflects the greater extent of total drug uptake by this cell line (Fig. 3).

DISCUSSION

These results show that adriamycin effectively reduces the viability of MTX-resistant cells whether the resistance is due to an elevated level of dihydrofolate reductase or to impaired MTX transport. The effectiveness of adriamycin in killing cells increases both with time and dose. These findings are compatible with previous studies which have demonstrated (i) collateral sensitivity between adriamycin and MTX in an MTX-resistant subline of L1210 leukaemia *in vivo* [27], and (ii) a reduced effectiveness in killing HeLa cell colony forming units when adriamycin is given over short periods as compared with longer exposure times [28, 29].

Transport studies with tritiated-adriamycin show that drug uptake occurs against a concentration gradient, is temperature dependent, and is reduced in the presence of various metabolic inhibitors. These findings confirm previous work which demonstrated that adriamycin is actively transported into cells [24, 25, 30]. Most of the adriamycin, after a 1 hr incubation at 37°C, is associated with the TCA-soluble fraction (approximately 70%). The majority of the ^{3}H-adriamycin associated with the TCA-insoluble fraction is bound to the DNA and relatively little drug is associated with cellular protein. These findings are in agreement with earlier observations that these anthracycline antibiotics bind much more strongly to DNA [31–33] than to RNA [31, 34, 35] and exert a major adverse effect on DNA synthesis, a slightly lesser effect on RNA synthesis and essentially no effect on protein synthesis [29, 30].

Although the uptake of adriamycin is markedly reduced at 4°C, there is still a significant amount of drug associated with the cells at this low temperature, which cannot be removed by further washing. This component may represent surface absorption, but there is evidence that some cellular binding occurs independent of temperature [30]. The kinetics of initial drug uptake at 37°C are comparable in all cell lines, however a "steady-state" concentration of adriamycin is only achieved after approximately 150 min of incubation. This prolonged course of uptake of adriamycin has been noted previously and is in contrast to the much more rapid uptake of Daunorubicin [25, 30].

The MTX-resistant L5178Y cells take up more ^{3}H-adriamycin than the sensitive line and this is reflected in the greater amount of drug bound to DNA. The cytotoxicity may be correlated with uptake since the MTX-resistant L5178Y cells accumulate the highest intracellular concentration of adriamycin and are most readily killed by the drug, whilst in the MTX-sensitive L5178Y cells the extent of drug uptake is lower and a higher extracellular concentration is required for a comparable extent of cell kill. Furthermore, in both L1210 cell lines, both the extent of uptake and the effect on cell viability are comparable (see Table 2).

Efflux of adriamycin from cell "preloaded" with the drug is comparable in both L5178Y cell lines and the rate of release of drug is similar to that reported in cultured rat fibroblasts [25].

These experimental studies indicate that adriamycin may be a valuable agent for treating neoplasms resistant to MTX and that a combination of adriamycin and MTX might be valuable for treating mixed populations of tumour cells.

REFERENCES

1. A. Dimarco, M. Gaetani and B. Scarpinato, Adriamycin (NSC–123127): A new antibiotic with antitumour activity. *Cancer Chemother. Rep.* **53,** 33 (1969).
2. A. Dimarco, L. Lenaz, A. M. Casazza and B. Scarpinato, Activity of adriamycin (NSC–123127) and daunomycin (NSC–82151) against mouse mammary carcinoma. *Cancer Chemother. Rep.* **56,** 153 (1972).
3. J. S. Sandberg, F. L. Howsden, A. Dimarco and A. Goldin, A comparison of the anti-leukemic effect in mice of adriamycin (NSC–123127) with daunomycin (NSC–82151). *Cancer Chemother. Rep.* **54,** 1 (1970).
4. A. Dimarco, A. M. Casazza, T. Dasdia, F. Giuliani, L. Lenaz, A. Necco and C. Soranzo, Cytotoxic, antiviral and antitumour activity of some derivatives of daunomycin (NSC 82151). *Cancer Chemother. Rep.* **30,** 269 (1973).

5. G. Bonadonna, S. Monfardini, M. Delena, F. Fossati-Bellani and G. Beretta, Phase I and preliminary Phase II evaluation of Adriamycin. *Cancer Res.* **30,** 2572 (1970).
6. E. Middleman, J. Luce and E. Frei III, Clinical trials with Adriamycin. *Cancer (Philad.)* **28,** 844 (1971).
7. G. Bonadonna, S. Monfardini, M. Delena and F. Fossati-Bellani, Clinical evaluation of Adriamycin, a new antitumour antibiotic. *Brit. Med. J.* **3,** 503 (1969).
8. G. Bonadonna, S. Monfardini, M. Delena, F. Fossati-Bellani and G. Beretta, Clinical trials with adriamycin: Results of a three-year study. 1*st. International Symposium on Adriamycin* 1971. Springer-Verlag, Berlin p. 139 (1972).
9. S. Lowenbraun, Cycle-non specific preceding cycle-specific chemotherapy in metastatic lung carcinoma. *Proc. Amer. Soc. clin. Oncology Abs.* **15,** 712 (1974).
10. G. Rosen, S. Suwansirikul, C. Kwon, C. Tan, S. Wu, E. Beattie and M. Murphy, High dose methotrexate with citrovorum factor rescue and adriamycin in childhood osteogenic sarcoma. *Cancer (Philad.)* **33,** 1151 (1974).
11. D. Guthrie and S. Way, Treatment of advanced carcinoma of the cervix with adriamycin and methotrexate combination. *Obstet. Gynaecol.* **44,** 586 (1974).
12. J. H. Goldie, M. E. Furness and L. A. Price, Comparison of the effects of methotrexate and pyrimethamine on L5178Y lymphoblasts in culture. *Europ. J. Cancer* **9,** 709 (1973).
13. B. T. Hill, J. H. Goldie and L. A. Price, Studies concerned with overcoming resistance to methotrexate: A comparison of the effects of methotrexate and 2,4-diamino-5-(3′4′-dichlorophenyl)-6-methylpyrimidine (BW 50197) on the colony forming ability of L5178Y cells. *Brit. J. Cancer* **28,** 263 (1973).
14. G. A. Fischer, Defective transport of Amethopterin methotrexate as a mechanism of resistance of the anti-metabolite in L5178Y leukaemia cells. *Biochem. Pharmacol.* **11,** 1233 (1960).
15. K. R. Harrap, B. T. Hill, M. E. Furness and L. I. Hart, Sites of action of amethopterin: intrinsic and acquired drug resistance. *Ann. N.Y. Acad. Sci.* **186,** 312 (1971).
16. J. H. Goldie, S. I. Harrison, L. A. Price and B. T. Hill, Impaired responsiveness to folinic acid protection in methotrexate-resistant L5178Y cells. *Europ. J. Cancer* **11,** 627 (1975).
17. R. C. Jackson, Personal communication.
18. M. Y. Chu and G. A. Fischer, The incorporation of ^{3}H-Cytosine arabinoside and its effect on murine leukaemic cells (L5178Y). *Biochem. Pharmac.* **17,** 753 (1968).
19. J. P. Scott, A. P. Fraccastoro and E. B. Taft, Studies in histochemistry. I. Determination of nucleic acids in microgram amounts of tissue. *J. Histochem. Cytochem.* **4,** 1 (1965).
20. K. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
21. A. Fleck and H. N. Munro, The precision of ultraviolet absorption measurements in the Schmidt–Thannhauser procedure for nucleic acid estimation. *Biochim. biophys. Acta.* **55,** 571 (1962).
22. O. Lowry, N. J. Rosebrough, A. L. Farr and J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
23. B. T. Hill, L. A. Price and J. H. Goldie, Methotrexate resistance and uptake of DDMP by L5178Y cells: Selective protection with folinic acid. *Europ. J. Cancer* **11,** 545 (1975).
24. E. Frei III, Combination chemotherapy. *Proc. Roy. Soc. Med.* **67,** 425 (1974).
25. G. Noel, A. Trouet, A. Zenebergh and P. Tulkens, Comparatice cell pharmacokinetics of daunomycin and adriamycin. In *Adriamycin Review* (Edited by the European Organization on Treatment of Cancer), p. 99, European Press Medikon, Gent (1975).
26. G. N. Wilkinson, Statistical estimations in enzyme kinetics. *Biochem. J.* **80,** 324 (1961).

27. A. Hoshino, T. Kato, H. Amo and K. Ota, Antitumour effects of adriamycin on Yoshida rat sarcoma and L1210 mouse leukaemia—Cross-resistance and combination chemotherapy. 1*st International Symposium on Adriamycin* (Edited by S. K. Carter, A. Dimarco, M. Gluone, I. H. Krakoff and G. Mathe) Springer-Verlag p. 47 (1972).
28. R. Silvestrini, C. Gambarucci and T. Dasdia, Biological activity of adriamycin "in vitro". *Tumori* **56,** 137 (1970).
29. S. H. Kim and J. H. Kim, Lethal effect of adriamycin on the division cycle of HeLa cells. *Cancer Res.* **32,** 323 (1972).
30. W. D. Meriweather and N. R. Bachur, Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukaemia. *Cancer Res.* **32,** 1137 (1972).
31. E. Calendi, A. Dimarco, M. Regiani, B. Scaripinato and L. Valentini, On physico-chemical interactions between daunomycin and nucleic acids. *Biochim. biophys. Acta.* **103,** 25 (1965).
32. W. Kersten, H. Kersten and K. W. Szybols, Physicochemical properties of complexes between deoxyribonucleic acid and antibiotics which affect RNA synthesis (actinomycin, daunomycin, cinerubin, nogalomycin, chromoycin, mithramycin and olivomycin), *Biochemistry* **5,** 236 (1966).
33. A. Dimarco and L. Lenaz, Daunomycin and adriamycin In: *Cancer Medicine.* (Edited by J. F. Holland and E. Frei) p. 826. Lea and Febiger, Philadelphia (1973).
34. B. K. Bhuyan and C. G. Smith, Differential interaction of Nogalomycin with DNA of varying base composition, *Proc. Nat. Acad. Sci. (Wash.)* **54,** 566 (1965).
35. D. C. Ward, E. Reich and J. H. Goldberg, Base specificity in the interaction of polynucleotides with antibiotic drugs. *Science* **149,** 1259 (1965).

Europ. J. Cancer Vol. 12, pp. 551–555. Pergamon Press 1976. Printed in Great Britain

Changes in Proliferation Rate of Human Bone Marrow Colony-Forming Cells Measured by a Cytosine Arabinoside-Diffusion Chamber Method*

M. Y. GORDON and I. D. C. DOUGLAS

Division of Biophysics, Institute of Cancer Research, Sutton, Surrey, and Department of Haematology, Royal Marsden Hospital, Sutton, Surrey, U.K.

Abstract—*A cytosine arabinoside-diffusion chamber method of measuring changes in the proliferation rate of bone marrow colony-forming cells has been tested using normal and rapidly proliferating cells from C57Bl mice. Comparable changes have been found in human marrow after chemotherapy. The method may therefore be useful for monitoring the proliferation rate of human bone marrow during treatment with cytotoxic drugs.*

INTRODUCTION

SEVERAL important aspects of drug action on normal haemopoietic precursor cells have been revealed by studies on mouse bone marrow. van Putten [1] and Valeriote and van Putten [2] have reviewed the data showing that the effect of certain drugs is restricted to one particular phase in the cell cycle (e.g., DNA-S). It has been shown that the effect of many drugs depends also on the proliferation rate of the target cells [3–5]. Furthermore, the proliferation rate of haemopoietic precursor cells is increased during recovery from chemotherapy [6] and this may result in a greater sensitivity to drugs during this period. This has important implications for the timing of repeated chemotherapy where cyclical changes in proliferation rate, and therefore in drug sensitivity, may be expected to occur as a result of repeated drug administration. Since myelotoxicity often limits chemotherapy, changes in the proliferation rate and drug sensitivity of human marrow during recovery are important in timing successive doses.

This paper describes the development of a method using cytosine arabinoside (ara-C) to detect changes in the proliferation rate of granulopoietic precursor cells which are capable of forming colonies in agar in diffusion chambers [7].

The responses of normal and rapidly proliferating mouse bone marrow to ara-C, both *in vivo* and *in vitro*, and of human bone marrow before and after cytotoxic chemotherapy have been studied. The potential applications of this method to clinical management are discussed.

MATERIAL AND METHODS

1. *Mouse bone marrow*

Male C57Bl mice, 2–3 months old, were used as mouse marrow donors and as hosts for the diffusion chambers.

Continuous irradiation from a ^{137}Cs source, at a dose rate of 30 rad/day for a period of 2 weeks, reduces the number of colony forming cells to 20% of the unirradiated control value and increases the proliferation rate of the surviving cells [8]. This has been used as a model for rapidly proliferating marrow in man.

(i) *Exposure of mouse femoral cells to ara-C* in situ *in the donor mouse.* The effect of a single intravenous injection of 100, 200 or 300 mg kg^{-1} ara-C on the bone marrow of normal and continuously irradiated (CI) mice was determined using the agar diffusion chamber technique as an assay system [9, 10]. Two hours after injection of the drug, marrow was

Accepted 21 January 1976.

*Work supported by a grant from the National Cancer Institute, contract No. N.C.I.-CM-23717.

flushed from the femurs of these mice, plated in agar in diffusion chambers and incubated in the peritoneal cavities of host mice for 6–7 days. The survival of granulocytic precursor cells, following exposure to the drug, was determined by their ability to form colonies in the agar, and calculated in terms of colony forming cells per femur.

(ii) *Exposure of mouse femoral cells to ara-C after plating in agar diffusion chambers and implantation into host mice.* Femoral cells from normal or CI mice were plated in agar diffusion chambers and implanted into host mice. The chamber hosts were given an intravenous injection of 100, 200 or 300 mg kg^{-1} ara-C and two hours later the chambers were retransplanted into secondary diffusion chamber hosts. The number of colonies were scored after 6–7 days intraperitoneal incubation.

2. *Human bone marrow*

Two to five millilitres of human bone marrow was aspirated from the iliac crest and cultured in semi-solid agar-medium in diffusion chambers. Samples were taken before chemotherapy, within a few days after treatment, and, in 2 cases, 2–3 weeks later.

The timing of ara-C treatment and chamber retransplantation was identical to that for mouse bone marrow. However, for human bone marrow, the secondary chamber recipients received 900 rad ^{60}Co γ radiation 1–2 hr before chamber implantation, since successful growth of human bone marrow colonies requires host pretreatment. The human bone marrow colonies were scored after 8–9 days intraperitoneal incubation, and expressed as the number of colonies per 2×10^5 cells cultured.

RESULTS

1. *Treatment of mouse bone marrow* in situ

The effect of graded doses of ara-C on bone marrow colony forming cells from normal and CI mice are shown in Fig. 1. The dose response curves had distinct plateaux, the survival for the normal cells being approximately 80% of the control levels and for the rapidly proliferating cells, from CI mice, between 20% and 30%.

2. *Treatment of mice bearing chambers containing normal mouse or human bone marrow cells*

The purpose of these experiments was to determine the effect, on colony precursor survival, of the time interval between chamber implantation and injection of the host mouse with ara-C. Experiments using chambers containing mouse or human bone marrow cells were done in parallel and in all cases the chambers were retransplanted 2 hr after injection of the drug. The surviving fraction of the colony forming cells, following exposure to ara-C, was constant over the first two hours after implantation into the primary host (Fig. 2), thereafter falling to a nadir 8 hr after implantation. Since this change in the sensitivity

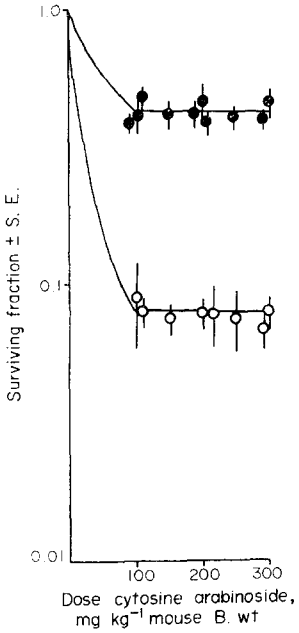


Fig. 1. The sensitivities of (●) normal and (○) rapidly proliferating mouse bone marrow colony forming cells 60 in vivo *administration of cytosine arabinoside.*

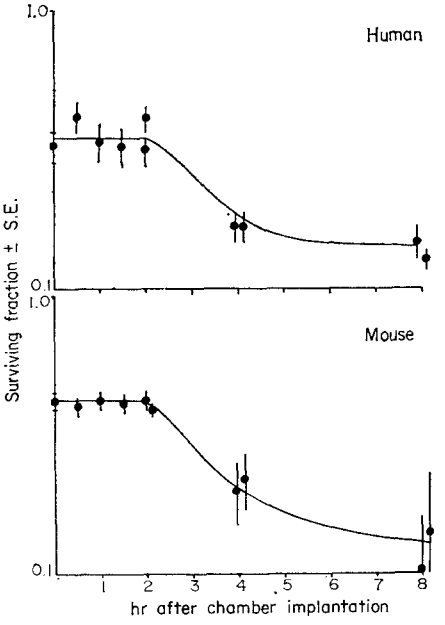


Fig. 2. The effect of injecting cytosine arabinoside into chamber bearing mice, at different times after chamber implantation, on mouse and human colony forming cells held in the diffusion chambers.

of the cells may indicate a change in their proliferative state, which cannot be related to their proliferation rate *in vivo*, all injections in other experiments were given within 2 hr after chamber implantation.

3. *Treatment of mice bearing chambers containing normal or rapidly proliferating mouse bone marrow*

These experiments were carried out to determine whether the differential sensitivity to ara-C was maintained when normal and rapidly proliferating mouse marrow cells were exposed to ara-C by treatment of the diffusion chamber host. The survival of rapidly proliferating colony precursors was much lower than that of normal cells (Fig. 3) and a substantial differential kill was maintained. These results from mouse bone marrow indicate that decreased survival of human bone marrow cells,

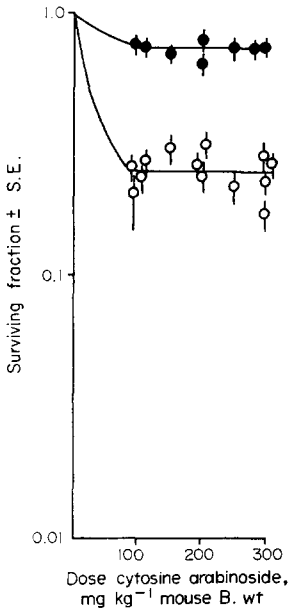


Fig. 3. *The sensitivites of colony forming cells from* (●) *normal and* (○) *rapidly proliferating mouse bone marrow in diffusion chambers to cytosine arabinoside administered to the chamber host.*

following exposure to ara-C in this way, will suggest an increased *in vivo* proliferation rate.

4. *Treatment of mice bearing chambers containing marrow taken before and after chemotherapy*

The survival curves in Fig. 4 are from measurements made on patients' marrow before and after chemotherapy. In each case there was an increase in the sensitivity of the cells following treatment of the patients which, by analogy with the mouse data, indicates an increase in their proliferation rate 2–5 days after chemotherapy, with a return to normal by the 17th day.

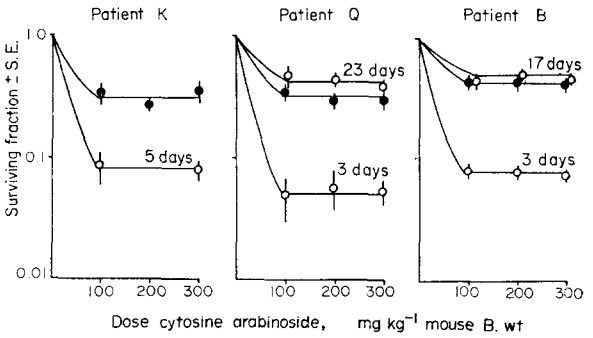


Fig. 4. *The changes in sensitivity of human bone marrow cells following chemotherapy.* (●) *marrow sampled before treatment* (○) *at specified time after treatment.*

The clinical details of the patients and the colony forming abilities of their marrow are given in Table 1. Increases in proliferation rate were found 2–5 days after treatment with both COPAM and cyclophosphamide with a return to normal by 17–23 days. Although there were no dramatic changes in the incidence of colonies per 2×10^5 cells, the cellularity of the aspirates was lower 2–5 days after treatment resulting in a lower yield of colonies per ml of the marrow sample. When the results from the two patients investigated 17 and 23 days after chemotherapy are also expressed as the number

Table 1. *Clinical details and colony-forming abilities of the patients used in this study*

	Patient K	Patient Q	Patient B
Diagnosis	Oat cell Ca bronchus	Teratoma	Teratoma
Treatment	COPAM (cyclophosphamide; adriamycin; vincristine; prednisone; methotrexate)	Cyclophosphamide	Cyclophosphamide
Colonies/2 × 10^5 cells	Day 0 30·8 Day 5 49·7	Day 0 31·5 Day 3 21·0 Day 23 38·2	Day 0 15·1 Day 2 6·3 Day 17 54·6
Colonies/ml of aspirate	Day 0 1656 Day 5 534	Day 0 3292 Day 3 1113 Day 23 25365	Day 0 868 Day 2 607 Day 17 29484

of colonies per ml of marrow substantial increases in the number of colony forming cells are evident.

DISCUSSION

Studies of changes in the colony forming capacity of patients' bone marrow during treatment and recovery require a reliable and reproducible culture system. The agar diffusion chamber technique for growing colonies from bone marrow aspirates provides favourable conditions for such investigations, particularly since it is not subject to variations in the production and activity of the colony stimulating factor used for bone marrow culture *in vitro* [7]. However, interpretation of results based on colony number alone may be distorted by factors such as variable dilution of the sample with peripheral blood during aspiration or changes in the distribution of active marrow. Measurement of changes in the proliferation rate of the precursor cells is independent of colony incidence and, consequently, independent of these variables. This parameter may be of relevance to the planning of courses of chemotherapy since the sensitivity of bone marrow cells to many cytotoxic drugs is related to their proliferation rate.

The "thymidine suicide" technique has been widely used to detect changes in proliferation rate. Alternative agents have been suggested for measuring these changes; Eaves and Bruce [11] found that the sensitivity of haemopoietic stem cells (CFU-S) to 5-fluorouracil increased following endotoxin, cyclophosphamide or irradiation and attributed these changes to an increase in proliferation rate during recovery. Millard and Okell [5] measured changes in the sensitivity of mouse marrow to ara-C after a single dose of myleran and found an increase in the proliferation rates of both *in vivo* (CFU-S) and *in vitro* (CFU-C) colony forming cells. Therefore, in addition to our findings, the experience of others suggests that sensitivity to ara-C provides a useful means of measuring the proliferative state of bone marrow cells.

An important point in the interpretation of proliferation rates using phase specific drugs is that the dose response curve should have a plateau and only doses on the plateau should be used. Over a wide range of doses, Blackett, Millard and Belcher [8] were unable to show a distinct plateau for ^{3}HTdR whereas a plateau for ara-C is more easily demonstrable [5]. The presence of a dose independent plateau on the survival curve is a consequence of the phase specific action of ara-C; its level is a function of the duration of the sensitive phase of the cells and of the availability time of the active drug. Both the absolute and relative sensitivities of normal and rapidly proliferating mouse cells to ara-C administered to the chamber-bearing host mouse were greater than the effects observed when the cells were exposed to the drug *in situ* in the donor mouse (Fig. 1 and 3). This discrepancy indicates that the cells in diffusion chambers may be exposed to ara-C for longer than the cells in the femur.

Greenberg *et al.* [6] used the *in vitro* thymidine suicide technique to investigate the proliferative status of *in vitro* colony forming cells in the marrow of acute leukaemic and lymphoma patients through several courses of chemotherapy. They found an initial decrease in the proportion of cycling colony precursor cells followed by a sharp increase and an oscillatory return to normal levels by 3–4 weeks post-treatment.

Our results from experiments on human bone marrow, from patients without haematological disease, also show an increase in the proliferation rate of the colony precursor cells following chemotherapy. Although there were no dramatic changes in the incidence of colony forming cells at the times investigated, their numbers per ml of aspirate showed a reduction 2–5 days after treatment followed by an overshoot in Patients Q and B. These data are consistent with the interpretation that depletion of bone marrow precursor cells by cytotoxic drugs is followed by a recovery phase during which their proliferation rate is accelerated.

Information on the timing of changes in the proliferation rate of haemopoietic precursor cells may aid the design of chemotherapeutic regimes attempting to minimise bone marrow toxicity. Accordingly, the cytosine arabinoside-diffusion chamber technique is now being applied to longer term studies of the kinetics of human bone marrow precursor cells over repeated courses of chemotherapy.

Acknowledgements—The authors wish to thank Professor L. F. Lamerton and Dr. N. M. Blackett for their help in the preparation of the manuscript, and Mrs. B. M. J. Pickering for her expert technical assistance.

REFERENCES

1. L. M. VAN PUTTEN, Are cell kinetic data relevant for the design of tumour chemotherapy schedules? *Cell Tissue Kinet.* **7,** 493 (1974).

2. F. VALERIOTE and L. VAN PUTTEN, Proliferation dependent cytotoxicity of anticancer agents: a review. *Cancer Res.* **35,** 2619 (1975).
3. W. R. BRUCE and B. E. MEEKER, Comparison of the sensitivity of hematopoietic colony-forming cells in different proliferative states to 5-fluorouracil. *J. nat. Cancer Inst.* **38,** 401 (1967).
4. W. O. DEWYS, A. GOLDIN and N. MANTEL, Hematopoietic recovery after large doses of cyclophosphamide: Correlation of proliferative state with sensitivity. *Cancer Res.* **30,** 1092 (1970).
5. R. E. MILLARD and S. F. OKELL, The effect of cytosine arabinoside *in vitro* on agar colony forming and spleen colony forming cells of C57Bl mouse bone marrow. *Cell Tissue Kinet.* **8,** 33 (1975).
6. P. GREENBERG, I. BAX, B. MARA, S. SCHRIER, Alterations of granulopoiesis following chemotherapy. *Blood* **44,** 375 (1974).
7. M. Y. GORDON, N. M. BLACKETT and I. D. C. DOUGLAS, Colony formation by human haemopoietic precursor cells cultured in semi-solid agar in diffusion chambers. *Brit. J. Haematol.* **31,** 103 (1975).
8. N. M. BLACKETT, R. E. MILLARD and H. N. BELCHER, Thymidine suicide *in vivo* and *in vitro* of spleen colony forming cells and agar colony forming cells of mouse bone marrow. *Cell Tissue Kinet.* **7,** 309 (1974).
9. M. Y. GORDON, Quantitation of haemopoietic cells from normal and leukaemic RFM mice using an *in vivo* colony assay. *Brit. J. Cancer* **30,** 421 (1974).
10. M. Y. GORDON and N. M. BLACKETT, Stimulation of granulocytic colony formation in agar diffusion chambers implanted in cyclophosphamide pretreated mice. *Brit. J. Cancer* **32,** 51 (1975).
11. A. C. EAVES and W. R. BRUCE, Altered sensitivity of haematopoietic stem cells to 5-fluorouracil following endotoxin, cyclophosphamide or irradiation. *Cancer Chemother. Rep.* **58,** 813 (1974).

Europ. J. Cancer Vol. 12, pp. 557–561. Pergamon Press 1976. Printed in Great Britain

Spontaneous Transformation of Human Fibroblast Cultures Derived from Bronchial Carcinomata

BRUNO AZZARONE and DELFINA PEDULLÀ
Institute of Medical Microbiology, University of Genoa, Viale Benedetto XV, 16132 *Genoa, Italy*

Abstract—*Stromal fibroblasts derived from human tumours have been always considered normal cells. In this report it is shown that fibroblasts derived from lung cancers, regularly undergo spontaneous transformation when cultured in crowded growth conditions, while fibroblasts derived from non-neoplastic tissues do not transform. This particular behaviour may reflect a preneoplastic state of stromal fibroblasts derived from lung cancers.*

INTRODUCTION

One of the greatest difficulties in establishing permanent cell lines derived from human tumours is the presence in the explants of stromal fibroblasts, which outgrow abnormal cells [1, 2]. Since stromal fibroblasts have morphological and growth patterns, which seem to fulfill the criteria for normal fibroblasts *in vitro* established by Hayflick [3], they are considered normal cells and sometimes used as control cells.

The biological behaviour of these fibroblasts has not been fully explored. In the present study we report evidence suggesting that the *in vitro* behaviour of stromal fibroblasts from human lung cancers differ from that of fibroblasts derived from non-neoplastic tissues.

MATERIAL AND METHODS

Tissue sources

Four biopsy specimens of non-irradiated lung tumours were obtained from different patients (Table 1). Six biopsies from patients without neoplasia were obtained from skin, kidney and lung. One diploid cell strain (WI38) was also carried as a control.

Accepted 21 January 1976.

This work has been performed within the frame of a EORTC–T.C.C. research project.

Cell and culture methods

Fragments of surgical specimens were minced in phosphate buffered saline without calcium and magnesium containing trypsin 0·125% and EDTA 0·002%. The fragments were repeatedly pipetted to obtain a cell suspension which was sedimented by low speed centrifugation, and re-suspended in 2 ml of growth medium. The culture medium was Eagle's minimum essential medium (MEM) supplemented with non-essential aminoacids, 20% fetal calf serum (FCS), penicillin (100 I.U./ml), streptomycin (100 μg/ml) and fungizone (2·5 μg/ml). The cell suspension was seeded in plastic flasks, erythrocytes were washed-off when cells had attached and started to grow.

Table 1. *Origin of tumor cell lines*

Cell Strains	Sex	Age	Diagnosis
ORG	Male	50	Epidermoid carcinoma Slightly differentiated
FERG	Male	59	Epidermoid carcinoma + Fusiform cells
BC	Male	60	Epidermoid carcinoma Moderately differentiated
MAN	Male	61	Anaplastic carcinoma

As soon as monolayers were obtained, the cells were subcultured by 1:2 splits; medium was replaced every third or fourth day.

Selection of transformed cells

At each subculture one culture was maintained in crowded growth conditions with daily replacement of medium, until foci of altered cells were noted, or until cells died. Cultures which did not alter after three months of crowded culture conditions were discarded and considered normal. Foci of altered cells were picked up with a Pasteur pipette, trypsinized and subcultured in MEM supplemented with 10% FCS.

Doubling time and saturation density

Replicate 60 mm plastic Petri dishes were seeded with 2×10^5 cells in 5 ml per dish of growth medium. The medium was replaced 3 times weekly. To estimate doubling time the cells in duplicate dishes were counted 3–5 times weekly. For determination of saturation density, dishes were counted during about a 5 day period after stationary growth phase was reached.

Plating efficiency

For determination of the plating efficiency, about 200 cells were plated in 5 ml of medium in 60 mm plastic dishes. Colonies were fixed and counted at day 6. Only colonies consisting of more than 10 cells were scored.

Concanavalin A (Con A) agglutination

Agglutination assay was done according to Inbar and Sachs [4].

Growth in soft agar

A previously described technique was used [5].

Cell growth in vivo. *In vivo* determination of tumour producing capacity has been studied only on ORG and ORG(T) cells; 1×10^6 cells were injected subcutaneously into newborn Swiss NIH mice. Tumours were macroscopically visible after 10 days. The tumours were characterized by chromosomal analysis.

Chromosomes studies

A standard procedure using colcemid and acetic acid orcein staining was used.

RESULTS

Primary cultures derived from neoplastic tissues contained heterogeneous population. Groups of epithelioid cells were surrounded by more abundant fibroblast-like population. During first subcultures the epithelioid population progressively showed cytoplasmic granularity, swelling and detachment from the plastic surface.

These cells were recovered from the supernatant and were found to be non-viable by Trypan-blue staining. Cultured cells derived from subsequent subcultures grew out as monolayers of fibroblast like cells. At this stage the cells were contact-inhibited and other tests

Table 2. Biological characteristics of non-transformed and transformed cell lines derived from normal and malignant tissues

Cell line		Transformation event					
Origin	Designation	Present (+) Absent (−)	Days after culture initiation	Passage level	Doubling time (hr)	Colony formation in agar	Agglutination by Con. A
Tumor specimens	ORG	−	68	XVII*	40	−	−
	FERG	−	55	XVII*	38	−	−
	BC	−	75	XXII*	42	−	−
	MAN	−	70	XX*	46	−	−
	ORG(T)	+	63†	VI	20	+	+
	FERG(T)	+	72†	III	22	+	+
	BC(T)	+	56†	VIII	20	+	+
	MAN(T)	+	90†	II	28	+	+
Normal tissues	SKIN 1	−	90†	XXX	n.t.	−	−
	SKIN 2	−	43†	XXXIV	36	−	−
	KIDNEY 1	−	50†	XVIII	n.t.	−	−
	KIDNEY 2	−	68†	XXI	39	−	−
	WI38	−	90†	XXXVII	24	−	−

*The cells were grown in crowded culture conditions.

†At this passage level cells ceased dividing. Thereafter they were observed till total degeneration of the culture.

(Table 2) also failed to give evidence for their malignant origin.

After variable periods of (Table 2) growth in crowded culture conditions, foci of cells with altered morphology were noted on the monolayers of ORG, BC, FERG and MAN cells; while no similar changes were evident in crowded cultures from normal tissues or non-crowded cultures from neoplastic origin. The abnormal cells appeared initially in small focal areas. They were larger than the original fibroblast like cells, showed criss-cross growth and rapidly outgrew the untransformed cells. Loss of contact inhibition was shown by continued growth which caused overlapping, and piling-up of cells. The new cell lines obtained were identified by adding a (T) to the original designation. Initially the new lines formed focal aggregates. In newly transferred cultures they formed homogeneous multilayers. All four cell lines were agglutinated by Concanavalin A, formed colonies in soft agar (Table 2) and were heteroploid (see Chromosome Study) complete characterization including the determination of growth rate, saturation density, plating efficiency, *in vivo* growth and virus induction, was only done for ORG(T) cells. ORG cells that have been stored in liquid nitrogen were subjected recently to the crowded conditions. The new transformed cell lines obtained were termed ORG [2] and ORG [3] respectively.

Growth in soft agar

Transformed cells formed colonies (approximately 0·3 in diameter, average 30 colonies per dish) at 6×10^4 cells per 60 mm-plate, when the non transformed cells did not at 250×10^5 cells per plate. The colonies obtained from agar plates were picked up into liquid medium and yielded cells which could be serially subcultivated and resembled the parent cells.

Rate of growth saturation density and plating efficiency

The average doubling-time of ORG(T) cells was 20 hr. The doubling-time measured several times in several culture transfers, was constant, ORG(T) cells attained high cell density at stationary growth phase (2×10^5 cells/cm^2). In contrast the original fibroblast-like cells attained maximum cell densities approximately 8-fold lower. Plating efficiency was determined in two experiments with values of about 60% Plating efficiency of normal cells resulted < 0·001%.

Untransformed cells

	Passage/ mitoses scored	Chromosome distribution										
		< 45	45	46	47	48/49	90†	92†	94	165	180	184
ORG	IV/51		13	37			1					
	VII/53		21	18	1	1	4	8				
FERG*	XI/45	11	6	11		7	3	5		2		
	VIII/75	7	5	35	1	6	5	13	1		2	
BC	XI/11		1	2		1	1				6	
	VIII/45	10	6	20			6	2				1
MAN	X/73	6	7	37	2	5	6	2				1

*Metaphases with 45 and 90 chromosomes are lacking chromosomes of G group.
†Many metaphases scored were endoreduplications.

Transformed cells

Lines	Mitoses scored	Chromosome distribution						
		46	50/60	60/70	80/90	90/100	+100	+200
ORG(T)	50	1	2	40	7			
FERG(T)	50		17	13			19	1
BC(T)	50		6	34			10	
MAN(T)	50					13	31	6

In vivo *growth*

In mice inoculated with ORG(T) cells, large masses appeared 10 days after inplantation. The tumours were transplanted into newborn mice and then were again established in culture. The cell lines thus obtained resembled the original cell line in morphology and in growth patterns and showed the same karyotype.

Chromosome study

Cytogenetic analysis revealed the chromosome distribution in untransformed and transformed cells as shown on p. 3.

DISCUSSION

Four cell lines are described, each one isolated from bronchial tumor biopsies. The primary cultures consisted of mixed epitheloid-fibroblast cell populations. Subculture led to progressive loss of the epitheloid element, yielding apparently normal contact inhibited fibroblast strains. The cytogenetic analysis performed on untransformed cells has, however, revealed during the phase of active growth the presence of variable degree of aneuploidy, which can be considered significative if compared with the data of Chen and Ruddle [6] on WI38 (0·02 of aneuploidy in phase II and 0·3 in phase III). However, maintenance of these cells under crowded culture conditions reproducibly led to the appearance of foci of altered cells from which cell lines could be generated which met all available criteria of malignancy, such as: (i) high saturation density, (ii) high plating efficiency, (iii) multilayered growth, (iv) colony formation in soft agar, (v) agglutination by concanavalin A, (vi) heteroploidy, (vii) tumor induction *in vivo* in mice.

Several hypotheses can be put forward to explain spontaneous transformation as seen in our study.

(i) Cells may have been transformed by accidental contamination with oncornaviruses. This possibility seems unlikely because no such viruses nor any other cell line was being carried in our laboratory at the time when transformation occurred. Secondly one would have to assume that normal fibroblasts derived from tumor specimens have a higher susceptibility to viral or spontaneous transformation such as described for certain genetically anomalous human cells [7].

(ii) A few tumor cells stayed present throughout the first subcultures and overgrew the fibroblasts when conditions were favorable. This is a reasonable hypothesis which cannot be ruled out.

(iii) Apparently normal fibroblastic cells derived from the tumor specimens contain oncogenic information, e.g. carried by an endogenous oncornavirus. Under crowded culture conditions this information is switched on and leads to transformation. Such events have been described for mouse 3T3-cells [8] and for certain cultures derived from brain tumors [9]. In these systems the transformed cells also released C-type virus particles. With the techniques available to us, we were unable to demonstrate the activation of such viruses in our cultures.

REFERENCES

1. D. J. Giard, S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik and W. P. Parks, *In vitro* cultivation of human tumours: establishment of cell lines derived from a series of solid tumours. *J. nat. Cancer Inst.* **51,** 1417 (1973).
2. S. Rashead, W. A. Nelson-Rees, E. M. Toth, P. Arnstein and M. B. Gardner, Characterization of a newly derived human sarcoma cell line (HT 1080). *Cancer (Philad.)* **33,** 1027 (1974).
3. L. Hayflick, The limited *in vitro* life time of human diploid cell strains. *Exp. Cell. Res.* **37,** 614 (1965).
4. Inbar and L. Sachs, Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc. nat. Acad. Sci. (Wash.)* **63,** 1418 (1969).
5. R. M. McAllister, G. Reed and R. J. Huebner, Colonial growth in agar of cell derived from adenovirus-induced hamster tumours. *J. nat. Cancer Inst.* **39,** 43 (1967).
6. F. H. Ruddle and T. R. Chen, 19th Annual Meeting of the Tissue Culture Association, Abstract no. **141,** 62 (1968).

7. R. W. MILLER and J. TODARO, Viral transformation of cells from persons at high risk of cancer. *Lancet* **i,** 81 (1969).
8. W. T. HALL, W. F. ANDRESEN, K. K. SANFORD, V. EVANS and J. W. HARTLEY, Virus particles and murine leukemic virus complement fixing antigen in neoplastic and non neoplastic cell lines. *Science* **156,** 85 (1967).
9. J. HOOKS, C. J. GIBBS, H. COPRA, M. LEWIS and D. C. GAYDUSEK, Spontaneous transformation of human brain cells grown *in vitro* and description of associated virus particles. *Science* **176,** 1420 (1972).

Europ. J. Cancer Vol. 12, pp. 563–565. Pergamon Press 1976. Printed in Great Britain

Norethisterone Acetate in the Treatment of Advanced Breast Cancer

R. D. RUBENS, R. K. KNIGHT and J. L. HAYWARD
Imperial Cancer Research Fund, Breast Cancer Unit, Guy's Hospital, London, SE1, U.K.

Abstract—*Twenty-four patients with advanced breast cancer who had relapsed after both primary and secondary endocrine therapy and chemotherapy were treated with norethisterone acetate. Objective regressions were obtained in 6 patients (median duration 3·5 months), 4 of whom had previously responded to hypophysectomy. The best responses were obtained with cutaneous lesions, but improvement was also seen in breast, lymphatic and skeletal disease; visceral metastases were resistant. Toxicity was negligible.*

INTRODUCTION

NORETHISTERONE acetate (SH420, Schering) is a progestogen with therapeutic activity in breast cancer [1, 2]. Its mode of action may be related to its inhibitory effect on gonadotrophin secretion [3]. Unlike androgens and oestrogens, the use of progestogens is rarely associated with undesirable side-effects. They have, however, been relatively little used in comparison with other hormonal treatments, although a preliminary report of a controlled trial showed that they were less effective than either oestrogens or androgens in treating disseminated disease [4]. We report here on the use of norethisterone acetate in patients who have relapsed after treatment by both primary (ovarian ablation, androgens or oestrogens) and secondary (hypophysectomy or prednisone) endocrine therapy and cytotoxic chemotherapy.

MATERIAL AND METHODS

Twenty-four patients with advanced breast cancer and evidence of progressive disease were treated with norethisterone acetate 20 mg three times daily administered orally. All patients had previously been considered for endocrine treatment and, subsequently, chemotherapy which had been given unless there were specific contraindications. The characteristics of these patients with regard to age, menopausal status, disease-free interval, previous therapy and time from diagnosis to start of treatment with norethisterone acetate are shown in Table 1. Before treatment was started all patients were assessed. Palpable and visible lesions were measured and photographs of superficial lesions and radiographs of chest, skull, total spine and pelvis taken. After treatment was started patients were seen at four-weekly intervals for assessment.

Table 1. Characteristics of patients. Age: Median 58·5 yr (Range 35–70)

	No. of patients
Menopausal status at diagnosis	
Premenopausal	13
Postmenopausal (1–5 yr)	3
Postmenopausal (> 5 yr)	8
	24
Disease-free interval	
0–2 yr	18
> 2 yr	6
	24
Stage at first diagnosis	
Operable (I, II)	11
Primarily advanced (III, IV)	13
	24
Previous treatment for advanced disease	
Ovarian ablation	8
Androgens	13
Oestrogens	4
Hypophysectomy	19
Prednisone	3
Chemotherapy	21
Radiotherapy	13
Time from diagnosis to start of treatment with norethisterone acetate	
Mean	51 months
Median	38 months
Range	14–184 months

Accepted 21 January 1975.

Strict criteria* of tumour regression were followed. Categories of response are defined as follows:

1. *Objective regression*

(i) *Complete response.* Disappearance of all known disease.

(ii) *Partial response.* Greater than 50% decrease in the product of perpendicular diameters of measurable lesions and objective improvement in other evaluable lesions. No new lesions appearing.

2. *No change*

Less than 50% decrease or less than 25% increase in the size of measurable lesions.

3. *Progressive disease*

(i) *Mixed.* Some lesions regress while others progress or new lesions appear.

(ii) *Failure.* Progression of some or all lesions and/or appearance of new lesions. No lesions regressing.

Duration of response in a patient achieving a regression has been dated from the start of therapy until either new lesions appeared, any one existing lesion increased by 25% or more above the smallest size recorded, or non-measurable, but evaluable lesions deteriorated.

RESULTS

The responses obtained and their duration are summarised in Table 2. It is seen that, even in this group of patients with highly resistant disease, 6 patients (25%) achieved partial regressions on the strictly applied criteria. The regressions occurred only in lesions in non-visceral soft-tissues and bone; visceral lesions did not respond (Table 3). In one patient whose disease progressed (mixed response) whilst on treatment, there was a regression of disease (partial response) lasting 3 months when the drug was stopped. Two patients appeared to have an acceleration of tumour growth on norethisterone acetate. Four of the 6 patients with objective regressions had had previous hypophysectomies, to which they had responded and subsequently relapsed. Toxic effects from the drug were not seen although some patients had modest weight increases due to fluid retention.

Table 2. Response to norethisterone acetate

	No. of patients	Duration (months)
Objective regression		
Complete response	0	—
Partial response	6	3·5 (3–6+)
No change	1	4
Progressive disease		
Mixed	4	—
Failure	13	—
	24	

Table 3. Responses at different sites

Site	Regressions*
Breast	2/11
Lymph nodes	3/15
Skin	6/9
Bone	3/11
Pleura	0/2
Liver	0/2
Peritoneum	0/1

*Denominators indicate the number of patients with involvement of the stated site at the start of treatment with norethisterone acetate and numerators show the number with a regression at that site.

DISCUSSION

In this small series, norethisterone acetate has been shown to be capable of inducing worthwhile regressions in 25% of patients with highly resistant advanced breast cancer, at a time when no other endocrine treatment is effective. The best responses were obtained with cutaneous lesions, but improvement in breast, lymphatic and skeletal disease was also seen; visceral metastases were resistant. The "withdrawal effect" seen in one patient has been previously noted with this agent [6]. The mechanism of action of progestogens is not clearly understood, but inhibition of gonadotrophin secretion is believed to be important. However, in this series, 4 of the 6 patients with objective regressions had previously responded to hypophysectomy, and an earlier report has shown that a combination of oestrogen and progestogen can benefit patients after adrenalectomy or hypophysectomy [7]. It is now

*These criteria have been formulated by an international committee which met at the UICC in June 1975. The detailed document is currently being circulated for comment, with a view to it being recommended for widespread use.

Because of the short duration of response in this study, the criteria of the British Breast Group [5] were not used.

known that complete ablation of pituitary function is unusual after hypophysectomy [8] and so pituitary inhibition could still explain the activity of norethisterone acetate in these patients. Furthermore, progestogens may exert a direct inhibitory effect on the tumour [9].

There is evidence that progestogens can improve the results of chemotherapy in breast cancer [10], and, in view of the lack of toxicity and comparability of their therapeutic effects with other hormonal treatments, they warrant further study in the treatment of this disease.

REFERENCES

1. J. L. HAYWARD, Hormones and human breast cancer. In *Recent Results in Cancer Research.* Vol. 24. Springer, Berlin (1970).
2. B. A. STOLL, Breast cancer—endocrine therapy. In *Endocrine Therapy in Malignant Disease.* (Edited by B. A. STOLL). W. B. Saunders, Philadelphia (1972).
3. I. MARTIN and K. CUNNINGHAM, Suppression of pituitary gonadotrophins by 17-ethinyl-19-nortestosterone in patients with metastatic carcinoma of the breast. *J. clin. Endocrinol.* **20,** 529 (1960).
4. V. JONES, C. A. F. JOSLIN, R. E. JONES, D. K. L. DAVIES, MAUREEN M. ROBERTS, E. N. GLEAVE, H. CAMPBELL, A. P. M. FORREST, Progestogens and Advanced Breast Cancer. *Lancet* **i,** 1049 (1971).
5. British Breast Group, Assessment of response to treatment in advanced breast cancer. *Lancet* **ii,** 38 (1974).
6. S. CURWEN, The treatment of advanced carcinoma of the breast with SH420. *Clin. Radiol.* **21,** 219 (1970).
7. R. L. LANDAU, E. N. EHRLICH and C. HUGGINS, Estradiol benzoate and progesterone in advanced human-breast cancer. *J. Amer. med. Ass.* **182,** 632 (1962).
8. T. BATES, R. D. RUBENS, R. D. BULBROOK, P. R. GOODWIN, D. Y. WANG, M. A. FALCONER, L. F. W. SALMON, P. H. SCHURR, O. H. SHAHEEN, R. K. KNIGHT and J. L. HAYWARD, Comparison of pituitary function and clinical response after transphenoidal and transfrontal hypophysectomy for advanced breast cancer. *Brit. J. Surgery* **62,** 654 (1975).
9. B. A. STOLL, Investigation of organ culture as an aid to the hormonal management of breast cancer. *Cancer (Philad.)* **25,** 1228 (1970).
10. W. G. TUCKER, P. B. STOTT, L. ZELKOWITZ and M. KUKRIKA, Medoxyprogesterone acetate in combination with chemotherapeutic agents for the treatment of metastatic breast cancer. *Abstracts XI Int. Cancer Cong.* **3,** 600 (1974).

Europ. J. Cancer Vol. 12, pp. 567–573. Pergamon Press 1976. Printed in Great Britain

The Metabolism of Cytosine Arabinoside as a Predictive Test for Clinical Response to the Drug in Acute Myeloid Leukaemia

J. F. SMYTH, A. B. ROBINS and C. L. LEESE

Department of Applied Biochemistry, Division of Medicine, Institute of Cancer Research, Clifton Avenue, Belmont, Sutton, Surrey, U.K.

Abstract—*The uptake, deamination (D) and phosphorylation (K) of cytosine arabinoside (AraC) by acute myeloid leukaemic blast cells in short term culture has been studied. There was a wide variation in these parameters but no correlation was found between the values of K, D or K/D with the clinical response of patients to an induction course of chemotherapy utilising a combination of AraC, daunorubicin and adriamycin. Hence measurement of these parameters is of little value in predicting the response of patients to this drug combination.*

INTRODUCTION

1-β-D-ARABINOFURANOSYL cytosine (AraC) is widely used in the treatment of acute myeloid leukaemia (AML) [1–3]. The antitumour activity of this compound requires intracellular phosphorylation, initially by deoxycytidine phosphokinase, and thence by further nucleotide phosphokinase activity to cytosine arabinoside-5′-triphosphate (AraCTP). This drug metabolite inhibits DNA polymerase by competing with deoxycytidine triphosphate, one of the endogenous substrates for this enzyme [4]. Conversely AraC is inactivated intracellularly by cytidine deaminase to the non-cytotoxic 1-β-D-arabinofuranosyl uracil (AraU) [5]. Thus the therapeutic efficacy of AraC will be influenced under pharmacological conditions a) by the total amount of drug transported into the target cells and b) by the relative proportions of the drug activated by phosphorylation (K) and inactivated by deamination (D). Reports in the literature suggest that responsiveness to AraC in acute myeloid leukaemia may be related to the net effect of these two opposing metabolic pathways in leukaemic blast cells. Steuart and Burke [6] equated clinical drug resistance and decreasing duration of remission with raised levels of cytidine deaminase, whereas Kessel *et al.* [7] and Tattersall *et al.* [8] found that response to AraC correlated broadly with the ability of blast cells to phosphorylate deoxycytidine.

In order to evaluate the contribution of these opposing metabolic pathways, and assess their relevance in predicting the response of patients treated with AraC, we have devised a rapid test based on the uptake and fate of AraC when intact peripheral AML blast cells are exposed to pharmacologically relevant levels of labelled AraC under short term culture conditions. The analytical techniques permitted an assessment of drug uptake, phosphorylation and deamination (expressed as K/D ratio) and an examination of drug distribution between its various phosphate ester derivatives. All patients were studied at presentation prior to chemotherapy. In some cases it was possible to re-examine these parameters in patients who, following a period of remission, subsequently relapsed.

MATERIAL AND METHODS

Patients

The patients included in this study were under the care of the Physicians at the Royal Marsden Hospital, Surrey, U.K. Peripheral blood samples, from which leukaemic blast cells were subsequently separated (see methods)

Accepted 20 January 1976.

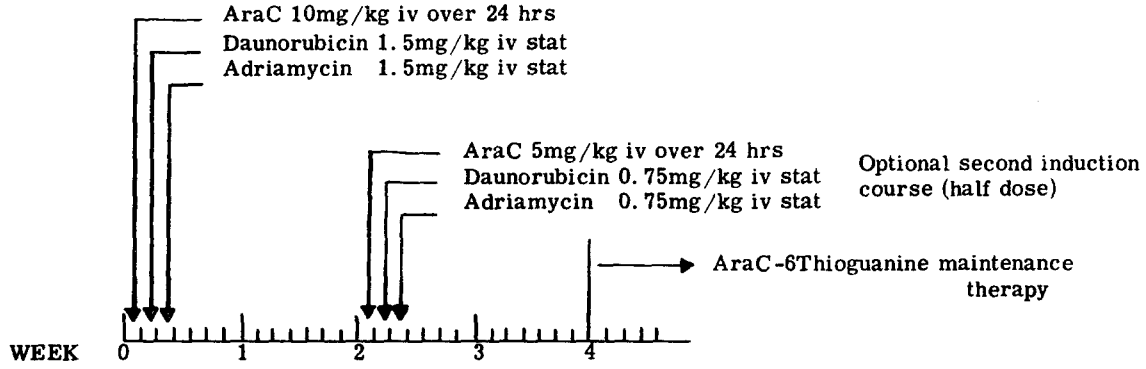


Fig. 1. *Induction chemotherapy schedule for AML.*

were taken from all patients prior to chemotherapy which was administered according to the protocol outlined in Fig. 1.

The response of patients to induction therapy was assessed according to the following criteria:

1. *Complete remission (CR).* Where blast cells were eliminated from the peripheral blood, the bone marrow contained less than 5% of normal blast cells and there was an adequate peripheral circulatory count of polymorphonuclear leucocytes and platelets.

2. *Partial remission (PR).* Where there was a response to treatment indicated by a fall in the blast cell count but the response did not reach the criterion required for CR. This group of patients was further sub-divided into a group PR I, who subsequently showed further improvement following induction therapy and a second group PR II, who deteriorated after an initial partial response.

3. *No remission (NR).* Where there was no response following an adequate trial of therapy.

A summary of the patients included in this study is shown in Table 1.

MATERIAL

Ficoll and Triosil were obtained from Pharmacia Ltd. (London, U.K.) and Nyegaard and Co., Oslo (U.K. agents, Vestric Ltd., London) respectively. Unlabelled AraC (Cytosar) was the product of Upjohn Ltd. (Crawley, Sussex, U.K.) and the nucleotides AraCMP and AraCTP were purchased from Sigma Chemical Co. Ltd. (Kingston-upon-Thames, Surrey, U.K.). Other analytical reagent grade chemicals were purchased either from British Drug Houses Ltd. (Poole, Dorset, U.K.) or Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.). Fischer's Medium with L-Glutamine was the product of Gibco Bio-Cult (Glasgow, Scotland).

Tritiated AraC (1-β-D-arabinofuranosyl[5-^{3}H]cytosine; specific activity 14 Ci/mmol) was obtained from the Radiochemical Centre Ltd. (Amersham, Buckinghamshire, U.K.).

Non-labelled AraU was prepared by nitrous acid deamination of AraC in dilute acetic acid and freed from unchanged drug by chromatography on Dowex-50W resin; H^+ form [7].

Table 1. *Clinical response of* 29 *AML patients to induction therapy*

	Complete remission CR	Partial remission PR		No remission NR
		PR I	PR II	
Number	14	5	4	6
(Males	(7)	(2)	(0)	(4)
(Females)	(7)	(3)	(4)	(2)
Mean age	53·6	57·6	47·6	54·0
(Range)	(42–73)	(31–66)	(31–61)	(34–72)

Purification of tritiated AraC

Commercially available tritiated AraC contained up to 5% of ^{3}H-AraU, an impurity which increased on storage presumably due to radiolytic deamination. The compound was purified by application of 100 μl of commercial solution (100 μCi) as a streak to carboxymethyl cellulose paper (Whatman CM82) together with 2 μl of 10 mM AraC solution as a marker. Ascending chromatography with water as the mobile phase for 1 hr effected an adequate separation of ^{3}H-AraC (R_f 0·1) from ^{3}H-AraU (R_f 0·8). The region of the chromatogram visualised by uv light absorption of marker was removed and the drug eluted for 30 min with 1·0 ml 0·1 M hydrochloric acid. The purified drug solution was diluted with 100 μl of unlabelled AraC (10 mM) and 9 ml Fischer's culture medium; a final adjustment to pH 7·4 was made by addition of sodium bicarbonate.

Cell culture

AML blast cells were prepared from fresh heparinised blood by centrifugation through a Ficoll–Triosil mixture [9]. The blast cells accumulated at the interface and were removed with a Pasteur pipette and washed twice by centrifugation from normal saline. The cells (95% blast cells or greater) were suspended in Fischer's medium (without protein additives) at a concentration of 4×10^6 cells/ml, and finally equilibrated at 37°C.

Aliquots of this suspension (0·5 ml) were mixed with an equal volume of Fischer's medium containing ^{3}H-AraC to give final concentrations of 2×10^6 cells/ml and 0·05 mM respectively. Each determination was performed in quadruplicate. The cell suspensions were incubated at 37°C for 30 min.

Extraction and analysis of AraC metabolites

At the end of the incubation period the cells were recovered by centrifugation (400 ***g***, 10 min) at 4°C. To avoid loss through rapid efflux of nucleosides [10] the cells were not washed but the pellet was drained as completely as possible after decanting the supernatant and any excess extraneous fluid removed with the aid of tissues. The cells were treated immediately with 10 µl of 0·1 M PCA at 0°C and centrifuged at 2000 ***g*** for 15 min at 4°C.

Aliquots of the supernatant (2 µl) were applied to sheets of Whatman No. 1 chromatography paper (23 × 57 cm) previously moistened with 0·05 M sodium citrate adjusted to pH 3·75 with acetic acid, together with 2 µl of each of 10 mM aqueous marker solutions of AraC, AraU, AraCMP and AraCTP. The mixtures were separated by high voltage electrophoresis in a flat bed apparatus (4 kV, 80 mA, 40 min; Shandon-Southern, model L24). The electrode compartments contained the same buffer solution.

Following separation the paper was dried and the location of the components detected under uv light. The appropriate areas were removed, and eluted with 0·5 ml water for 30 min prior to the addition of 10 ml of scintillant. Activity associated with each fraction was measured in an intertechnique counter (SL30 liquid scintillation spectrometer) at 40% efficiency. In the absence of non-labelled marker AraCDP areas of peak radioactivity located between AraCMP and AraCTP, were recovered from the electrophoretograms to estimate the amount of the arabinoside diphosphate formed in each case. Electrophoresis under the above conditions permitted the clear resolution of AraC, AraU, AraCMP, AraCTP, AraUDP and AraUTP in order of increasing anodal mobilities. AraCDP and AraUMP had closely similar mobilities lying between those of AraCMP and AraCTP and could not be resolved from each other by this method.

The concentration of each component was calculated as nmoles per 10^9 cells per 30 min.

RESULTS

Total uptake of ^{3}H-AraC by AML blast cells

The total uptake of tritiated AraC by cells from patients within the four clinical subgroups is presented in Table 2. The values include that proportion converted to AraC metabolites during the incubation period. No corrections were routinely made for labelled components present in the extracellular space within the cell pellet which was assumed to be constant and to contain only nucleoside derivatives. Cells incubated with lower concentrations of AraC gave lower levels of phosphorylated derivatives although the percentage AraC converted to its phosphate derivatives increased.

Phosphorylation and deamination of ^{3}H-AraC by AML blast cells

The extent of total phosphorylation of AraC and of the formation of AraCTP, the inhibitor

Table 2. Uptake and metabolism of ^{3}H-AraC by AML blast cells. Values are nmoles/10^9 cells/30 min ± 1 standard deviation

Clinical sub-group	Uptake	Phosphorylation		Deamination
	Total	Total*	AraCTP	AraU
CR	67 ± 34	8·8 ± 6·0	4·6 ± 4·2	3·0 ± 2·7
PR I	66 ± 25	2·7 ± 2·5	1·8 ± 2·0	2·9 ± 2·4
PR II	74 ± 6	11·0 ± 6·0	4·4 ± 1·5	6·0 ± 5·0
NR	63 ± 7	11·1 ± 4·0	3·8 ± 3·6	2·3 ± 2·1

*See results.

Table 3. K/D Ratios for cells from AML patients in various clinical sub-groups. Values are quoted ± 1 standard deviation with ranges given in parentheses

Clinical sub-group	K/D (AraCTP)		K/D* (Total phosphorylation)	
CR	2·7 ± 2·5	(0·1– 6·5)	5·0 ± 3·5	(0·3–10·0)
PR I	0·6 ± 0·25	(0·2– 0·8)	1·1 ± 0·6	(0·4– 1·9)
PR II	3·1 ± 3·7	(0·5– 5·7)	3·5 ± 4·2	(0·6– 6·5)
NR	4·0 ± 6·2	(0·3–16·4)	5·2 ± 7·4	(0·4–20·0)

*See results.

of DNA polymerase, is shown in Table 2. No significant differences were found in the ability of AML blast cells from patients within the four clinical sub groups to phosphorylate the drug. A similar absence of correlation was found between the ability of blast cells to deaminate AraC and the overall response of patients to induction therapy. Values for the total phosphorylation of AraC, and hence the K/D values derived from the total phosphorylation data (Tables 2, 3 and 6) may be somewhat elevated due to the inability to resolve AraCDP and AraUMP by the electrophoretic technique described above. AraUMP could be formed from either AraC by deamination and subsequent phosphorylation of AraU or by deamination of AraCMP by intracellular deoxycytidylate deaminase activity. Incubated with ^{3}H-AraU AML blast cells showed little tendency to phosphorylate this compound. However, due to the clear resolution of AraCTP by the analytical technique phosphorylation and K/D data derived from the formation of this AraC derivative is a much more reliable measure of the overall phosphorylation activity of the cells.

K/D ratios

The values of the K/D ratio (derived from the parameters summarized in Table 2) are presented in Table 3. K/D ratios were calculated both on the basis of the total extent of phosphorylation by AML blast cells and also on the basis of AraCTP formation. In neither case could a predictive value be ascribed to these ratios in distinguishing those patients who subsequently responded well to induction therapy from those who did not, either on a group or an individual basis.

Data on all these parameters derived from individual patients is presented in Fig. 2 with K and D values expressed as a percentage of total AraC uptake.

Metabolism of ^{3}H-AraC in blast cells of AML patients in relapse

The ability of blast cells to phosphorylate and deaminate AraC (and the K/D values derived from these parameters) in patients who relapsed after a period of remission is presented in Table 4. The data at relapse is compared with that obtained from the patients' blast cells prior to the first induction course of chemotherapy.

DISCUSSION

The objective of the present work was to devise a simple rapid test which, hopefully, by determining the sensitivity of blast cells

Table 4. Phosphorylating and deaminating ability of AML blast cells from patients in relapse following an initial period of complete or partial remission. Values of K and D are given as nmoles/ 10^9 cells/30 min

Patient No.	Clinical sub-group	Pretreatment			1st relapse		
		K	D	K/D	K	D	K/D
1	CR	0·18	0·03	6·0	0·14	0·03	4·7
2	CR	0·10	0·02	5·0	0·30	0·01	30·0
3	CR	0·08	0·04	2·0	0·10	0·04	2·5
4	CR	0·02	0·004	5·0	0·14	0·008	17·5
5	PR I	0·03	0·02	1·5	0·013	0·01	1·3

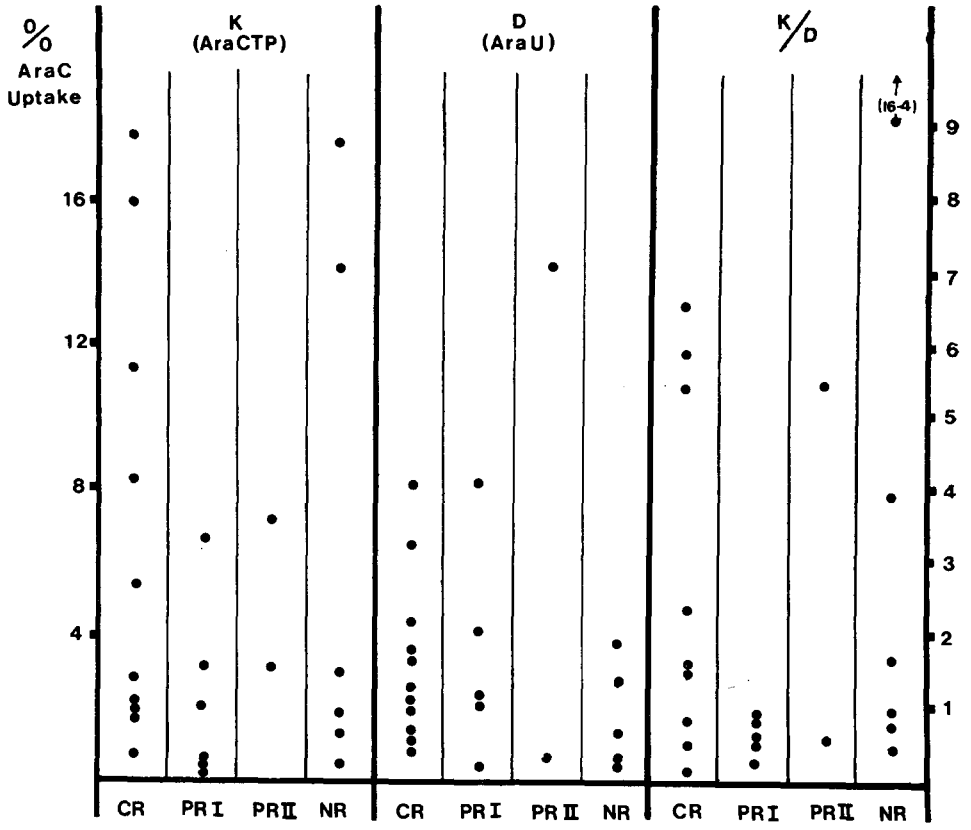


Fig. 2. Values for phosphorylation (formation of AraCTP) and deamination; both expressed as a percentage of total AraC uptake and of the K/D ratios for individual patients.

to one component of a multidrug regime, would predict the individual patient response to the combination therapy used for remission–induction. Conditions were chosen such that the *in vivo* situation was approximated as closely as possible in that during the short term culture experiment *intact* blast cells were exposed to AraC at a drug concentration of 50 μM. This was a chosen compromise between serum levels expected in patients in the early stages following a single intravenous drug administration and the more sustained serum levels achieved by intravenous infusion [11, 12]. AraC is known to be susceptible to hepatic deamination and rapid renal clearance following single dose administration, or at the termination of a period of infusion, with a serum half-life of 12–20 mins [11, 13].

Below concentrations of 1 mM, uptake of AraC into various cells at 37°C has been shown to be a facilitated diffusion process with rapid equilibration between extra- and intracellular concentrations [10, 13]. This is consistent with the present observations where in each case the total intracellular concentration of drug and its metabolites exceeded the extracellular AraC concentration, the excess being due to the formation of phosphorylated drug derivatives not able to permeate across the cell membranes to the extracellular space.

Table 5. The effect of freeze-thawing prior to treatment of cells with PCA compared to cells treated with PCA alone

	No. of patients	Ratio of activity Freeze-thawed + PCA/PCA alone
Deamination	19	20·6 ± 16·5
Phosphorylation	19	1·0 ± 0·47

The method of isolating and disrupting cells from culture was found to be critical. In preliminary studies cell pellets were subjected to a rapid six-cycle freeze-thawing between −70°C and +37°C prior to treatment with perchloric acid. Observed levels of AraC deamination were considerably elevated (20-fold) over those found in cells treated directly with PCA (Table 5) due presumably to the release of "latent" deaminase in the course of freeze-thawing. Freeze-thawing prior to PCA treatment had no effect on the degree of total

Table 6. The effect of freeze-thawing prior to PCA extraction on the distribution of AraC between its phosphorylated derivatives. Values quoted represent the concentration of each component as a percentage of total AraC phosphates ± 1 standard deviation

Extraction method	No. of patients	AraC phosphates (% total phosphates)		
		AraCMP	AraCDP*	AraCTP
PCA	12	12 ± 15	18 ± 12	70 ± 20
Freeze thaw +PCA	13	62 ± 18	21 ± 9	16 ± 18

*See results.

phosphorylation (Table 5). However, on examination of the distribution of AraC between its phosphate esters AraCMP was found to predominate (Table 6). In contrast, where cells were treated directly with PCA, AraCTP was the major drug nucleotide present in the cell extract. Loss of AraCTP in the course of freeze-thawing was due, presumably, to release of phosphorylase or pyrophosphorylase activity.

Because of these anomalies introduced by preliminary freeze-thawing all the data presented in the results were derived from unwashed cell pellets treated directly with PCA.

In the present studies no correlation could be observed between the levels of drug deamination and the clinical response of patients receiving an adequate course of therapy. This is in contrast to the report of Steuart and Burke [6] who employed 10,000 *g* supernatant fractions from leukaemic blast cells obtained from bone marrow aspirates. Using the natural substrates cytidine and deoxycytidine these authors claimed that pyrimidine nucleoside deaminase activity in these preparations reflected both intrinsic and acquired resistance to AraC. Coleman and Chabner [14], again employing broken cell preparations, found a wide variation in deaminase levels in three patients with AML.

Employing intact cells at various drug concentrations Kessel *et al.* [7] and Ho [15] found a wide range in the ability of normal and leukaemic human cells to phosphorylate the drug. Resistance to AraC in these cells was equated with a reduced, but non-significant, ability of the cells to phosphorylate the drug. Similar conclusions were drawn by Tattersall *et al.* [8] working with sonicates of AML bone marrow blast cells and deoxycytidine as substrate, and by Coleman and Chabner [14]. The broad spectrum of phosphorylating ability in AML cells is confirmed by the present work whether this activity be measured on the basis of total AraC phosphates formed or on the production of the specific DNA polymerase inhibitor AraCTP.

The broad range of deaminase and phosphorylating ability of AML cells is reflected in the variability of the derived K/D ratios both in terms of data on individual patients (Fig. 2) and of the data relating to the four clinical sub-groups studied in the present work (Table 3). On limited data Coleman and Chabner [14] reached similar conclusions, namely that K/D measurements were of little value in predicting the response of AML patients to AraC concluding that measurements in crude extracts were unreliable due to the presence of enzymes interfering with accurate measurement of both K and D. One clinical situation where K/D measurements have been shown to have some value in predicting responsiveness to AraC is in human metastatic melanoma; 3/13 patients with high K/D ratios in extracts of metastatic melanotic lesions responded to repeated AraC infusions [16]. In contrast Barranco *et al.* [17] showed that the K/D ratio of four different human melanoma cell lines derived from the same lesion and maintained *in vitro* bore little relationship to the sensitivity of these lines to the drug.

In the present series no consistent change in either K, D or K/D values could be determined in patients in relapse following a mean period of six months in remission.

Predictive response to treatment in the clinical situation is of value if a) there is an alternative and equally effective mode of therapy and b) if the predictive test data permits the dosage and scheduling of the drug to be tailored to the requirements of the individual patient. For the initial treatment of AML at the present time there is no drug that has been shown to be superior to AraC in

combination with adriamycin and/or daunorubicin; thus the predictive value of AraC responsiveness may only be pertinent to the second criterion mentioned above. The lack of correlation between the K/D values with clinical response suggests that other factors e.g., the other drugs employed in the combination and host factors such as age, presence or absence of infection, platelet count etc. contribute as much if not more than the effect of a single drug component to the prognosis for the individual patient.

Acknowledgements—This work was supported under MRC/CRC Grant No. G973/72. J. F. S. gratefully acknowledges the receipt of a Cancer Research Campaign Fellowship. The authors wish to thank Dr. T. J. McElwain and the staff of the Leukaemia Unit, Royal Marsden Hospital, Sutton, Surrey, for their co-operation in allowing us to study patients under their care.

REFERENCES

1. D. Crowther, R. L. Powles, C. J. T. Bateman, M. E. J. Beard, C. L. Gauci, P. F. M. Wrigley, J. S. Malpas, G. Hamilton Fairley and Sir Ronald Bodley Scott, Management of adult acute myelogenous leukaemia, *Brit. med. J.* **i,** 131 (1973).
2. R. R. Ellison, J. F. Holland, M. Weil, C. Jacquilat, M. Boison, J. Herbard, A. Sawitsky, F. Rosner, B. Gussoff, R. T. Silver, A. Karanas, J. Cuttner, C. L. Spurr, D. M. Hayes, J. Blom, L. A. Leon, F. Haurani, R. Kyle, J. L. Hutchison, R. J. Forcier and J. H. Moon, Arabinosyl cytosine: a useful agent in the treatment of acute leukaemia in adults, *Blood* **32,** 507 (1968).
3. G. P. Bodey, E. J. Freireich, R. W. Monto and J. S. Hewlett, Cytosine arabinoside (NSC 63878) therapy for acute leukaemia in adults, *Cancer Chemother. Rep.* **53,** 59 (1969).
4. J. J. Furth and S. S. Cohen, Inhibition of mammalian DNA polymerase by the 5′-triphosphate of 1-β-D-arabinofuranosylcytosine and the 5′-triphosphate of 9-β-D-arabinofuranosyladenine, *Cancer Res.* **28,** 2061 (1968).
5. W. A. Creasey, R. J. Papac, M. E. Markiw, P. Calabresi and A. D. Welch, Biochemical and pharmacological studies with 1-β-D-arabinofuranosyl cytosine in man, *Biochem. Pharmacol.* **15,** 1417 (1966).
6. C. D. Steuart and P. J. Burke, Cytidine deaminase and the development of resistance to arabinosyl cytosine, *Nature (Lond.)* **233,** 109 (1971).
7. D. Kessel, T. C. Hall and D. Rosenthal, Uptake and phosphorylation of cytosine arabinoside by normal and leukemic human blood cells *in vitro*, *Cancer Res.* **29,** 459 (1969).
8. M. H. N. Tattersall, K. Ganeshaguru and A. V. Hoffbrand, Mechanisms of resistance of human acute leukemia cells to cytosine arabinoside, *Brit. J. Haematol.* **27,** 39 (1974).
9. A. Bøyum, Isolation of mononuclear cells and granulocytes from human blood, *Scand. J. clin. lab. Invest.* Suppl. **21,** 97 (1968).
10. D. Kessel and S. B. Shurin, Transport of two non-metabolised nucleosides, deoxycytidine and cytosine arabinoside, in a sub-line of the L1210 murine leukaemia, *Biochem. biophys. Acta* **163,** 179 (1968).
11. R. L. Momparler, A. Labitan and M. Rossi, Enzymatic estimation and metabolism of 1-β-D-arabinofuranosylcytosine in man, *Cancer Res.* **32,** 408 (1972).
12. R. L. Momparler, A model for the chemotherapy of acute leukemia with 1-β-D-arabinofuranosylcytosine, *Cancer Res.* **34,** 1775 (1974).
13. J. H. Mulder and K. R. Harrap, Cytosine arabinoside uptake by tumour cells *in vitro*, *Europ. J. Cancer* **11,** 373 (1975).
14. C. N. Coleman and B. A. Chabner, Deoxycytidine kinase (K) and deaminase (D) in human leukaemic cells: separation and properties, *Proc. Amer. Ass. Cancer Res.* **15,** 54 (1974).
15. D. H. W. Ho, Distribution of kinase and deaminase of 1-β-D-arabinofuranosylcytosine in tissues of man and mouse, *Cancer Res.* **33,** 2816 (1973).
16. J. B. Hart, D. H. Ho, S. L. George, P. Salem, J. A. Gottlieb and E. Frei, Cytokinetic and molecular pharmacology studies of arabinosylcytosine in metastatic melanoma, *Cancer Res.* **32,** 2711 (1972).
17. S. C. Barranco, D. H. W. Ho, B. Drewinko, M. M. Romsdahl and R. M. Humphrey, Differential sensitivities of human melanoma cells grown *in vitro* to arabinosylcytosine, *Cancer Res.* **32,** 2733 (1972).

Europ. J. Cancer Vol. 12, pp. 575–579. Pergamon Press 1976. Printed in Great Britain

Stereological Measurements of Bladder Tumour Morphology*

M. J. FULKER,§ S. J. ADAMTHWAITE† and C. K. ANDERSON‡
†*Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds LS2 9NL, Yorkshire, England*
‡*Department of Urology, The General Infirmary, Leeds, Yorkshire, England*

Abstract—*Stereological and image analyser methods have been used to measure parameters of the subcellular morphology of a series of human bladder tumours and specimens of normal bladder epithelium.*

The interdigitation of the plasma membrane of adjoining cells, the nuclear volume fraction, and the area and shape factors of nuclear profiles in sections for the light microscope and for the electron microscope, have been measured. Some tumours show high values of nuclear profile areas and have less invagination of the nuclear membrane than normal bladder epithelium.

NOMENCLATURE

T	Plasma membrane interdigitation factor.
S_v	Plasma membrane surface density.
$\bar{A}_L$	Mean nuclear profile area for light microscopic sections.
$\bar{P}_L$	Mean nuclear profile perimeter for light microscope sections.
$\left(\overline{\frac{P^2}{A}}\right)_L$	Mean nuclear profile shape factor for light microscope sections
$\bar{A}_E$	Mean nuclear profile area for E.M. sections.
$\bar{P}_E$	Mean nuclear profile perimeter for E.M. Sections.
$\left(\overline{\frac{P^2}{A}}\right)_E$	Mean nuclear profile shape factor for E.M. sections.
$\bar{V}_v$	Nuclear volume fraction = Mean nuclear point count per frame / Mean epithelial cell point count per frame
A_n	Projected nuclear area, exfoliative cytology.
A_c	Projected cell area, exfoliative cytology.
N/C	$= (A_n/A_c)$ nuclear cytoplasm projected area ratios, exfoliative cytology.

Accepted 4 September 1975.

*Supported by the Yorkshire Cancer Research Campaign.

§Present address: British Nuclear Fuels Ltd., Sellafield, Seascale, Cumbria, CA20 1PG England.

INTRODUCTION

IN SEVERAL tissues, [1–3], there is evidence from exfoliative cytology that neoplastic change is accompanied by variation in cell size and shape, nuclear size and shape, and in staining. While differentiation of individual cells and their spatial arrangement can be related to the growth and behaviour of tumours, no single parameter can provide a complete morphological and functional description of a tissue. The purpose of the present work was to provide stereological data for a series of human bladder tumours and normal bladder epithelium, and to examine possible relationships between subcellular morphology and the overall differentiation of a tissue. The size and shape of nuclear profiles in sections for the light microscope and for the transmission electron microscope, and the nuclear volume fractions are compared with the pathologist's grading of differentiation using conventional histological techniques. In addition, for a subgroup of specimens, the plasma membrane interdigitation of adjoining cells has been measured, because earlier studies [4, 5] suggested a possible relationship between interdigitation and differentiation.

EXPERIMENTAL PROCEDURE

Tumour specimens were obtained by transurethral endoscopic resection using isotonic glycine as the bladder irrigating fluid. Bladder epithelium was taken from non-tumour bearing areas of patients with bladder cancer or at the time of bladder surgery for non-malignant disease. Tissue was dissected into 1 mm cubes, avoiding damaged areas, and fixed for 4 hr in 2% glutaraldehyde in sodium cacodylate buffer, tonicity 360 m osmoles kg^{-1}. Tissue blocks were washed overnight in sodium cacodylate buffer with added sucrose, and post-fixed in 1% osmium tetroxide also in sodium cacodylate buffer. After dehydration in graded alcohols the blocks were embedded in Araldite and thin sections cut from at least five blocks of each specimen were mounted on "200" size copper grids (hole width 94 μm) and stained with lead tartrate.

The bladder specimens were graded using H & E stained sections, on the scale: 0 normal, 1 well differentiated, 2 intermediate differentiation, 3 poorly differentiated.

MEASUREMENTS

The interdigitation factor T is a measure of the interlocking of the plasma membrane of adjoining cells, and is defined by: $T = B/(A+B)$ where A and B are the number of intersections of the test system with plasma membranes which are: (i) type A, non-folded, (ii) type B, folded. The measurement of A and B using a specimen scanner on the electron microscope has been described in a previous paper [6]. For each specimen sufficient measurements were made from 4 or 5 thin sections to reduce the coefficient of variation of T to about 10%.

For the analysis of nuclear profiles, a systematic set of 30–55 electron micrographs on 35 mm film (Kodak fine grain positive) were obtained from each specimen. Contact positives made on Kodalith sheet film, developed in Kodak DG 10, were projected at a final magnification of 5933 and the epithelial nuclei traced in ink. The area and perimeter of each tracing was measured on a Quantimet 720 fitted with a shading corrector and MS3 computer module, the data being recorded on punched tape. Nuclear tracings were also made from semi-thin (1 μm) sections, stained with toluidine blue, projected by a Zeiss Microprojector at a final magnification of 1300. The area and perimeter of these tracings were measured on the Quantimet using the light pen to detect individual nuclear profiles.

Both sets of data were analysed on an I.C.L. 1906A computer to give the mean areas $\bar{A}_L$ and $\bar{A}_E$, and the shape factors $\overline{(P^2/A)}_L$ and $\overline{(P^2/A)}_E$.

The mean shape factor of the nuclear profiles $\overline{(P^2/A)}$ is independent of profile area and is related to mean perimeter ratio $F_{3\,3} = \overline{(L_P/L_{eP})}$ devised by Fischmeister [7] where L_P is the actual perimeter of the profile, equal to P in this case, and L_{eP} is the perimeter of the equivalent circle (the circle with the same area).

The nuclear volume fraction $\bar{V}_v$, the fraction of epithelial tissue occupied by the nucleus, was measured on toluidine blue stained 1 μm sections by point counting 40–50 fields on a 100 point eye piece grid at a magnification of 2000 using phase contrast, and the mean value $\bar{V}_v$ calculated from the ratio of the mean nuclear count per field to the mean epithelial cell count.

RESULTS

The plasma membrane interdigitation coefficient, T, was determined for 9 specimens. In other specimens the level of intercellular oedema was too great to allow meaningful measurement of interdigitation. The measurement of T, shown in Table 1, did not show significant correlation with grade of differentiation.

Table 1. *Plasma membrane interdigition coefficient* T *and specimen grade*

Specimen	Grade	T (Mean value)	T range (±2 S.E.)
1	0	0·61	0·59–0·63
2	1	0·31	0·29–0·33
3	1	0·37	0·33–0·41
4	1	0·48	0·46–0·50
5	2	0·50	0·48–0·52
6	2	0·28	0·25–0·31
7	2	0·28	0·25–0·31
8	3	0·53	0·50–0·56
9	3	0·33	0·32–0·34

Pearson correlation coefficient of T with grade—0·30, significance $< 0{\cdot}44$.

Table 2 shows for 20 specimens the values of the areas $\bar{A}_L$ and $\bar{A}_E$, the shape factors $\overline{(P^2/A)}_L$ and $\overline{(P^2/A)}_E$, the nuclear volume fraction $\bar{V}_v$ and the ratio $[\bar{A}_L/\overline{(P^2/A)}_E]$. The mean profile area of the light microscope nuclear tracings, $\bar{A}_L$ plotted against grade of differentiation is shown in Figure 1.

Table 2. Nuclear profile parameters and specimen grade

Specimen	Grade	$\bar{A}_L$ (μm^2)	$\bar{A}_E$ (μm^2)	$\left(\overline{\frac{P^2}{A}}\right)_L$	$\left(\overline{\frac{P^2}{A}}\right)_E$	$\bar{V}_v$	$\frac{\bar{A}_L}{\left(\overline{\frac{P^2}{A}}\right)_E}$
1	0	52·7	22·0	13·2	30·5	0·21	1·73
10	0	35·0	11·9	19·9	23·5	0·22	1·49
11	0	21·9	5·47	20·9	23·1	0·21	0·95
2	1	34·2	17·4	17·2	18·9	0·24	1·81
3	1	40·4	26·2	19·0	21·1	0·24	1·91
4	1	32·8	23·8	18·1	16·1	0·18	2·04
12	1	36·9	18·8	18·5	20·0	0·19	1·85
17	1	50·3	24·2	17·1	19·5	0·23	2·58
19	1	49·7	27·2	17·5	17·9	0·21	2·78
5	2	31·2	16·5	13·6	17·1	0·22	1·82
6	2	35·8	27·1	17·5	16·1	0·21	2·33
7	2	58·0	35·0	17·6	22·2	0·23	2·61
13	2	36·4	20·0	19·3	27·3	0·15	1·33
15	2	42·2	23·1	16·9	19·3	0·25	2·19
8	3	53·1	34·8	16·9	18·9	0·25	2·81
9	3	64·5	44·4	18·1	21·1	0·33	3·06
14	3	51·2	27·0	18·0	18·9	0·20	2·70
16	3	59·6	33·0	16·3	18·0	0·20	3·30
18	3	57·2	31·5	18·5	19·8	0·17	2·89
20	3	69·3	41·3	17·0	23·3	0·28	2·93
Pearson correlation coefficient with grade		0·63	0·76	−0·14	0·32	0·31	0·75
Significance		0·003	0·001	0·55	0·16	0·19	0·001

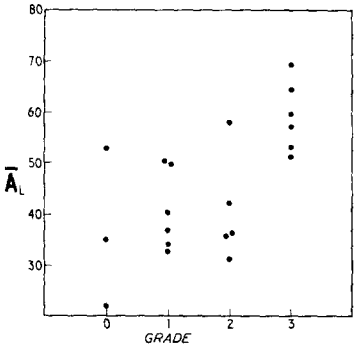


Fig. 1. Variation of mean nuclear profile area $\bar{A}_L$ (in μm^2) with grade.

The mean values of the electron microscope nuclear profiles $\bar{A}_E$ are smaller than the corresponding $\bar{A}_L$ values because the E.M. sections, being thinner, intersect more small profiles. Neither of these profile area measurements provides a significant correlation with specimen differentiation.

The light microscope nuclear shape factor $\overline{(P^2/A)}_L$ shows a wide spread of values for each grade. The E.M. shape factor $\overline{(P^2/A)}_E$, which takes into account small invaginations of the nuclear membrane not resolvable by the light microscope, shows high values for the three normal specimens confirming the impression that normal bladder epithelium has highly invaginated nuclei.

The nuclear volume fraction $\bar{V}_v$ results shows high $\bar{V}_v$ values for some, but not all, of the grade 3 tumours, with an increasing spread as the grade increases.

The ratio $[\bar{A}_L/\overline{(P^2/A)}_E]$ (Fig. 2) shows values which increase with tumour grade, and the Pearson correlation coefficient ($r = 0{\cdot}75$, $\alpha < 0{\cdot}001$) shows better correlation with grade than either $\bar{A}_L$ or $\overline{(P^2/A)}_E$ alone.

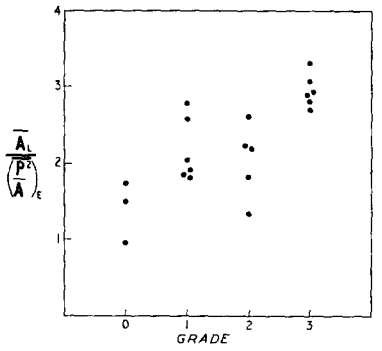


Fig. 2. Variation of $\frac{\bar{A}_L}{\left(\overline{\frac{P^2}{A}}\right)_E}$ with grade.

DISCUSSION

There have been many quantitative studies of whole cells from the human cervix. Cancer and dysplasia were found by Reagan, Hamonic and Wentz [8] to be often associated with a decrease in cell area A_c, an increase in nuclear area A_n and an increase in the nuclear/cytoplasmic ratio N/C. Tolles and Bostrom [9] also found an increase in N/C. McMaster [10] measured six nuclear parameters and combined them in an optimised discriminant function for normal and malignant cells. Some malignant cells showed increased A_n as well as slight increases in the irregularity and elongation of the projected nuclear outline.

The nuclear diameters of whole cell preparations of bladder tumours have been compared with DNA measurements by Levi *et al.* [11], where it was found that different tumours having similar DNA contents showed a considerable range in nuclear diameters.

Sections of human cervix were used by Wiernik *et al.* [12], who found that profile areas, $\bar{A}_L$, for 4 μm sections were larger for many tumour specimens than for normal cervix. The plasma membrane surface density S_{Vi}, the surface area of plasma membrane per unit tissue volume, was decreased in some tumours, although there was considerable overlap between the distribution of S_{Vi} for normal and tumour, as in the case of the plasma membrane parameter T in the present work.

These results for cervix and bladder suggest that the surface volume ratio of individual cells, whether tumour or normal, can vary widely, and perhaps change rapidly in response to stimuli.

The increase in the mean light microscope profile area $\bar{A}_L$ for high grade tumours (Fig. 1) is in general agreement with the cytological finding that cancer cell nuclei occupy a greater volume, and have larger projected area, A_n, than their benign counterparts. Since the average diameter of the light microscope nuclear profiles is large compared with the 1 μm thickness of the sections, many of the profile areas $\bar{A}_L$ will be smaller than the corresponding average nuclear area A_n of whole nuclei.

Our results suggest that grade 3 tumours have larger nuclear profiles $\bar{A}_L$ than normal or well differentiated (grade 1) tumours and some of the grade 3 tumours also show higher values of nuclear volume fraction $\bar{V}_v$ than normal epithelium, these two findings for bladder epithelium are consistent with increase in A_n, N/C and $\bar{A}_L$ found by other workers for tumours of the cervix.

The nuclear shape factors $\overline{(P^2/A)}_L$ and $\overline{(P^2/A)}_E$ for bladder are not in accord with the increased nuclear irregularity and elongation found by McMaster [10] in malignant cervical cells, and our work does not provide any basis for the use of increased nuclear irregularity as a criterion for bladder neoplasia, but suggests that the less differentiated tumours have nuclei closer to a spherical shape, with less invagination of the nuclear membrane. It is unlikely that stereological measurements on tissue sections will lead to significant improvements in diagnostic pathology, nevertheless it is important, for the better understanding of the morphological and functional changes in neoplasia, that stereological methods be used to evaluate the, often conflicting, subjective impressions.

Acknowledgements—The authors thank the Yorkshire Cancer Research Campaign for financial support, Dr. T. E. Kenny for computer programming, Mr. R. E. Williams, Leeds General Infirmary, for clinical specimens, and Professor A. Williams of the Department of Fuel and Combustion Science for use of the Quantimet Image Analyser.

REFERENCES

1. L. G. Koss, *Diagnostic Cytology*. Lippincott, Philadelphia (1968).
2. J. R. Meyer-Arendt and D. M. Humphreys, Quantitative morphology of cancer cells. *Acta Histochem.* **44,** 41 (1972).
3. M. R. Melamed, Diagnostic cytology of urinary tract carcinoma. *Europ. J. Cancer* **8,** 287 (1972).
4. M. J. Fulker, E. H. Cooper and T. Tanaka, Proliferation and ultrastructure of papillary transitional cell carcinoma of the human bladder. *Cancer (Philad.)* **27,** 71 (1971).
5. G. B. Dermer and W. H. Kern, Changes in the affinity of phosphotungstic acid and positively charged colloidal particles for the surface of malignant human transitional epithelium of the urinary bladder. *Cancer Res.* **34,** 2011 (1974).

6. M. J. FULKER, A specimen scanner for stereology with the electron microscope. *J. Micros.* **98,** 57 (1973).
7. H. F. FISCHMEISTER, Shape factors in quantitative microscopy. *Z. für Metallkunde* **65,** 558 (1974).
8. J. W. REAGAN, M. J. HAMONIC and W. B. WENTZ, Analytical study of the cells in cervical squamous-cell cancer. *Lab. Invest.* **6,** 241 (1957).
9. W. E. TOLLES and R. C. BOSTROM, Automatic screening of cytological smears for cancer: the instrumentations. *Ann. N.Y. Acad. Sci.* **63,** 1211 (1956).
10. G. W. MCMASTER, A measurement of the pattern of normal and malignant cervical cells. *Acta cytol. (Philad.)* **12,** 9 (1968).
11. P. E. LEVI, E. H. COOPER, C. K. ANDERSON and R. E. WILLIAMS, Analysis of DNA content, nuclear size and cell proliferation of transitional cell carcinoma in man. *Cancer (Philad.)* **23,** 1074 (1969).
12. R. WIERNIK, S. BRADBURY, M. PLANT, R. H. COWDELL and E. A. WILLIAMS, A quantitative comparison between normal and carcinomatous squamous epithelia of the uterine cervix. *Brit. J. Cancer* **28,** 488 (1973).

Europ. J. Cancer Vol. 12, pp. 581–582. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Cellular "Modulation" in Breast Tumour Cultures

N. P. BISHUN, D. C. WILLIAMS and J. REED
Research Department, Marie Curie Memorial Foundation, The Chart, Oxted, Surrey, England

CARCINOMA of the breast is the most widespread malignancy in the female and may account for as much as 25% of total female cancer. Attempts to check the spread of these tumours have been numerous, and several new techniques have been developed in order to assess methods whereby these cancers can be treated.

One of these developments has been the advent of tissue culture methods which have helped enormously to elucidate the mechanism of biological processes at the cellular level. With tissue culture, however, the basic problem lies in establishing reliable techniques for growing tumour cells and maintaining them in a functional state during their life span in culture.

In our initial attempts to establish permanent cell lines from carcinoma of human breast, a variety of culture methods and media were tried. These included explant and trypsinisation methods with various added constituents in the media [1].

As reported by us in a previous communication [1], enzymatic digestion of the fragments proved to be unsatisfactory and had to be discarded. We have persevered with the explant method from which both fibroblastic and epithelial like cells proliferated. Culture methods have been reported previously [2], but basically the medium consists of M.E.M. (Minimum Essential Medium) plus 10% fetal calf serum with added antibiotics, penicillin and streptomycin (50 μg/ml) with 5 μg/ml of 17-β-estradiol.

To date, we have cultured over 100 tumours from human breasts and, the object of this communication, is to report recent observations that certain cultures alter their fibroblastic morphology to a considerable extent (see figures).

About 40% of our cultures produce fibroblastic like cell growth and about the same percentage, epithelial like cells. We have been able to culture both types of cells for up to 9 months although they are predominantly slow growing.

In about 5% of our cultures, however, certain peculiarities have been observed in which normal fibroblastic growth was apparent at the onset (Fig. 1). In these cultures, after about a week, the fibroblasts became very flattened (Fig. 2), granular, tended to lose contact inhibition, and grow in a criss cross fashion. We ascertained that these cells have now become malignant in view of their clonal appearance, multilayeredness, high nuclear cytoplasmic ratios and high refringence of the cytoplasm. These are all features which we have previously described as representing a cancerous cell [2]. Investigations into tumour cell markers have been carried out to a limited extent. All tumours are assessed for oestrogen receptors before being set up in culture, but we have not found any correlation between the presence or absence of receptors and the growth response in culture. We have not examined the cells for other markers.

After about 2 weeks in culture, the cells have completely lost their fibroblastic appearance (Fig. 3), tend to pile up on each other, have an epithelioid appearance with large distinct nuclei and the formation of intercellular matrix. Within 9 months the cells assume a distinct epithelioid appearance and form colonies. They form in clumps, are slow growing and have a life span of about a further 3 months. Since this change in morphology of the cultures has only been observed in a small minority of our breast tumours it has been ruled out as being due to any additives or culture methods, since all our cultures are treated identically. Cross contamination by

Accepted 5 February 1976.

other cell lines can be ruled out as other human cell lines were not being cultured at the time of these observations. We have eliminated contamination by epithelial cells from the original tumour since, in our experience, all the cultures containing a mixed fibroblast and epithelial cells make up, eventually resort to a fibroblastic one. The fibroblast cells tend to overgrow and select out the epithelial ones. It is our contention that had there been epithelial cells at the commencement of the cultures they would be overgrown, and not overgrow the fibroblastic type.

Two possible explanations are offered for these observations. The first being that a virus, which we have been unable to detect, being responsible for the transformation from the fibroblastic to epithelioid state. However, if this were the case, one would have expected more of our cultures to show this phenomenon and the process to have been more prolonged. The second explanation, which seems more probable, is that the cells undergo differentiation in culture and become transformed into the epithelial cells. However, both these speculations would be difficult to substantiate although long term cultures have been established and it is hoped that more extensive studies will elucidate the problem.

In view of the enormous amount of work being undertaken in characterising breast cancer cells in culture, we consider this unusual observation in culture to be of sufficient scientific interest to warrant this report.

REFERENCES

1. N. Bishun, J. Mills and D. C. Williams, Culturing human breast tumours. *Cytologia* **38,** 651 (1973).
2. N. Bishun, J. Mills and D. C. Williams, A tissue culture device for culturing breast tumours and gynaecological tissues. *Cytologia* **40,** 91 (1975).

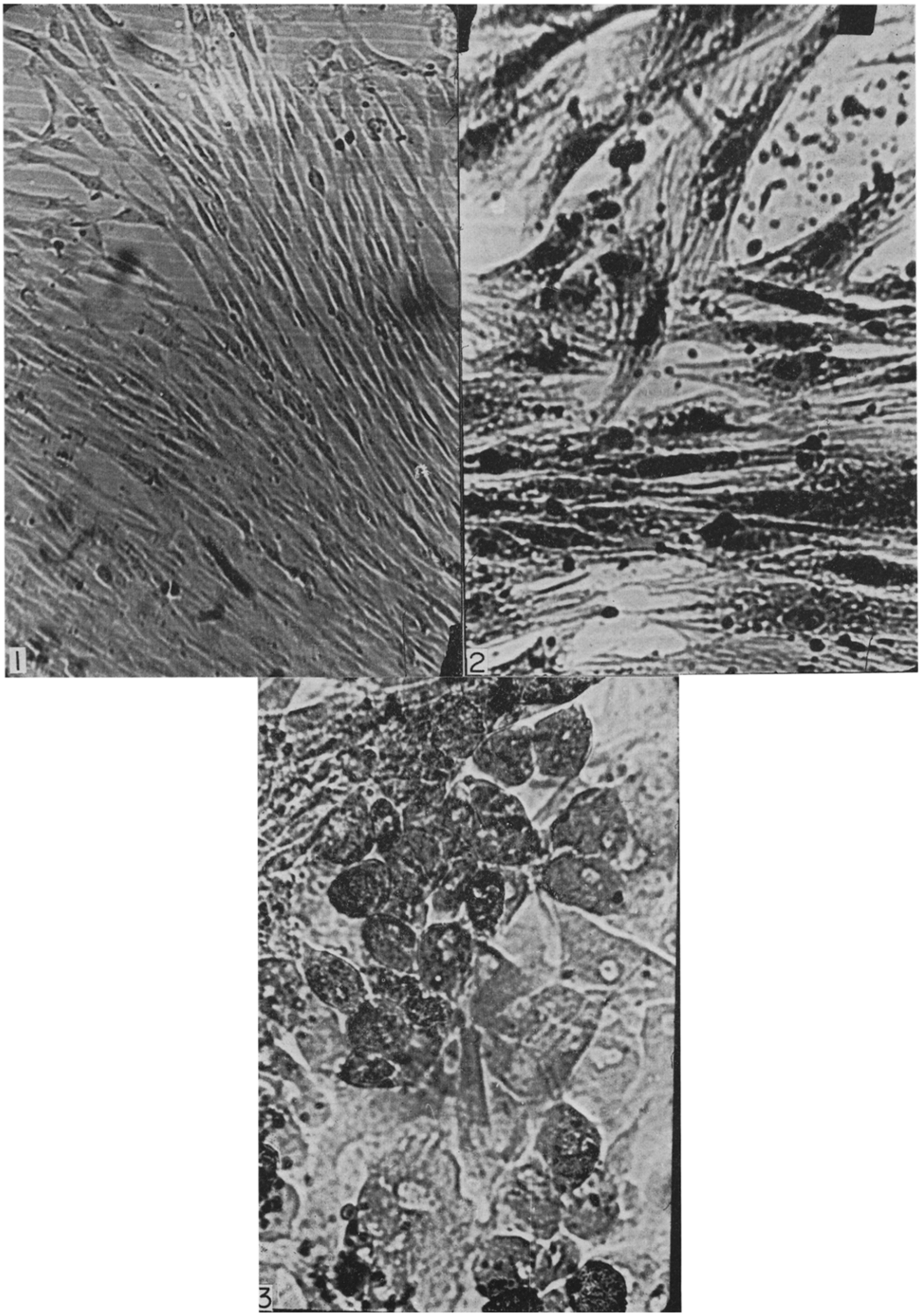


Fig. 1. *Normal fibroblastic growth appearing from primary explant: they show monolayer characteristics, contact inhibition, and no contamination with other cell types.*

Fig. 2. *Change in morphology of fibroblasts showing granular and criss cross appearance. Nuclear material is dark staining and cytoplasmic bridges are apparent. In certain areas of these cultures criss crossing is apparent.*

Fig. 3. *Multilayered, epithelioid appearance of apparently granular cells showing a high nuclear cytoplasmic ratio, very little cytoplasm which is birefringent, clumping of cells and the apparent formation of "mounds".*

(to face p. 582)

Europ. J. Cancer Vol. 12, p. 583. Pergamon Press 1976. Printed in Great Britain

Announcement

EUROPEAN ORGANIZATION FOR RESEARCH ON TREATMENT OF CANCER
(E.O.R.T.C.)

SYMPOSIUM ON RECENT ADVANCES IN CANCER TREATMENT

at the Institut Jules Bordet, Brussels
September 23–24, 1976

With the participation of:

P. ALEXANIAN (Houston, U.S.A.), G. BONADONNA (Milano, Italy), J.H. BURCHENAL (New York, U.S.A.), V.T. DEVITA (Bethesda, (U.S.A.), I. DJERASSI (Darby, U.S.A.), G.H. FRIEDELL (Worcester, U.S.A.), S. GARATTINI (Milano, Italy), A. GOLDIN (Bethesda, U.S.A.), J.C. HEUSON (Bruxelles, Belgium), N. JAFFE (Boston, U.S.A.), Y. KENIS (Bruxelles, Belgium), J. KLASTERSKY (Bruxelles, Belgium), G. MATHE (Paris, France), C.G. MOERTEL (Rochester, U.S.A.), F.M. MUGGIA (Bethesda, U.S.A.), H.M. PINEDO (Utrecht, The Netherlands), M. STAQUET (Bruxelles, Belgium), P. STRYCKMANS (Bruxelles, Belgium), H.J. TAGNON (Bruxelles, Belgium), M.H.N. TATTERSALL (London, Great Britain), L.M. VAN PUTTEN (Rijswijk, The Netherlands), M.D. WALKER (Baltimore, U.S.A.), R.C. YOUNG (Bethesda, (U.S.A.)

Number of Participants is Limited to 200.

For details and registration, write to Dr. M. STAQUET, EORTC Coordinating Office, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Bruxelles, Belgium.

The Symposium will be held 2, rue Evers, 1000 Bruxelles (1st floor).

Registration fee: 2000 Belgian francs (to be paid in advance)

Europ. J. Cancer Vol. 12, p. 584. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

British Journal of Cancer — *July, 1976*

J. A. V. Pritchard, J. L. Moore, W. H. Sutherland and C. A. F. Joslin: The clinical assessment of the MOD–MEM cancer test, using controls hospitalised with non-malignant diseases.

A. Shaw, G. Ettin and T. A. McPherson: The macrophage electrophoretic mobility (MEM) test: Positive responses in cancer patients to basic proteins not just a function of protein charge.

B. M. Jones: Mechanisms of leukocyte migration inhibition by breast tumour cell fractions.

Suzanne A. Eccles, B. Bandlow and P. Alexander: Monocytosis associated with the growth of transplanted syngeneic rat sarcomata and its relation to the immunogenicity of the tumour.

R. D. Barnes, Maureen Tuffrey, Jean Holliday, J. H. M. Hilgers and T. Souissi: Murine leukaemia virus group specific antigen in tumour resistant tetraparental AKR ↔ CBA/H-T6 chimaeras.

R. D. Barnes, Maureen Tuffrey, E. J. Wills, G. Mahouy and Jacqueline Lasneret: The innate resistance of CBA mice to endogenous murine leukaemia virus infection.

V. Doreen Courtenay: An *in vitro* colony assay in soft agar for the Lewis lung tumour and the B16 melanoma taken directly from the mouse.

W. L. Wiseman, J. S. Senn, R. G. Miller and G. B. Price: Stem cell characterization of neutropenia: Velocity sedimentation and mass culture analysis.

B. J. Smith and D. Kundu: A case of acquired resistance to daunorubicin and cytosine arabinoside in a patient with acute myelogenous leukaemia.

S. Christakos, D. Sinha and T. L. Dao: Effects of neonatal modification of endocrine functions on mammary carcinogenesis in the remale rat.

D. S. Alberts and T. van Daalen Wetters: Rubidazone vs Adriamycin: An evaluation of their differential toxicity in the spleen colony assay system.

I. Leck, Jillian N. Birch, H. B. Marsden and J. K. Steward: Methods of classifying and ascertaining children's tumours.

D. Trichopoulos, G. Papaevangelou, M. Violaka, C. Vissoulis, L. Sparros and O. Manoussos: Geographic correlation between mortality from primary hepatic carcinoma and prevalence of hepatitis B surface antigen in Greece.

Brief Communications

K. Kudo, Y. Hanaoka and H. Hayashi: Purification of a tumour cell aggregation promoting factor associated with rat ascites hepatoma cell surface.

Bela Toth and D. Nagel: Tumour induction study with allyhydfazine hydrochloride in Swiss mice.

B. M. Jones, C. E. Connolly, P. Isaacson, D. T. L. Turner and A. R. Turnbull: Horizontal studies of tumour directed leucocyte migration in patients with operable mammary carcinoma. Additional clinical correlations.

Europ. J. Cancer Vol. 12, pp. 585–594. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

The Biology and Treatment of Malignant Melanoma

ALISTAIR J. COCHRAN
The University of Glasgow Department of Pathology, The Western Infirmary, Glasgow G11 6NT, Great Britain.

SINCE ITS recognition as a clinical and pathological entity in the early 19th century, malignant melanoma has commanded interest disproportionate to its relatively low frequency. Clinical concern and unflagging enthusiasm among biologists for pigment cell research have ensured a massive literature on pigment cell-related topics, a series of highly successful International Pigment Cell Congresses, a new Journal, Pigment Cell, and current moves to establish a multidisciplinary International Pigment Cell Society. This paper summarizes some pertinent findings from recent studies of clinical, pathological and biological aspects of the melanocytic tumours.

Melanoma has a particularly sinister reputation which is entirely justified in individual cases, but is excessive if one compares the fate of melanoma patients as a whole with that of patients with, for instance, bronchogenic carcinoma or gastric carcinoma.

The need for an understanding of the mechanisms of malignant transformation of melanocytes, the factors involved in metastasis formation and the development of improved treatment is underlined by a steady increase in the frequency of melanoma and of deaths from it. Reports of an increasing incidence come from areas as diverse as Norway [1], Australia [2], the United States of America, and England and Wales [3]. There is some increase in all age groups, but adults under 65 seem maximally affected and the increase involves men and women equally. The disease has tended to increase most in areas of affluence and in higher socio-economic groups.

The reasons for this increase are complex but various factors may have an aetiological role. Sunlight-derived radiant energy has been much discussed. Black people seldom develop melanoma, the incidence in U.S. blacks being around 5 per 100,000. Conversely albinos and individuals of Northern European stock, who tan poorly or freckle, seem specially susceptible. The maximum incidence is seen in those of Northern European stock who live in sunny areas such as Australia and South Africa, the peak reported incidence in this group being 265 per 100,000 in Queensland. The frequency of melanoma and death rate from it increase progressively as the equator is approached, both in Australia [4, 5] and in the U.S. [6]. Involvement of solar radiation is further suggested by the observed differences of primary melanomas of different sites in men and women, which correlate with dress habits. The high frequency of primaries on the lower leg in women, for example, fits with the exposure of the leg associated with the wearing of skirts.

The situation is complicated by the observation that unlike other skin cancers, the increased frequency of melanoma in Australia involves all areas of the body, not only those exposed to sunlight. Lee and Merrill [7], therefore, postulate that sun-damaged melanocytes may release materials (solar circulating factor) which circulate and may cause malignant transformation of melanocytes in areas remote from those exposed to the sun. There is as yet no evidence to support (or refute) this interesting speculation. A simpler possibility is that light clothing may not adequately screen out carcinogenic rays. This certainly appears to be the case with stockings and tights. Another paradox is that many melanoma patients do not have especially "outdoor" occupations and analysis of leisure activities

has so far failed to show a totally convincing correlation with melanoma incidence. It is possible that acute exposure of the skin, especially that which tans poorly, by injudicious sunbathing is more dangerous than chronic exposure of conditioned skin which tans effectively [8].

The role of physical trauma in melanoma remains enigmatic. Specific traumatic events recorded by a minority of patients are probably examples of "traumatic determinism". However melanomas of the sole of the foot do occur frequently in populations who go barefooted [9].

Endocrine factors may also be important. Melanoma is more common in women of child-bearing age than in men of comparable age. However, in such women the disease appears somewhat less aggressive. The view that melanoma is more common during pregnancy has not been substantiated by recent studies, but in individual cases pregnancy is associated with an increased rate of tumour growth and extension. It seems likely that endocrine factors are not directly carcinogenic, but act as cofactors. Endocrine contraceptive agents may well influence melanocytes, and definitive studies of these materials in melanocyte biology and their effect on the incidence of melanomas are desirable. Recent reports of therapeutic successes with hormones are of interest, and it seems that this approach is due for re-examination [10].

The role of viruses in human carcinogenesis is under detailed scrutiny and studies of this possibility in melanoma have been reported. Birkmayer *et al.* [11] found viral particles in human melanomas which share characteristics with the oncorna viruses of animal melanomas. These workers also identified reverse transcriptase and 70S RNA in virus containing human melanomas, characteristics of oncogenic RNA viruses in more defined animal situations. They also reported positive nucleic acid hybridization studies with virus-specific DNA and material from human melanomas, but not with other skin cancers. These exciting findings require confirmation and even if this is forthcoming the problem of proving a causal association remains. However reports of similar antigens on different melanomas (see below) do reflect the situation in animal tumours induced by viruses.

Genetic factors are involved. I have discussed the susceptibility of certain ethnic groups to melanoma, and the persistence of high melanoma incidence in the descendents of immigrants from Northern Europe to Australia indicates that melanoma susceptibility is heritable. Additionally there are families in which melanomas are specially frequent, develop relatively early in life and may be multiple. In a study in Queensland the risk of a first degree relative of a melanoma patient developing this tumour was roughly twice that of the general population. However, these individuals also have an increased likelihood of developing cancers other than melanoma. The nature of the genetic defect remains unknown. Wallace and Exton [12] merely state that it is polygenic, but Sutherland *et al.* [13] interpret their initial data on familial melanoma as in keeping with the effects of an autosomal gene with incomplete penetrance.

Diagnosis remains a problem. In contrast with other skin malignancies, which are often suspected to be malignant and assigned to the correct diagnostic category, melanoma is correctly diagnosed in less than 50% of cases. This is no doubt due to a low frequency of heavily melanized melanomas and the absence of clinically detectable pigment in some (although less than 1% are amelanotic on cytochemical examination). Accuracy of diagnosis depends on a willingness to consider melanoma in the differential diagnosis of even totally non-pigmented skin lesions, a careful clinical history with emphasis on the nature and colour of any lesion which previously existed at the tumour site and careful examination of the tumour and the skin around it for any alterations of colour and not just for brown or black pigmentation. This is highlighted by Mihm *et al.* [14] in their excellent diagnostic atlas of melanoma and by the WHO pamphlet, "Clinical Diagnosis of Malignant Melanoma" [15]. In cases where, despite careful inspection, the diagnosis remains in doubt, magnification by a hand lens or operating microscope [16] may be of assistance.

Careful clinical examination and history taking will indicate the diagnosis in all but a few melanomas, and any doubts are settled by histological examination of biopsy. The biopsy should, whenever possible, be an excision biopsy, but there is little convincing evidence that incision biopsy is harmful, if followed promptly by excisional surgery. Ingenious non-operative aids to diagnosis have been introduced and in the hands of enthusiasts have had some success. These include the uptake of ^{32}P [17 18], thermography [19] and the uptake of radio-labelled quinolines with affinity for melanin [20]. In the face of the diagnostic efficiency and simplicity of careful clinical observation, and the accessibility of this tumour

for biopsy, it is difficult to see the place of such technically complex procedures in melanoma diagnosis.

Diagnosis by estimation of tumour cell products has attracted interest recently. Estimation of urinary melanogens and dopa oxidase or tyrosinase in the blood have insufficient specificity to be really useful, especially in patients with early tumours. A promising approach is the estimation of 5-S-cysteinyldopa, a catechol precursor of phaeomelanin, in the urine [21]. This is excreted by normal individuals, but levels are raised in the urine of melanoma patients. Interpretational problems exist, the level of excretion being raised, for example, after sunbathing, but further study may well indicate a role for estimation of 5-S-cysteinyldopa, at least as a monitor for recurrent melanoma.

Hopes for immunodiagnosis of this and other tumours have not yet been realized. However, studies of immunological aspects of melanoma have yielded much of interest. In 1967 Lewis [22] found that serum from patients with slowly evolving melanomas killed autologous tumour cells *in vitro*, an effect not seen with serum from patients with rapidly progressing melanomas.

Apparently melanoma-specific antibodies were detected by Morton *et al.* [23], Lewis *et al.* [24], Muna *et al.* [25] and Romsdahl and Cox [26] and subsequently by numerous other authors. These were directed against antigens in two sites: the cytoplasm and the cell membrane. There is agreement that antibodies to cytoplasmic components cross-react with allogeneic melanomas, however there is less unanimity about the specificity of membrane-reactive antibodies. Lewis *et al.* [24], and more recently Bodurtha *et al.* [27], claim that the antibodies to the membrane antigens are individual-specific while others find these too cross-reactive. This disagreement remains to be explained, but is at least partly due to differences in technique. A nucleolar antigen has also been described [28] against which a proportion of patients develop antibodies. The possession of this nucleolar antigen indicates a poor prognosis. Melanoma cell killing and cytostasis by specifically sensitized lymphocytes and non-sensitized lymphocytes and macrophages have been demonstrated *in vitro*, and such cells seem likely to be active against tumour cells *in vivo*. Specifically sensitized lymphocytes have been demonstrated by *in vitro* cytotoxicity [29, 30], tumour-antigen mediated transformation [31, 32] and tumour-antigen mediated inhibition of leucocyte migration [33, 34]. Sensitization of a proportion of melanoma patients to melanoma-derived materials has also been demonstrated by skin reactivity studies [35–37]. The exact specificity of these reactions is as yet unclear and it is possible that some may be reactions against foetal antigens rather than tumour-associated antigens with a rejection potential. Studies of lymphocytes subpopulations in melanoma patients [38] have been reported and indicate variations in, for instance, T cells capable of forming rosettes with sheep erythrocytes under suboptimal conditions [39].

Tumour-directed antibodies and lymphoid cells have been most readily detected by most workers in early malignancy when the tumour volume is small but even in patients with advanced disease autoimmunisation with tumour cells will cause the transient reappearance of tumour-directed antibodies [40] and lymphoid cells [41]. The mechanisms for production of tumour immunity thus remain intact, though eclipsed, in advanced disease. In the case of tumour-directed antibodies Lewis *et al.* [42] advance the interesting suggestion that anti-idiotypic antibodies are produced in advanced melanoma which react with tumour-directed antibodies and neutralize them.

With mounting evidence of immune reactions to melanoma-associated antigens the question has to be asked whether such reactions influence tumour progression or regression or are merely epiphenomena. There is as yet no clear answer to this. Indices of anti-tumour reactivity decline with advancing disease, but whether this is the cause of tumour progression or secondary to it remains unknown. Serial studies of patients may shed light on this problem, but although loss of tumour-immune reactivity may precede and indeed predict clinically apparent metastases in some patients the findings are not sufficiently clear cut to allow their routine clinical use. The situation is complicated by the observation that the longer a patient remains tumour-free after removal of a melanoma the less likely is it that tumour-directed reactivity will be detectable [43].

There is also much interest in means whereby tumour cells may evade cytotoxic immune mechanisms. Most studies have concerned themselves with "blocking" mechanisms. It was initially thought that non-cytotoxic tumour-directed antibodies might coat tumour cells and exclude cytotoxic lymphocytes and possibly cytotoxic non-lymphocytic mononuclear cells (Fig. 1-A). There followed a period in which

the role of antibodies was discounted in favour of the involvement of antigen–antibody complexes and free tumour antigen. Interest in tumour directed antibodies has been restimulated by the observations of Keisari and Witz [44] that lysosomal enzymes of tumour cells can cleave tumour directed antibodies leaving Fab fragments, which would not be detectable by most anti-Ig sera. However this may be, the majority of workers believe that complexes of tumour-derived antigen and tumour-directed antibody impede effector cells in a manner which varies with the proportions of antigen

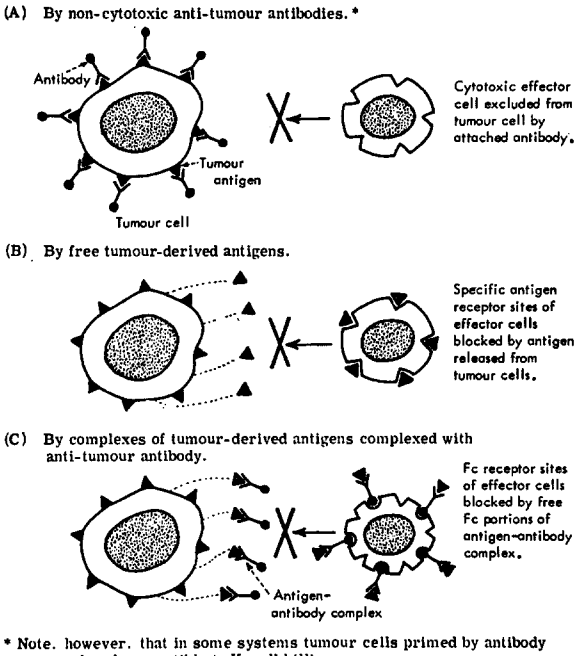


Fig. 1. *Possible mechanisms whereby effector cells may be denied access to tumour cells, or rendered inactive.*

and antibody present (Fig. 1-C). Currie *et al.* [45, 46] present strong evidence for their hypothesis that free tumour-derived antigens may also react with potential effector cells (Fig. 1-B) and this hypothesis is made the more attractive by their report [46] that tumour cells which readily and continually shed their antigens are those which are weakly immunogenic and are more likely to metastasize than those with more stable membrane antigens.

Most studies of host response to tumours have concerned the role of the lymphocyte. However, attention is increasingly directed to the activities of macrophages and certainly tumours with a high content of macrophages are those which are relatively highly immunogenic and exhibit a lower tendency to metastasize [47]. The intimate relationship between lymphocytes and macrophages is indicated by the finding that macrophage accumulation depends upon an intact T-cell system. Gauci [48] has shown that the macrophage content of early melanomas is higher than that in metastases. It is also possible that some of the cells which kill antibody-coated target cells [49] (K cells) are derived from the macrophage series.

One of the earliest findings suggestive of a melanoma-associated immune response was the observation of cellular infiltrates in and around melanomas. These appear to consist, on light microscopy, of lymphocytes, macrophages and occasionally plasma cells. However, Cesarini and Roubin [50] have presented ultrastructural evidence that the great majority of such cells are monocytoid. These infiltrates are present maximally in the early stages of tumour development, decline as the vertically invasive tumour develops and are virtually absent from metastases. The limited studies of such infiltrates have *not* found them strongly cytotoxic to tumour cells and Carr and Underwood [78] thought that they were cells undergoing instruction rather than active effector cells. Unlike gastric cancer [51] and medullary carcinoma of the breast [52] infiltration of a melanoma by mononuclear cells does not, in the view of most authors, presage a good outcome. In a personally studied series, however, local recurrences were less frequent in patients with infiltrated tumours, perhaps indicating enhanced local immunity [53].

Interest in morphological aspects of malignant melanomas has remained high. The work of Clark [54] and McGovern [55] has produced a better understanding of the histogenesis of malignant melanoma. On the basis of the experience of Clark and his associates, Cochran [53], McGovern [55], Little [57] and others, an attempt was made in 1973 during the VIIIth International Pigment Cell Conference in Sydney to produce guidelines for a clinicopathological classification of primary melanomas. The main points recommended for consideration are summarized in the Table. It is hoped that this will be exploited during the next decade when, on the basis of the experience gained, a new and better classification will then be produced. The pathologists of the World Health Organisation Melanoma Reference Centre have also produced a new classification based on their extensive histological material and it will be of interest to see how this compares with the "Sydney" classification. The significance of Mishima's interesting division of melanomas into melanocytic and naevocytic types [56] deserves more attention

than it has yet received. Although there is an extensive and fascinating literature on ultrastructural studies of melanocytes and melanomas the main practical place of electron microscopy remains the identification of melanosomes in doubtful tumours.

Those concerned with the histological diagnosis of pigmented tumours have had much to ponder with renewed interest in the halo naevus, the description of desmoplastic malignant melanoma [58], the separation of the Spitz juvenile melanoma into spindle cell and epithelioid cell naevi, the realization that these lesions occur in adults and the account of "delayed maturation naevi" [59]. There is an increasing, if grudging, acceptance that frozen section diagnosis of pigmented tumours is practical, although the real exponents and proponents of this difficult diagnostic service are, not surprisingly, those who see the greatest number of pigmented tumours [60, 61]. My own very limited experience suggests that the separation of melanocytic from non-melanocytic lesions on frozen sections is practical and worthwhile. For the exact diagnosis of melanocytic lesions I much prefer conventionally fixed and stained sections.

The optimal treatment of malignant melanoma remains debatable. The early and superficial tumours are well treated by surgery with survival rates of between 80 and 90% at 5 years, although it is true that some of the survivors at 5 years will subsequently develop metastases and die. Yet even at this stage there is debate about the margin of excision necessary and the extent to which an "adequate" excision necessitates grafting. The case for elective nodal dissection has weakened over the years and although enthusiasm for this technique remains in some quarters it seems that, in the face of clinically impalpable nodes, most surgeons choose to wait and see. The paper by Conrad [62] for instance, certainly suggests that prophylactic dissection of impalpable nodes was not helpful. It is to be hoped that the major study of this problem by the World Health Organization Melanoma Reference Centre, at present approaching a stage where definitive analysis will be possible, will finally settle this difficult problem. It is, of course, possible that while elective nodal dissection is not the treatment of choice for most patients, it may be applicable to those whose tumours, by virtue of site or pathological type, have a specially unfavourable prognosis.

Treatment by surgery becomes dramatically inadequate in the face of overt node involvement or visceral metastases. In this situation the available alternatives are chemotherapy, radiotherapy, and the recently introduced techniques of immune stimulation.

Chemotherapy has never been satisfactory in melanoma, with most agents achieving, at best, a transient response in 8–10% of patients. The relatively new agent imidazole carboxamide (DTIC) has done better, producing remissions in up to 30% of patients [63], but even this seems little return for therapy which has of itself powerful and undesirable side effects. Combinations of up to five agents have not improved on DTIC alone and newer agents such as dibromodulcitol show no magical specificity in early trials. New and very specific chemotherapeutic agents or striking synergy with immunotherapy are necessary if chemotherapy is to achieve any lasting place in the therapy of melanomas. The technically difficult techniques of regional perfusion have produced some improvement in survival in the hands of experts but seem unlikely to find general application.

Radiotherapy continues to be used in most Western countries primarily in palliation of the advanced patient, a function which it performs well in some cases. However, in occasional situations such as localized inoperable tumours, tumours in patients unfit for surgery or unwilling to accept such treatment and mucosal melanomas radiotherapy may achieve a good if transitory response [64]. Radiotherapy remains relatively more popular as a treatment for melanoma in the Eastern European Countries and, in the hands of experienced therapists, is reportedly effective. We have been impressed by apparent synergy between radiotherapy and non-specific immune stimulation by BCG in a small number of cases and this is worthy of further study. Endolymphatic radiolipiodol has been examined in a number of centres, but has been relatively disappointing.

The demonstration of melanoma associated antigens (MAA) and immune responses to them allows one to ask whether treatment by immunological means, immunotherapy, is possible. Two main approaches exist: *active immunotherapy*, which endeavours to stimulate endogenous anti-tumour reactivity and *passive immunotherapy* which administers anti-tumour factors raised in animals or other humans. Based on the studies of Mathé [65] attempts are in progress to stimulate, in a non-specific way, the patient's immunological system by agents such as Bacille Calmette–Guérin, Corynebacterium parvum, vaccinia virus and Levamisole. Some workers attempt to add an

element of specificity by giving autologous or allogeneic (usually sublethally irradiated) tumour cells.

Passive immunotherapy has been attempted (or is being considered) by cross immunizing cancer patients and subsequently exchanging their sera [66], by transfusing sera from patients whose melanomas have regressed [67] by giving "transfer factor" from regressed cases or non-melanomatous family members [68], by the administration of "immune" RNA [69] and by the administration of non-sensitized lymphocytes or lymphocytes sensitized to MAA *in vivo* or *in vitro*. Golub [70] has certainly shown *in vitro* sensitization to be feasible. Interest has also focused on the use of tumour-specific antibodies as carriers of chemotherapeutic agents [71] and this theoretically attractive, though technically difficult approach is being actively investigated. Synergy of immunotherapy with chemotherapy is presently under investigation by Gutterman *et al.* [72] with initially promising results.

Local immunotherapy, the application to accessible melanomas of materials which induce delayed hypersensitivity responses, has yielded interesting results, especially the use of intralesional BCG by Morton *et al.* [73] and of dinitrochlorobenzene by Malek-Mansour and her associates [74]. The regression of non-injected cutaneous secondaries and very occasionally of visceral metastases (Malek-Mansour, personal communication) suggests that this may in fact be more than "local" immunotherapy.

In summary, much remains to be done to establish the place of immunotherapy in the treatment of melanoma (and other malignancies). Some patients have obtained significant clinical benefit, but we know little of the best agents to use, the optimum routes of administration, acceptable doseages of the various adjuvants, methods of monitoring adjuvant-induced effects and how to select those patients most likely to respond. The morbidity and mortality of immunotherapy has mainly been related to hypersensitivity to and infection [75] by living adjuvants, indicating the desirability of non-living adjuvants. Information leading to the more scientific use of immunotherapy will most probably be obtained from properly designed trials and those contemplating such treatment should, in my view, consider very seriously collaborating within the existing trials of EORTC, WHO or the American Clinical Cooperative groups [76].

Table 1. *The form of histological report of an invasive malignant melanoma recommended in the paper by McGovern* et al. [77]

A. *Diagnosis according to histogenetic pattern*
1. Malignant melanoma, invasive, with adjacent intra-epidermal component of Hutchinson's melanotic freckle type or,
2. Malignant melanoma, invasive, with adjacent intra-epidermal component of superficial spreading type, or
3. Malignant melanoma, invasive, with adjacent intra-epidermal component, unclassifiable, or,
4. Malignant melanoma, invasive without adjacent intra-epidermal component.

B. *Cross-sectional profile of section on slide**
1. flat or uneven
2. convex or plateau of measurable height
3. polypoidal.

C. *Levels of invasion*
1. intra-epidermal
2. papillary-dermal
3. papillary-reticular interface
4. reticular-dermal
5. subcutaneous fat.

D. *Ulceration, more than* 6 *mm in extent*

E. *Vascular invasion*
1. lymphatics
2. blood vessels.

F. *Mitotic Activity, assessed on at least* 10 *high power* (× 300) *fields* (*h.p.f.*)
1. fewer than 1/5 h.p.f.
2. between 1/5 h.p.f. and 1/h.p.f.
3. 1/h.p.f. and over.

G. *Other parameters* such as cytology, pigmentation, "lymphocytic" infiltrates, evidence of spontaneous regression, associated naevi and solar changes in the dermis are mainly of research interest at present, and their recording is left to the individual histologist's discretion.

*Breslow's measurement of thickness [79] is clearly superior to this crude examination.

REFERENCES

1. K. Magnus, Incidence of malignant melanoma of the skin in Norway 1955–1970. *Cancer (Philad.)* **32,** 1275 (1973).
2. J. J. McGovern and M. M. Lane Brown, In *The Nature of Melanoma*, p. 113. Charles C. Thomas, Springfield, 1969.
3. J. A. H. Lee and Anne P. Carter, Secular trends in mortality from malignant melanoma. *J. nat. Cancer Inst.* **45,** 91 (1970).

4. H. O. LANCASTER, Some geographical aspects of the mortality from melanoma in Europeans. *Med. J. Aust.* **1,** 1082 (1956).
5. N. C. DAVIS, J. J. HERRON and G. R. MCLEOD, Malignant melanoma in Queensland. *Lancet* **ii,** 407 (1966).
6. F. BURBANK, Patterns in cancer mortality in the United States: 1950–67. In N.C.I. Monograph 33, p. 370. Washington USDHEW, (1971).
7. J. A. H. LEE and J. M. MERRILL, Sunlight and the aetiology of malignant melanoma: a synthesis. *Med. J. Aust.* **2,** 846 (1970).
8. J. A. H. LEE, Recent changes in the incidence of malignant melanoma in the white populations of the U.S. and Canada. In *Pigment Cell.* (Edited by V. RILEY Karger, Basel. In press.
9. K. SHANMUGARATNAM and E. B. LA'BROOY, Skin cancer in Singapore. *Nat. Cancer Inst. Monogr.* **10,** 153 (1963).
10. L. SADOFF, J. WINKLEY and S. TYSON, Is malignant melanoma an endocrine-dependent tumour? *Oncology* **27,** 244 (1973).
11. G. D. BIRKMAYER, B.-R. BALDA and F. MILLER, Oncorna-viral information in human melanomas. *Europ. J. Cancer* **10,** 419 (1974).
12. D. C. WALLACE and L. A. EXTON, Genetic predisposition to development of malignant melanoma. In *Melanoma and Skin Cancer,* p. 65. Government Printer, Sydney (1972).
13. C. M. SUTHERLAND, H. W. KLOEPFER, P. W. A. MANSELL and E. T. KREMENTZ, Familial melanoma. In *Pigment Cell.* (Edited by V. RILEY). Karger, Basel. In press.
14. M. C. MIHM, JR., T. B. FITZPATRICK, M. M. LANE-BROWN, J. W. RAKER, R. A. MALT and J. S. KAISER, Early detection of primary cutaneous malignant melanoma. *New Engl. J. Med.* **289,** 989 (1973).
15. Clinical diagnosis of malignant melanoma. WHO, International Reference Centre for the Evaluation of Methods of Diagnosis and Treatment of Melanoma, Milan (1974).
16. R. M. MACKIE, An aid to the preoperative assessment of pigmented lesions of the skin. *Brit. J. Derm.* **85,** 232 (1971).
17. F. K. BAUER and C. G. STEFFEN, Radioactive phosphorus in the diagnosis of skin tumours. *J. Amer. med. Ass.* **158,** 563 (1955).
18. R. L. IKONOPISOV, Malignant melanoma of the skin. *Neoplasma* **8,** 377 (1960).
19. T. VENKEI and L. BAKOS, Early diagnosis of melanoblastoma by thermo-difference. *Acta Un. int. Cancr.* **120,** 1833 (1962).
20. W. H. BEIERWALTES, V. M. VARMA, L. M. LIEBERMAN, R. E. COUNSELL and J. O. MORALES, Scintillation scanning of malignant melanomas with radio-iodinated quinoline derivatives. *J. lab. clin. Med.* **72,** 485 (1968).
21. G. AGRUP, B. FALCK, H. RORSMAN, A. M. ROSENGREN and E. ROSENGREN, Sun exposure and urinary excretion of 5-S-Cysteinyldopa. In *Pigment Cell.* (Edited by V. RILEY), Karger, Basel. In press.
22. M. G. LEWIS, Possible immunological factors in human malignant melanoma. *Lancet* **ii,** 921 (1967).
23. D. L. MORTON, R. A. MALMGREN, E. C. HOLMES and A. S. KETCHAM, Demonstration of antibodies against human malignant melanoma by immunofluorescence. *Surgery* **64,** 233 (1968).
24. M. G. LEWIS, R. L. IKONOPISOV, R. C. NAIRN, T. M. PHILLIPS, G. HAMILTON FAIRLEY, D. C. BODENHAM and P. ALEXANDER, Tumour specific antibodies in human malignant melanoma and their relationship to the extent of the disease. *Brit. med. J.* **2,** 547 (1969).
25. N. M. MUNA, S. MARCUS and C. SMART, Detection by immunofluorescence of antibodies specific for human malignant melanoma. *Cancer (Philad.)* **23,** 88 (1969).
26. M. M. ROMSDAHL and I. S. COX, Human malignant melanoma antibodies demonstrated by immunofluorescence. *Arch. Surg.* **100,** 491 (1970).
27. A. BODURTHA, D. CHEE, J. F. LAUCIUS, M. MASTRANGELO and M. PREHN, Clinical and immunological significance of human melanoma cytotoxic antibody. *Cancer Res.* **35,** 189 (1975).
28. C. M. MCBRIDE, J. M. BROWN, L. L. DMOCHOWSKI, Antinucleolar antibodies in the sera of patients with malignant melanoma. *Surg. Forum* **23,** 92 (1972).
29. I. HELLSTRÖM, K. E. HELLSTRÖM, H. O. SJOGREN and G. A. WARNER, Demonstration of cell mediated immunity to human neoplasms of various histological types. *Int. J. Cancer* **7,** 1 (1971).

30. J. E. DE VRIES, P. RÜMKE and J. L. BERNHEIM, Cytotoxic lymphocytes in melanoma patients. *Int. J. Cancer* **9,** 567 (1972).
31. U. M. JEHN, L. NATHANSON, R. S. SCHWARTZ and M. SKINNER, *In vitro* lymphocyte stimulation by a soluble tumor antigen in malignant melanoma. *New Engl. J. Med.* **283,** 329 (1970).
32. E. M. HERSH, J. U. GUTTERMAN, G. M. MAVLIGIT, C. H. GRANATEK, R. C. REED, U. AMBUS and C. M. MCBRIDE, Approaches to the study of tumor antigens and tumor immunity in malignant melanoma. *Behring Inst. Mitt.* **56,** 139 (1975).
33. A. J. COCHRAN, W. G. S. SPILG, R. M. MACKIE and C. E. THOMAS, Post operative depression of tumour directed cell-mediated immunity in patients with malignant disease. *Brit. med. J.* **4,** 67 (1972).
34. A. J. COCHRAN, R. M. MACKIE, C. E. THOMAS, R. M. GRANT, D. E. CAMERON-MOWAT and W. G. S. SPILG, Cellular immunity to breast carcinoma and malignant melanoma. *Brit. J. Cancer* **28,** Suppl. I, 77 (1973).
35. L. FASS, R. B. HERBERMAN, J. L. ZIEGLER and J. W. KIRYABWIRE, Cutaneous hypersensitivity reactions to autologous extracts of malignant melanoma cells. *Lancet* **i,** 116 (1970).
36. A. Z. BLUMING, C. L. VOGEL, J. L. ZIEGLER and I. W. KIRYABWIRE, Delayed cutaneous hypersensitivity reactions to extracts of autologous malignant melanoma: A second look. *J. nat. Cancer Inst.* **48,** 17 (1972).
37. R. B. HERBERMAN, A. HOLLINSHEAD, D. CHAR, R. OLDHAM, J. MCCOY and M. COHEN, *In vivo* and *in vitro* studies of cell mediated immune response to antigens associated with malignant melanoma. *Behring Inst. Mitt.* **56,** 131 (1975).
38. J. J. BOURGOIN, M. VITRIS, J. RIFA and J. GENEVE, Study of peripheral human lymphocytes forming spontaneous rosettes with sheep erythrocytes in malignant melanomas. *Behring Inst. Mitt.* **56,** 263 (1975).
39. J. WYBRAN and L. E. SPITLER, Rosette-forming cells and melanoma. *Clin. Res.* **21,** 655 (1973).
40. R. L. IKONOPISOV, M. G. LEWIS, I. D. HUNTER-CRAIG, D. C. BODENHAM, T. M. PHILLIPS, I. C. COOLING, J. PROCTOR, G. H. FAIRLEY and P. ALEXANDER, Autoimmunisation with irradiated tumour cells in human malignant melanoma. *Brit. med. J.* **2,** 752 (1970).
41. G. A. CURRIE, F. LEJEUNE and G. H. FAIRLEY, Immunisation with irradiated tumour cells and specific lymphocyte cytotoxicity in malignant melanoma. *Brit. med. J.* **2,** 305 (1971).
42. M. G. LEWIS, T. M. PHILLIPS, K. B. COOK and J. BLAKE, Possible explanation for loss of detectable antibody in patients with disseminated malignant melanoma. *Nature (Lond.)* **232,** 52 (1971).
43. A. J. COCHRAN, C. E. ROSS, R. M. MACKIE, R. M. GRANT and D. E. HOYLE, The immune status of patients with malignant melanoma. *Behring Inst. Mitt.* **56,** 125 (1975).
44. Y. KEISARI and I. P. WITZ, Degradation of xenogeneic, cytotoxic anti-tumor antibodies by tumor-derived lysosomal enzymes and the generation of products blocking humoral cytotoxicity. *Behring Inst. Mitt.* **56,** 19 (1975).
45. G. A. CURRIE, *Cancer and the Immune Response*, p. 65. Arnold, London (1974).
46. G. A. CURRIE and P. ALEXANDER, Spontaneous shedding of TSTA by viable sarcoma cells. its possible role in facilitating metastatic spread. *Brit. J. Cancer* **29,** 72 (1974).
47. R. EVANS, Macrophages in syngeneic animal tumours. *Transplantation* **14,** 468 (1972).
48. C. L. GAUCI, The macrophage content of human malignant melanomas. *Behring Inst. Mitt.* **56,** 73 (1975).
49. J.-C. CERROTINI and K. T. BRUNNER, Cell mediated cytotoxicity, allograft rejection and tumor immunity. *Adv. Immunol.* **18,** 67 (1974).
50. J.-P. CESARINI and R. ROUBIN, Host reaction against tumour aggression in cutaneous primary human malignant melanoma of the superficial spreading type: macroscopic, microscopic and ultrastructural features. *Behring Inst. Mitt.* **56,** 65 (1975).
51. M. M. BLACK, S. R. OPLER and F. D. SPEER, Microscopic structure of gastric carcinomas and their regional lymph nodes in relation to survival. *Surg. Gynec. Obstet.* **98,** 725 (1954).

52. M. M. Black, S. R. Opler and F. D. Speer, Survival in breast cancer cases in relation to the structure of the primary tumour and regional lymph nodes. *Surg. Gynec. Obstet.* **100,** 543 (1955).
53. A. J. Cochran, Histology and prognosis in malignant melanoma. *J. Path.* **97,** 459 (1969).
54. W. H. Clark, Jr., A classification of malignant melanoma in man correlated with histogenesis and biological behaviour. In *Advances in Biology of the Skin.* (Edited by W. Montagna and F. Hu), Vol. 8, p. 621. Pergamon Press, Oxford (1967).
55. V. J. McGovern, The classification of melanoma and its relationship with prognosis. *Pathology* **2,** 85 (1970).
56. Y. Mishima, Melanocytic and nevocytic malignant melanomas: cellular and sub-cellular differentiation. *Cancer (Philad.)* **20,** 632 (1967).
57. J. H. Little, Histology and prognosis in cutaneous malignant melanoma. In *Melanoma and Skin Cancer,* p. 95. Australian Government Printer, Sydney (1972).
58. J. Conley, R. Lattes and W. Orr, Desmoplastic malignant melanoma (a rare variant of spindle cell melanoma). *Cancer (Philad.)* **28,** 914 (1971).
59. A. J. Cochran and K. M. Cochran, Naevi in childhood. In *Pigment Cell.* (Edited by V. Riley). Vol. 1, p. 276. Karger, Basel (1973).
60. E. Hirst, S. W. McCarthy and P. M. Bale, Frozen section diagnosis of cutaneous malignancy. In *Melanoma and Skin Cancer,* p. 185. Government Printer, Sydney, (1972).
61. J. H. Little and N. C. Davis, Frozen section diagnosis of suspected malignant melanoma. *Cancer (Philad.)* **34,** 1163 (1974).
62. F. G. Conrad, Treatment of malignant melanoma. Wide excision alone vs. lymphadenectomy. *Arch. Surg.* **104,** 587 (1972).
63. J. H. Moon, S. Gailani, M. R. Cooper, D. M. Hayes, V. B. Rege, J. Blom, G. Falkson, P. Maurice, K. Brunner, O. Glidewell and J. F. Holland, Comparison of the combination of 1,3-BIS(2-chloroethyl)-1-nitrosourea (BCNU) with two dose schedules of 5-(3,3-dimethyl-1-triazeno). Imidazole 4-carboxamide (DTIC) in the treatment of disseminated malignant melanoma. *Cancer (Philad.)* **35,** 372 (1975).
64. R. Regnier, Utilité de la radiothérapie dans le traitement du mélanome cutané. *Acta chir. belg.* **73,** 237 (1974).
65. G. Mathé, Active immunotherapy. *Adv. Cancer Res.* **14,** 1 (1971).
66. H. Willoughby, J. P. A. Latour, E. J. Tabah and H. R. Shibata, Cross implantations of tumor tissue and cross transfusions in patients with advanced cancer. *Amer. J. Obstet. Gynec.* **108,** 889 (1970).
67. W. C. Sumner and A. G. Foraker, Spontaneous regression of human melanoma. *Cancer (Philad.)* **13,** 79 (1960).
68. L. E. Spitler, J. Wybran, H. H. Fudenberg, A. S. Levin and M. G. Lewis, Transfer factor therapy of malignant melanoma. *Clin. Res.* **21,** 221 (1973).
69. Y. H. Pilch, D. Fritze and D. H. Kern, Immune cytolysis of human melanoma cells mediated by immune RNA. *Behring Inst. Mitt.* **56,** 184 (1975).
70. S. H. Golub and D. L. Morton, Sensitisation of lymphocytes *in vitro* against human melanoma-associated antigens. *Nature(Lond.)* **251,** 161 (1974).
71. T. Ghose, S. T. Norvell, A. Guclu, D. Cameron, A. Bodurtha, and A. S. McDonald, Immunochemotherapy of cancer with chlorambucil-carrying antibody. *Brit. med. J.* **3,** 495 (1972).
72. J. U. Gutterman, G. Mavligit, J. A. Gottlieb, M. A. Burgess, C. M. McBride, L. Einhorn, E. J. Freireich and E. M. Hersh, Chemoimmunotherapy of disseminated malignant melanoma with dimethyl triazeno imidazole carboxamide and Bacillus Calmette-Guérin. *New Engl. J. Med.* **291,** 592 (1974).
73. D. L. Morton, F. R. Eilber, E. C. Holmes, J. S. Hunt, A. S. Ketcham, M. J. Silverstein and F. C. Sparks, BCG immunotherapy of malignant melanoma: summary of a seven-year experience. *Ann. Surg.* **180,** 635 (1974).
74. S. Malek-Mansour, S. Castermans-Elias and C. M. Lapiere, Régression de metastases de mélanome après thérapeutique immunologique. *Dermatol (Basel)* **146,** 156 (1973).
75. R. M. Grant, R. M. Mackie, A. J. Cochran, E. L. Murray, D. E. Hoyle and C. E. Ross, Results of the administration of BCG to melanoma patients. *Lancet* **ii,** 1096 (1974).

76. For information on current trials contact Dr. Dorothy Windhorst, Coordinator, International Registry of Tumor Immunotherapy, National Institutes of Health, Building 10, Room 4B15, Bethesda, Md. 20014, U.S.A.
77. VINCENT J. MCGOVERN, MARTIN C. MIHM, JR., CHRISTIANE BAILLY, JOAN C. BOOTH, WALLACE H. CLARK, JR., ALISTAIR J. COCHRAN, E. G. HARDY, J. D. HICKS, ARNOLD LEVENE, MARTIN G. LEWIS, J. H. LITTLE and G. W. MILTON, The classification of malignant melanoma and its histologic reporting. *Cancer (Philad.)* **32,** 1446 (1973).
78. I. CARR and J. C. E. UNDERWOOD, The ultra structure of the local cellular response to neoplasia. *Int. Rev. Cytol.* **37,** 329 (1974).
79. A. BRESLOW, Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann. Surg.* **172,** 902 (1970).

Europ. J. Cancer Vol. 12, pp. 595–598. Pergamon Press 1974. Printed in Great Britain

Demonstration of the Stimulation of the Reticulohistiocytic System (RHS) of Mice by Treatment with BCG by Means of Biometric and Histochemical Techniques

F. FEY, W. ARNOLD and A. GRAFFI
Academy of Sciences of the GDR, Central Institute of Cancer Research, Lindenberger Weg 80, 1115 *Berlin, GDR*

Abstract—*The efficiency of different intravenously-given BCG doses on the RHS was investigated in the present paper. A time- and dose-dependent increase in the weight of liver, spleen, and lungs, was demonstrated in BCG-treated animals. It was shown, by means of histochemical methods, that the weight increase is due to an enhanced proliferation of cells with acid phosphatase reaction. There is a good correlation between the results obtained with biometric and histochemical methods. The question as to whether there is a direct connection between the proliferation of phosphatase-positive cells of the RHS and an increase in tumour defense especially in the model of metastasizing is under discussion.*

INTRODUCTION

BCG PLAYS at present the most important role among the nonspecific stimulants of tumour defense. A tumour inhibiting effect could be demonstrated both in animal experiments [1–4] and for the therapy of some special human neoplasias [5]. Experiments carried out in the last 15 years have shown that several mechanisms, some of them not yet known in detail are responsible for this inhibition [6, 7]. Previous investigations performed by us revealed a clear dose-dependent efficiency in the prevention of metastases in a syngeneic murine test model [4]. Like other groups [8], we observed further a significant increase in the weight of the organs of the RHS, especially of liver and spleen, which was directly dependent on the dose and the way of application [4]. This increase is possibly a result of the stimulation of the reticulo-histiocytic system by BCG. Since the extent of the stimulation allows to draw conclusions with regard to the defense status of the organism against metastases we tried to obtain a measure of the degree of stimulation by means of histochemical techniques. It was attempted to show a stimulation of the RHS by means of the presentation of the acid phosphatase because most RHS cells have a distinct acid phosphatase activity [9].

Accepted 12 March 1976.

MATERIAL AND METHODS

Animals

We used male animals of the mouse inbred strain XVII/Berlin of an average weight of 20 g and an age of about 3 months. The animals showed no symptoms of disease and were in a good constitution. They were kept in plastic cages by tens at constant room temperature of 22°C. Their food was standardized (recipe R 9) and tap water, both *ad libidum*.

BCG treatment

For the treatment with the nonspecific stimulant we used the BCG vaccine from the BCG-Institute at Jena which is composed of lyophilized bacteria (*Mycobacterium bovis*, BCG strain, S4 substrain). The amount of germ units is about 20 million per mg semi-dry substance with a proportion of about 20% viable ones [10]. The dried vaccine was resuspended in isotonic

Table 1. *Relative organ weights (in % of body weights) registered* 10, 20 *and* 30 *days after intravenous application of different BCG doses*

Organs	Days after injection	Control	0·1 mg	1 mg	3 mg	5 mg BCG
Liver	untreated	6·02 ± 0·62	—	—	—	—
	10	6·42 ± 0·58	5·91 ± 1·27	7·05 ± 0·83	7·39 ± 0·76*	7·43 ± 1·35
	20	6·60 ± 1·24	6·50 ± 0·91	7·06 ± 0·87	9·26 ± 1·15*	9·34 ± 1·69*
	30	6·36 ± 0·86	6·36 ± 0·66	7·61 ± 0·58*	8·43 ± 0·61*	8·41 ± 0·83*
Spleen	untreated	0·94 ± 0·35	—	—	—	—
	10	1·37 ± 0·46	0·93 ± 0·57	1·52 ± 0·72	2·02 ± 1·09	1·71 ± 0·55
	20	0·89 ± 0·43	1·30 ± 0·32	1·52 ± 0·58	2·34 ± 0·62*	2·58 ± 0·75*
	30	1·16 ± 0·66	1·21 ± 0·60	2·03 ± 0·53	2·19 ± 0·55*	2·49 ± 0·61*
Lungs	untreated	0·89 ± 0·09	—	—	—	—
	10	0·91 ± 0·21	0·94 ± 0·18	1·02 ± 0·26	1·27 ± 0·16*	1·56 ± 0·17*
	20	0·91 ± 0·14	0·99 ± 0·19	0·86 ± 0·26	1·39 ± 0·37*	1·73 ± 0·35*
	30	0·94 ± 0·10	0·93 ± 0·15	1·16 ± 0·37	1·35 ± 0·17*	1·39 ± 0·20*

*Statistically verified for $\alpha = 0{\cdot}01$ by means of the u-test according to Mann and Whitney [14].

saline and immediately intravenously injected into the animals. Doses between 0·1 mg and 5 mg of BCG in 0·5 ml physiological saline per animal were used for the application. Control animals received i.v. injections of 0·5 ml saline.

Estimation of weight

Ten animals of each treated group were killed at intervals of 10, 20 and 30 days after BCG application; respectively, in the second experiment, 20 animals were killed after 2, 10, 15, 20, 30 and 60 days. Each time, we registered the body weight and the absolute and relative fresh weights of liver, spleen, and lungs. The relative organ weights represent the absolute weights of the organs in percentage of body weights.

Histochemistry

Cryostate sections were prepared from the organs immediately after excision and fixed in acetone for 10 min. The demonstration of acid phosphatase was performed by means of the azo-cuppling reaction according to Gössner [11] with naphtole–AS–BS–phosphate as substrate and fast red salt ITR at pH 6·5. After counter staining with hematoxylin, the samples were covered with glycerolgelatine. As controls heat-inactivated samples were used and samples from control mice which had been treated only with saline.

RESULTS

In the first experiment, the dose dependence was checked for doses between 0·1 mg and 5 mg BCG (according to Table 1) whereas in the second experiment the time dependence with a dose of 3 mg BCG was investigated (according to Table 2).

1. *Weights of RHS organs after BCG treatment*

Compared with control animals which had been tested parallely BCG-treated mice showed

Table 2. *Relative organ weights (in % of body weights) registered* 2, 10, 15, 20, 30 *and* 60 *days after intravenous injection of* 3 *mg BCG*

Days after BCG treatment	Liver		Spleen		Lungs	
	Control	BCG	Control	BCG	Control	BCG
Untreated	6·35 ± 0·54	—	0·97 ± 0·25	—	0·90 ± 0·10	—
2	6·33 ± 0·94	6·21 ± 0·86	0·57 ± 0·11	0·55 ± 0·16	0·83 ± 0·11	1·26 ± 0·35*
10	6·48 ± 0·50	7·19 ± 0·96*	1·26 ± 0·43	1·75 ± 0·76	0·92 ± 0·17	1·35 ± 0·22*
15	6·49 ± 0·65	9·17 ± 1·01*	0·85 ± 0·51	2·21 ± 0·51*	0·88 ± 0·11	1·52 ± 0·32*
20	6·10 ± 1·08	9·43 ± 1·13*	0·76 ± 0·35	2·47 ± 0·55*	0·89 ± 0·10	1·46 ± 0·29*
30	6·33 ± 0·69	8·29 ± 0·67*	1·01 ± 0·57	2·28 ± 0·42*	0·95 ± 0·14	1·32 ± 0·19*
60	6·55 ± 0·46	8·25 ± 0·45*	0·73 ± 0·25	1·86 ± 0·29*	0·85 ± 0·07	1·15 ± 0·07*

*Statistically verified for $\alpha = 0{\cdot}01$ by means of the u-test according to Mann and Whitney [14].

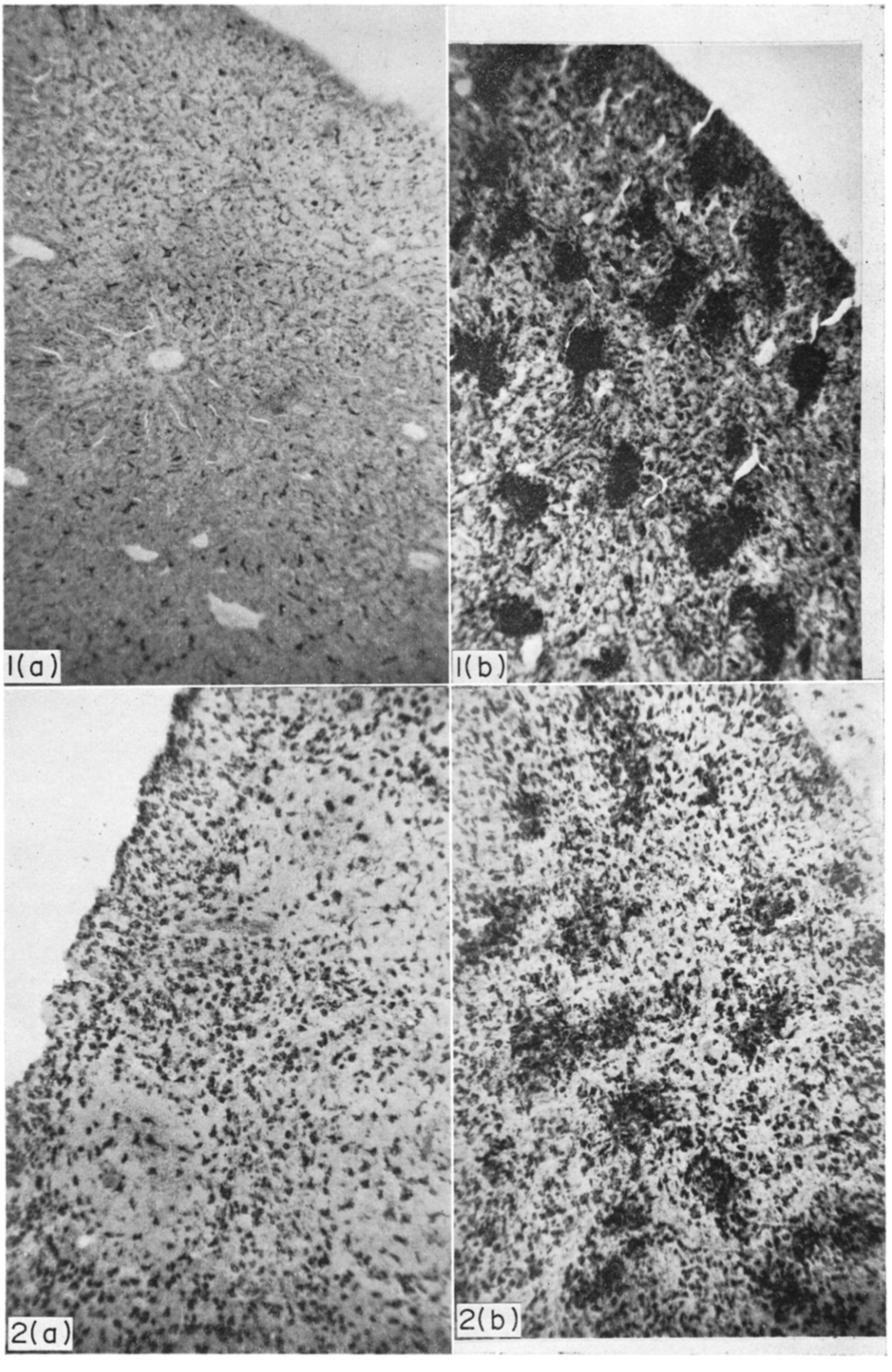


Fig. 1. *Demonstration of acid phosphatase in the liver of a control animal* (*a*) *and a mouse treated intravenously with* 3 *mg BCG on the* 30*th day after BCG application* (*b*). 150×.

Fig. 2. *Demonstration of acid phosphatase in the spleen of a control animal* (*a*) *and a mouse treated intravenously with* 3 *mg BCG on the* 30*th day after BCG application* (*b*). 150×.

(to face p. 596)

a significant increase in the fresh weights of liver, spleen, and lungs. The weight increase was directly proportional to the applicated BCG dose (Table 1). In addition to the dose dependence, we registered a time-dependent increase in the organ weights (Table 2). In the groups treated with the highest dose (3 mg and 5 mg BCG per animal) the maximum was reached about 20 days after BCG treatment. By this time the relative spleen weight had trebled. In the group treated with 1 mg BCG a maximum was observed only after 30 days. But the increase was significantly below that found in the 3 mg- and 5 mg-BCG-groups. A comparison of the three investigated organs showed that the spleen weight had increased most strikingly both in absolute and relative organ weight.

2. *Histochemistry*

Liver. In the controls treated with saline a slight activity was observed only in the Kupffer cells (Fig. 1a). Ten days after BCG treatment the livers show a significantly stronger enzyme reaction especially in the region of the central veins. The capillaries are well presented by the acid phosphatase reaction. Twenty days after BCG treatment the liver shows a strong enzyme reaction. The RHS is stimulated to a maximum, the enzyme-active zones are confluent and show big positive areas. Thirty days after BCG treatment maximum stimulation is still largely present (Fig. 1b). After 60 days the stimulation is slightly decreased.

Spleen. In the saline-treated controls a clear enzyme activity is observed in the red pulpe. The white pulpe is noticeably omitted. The activity is mainly bound to the border cells of the sinusoid (Fig. 2a). Ten days after BCG treatment the spleens show a significantly stronger enzyme activity. The white pulpe is no longer noticeably marked off. Twenty and thirty days after BCG treatment maximum stimulation is demonstrated by enzyme reaction. Similar to the liver a formation of areas is obtained by the confluence of positive parts (Fig. 2b).

Lungs. A slight enzyme activity is found in the saline controls appearing mostly in an annular form round the bronchioles. In contrast to the liver and the spleen, already 2 days after injection a noticeable reaction was observed like in the weight (Table 2). Ten days after BCG treatment the activity increases and overlaps on the whole respiratory parenchyme. Twenty days after BCG application a strong enzyme activity is observed which slightly once again increases 30 days after treatment. Positive reactions are found all over the lung tissue, but here too areas are formed by confluence in some regions.

A second series of experiments showed that in the three tested organs higher BCG doses produced an increase in the intensity and extent of the phosphatase reaction.

DISCUSSION

The results presented showed a good correlation between the increase of organ weights and the extent of acid phosphatase activity in the stimulated cells of RHS. A time- and dose-dependence could be demonstrated with both methods. The method used mostly hitherto for measuring the stimulation of RHS in distinct organs or in the whole organism was the investigation of an increased ability of phagocytosis or storage of special dyes or radioisotopes by the cells of the RHS. Since these methods are difficult and produce sometimes wrong values, especially for small experimental animals, we used the histochemical demonstration of the stimulation. The increase in acid phosphatase activity in the quoted organs after BCG treatment shown in our experiments can be taken as a real measure of the RHS stimulation since it is known that the Kupffer cells of the liver, the border cells of the sinusoid as well as the macrophages and histiocytes [9], which represent the main cellular parts of the RHS, are characterized by a distinct acid phosphatase activity. Following the application of BCG, several investigators showed an increase of intracellular acid phosphatase activity in macrophages [12, 13].

The period investigated is characterized by successive decreases of acid-resistant bacteria in lungs whereas in spleen and in liver such a decrease was not proved. For the purpose of this examination the tested organs had been embedded in paraffin and stained according to Ziehl–Neelsen. Two days after i.v. injection of BCG increased amounts of acid-resistant bacteria were found in lungs, a smaller quantity in spleen and in liver. After 20, 30 and 60 days all bacteria were found to be phagocytized by macrophages in lungs. In spleen and in liver 10, 20, 30 and 60 days after injection the bacteria were seen only rarely, and then mostly in proliferating centres, but a considerable number of centres is absolutely free of bacteria. A tissue-reaction outside the RHS is strikingly low. Inflammatory processes and necroses never were found. On the basis of these results a persistent infection finally cannot be ex-

cluded, but we suggest that this infection is not directly related to the RHS stimulation. In order to clarify this question additional investigations are in progress.

The increased proliferation of the RHS cells which shows a strong dependence on the BCG dose used allows the conclusion that there is a direct correlation between the degree of stimulation of the RHS and the defense status in the organism. In accordance with that we had shown in previous investigations with a metastatic model that high BCG doses [3–5 mg BCG per animal intravenously) produced a significantly stronger preventive effect than doses of 0·5 mg or 1 mg BCG [4]. This metastatic model is characterized by i.v. applications of syngeneic ascitic tumour cells of a 3,4-benzopyrene-induced sarcoma in the mouse. The metastases occur mainly in subcutis, musculature, lymph nodes, kidney and adrenal gland, whereas metastases in lungs seldom appear. The inhibition of metastasizing which is proportional to the BCG doses is not only found for lung metastases but for the experimental metastasizing in general. The results of our investigations justify the assumption that there is a direct relation between the degree of stimulation of the RHS demonstrated by means of the acid phosphatase activity and the extent of the defense efficiency against the experimental metastasizing in a syngeneic test model of mice.

REFERENCES

1. L. J. Old, D. A. Clarke and B. Benacerraf, Effect of Bacillus Calmette–Guérin (BCG) infection on transplanted tumours in the mouse. *Nature (Lond.)* **184,** 291 (1959).
2. R. W. Baldwin and M. V. Pimm, Influence of BCG infection of growth of 3-methylcholanthrene-induced rat sarcomas. *Europ. J. clin. biol. Res.* **16,** 875 (1971).
3. G. Mathé, O. Halle-Pannenko and Ch. Bourut, BCG in cancer immunotherapy: Results obtained with various BCG preparations in a screening study for systemic adjuvants applicable to cancer immunoprophylaxis or immunotherapy. *Nat. Cancer Inst. Monogr.* **39,** 107 (1973).
4. W. Arnold, T. M. Muresian, I. Kiricuta and A. Graffi, Inhibition of experimental metastatic spread by non-specific stimulants. *UICC-Symposium on Experimental Models in Cancer Immunotherapy, Bucharest,* 29–31 May (1975) Plenum, London. (In press).
5. G. Mathé, R. Weiner, P. Pouillart, L. Schwarzenberg, C. Jasmin, M. Schneider, M. Hayat, J. L. Amiel, F. De Vassal and C. Rosenfeld, BCG in cancer immunotherapy: Experimental and clinical trials of its use in treatment of leukemia minimal and or residual disease. *Nat. Cancer Inst. Monogr.* **39,** 165 (1973).
6. P. Alexander, Activated macrophages and the antitumor action of BCG. *Nat. Cancer Inst. Monogr.* **39,** 127 (1973).
7. S. R. Rosenthal, BCG and the lympho-reticuloendothelial system. *Nat. Cancer Inst. Monogr.* **39,** 91 (1973).
8. L. J. Old, D. A. Clarke, B. Benacerraf and M. Goldsmith, The reticuloendothelial system and the neoplastic process. *Ann. N.Y. Acad. Sci.* **88,** 264 (1960).
9. H. E. Schäfer und R. Fischer, Enzymhistochemische Untersuchungen an den Gewebeleukozyten im Vergleich zu Blut- und Bindegewebszellen bei Maus und Ratte. *Virchows Arch. path. Anat.* **338,** 130 (1964).
10. A. Zureck (head of the BCG-Institute at Jena), personal communication.
11. W. Gössner, Histochemischer Nachweis hydrolytischer Enzyme mit Hilfe der Azofarbstoffmethode. *Histochemie* **1,** 65 (1957).
12. A. M. Dannenberg, Cellular hypersensitivity and cellular immunity in the pathogenesis of tuberculosis: Specificity, systemic and local nature, and associated macrophage enzymes. *Bact. Reviews* **32,** 85 (1968).
13. S. Kwiek, J. Wyszomirska and L. Rzucido, Acid phosphatase activity of exudative cells of peritoneal cavity of guinea pigs infected with tubercule and BCG bacilli. *Acta microbiol. pol.* **17,** 319 (1968).
14. E. Weber, *Grundriss der biologischen Statistik.* Fischer Verlag, Jena (1967).

Europ. J. Cancer Vol. 12, pp. 599–604. Pergamon Press 1976. Printed in Great Britain

Tumor-Enhancing and Tumor-Inhibiting *In Vivo* Effects of Phytohaemagglutinin

Study on Proliferation of Transplantable Mouse Melanoma

G. SCHWARZE and A. PAPPAS*
Department of Medicine, University Hospital, 665 Homburg/Saar, (Fed. Rep. Ger.)

Abstract—*Phytohaemagglutinin (PHA)-induced stimulation, as well as inhibition of tumor growth in mice transplanted with Harding–Passey melanoma, has been demonstrated in the present study. Tumor proliferation could be modulated depending upon the dose of PHA and its time of administration with respect to tumor implantation. Once a critical dose of PHA was exceeded or tumor implantation has been performed, a shift from tumor-enhancing to tumor-inhibiting activity took place. Furthermore, experimental conditions which enhanced tumor proliferation, increased the rate of "tumor takes" and vice versa. While the inhibitory effect of PHA was maintained in re-transplanted tumors, PHA-induced enhancement of tumor growth was no longer observed during successive passages of the melanoma in untreated recipients. In addition, spleen cells of melanoma bearing mice were tested for their ability to respond to sheep red blood cells (SRBC). SRBC-specific responses decreased progressively during the course of tumor-bearing and additional treatment with PHA including tumor-enhancing treatment was associated with a further depression of splenic immunocompetence to this antigen.*

Our results, suggesting a dose and time dependent different action of PHA on tumor growth, indicate how critically experimental parameters must be controlled for applying immunotherapy with this mitogen.

INTRODUCTION

SEVERAL studies provide data on the influence of phytohaemagglutinin (PHA) on the growth of different experimental animal tumors. A survey of the literature, however, reveals contradictory results. While Robinson and Mekori [1, 2] described significantly reduced volumes of Ehrlich ascites carcinoma in mice treated with PHA, Datta *et al.* [3] and Rubio and Unsgaard [4] did not find this. In contrast, it was shown in our previous report [5] that administration of PHA to mice inoculated with this tumor increased intraperitoneal tumor growth. Similarly, enhanced growth of methylcholanthrene-induced fibrosarcoma [6] and Sarcoma-180 [7] in mice treated with PHA has been described in recent reports.

As a result of the current interest in the use of adjuvants in tumor therapy we felt obliged to confirm our previous observations and to gain further information relevant to the influence of PHA on tumor proliferation. In the present study, the effect of different dose- and time-schedules of PHA on the growth of transplanted Harding–Passey melanoma and the concomitant primary immune response to sheep red blood cells (SRBC) has been investigated in mice.

We demonstrate that both, tumor-enhancing and tumor-inhibiting effects of PHA will occur under certain conditions.

Accepted 5 March 1976.

*Supported by a grant from the "Deutsche Forschungsgemeinschaft" (Pa 129/3).

MATERIAL AND METHODS

(1) *Animals and tumor transplantation*

Harding–Passey melanoma, originally obtained from Dr. D. Gericke* has been kept alive by serial transfers in random bred, female Swiss mice aged 4–6 weeks (Broekmann, Helmond, Netherlands). For tumor transplantation, 1·5 g tumor tissue was removed aseptically from freshly killed animals, cut into small pieces, minced, cytosieved, and suspended in 3 ml of phosphate-buffered saline (PBS). Mice were injected s.c. into the neck with 0·2 ml of the prepared tumor suspension. The percentage of "tumor takes" was 80–85% from one passage to another.

(2) *PHA-treatment and primary immunization with sheep red blood cells (SRBC)*

PHA (PHA-purified, Burroughs–Wellcome) was dissolved in saline and injected into the tail vein. Dosage and schedule of administration of this agent were varied as indicated below. In parallel groups, tumor-bearing animals were sensitized to SRBC (Behring–Werke, Marburg/Lahn, Fed. Rep. Ger.) 4 days before tumor removal. Immediately prior to use, SRBC were washed three times in PBS and finally diluted to contain 8×10^8 cells/ml. For primary immunization mice were injected with 4×10^8 SRBC i.p.

(3) *Tumor measurement and assay of antibody production to SRBC*

Melanomas grew only at the site of injection and could be removed easily at the times indicated. For tumor growth data the individual weights were determined.

Spleens from animals primed with SRBC 4 days before tumor removal were assayed for their numbers of SRBC-specific haemolysin plaque-forming cells (PFC) according to the technique of Jerne and Nordin [8] as modified by Mishell and Dutton [9]. Results are given as direct anti-SRBC-PFC/spleen.

(4) *Statistical analysis*

Arithmetic mean and standard deviation (SD) of the mean were calculated for 5-week tumor weights of each experimental group. Differences between groups were considered significant only when the p values of comparison were 0·05 or smaller (Student's t test).

*Farbwerke Hoechst AG, Frankfurt/M, Fed. Rep. Ger.

(5) *Experimental plan and injection schedule*

All mice received tumor implants on day 0 of the experimental period, and were divided into the following groups:

Group I. (a) Growth of Harding–Passey melanoma in untreated control mice during the time course of 6 weeks after implantation. Five to eight mice were killed per week and individual tumor weights determined.

(b) Tumor growth and concomitant PFC responses to SRBC during the time course of 6 weeks after tumor implantation. Weekly subgroups of 3–5 mice were assayed for both, individual tumor weights and splenic PFC responses against SRBC.

Group II. Effect of PHA on tumor growth and concomitant splenic PFC production against SRBC in mice treated with daily injections of 10, 5 or 2 mg PHA/kg b.w. for 4 days: (A) before (day -4 to -1), (B) starting together with (day 0 to $+3$), or (C) after (day $+4$ to $+7$) tumor implantation. Weekly subgroups of 4–6 mice of animals, treated with 4×10 mg PHA/kg b.w. were assayed for tumor weights, 3–5 of them simultaneously for their anti-SRBC responses. Similarly, weekly subgroups of 5–12 mice of animals, treated with 4×5 mg PHA/kg b.w. were assayed for individual tumor weights, 3–5 of them simultaneously for their anti-SRBC responses. Six to seven mice of animals, injected with 4×2 mg PHA/kg b.w. were killed 5 weeks after tumor implantation and only individual tumor weights determined.

Group III. Serial transfer of enhanced or inhibited grown melanomas in mice treated with PHA to normal recipients. Equal parts of 3 enucleated tumors which were representative for mean tumor weight were pooled and tumor suspensions prepared for re-transplantation. Tumor weights were determined at the end of every 5-week interval of 3 successive passages, each including 5 to 7 animals.

RESULTS

(1) *Growth pattern of transplanted Harding–Passey melanoma and concomitant splenic PFC responses to SRBC in untreated mice (Group I)*

Mean tumor weights of melanoma grown in untreated control mice and in aminals primed with SRBC 4 days before tumor removal were not significantly different throughout the whole experimental period. In addition, the

rate of "tumor takes" was similar for both groups (83 and 81 % respectively). Therefore, tumor growth values were combined into a single group. As illustrated in Fig. 1, mean tumor weights increased rapidly and approximately linear until the 5th week after tumor implantation and then slowed slightly until the 6th week. The analysis of concomitant PFC responses to SRBC demonstrated that the progression in tumor growth was accompanied by a continuous decline of PFC numbers up to about 25 % at 6 weeks after tumor implantation.

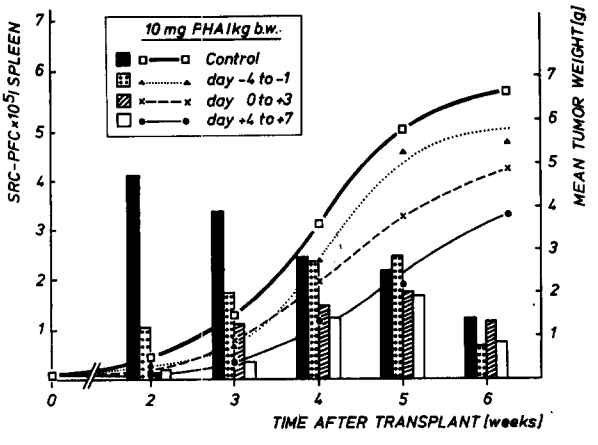


Fig. 1. Proliferation of Harding–Passey melanoma (curves) and concomitant PFC responses to SRBC (columns) in untreated controls and mice treated i.v. with daily injections of 10 mg PHA/kg b.w. for 4 days in relation to the time of tumor implantation. Each symbol represents the mean tumor weight of 5–8 untreated and 4–6 treated animals, respectively. Mean numbers of SRBC-specific PFCs were simultaneously determined in the spleen of 3–5 mice treated with PHA or not. For primary immunization mice were injected with 4 × 10⁸ SRBC i.p. 4 days before tumor removal.

(2) *Tumor proliferation and concomitant PFC responses to SRBC in mice treated with PHA (Group II)*

Since no statistically significant differences between tumor weights of PHA-treated mice whether immunized with SRBC or not, were noted at any time of the experimental period, again, values were combined into a single group.

(A) *Treatment with PHA before tumor implantation (day −4 to −1).* At all 3 dose levels of PHA the percentage of mice with growing tumor transplants was increased up to 90·5–97 %. The effect on tumor growth, however, was quite different. The growth curve of tumors in animals treated with 4 × 10 mg PHA/kg b.w. ran parallel to the control group; differences were not statistically different (Fig. 1). In contrast, the proliferation of tumor implants in mice injected with 4 × 5 mg PHA/kg b.w. was markedly enhanced (Fig. 2). The increase in

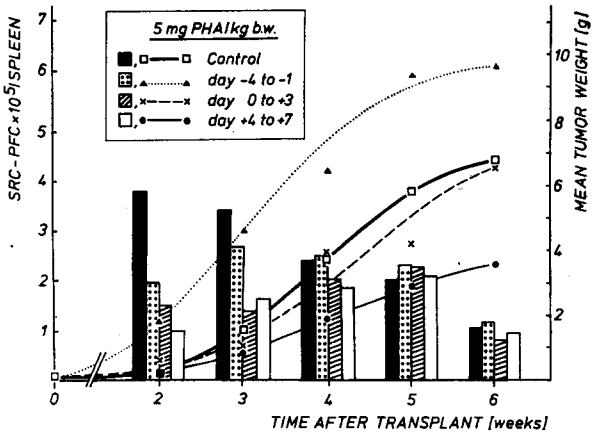


Fig. 2. Proliferation of Harding-Passey melanoma (curves) and concomitant PFC responses to SRBC (columns) in untreated controls and mice treated i.v. with daily injections of 5 mg PHA/kg b.w. for 4 days in relation to the time of tumor implantation. Each symbol represents the mean tumor weight of 5–8 untreated and 5–12 treated animals, respectively. Mean numbers of SRBC-specific PFCs were simultaneously determined in the spleen of 3–5 mice treated with PHA or not. For primary immunization mice were injected with 4 × 10⁸ SRBC i.p. 4 days before tumor removal.

mean tumor weights, already evident at 2 weeks, was greatest ($P < 0{\cdot}0025$) 5 weeks after implantation (9·39 g ± 1·97 S.D. vs 5·78 g ± 1·6 S.D. for control mice). Administration of 4 × 2 mg PHA/k b.w. led to a further augmentation of tumor growth, the 5-week value being 11·4 g ± 2·36 S.D. The result of this experiment together with 5-week values of mice treated with 4 × 10 or 4 × 5 mg PHA/kg b.w. is illustrated in Fig. 3. Data are expressed as percentage-change in mean tumor weight as compared to the control group. Concomitant PFC responses to SRBC in tumor-bearing mice which had received 4 × 10 mg or 4 × 5 mg PHA/k b.w. were strongly depressed until 3 weeks after tumor implantation and, thereafter, no longer different from the controls (Fig. 1 and 2).

(B) *Treatment with PHA starting together with tumor implantation (day 0 to +3).* Percentages of "tumor takes" varying between 77 and 85 % in animals injected with daily doses of 10, 5 or 2 mg PHA/k b.w. were not significantly different from the control group. Concerning tumor growth, the proliferation of melanomas in mice treated with 4 × 10 mg PHA/kg b.w. slowed with time (Fig. 1) indicating a distinct anti-tumor effect (5-week value of 3·64 ± 0·74 S.D. vs 5·78 g ± 1·6 S.D. of the respective

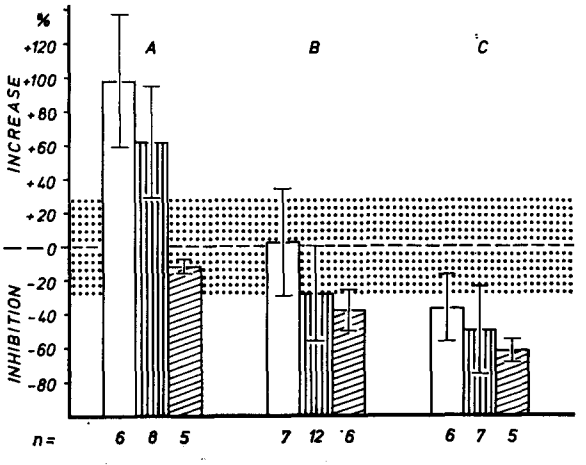


Fig. 3. Effect of varying doses of PHA on the proliferation of Harding–Passey melanoma in mice. Data representing means ± S.D. are expressed as percentages of change in mean tumor weights 5 weeks after tumor implantation in comparison to untreated controls (shaded area). Experimental mice were treated i.v. with daily injections of 2 mg (□), 5 mg (▥) or 10 mg PHA/kg b.w. (▨): A) before (day −4 to −1), B) starting together with (day 0 to +3) or C) after (day +4 to +7) tumor implantation.

control; $P < 0{\cdot}05$). At lower doses of 4 × 5 or 4 × 2 mg/kg b.w. tumor implants grew approximately to the same size as compared to untreated control mice (Fig. 2 and 3). The results of assaying the splenic PFC response to SRBC in tumor-bearing mice injected with 4 × 10 or 4 × 5 mg PHA/kg b.w., again, showed a depressed PFC production during the first 3 weeks after tumor implantation. With time, SRBC-specific responses were no longer different from control values (Fig. 1 and 2).

(C) *Treatment with PHA a few days after tumor implantation (day +4 to +7).* The percentages of "tumor takes" varying between 65 and 70% in mice treated with 4 × 10, 4 × 5 or 4 × 2 mg PHA/kg b.w. were considerably lower than in the untreated control group (82%). The effect of PHA on tumor growth is shown in Fig. 1–3. While treatment with 4 × 2 mg PHA/k b.w. did not significantly alter the proliferation of tumor implants, markedly reduced tumor weights were found in mice injected with 4 × 5 or 4 × 10 mg PHA/kg b.w., the mean values at 5 weeks were 2·87 g ± 1·47 S.D. ($P < 0{\cdot}005$) and 2·2 g ± 0·39 S.D. ($P < 0{\cdot}002$) respectively. Splenic PFC responses to SRBC which were determined in tumor-bearing animals treated with the 2 latter doses of PHA, again, were strongly depressed during the first weeks of the experimental period. Responses had risen to the control level at 5 weeks after tumor implantation (Fig. 1 and 2).

(3) *Serial transfer of tumors from PHA-treated mice to normal recipients (Group III)*

Tumor tissue from mice bearing enhanced grown melanomas after treatment with daily injection of 2 mg PHA/kg b.w. before tumor implantation, or bearing inhibited grown melanomas after treatment with daily injections of 10 mg PHA/kg b.w. from day +4 to +7 after tumor implantation, were inoculated into normal recipients receiving no further treatment. Mean tumor weights, determined at the end of every 5-week interval of 3 successive passages, are illustrated in Fig. 4, results are expressed as percentage-change of the control value. Apparently, PHA-induced enhancement of tumor growth was no longer detectable, however, PHA-induced inhibition of tumor growth was consistently found during all 3 transplant generations.

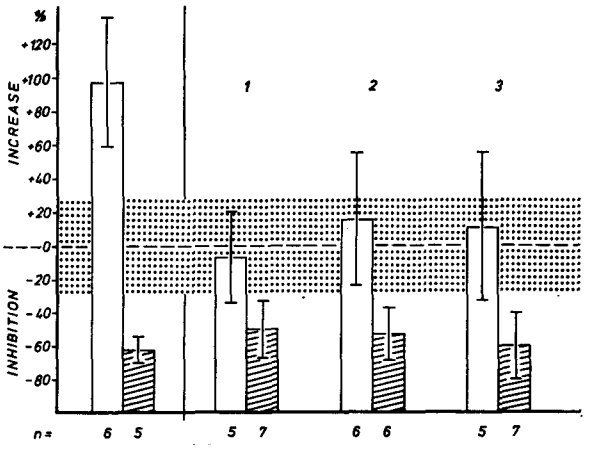


Fig. 4. Transfer of Harding–Passey melanoma grown for 5 weeks in PHA-treated mice to normal recipients. Tumor implants from animals challenged with daily injections of 2 mg PHA/kg b.w. (□) before (day −4 to −1) or treated with daily injections of 10 mg PHA/kg b.w. (▨) after (day +4 to +7) tumor implantation were followed up in 3 transplant generations (1–3) each lasting 5 weeks. Growth rates are expressed as percentages of change in mean tumor weights as compared with untreated controls (shaded area).

DISCUSSION

PHA-induced stimulation as well as inhibition of tumor growth in mice transplanted with Harding–Passey melanoma has been demonstrated in the present study. These effects clearly depend upon the dose of PHA and its time of administration with respect to tumor implantation. When high PHA doses were injected immediately after or a few days after tumor implantation the growth of melanoma cells was inhibited, while enhanced tumor growth was found in mice treated with low doses of PHA before inoculation of melanoma cells.

After stimulation with PHA, T lymphocytes have been shown to exert different activities, such as transformation into blast-like cells, suppression or augmentation of antibody production and induction of lymphocyte-mediated cytotoxicity, i.e., lysis of target cells (Review [10]). The latter effect can be expressed on autologous, allogeneic or heterologous target cells, including various malignant cells [11–15]. Further experiments established that (a) lytic effects on target cells are caused only by cytotoxic effector T cells and not by all mitogen activated T cells [15], and (b) only in the presence of certain agglutinants, such as PHA or concanavalin A, mitogen-induced cytotoxic cells are able to lyse target cells, indicating the requirement of a close contact with the target [15–17]. Since this reaction is thought to be analogous to rejection processes *in vivo*, the inhibitory effect of PHA on tumor growth has been explained by such a mechanism. Alternatively, Robinson and Mekori suggested that this mitogen may act *in vivo* by destroying malignant cells directly [1]. Our present experiments demonstrate that the inhibition of tumor growth is maintained in re-transplanted melanomas. Although an involvement of PHA-induced cytotoxicity of lymphocytes *in vivo* cannot be excluded, this behavior of re-transplanted tumors provides strong evidence for a direct antitumor effect of PHA.

Concerning PHA-induced stimulation of tumor growth, different explanations have been proposed. Unspecific toxic effects of this mitogen on mice leading to general malaise and enhancing tumor proliferation, or the possibility that the phenomenon of immunological enhancement due to blocking antibodies might be involved, has been discussed by Wagener [6]. Since immunosuppressive activities of PHA in various types of T cell-dependent immune phenomena (delayed hypersensitivity [18], graft rejection [19, 20] and graft-vs-host disease [21, 22]) have been demonstrated, enhanced tumor proliferation and higher acceptance of tumor implants in mice treated with PHA were interpreted as an unspecific, mitogen-induced immune deficiency of the host thus, facilitating the establishment and the proliferation of the tumor implant [5, 6]. Our present findings are consistent with the last alternative. When a direct inhibitory effect of PHA of tumor cell growth was minimal (low-dose treatment before tumor implantation), a considerable enhancement of melanoma growth was found. Moreover, all three doses of PHA suppressed the T cell-dependent (humoral) immune response against SRBC. Finally, PHA-induced augmentation of tumor proliferation was no longer observed in re-transplanted tumors. From these observations we conclude that the increased growth of melanoma cells in mice pretreated with PHA results most likely from an inhibition of the surveillance mechanisms against the implanted neoplastic cells.

So far the effect of PHA on tumor growth is a variable one and—if the results obtained here in an animal model system may be considered to be of clinical value—they indicate how critically experimental conditions must be controlled for applying immunotherapy with this mitogen.

Acknowledgements—The authors thank Dr. C. Doenecke for help in statistical evaluation of the data and Miss R. Houy for expert technical assistance.

REFERENCES

1. E. Robinson and T. Mekori, Studies on the effect of phytohaemagglutinin on ascites tumor in mice. *Israel J. med. Sci.* **7,** 83 (1971).
2. E. Robinson, Phytohaemagglutinin and cancer. *Lancet* **i,** 213 (1967).
3. S. P. Datta, M. Cerini, T. Ghose and J. Cerini, Effect of phytohaemagglutinin on Ehrlich ascites carcinoma. *Brit. J. Cancer* **23,** 616 (1969).
4. C. A. Rubio and B. Unsgaard, Immunology and phytohaemagglutinin in cancer. *Lancet* **ii,** 1191 (1966).
5. A. Pappas, G. Schwarze, C. Wolff and P. G. Scheurlen, Enhanced tumor proliferation in mice treated with Phytohaemagglutinin. *Z. Immun.-Forsch.* **145,** 449 (1973).
6. D. J. T. Wagener, Enhanced growth of fibrosarcoma in mice treated with phytohaemagglutinin. *Cancer Res.* **33,** 1295 (1973).
7. T. Mekori and E. Robinson, Further observations on the effect of PHA in conjunction with radiotherapy and chemotherapy on S–180 in Swiss mice. *Europ. J. Cancer* **10,** 67 (1974).
8. N. K. Jerne and A. A. Nordin, Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405 (1963).

9. R. J. Mishell and R. W. Dutton, Immunization of dissociated spleen cell cultures from normal mice. *J. exp. Med.* **126,** 423 (1967).
10. G. Möller (ed.), Lymphocyte Activation by Mitogens. *Transpl. Rev.* **11,** (1972).
11. G. Holm, P. Perlmann and B. Werner, Phytohaemagglutinin induced cytotoxic action of normal lymphoid cells on cells in tissue culture. *Nature (Lond.)* **203,** 841 (1964).
12. E. Möller, Contact induced cytotoxicity by lymphoid cells containing foreign isoantigens. *Science* **147,** 873 (1965).
13. P. Perlmann and G. Holm, Cytotoxic effects on lymphoid cells *in vitro*. *Adv. Immunol.* **11,** 117 (1969).
14. M. Greaves and G. Janossy, Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. *Transpl. Rev.* **11,** 87 (1972).
15. M. J. Bevan and M. Cohn, Cytotoxic effects of antigen- and mitogen-induced T cells on various targets. *J. Immunol.* **114,** 559 (1975).
16. G. Möller, O. Sjöberg and J. Andersson, Mitogen-induced lymphocyte-mediated cytotoxicity *in vitro*: effects of mitogens selectively activating T or B cells. *Europ. J. Immunol.* **2,** 586 (1972).
17. G. L. Asherson, J. Ferluga and G. Janossy, Non-specific cytotoxicity by T cells activated with plant mitogens *in vitro* and the requirement for plant agents during the killing reaction. *Clin. exp. Immunol.* **15,** 573 (1973).
18. J. E. Stevans and D. A. Willoughby, Phytohaemagglutinin and cell-mediated hypersensitivity in rats. *Nature (Lond.)* **215,** 967 (1967).
19. K. Markley, G. Evans and E. Smallman, Prolongation of skin graft survival time by phytohaemagglutinin. *Transplantation* **5,** 1535 (1967).
20. S. S. Stefani and C. D. Moore, Effect of phytohaemagglutinin on skin allograft survival in mice. *J. Immunol.* **104,** 780 (1970).
21. R. L. Hunter, R. B. Millmann and E. M. Lerner, Suppression of graft-versus-host disease and antibody formation by phytohaemagglutinin. Example of antigen competition. *Transplantation* **8,** 413 (1969).
22. Z. Markus, D. A. Rigas and B. V. Siegel, Suppression of graft-versus-host reaction by phytohaemagglutinin. *Experientia* **24,** 836 (1968).

Europ. J. Cancer Vol. 12, pp. 605–610. Pergamon Press 1976. Printed in Great Britain

Immunogenicity and Concanavalin A agglutinability in Transplantable Mouse Lymphosarcoma and Human Leukemia

L. A. SMETS and Ch. HOMBURG

Department of Experimental Cytology, The Antoni van Leeuwenhoek Laboratory, Sarphatistraat 108, *Amsterdam, The Netherlands*

Abstract—*After transfer to tissue culture conditions, transplantable mouse lymphosarcoma cells showed a parallel increase in immunogenicity and in concanavalin A (Con A) directed agglutinability. Injections with cultured tumor cells gave better protection against a challenge with untreated tumor cells than did unmodified, X-ray inactivated cells or cells pretreated with neuraminidase, glutaraldehyde or heat.*

The preservation of cell integrity following i.p. injection was highest for cultured and fixed tumor cells, but neuraminidase or heat-treated cells were rapidly cleared from the intraperitoneal cavity.

The results indicate that the immunogenicity of mouse lymphosarcoma cells was correlated with: (a) the agglutinability with Con A and (b) the persistance of cell integrity following injection.

Human leukemia cells transferred in vitro *acquired also increased agglutinability with concanavalin A, suggesting a concomitant increase in antigenicity.*

INTRODUCTION

NUMEROUS papers report on the use of inactivated tumor cells as a specific vaccine against the tumor of origin in animal model systems as well as in human patients. In many of these studies, the optimal conditions of tumor cell inactivation with preservation of its immunogenic properties are explored. In others, attempts are made to enhance antigen expression by modification of the cell surface, for instance by treatment with neuraminidase [1–3] or iodoacetate [4].

In a previous report [5], we demonstrated that transplantable mouse lymphosarcoma cells acquired increased immunogenicity if propagated in tissue culture for some days, resulting in rejection by untreated hosts following reinoculation. The augmentation of immunogenicity in tissue culture was accompanied by a parallel increase in agglutinability with the plant lectin Concanavalin A (Con A). These results suggested that antigenic expression could be monitored *a priori* in these cells by the agglutination response in the presence of Con A. Moreover, the observation indicated that tumor cells, modified by tissue culture conditions, could probably serve as a vaccine against the tumor of origin (compare ref. [6]).

In the present paper, experiments will be reported regarding the immunogenicity of mouse lymphosarcoma cells propagated in tissue culture as compared to ascites tumor cells inactivated by X-rays. In addition, the immunogenic properties of tumor cells modified in various ways were related to their agglutinability with Con A and the persistence as intact cells *in vivo* following reinoculation. Finally, human leukemia cells were transferred to *in vitro* conditions to investigate whether they underwent changes in Con A agglutinability similar to those observed in mouse lymphosarcoma cells.

Accepted 4 March 1976.

MATERIAL AND METHODS

Cell strains and animals

MB VIA mouse lymphosarcoma cells were grown as transplantable ascites tumor in syngeneic (C_{57} Black × DBA) F_1 hybrid mice and passaged weekly. This transplantable strain has been established from the MB VIA cells propagated in tissue culture as described earlier [5]. MB VIA cells serially transplanted *in vivo* are highly tumorigenic and less than 10 cells will kill all recipients. The MB VIA cells grown *in vitro*, on the other hand, are rejected by untreated hosts but induce tumors if injected in the eye-chamber or in X-irradiated animals.

The non-malignant MB III strain, selected from MB VIA *in vitro* [7], does not induce tumors in conditioned hosts and is negative for a change in glycoproteins accompanying malignancy [8].

Human leukemia cells were obtained from defibrinated blood from patients not subjected to chemotherapy containing over 90% tumor blasts. Purified tumor cells were collected from the interphase of Ficoll–Isopaque gradients which did not affect the agglutinability with Con A [5]. The cells were cultured in RPMI 1640 medium at a density of $4 \cdot 10^6$ cells/ml for 3 days. The cell number did not change in this period.

Immunization procedures

Syngeneic female mice were immunized by two i.p. injections of cells in 0·4 ml saline. Immunizing and challenge injections were administered with intervals of one week. The recipient animals were inspected daily for the development of lethal ascites tumors. Animals surviving for 60 or more days were scored tumor-free. In preliminary experiments, optimal conditions for immunization were explored. Increasing the cell number per immunization dose over $2 \cdot 10^6$ cells did not improve the results. The optimal number of injections was 2–3 and animals injected with 5 or more doses did not survive a subsequent challenge. This phenomenon, which was not explored further, probably reflects some form of immunoparalysis.

Agglutination assay

Con A mediated agglutination was assayed using the procedures described earlier [5]. Agglutinability was expressed quantitatively by the Con A concentration required for half-maximal agglutination in 20 min. The lectin was assayed in graded concentrations and the midpoints were estimated by intrapolation.

Modification of tumor cells

Vibrio comma neuraminidase (Behringwerke AG, Marburg) was used at a concentration of 50 U per ml, pH 5·5 at 37°C. Fixation in glutaraldehyde was performed at 0°C by mixing the cells in saline with an equal volume of 0·250% glutaraldehyde. After 60 min the fixative was removed by centrifugation. X-ray inactivation was carried out with a 250 KeV unit, operated at 8 mA at a dose-rate of 400 roentgen per min and heat-inactivation in a shaking waterbath of 42·5 ± 0·5°C. Concentration of cells during all treatments was $4 \cdot 10^6$ cells/ml. At the end of the treatment, trypan blue was excluded by more than 95% of the cells except for neuraminidase-treatment which caused staining of 20% of the cells.

Recovery studies

Since low numbers of injected cells are difficultly recovered quantitatively from the intraperitoneal cavity, survival of modified cells after injection was determined in per cent of simultaneously injected untreated cells. To this end, tumor-bearing animals were injected i.p. with either ^{3}H- or ^{14}C-labelled thymidine. After 18 hr the ^{3}H-labelled cells were harvested from the donors and subjected to the various treatments. Next, equal numbers of untreated, ^{14}C-labelled cells were added and the mixture containing $5 \cdot 10^6$ treated and $5 \cdot 10^6$ untreated cells was injected. After 5 hr the i.p. cavity was washed three times with saline and the cells were collected by centrifugation. Cell pellets from the original mixture and from the combined washings were dissolved and the ^{3}H- and ^{14}C-radioactivity was counted separately. Recovery of ^{3}H-labelled cells (treated) was calculated in per cent of ^{14}C-labelled cells (untreated).

RESULTS

Immunization with cultured tumor cells

Mice that had received 2 injections of $2 \cdot 10^6$ cells from the *in vitro* grown strain, became resistant against a subsequent challenge with cells from the transplantable *in vivo* strain as demonstrated in Figs. 1 and 2.

Mice immunized with cells from the non-malignant MB III strain did not survive a challenge with transplantable MB VIA cells (Fig. 1) nor did this treatment prolong the latency period of lethal tumors (Fig. 2). Apparently, the immunity acquired by injection of *in vitro* grown malignant cells was directed against tumor-associated antigens and not

against components derived from tissue culture conditions, i.e., membrane-associated calf serum proteins. Injections with MB VIA cells solubilized by sonication failed to induce a detectable level of immunity.

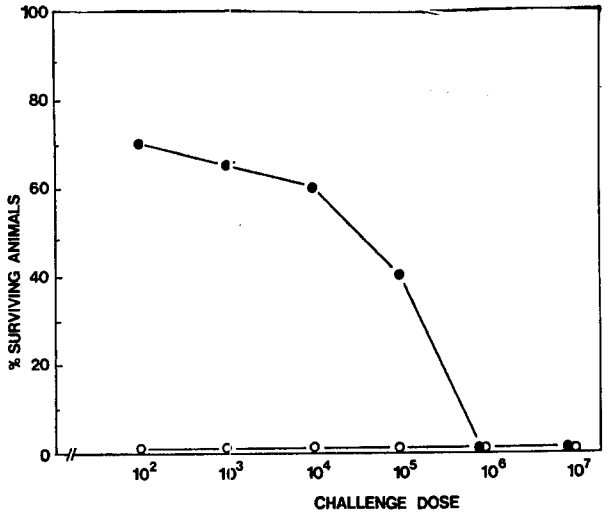


Fig. 1. Immunity of mice against a challenge with transplantable MB VIA tumor cells following immunization with malignant MB VIA or non-malignant MB III cells grown in vitro: *effect of immunization on survival.*

● *Immunized with MB VIA cells*
○ *Immunized with MB III cells*

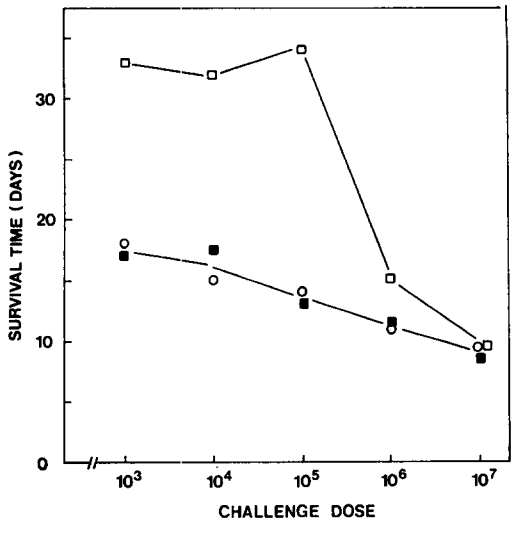


Fig. 2. The same legend as in Fig. 1: effect on surviving time of animals developing lethal tumors.

□ = *Immunized with MB VIA*
■ = *Immunized with MB III*
○ = *Saline controls*

The effect of surface modifications on agglutinability and immunogenic properties

Transplantable tumor cells were harvested from 6-day old ascites tumors and exposed to X-rays, incubated with neuraminidase, fixed in glutaraldehyde, incubated at 42·5°C, or transferred to tissue culture conditions. Surface changes induced by the various treatments were monitored by determination of the Con A mediated agglutinability. As shown in Fig. 3, heat exposure, tissue culture conditions, and treatment with neuraminidase, caused the agglutinability to increase resulting in a 25-fold reduction in the lectin concentration required for half-maximal agglutination. Tumor cells exposed to X-rays and incubated for some hours in tissue culture, did not change their (low) agglutinability (Fig. 3), nor did cells fixed in glutaraldehyde (not shown but compare ref. [9]).

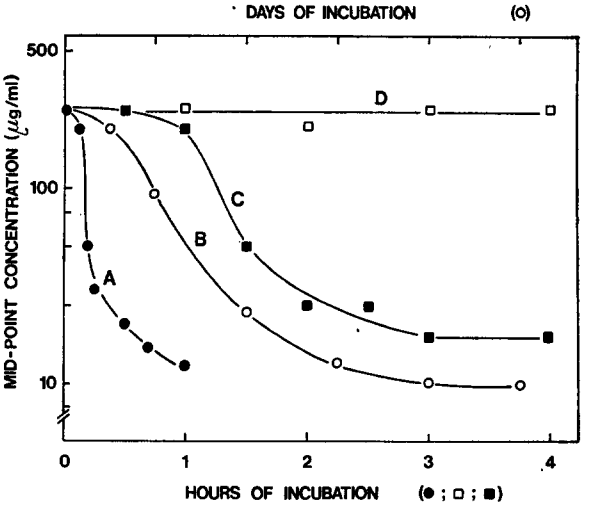


Fig. 3. The effect of various treatments on the Con A agglutinability of MB VIA tumor cells. Agglutinability is expressed by the lectin concentration required for half-maximal agglutination. Curve A: cells incubated in neuraminidase; curve B: explanted in vitro*; curve C: incubated at 42·5°C; curve D: exposed to 8·000 R.*

In a series of experiments, groups of 6 mice were immunized with two doses of $2 \cdot 10^6$ treated cells each and subsequently challenged with 10^4 untreated cells. The conditions of the various treatments were chosen to obtain the maximal degree of agglutinability according to the results in Fig. 3, except for tissue culture cells which were obtained from the *in vitro* grown strain. The outcome of the experiments is summarized in Table 1.

Tumor cells grown in tissue culture imparted a high degree of immunity to the hosts as reflected by a 66% survival rate and almost doubling of the survival time of tumor-positive animals. Irradiated tumor cells were much less effective in protecting mice against the challenge and the latency period of lethal tumors was prolonged only with an average of 2 days. With glutaraldehyde- or neuraminidase-treated cells, no protection at all could be obtained in

Table 1. *The effect of pretreatment on the immunogenic properties of MB VIA tumor cells*

	Pretreatment of cells	Surviving controls* ns/nt†	%	Surviving immunized animals‡ ns/nt	%	Prolongation of survival time (days) §
A.	Tissue culture	60/60	100	20/30	66	15
B.	X-irradiation (8000 Rö)	30/30	100	5/30	17	2
C.	Glutaraldehyde; 0·125%, 1 hr	12/12	100	0/12	0	0
D.	Neuraminidase; 1 hr, 50 U/ml	12/12	100	0/12	0	0
E.	42·5°C; 3 hr	0/12	0	N.T.	—	—

*Immunized twice with $2 \cdot 10^6$ treated cells but not challenged with ascites tumor cells.
†Number of surviving animals/number of animals.
‡Challenged with 10^4 untreated ascites tumor cells.
§Of the non-surviving animals.

these experiments. All unchallenged control animals injected with heat-treated cells developed lethal tumors indicating insufficient inactivation and low immunogenicity by this treatment, in spite of its reported effect on the viability of murine leukemia cells [20].

Next, it was investigated how the various treatments of tumor cells affected their stay in the intraperitoneal cavity following i.p. injection. As shown in Table 2, cellular integrity was best retained by glutaraldehyde fixed cells or by the *in vitro* grown tumor cells, whereas most of the heat or neuraminidase-treated cells had disappeared from the intraperitoneal cavity within 5 hr following injection. These differences can not be inferred from the effects on trypan blue exclusion (see Material and Methods).

Table 2. *Recovery of pretreated MB VIA ascites tumor cells*

Pretreatment conditions	% intact cells recovered after 5 hr *in vivo**
A. 42·5°C; 1 hr	13%
B. Neuraminidase 1 hr, 50 U/ml	35%
C. X-rays; 8·000 Rö	72%
D. Glutaraldehyde; 1 hr, 0·125%	88%
E. Tissue culture	76%

*In per cent of admixed untreated tumor cells.

Agglutinability of human leukemic cells in vivo *and* in vitro

Tumor cells collected from the peripheral blood of a number of patients with leukemia or leukemic transformation of lymphosarcoma were assayed for Con A agglutinability immediately following isolation or after 3 days of tissue culture. The results, including measurements of mouse lymphosarcoma, are shown in Table 3. Increased agglutinability was observed

Table 3. *The effect of tissue culture on the agglutinability of various human leukemia cells*

Patient	Diagnosis*	Con A concentration† (μg/ml) Fresh cells	3 days *in vitro*
1. v.D.	AML	> 500	30–40
2. Co.	ALL	> 500	> 500
3. Bo.	CML	> 500	20–30
4. Sch.	CLL	> 250	> 250
5. Go.	ALL	> 500	50
6. Teu.	AML	> 1000	50
7. Ha.	LS	> 500	50
MB VIA mouse lymphsarcoma		250	10–15

*AML: acute myeloid leukemia.
ALL: acute lymphocytic leukemia.
CML: chronic myeloid leukemia.
CLL: chronic lymphocytic leukemia.
LS: lymphosarcoma (leukemic transformed).
†Lectin concentration required for half-maximal agglutination.

in 5 out of 7 cases of leukemia. The cells of the 2 negative cases (pats. 2.4) were obtained from terminal patients with a high white cell number exceeding 10^5 cells/mm^3. These cells were very small and metabolically inactive since

they did not change the pH of the culture medium. The failure to acquire a high level of agglutinability in these cases was probably not related to the type of the disease but to the condition of the cells. The relative low initial agglutinability of the CLL case as compared to reported values [10] may be ascribed to the same bad condition of the cells.

DISCUSSION

In the present report, we have demonstrated that the MB VIA tumor cells, grown *in vitro*, are a potent cellular vaccine directed against the homologous tumor cells. The immunizing potential of these cells was far better than of ascites tumor cells inactivated by X-irradiation (Table 1) and is most likely due to changes at the cell periphery resulting from tissue culture conditions.

Most tumor cells *in vitro* as well as *in vivo* are characterized by increased levels of proteolytic enzymes at or near the cell surface [11, 12]. These membrane-associated enzymes are capable of modifying the surface of neighbouring cells [13] and cell contact dependent changes in agglutinability with Con A have been demonstrated in transformed fibroblasts [14]. It is significant for the depicted concept of mutual modification of MB VIA cells that the acquisition of high agglutinability and high immunogenicity in tissue culture occurs only under conditions of intensive cell interactions [5].

Con A is a known mitogenic compound whose interaction with its receptor in many respects resembles the antigen-antibody reaction [15]. Therefore, the enhanced agglutinability of tumor cells kept *in vitro* may well be representative for the increased efficiency with which these cells are processed by the immune system. In mouse adenocarcinoma cells, similar correlations between Con A agglutinability on the one hand and transplantability cq. antigen-expression on the other hand have been observed [16, 17].

However, as shown in Table 2, high agglutinability alone is not a sufficient condition for successful immunization. In our assay, neuraminidase-treated cells were ineffective although many successful applications of this pretreatment have been reported [1–3]. The rapid clearance of these cells (Table 2) indicates that the presence of intact cells for prolonged periods is necessary for optimal immunization. The disappearance of neuraminidase-treated cells is most probably due to membrane changes induced by the enzyme [18] and to increased lysis by normal serum of murine lymphoid cells [19]. Similarly, the rapid clearance of heat-treated cells apparently prevented the development of immunity against surviving cells which killed the animals during immunization (Tables 1 and 2). On the other hand, the immobilized or chemically modified antigenic sites on glutaraldehyde-fixed cells were probably insufficiently accessible for immunorecognition in spite of longer stay of intact cells in the animal.

Thus, it appears from these studies that MB VIA cells *in vitro* are subjected to subtile membrane changes that are recognized as increased agglutinability with Con A and that potentiate the immune response. At the same time, cellular integrity is retained to provide for prolonged survival *in vivo* following injection. The possible involvement of virus particles in the increased immunogenicity of MB VIA cells grown *in vitro* [7] is not excluded. Studies of these membrane modulations may be valuable in the design of optimal cellular vaccines. The possible clinical value of the Con A assay is indicated in the pilot studies with human leukemia cells (Table 3). Human leukemia cells pretreated to enhance the immunogenicity for immunotherapeutic purposes, may be successfully monitored first by the relatively simple agglutination assay.

REFERENCES

1. G. A. Currie and K. D. Bagshawe, The role of sialic acid in antigenic expression: further studies of the Landschütz ascites tumor. *Brit. J. Cancer* **22,** 843 (1968).
2. J. G. Bekesi, G. St. Arneault and J. F. Holland, Increase of leukemia L–1210 cells immunogenicity after neuraminidase treatment. *Amer. Soc. Hematol.*, 13th Ann. Mtg. Program, No. 115,78 (1970).
3. A. Rios and R. L. Simmons. Immunospecific regression of various syngeneic mouse tumours in response to neuraminidase-treated tumor cells. *J. nat. Cancer Inst.* **51,** 637 (1973).
4. C. A. Apffel and B. G. Arnason, Introduction of tumour immunity with tumour cells treated with iodoacetate. *Nature (Lond.)* **209,** 694 (1966).

5. L. A. Smets and J. Broekhuyzen-Davies, Shielding of antigens and concanavaline A agglutination sites by a surface coat of transplantable mouse lymphosarcoma cells. *Europ. J. Cancer* **8,** 541 (1972).
6. J. Moore, J. Keiler and B. Biczowa, Comparative studies of a near tetraploid and a near diploid line of Ehrlich ascites tumor propagated *in vivo* and *in vitro*. II. Cytology and transplantability. *Eur. J. Cancer* **4,** 18 (1968).
7. W. de Bruyn and E. Benedetti, Studies on non-tumor and tumor producing cell-lines derived from transplantable mouse lymphosarcoma MB T86157. *Proc. 9th Congr. Europ. Soc. Hemat.*, Vol. II, 663, Karoer, Basle (1963).
8. W. P. van Beek, L. A. Smets and P. Emmelot, Increased sialic acid density in surface glycoprotein of transformed and malignant cells—a general phenomenon? *Cancer Res.* **33,** 2913 (1973).
9. Tateshi Kataoka, Shigeru Tsukagoshi and Yoshio Sakurai, Transplantability of L-1210 cells in BALB/c × DBA/2 F_1 mice associated with cell agglutinability by concanavalin A. *Cancer Res.* **35,** 531 (1975).
10. U. Mintz and L. Sachs, Membrane differences in peripheral blood lymphocytes from patients with chronic lymphocytic leukemia and Hodgkin's disease. *Proc. Acad. Sci.* **72,** 2428 (1975).
11. B. Sylvén and S. Bois-Svensson, On the chemical pathology of interstitial fluid. I. Proteolytic activities in transplanted tumours. *Cancer Res.* **25,** 468 (1965).
12. B. Sylvén, O. Snellman and P. Straüli, Immunofluorescent studies on the occurrence of cathepsin B1 at tumor cell surfaces. Virchows Arch., B, *Cell Path.* **17,** 97 (1974).
13. M. Burger, Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature (Lond.)* **227,** 170 (1970).
14. L. A. Smets, L. de Ley and J. G. Collard, Contact-mediated changes in cytoagglutination. *Exp. Cell Res.* **95,** 95 (1975).
15. G. Nicolson, The interactions of lectins with animal cell surfaces. *Int. Rev. Cytol.* **39,** 89 (1974).
16. S. Friberg Jr., Comparison of an immunoresistant and an immunosusceptible ascites subline from Murine Tumor TA3. I. Transplantability, Morphology, and some physicochemical characteristics. *J. nat. Cancer Inst.* **48,** 1463 (1972).
17. S. Friberg Jr., Comparison of an immunoresistant and an immunosusceptible ascites subline from murine tumor TA3. II. Immunosensitivity and antibody-binding capacity *in vitro* and immunogenicity in allogeneic mice. *J. nat. Cancer Inst.* **48,** 1477 (1972).
18. T. S. Hauschka, L. Weiss, B. A. Holdridge, T. L. Cudney, M. Zumpft and J. A. Planinsek, Karyotypic and surface features of murine TA_3 carcinoma cells during immunoselection in mice and rats. *J. nat. Cancer Inst.* **47,** 343 (1971).
19. P. K. Ray and R. L. Simmons, Unmasking of xenogeneic neoantigens on mouse lymphoid cell surfaces by vibrio cholera neuraminidase. *Proc. Soc. exp. Biol. (N.Y.)* **142,** 217 (1973).
20. B. Giovanella, W. Lohman and Ch. Heidelberger, Effects of elevated temperatures and drugs on the viability of L1210 leukemia cells. *Cancer Res.* **30,** 1623 (1970).

Europ. J. Cancer Vol. 12, pp. 611–616. Pergamon Press 1976. Printed in Great Britain

Deoxycytidylate Deaminase Activity and its Template Stability in Human Acute Lymphoblastic and Myeloblastic Leukaemic Cells*

MARIA GRAZIA BERNENGO and LUIGI PEGORARO
Sezione di Ematologia, Clinica Medica Generale dell'Universita di Torino, Torino, Italia

Abstract—*The activity of deoxycytidylate (dCMP) deaminase, an enzyme of the main pathway of the DNA synthesis and the effects of actinomycin D upon its template stability were studied in the peripheral blast cells of 11 acute leukaemia patients. The relationship between enzyme activity, template stability, cytochemical characteristics and proliferative activity of the blast cells was also investigated.*

By employing current cytochemical techniques, 6 of the 11 cases were classified as lymphoblastic and 5 as myeloblastic. The proliferative activity was evaluated with autoradiographic technique following short incubation with ^{3}H-thymidine. Acute myeloblastic leukaemia (AML) cells displayed higher enzyme activity than acute lymphoblastic leukaemia (ALL) cells. In addition, a direct correlation between the enzyme activity and the proliferative activity was shown in AML cells.

dCMP deaminase template stability was assayed at the end of a 9 hr culture time in presence of actinomycin D at a dose which inhibited 90% of ^{3}H-uridine incorporation into the RNA of both AML and ALL cells. While none of the cases of AML showed significant change of the dCMP deaminase activity after actinomycin D treatment, in all cases of ALL a marked decrease of the enzyme activity was observed, suggesting that, at least in the steady state conditions, the AML and the ALL cells have a different rate of degradation of the RNA templates specific for the dCMP deaminase and that the rate of degradation is related to the proliferative activity of the cells. In fact, in the ALL cases a significant correlation was observed between the proliferative activity and the decrease of the enzyme activity induced by actinomycin D.

INTRODUCTION

THE CLASSIFICATION of the myeloblastic and lymphoblastic forms of human acute leukaemias is mainly based upon morphological and cytochemical criteria. In addition, the blast cells of these forms have been shown to exhibit different cytogenetic [1, 2] and kinetic [3, 4] characteristics. However, with few exceptions [5], biochemical studies on human acute leukaemias have been done without taking into separate consideration the lymphoblastic and the myeloblastic forms.

Because of their relationship to chemotherapy the enzymes of the DNA synthetic pathway have been extensively investigated in the acute leukaemias and thoroughly reviewed [6]. The main aim of these studies was the search for either differences between normal leukocytes and leukaemic cells or for the changes induced by chemotherapic agents in the activity of these enzymes in leukaemic cells. On the other hand, since the treatments for ALL and for AML are becoming increasingly specific, the finding of differences between these two types of leukaemia might lead to a more rational design of therapeutic schemes.

dCMP deaminase is an enzyme of the main pathway leading to the synthesis of thymidylate. In the present communication we report on studies on the activity of this enzyme in acute leukaemia cells and on changes of this activity after actinomycin D treatment. Acute leukaemia cases were classified, according to

Accepted 20 February 1976.

*Supported by Grant 71.00823.04 from CNR Rome.

the current morphological and cytochemical criteria, as ALL or AML. In each case the dCMP deaminase activity was compared both with cytochemical characteristics and with the proliferative activity of the blast cells.

MATERIAL AND METHODS

The cells studied were obtained from the peripheral blood of 11 acute leukaemia untreated patients. The age of the patients ranged between 4 and 75 y. Peripheral leukocyte levels ranged from 30,000 to 274,000 of which more than 67% were blast cells. The heparinized venous blood was allowed to sediment at 37°C and the leukocyte layer was separated, diluted with MEM (Gibco) containing 20% of autochtonous plasma to obtain a cell concentration of $3–6 \times 10^6$ cells/ml, and distributed in 7 ml aliquots into glass screw cap flasks of 25 ml total capacity. The cultures were incubated at 37°C for 9 hr in atmosphere of 95% balanced air and 5% CO_2. Actinomycin D (a gift from Merck, Sharp & Dohme), when used, was added to a final concentration of 0·5 μg/ml. All cultures were done in triplicate. The viability of the cells when the cultures were arrested was checked by their ability to exclude the dye trypan blue [7]. The cultures were harvested by centrifugation and contaminating erythrocytes lysed by osmotic shock [8]. This treatment did not produce any significant change in the enzyme activity.

For the determination of deoxycytidylate deaminase activity [9] approximately 4×10^7 cells were homogenized in 0·5 ml of 0·15 M KCl, 3 mM 2-mercaptoethanol, 10 mM Tris–HCl pH8 at 20°C, 0·31 mM dCTP, with a motor driven glass homogenizer of 1 ml total capacity. The homogenate was centrifuged at 30,000 *g* at 4°C for 15 min in a Sorvall refrigerated ultracentrifuge and the supernatant used as the source of the enzyme. The reaction contained, in a final volume of 0·25 ml: dCTP (Sigma) 20 nmoles, $MgCl_2$ ·5 μmoles, 2-mercaptoethanol 0·75 μmoles, NaF 3·7 μmoles, Tris–HCl (pH 8 at 20°C) 21 μmoles, ^{3}H-dCMP (Amersham) 25 μmoles ($2·77 \times 10^6$ dis/min) and enzyme extract consisting of 20–80 μg of protein. After 10 min of incubation at 37°C the reaction was stopped by addition of 0·25 ml of 0·3 N perchloric acid. The enzyme activity was linear up to 60 min and was proportional to a broad range of protein concentration.

dCMP and dUMP were separated by descending chromatography on DEAE 81 Whatman paper strips with 4 N formic acid and 0·1 N ammonium formate as the solvent. The strips were cut into 2 cm sections and counted in omnifluortoluene (NEN). The protein concentration of the enzyme extract was determined by the method of Lowry *et al.* [10].

In order to study RNA synthesis cultures were incubated for 9 hr with and without actinomycin D. Uridine ^{3}H-5-T (24·6 Ci/mM) was added 2 hr before harvesting the cultures at a concentration of 10 μCi/ml. The cells were harvested and washed twice with cold buffered saline solution. The pellet was then suspended in 5% cold TCA. After 30 min at 0°C the precipitate was filtered through a millipore filter and the filters washed 3 times with 5% TCA. The filters were dissolved in 1 ml cellosolve and mixed with 15 ml of liquifluor for the determination of the radioactivity in a Nuclear Chicago scintillation spectrometer model Mark 1.

For the determination of the labelling indices 1 ml aliquots of peripheral blood were incubated for 1 hr with ^{3}H-thymidine (Amersham, 6·7 Ci/mM) at the concentration of 1 μCi/Ml. Slides were processed for autoradiography [11] and the fraction of cells in S determined on at least 1000 cells.

The peroxidase reaction was determined by the method of Hayhoe [12], the Sudan black reaction by the method of Sheehan and Storey [13], and the periodid acid Schiff(PAS) reaction by the method of Hayhoe *et al.* [12]. Positive peroxidase reactions were considered those where weak diffuse granules or a tinge confined to Auer rods were present in the blast cells; positive Sudan black reactions those where blast cytoplasm showed variable amounts of sudanophilic granules or else only Sudan positive Auer rods; positive PAS reactions those where there was a crown of cytoplasmic granules or a single block of glycogen even if this particular type of staining reaction was present in only a very small number of immature cells. On the contrary, if blast cells were completely negative or if they showed a weak diffuse tinge or, more exceptionally, if their cytoplasm contained PAS positive Auer rods, PAS reactions were considered negative [12].

RESULTS

The main features of the cases used for the present study are listed in Table 1. Leukaemias were classified as lymphoblastic or myeloblastic according to the following criteria of recognition. The group of lymphoblastic leukaemias included those with a high nucleocytoplasmic ratio, absence of Auer rods and of

Table 1. *Clinical and cytochemical data of the acute leukaemia cases*

	Case No.	Patient	Age (years)	Sex	WBC/ cu mm $\times 10^3$	% of blasts	Sudan black	Peroxidase	P A S
Acute	1	A. P.	6	F	66	90	+	+	—
myeloblastic	2	C. V.	22	F	274	87	+	+	—
leukaemia	3	S. E.	10	M	14	84	+	+	—
	4	R. A.	70	F	184·6	86	+	+	—
	5	C. M.	70	F	15	67	+	+	—
	6	P. F.	75	F	51	85	—	—	+
Acute	7	A. V.	6	F	40	97	—	—	+
lymphoblastic	8	C. P.	4	M	16	86	—	—	+
leukaemia	9	R. M.	45	M	16	93	—	—	+
	10	R. A.	65	F	171	84	—	—	+
	11	M. O.	4	F	235	93	—	—	+

other intracellular structures, negative peroxidase and Sudan black reactions and positive PAS reaction. The myeloblastic forms were considered those with a lower nucleo-cytoplasmic ratio, presence of Auer rods and/or of azurofil granulations, positivity of peroxidases and Sudan black reactions and negativity of PAS reaction.

The results of the determination of dCMP deaminase activity and of the effect of actinomycin D upon the enzyme activity are shown in Table 2. The values of the average labelling index of the blast cells population of each case are also reported. The effect of actinomycin D is shown in the table as the percentage of inhibition of enzyme activity, taking as 100 the enzyme activity of the untreated cultures. The effectiveness of the dose employed was checked by its ability to reduce uridine incorporation into RNA. In both lymphoblastic and myeloblastic leukaemias actinomycin D inhibited uridine incorporation more than 90%. Furthermore, under these conditions, actinomycin D did not produce any gross morphological change in the cells nor differences in the cell viability, as judged by the trypan blue exclusion test, between treated and control cultures.

Table 2 shows that the dCMP deaminase activity is significantly ($P < 0{\cdot}05$) higher in AML than in ALL cells. In addition, a close correlation was observed between dCMP deaminase activity and the percentage of labelled cells in the AML forms (Fig. 1). Enzyme activity was directly proportional to the proliferative activity. On the contrary, in acute

Table 2. *Deoxycytidylate deaminase activity and inhibition of the enzyme activity following actinomycin D treatment*

	Case	*mμmoles of dCMP deaminated/min/ mg of protein	% of inhibition	†Labelling indices
	1	13·0 ± 1.	0	2·6
Acute	2	20·8 ± 0·7	0	3·1
myeloblastic	3	27·4 ± 0·1	0	3·7
leukaemia	4	35·9 ± 19·4	0	4·3
	5	48·6 ± 7·1	4	5
	6	24 ± 1·3	73	1
Acute	7	23·6 ± 3·5	68·5	2.4
lymphoblastic	8	4·8 ± 2·5	68	2·2
leukaemia	9	2·5 ± 1·1	42	9·8
	10	12·4 ± 0·3	14	18·4
	11	9·4 ± 0·3	10·3	19·6

*Each number is the average of three determinations.

†Percentage of ^{3}H–TdR labelled blast cells.

lymphoblastic leukaemia dCMP deaminase activity exhibited a greater range of variability and was not related with the cell proliferative rate.

The effect of actinomycin D upon the activity of the enzyme revealed different behaviour pattern between the two types of leukaemias. dCMP deaminase activity in all cases of AML was not affected by the 9-hr actinomycin D treatment, while in all cases of ALL a decrease of enzyme activity was observed. Since the degree of inhibition varied from case to case a possible relationship of the actinomycin effects with the proliferative activity of the ALL cells was investigated. A close correlation (Fig. 2) was observed between the template stability and the labelling indices.

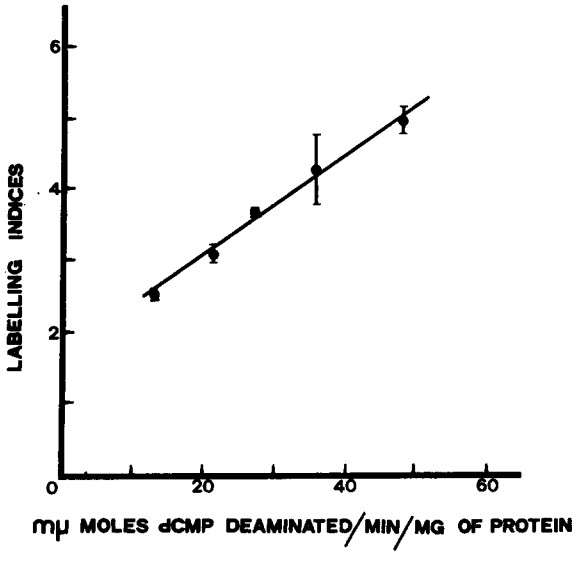


Fig. 1. Correlation between the percentage of labelled blast cells (ordinate) and the dCMP deaminase activity (abscissa), expressed in mμmoles deaminated/minute/mg of protein, in the AML cases.

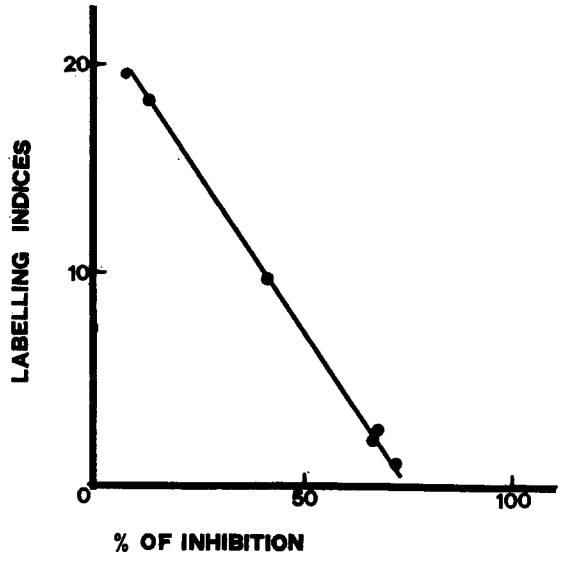


Fig. 2. Correlation between the percentage of labelled blast cells (ordinate) and the percentage of inhibition of the dCMP deaminase activity by actinomycin D (abscissa) in the ALL cases. (σ — 0·15%).

DISCUSSION

The results of this study show that the blast cells of AML and of ALL possess a different ability to deaminate dCMP. In fact, the activity of dCMP deaminase was found to be higher in the myeloblastic than in the lymphoblastic forms in spite of the fact that the latter possess a higher proportion of cells in the S phase of the cell cycle. In AML a direct correlation was found between the proliferative activity and the enzyme activity of the blast cells.

Increased activity of some of the enzymes of the DNA synthetic pathway, including dCMP deaminase have been shown to occur preceding or concomitantly with DNA synthesis in a number of systems [14, 15]. It is not surprising therefore that those AML cases with a higher fraction of cells in S and hence, with a higher demand of TMP synthesis, also possess a higher dCMP deaminase activity. It is not clear, however, why in the ALL forms such correlation is absent. It is possible that this represents an imbalance in the regulation of the enzyme molecules synthesis or activity. However, one must also consider the possibility that the group of ALL is heterogeneous in the sense that many subgroups of ALL are included in it. Cytochemical investigations seems to support this view [16].

The main difference found in the present study between the AML and ALL forms concerns the stability of the enzyme activity to actinomycin D treatment. Actinomycin D, at the dose we used, almost completely inhibits RNA synthesis, in both AML and ALL cells, and hence prevents the synthesis of new templates. It is therefore likely that a decrease of dCMP deaminase activity in the treated cultures is due to depletion of the RNA templates specific for this enzyme. The fact that after 9 hr of actinomycin D treatment the activity of dCMP deaminase in AML cells it is unchanged while in ALL cells it is strongly decreased suggests that the former possess a more stable template for this enzyme. The stability of the templates of the enzymes of the DNA synthetic pathway has been extensively studied, with the aid of actinomycin D, both in systems of stimulated DNA synthesis and in continuously

dividing normal and neoplastic cells. In quiescent or G_0 cells RNA templates were found, in most instances, to be more stable than in cells stimulated to undergo to DNA synthesis [17–21]. We have shown that the treatment of PHA stimulated normal lymphocytes with actinomycin D for 9 hr preceding the maximum DNA synthesis decreases to 50% of the dCMP deaminase activity. Conversely, in unstimulated lymphocytes, treatment for 9 hr with actinomycin D does not affect the enzyme activity [22]. The stability of dCMP deaminase activity to actinomycin D treatment has also been found to be greater in normal than in regenerating liver [17]. In rapidly proliferating normal and neoplastic cells little or no effect of actinomycin D has been shown upon the activity of the enzymes of the DNA synthetic pathway [23–25].

In the present study AML cells, when treated with actinomycin D, behave in the same way as Novikoff hepatoma and Ehrlich ascites cells, while, in most cases, ALL cells are very similar to cells stimulated to DNA synthesis. Furthermore, in the ALL a correlation between the dCMP deaminase template lability and the proliferative activity of the blast cells was found, suggesting that the half life of the RNA templates is directly proportional to the percent of cycling cells. Since the enzyme level within a cell is dependent not only upon the stability of its templates but also upon the turnover rate of the enzyme protein itself, the difference between AML and ALL cells could depend upon a different half life of the dCMP deaminase molecules. The fact that other systems such as normal adult and regenerating liver possess equally stable thymidine kinase but different turnover for the template of this enzyme may suggest that also AML and ALL cells have a similar pattern [26]. Unfortunately, our attempts to evaluate the half life of dCMP deaminase with cycloeximide treatment in acute leukaemic cells were unsuccessful. We cannot therefore assert from our results whether the difference between the ALL and the AML cells lies at the level of transcription or at the level of degradation of the enzyme molecules. Our data concerning the dCMP deaminase have shown differences between AML and ALL both in the activity of the enzyme and in effects of actinomycin D upon this activity. It may be worth further studies to see whether this difference is limited to dCMP deaminase or is it common to other enzymes of the DNA synthetic process.

REFERENCES

1. A. A. Sandberg, T. Ishihara, Y. Kikuchi and L. H. Crosswhite, Cromosomal differences among the acute leukemias. *Ann. N.Y. Acad. Sci.* **113,** 663, (1964).
2. L. E. Reisman, W. W. Zuelzer and R. I. Thompson, Further observation on the role of aneuploidy in acute leukemia. *Cancer Res.* **24,** 1448 (1964).
3. F. Gavosto, A. Pileri, A. Ponzone, P. Masera, R. P. Tarocco and V. Gabutti, Different blast kinetics in acute myeloblastic and lymphoblastic leukaemia. *Acta. Haemat.* **41**, 215 (1969).
4. F. Gavosto, Observations on the kinetics of acute lymphoblastic leukaemia in relation to its division into subvarietes. In *Recent Results in Cancer Research.* (Edited by G. Mathe, P. Pouillart and L. Schwarzenberg) Vol. 43, p. 76 (1972).
5. D. Roberts and T. C. Hall, Enzyme activities and deoxynucleoside utilization of leukemic leukocytes in relation to drug therapy and resistance. *Cancer Res.* **29,** 166 (1969).
6. R. C. Gallo, Synthesis and metabolism of DNA and DNA precursors by human normal and leukemic leukocytes. *Acta Haemat.* **45,** 136 (1971).
7. A. M. Pappenheimer, Experimental studies upon lymphocytes. I. The reactions of lymphocytes under various experimental conditions. *J. exp. Med.* **25,** 633 (1916).
8. Y. Rabinowitz, DNA polymerase and carbohydrate metabolizing enzyme content of normal and leukemic glass column separated leukocytes. *Blood* **27,** 779 (1962).
9. F. Maley and G. F. Maley, Nucleotide interconversions. II. Elevation of deoxycytidylate deaminase and thymidylate synthetase in regenerating rat liver. *J. biol. Chem.* **235,** 2968 (1960).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).

11. F. Gavosto, A. Pileri, C. Bachi and L. Pegoraro, Proliferation and maturation defect in acute leukaemia cells. *Nature (Lond.)* **203,** 92 (1964).
12. F. G. J. Hayhoe, D. Quaglino and R. Doll, The cytology and cytochemistry of acute leukaemias. *M. R. C. Special Report Series* n. 304, p. 16, London (1964).
13. H. L. Sheehan and G. W. Storey, An improved method of staining leukocyte granules with Sudan black. *J. Path. Bact.* **59,** 336 (1947).
14. A. S. Gelbard, J. H. Kim and A. G. Perez, Fluctuations in deoxycytidine monophosphate deaminase activity during the cell cycle in synchronous populations of HeLa cells. *Biochim. biophys. Acta* **182,** 564 (1969).
15. E. Stubblefield and S. Murphree, Synchronized mammalian cell cultures. II. Thymidine kinase activity in Colcemid synchronized fibroblasts. *Exp. Cell Res.* **48,** 652 (1967).
16. G. Mathe, P. Pouillart, M. Sterescu, J. L. Amiel, L. Schwarzenberg, M. Schneider, M. Hayat, F. De Vassal, C. Jasmin and M. Lafleur, Subdivision of classical varieties of acute leukemia. Correlation with prognosis and cure expectancy. *Rev. franc. Etud. clin. biol.* **16,** 554 (1971)
17. I. Lieberman, R. Abrams and P. Ove, Changes in the metabolism of ribonucleic acid preceding the synthesis of deoxyribonucleic acid in Mammalian cells cultured from the animal. *J. biol. Chem.* **238**, 2141 (1963).
18. R. L. Holtzer, A. Oda and M. Chiga, Effect of actinomycin D on deoxycytidylate deaminase activity of rat liver after partial hepatectomy. *Lab. Invest.* **13,** 1514 (1964).
19. H. C. Pitot, C. Peraino, Lamar Jr. and A. L. Kennan, Template stability of some enzymes in rat liver and hepatoma. *Proc. nat. Acad. Sci. (Wash.)* **51,** 810 (1964).
20. G. F. Maley, M. G. Lorenson and F. Maley, Inhibitors of protein synthesis: effect on the levels of deoxycytidylate deaminase, thymidylate synthetase, and thymidine kinase in regenerating rat liver. *Biochem. biophys. Res. Comm.* **18**, 364 (1965).
21. L. Pegoraro and R. Baserga, Time of appearance of deoxythymidylate kinase and deoxythymidylate synthetase and of their templates in isoproterenol-stimulated deoxyribonucleic acid synthesis. *Lab. Invest.* **22,** 266 (1970).
22. L. Pegoraro and M. G. Bernengo, Thymidine kinase, deoxycytidine kinase and deoxycytidylate deaminase activities in phytohaemagglutinin stimulated human lymphocytes. *Exp. Cell Res.* **68,** 283 (1971).
23. J. S. Roth, Increased stability of templates for some enzymes of DNA synthesis in tumors: Deoxycytidylate deaminase, thymidine and thymidine kinase. *Life Sci.* **3,** 1145 (1964).
24. R. Baserga, R. D. Estensen, R. O. Petersen and J. P. Layde, Inhibition of DNA synthesis in Ehrlich ascites cells by Actinomycin D. I. Delayed inhibition by low doses. *Proc. nat. Acad. Sci. (Wash.)* **54,** 745 (1956).
25. E. Bresnick, S. S. Williams and H. Mosse, Rates of turnover of deoxythymidine kinase and of its template RNA regenerating and control liver. *Cancer Res.* **27,** 469 (1967).
26. E. Bresnick and S. S. Burleson, Rates of turnover of deoxythymidine kinase and of its template RNA in Novikoff Hepatoma and neonatal liver. *Cancer Res.* **30,** 1060 (1970).

Europ. J. Cancer Vol. 12, pp. 617–623. Pergamon Press 1976. Printed in Great Britain

Sequential Use of Methotrexate, Folinic Acid, and Cytosine Arabinoside in the Treatment of Acute Leukemia*

MICHAEL L. GREENBERG† and SAMUEL WAXMAN
Divisions of Hematology and Oncology, Dept. of Medicine, Mt. Sinai School of Medicine, New York, N.Y.

Abstract—*Methotrexate, folinic acid and cytosine arabinoside were used in sequence over 48 hr in 12 courses of treatment in 3 patients with lymphoblastic and 2 with myelomonoblastic leukemia, all of whom had become resistant to conventional chemotherapy, in an attempt to develop a means of synchronizing and/or recruiting blast cells for more effective chemotherapeutic effect. Three patients had partial, and two had complete remissions. Serial marrow specimens were studied. Methotrexate* in vivo *and* in vitro *blocked* de novo *DNA synthesis; most patients had a compensatory increase in the salvage pathway. The labeling index was unchanged or increased by methotrexate* in vivo, *and the mitotic index was markedly decreased. Folinic acid "rescue" incompletely restored* de novo *DNA synthesis but usually increased the labeling index. Cytosine arabinoside then markedly decreased the labeling index. The recovery of labeling and mitotic indices to pretreatment levels took 1–8 days after treatment. These studies demonstrate the biochemical, cellular kinetic and clinical feasibility of this approach to therapy.*

INTRODUCTION

MANY of the drugs used in the treatment of the acute leukemias have a major effect on one phase or another of the cell replicative cycle, as has been recently reviewed [1, 2]. A current model of leukemic blast cell replication suggests that only a portion of the blasts are in the active proliferative cycle [1–3]. The quiescent cells may be end stage cells which are destined to die, G_0 cells which will divide again under proper stimulation, cells with a long postmitotic rest time (G_1), or a combination of these. Destruction of the cells in one phase of the cell cycle may result in (1) synchronization of the remaining cells, i.e., a greater than usual fraction will now be in a given phase of the cycle, and/or (2) recruitment, i.e., quiescent cells in a G_0 or long G_1 phase will be stimulated into active replication. A decrease in the number of tumor cells or products of their destruction may actively stimulate replication of the remaining tumor cells or may remove an inhibitory factor. The distinction between G_0 and prolonged G_1 is presently not experimentally definable.

Several groups [3–6] have attempted to use the above principles combined with a knowledge of the effect of various drugs on the cell cycle (see Discussion) in the treatment of human leukemia. Various schedules of drugs given in sequence have been designed to induce and then take advantage of synchronization and recruitment, although some animal experiments [7] showed that drugs which caused the greatest tumorical effect were also the most effective at recruiting normal stem cells.

In this report five patients with acute leukemia were treated sequences of drugs based upon cellular kinetic and pharmacologic principles. This therapy, shown graphically in the figure, consisted of a 24 hour infusion of methotrexate (MTX), 100–130 mg/square meter body surface area, immediately followed by folinic acid (FA) and 8-hr later by a 16-hr infusion of cytosine arabinoside (ARA-C), 200 mg/square meter. FA was usually given as follows: 24 mg intramuscularly and 24 mg orally at the end of the MTX infusion and

Accepted 18 March 1976.
*Supported in part by NIH Grants CA-10515 and AMI-6690-01, Chemotherapy Foundation of New York, and United Leukemia Fund.
†MLG was a scholar of Leukemia Society of America during part of this work.

24 mg orally 3 and 6 hr later and then every 6 hours for 8 more doses.

The rationale behind the scheduling of drugs was as follows. The MTX, by inhibiting nucleotide thymidylate formation, was to kill a certain fraction of leukemic cells while merely blocking others. Killing a fraction of cells should recruit new cells into active replication. The FA was then to reverse the MTX effect, allowing those cells which were only damaged to proceed through DNA synthesis, so that the cell population might be at least partly synchronized. The ARA-C was chosen 8 hr after FA, which allow considerable effect from the FA but is less than the DNA synthesis time for leukemic cells. If the interval were too long, the synchronized cells might have passed through DNA synthesis and no longer be sensitive to ARA-C.

MATERIAL AND METHODS

The five patients studied were in relapse after prior treatment. BA, a 9-year-old boy with lymphoblastic leukemia, had received vincristine, prednisone, dexamethasone, 6-mercaptopurine, daunomycin and MTX. BL, a 19-year-old boy with lymphoblastic leukemia had received vincristine, prednisone, L-asparaginase, daunomycin and MTX. SA, a 53-year-old woman with lymphoblastic leukemia, had received vincristine, prednisone, 6-mercaptopurine, MTX, cyclophosphamide, ARA-C, daunomycin, BCG vaccine, cranial irradiation, and irradiation of leukemic skin nodules. TO, a 34-year-old man with myelomonoblastic leukemia, had received daunomycin, ARA-C, and 6-thioguanine. RO, a 51-year-old man with myelomonoblastic leukemia, had received ARA-C, daunomycin and guanazole. RO had never gone into remission. Each of the other patients had had at least one complete remission and received the present therapy after having been retreated without achieving a complete remission again (SA, BA, TO) or after having had two relapses (BL).

Patient BA received one course of the present therapy, BL two such courses, SA and TO four courses, and RO died just before ARA-C was to start on his second course. In the last course for patient SA 2 mg vincristine was given intravenously three days after the ARA-C. Courses were given 5–14 days apart.

Bone marrow specimens were taken during at least one course in each patient before therapy, at the end of the MTX infusion, just before ARA-C was given, at the end of the ARA-C infusion, and 24 hours and 7–8 days after ARA-C infusion. A portion of the marrow was anticoagulated with 0·5% EDTA in saline, incubated with ^{3}H-thymidine and processed for autoradiography to determine the fraction of blasts in DNA synthesis (labeling index), as described previously [8]. The fraction of blasts in mitosis (mitotic index) was determined from the same specimen.

Another portion of each marrow specimen was collected in heparinized Hank's balanced salt solution and processed for several studies as described previously [9, 10]. One fraction was used to evaluate the *de novo* pathway of DNA–thymine synthesis (in which thymidylate is produced from deoxyuridine) by measuring the ability of exogenously added deoxyuridine to suppress the incorporation of subsequently added ^{3}H-thymidine into DNA [9], a modification of Killmann's original technique [11]. In marrow cultures from normal individuals suppression exceeds 90%. Less suppression is abnormal; this indicates that the *de novo* pathway is functioning to a lesser degree than is normal and is a sensitive indicator of a deficiency of reduced folate, e.g., due to MTX. This procedure tests this pathway before therapy and tests the effect of MTX and FA *in vivo* after therapy has begun. Another fraction was used to evaluate the effect of the *in vitro* addition of MTX (10^{-5} M) on the ability of MTX to prevent deoxyuridine suppression of ^{3}H-thymidine incorporation into DNA [10]. In patients who had received MTX therapy this procedure tests whether the *in vivo* MTX has produced a maximal biochemical effect. Still another fraction was used to evaluate the salvage pathway for DNA synthesis (in which thymidylate is produced from thymidine) by measuring the incorporation of ^{3}H-thymidine into DNA [10]. To compare the different specimens on the basis of a constant number of cells in DNA synthesis the counts/min per 10^{9} cells were multipled by 100/labeling index of the blasts (which formed at least 90% of the DNA synthesizing cells in all specimens).

RESULTS

Clinically, patients BA, BL, and RO each had a partial response with marked decreases in blood and bone marrow blasts. In patients BA and BL palpable splenomegaly disappeared, while in patient RO marked hepatosplenomegaly decreased dramatically. Seven days

after the first course, patient SA had a hypocellular marrow with a few residual primitive cells which persisted through 3 more courses. When therapy was stopped the marrow regenerated with a normal population; a complete remission persisted for 4 weeks. Patient TO had a complete remission after 4 courses as judged by the peripheral blood (he did not allow a marrow aspiration), but relapse was evident 4 weeks later.

In the following a number after the patient's name refers to the course of therapy for the patient.

In the study of the bone marrow labeling and mitotic indices relatively complete data was obtained for BL-1, BL-2, BA, RO-1, SA-1 and TO. Initially, labeling indices were 43, 29, 35, 12·5 and 12, and 26%, respectively. The data for each patient in the figure is normalized to the mean initial labeling index of 26·3% in this group of studies to aid the clarity of presentation. Similarly, data for mitotic indices is normalized in the chart to the mean initial value of 0·78%. At the end of the MTX infusion (Fig. 1) the labeling index was significantly increased over the initial values in BL-2, RO-1 and TO, but not substantially altered in the others. Eight hours after beginning FA the labeling index increased markedly in BL-2, slightly in all others except RO-1 (fell slightly from a very high level). At the end of the ARA-C infusion the labeling index had fallen markedly to levels far below the initial level in BL-1, BL-2 and SA-1 but fell only moderately in BA and TO (not measured in RO-1). A day later the labeling index was above the initial value in BL-2 and RO-1, had risen but was still low in BL-1 and SA-1, and was not measured in BA or TO. A week later all values broadly scattered about the initial levels (not measured in RO-1). The fraction of large blasts cells correlated closely with the overall labeling indices presented. Mitotic indices fell markedly by the end of the MTX infusion and remained low in 3 of 4 patients studied 24 hr after the cessation of therapy.

Table 1, column A, presents the data on the *de novo* pathway for DNA-thymine synthesis without added MTX. Initially there was a slight block in this pathway in RO-1 and TO (no previous MTX; folate deficient?) and a greater block in SA-2 (previous MTX given). In all patients tested, including those who had a prior block, there was a marked blocking effect on that pathway by the end of the MTX infusion. By the time ARA-C was begun 8 hr later there was only partial, if any, "rescue" by FA, but there was considerable, usually incomplete, "rescue" by the time FA had been given for 24 hr. The last column generally shows a further subsequent recovery of that pathway.

Table 1, column B, shows the results of the tests of the *de novo* pathway for DNA–thymine synthesis in the presence of MTX added to each culture. By comparing with column A

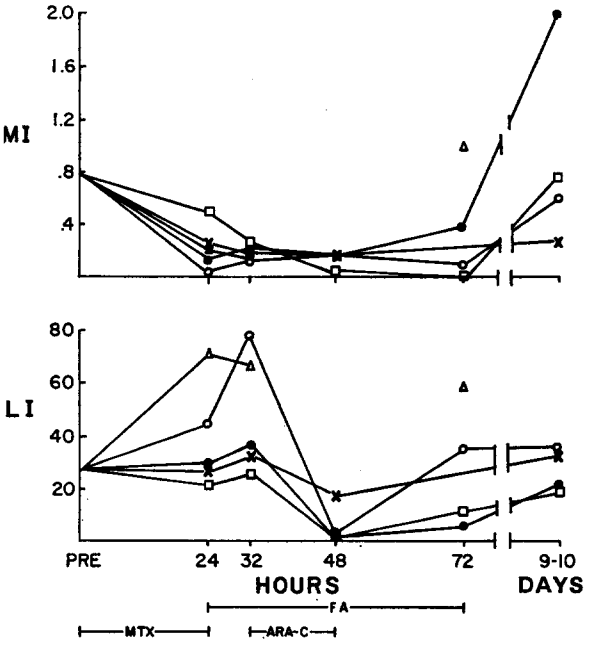


Fig. 1. All data is normalized to the initial mean of the group

Upper panel. MI = mitotic index(%)
Lower panel. LI = labeling index(%)

Data is shown for patients BL-1 (●——●), BL-2 (○——○), BA (x——x), RO-1 (△——△), SA-1 (□——□), and TO (■——■).

MTX = methotrexate
FA = folinic acid
ARA-C = cytosine arabinoside

before the MTX infusion was given, one notes that all tested patients had biochemical sensitivity to MTX. All patients had only slight or no additional effect from MTX added *in vitro* when they were tested at the end of the MTX infusion. After FA was begun there was a return of sensitivity to MTX after variable periods of time in those patients who had serial testing.

Table 2 shows the results of the incorporation of ^{3}H-thymidine in the evaluation of the salvage pathway for DNA synthesis. In 3 of 6 studies there was enhancement of that pathway following the MTX infusion. Eight hours of FA

Table 1. De novo *pathway of DNA—thymine with and without added MTX (% Deoxyuridine suppression)*

Patient	Pre MTX		End of MTX		Immediately before ARA-C		Immediately after ARA-C		Time after end of ARA-C		
	A	B	A	B	A	B	A	B		A	B
BL-1	90	10	28	28	16	16	71	N.D.*	1 day –	90	0
									8 days–	95	35
BL-2	95	30	38	40	5	0	70	0	1 day–	92	20
									7 days–	97	65
BA	95	43	50	50	66	65	65	75		N.D.	N.D.
RO-1	86	0	17	10	22	15	60	0	1 day–	90	0
									3 days–	72	0
RO-2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+‡			+	+
SA-1	95	65	N.G.†	N.G.	70	70	69	55	1 day –	60	63
									2 days–	78	10
									7 days–	60	40
SA-2	60	40	37	25	N.D.	N.D.	N.D.	N.D.		N.D.	N.D.
SA-3,4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		N.D.	N.D.
TO	74	0	31	26	69	50	94	82		N.D.	N.D.

The headings over columns A and B refer to the drug therapy given to the patients.
Column A—No MTX added to culture.
Column B—MTX added to culture.
*N.D.—Not done.
†N.G.—Technical problem—culture not good.
‡+—Patient died.

Table 2. *Salvage pathway of DNA synthesis–(^{3}H-thymidine CPM/10^9 cells)* (100/*LI*)

Patient	Pre MTX	End of MTX	Immediately before ARA-C	Immediately after ARA-C	Time after end of ARA-C	
BL-1	30·2	84·2	25·0	1700	1 day –	55·5
					8 days–	185·7
BL-2	224	85·7	2·9	87·5	1 day –	55·5
					7 days–	128
BA	68·6	294	267	62·2		N.D.*
RO-1	9·6	4·4	9·4	N.D.	1 day –	1·6
					3 days–	N.D.
RO-2	N.D.	N.D.	N.D.	+‡		+
SA-1	19·2	N.G.†	26·7	600	1 day –	6·9
					2 days–	44·5
					7 days–	144
SA-2	144	60·2	N.D.	N.D.		N.D.
SA-3,4	N.D.	N.D.	N.D.	N.D.		N.D.
TO	2·8	6·8	8·1	9·2		N.D.

*N.D. = not done.
†N.G. = technical problem—culture not good.
‡+ = patient died.

diminished the pathway in 3 of the 5 studies in which its effect was tested. ARA-C increased the pathway in 4 of 5 studies.

DISCUSSION

MTX given to leukemic patients in a single i.v. dose, 1 mg/kg or less, was associated with an increase in the labeling index thought to be due to a block of cells in the DNA synthetic state with little or no continued production of DNA [12, 13]. It was felt that partial synchronization of the cells occurred [13] or that there was a recruitment of cells into the DNA synthetic phase [12]. No block of cells coming into DNA synthesis was found [12, 13]. In our patients who had a similar increase in the labeling index (Fig. 1) that explanation seems likely. The lack of rise of labeling index in some patients may be due to the death rather than merely blockage of at least some of the cells, probably because our dose of MTX was much higher.

Since all of our patients had evidence of a pharmacologic effect of the MTX both *in vitro* and *in vivo* (Table 1), a biological effect of the drug is likely. The mixed effect of MTX on the salvage DNA pathway (Table 2) may be explained as a balance between some cells which had a compensatory increase in that pathway, as expected, some cells which were undamaged but unable to have a compensatory increase, and other cells so severely damaged by the MTX that only slight DNA synthesis is still possible (although all of that DNA synthesis may be by the salvage pathway). Variable effects on the thymidylate pool by the MTX may also alter the amounts of ^{3}H-thymidine incorporated. Recruitment of resting cells into DNA synthesis may well be enhanced by partial destruction of the population [13] so that higher doses of MTX may be more useful than smaller doses. The block of the *de novo* pathway by MTX has been shown to be more likely in leukemic patients who are clinically sensitive to the drug than those resistant to it, but the test has not been specific enough to give a reliable prediction of the clinical outcome in all patients [7, 14–16]. In this study (Table 1) the pretreatment effect of MTX on the *de novo* pathway *in vitro* was profound, as was its subsequent effect *in vivo*. Furthermore, the lack of significant further blockade of the *de novo* pathway by the addition of MTX *in vitro* at the end of the MTX infusion demonstrates the specificity and reliability of the deoxyuridine suppression test in these patients.

FA has been shown to limit the toxicity of the MTX [15, 17, 18], allowing much larger doses to be given than would be possible otherwise. Normal marrow cells were rescued from MTX effect before tumor cells in one group of patients [19]. FA allows the *de novo* pathway to proceed by converting tetrahydrofolate to N^5, N^{10}-methylene tetrahydrofolate at a step distal to the block caused by MTX [15]. The early increases in labeling index seen in most of our studies after starting FA (Figure) must represent synchronization and/or recruitment. The magnitude of those effects cannot be completely judged since an unknown number of cells which had been in DNA synthesis, some of which would be expected to still be in that phase, are thought to have been destroyed by the MTX. Also, it is obvious from the tables that the pharmacologic effect of MTX has been only partly reversed by the FA by the time ARA-C is begun.

ARA-C is known to cause a considerable decrease in the labeling index with ^{3}H-thymidine [5, 13, 20, 21] as was seen in most of our patients (Figure). The relatively small decreases in BA and TO may be due to relative resistance to the drug. ARA-C is thought to arrest cells in DNA synthesis by inhibiting DNA polymerase and by depressing the formation of deoxycytidine diphosphate from cytidine diphosphate without interfering with the other portions of the cell cycle. A subsequent rise in the labeling index, usually 24–28 hr later, may be associated with at least partial synchronization and perhaps recruitment; although we may be merely observing recovery. All of these possibilities are consistent with our data. Again, there is an unmeasured effect on the labeling index due to death of a least part of the cells which had been in DNA synthesis. It is not clear whether the very high values (Table 2) for ^{3}H-thymidine incorporation in BL-1 and SA-1 are real or not because of the statistical inaccuracies of very low labeling indices and counts per minute of tritium. On the other hand, the few cells in DNA synthesis may be heavily dependent on the salvage pathway for unknown reasons.

In all of these studies the mitotic indices (Figure) fell dramatically with therapy, and the return of mitoses generally lagged behind the labeling index. Since both MTX and ARA-C block cells in DNA synthesis, it is clear that there are few cells which will pass into mitosis and that recovery of DNA synthesis must precede mitosis.

The expected kinetic and pharmacologic effects, explained in the Introduction, were

demonstrated only in part. In certain points the rationale offers only one of several possible explanations for the data. Nonetheless, the clinical results were quite promising when one considers the advanced disease present in this small group of previously heavily treated patients.

Other groups using similar principles have devised other schedules of drugs which should be compared to ours. Lampkin *et al.* [13] used ARA-C followed by MTX. We felt that since FA was available to reverse the block of the MTX that we might be able to synchronize the cells more predictably than one does with ARA-C wherein one depends upon spontaneous recovery of damaged cells. Other investigators have used serial courses of MTX followed by FA [15, 17, 18, 22]. In this case the MTX serves the same function as did ARA-C in our patients if the timing is proper. The clinical results reported by Hryniuk and Bertino were strikingly good [15], and one could wonder whether the clinical effects which we saw might actually be due solely to the MTX and FA. Our data on the labeling indices, however, demonstrates that ARA-C had a pharmacologic effect. Whether repetition of cycles of therapy at more frequent intervals than we used would be of greater benefit is uncertain, nor is it known whether one need wait for complete recovery of the labeling index to achieve maximal effect. Other types of sequential use of drugs have also been used [2–4, 13, 23] but are not directly comparable.

In another report ARA-C and MTX have been used in association with cyclophosphamide in the acute leukemias with good clinical results [24], but since the chemotherapeutic drugs were given simultaneously (except that MTX was given over 24 hr, the other 0·5 hr) that combination is also not comparable. It is interesting, however, that in a culture system with murine lymphoma cells there is evidence of antagonism when MTX and ARA-C are used simultaneously or if MTX is given at certain intervals preceding the ARA-C, probably due to an elevation of intracellular deoxycytidine triphosphate, induced by MTX for unknown reasons [25]. This antagonism was not present clinically in our own patients or those above [24], and the changes seen in the labeling of our patients when they were given ARA-C demonstrate that either such antagonism was of no importance or that it was prevented by the FA.

The combined use of autoradiography, biochemistry, and clinical response allowed us to learn more about this type of therapy than would have been possible with only one or two of those types of observations. We believe that we have demonstrated the feasibility of a sequential drug therapy in the acute leukemias and that further exploration of this form of treatment is warranted.

REFERENCES

1. S. A. Killmann, Effect of cytostatic drugs on the kinetics of leukemic blast cells in man. *Schweiz. med. Wschr.* **104,** 278 (1974).
2. B. C. Lampkin, N. B. McWilliams and A. M. Mauer, Cell kinetics and chemotherapy in acute leukemia. *Semin. Hematol.* **9,** 211 (1972).
3. P. Pouillart, G. Mathé and L. Schwarzenberg, Essai de recrutement cellulaire par synchronisation partielle pour l'estabissement d'une combinason chimiotherapique. *Bull. Cancer* **60,** 187 (1973).
4. P. J. Burke and A. J. Owens, Jr, Attempted recruitment of leukemic myeloblasts to proliferative activity by sequential drug treatment. *Cancer* **28,** 830 (1971).
5. B. C. Lampkin, T. Nagao and A. M. Mauer, Drug effect in acute leukemia. *J. clin. Invest.* **48,** 1124 (1967).
6. W. R. Vogler, L. E. Cooper and D. P. Groth, Correlation of cytosine arabinoside induced increment in growth fraction of leukemic blast cells with clinical response. *Cancer (Philad.)* **33,** 603 (1974).
7. L. M. VanPutten, Recrutement arme a double tranchant dans la chimiotherapie du cancer. *Bull. Cancer* **60,** 131 (1973).
8. M. L. Greenberg, A. D. Chanana, E. P. Cronkite, G. Giacomelli, K. R. Rai, L. M. Schiffer, P. A. Stryckmans and P. C. Vincent, The generation time of human leukemic myeloblasts. *Lab. Invest.* **26,** 245 (1972).
9. J. Metz, A. Kelly, V. C. Swett, S. Waxman and V. Herbert, Deranged DNA synthesis by bone marrow from vitamin B12 deficient humans. *Brit. J. Haem.* **14,** 575 (1968).

10. S. WAXMAN, J. J. CORCINO and V. HERBERT, Drugs, toxins and amino acids affecting **B12** and folic acid absorption or utilization. *Amer. J. Med.* **48,** 599 (1970).
11. S. A. KILLMANN. Effect of deoxyuridine on incorporation of tritiated thymidine: difference between normoblasts and megaloblasts. *Acta med. Scand.* **175,** 483 (1964).
12. P. ERNST and S. A. KILLMANN, Effect of antileukemic drugs on cell cycle of human leukemic blast cells *in vivo*. *Acta med. scand.* **186,** 239 (1969).
13. B. C. LAMPKIN, T. NAGAO and A. M. MAUER, Synchronization and recruitment in acute leukemia. *J. clin. Invest.* **50,** 2204 (1971).
14. A. V. HOFFBRAND, E. TRIPP, D. CATOVSKY and K. C. DAS, Transport of methotrexate into normal hemopoietic cells and into leukemic cells and its effect on DNA synthesis. *Brit. J. Haem.* **25,** 497 (1973).
15. W. M. HRYNIUK and J. R. BERTINO, Treatment of leukemia with large doses of methotrexate and folinic acid. Clinical-biochemical correlates. *J. clin. Invest.* **48,** 2140 (1969).
16. T. F. NECHELES, A. MANIATIS and D. M. ALLEN, Parameters of clinical response to methotrexate in acute leukemia. *Clin. Res.* **16,** 310 (1968).
17. I. D. C. DOUGLAS and L. A. PRICE, Bone marrow toxicity of methotrexate: A reassessment. *Brit. J. Haem.* **24,** 625 (1973).
18. J. H. GOLDIE, L. H. PRICE and K. R. HARRAP, Methotrexate toxicity; Correlation with duration of administration, plasma levels, dose and excretion pattern. *Europ. J. Cancer* **8,** 409 (1972).
19. E. FREI, III, N. JAFFE, M. H. N. TATTERSALL, S. PITMAN and L. PARKER, New approaches to cancer chemotherapy with methotrexate. *N. Engl. J. Med.* **292,** 846 (1975).
20. P. ERNST, A. FAILLE and S. A. KILLMAN, Perturbation of cell cycle of human leukaemic myeloblasts *in vivo* by cytosine arabinoside. *Scand. J. Haem.* **10,** 209 (1973).
21. A. FAILLE, Y. NAJEAN et J. BERNARD, La cinetique des myeloblastes leucemiques humains apres cytosine-arabinoside. *Acta Haem.* **49,** 6 (1973).
22. G. MATHÉ, Le facteur temps en chimiotherapie cancerostatique. *Bull. Cancer* **60,** 179 (1973).
23. H. O. KLEIN, K. J. LENNARTZ, W. TEICHMULLER and R. GROSS, Quoted in reference 2.
24. R. T. SKELL, J. C. MARSH, R. C. DECONTI, M. S. MITCHELL, S. HUBBARD and J. R. BERTINO, Development of a combination chemotherapy program for adult acute leukemia: CAM and CAM-L. *Cancer (Philad.)* **32,** 76 (1973).
25. M. H. N. TATTERSALL and K. R. HARRAP, Combination chemotherapy: The antogonism of methotrexate and cytosine arabinoside. *Europ. J. Cancer* **9,** 229 (1973).

Europ. J. Cancer Vol. 12, pp. 625–631. Pergamon Press 1976. Printed in Great Britain

Correlation of Skin Test Reactivity and *In Vitro* Lymphocyte Stimulation by Soluble Antigens in Normal Subjects and Leukemic Patients

J. P. GMÜR, G. DELUIGI, CHR. SAUTER and P. W. STRAUB
University of Zürich, Department of Internal Medicine, Zürich, Switzerland

Abstract—*In this study, immunocompetence of normal subjects and leukemic patients was investigated using conventional skin testing and an* in vitro *lymphocyte stimulation micromethod. As antigens for both tests, Purified protein derivative (PPD) of tuberculine and a newly developed influenza-A-antigen (INF) were utilized. Test conditions for lymphocyte stimulation were adapted to give optimal* in vitro *results (1×10^5 lymphocytes per culture, 0·2 ml final volume, broad range of antigen concentration, 5 days incubation time).*

In vitro *lymphocyte stimulation allowed statistically significant discrimination between skin-test positive and negative normal subjects and leukemic patients. Furthermore a significant quantitative correlation could be established between these two test methods.*

If, in leukemic patients, care is taken to minimize the influence of chemotherapy and contamination of tested lymphocyte suspensions by residual blast cells, in vitro *lymphocyte stimulation appears to be superior to the skintests with respect to quantification and objectivity. The combined use of these test methods may be helpful for the study of the cellular immunocompetence of patients with acute leukemia undergoing specific immunotherapy.*

INTRODUCTION

IN THE past few years a number of studies have been devoted to immunotherapy of acute leukemia [1–3], a therapeutic approach which has reawakened interest in the immunologic status and competence of such patients [4–6]. The PPD skin-test as a measure of cell-mediated immunity in humans was the usual way to to investigate the cellular immunity until Permain [7] and Schreck [8] demonstrated in 1963 that lymphocytes of subjects with positive PPD skin-tests underwent specific lymphocyte transformation when cultured in the presence of PPD. Subsequent investigations of this *in vitro* phenomenon and its correlation with the skin-test however showed contradictory results [9–14].

If it could be shown that the results of an *in vitro* lymphocyte stimulation test correlate with the results of conventional skin-testing with various antigens; the combined use of such an objective and quantitative *in vitro* test with the more subjective and less quantitative skin-test methods might be helpful for the study of the cellular immunocompetence of patients with acute leukemia undergoing specific immunotherapy. We have used an adapted micromethod for lymphocyte stimulation by soluble antigens, e.g., PPD and INF, to study adult normal and leukemic patients and have correlated the results with those simultaneously obtained using skin testing with the same two antigens. In this report, we present the findings in normal subjects and in patients with acute leukemia not undergoing immunotherapy.

Accepted 11 March 1976.

MATERIAL AND METHODS

1. *Skin test antigens*

Skin tests were done with PPD commercially available from Institut Sérologique et Vaccinal

Suisse, Berne, at a concentration of 10 tuberculin units (TU) in 0·1 ml as well as with INF. For preparation of INF virus of A/Turkey/England/63, Havl Nav 3, Langham strain was grown in chicken embryo fibroblast cultures. The supernatant from virus infected cultures was harvested at 24–48 hr after infection and centrifuged at 1000 *g* for 30 min at 4°C. The supernatants were then exposed to U.V. (10 min, 10 cm, 30 w) in order to inactivate the virus. The concentration of the viral hemagglutinin antigen was adjusted to 160 hemagglutinating units (HAU) per 1 ml in Eagle's minimum essential medium (Grand Island Biological Company, (Gibco), USA) with 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco) and stored at −20°C until use.

2. *Skin testing*

Skin tests were performed and readed according to the recommendation of the World Health Organization [15]. Disposable 1-ml syringes with 30 ½-gauge needles were used. Induration of 5–5 mm or more in two perpendicular diameters after 24 and/or 48 hr was considered as positive.

3. *Culture medium and soluble antigens for lymphocyte stimulation*

The culture medium consisted of RPMI 1640 (Gibco) with 20% decomplemented, pooled human plasma of blood group AB donors. Penicillin (50 U/ml), streptomycin (50 μg/ml) and heparin (10 I.U./ml) were added.

For PPD stimulation lyophilized PPD was dissolved in culture medium and adjusted to the concentrations 1000, 500, 100, and 10 TU per ml.

For INF stimulation, the initial influenza-A-antigen solution with 160 HAU/ml was diluted to the concentrations 80, 20, 8 and 0·8 HAU/ml with culture medium.

4. *Culture technique*

The micromethod described by Bach *et al.* [16] for mixed leukocyte culture was adapted for soluble antigen stimulation. Venous blood was drawn in a plastic syringe, immediately transferred to glass vials containing 50 I.U. heparin per ml blood and diluted with the same volume of isotonic saline. Lymphocyte separation was performed on a discontinuous gradient as described by Boyum [17]. Between 90 and 98% of the harvested cells were mononuclear cells (mainly lymphocytes). Viability was over 95% as tested by dye exclusion. From 1 ml of blood at least 1 million of mononuclear cells could be collected. Subsequently, these lymphocytes were washed 3 times in HEPES buffered TC 199 (Gibco) and finally resuspended in culture medium at a concentration of $1–10^6$/ml. Standard cultures were set up with $1–10^5$ lymphocytes per culture in a final volume of 0·2 ml of culture medium. Distribution on microtiter plates (Falcon 3040 micro test II, USA) was performed by Hamilton microliter syringes. Each assay consisted of the following cultures, each in triplicate: (1) lymphocytes + soluble antigen; (2) negative control: lymphocytes alone; (3) positive control: lymphocytes + phytohemagglutinin (PHA–M, Difco, 0·1 ml of a final dilution of 1:100 in culture medium) as well as positive control with $2–10^5$ allogeneic, mitomycin C-inactivated lymphocytes. The plates were incubated in a humidified 95%-air, 5%-CO_2 atmosphere for 5 days, when 2 μCi of tritiated thymidine (specific activity 2 Ci/mMol, Amersham, England) in 0·05 ml of culture medium was added to each culture. At the end of an additional incubation time of 14 to 16 hr, the cultures were removed from the incubator and immediately precipitated on glass fiber filters according to the method of Hartzmann *et al.* [18]. The dried filters were introduced into 10 ml of scintillation liquid and counted for one minute in a liquid scintillation spectrometer (Packard Model 3330). The net counts/min were expressed as the average counts/min of stimulated cultures minus the average counts/min of the corresponding negative control culture, e.g., cultures containing unstimulated lymphocytes. All cultures showed positive controls, e.g., lymphocytes stimulated by PHA or allogeneic lymphocytes of at least 10,000 counts/min. A few negative control cultures of leukemic patients showed 1500 counts/min or more, probably due to the presence of some blast cells, and these experiments were therefore excluded.

5. *Test subjects*

Skin testing and *in vitro* lymphocyte stimulation were performed on the same day and in two different groups of subjects (see Table 1): 29 healthy normal members of hospital staff, mostly PPD skin-test positive subjects. All 9 PPD negative subjects were selected from new employees whose routine PPD skin-test with 100 tuberculin units had been negative. The second group consisted of 20 patients with various forms of acute leukemia admitted during 1974. *In vitro* tests of these patients were done only after complete remission was achieved and at the earliest 3 weeks after the last cycle of chemotherapy was completed.

Table 1. *Skin test in normal subjects and leukemic patients with PPD,* 10 *TU in* 0·1 *ml and INF,* 16 *HAU in* 0·1 *ml intracutaneously*

Antigen	Age range, (yr)	PPD		Influenza	
Skin test		Positive	Negative	Positive	Negative
Normal subjects	18–36	20	9	26	3
Leukemic patients	19–43	14	6	16	4

RESULTS

As outlined in Fig. 1 and 2 as well as in Tables 2 and 3, the following culture conditions can be chosen as optimal for lymphocyte stimulation by soluble antigens: antigen concentration range of 10 to 100 TU per culture for PPD and 1·6 to 16 HAU per culture for INF, incubation time 5 days, lymphocyte concentration $1 = 10^5$ per culture. Declining

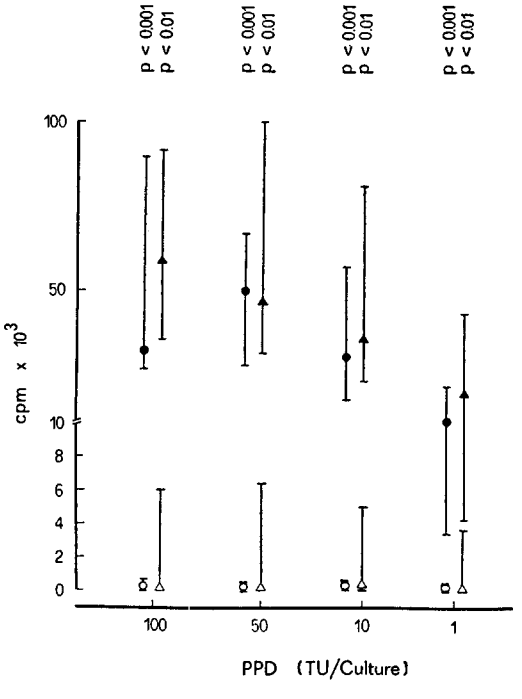


Fig. 1. *Tritiated thymidine uptake by lymphocyte cultures stimulated by PPD* (1, 10, 50 *or* 100 *TU per culture*) *in PPD skin test positive normal subjects* (●) *and leukemic patients* (▲), *PPD skin test negative normal subjects* (O) *and leukemic patients* (△).

counts/min of lymphocyte stimulation were achieved by higher antigen concentrations (PPD: 200 up to 1000 TU per culture; INF: 40 up to 80 HAU per culture) independently of skin-test reactivity. Therefore, all further experiments were performed under the above mentioned optimal culture conditions.

1. *Lymphocyte stimulation and skin-tests with PPD*

Estimation of lymphocyte stimulation by PPD in skin-test positive and negative individuals showed a significant difference at all 4 tested antigen concentrations in normal subjects ($P < 0{\cdot}001$) as well as in leukemic patients ($P < 0{\cdot}01$) (see Fig. 1). All tested individuals with a positive PPD skin-test (20 normal subjects and 14 leukemic patients) showed a lymphocyte stimulation of more than 5000 counts/min in the optimal range of PPD.

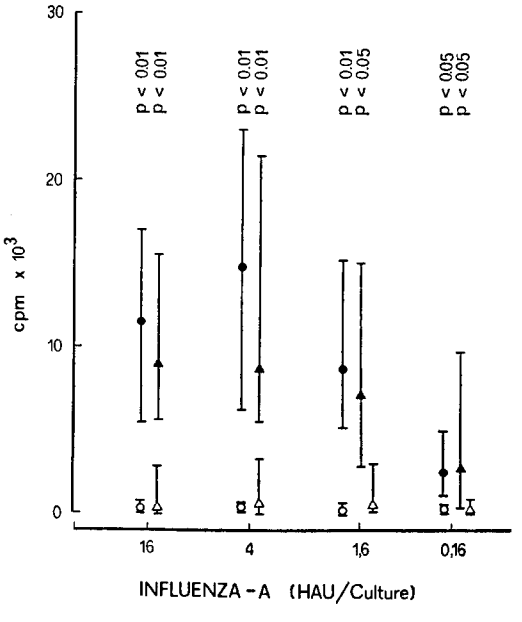


Fig. 2. *Tritiated thymidine uptake by lymphocyte cultures stimulated by INF* (0·16, 1·6, 4 *and* 16 *HAU per culture*) *in INF skin test normal subjects* (●) *and leukemic patients* (▲), *respectively INF skin test negative normal subjects* (O) *and leukemic patients* (△).

There was no significant difference between skin-test positive normal subjects and skin-test positive leukemic patients. On the other hand, 9 of 9 skin-test negative normal subjects and 5 of 6 skin-test negative leukemic patients had a stimulation of less than 1500 counts/min at the same PPD-concentrations. The remaining patient with 25,701 counts/min in lympho-

Table 2. Influence of incubation time on ^{3}H-thymidin uptake of lymphocyte cultures of normal subjects stimulated by PPD (50 TU per culture) and INF (4 HAU per culture). Mean value and SD from 5 experiments

Antigen	Skin test	Counts/min 4 days	5 days	7 days
PPD	Positive	13,646 ± 16,015	39,698 ± 56,824	18,173 ± 13,490
	Negative	213 ± 278	338 ± 405	868 ± 784
INF	Positive	3452 ± 2853	9081 ± 6977	15,295 ± 8829
	Negative	ND	246 ± 145	ND

Table 3. Influence of cell concentration on ^{3}H-thymidin uptake of lymphocyte cultures of normal subjects stimulated by PPD (50 TU per culture) and INF (4 HAU per culture). Mean value and SD from 5 experiments

Antigen	Skin test	Lymphocytes per culture $\times 10^5$ 0·25	0·5	1·0 (counts/min)	2·0
PPD	Positive	112 ± 107	3337 ± 2597	28,628 ± 18,193	65,700*
	Negative	6 ± 1·5	71 ± 61	87 ± 75	206 ± 26
INF	Positive	26 ± 18	1091 ± 575	8253 ± 5432	ND
	Negative	ND	ND	246 ± 145	ND

*Single value.

cyte stimulation explains the high variation in this group of skin-test negative leukemic patients (Fig. 1).

The correlation between the intensity of the skin-test reactivity (dia. in mm) and the lymphocyte stimulation (log of counts/min $\times 10^3$) of skin-test positive normal subjects and leukemic patients is presented in Fig. 3A and B. The quantitative correlation was significant for the normal subjects ($r = 0{\cdot}58415$, $P < 0{\cdot}01$) but not for the leukemic patients ($r = 0{\cdot}09585$, $P > 0{\cdot}10$).

2. *Lymphocyte stimulation and skin-tests with INF*

Comparison of *in vitro* lymphocyte reactivity to INF between skin-test positive and negative individuals (Fig. 2) showed a significant difference for all four antigens concentrations in normal subjects and for the three higher concentrations in leukemic patients.

Lymphocyte stimulation in the 26 skin-test positive normal subjects varied widely (2000–38,000 counts/min). In the 16 skin-test positive leukemic patients tested, one patient showed no stimulation, whereas the remaining 15 had at least 3000 counts/min in the optimal range of antigen concentration. There was no significant difference between INF skin-test positive normal subjects and leukemic patients. All 3 skin-test negative-normal subjects showed a stimulation of less than 500 counts/min. Out of 4 skin test negative leukemic patients, one showed a stimulation of 4000 counts/min, the others were also below 500 counts/min.

The quantitative correlation between the intensity of skin test reactivity (dia. in mm) and the lymphocyte stimulation (log of counts/

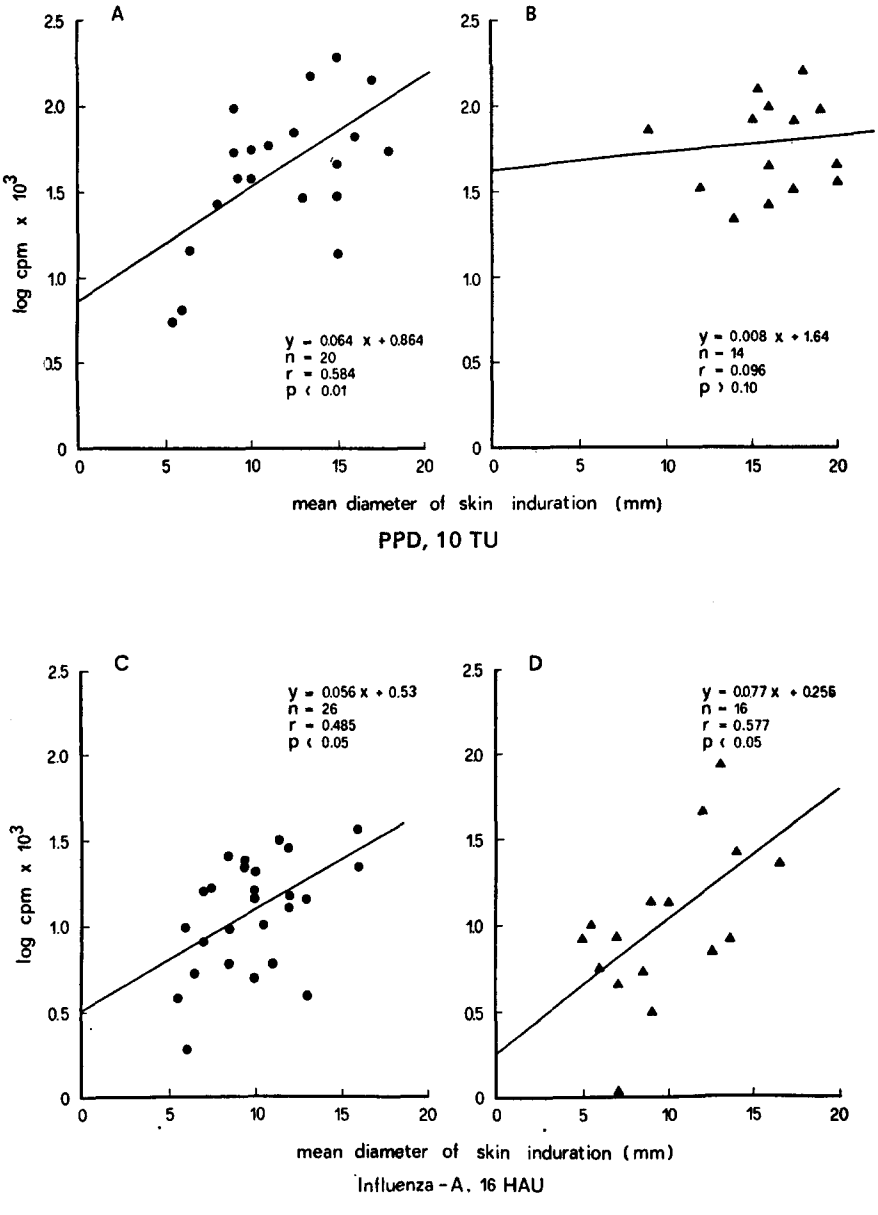


Fig. 3. Relationship between degree of lymphocyte stimulation expressed as the log of cpm $\times 10^3$ *and the diameter of skin test expressed in mm: (A) = PPD, normal subjects. (B) = PPD, leukemic patients. (C) = INF, normal subjects. (D) = INF, leukemic patients.*

min $\times 10^3$) of skin test positive normal subjects and leukemic patients was significant in both normal subjects and leukemic patients (Fig 3C and D, $P < 0{\cdot}05$).

DISCUSSION

In our studies on cellular immunocompetence of normal subjects and leukemic patients we first investigated optimal culture conditions for *in vitro* lymphocyte stimulation by soluble antigens. We used as antigens PPD and a newly developed influenza-A-antigen (INF) since a high proportion of normal subjects showed positive skin-tests to these two antigens. A micromethod of lymphocyte stimulation was modified as to give an optimal discrimination between maximally and minimally stimulated cultures in as short an incubation time as 5 days and with only 1×10^5 lymphocytes/culture (see Tables 2 and 3). The wide variation in the reaction kinetic of *in vitro* lymphocyte stimulation as postulated by others [14] appeared to play a minor role in our experimental system. Although we used only one incubation time, 4 different antigen concentrations were used simultaneously for each antigen in order to avoid false negative results in weakly positive subjects as described by Nilsson [14]. The range of antigen concentrations used could be shown to be optimal, the lowest

antigen concentration regularly giving lower values than the next higher concentration. On the other hand higher concentrations of PPD or INF did not provoke stimulation at all, probably due to a toxic effect of the concentrated antigens or their preservative, or to an exhaustion of culture medium.

Under our standard test conditions we found a highly significant difference of *in vitro* lymphocyte stimulation between skin-test positive and negative subjects in all tested antigen concentrations. Furthermore, a statistically significant quantitative correlation between the degree of skin-test reactivity and *in vitro* lymphocyte stimulation could be demonstrated in skin-test positive normal subjects and for INF also in leukemic patients. This is in agreement with most other investigations [10, 13, 19]. However, some publications have reported that lymphocytes from PPD-skin-test negative donors may react *in vitro* [9, 20]. It is interesting to see that in the studies of Nilsson [9] the discrepancy of a negative skin-test to PPD and an intermediate *in vitro* lymphocyte stimulation was found only in cultures stimulated by high doses of PPD and after prolonged incubation time. Since no results were reported on unspecific thymidine uptake by unstimulated control-cultures, an artefact cannot be excluded. Thus, we regularly observed a 2- to 3-fold increase of uptake in unstimulated control cultures when the incubation time was prolonged from 5 to 7 days. The PPD-stimulated cultures of PPD-skin-test negative subjects showed the same tendency, but the difference to the PPD-skin-test positive subjects continued to be significant also after longer incubation. Out of the variables affecting DNA-synthesis in stimulated lymphocyte cultures [10, 21], at least the influence of contaminating granulocytes [21] was greatly reduced by Boyum's lymphocyte separation technique. Bach *et al.* [16] proved the superiority of their microtechnique as compared to the macrotechnique in mixed leukocyte cultures. Since we used the same microtechnique, adapted to the stimulation by soluble antigens, the improvement of the correlation between *in vitro* and *in vivo* tests, as compared to the earlier studies, may therefore well be due at least, in part, to an improvement in culture technique. Furthermore, this microtechnique permitted saving of blood, medium, and time.

In contrast to these observations in normal subjects, skin-test reactivity and *in vitro*-lymphocyte stimulation in leukemic patients did qualitatively correlate in only 92% of tests performed, and the quantitative correlation between both methods was statistically significant only for INF-positive, but not for PPD-positive leukemic patients. In addition to influences of culture conditions even in normal subjects [10, 20, 21], several not well defined factors may influence the results in leukemic patients, such as chemotherapy, marrow status and peripheral blood picture. Since leukemic cells and normal lymphocytes cannot always be well distinguished in cell suspensions, this point seems of special interest in patients with acute lymphoblastic leukemia. Leukemic blast cells may show a spontaneous incorporation of labeled thymidine and/or be immunologically incompetent. The first assumption would explain why two negative control cultures in leukemic patients showed a thymidine incorporation of more than 1500 counts/min, whereas the second would explain the discrepancy between a positive skin test and a negative *in vitro* test observed in 1 patient. In this study, *in vitro* lymphocyte stimulation was not performed before patients achieved a complete remission. A variable contamination of lymphocyte suspensions by some remaining blast cells could not be ruled out nevertheless as documented in several cytocentrifuge blood pictures done in leukemic patients in apparently complete remission. This disadvantage of *in vitro* lymphocyte stimulation test can only be overcome by very careful controls of the composition of tested lymphocyte suspensions.

Results with the newly developed virus antigen INF were satisfactory since a high rate of positive skin tests (Table 1), and a good qualitative and quantitative correlation between skin test reactivity and *in vitro* lymphocyte stimulation could be demonstrated in all tested normal subjects and leukemic patients. The high rate of positive tests is not surprising since this avian virus shares the A antigen with influenza A virus occurring very commonly in humans. This antigen is easy to handle since it can be standardized by its property to agglutinate erythrocytes.

The present *in vitro* technique has shown to give results closely correlated with skin test reactivity using PPD, but particularly using an influenza antigen so far not used for this purpose. If adequate care is taken to minimize the influence of chemotherapy or residual blast cells, it appears to be superior to the skin tests with respect to quantification and objectivity. On the other hand, the results also suggest that skin testing is more valuable for the assessment of cellular immunocompetence than was previously thought.

REFERENCES

1. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat and F. de Vassal, Active immunotherapy for acute lymphoblastic leukemia. *Lancet* **i,** 697 (1969).
2. D. Crowther, R. L. Powles and C. J. T. Batheman, Management of adult acute myelogenous leukemia. *Brit. med. J.* **1,** 131 (1973).
3. C. B. Freeman, R. Harris, C. G. Geary, M. J. Leyland, J. E. McCivier and I. W. Delamore, Active immunotherapy used alone for maintenance of patients with acute myeloid leukemia. *Brit. med. J.* **4,** 571 (1973).
4. M. E. Oren and R. B. Herberman, Delayed cutaneous hypersensitivity reactions to membrane extracts of human tumor cells. *Clin. exp. Immunol.* **9,** 45 (1971).
5. R. B. Herberman, R. G. Halterman and B. G. Leventhal, Cellular immune reactions to human leukemia. *Nat. Cancer Inst. Monogr.* **35,** 259 (1972).
6. J. U. Gutterman, R. D. Rossen, W. T. Butler, K. B. McCredie, G. P. Bodey, E. J. Freireich and E. M. Hersh, Immunoglobulin on tumor cells and tumor-induced lymphocyte blastogenesis in human acute leukemia. *New Engl. J. Med.* **288,** 169 (1973).
7. G. Permain, R. R. Lycette and P. H. Fitzgerald, Tuberculine induced mitosis in peripheral blood leukocytes. *Lancet* **i,** 637 (1963).
8. R. Schreck, Cell transformation and mitosis produced *in vitro* by tuberculin purified protein derivate in human blood cells. *Amer. Rev. resp. Dis.* **87,** 734 (1963).
9. D. W. Gump, F. R. Fekety, J. Urbanetti and C. Nosenzo, Studies of human leukocyte culture as an *in vitro* test of delayed hypersensitivity reaction to tuberculin. *Amer. Rev. resp. Dis.* **95,** 470 (1967).
10. G. R. Kerby, Correlation of tuberculin skin reactions with *in vitro* lymphocyte transformation. *Amer. Rev. resp. Dis.* **97,** 904 (1968).
11. S. D. Chaparas, J. N. Sheagren, A. De Meo and S. Hedrick, Correlation of human skin reactivity with lymphocyte transformation induced by mycobycterial antigens and histoplasmin. *Amer. rev. Resp. Dis.* **101,** 67 (1970).
12. J. W. Thomas, D. Clements and S. Grzybowski, *In vitro* lymphocyte responses and skin test reactivity following BCG vaccination. *Clin. exp. Immunol.* **9,** 611 (1971).
13. S. D. Miller and H. E. Jones, Correlation of lymphocyte transformation with tuberculin skin-test sensitivity. *Amer. Rev. resp. Dis.* **107,** 531 (1973).
14. B. S. Nilsson, The response of lymphocytes from tuberculin-negative or positive humans to various doses of PPD-tuberculin *in vitro*. *Cell. Immunol.* **3,** 493 (1972).
15. WHO/OMS: Primary immuno deficiencies. *Bull. Wld. Hlth. Org.* **45,** 125 (1971).
16. R. J. Hartzmann, M. Segall, M. L. Bach and F. H. Bach, Miniaturization of the mixed leukocyte culture test: A preliminary report. *Transplantation* **11,** 268 (1971).
17. A. Böyum, Blood cell separation by gradients. *Scand. J. clin. lab. Invest. Suppl.* **97,** 21 (1968).
18. R. J. Hartzmann, M. L. Bach, F. H. Bach, G. B. Thurman and K. W. Sell, Precipitation of radioactively labelled samples: a semi-automatic multiple sample processor. *Cell. Immunol.* **4,** 182 (1972).
19. W. McFarland and D. Heilman, Comparison of lymphocyte transformation and intradermal reactions to tuberculin. *Amer. Rev. resp. Dis.* **93,** 742 (1966).
20. B. S. Nilsson, *In vitro* lymphocyte transformation to tuberculin. *Scand. J. resp. Dis.* **52,** 39 (1971).
21. M. L. Bach, F. H. Bach, M. Widmer, H. Oranen and W. H. Wolberg, The effect of polymorphnuclear leukocytes on lymphocyte response. *Transplantation* **12,** 283 (1971).

Europ. J. Cancer Vol. 12, pp. 633–637. Pergamon Press 1976. Printed in Great Britain

Transfection of Mouse Bone Marrow Cells with Rauscher Virus Proviral DNA*

KEES NOOTER, KOR VAN DEN BERG, JAN DE VRIES and PETER BENTVELZEN
Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk (ZH), The Netherlands

Abstract—*Mouse bone marrow cells were transfected with DNA isolated from Rauscher virus-induced leukemic spleen cells. Successful transfection was manifested by virus production and transformation.*

Virus production by the transfected bone marrow cells was shown by the XC test. Injection of transfected cells into syngeneic hosts led to the development of erythroblastosis, probably due to the spread of virus reproduced by infectious proviral DNA.

The transforming potential of the infectious viral DNA was demonstrated by the formation of erythroid colonies in vitro *without the addition of erythropoietin to the culture medium.*

INTRODUCTION

TRANSFECTION has been defined by Hill and Hillowa [1] as a phenotypic change in cells after exposure to exogenous DNA. Most work in this area of research is done with DNA isolated from cells transformed by avian tumour viruses [1], but mammalian cells have also been transfected with proviral DNA of the mouse sarcoma virus [2]. The term transfection has also been used when exposure of cells to proviral DNA of leukemia viruses leads to virus replication only [3, 4]. Phenotypic changes in cells induced by incubation with proviral DNA has not yet been described for leukemia viruses. This is probably due to the lack of suitable transformation assay systems for these viruses.

We recently developed an *in vitro* system which selectively supports the growth of transformed erythroid progenitor cells from bone marrow (BM) of Rauscher leukemia virus (RLV)-infected mice [5]. In contrast to normal erythroid colony forming cells, the transformed cells do not require erythropoietin (EP) for colony formation. Incubation of mouse BM cells with purified RLV leads to transformation *in vitro* [6]; the erythroid cells show a similar hormone-independency as found with cells from leukemic mice. A comparable *in vitro* transformation system has been developed for the Friend murine leukemia virus [7].

In the present study, we show that mouse BM cells can be transfected by Rauscher virus proviral DNA. The transfected BM cells proved to be positive for virus production as assayed in the XC test and were shown to be phenotypically changed in our *in vitro* system. Transfected cells induced leukemia in mice.

MATERIAL AND METHODS

DNA isolation

Four-week-old female BALB/c mice were injected i.p. with 0·1 ml of a crude cell-free extract of RLV from leukemic spleens [5]. When the spleens reached a weight of about 2 g, these organs were used for the isolation of DNA. Spleens from mice treated with phenylhydrazine 5 days earlier were used as controls. These spleens contain many immature erythroid cells owing to the phenylhydrazine-induced anemia. DNA was isolated from spleens according to established procedures [8] with some modifications. The spleens were first made into a single cell suspension and washed twice with phosphate buffered saline. The cell pellet was resuspended in 0·01 M Tris buffer, pH 8·1, containing 0·01 M EDTA and 500 μg/ml pronase (inactivated). The cells were lysed by addition of sodium dodecyl sulphate to a final

Accepted 3 March 1976.

*This work was supported by the Netherlands Organization for Fundamental Medical Research.

concentration of 0·5% and incubated for 1 hr at 37°C. The lysate was extracted twice with phenol. On addition of two volumes of ethanol, DNA was precipitated and redissolved in 0·1 SSC (15 mM sodium chloride, 1·5 mM sodium citrate pH 7·0). In some cases, DNA was further purified by incubation with RNase (Sigma, protease free, 50 μg/ml) and RNase T_1 (Sigma, 50 μg/ml) for 1 hr at 37°C and an additional incubation with pronase (Calbiochem, nuclease free, 100 μg/ml) for 1 hr at 37°C. The solution was extracted twice with chloroform-isoamyl alcohol (24:1 v/v), dialysed against 0·1 SSC and concentrated over polyethylene glycol (MW 6000). The DNA concentration was determined both from its absorption at 260 nm and chemically [9]. The ratio of absorbance at 260 and 280 nm was more than 1·8. The RNA and protein contamination of the DNA was less than 5%. The sedimentation coefficient of the DNA preparations was between 30 and 70 S, as determined by band sedimentation in an analytical ultracentrifuge.

DNA incubation

Bone marrow was prepared from femurs of adult BALB/c mice [5]. The cells were washed in Hanks Balanced Salt Solution and the pellet was resuspended in 0·5 ml Dulbecco's Modified Minimal Essential Medium (MEM). Prior to use, the DNA was diluted (at least 50×) in Hepes phosphate buffer and treated with a high calcium concentration according to the method of Graham and van der Eb [10]. The final calcium concentration was adjusted to 125 mM and the resulting precipitate was allowed to form over a period of 20 min at room temperature. A 0·5 ml sample of DNA precipitate was added to the cell suspension (10^7 BM cells/ml) and the mixture was kept at room temperature for one-half hr with repeated stirring. The final volume was then adjusted to 4 ml with MEM plus 2% fetal bovine serum (FBS) and incubated in a humidified CO_2-incubator with 5% CO_2 in air for 4 hr at 37°C. Control cultures were incubated under the same conditions.

XC Cytophatogenicity assay

With the aid of the modified XC-test for haemopoietic cells [11] virus production by these DNA-incubated BM cells was measured. DNA-incubated BM cells instead of virus-infected mouse embryo fibroblasts were plated over an XC cell underlayer in order to induce syncytia. The change in plating sequence is in favour of BM cells, of which only a selected part adheres to the culture surface. Besides this, the XC underlayer provides an appropriate environment for BM cells. Small 50 mm plastic petri dishes (Greiner und Sohnen, Nürtinger, Germany) were seeded with 2×10^5 XC cells in 3 ml MEM on day one. On day 2, BM cells were added to the dishes (3×10^5 in 3 ml) after removal of the medium. The dishes were stained with May–Grünwald–Giemsa on day 5.

Transformation in vitro *assay*

The culture technique used here for the BM erythroid colony-forming cell (CFU–E) is described in detail elsewhere [5, 12, 13]. Briefly, 2×10^5 BM cells are mixed with 0·8% methylcellulose, 30% fetal calf serum (Flow Laboratories, Scotland) and erythropoietin (EP) (1.I.U./ml; step III Connaught, Toronto) and plated in 35 mm plastic petri dishes. After 5 days incubation at 37°C in a humidified incubator with 5% CO_2 in air, the number of erythroid colonies are counted by adding a few drops of 0·2% dimethoxybenzidine in 0·5 M acetic acid with 0·5% hydrogen peroxide to the plates. Only the blue-coloured aggregates of more than 8 cells were counted.

CFU–E's that form colonies in the absence of exogenous Ep are regarded as being transformed and this spontaneous growth is used as an assay for the presence of transforming proviral DNA in transfected BM cells.

Bioassay

Four-week-old BALB/c female mice were injected intravenously with 10^5 syngeneic BM cells previously incubated with a DNA solution. The animals were examined twice a week for the occurrence of leukemia. Blood smears were made with blood from the orbital plexus. Material for histological examination was fixed in 10% buffered formalin.

RESULTS

BM cells incubated with proviral DNA isolated from cells of RLV-induced erythroblastotic spleens gave rise to the formation of syncytia of XC cells (Table 1). This means that ecotropic C-type oncorna viruses were released by the BM cells. BM cells incubated with DNA isolated from phenyl hydrazine-treated control mice failed to produce syncytium counts above control values.

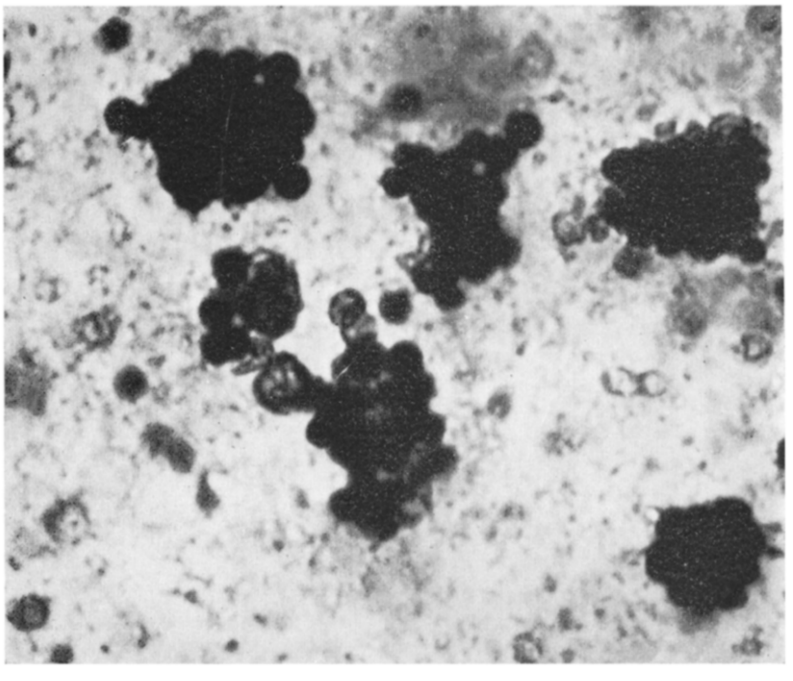


Fig. 1. *Spontaneous erythroid colonies in methylcellulose formed by BM cells transfected with Rauscher virus proviral DNA. The colonies were stained* in situ *with a* 0·2% *benzidine solution.* (× 380)

(to face page 634)

Table 1. *XC cell syncytium-inducing capacity of BM cells transfected with proviral DNA*

XC cells overlayered with BM cells incubated with	Number of syncytia*
10 μg DNA isolated from leukemic spleens	198
10 μg DNA isolated from control spleens†	18
No overlayer	15

*Mean of 4 dishes; counted with the inverted microscope (×75).

†Control donor BALB/c mice were treated with phenylhydrazine (0·5 mg i.p.) and sacrificed 5 days later.

Table 2. *Assay for the presence of transforming proviral DNA*

Incubation	Number of spontaneous erythroid colonies per dish
RLV-spleen DNA 10 μg	744
rat spleen DNA 10 μg	22
RLV-spleen DNA 10 μg + DNase*	16
High calcium buffer only	20

*Incubated for 60 min at 37°C with 50 μg/ml DNase before the addition of calcium.

Table 3. *The induction of leukemia by transfected BM cells*

Incubation		Leukemia incidence*
RLV-spleen DNA 10 μg	exp. 1	10/10
	exp. 2	3/4
	exp. 3	6/6
	exp. 4	2/5
High calcium buffer only	exp. 1	0/10
	exp. 2	0/4

*Number of animals with leukemia/total number of animals inoculated; the animals were sacrificed 4 months after inoculation. The spleen weights of the leukemic mice ranged between 1500–2200 mg; hematocrits, between 11–25%.

Incubation of normal BM cells with proviral DNA isolated from leukemic spleens induced transformation of erythroid cells to a hormone-independent state (Fig. 1). The number of spontaneous erythroid colonies formed by transfected BM cells *in vitro* is far greater than the number of colonies produced in the control experiments involving digestion of proviral DNA by DNase or incubation with normal rat DNA.

Twenty of the 25 mice inoculated with syngeneic BM cells previously incubated with proviral DNA developed leukemia after about 3 months (Table 3).

The animals were killed at 4 months after injection. None of the control mice, which received BM cells incubated with the high-calcium buffer only, showed signs of leukemia. Histological examination of the diseased animals indicated a true erythroblastosis. The hematocrits of the leukemic mice were extremely low (between 11 and 25%); the spleens were greatly enlarged and weighed between 1550 and 2200 mg.

Peripheral blood smears contained many erythroblasts. Spleen cells of the erythroblastotic mice were injected subcutaneously into newborn BALB/c mice. In no case was there a local outgrowth of tumour cells. Spleen cells were cocultured with XC cells and induced a high number of large syncytia. Cell-free extracts of the spleens of the leukemic animals were prepared and added to 24-hr cultures of BALB/3T3 cells. One week later, immunofluorescence studies with a rat antiserum to RLV [14] demonstrated the presence of RLV-specific antigens.

DISCUSSION

Exposure of normal cells to cellular DNA isolated from cells transformed by an oncornavirus may lead to neoplastic transformation and/or virus replication [2, 3, 15, 16]. Virus production can be shown relatively easy, e.g., by means of reverse transcriptase activity, electron microscopy, the presence of virus-specific proteins and bioassays. Transformation *in vitro* is a more delicate problem as far as the mouse leukemia viruses are concerned.

A few culture systems which allow the *in vitro* detection of transformed cells induced by murine leukemia viruses have been recently developed [7, 17, 18].

In the present study one of these assays was used to show the transforming character of infectious proviral DNA extracted from RLV-infected spleen cells. We applied the so-called calcium method for transformation of cells by viral DNA as developed by Graham and van der Eb [10].

This method has proved to be superior in transformation efficiency as compared to the widely used DEAE-dextran method in several

systems [16, 19, 20]. This is probably due to protection of the calcium–phosphate–DNA complex from the action of nucleases (van den Berg, unpublished results). BM cells transfected with Rauscher proviral DNA release a murine ecotropic C-type virus as revealed by the XC test.

When these transfected BM cells are injected into syngeneic hosts, the animals develop an erythroblastosis with the same characteristics as the original virus induced disease: splenomegaly with infiltration of blasts into liver and peripheral blood accompanied by anemia. These leukemias are probably not so much caused by proliferation of transformed cells as by spread of virus which is produced by infectious proviral DNA. This becomes evident from the repeated failure to transplant leukemic spleen cells and the high virus content of the spleens from the leukemic animals. The bioassay data strongly suggest that the virus released by the transfected BM cells is the original RLV. The late appearance of the erythroblastosis (about 3 months) might be due to the small number of virus releasing cells in the inoculum. However, a large number of transformed hormone-independent erythroid colonies are found after transfection with RLV proviral DNA. A similar number of erythroid colonies is formed by normal BM cells in the presence of erythropoietin. This finding might suggest that a large number of target cells becomes transfected by RLV proviral DNA. The procedure utilized here to isolate DNA gave a range of molecular weights between 20×10^6 and 200×10^6 daltons. Cooper and Temin [16] found the minimum molecular weight for the avian Rous sarcoma virus proviral DNA isolated from transformed cells to be about 6×10^6.

Nonintegrated viral DNA produced soon after infection of mouse cells with Molony murine leukemia virus must have approximately the same molecular weight in order to be infectious [20]. It is possible, however, that a small fragment of the sarcoma virus genome carries transforming activity to fibroblasts. [21].

Acknowledgements—We wish to thank Drs. A. C. Ford for aid in the preparation of the manuscript, Ch. Zurcher for the histological examinations of the leukemias and A. J. van der Eb for determination of sedimentation coefficients.

REFERENCES

1. M. Hill and J. Hillova, RNA and DNA forms of the genetic material of C-type viruses and the DNA form in the cellular chromosome. *Bioch. biophys. Acta* **35,** 7 (1974).
2. A. Karpas and C. Milstein, Recovery of the genome of murine sarcoma virus (MSV) after infection of cells with nuclear DNA from MSV transformed non-virus producing cells. *Europ. J. Cancer* **9,** 295–299 (1973).
3. M. Brunner, Transfection of XC plaques with DNA from murine leukemia virus producer cells. *Biochem. biophys. Res. Commun.* **66,** 397 (1975).
4. E. Scolnick and S. Bumgarner, Isolation of infectious xenotropic mouse type C virus by transfection of a heterologous cell with DNA from a transformed mouse cell. *J. Virol.* **15,** 1293 (1975).
5. K. Nooter and R. Ghio, Hormone-independent *in vitro* erythroid colony formation by bone marrow cells from Rauscher virus-infected mice. *J. nat. Cancer Inst.* **55,** 59 (1975).
6. K. Nooter and P. A. J. Bentvelzen, *In vitro* transformation of murine erythroid cells by Rauscher leukemia virus. *Cancer Lett.* **1,** 155–160 (1976).
7. B. Clarke, A. Axelrad, M. Shreeve and D. McLeod, Erythroid colony induction without erythropoietin by Friend leukemia virus *in vitro*. *Proc. nat. Acad. Sci.* **72,** 3556 (1975).
8. J. Marmur, A procedure for the isolations of deoxyribonucleic acid from microorganisms. In: *Methods in Enzymology* (Edited by S. P. Colowick and N. D. Kaplan), **6,** p. 726, Academic Press, New York, (1963).
9. K. W. Giles and A. Myers, An improved diphenylamine method for the estimation of deoxy ribonucleic acid. *Nature (Lond.)* **206,** 93 (1965).
10. F. Graham and A. Van der Eb, Transformation of rat cells by DNA of human adenovirus 5. *Virology* **54,** 536 (1973).
11. K. Nooter and D. Aarssen, Cytopathogenic XC assay for bone marrow from mice infected with murine leukemia viruses. *Biomedicine* **23,** 295 (1975).
12. J. Stephenson, A. Axelrad, D. McLeod *et al.*, Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro*. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1542 (1971).

13. F. Iscove and F. Sieber, Erythroid progenitor in mouse bone marrow detected by macroscopic colony formation in culture. *Exp. Hematol.* **3,** 32 (1975).
14. C. Zürcher, J. Brinkhof, P. A. J. Bentvelzen and J. De Man, C-type virus antigens detected by immunofluorescence in human bone tumour cultures. *Nature (Lond.)* **254,** 457 (1975).
15. M. Hill and J. Hillova, Virus recovery in chicken cells treated with Rous sarcoma cell DNA. *Nature New Biology* **237,** 35 (1972).
16. G. Cooper and H. Temin, Infectious Rous sarcoma virus and reticuloendotheliosis virus DNAS. *J. Virol.* **14,** 1132 (1974).
17. A. Hacket and S. Sylvester, Cell line derived from BALB/3T3 that is transformed by murine leukemia virus: a focus assay for leukemia virus. *Nature New Biology* **239,** 164 (1972).
18. M. Rozenberg, D. Baltimore and Ch. Scher, *In vitro* transformation of lymphoid cells by Abelson murine leukemia virus. *Proc. nat. Acad. Sci. (Wash.)* **72,** 1932 (1975).
19. J. Hillova, R. Mariage and M. Hill, Infectious DNA coding for a temperature sensitive DNA polymarase of the coordinate LA335 mutant of Rous sarcoma virus (RSV). *Virology* **67,** 292 (1975).
20. D. Smotkin, A. Gianni, S. Rozenblatt and R. Weinberg, Infectious viral DNA of murine leukemia virus. *Proc. nat. Acad. Sci. (Wash.)* **72,** 4910 (1975).
21. P. Neiman, S. Wright, C. McMillin and D. MacDonnell, Nucleotide sequence relationships of Avian RNA tumor viruses: measurement of the deletion in a transformation defective mutant of Rous sarcoma virus. *J. Virol.* **13,** 827 (1972).

Europ. J. Cancer Vol. 12, pp. 639–649. Pergamon Press 1976. Printed in Great Britain

Reactivity of Peripheral Blood Leucocytes against Human Fetal Cells*

BRENT M. VOSE and MICHAEL MOORE
Department of Immunology, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England

Abstract—*Cell cultures from different organs of two 13-week-old fetuses were exposed in microplate wells to leucocyte preparations from healthy donors, cancer patients and patients with non-malignant chest conditions. Leucocytotoxicity was observed for the majority of combinations tested in which survival of target cells was compared with those incubated in medium alone. Mean cytotoxic indices against target cells were in general significantly higher in wells exposed to leucocytes from patients with neoplasia or non-malignant conditions than those from healthy donors but there was considerable overlap in reactivity and the difference between groups was one of degree rather than of specificity. A similarly elevated reactivity was demonstrable against the long term bladder carcinoma cell line T24 but not short term cultures of lung and colonic neoplasias.*

Some variation in susceptibility to leucocytotoxicity was also encountered among the cells from different organs, those of lung and gut showing greatest sensitivity, and those of heart and brain least sensitivity. The phenomenon did not appear to be related to the proportion of non-lymphocytic cells in the effector population, nor was there clear evidence of an induction of nutritional deficiency in the medium during the course of the micro-assay. The implication of these findings for the detection of fetal antigens putatively expressed on human malignant cells is discussed.

INTRODUCTION

PREVIOUS studies in this laboratory [1, 2] have revealed that cells cultured from 13 to 14-week-old fetal tissues show a high susceptibility to the cytotoxic potential of leucocytes from cancer patients as assessed in the microassay method of Takasugi and Klein [3]. Leucocytes from healthy donors, although having some reactivity in these tests, were less effective than those of the cancer patients in reducing the number of remaining adherent cells. These results are superficially not dissimilar to findings in other laboratories [4, 5] and their implication is that high fetal cell susceptibility to activity in cancer patient leucocytes is a reflection of the development of cell-mediated sensitivity to antigens putatively expressed on tumor cells and shared by embryonic tissues, i.e., fetal antigens.

With the current controversy regarding the capacity of the cytotoxicity test to indicate specific cell-mediated responsivity particularly in cancer patients such interpretations must be regarded with caution. Leucocytes from healthy donors continue to reveal high cytotoxicity for a range of cultured target cells [2, 6, 7] such that the definition of specific reactivity in cancer patients to tumor-associated antigens by this assay would appear unlikely.

With these assay limitations in mind it was the intention of this study to investigate further the consistent elevation of cytotoxicity in cancer patient leucocytes noted in the previous reports. The reactivity of leucocytes from patients with malignant and non-malignant disorders against cultured fetal cells was compared with that of leucocytes from normal individuals. In order to promote some uniformity at the target cell level and thus permit meaningful comparisons of cell survival in the presence of leucocytes from various donors, cultures were initially limited to the organs of two fetuses and tested after identical periods in tissue culture. The specificity of the observed cytotoxicity was then amenable to examination on a series of closely related cultures in which genetically determined antigens were common although quantitative

Accepted 4 February 1976.
*This study was supported by grants from the Medical Research Council and the Cancer Research Campaign.

variation in the expression of these, or of antigens which might be acquired in tissue culture cannot be excluded.

MATERIAL AND METHODS

Fetal tissue

Fetal tissue was obtained from two patients undergoing hysterotomy for the therapeutic termination of pregnancy. The period of gestation was estimated to be 13 weeks from fertilization on the basis of fetal size and appearance as described by Moore [8]. Tissue samples were dissected from the following organs: brain, lung, heart, kidney, liver, thymus, gut and skin with underlying muscle. They were transported to the laboratory for the initiation of tissue cultures within 2 hr of surgery.

Preparation of tissue cultures

Tissue samples were finely minced with scissors and fragments (1–2 mm^3) were allowed to adhere to the surface of 25 cm^2 Falcon flasks in Waymouth's medium supplemented with 20% fetal calf serum (FCS), 200 μg/ml ampicillin, 100 μg/ml streptomycin and 2·5 μg/ml Amphotericin B. The monolayer cultures were maintained by serial passage and used as targets for leucocytotoxicity tests between the first and fourth passages.

Patients

Blood samples (20 ml) were obtained from patients attending hospitals in this region for confirmed malignancy (Table 1). Patients with carcinoma of the lung, breast, colon and bladder comprised a largely untreated group with samples being taken pre-operatively or before the first exposure to local radiotherapy. Other samples were taken at various times during a local radiotherapy regimen comprising a series of fractionated dosages. Specimens from healthy individuals were obtained from the National Blood Transfusion Service or from laboratory personnel. Samples were also obtained from patients with a variety of heart and chest conditions attending a local outpatient clinic.

Leucocytes

Leucocytes were prepared by centrifugation (800 ***g*** for 20 min) of samples of heparinised blood on gradients of Ficoll 400 (Pharmacia, Uppsala, Sweden) and Triosil (Nyegaard and Co., Oslo, Norway) of specific density 1·077.

Table 1. *Leucocyte donors*

	No. tested
Healthy donors	*62*
Cancer patients	
Lung	21
Breast	20
Head and Neck	8
Colon	5
Bladder	4
Miscellaneous (Melanoma, Wilms, Lymphoma)	11
Total	*69*
Patients with non-malignant conditions	
Chest infection including tuberculosis	8
Heart disease	4
Bronchitis and Emphysema	4
Miscellaneous chest conditions	9
Total	*25*

Microcytotoxicity tests

The cytotoxicity of peripheral blood leucocytes from patients and healthy individuals for cultured target cells was measured by the microassay method of Takasugi and Klein [3] using a leucocyte:target ratio of 75:1. Briefly 200 target cells were seeded into the wells of Microtest plates (No. 3034; Falcon Plastics Co., Los Angeles) in Eagle's MEM buffered with 40 mM HEPES (N-2 hydroxyethyl-piperazine-N′-ethanesulphonic acid) supplemented with 10% FCS. After 4–24 hr incubation at 37°C to allow cell attachment the medium was replaced by 10 μl aliquots of leucocyte suspensions ($1·5 \times 10^6$/ml). After a further 2 days incubation the plates were washed with saline to remove non-adherent cells, fixed with methanol and stained with 0·5% crystal violet. The degree of cytotoxicity was calculated as the percentage reduction of cell survival (cytotoxic index) in those wells treated with leucocyte suspensions as compared with wells which received medium alone. The significance of the difference was determined by the Student's *t* test. Reductions of cell survival of greater than 15% were found to be significant at at least the 95% level. In all tests in which the cytotoxicity of leucocytes from a single individual was assessed against cultures derived from different fetal organs, the cultures had been maintained for comparable periods in tissue culture. Only cells which showed unequivocal

target cell morphology were counted, i.e., adherent fibroblasts so that any contributions of adherent leucocytes to the survival count was excluded.

RESULTS

Cytotoxic reactions against cells from Embryo 37

Leucocyte preparations from both healthy donors and cancer patients were tested for cytotoxicity against tissue cultures derived from a number of organs of the same fetus (Fig. 1–5). By comparison with those cells remaining in wells after treatment with medium alone the number of targets surviving exposure to leucocytes was frequently but variably reduced so

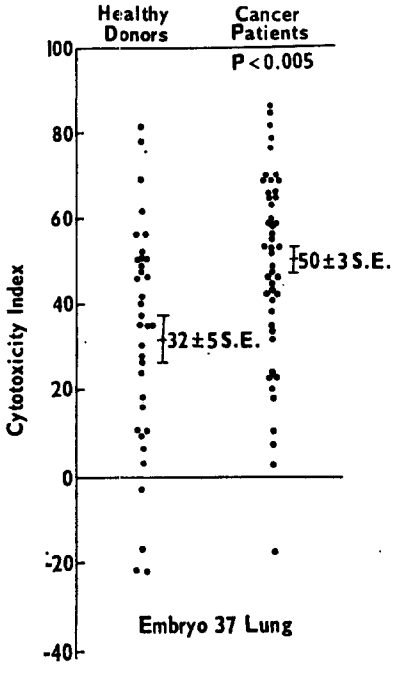


Fig. 1. *Cytotoxic indices of peripheral blood leucocytes from healthy donors and cancer patients for target cells cultured from Embryo* 37 *lung. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.*

that a range of cytotoxic indices were derived against all the targets examined. Cytotoxic potential was determined in both the healthy donor and cancer patient groups so that differences were of degree rather than specificity. However, taking the reactivity of the healthy donors as a group as control, a significant elevation of cytotoxicity was evident against each cultured target cell type ($P < 0{\cdot}01$) in the cancer patients. This difference was also maintained when cells cultured from the

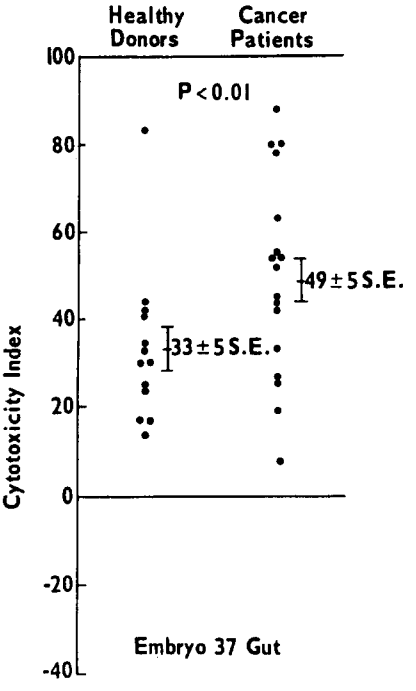


Fig. 2. *Cytotoxic indices of peripheral blood leucocytes from healthy donors and cancer patients for target cells cultured from Embryo* 37 *gut. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.*

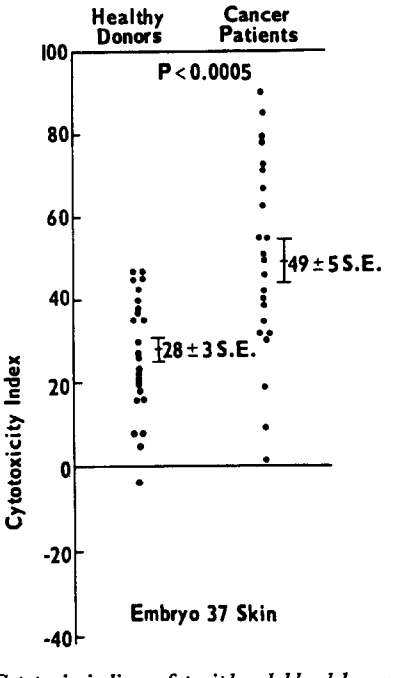


Fig. 3. *Cytotoxic indices of peripheral blood leucocytes from healthy donors and cancer patients for target cells cultured from Embryo* 37 *skin. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.*

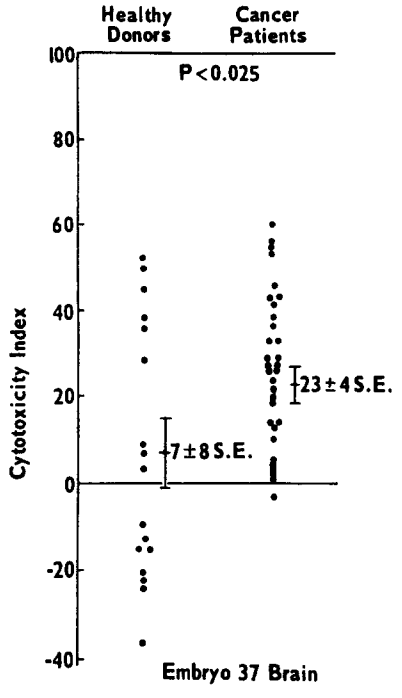


Fig. 4. Cytotoxic indices of peripheral blood leucocytes from healthy donors and cancer patients for target cells cultured from Embryo 37 brain. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

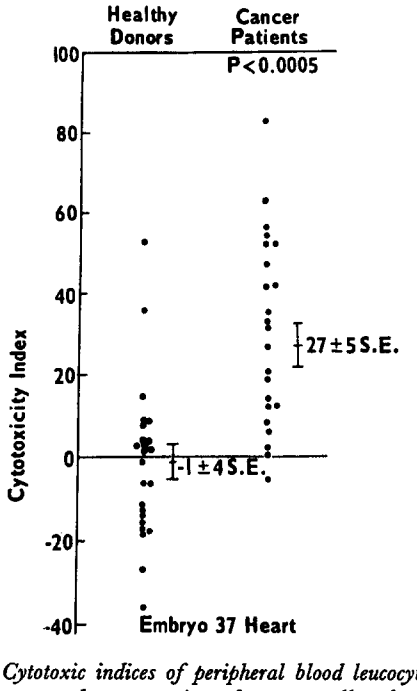


Fig. 5. Cytotoxic indices of peripheral blood leucocytes from healthy donors and cancer patients for target cells cultured from Embryo 37 heart. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

thymus, liver and kidney of this fetus were used as targets (figures not shown).

Since differences in survival such as those described here are likely to result at least in part from the substantial change in cultural conditions of target cells with and without added leucocytes, tests were designed so that any microtest plates contained at least 1 healthy donor preparation as well as the medium alone control. Cytotoxic indices could then also be calculated by reference to the survival of cells in wells treated with the least active healthy donor preparation on a given plate. Results calculated in this way revealed that although some healthy donor preparations were significantly cytotoxic by comparison with others (Table 2, tests 5 and 6) in general their reactivity was significantly less than the cancer patients tested at the same time. For the sake of brevity only examples of such calculations with target cells derived from fetal lung are presented (Table 2) but all other targets showed similar patterns of reaction. A further feature of the experimental design was that when possible each leucocyte preparation was tested for cytotoxicity against a number of fetal target cell cultures and "adult" cultures (the long term bladder carcinoma cell line T24 and the short term cultures L6 and CO32 derived from lung and colon carcinomas respectively). It was thus possible to assess both the relative susceptibility of the fetal cultures to leucocytotoxicity and the specificity of the reaction. Table 3 shows representative examples of the patterns of reactivity observed in this study against cells of various derivations. While some leucocyte preparations from healthy donors failed to induce cytotoxicity (Patients 2, 3 and 5) and others showed selective but occasionally high cytotoxicity, those from cancer patients were almost without exception toxic for all the fetal targets against which they were tested (Patients 13, 15–23, 25–26). A comparison of the sensitivity of the different target cells to a reduction of survival by the same leucocytes showed that cultures derived from lung, gut, skin and kidney were significantly ($P < 0{\cdot}05$) more reactive than those from heart, brain, thymus an liver. These differences were apparent with leucocytes from both healthy donors and cancer patients. Among those cultures which were susceptible to leucocytotoxicity, none revealed preferential sensitivity to any group of cancer patients whose disease originated in the same organ, i.e., reactivity was a reflection of culture sensitivity rather than an organ-related phenomenon. Fetal lung cells were then no more or less

Table 2. Cytotoxicity of peripheral blood leucocytes from healthy donors and cancer patients for fetal lung cells

Test no.	Patient	No. of cells surviving in wells treated with: Medium alone	Leucocytes	Cytotoxic index wrt medium	Cytotoxic index wrt healthy donor
1.	Healthy donor	63 ± 2	41 ± 7	35*	
	Colon Ca		49 ± 4	23*	18
	Breast Ca		20 ± 2	68*	52*
	Melanoma		21 ± 3	65*	47*
	Healthy donor		31 ± 3	50*	23
2.	Healthy donor	171 ± 12	210 ± 7	−22	
	Ca larynx		203 ± 8	−18	3
	Ca bladder		113 ± 9	34*	46*
	Ca oesophagus		72 ± 12	58*	66*
	Ca oesophagus		91 ± 11	47*	57*
3.	Healthy donor	105 ± 9	68 ± 10	35*	
	Ca larynx		57 ± 8	46*	17
	Ca pituitary		39 ± 3	63*	43*
	Post cricoid Ca		42 ± 5	60*	39*
4.	Healthy donor	96 ± 6	37 ± 4	62*	
	Hodgkin		39 ± 5	59*	−7
	Hodgkin		29 ± 3	70*	21*
	Ca bladder		90 ± 7	7	−142
5.	Healthy donor	43 ± 2	37 ± 2	16*	
	Lung		20 ± 1	55*	46*
	Lung		28 ± 1	35*	23*
	Lung		18 ± 1	59*	51*
	Healthy donor		27 ± 1	37*	25*
6.	Healthy donor	109 ± 5	78 ± 4	28*	
	Bladder		56 ± 4	48*	28*
	Lung		51 ± 3	53*	35*
	Lung		38 ± 3	65*	52*
	Healthy donor		53 ± 3	51*	32*
7.	Healthy donor	103 ± 4	56 ± 5	46*	
	Bladder		49 ± 1	53*	13
	Colon		50 ± 2	52*	12
	Lung		25 ± 2	76*	55*

* < 0·05 wrt = with respect to.

susceptible to leucocytes from patients with pulmonary neoplasia than those of any other group of patients.

Tests against "adult" cultures of malignant derivation were somewhat disparate. A battery of leucocyte preparations from healthy donors and cancer patients were tested variously against cells cultured from embryo lung, T24, L6 and CO32 (Table 4). The pattern of reactivity noted with the embryo lung cultures as targets i.e. elevated cytotoxicity in the cancer patient group was also apparent with the long term culture T24. By contrast, this difference was not present against the short term cultures L6 and CO32. Leucocyte preparations from cancer patients with malignancies at sites corresponding to the selected target cells, e.g., lung carcinoma against L6 were excluded from consideration in each case.

Cytotoxic reactions against cells of Embryo 38

In order to characterise more fully the occurrence and reproducibility of the cytotoxic reactivity demonstrated against cells from E37, a further series of healthy donors, cancer patients and, in addition, donors with non-malignant chest and heart conditions were tested against cells cultured from the lung, gut, liver and brain of a second 13-week-old human fetus (Fig. 6–9). A range of cytotoxic indices against target cells with leucocytes from all groups of donors examined was again a major feature of these studies. In common with results

Table 3. *Cytotoxicity of peripheral blood leucocytes from healthy donors and patients with malignancy for cultured fetal tissues*

No.	Diagnosis	Sex	Cytotoxic index against:— Lung	Skin	Gut	Thymus	Heart	Brain	Liver	Kidney
1.	Healthy donor	M	28	28		30	2	−24		
2.		M	−22	−4		6	−27	−15		
3.		M	3	8		−1	4	9		
4.		M	46	38		12	−17	−22		
5.		M	−17	5		2	−36	−68		
6.		M	10	−18		15	−18	−37		
7.		F	35			22	15			
8.		F	49	37	25	35	9		53	25
9.		F	35	22		51	4	45	53	
10.		F	−22	23		−9	−16	−10		
11.		F	30	20		−7	3			
12.		F	47	27		6	8			
13.	Lung carcinoma	M	38		54	48				84
14.		M	42	19		15	33	10		
15.		F	55	63				53	37	
16.		M	53	67		51	52	20		
17.		F	77	85		76	52	55		
18.	Breast carcinoma	F	69	78		62	83	43		
19.		F	40		55	60				61
20.		F	56	79		56	56	60		
21.	Bladder carcinoma	M	48	55		36	41	26		
22.		F	34	40		40	12	24		
23.	Colon carcinoma	F	23	35		19	47	43		30
24.		M	10	1		−9	2	1		
25.	Melanoma	M	66	54		46	30	2		4
26.	Oesophageal carcinoma	F	58	39		49	19	13		

Table 4. *Cytotoxicity of peripheral blood leucocytes from healthy donors and cancer patients for cells cultured from human malignancies and fetal lung*

Target cells	Cytotoxic indices with leucocytes from: Healthy donors	Mean	Cancer patients	Mean	$P <$
T24	34, 47, 37, 54, 40, 27 15, 22, 30, 10, 7, 18, 22, 19.		22, 76, 87, 88, 67, 65 80, 47, 48, 87, 83, 92, 48, 25.		0·0005
L6	29, 28, 8, 42.	27	24, 58, 63, 32, 4, 34 24.		0·30
CO32	53, 0, 37, 5, 24, 20 −10, 6, 45.		48, 50, 45, 5, 16, 23 13, −3, 13.		0·40
Embryo lung	24, 51, 28, 46, 25, 20 23, 7, 40, 27, −22, −22, 18, 42, −3.		43, 42, 38, 53, 65, 45 48, 53, 52, 75, 55, 63, 42, 44, −19, 34, 58, 47, 10.		0·0025

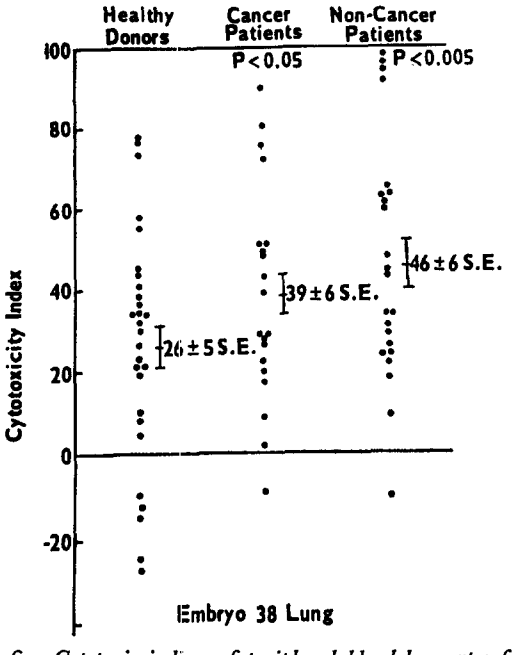


Fig. 6. Cytotoxic indices of peripheral blood leucocytes from healthy donors, cancer patients and non-cancer patients for Embryo 38 lung. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

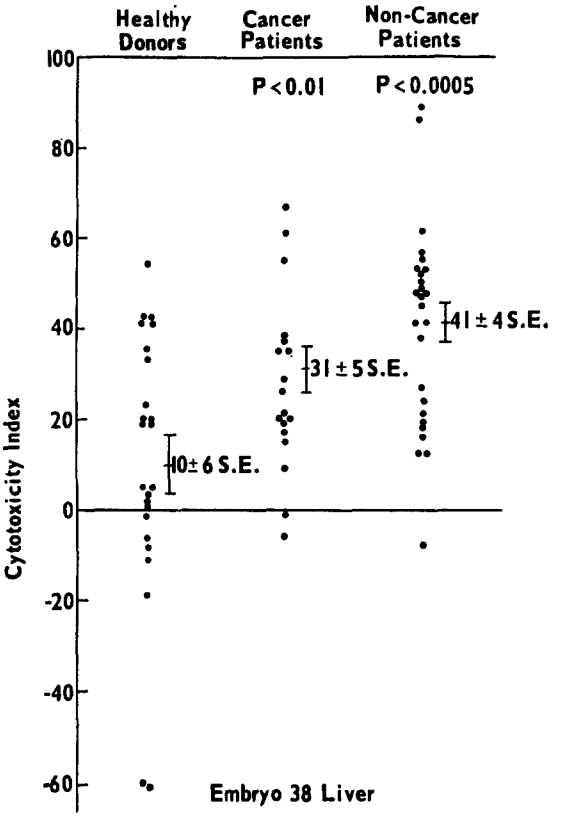


Fig. 8. Cytotoxic indices of peripheral blood leucocytes from healthy donors, cancer patients and non-cancer patients for Embryo 38 liver. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

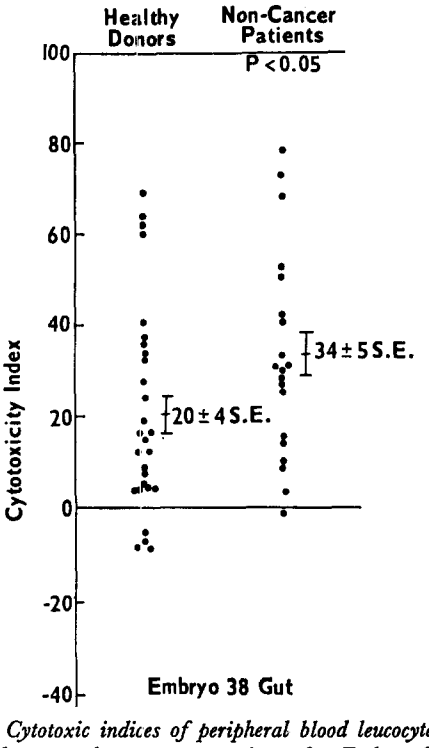


Fig. 7. Cytotoxic indices of peripheral blood leucocytes from healthy donors and non-cancer patients for Embryo 38 gut. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

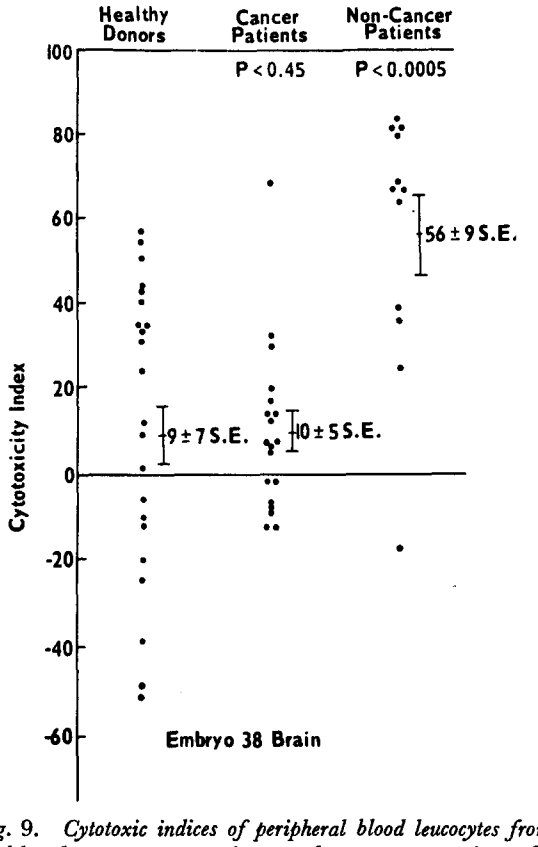


Fig. 9. Cytotoxic indices of peripheral blood leucocytes from healthy donors, cancer patients and non-cancer patients for Embryo 38 brain. Cytotoxic index was calculated by reference to the number of target cells surviving in wells incubated with growth medium alone.

from E37, leucocytes from cancer patients showed significantly greater reactivity against fetal lung and liver cells than those from healthy donors though this was not apparent against E38 brain cells. Furthermore, leucocytes from patients with non-malignant conditions exhibited reactivity which not infrequently exceeded that of the cancer patients and was invariably greater than that of the healthy donors. Again there was no evidence of any organ-related reactivity with this embryo.

Nature of the cytotoxic effect

Leucocyte preparations used in this study usually comprised over 90% lymphocytes with variable granulocyte and monocyte and platelet contamination. Occasional preparations had relatively fewer lymphocytes (Table 5). Such preparations could not be predicted and no attempt was made to exclude these from consideration in the preceding experiments. No clear correlation existed between the level of non-lymphocytic contamination and cytotoxicity (Table 5). Thus preparations with high contamination might show low cytotoxic potential and vice versa. Attempts were made to select further for lymphocytic elements by incubation of the leucocyte preparations on a large glass surface. Such a procedure routinely elevated the population of lymphocytes in the sample. A significant ($P < 0{\cdot}025$) diminution of cytotoxic potential was apparent in preparations so treated compared with their reactivity prior to the depletion of adherent cells (Table 5). However, even amongst the healthy donor group these manipulations were not uniformly effective in reducing cytotoxicity to undetectable levels.

Dilution of the effector cell populations showed that cytotoxicity was relatively stable over a range of effector cell:target cell ratios (Table 6). Indeed, the reactivity of many leucocyte preparations did not decline until the ratio reached 6:1. Viable target cells were not discernable in the discarded supernatants at the termination of the cytotoxicity assay and the replating of such supernatants failed to initiate further attachment and growth of target cells. When the culture supernatants from higher effector cell:target cell ratios (400:1–100:1) were taken after the 2 day incubation of the microassay and freed of cells by centrifugation they occasionally had a reduced capacity to support the growth of further target cells when transferred (Table 6). Discarded supernatants from medium control wells or from lower cytotoxic effector:target cell ratios were capable of supporting such further growth for at least 2 days (Table 6). Cytotoxicity of leucocytes was then detectable in the absence of any notable reduction of the capacity of the medium in which they were suspended to support target cell growth.

Table 5. *Variation in the proportion of lymphocytes and cytotoxicity* against fetal skin of leucocyte preparations following depletion of adherent cells†*

	Leucocyte preparations			
	Before depletion		After depletion	
Patients	% lymphocytes	Cytotoxic index	% lymphocytes	Cytotoxic index
Healthy donors	65	28	96	0
	65	5	79	27
	75	49	96	2
	89	0	93	11
	90	0	97	0
	90	39	100	21
	93	55	100	27
	96	24	100	0
Cancer patients	78	48	100	28
	79	29	93	2
	89	23	100	20
	85	16	98	0
	98	40	100	44

*Cytotoxicity calculated by comparison with the survival of cells in wells treated with growth medium alone.

†Depletion of adherent cells by 1 hr incubation 37°C in a glass bottle.

Table 6. Comparison of the cytotoxicity of peripheral blood leucocytes from healthy donors and the supernatant of the microtest estimation of their cytotoxicity against fetal lung cells

	No. of cells surviving treatment with:-			
		Leucocytes		
	Medium alone	100:1	50:1	25:1
Preparation 1	70 ± 4	15 ± 2 (78)	13 ± 1 (82)	13 ± 2 (81)
Preparation 2	57 ± 4	19 ± 3 (66)	17 ± 2 (70)	16 ± 2 (72)
Preparation 3	115 ± 6	57 ± 4 (50)	84 ± 5 (27)	115 ± 5 (0)
Culture supernatant of preparation 1	54 ± 4	40 ± 4 (26)	49 ± 2 (9)	N.T.
Culture supernatant of preparation 2	51 ± 4	28 ± 4 (45)	55 ± 9 (−9)	58 ± 4 (−15)
Culture supernatant of preparation 3	66 ± 5	60 ± 5 (11)	77 ± 3 (−17)	87 ± 6 (−32)

Numbers in parentheses refer to the cytotoxic index of the test compared with a medium control. N.T. = not tested.

DISCUSSION

In agreement with previous studies [1, 2, 4, 5] it has been demonstrated that peripheral blood leucocytes from patients with malignancy have an elevated cytotoxic potential compared with that of leucocytes from healthy donors. Surprisingly in this study the heightened reactivity was also shared by leucocyte preparations from non-malignant pulmonary and coronary conditions. This latter finding suggests that cytotoxicity against fetal cells is a disease-related phenomenon rather than a cancer-specific event. As such the concept of expression of fetal antigens (a consistent feature of malignant transformation in animal tumor models [9, 10]) in human neoplasia requires reappraisal particularly when such antigens have been defined by a cellular cytotoxicity methodology such as that applied in the present study.

The extension of cytotoxicity outside the confines of malignant disease indicates that the high susceptibility of cells cultured from the human fetus may result from modification in the behaviour of leucocytes *in vitro* by deviation from the healthy state, such as might be effected for instance in a change in the absolute number or reactivity of individual leucocyte populations.

An alternative explanation of this high sensitivity to reduced survival in fetal targets induced by leucocytes from cancer patients, patients with non-malignant diseases and some healthy donors is that the differentiated state *in vivo* shows a high degree of instability [11–13] such that dedifferentiation with the expression of novel antigenic determinants is a common concomitant of disease and regeneration processes. Cellular sensitization to these antigens might account for cytotoxicity against fetal cells. Additionally, a high susceptibility to lysis in long-term cultures has been described [14] and is apparent from this study in that the long term culture T24 showed a "fetal" type of reactivity whereas L6 and CO32 were relatively resistant. Since a rapid and apparently irreversible dedifferentiation is known to occur in tissue culture [15] this could be interpreted as both an expression of temporally ectopic antigens following culture and an acquisition of high susceptibility to non-specific lysis. These two possibilities cannot be distinguished using a cytotoxicity system so that it must remain a matter for conjecture whether activity reflects an immune event or increased non-specific reactivity of leucocyte effector cells.

An increasing number of studies [2, 6, 7, 16] are currently appearing in the literature concerning the cytotoxicity of apparently healthy donors. The majority of such tests have used the survival of cells in wells treated with growth medium alone as control. We feel that this is justified because of the wide range of cytotoxic indices found in these normal donors. It is a necessary prerequisite in comparisons of reactivity of groups of patients that a stable baseline be used. By comparison with the survival in growth medium, leucocytes from healthy donors induced some cytotoxicity, which was unpredictable with respect to the

gross composition of the effector cells and often showed some target cell selectivity. Depletion of adherent cells in these populations reduced, but did not abolish cytotoxicity, suggesting the involvement of multiple factors in the phenomenon. At the effector target cell ratios used in this study there was no evidence of nutritional deficiency in the growth medium so that a more active cellular process is suggested for the "background" fall in cell survival.

Leucocytes from patients with malignancy and with non-malignant disorders showed raised reactivity compared with those of healthy donors. The cytotoxicity generally revealed little selectivity with regard to fetal target cell derivation but was evident in the calculation of cytotoxicity by comparison with both medium controls and the least active healthy donor preparation tested simultaneously. Cancer patient cytotoxicity then comprises the "background" reactivity which may vary and which is shared by the healthy donors and a superimposed further reactivity the nature of which has yet to be defined and to which long term cultures and fetal cells are exquisitely sensitive.

The further definition of the effector cell and nature of this cytotoxic effect will be obligatory in future investigations. Cytotoxicity testing in the allogeneic combination can no longer be considered capable of defining the presence of antigenic specificities on cultured target cells against which patients might become sensitized. Such tests, however, continue to show statistically significant differences between groups of patients and healthy donors which are of intrinsic interest. The acquisition of data by careful selection of control populations must be avoided since such data can only further confuse studies of the specificity of *in vitro* cytotoxicity.

Acknowledgements We are grateful to Ian Kimber and Roger Ferguson for skilled technical assistance.

Fetal tissue was obtained in compliance with the Local Ethical Committee of the University Hospital of South Manchester.

REFERENCES

1. B. M. Vose, M. Moore and G. D. Jack, Cell-mediated cytotoxicity to human pulmonary neoplasms. *Int. J. Cancer* **15,** 308 (1975).
2. B. M. Vose, M. Moore, P. F. Schofield and I. W. Dymock, Leucocytotoxicity in malignant and non-malignant colonic diseases. *Clin. exp. Immunol.* **22,** 393 (1975).
3. M. Takasugi and E. Klein, A microassay for cell-mediated immunity. *Transplantation* **9,** 219 (1970).
4. I. Hellström, K. E. Hellström and J. H. Sheperd, Cell-mediated immunity against antigens common to human colonic carcinomas and fetal gut epithelium. *Int. J. Cancer* **6,** 346 (1970).
5. G. Fossati, S. Canevari, S. and G. Della Porta, Cellular immunity in cancer patients against embryonal cells. *J. nat. Cancer Inst.* **51,** 667 (1973).
6. M. Takasugi, M. R. Mickey and P. I. Terasaki, Reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer Res.* **33,** 2898 (1973).
7. G. E. Pierce and B. L. Devald, Effects of human lymphocytes on cultured normal and malignant cells. *Cancer Res.* **35,** 1830 (1975).
8. K. L. Moore, *The Developing Human,* p. 78. W. B. Saunders, Philadelphia (1973).
9. R. W. Baldwin, M. J. Embleton, M. R. Price and B. M. Vose, Embryonic antigen expression on experimental rat tumours. *Transplant. Rev.* **20,** 78 (1974).
10. C. C. Ting, D. M. Lavrin, G. Shiu and R. B. Herberman, Expression of fetal antigens in tumor cells. *Proc. nat. Acad. Sci. (Wash.)* **69,** 1664 (1972).
11. E. D. Hay, Dedifferentiation and metaplasia in vertebrate and invertebrate regeneration. In *The Stability of the Differentiated State.* (Edited by H. Ursprung). Springer-Verlag, Berlin. p. 85 (1968).
12. N. G. Anderson and J. H. Coggin, Models of differentiation, retrogression and cancer. In *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* (Edited by N. G. Anderson and J. H. Coggin). U.S.A.E.C. p. 7 (1971).
13. G. I. Abelev, α-Fetoprotein as a marker of embryo-specific differentiations in normal and tumor tissues. *Transplant. Rev.* **20,** 3, (1974).

14. J. E. DE VRIES, M. MEXERING, A. VAN DONGEN and P. RÜMKE, The influence of different isolation procedures and the use of target cells from melanoma cell lines and short term cultures on the non-specific cytotoxic effects of lymphocytes from healthy donors. *Int. J. Cancer* **15,** 391 (1975).
15. I. R. KONIGSBERG, Clonal analysis of myogenesis. *Science* **140,** 1273 (1963).
16. H. H. PETER, J. PAVIE-FISCHER, W. H. FRIDMAN, C. AUBERT, J.-P. CESARINI, R. ROUBIN and F. M. KOURILSKY, Cell-mediated cytotoxicity *in vitro* of human lymphocytes against a tissue culture melanoma cell line (ICR3). *J. Immunol.* **115,** 539 (1975).

Europ. J. Cancer Vol. 12, pp. 651–655. Pergamon Press 1976. Printed in Great Britain

Size Distribution Patterns of Nuclei from Normal Rat Liver and from Two Hepatomas

EGON DURBAN* and ELISA M. DURBAN

Institut für Medizinische Molekularbiologie der Medizinischen Hochschule Lübeck 24 Lübeck, Ratzeburger Allee 160, Germany

Abstract—*Size distribution patterns of nuclei isolated from liver of rats of various ages and patterns of nuclei isolated from two Morris minimal deviation hepatomas were obtained with a Coulter Counter Size Distribution Plotter and were related to chromosome number and DNA content/nucleus. Nuclei from hepatoma 5123TC were found significantly larger than liver nuclei of 6 week old rats which correlates well with the larger number of chromosomes (49–52) found in hepatoma 5123TC as compared to 42 in rat liver and also correlates well with the higher DNA content per nucleus in this hepatoma. Nuclei of hepatoma 9121, on the other hand, were of the same size as rat liver nuclei which again correlates with our finding that nuclei of hepatoma 9121 and rat liver had the same number of chromosomes (42) and the same DNA content per nucleus. Finally, polyploidy could be easily demonstrated in nuclei of aging rats.*

INTRODUCTION

IT IS well known that the quality of a nuclei preparation is of primary importance in biochemical experiments with isolated nuclei. Furthermore, depending on the type of tissue, a number of factors must be considered as for example in the case of liver, the degree of liver parenchymal cell ploidy and the homogeneity of the preparation in terms of one kind of nuclei. Biochemical experiments involving normal rat liver and Morris minimal deviation hepatomas require characterization of the isolated nuclei preparation in order to make valid comparison studies.

A Coulter Counter has been previously used to count isolated rat liver nuclei [1] and to determine the size distribution of fractionated diploid and tetraploid rat liver nuclei [2] as well as for size distribution studies of whole nuclei populations [3]. We describe and evaluate in this paper a method for characterizing whole isolated nuclei preparations from rat liver and two hepatomas by means of size distribution patterns.

Accepted 5 February 1976.

*Present address: Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.

MATERIAL AND METHODS

Animals

Wistar rats 5–6 weeks, 3–4 months, 5–6 months, and 8–12 months old were used for preparing normal liver nuclei. Morris minimal deviation hepatomas 5123TC (generations 110–120) and 9121 (generations 60–70), maintained by intramuscular transplantation into Buffalo and ACI rats, respectively, were used for preparation of tumor nuclei.

Nuclei isolation

Nuclei from normal rat liver were isolated by the method of Widnell and Tata [4]. Briefly, livers were suspended in 0·32 M sucrose–3 mM $MgCl_2$, minced finely, and homogenized in a Potter S homogenizer (Braun, Melsungen, Germany) with six up and down movements and with a pestle speed of 1450 rev/min. Homogenate was filtered through three layers of gauze, diluted to 0·25 M sucrose with distilled water, and centrifuged at 700 *g* for 10 min, to get a crude nuclear fraction. These pellets were suspended in 2·4 M sucrose–1 mM $MgCl_2$ and centrifuged at 50,000 *g* for 1 hr.

Nuclei from Morris minimal deviation hepatomas were isolated by the method of Grunicke, Potter and Morris [5]. Briefly, tumors were excised and placed into 0·25 M sucrose–0·05 M Tris–HCl (pH 7·5)–0·025 M KCl–0·005 M $MgCl_2$, finely minced, and homogenized with 15 up and down movements at 1000 rev/min, in the Potter S homogenizer. The homogenate was filtered through three layers of gauze and centrifuged at 700 *g* for 10 min. Pellets were resuspended in 2·2 M sucrose–0·001 M citric acid–0·0004 M $CaCl_2$ (pH 5·9 with 0·1 M NaOH) and centrifuged for 1 hr at 100,000 *g*.

Preparation of purified nuclei for size distribution analysis

Purified pellets were carefully resuspended in 1 ml of 0·9% NaCl–0·25 M sucrose–3 mM $MgCl_2$–0·01 M Tris–HCl (pH 7·5). From this suspension, suitable dilutions were made for the Coulter counter (3000–5000 counts/4 sec/window in the peak region). Dilutions were analysed immediately after preparation since certain amount of clumping occurred after 15 min at room temperature. All dilutions were checked microscopically for structural integrity of the nuclei as well as for contamination. The diluent solution was filtered through a medium porosity glass filter to remove particulate debris and fibrous material.

Electronic particle counter

A Model B Coulter counter and Coulter automatic size distribution plotter (Coulter Electronics, Hialeah, Florida) were used. The instrument was previously described in detail by Brecher *et al.* [6]. Instrument settings of 1/APC = 0·707, 1/AMP = 2, and capillary aperture size of 50 μm were found to be satisfactory for size distribution patterns. Control patterns of the diluent solution were obtained each time and compared to the patterns of the samples. The instrument was calibrated with commercial latex particles (Coulter Electronics Ltd., England) of 6 μm dia or 113 μm^3 volume with the median in window 5, and 9·7 μm dia or 478 μm^3 volume with the median in window 19. Since the initial three plotter windows are primarily electronic noise, they were disregarded in any evaluation.

DNA determination

Purified nuclei pellets were dissolved in 50 ml 0·9% NaCl–0·24 M sucrose–3 mM $MgCl_2$–0·01 M Tris–HCl (pH 7·5) and filtered through 3 layers of gauze to eliminate clumps. Ten milliliter samples were brought to 4% perchloric acid, left at room temperature for 15 min, and centrifuged. The pellets were washed once with 4% perchloric acid and once with 1 ml of 1 M perchloric acid and extracted for 10 min at 70°C. The extraction step was performed twice. After extraction, DNA was determined by the method of Burton [7]. In addition, Coulter counter nuclear counts were performed on the samples used for DNA determination.

Chromosome preparation

Chromosomes from rat liver were prepared after the method of Sun and Chu [8]. Chromosomes from Morris minimal deviation hepatomas were prepared after Nowell *et al.* [9].

Statistical evaluation

First moments of the distribution curves describing the median were calculated as follows: $x = \sum (x_i f_i)/\sum f_i$ where f_i represents relative counts in window i at a distance x_i from the origin; so if a peak fell exactly into the 10th window, x was 9·5 cm.

RESULTS

Figure 1A shows representative distribution patterns of rat liver nuclei isolated from 6 week and 3–4 month old rats. Nuclei from 6 week-old-rats appear to give an approx. normal distribution curve with some tailing towards the higher size nuclei. The average first moment derived from 6 populations (considering windows 5–19) was 10·85 cm ($\pm$ 0·45 cm S.D.) corresponding to 282 μm^3 ($\pm$ 12 μm^3 S.D.). The nuclear size distribution patterns for the 3–4-month-old rats show a clear increase in number of nuclei of larger volume (from about window 14) when compared to the patterns of younger rats. However, as seen from the figure, this increase did not give a distinct second peak.

Nuclei size distribution patterns from 6-month- and 12-month-old rats are shown in Fig. 1B. The 6 month-old-rats do give a second nuclei population in addition to that at window 11. This second population cannot be described by first moment calculations since it overlaps to a large degree with the first population. However, from the size distribution patterns one can approx. place the median at

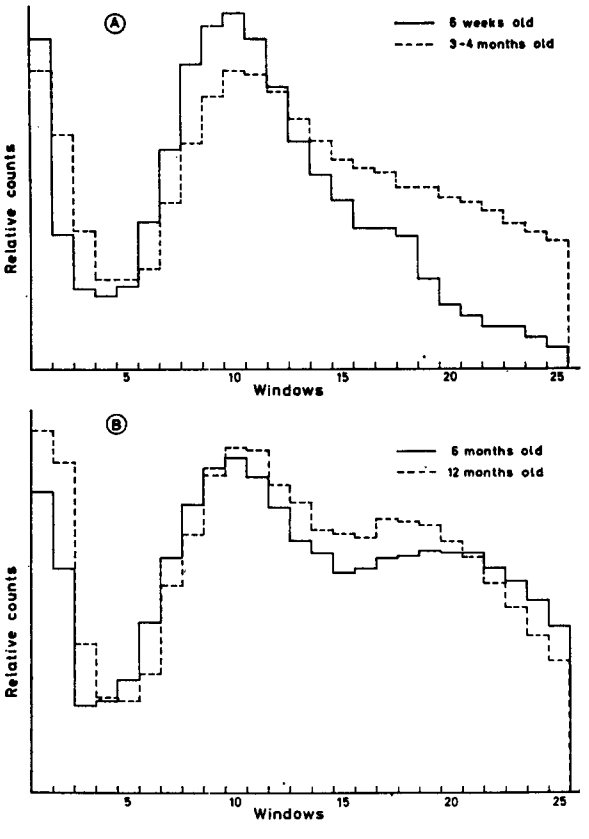


Fig. 1. *Size distribution patterns of rat liver nuclei.* (*A*) *Nuclei were isolated from livers of* 6 *week old rats* (——) *and* 3–4 *month old rats* (– – –). (*B*) *Nuclei isolated from livers of* 6 *month old rats* (——) *and* 12 *month old rats* (– – –). *Livers were homogenized in* 0·32 *M sucrose-*3 *mM* $MgCl_2$. *The homogenate was brought to* 0·25 *M sucrose concentration and centrifuged at* 700 **g** *for* 10 *min. Pellets were resuspended in* 2·4 *M sucrose-*1 *mM* $MgCl_2$ *and centrifuged for* 1 *hr at* 50,000 **g**; *pellets were then resuspended in* 0·9% *NaCl–*0·25 *M sucrose–*3 *mM* $MgCl_2$*–*0·01 *M Tris–HCl* (*pH* 7·5) *for counting.*

window 19. The 12-month-old rats give a nuclei distribution pattern very similar to that of 6-month-old rats. Furthermore, this is also true for rats between 6 and 12 months old.

Figure 2 illustrates typical nuclei distribution patterns from hepatomas 9121 and 5123TC. The average first moments derived from 6 populations were found to be 10·6 cm (± 0·68 cm S.D.) or 274 μm^3 (± 18 μm^3 S.D.) and 13·7 cm (± 0·72 cm S.D.) or 354 μm^3 (± 19 μm^3 S.D.) for hepatoma 9121 and 5123TC, respectively. It was of interest to determine whether differences between nuclei distribution patterns of 5123TC and rat liver nuclei (6 weeks old) were statistically significant. Using the Student's *t*-test, we calculated $t = 8{\cdot}2$; for 10 degrees of freedom and at the 1% significance level (highly significant difference), the table value is 3·169 as listed by Fisher and Yates [10]. One therefore can conclude that the 5123TC nuclei are different in size (with high significance) from rat liver nuclei. In a second *t*-test, a possible difference between rat liver nuclei and 9121 hepatoma nuclei was tested; *t* was found to be 0·75. Even at the 10% significance level (very low significance), the tabulated value for *t* is 1·812; since our calculated *t*-value was clearly below that value, there is no significant difference in size between nuclei of hepatoma 9121 and nuclei of rat liver.

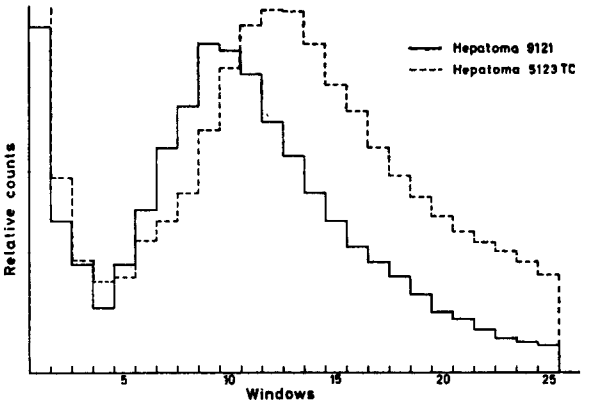


Fig. 2. *Size distribution patterns of nuclei isolated from Morris minimal deviation hepatomas* 9121 (——) *and* 5123*TC* (– – –). *Tumors were homogenized in* 0·25 *M sucrose–*0·05 *M Tris–HCl* (*pH* 7·5)*–*0·025 *M KCl–*0·005 *M* $MgCl_2$ *and centrifuged at* 700 **g**/10 min. *Pellets were resuspended in* 2·2 *M sucrose–*0·001 *M citric acid–*0·0004 *M* $CaCl_2$ *and centrifuged at* 100,000 **g** *for* 1 *hr. Nuclear pellets were resuspended in electrolyte buffer* (0·9% *pH* 7·5) *for counting.*

Results from chemical determination of DNA are summarized in Table 1. Nuclei from 6-week-old rats on the average contain somewhat less DNA per nucleus than those from older rats. Average DNA content per nucleus of hepatoma 9121 is similar to that of young rats whereas average DNA content per nucleus of hepatoma 5123TC is higher than that of young rats.

DISCUSSION

The size distribution patterns obtained with the Coulter counter from 6-week-old rat liver show a homogeneous population of approximately normal distribution. This population is most likely composed of parenchymal cell nuclei since other types of nuclei present in liver are of smaller size and would either cause skewing of the normal population or would be detected as a second smaller peak.

The size of parenchymal cell nuclei increased with age. This finding is in accordance with the well documented change from the diploid state of liver parenchymal cells at early age

Table 1. *Average DNA content and nuclear volumes as calculated from the size distribution patterns*

	Average DNA/nucleus* (gram × 10^{-12} ± stand. deviation)	Nuclear volumes (μ^3 ± stand. deviation)
Rat liver:		
1. 5–6 weeks	7·3 ± 0·6	282 ± 12
2. 7½ months		†
3. 12 months		†
Morris minimal deviation hepatoma 5123TC	11·2 ± 0·9	354 ± 19
Morris minimal deviation hepatoma 9121	7·6 ± 0·3	274 ± 18

*Means of DNA content per nucleus were obtained from 4 independent experiments. Per experiment, 3–5 livers or hepatomas were pooled; nuclei were isolated and DNA extracted as described under Materials and Methods.

†Two partially overlapping peaks (Fig. 1B), the first one lies approximately at 280 μm^3 and the second one at 470 μm^3.

to the tetraploid state at later age as studied by caryometry [11], microphotometrically [12, 13], sucrose gradient centrifugation [2, 14] and zonal centrifugation [1, 15]. Wannemacher *et al.* [3] reported qualitatively similar size distribution patterns obtained by a Coulter counter for liver nuclei of aging rats, however, their absolute values for nuclear volumes were apparently more than twofold lower than our values. This may possibly be due to the different ways of volume calibration. Wannemacher *et al.* [3] used erythrocytes whose size is actually very susceptible to e.g., osmotic changes while we used standard size Latex particles which are inert and independent of environmental influence such as e.g., variations in ionic strength, pH, or temperature. The size and DNA content of parenchymal liver nuclei reported by us agree well with those found by others [1, 2, 14, 15].

It was of interest to investigate whether the Coulter counter size distribution technique could also be employed for quick characterization of nuclei purified from hepatomas. Comparison of nuclei from 6-week-old rats with nuclei from Morris hepatoma 5123TC indicated that the latter nuclei were significantly larger. This finding correlated with the higher number of chromosomes present in nuclei of hepatoma 5123TC (49–52) as compared to 42 chromosomes present in diploid rat liver nuclei and is in accordance with the results of Nowell *et al.* [9] who reported this hepatoma in generation 42 to possess 49 chromosomes. Therefore, a change in the position of the peak indicates in this case an increase in chromosome number, i.e., in the amount of DNA per nucleus, as also seen from the average DNA estimation values. On the other hand, we did not find a difference in nuclear size (and in the position of the peak) between hepatoma 9121 and liver from 6-week-old rats. The chromosome number from this tumor in generation 64 was found to be, as for rat liver, 42 chromosomes. This is in accordance with chromosome counts in generation 7 reported by Nowell *et al.* [9]. Average DNA estimation values also support our observation that normal rat liver (6 weeks old) and hepatoma 9121 have nuclei of the same size.

In conclusion, the work described here demonstrates that distribution patterns obtained with the Coulter counter reliably show size differences among various nuclei populations. Furthermore, the significant difference in nuclear size found between hepatoma 5123TC and rat liver, systems often used for comparison, indicate the importance of controlling isolated nuclei preparations for biochemical experiments. The method described here is easy and can be performed quickly once the instrument has been calibrated and the proper settings and dilutions have been determined. We have also found it practical for controlling the quality and reproducibility of various nuclei preparations.

Acknowledgements—We wish to thank Prof. Dr. E. Harbers for his guidance throughout these studies as well as for providing the laboratory facilities. We also would like to thank Mrs. I. Fleischhauer and Miss U. Steinherr for skillful technical assistance and Mrs. G. Wegener for preparing the figures.

REFERENCES

1. I. R. Johnston, A. P. Mathias, F. Pennington and D. Ridge, The fractionation of nuclei from mammalian cells by zonal centrifugation. *Biochem. J.* **109,** 127 (1968).
2. R. J. Santen, Automated estimation of diploid and tetraploid nuclei with an electronic particle counter. *Exp. Cell Res.* **40,** 413 (1965).

3. R. W. WANNEMACHER, JR., K. MURAMATSU and W. K. COOPER, Size distribution and counting of liver nuclei by an electronic particle counter. *Proc. Soc. Exp. Biol. Med.* **129,** 899 (1968).
4. C. C. WIDNELL and J. R. TATA, A procedure for the isolation of enzymatically active rat-liver nuclei. *Biochem. J.* **92,** 313 (1964).
5. H. GRUNICKE, V. R. POTTER and H. P. MORRIS, Comparative studies on nuclei and chromatin of hepatomas and rat liver. *Cancer Res.* **30,** 776 (1970).
6. G. BRECHER, E. F. JAKOBEK, M. A. SCHNEIDERMAN, G. Z. WILLIAMS and P. J. SCHMIDT, Size distribution of erythrocytes. *Ann. New York Acad. Sci.* **99,** 242 (1962).
7. K. BURTON, A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
8. N. C. SUN and E. H. Y. CHU, An improved method for chromosome preparation from mouse liver. *Canad. J. Genet. Cytol.* **13,** 612 (1971).
9. P. C. NOWELL, H. P. MORRIS and V. R. POTTER, Chromosomes of "minimal deviation" hepatomas and some other transplantable rat tumors. *Cancer Res.* **27,** 1565 (1967).
10. R. A. FISHER and F. YATES, *Statistical Tables for Biological, Agricultural and Medical Research.* p. 46. Hafner, New York (1963).
11. C. NADAL and F. ZAJDELA, Polyploïdie somatique dans le foie de rat. I. Le rôle des cellules binucléées dans la genèse des cellules polyploïdes. *Exp. Cell Res.* **42,** 99 (1966).
12. H. NAORA, Microspectrophotometry of cell nuclei stained with the Feulgen reaction. IV. Formation of tetraploid nuclei in rat liver cells during postnatal growth. *J. biophys. biochem. Cytol.* **3,** 949 (1957).
13. M. ALFERT, Classification of liver cell nuclei by size and Feulgen reaction. In "Liver Function", (Edited by R. W. BRAUER), p. 11. American Institute of Biological Sciences, Washington (1958).
14. C. F. ALBRECHT, Fractionation of rat liver nuclei by sucrose gradient centrifugation. *Exp. Cell Res.* **49,** 373 (1968).
15. F. GONZALEZ-MUJICA and A. P. MATHIAS, Studies of nuclei separated by zonal centrifugation from liver of rats treated with thioacetamide. *Biochem. J.* **132,** 163 (1973).

Europ. J. Cancer Vol. 12, pp. 657–658. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Heterogeneity of Carcinoembryonic Antigen (CEA) in Human Serum*

BRANKA HERZOG†, J. C. HENDRICK‡ and P. FRANCHIMONT
Institute of Medicine, Radioimmunoassay Laboratory, University of Liége, Liége, Belgium

HETEROGENEITY of CEA was soon suspected following the demonstration by Coligan *et al.*, [1] that CEA extracted from tumours existed in at least 2 molecular sizes, which has the same immunoreactivity with their particular antiserum.

Furthermore, Plesh and Goldenberg [2] were able to detect a CEA molecule of molecular weight 370,000 Daltons in the plasma of patients with cancers of the colon, ovary, cervix and bronchus, whereas a molecule of molecular weight 200,000 Daltons was found in the plasma of patients with rectal tumours. In contrast, Pusztaszeri *et al.*, [3] found no differences in CEA immunoreactivity in plasma from patients with bronchial and colonic carcinoma.

To seek possible physiochemical and immunological heterogeneity of the CEA molecules as a function of the origin of the tumour 18 sera from patients with various cancers (colon: 6, lung: 9, breast: 1, kidney: 1, and ovary: 1) were investigated using gel chromatography on Sepharose 6B (4°C-1·5/90-Sorensen phosphate buffer 0·05 M pH 7·5 as eluant) and radioimmunoassay with two different antisera for immunological characterization [4].

The elution pattern of immunoreactive CEA illustrates the heterogeneity of this antigen in the serum of patients suffering from cancer. In no case was there a monophasic major peak of elution as measured using both antisera. In contrast, the main immunoreactive peak localized in the zone where unlabelled CEA migrated showed a multiphasic profile indicating the presence of a continuous spectrum of molecules with slightly different molecular weights. These molecular variants are presumably due to differences in the composition of carbohydrate moiety were also demonstrated by others [5, 6]. Moreover the two antisera did not necessarily detect the same degree of heterogeneity indicating that these different classes of molecules have different antigenic determinants and that our antisera are directed against different antigenic sites.

Further, the presence of immunoreactive material of high molecular weight in the region of the void volume (Fig. 1) was seen in four instances: 2 tumours of the lung, 1 of the colon and 1 of the kidney.

Thus, in our experiments, there was no relationship between the zone of elution of maximum immunoreactivity and the type of the tumour, in contrast to the findings of Plesh and Goldenberg [2].

The peak seen at the void volume does not appear to be due to an immune complex between endogenous CEA and immunoglobulin. Thus, when labelled CEA was incubated for prolonged periods with the sera or with the particular elution peak, there was no binding of the labelled CEA to immunoglobulins as detected by radioimmunoelectrophoresis or by selective precipitation with anti-human IgG, IgM, or IgA antisera.

This "big-CEA" could be an example of the same type of heterogeneity already described for hormones such as insulin, PTH, gastrin etc. (see review of Franchimont *et al.* [7]).

Accepted 24 February 1976.

*Work carried out with the help of grants No. 20305 of the "Fonds de la Recherche Scientifique Medicale" (FRSM) Belgium, and the "Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite", Belgium.

†Present address: Medical Faculty, Institute of Microbiology 61105 Ljubljana, Yugoslavia.

‡"Chargé de Recherches" at the "Fonds National de la Recherche Scientifique" (FNRS), Belgium.

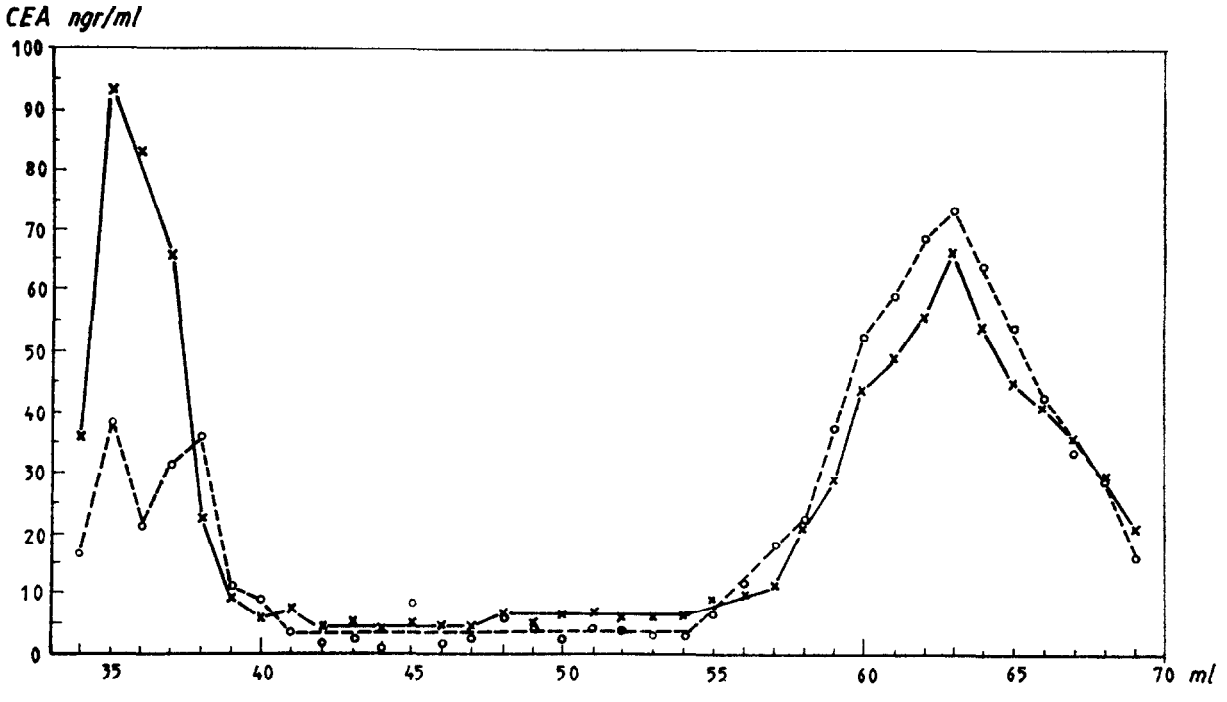


Fig. 1. *CEA levels in eluted fractions from serum chromatographed on sepharose 6B column (adenocarcinoma of colon)*
—×— *CEA detected with antiserum No. I*
– – ○ – – *CEA detected with antiserum No. II.*

REFERENCES

1. J. E. Coligan, J. T. Lautenschleger, M. L. Egan and C. W. Todd: Isolation and characterization of carcinoembryonic antigen. *Immunochemistry* **9**, 377 (1972).
2. Q. Pletsch and D. M. Goldenberg: Molecular size of carcinoembryonic antigen in plasma of patients with malignant disease. *J. nat. Cancer Inst.* **53,** 1201 (1974).
3. G. Pusztaszeri and J. P. Mach: Carcinoembryonic antigen (CEA) in non digestive cancerous and normal tissues. *Immunochemistry* **10,** 197 (1973).
4. P. Franchimont, M. L. Debruche, P. F. Zangerle, and J. Proyard, Radioimmunoassay of carcinoembryonic antigen (CEA). In *Radioimmunoassays and Related Procedures in Medicine.* Istanbul September, 1973, International Atomic Energy Agency **2,** 267 (1974).
5. G. T. Rogers, F. Searle and K. D. Bagshawe, Heterogeneity of carcinoembryonic antigen and its fractionation by con A affinity chromatography. *Nature (Lond.)* **251,** 519 (1974).
6. S. R. Harvey and T. M. Chu, Demonstration of two molecular variants of carcinoembryonic antigen by concavalin A sepharose affinity chromatography. *Cancer Res.* **35,** 3001 (1975).
7. P. Franchimont, U. Gaspard, A. Reuter and G. Heynen: Polymorphism of protein and polypeptide hormones. *Clin. Endocr.* **1,** 315 (1972).

Europ. J. Cancer Vol. 12, pp. 659–660. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *August, 1976*

Human Cancer

A. J. Cochran, R. M. Mackie, C. E. Ross, L. J. Ogg and A. M. Jackson: Leucocyte migration inhibition by cancer patients' sera.

S. H. Chan, T. S. Chew, E. H. Goh, M. J. Simons and K. Shanmugaratnam: Impaired general cell-mediated immune functions *in vivo* and *in vitro* in patients with nasopharyngeal carcinoma.

R. K. Oldham, J. H. Dean, G. B. Cannon, J. R. Ortaldo, G. Duntson, F. Applebaum, J. L. McCoy, J. Djeu and R. B. Herberman: Cryopreservation of human lymphocyte function as measured by *in vitro* assays.

R. Heimer and G. Klein: Circulating immune complexes in sera of patients with Burkitt's lymphoma and nasopharyngeal carcinoma.

M. Vuento, E. Engvall, M. Seppälä and E. Ruoslahti: Isolation from human gastric juice of an antigen closely related to the carcino-embryonic antigen.

A. W. Boddie, Jr., M. M. Urist, D. O. Chee, E. C. Holmes and D. L. Morton: Detection of human tumor-associated antigens by the leukocyte migration in agarose assay.

Experimental Cancer

G. A. Miller and J. D. Feldman: Genetic role of rat macrophage cytotoxicity against tumor.

J. Cerny, S. H. Fistel and P. A. Hensgen: Interactions of murine leukemia virus (MuLV) with isolated lymphocytes. I. Virus replication in lymphocytes infected with Friend virus and cultured in diffusion chambers *in vivo*.

J. Cerny, P. A. Hensgen, S. H. Fistel and L. Mastalir Demler: Interactions of murine leukemia virus (MuLV) with isolated lymphocytes. II. Infection of B and T cells with Friend virus complex in diffusion chambers and *in vitro*: Effect of polyclonal mitogens.

J. Cerny, M. Essex and D. B. Thomas: Interactions of murine leukemia virus (MuLV) with isolated lymphocytes. III. Alterations of splenic B and T cells in Friend virus-infected mice.

C.-C. Ting: Detection of antitumor antibody in virally induced tumors and its relationship to tumor growth.

M. A. Pierotti and M. I. Colnaghi: Natural antibodies directed against murine lymphosarcoma cells: Variability of level in individual mice.

J. P. Brown, G. van Belle and I. Hellström: Design of experiments using the microcytotoxicity assay.

N. A. Wivel and S. S. Yang: Murine neuroblastoma clones with varying degrees of C-type virus expression.

S. B. Pollack and K. Nelson: Production of tumor-specific inducer of cellular cytotoxicity by lethally irradiated mice.

J. W. Proctor, B. G. Auclair and C. M. Rudenstam: The distribution and fate of bloodborne ^{125}IUdR-labelled tumour cells in immune syngeneic rats.

British Journal of Cancer *August, 1976*

B. G. Achong, P. A. Trumper and B. C. Giovanella: C-type virus particles in human tumours transplanted into nude mice.

Nobuyoshi Ishikawa and Satoshi Hamada: Association of medullary carcinoma of the thyroid with carcino-embryonic antigen (CEA).

J. H. Marti and D. M. P. Thompson: Kinetics of anti-tumour immunity in malignant melanoma assessed by tube leukocyte-adherence inhibition assay.

D. J. Kirkland: Chemically induced transformation of Chinese hamster cells. I. A comparison of some properties of transformed cells.

D. J. Kirkland and S. Venitt: Chemically-induced transformation of Chinese hamster cells. II. Appearance of marker chromosomes.

V. M. Craddock and J. V. Frei: Induction of tumours by treatment of intact and of partially hepatectomised rats with ethyl methanesulphonate.

L. A. Smets, E. Mulder, F. C. de Waal, F. J. Cleton and J. Blok: Early responses to chemotherapy detected by pulse-cytophotometry.

A. Jacobs, A. Slater, J. A. Whittaker, G. Canellos and P. H. Wiernik: Serum ferritin concentration in untreated Hodgkin's disease.

H. Stott, R. J. Stephens, Wallace Fox and D. C. Roy: 5-year follow-up of a study of cytotoxic chemotherapy as an adjuvant to surgery in carcinoma of the bronchus.

E. S. Newlands, C. J. Oon, J. T. Roberts, P. Elliott, R. F. Mould, C. Topham, F. J. F. Madden, K. A. Newton and G. Westbury: A randomised clinical trial in disseminated malignant melanoma using combination chemotherapy and specific active immunotherapy.

R. F. Mould, T. Hearnden, M. Palmer and G. C. White: Distribution of survival times of 12,000 head and neck cancer patients who died with their disease present.

J. S. Crowther, B. S. Drasar, M. J. Hill, R. MacLennan, D. Magnin, S. Peach and C. H. Teoh-Chan: Faecal steroids and bacteria and large bowel cancer mortality in Hong Kong by socio-economic indicators.

Brief Communication

M. V. Pimm and R. W. Baldwin: Influence of whole body irradiation on BCG contact suppression of a rat sarcoma and the concomitant development of a tumour specific immune response.

Europ. J. Cancer Vol. 12, pp. 661–673. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Recent Trends in the Treatment of Non-Hodgkin's Lymphomas

GIANNI BONADONNA, ANGELO LATTUADA and ALBERTO BANFI
Istituto Nazionale Tumori, Milan, Italy

Abstract—*The results of new effective therapies as well as of new treatment approaches for non-Hodgkin's lymphomas are reviewed. Despite the limitation of a broad spectrum of obstacles precluding effective comparisons of case series, recent data allow to draw some practical therapeutic considerations. In early stages classified on pathological bases, megavoltage radiotherapy still represents the treatment of choice, and the cure rate after adequate doses and fields is achieved in about 60% of patients. One controlled study showed a significant improvement in the disease-free survival by adding chemotherapy to the irradiation. In advanced disease, a number of multiple drug treatments has produced a definite increase in the incidence of complete remission as well as a longer median survival compared to single agent therapy. Childhood lymphomas were shown to be controlled only by aggressive combined modalities. After either radiotherapy or chemotherapy patients free of disease at two years from the end of treatment can be considered cured if their primary disease was a diffuse histiocytic lymphoma. This definition of cure is less applicable to patients with lymphocytic lymphomas because of their pattern of continuous late relapse. Although the Rappaport histopathologic classification has demonstrated the prognostic importance of nodular and diffuse lymphomas, new functional classifications, if found applicable and reproducible, might improve in future the correlation between morphology and clinical findings. However, the progress is expected to be slow and difficult.*

THE TERM non-Hodgkin's lymphoma comprises a group of primary neoplasms of the lymphoreticular tissue which present a difficult challenge not only to clinicians but also to pathologists, cell biologists and immunologists. The present chaotic confusion surrounding this particular subject is due to a number of factors. However, it should be recognized that the terminology maze resulting from the problem of precise cytologic identification is probably more responsible for the increasingly difficult communications in this field than the many patterns of disease presentation and relapse, the lack of adequate staging system and the insufficient knowledge as how to properly use the available treatment tools.

Reprints requests to: Dr. Gianni Bonadonna, Istituto Nazionale Tumori, Via Venezian, 1, Milan 20133, Italy.

In the past few years, following conceptual parallelisms with the progress obtained for Hodgkin's disease, new treatments and new therapeutic approaches were devised for non-Hodgkin's lymphomas. The present review intends to summarize most of the important achievements despite the limitation that a broad spectrum of obstacles precludes effective comparisons of case series from different centres.

THE HISTOPATHOLOGY MAZE

It is well known that the morphologic identification and classification of malignant lymphomas have been the subjects of controversy and confusion for decades. For Hodgkin's disease, the revised classification of Lukes and Butler was favourably accepted on an international basis and systematically adopted by

clinicians for the past decade not only because of its simplicity and reproducibility, but because it bears a meaningful relationship with the natural history of disease.

For the other malignant lymphomas, pathologists from research centers realized, some years ago, that the widely used terminology of lymphosarcoma, reticulum cell sarcoma and giant follicular lymphoma was inadequate to characterize various processes morphologically. The histological classification that Rappaport proposed in 1956 (Table 1) was rediscovered and gradually applied to clinico-pathologic as well as to treatment studies, both retrospective and prospective. The modern understanding of the immune system, however, led to a proliferation of newly proposed functional classifications [1, 2]. Besides giving rise to a tremendous confusion of terminology and conceptual disputes, the value of new classifications has not yet been demonstrated in terms of (*a*) applicability and reproducibility, (*b*) improved prognostic value, (*c*) improved understanding of disease presentation, progression, and response to therapy, (*d*) correlation between morphologic designations with the results of immunologic studies.

Table 1. *The Rappaport histopathologic classification*

Nodular pattern		Diffuse pattern	
NLWD	Lymphocytic well differentiated	DLWD	Lymphocytic well differentiated
NLPD	Lymphocytic poorly differentiated	DLPD	Lymphocytic poorly differentiated
NH	Histiocytic	DH	Histiocytic
NM	Mixed histiocytic–lymphocytic	DU	Undifferentiated (non Burkitt)

The original Rappaport classification, although not up to date with the understanding of modern immunology, was found useful to clinicians since it has demonstrated the importance of nodular lymphomas. Furthermore, the cytological types of Rappaport were shown to be of prognostic significance in a number of clinico-pathologic studies [3–5]. However, the tendency of different authors to apply the same terms to different entities as well as the undoubted difficulties encountered to include some lymphoproliferative disorders in the original classification and to correlate some morphologic processes with the clinical course, has compounded the problem of communicating the diagnosis and of comparing results of therapy by using the current Rappaport classification in several occasions.

DISEASE PRESENTATION AND STAGING

The pleomorphic clinical presentation of non-Hodgkin's lymphomas is well known. Compared to Hodgkin's disease, this group of neoplasms presents frequently in extranodal sites, particularly in the gastrointestinal tract and Waldeyer's ring. The percentage difference between nodal (46–75%) and extranodal (16–58%) primary involvement as reported by various investigators may reflect either geographical factors or type of referred patients to research centers [1]. The clinical picture becomes even more complicated if nodular and diffuse histology are taken into consideration. Various centers report a different percentage incidence of nodular (11–45%) and diffuse (55–89%) patterns in both nodal and extranodal lymphoma. However, in general, the diffuse type is found more frequently than the nodular in extranodal lymphoma [1]. It should be recalled that sampling of tissues other than lymph nodes has undoubtedly influenced the morphologic interpretation. Both disease presentation and histology have affected in many ways the therapeutic approach as well as the survival rate and therefore, they make an adequate comparison of treatment results from different institutions more difficult.

Recent findings have proved that the number of occult lesions increases in proportion to the efforts made to determine the anatomical extent [1]. Briefly, the key diagnostic tests are represented by lymphography, bone marrow biopsy (needle or open) and surgical abdominal exploration (laparoscopy, laparotomy). Although at the present moment no satisfactory staging system is available, the four-stage

Ann Arbor classification adopted for Hodgkin's disease is temporarily utilized also for non-Hodgkin's lymphomas to assure a minimum of comparability among case series.

EFFECTS OF RADIOTHERAPY IN EARLY STAGES

Radiation therapy (RT) has traditionally represented the treatment of choice for the large majority of patients presenting with localized (stage I–II) disease, both nodal and extranodal. In the past decade, megavoltage irradiation has been introduced systematically into treatment programs. Table 2 presents the 5-year survival rates of various series published in recent years. Despite the above mentioned limitations of retrospective comparisons for heterogeneous case series, the therapeutic results in terms of cure were improved for the following reasons: (*a*) more accurate diagnosis of anatomical extent of disease; (*b*) systematic use of adequate doses (3500–4500 rads) delivered through megavoltage equipment; (*c*) adoption of RT programs which have included regional field irradiation as well as irradiation of adjacent and distant lymph node-bearing areas (extended field, subtotal, total nodal or lymphoid RT). With all techniques employed,

Table 2. Five-year survival in early stages treated with radiation therapy

Authors	Stage	Patients	Total (%)	Nodular (%)	Diffuse (%)
Fuller [6]	I–II	226	37	65	26
Tubiana [7]	I–II	123		55*	36*
	I (with lymphography)	21	60*		
	II (with lymphography)	36	37*		
Van Unnik [8]	I–II	180		55*	32*
	I	83	55		
	II	137	25		
Peckham [9]	I–II	51	63*	78*	50*
	I	19	73		
	II	32	56		
van der Werf-Messing [10]	I	79	66		
	II	63	43		
Hoerni [11]	I	69	42*		
	II	72	32*		
Musshoff [12]	I (nodal)	39	75*		
	II (nodal)	42	28*		
	I (extranodal)	13	60*		
	II (extranodal)	22	58*		
Dumont [13]	I–II	83	48*		
Patchefsky [14]	I–II	65		55*	58*
Milan† [15]	I	79	59		
	II	101	26		
	I–II	117		79	39
	I–II (nodal)	82	50		
	I–II (extranodal)	98	40		
	I–II (Waldeyer or GI tract)	68	33		
Wong [16]	I–II (head and neck)	128		83	DH: 30
	I–II (Waldeyer's ring)	83	46		other diffuse: 49
	I	55	58		
	II	73	37		
Cecchetti [17]	I (Waldeyer)	43	46		
	II (Waldeyer + regional nodes)	66	18		
Brugere [18]	I–II (Head and neck)	118	38		
	I (Head and neck)	50	40		
	II (Head and neck)	68	35		
Freeman [19]	I (Extranodal)	633	59		
	II (Extranodal + regional nodes)	834	28		
	I–II (Waldeyer)	199	33		

*Approximate.
†All patients evaluated with lymphography.

the 5-year-survival rate achieved with RT as primary treatment ranges from 33 to 50% for the group of patients classified as stage I and II (stage I: 40–79%, stage II: 18–56%). Fifty-five to 83% of patients with nodular histology survived 5 years compared to 26–50% of those with diffuse patterns.

The reasons for treatment failure may be numerous, and an exhaustive investigation on this particular point is not yet available. The initial relapse was noted to occur in the large majority of patients within the first two years from the end of irradiation. This was particularly true for patients with diffuse histology and/or extranodal lymphoma [1, 10, 15]. The true (local) recurrence is, in general, not a common finding, and was observed to occur particularly in patients with involvement of head and neck tissues and diffuse histology. For this reasons, modern irradiation approach employs in this type of patients tissues doses ranging from 5000 to 5500 rads.

The extension to non-irradiated areas represents the most frequent site of relapse for all histopathologic subgroups. The nodal extension is less predictable compared to Hodgkin's disease and often preceeds or is associated to extranodal dissemination. This prevents in most patients an effective use of prophylactic irradiation. Fuks *et al.* [3] reported that for stage I–II patients the initial relapse in non contiguous lymph node-bearing areas occurred preferentially in those with diffuse histology. Patients with a nodular pattern have a more indolent course with a pattern of continuous late recurrence beyond the second year, and relapse often occurs in lymph nodes. In primary extranodal lymphomas (e.g., head and neck, stomach, breast, testicle) the first site of relapse presents in regional nodes. The mediastinum is very seldom involved (10–15%) even in the presence of disseminated disease.

As far as the infradiaphragmatic relapse in stage I and II is concerned, the results of laparotomy indicate that treatment failure can be often accounted on the presence of occult mesenteric, splenic or hepatic involvement [1].

In an attempt to decrease the relapse rate, two similar controlled adjuvant studies were designed in the research centers of Milan [20] and Stanford [21]. The principal aim was to test the curative potential of 6 cycles of combination chemotherapy following irradiation in pathologic stage I–II patients. Fig 1 reports the updated results achieved in our Institute where patients with all cytologic types were treated either with radiotherapy (loco-regional fields) or radiotherapy plus cyclophosphamide, vincristine and prednisone (CVP). At the time of analysis, there is an advantage in adding chemotherapy to the irradiation (Fig. 1). However, the difference in the disease free survival is statistically significant only in the subgroup with lymphocytic lymphoma. No difference in favor of adjuvant chemotherapy was observed at Stanford where patients with unfavourable histology were treated with total lymphoid irradiation followed by CVP plus or minus bleomycin. The lack of increased cure rate might be due to the small number of evaluable patients (total 28), or to the fact that chemotherapy was started late after the completion of radiotherapy (60 days). Similar controlled studies designed by the EORTC group are in progress.

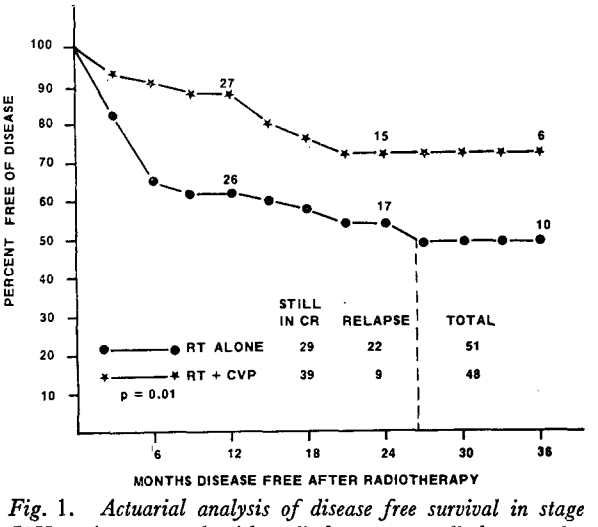


Fig. 1. Actuarial analysis of disease free survival in stage I–II patients treated with radiotherapy or radiotherapy plus combination chemotherapy.

EFFECTS OF CHEMOTHERAPY IN ADVANCED DISEASE

From the early years of cancer chemotherapy, all types of growth-inhibiting compounds were systematically evaluated in malignant lymphomas. Due to the particular susceptibility of lymphoid tissue to respond to the action of anticancer agents, malignant lymphomas were soon recognized as a group of signal diseases to test the therapeutic effects of new drugs.

Single agent chemotherapy

Until 1969, most patients with advanced non-Hodgkin's lymphomas were treated with single agent chemotherapy. Drugs were usually administered to the point of toxicity, and in responsive patients, treatment was continued with maintenance doses until relapse occurred. Upon progression, a drug of different class was tried next. Traditionally, chemotherapy was

started with one of the polyfunctional alkylating agents, followed in sequence by one of the vinca alkaloids, procarbazine and corticosteroids. In recent years the new antibiotics, adriamycin and bleomycin, the nitrosourea derivatives (BCNU, CCNU, Me-CCNU, streptozotocin), the epipodophyllotoxin derivatives (VM 26, VP 16) and imidazolcarboxamide were tested in patients resistant to conventional drugs.

Table 3. *Response rate to single drugs with advanced non-Hodgkin's lymphomas*

Drugs	Lymphocytic %	Histiocytic %
Prednisone (P)	60–80	20–30
Nitrogen mustard (HN2)	50–70	40–50
Cyclophosphamide (CTX)	50–70	50–70
Chlorambucil (CLB)	40–60	20–30
Procarbazine (PCZ)	40–50	20–35
Vinblastine (VLB)	15–25	20–35
Vincristine (VCR)	40–65	40–80
VM 26	50–60	30–35
VP 16	25–30	10–15
Adriamycin (ADM)	35–50	50–65
Bleomycin (BLM)	30–40	30–40
BCNU, CCNU, Me-CCNU	20–30	25–35
Streptozotocin (STZ)	20–30	10
Imidazole-carboxamide (DTIC)	20–25	10
Methotrexate (MTX)	15–25	15–25
Cytosine arabinoside (Ara-C)	20–25	15–20

Table 3 lists the drugs which are presently considered useful in non-Hodgkin's lymphomas. The response rate (complete plus partial remissions greater than 50%) as derived from the literature for lymphocytic and histiocytic types should be regarded as merely indicative for a number of reasons. In fact, the above mentioned difficulties concerning histopathology, the different proportions of previously treated and untreated patients included in the various case series, as well as the different criteria of drug response employed among investigators, rise obvious difficulties in comparing results from different institutions. However, some orientative conclusions can be drawn. First of all, CTX, VCR and ADM appear to be the most effective agents. It should be recalled that since most of the new drugs were tested in patients with disseminated disease refractory to conventional agents, available data might underestimate considerably their therapeutic potential. However, the results provide sufficient evidence of lack of cross resistance between the group of conventional drugs and that of new agents. With single agent chemotherapy, even in previously untreated patients, the overall complete remission rate (CR) was usually less than 20% [22]. However, Jones *et al.* [23] noted a CR of 28–48% in patients with nodular lymphomas given alkylating agents, while in the series of Monfardini *et al.* [24] nodular lymphomas treated with CTX achieved a 50% complete response. With all drugs employed, nodular lymphomas exhibited a higher response rate, as well as a longer duration of remission and median survival compared to diffuse lymphomas [22].

Combination chemotherapy

It is now recognized that the era of combination chemotherapy was officially started with the first report on the effects of MOPP in Hodgkin's disease [25]. As far as non-Hodgkin's lymphoma is concerned, the first study showing the usefulness of multiple drug treatment was that published by Hoogstraten *et al.* [26] in 1969. The authors reported that through a continuous combination of CTX, VCR and P for 6 weeks, they could obtain CR in 35% of patients with lymphocytic lymphoma and in 31% of those with histiocytic lymphoma, respectively. The concept of cyclical combination chemotherapy was then developed by the use of MOPP [25], and subsequently by COP [27] and CVP [28].

Table 4 reports some of the cyclical 3–4 and 5-drug combinations presently used for advanced non-Hodgkin's lymphomas. As already noticed for Hodgkin's disease [36], all multiple drug regimens, regardless of the number and types of drugs, doses and sequence, produced a remarkable improvement in the incidence as well as in the duration of CR, when retrospectively compared to single agent chemotherapy. All investigators noticed that the remission duration in complete responders was five to fifteen times longer than that observed in partial remitters.

The proliferation of combination regimens deserves some comments. Most, if not all, of them were designed on empirical basis, and the first treatments (COP, CVP, MOPP, C-MOPP, COPP) have employed conventional drugs. New combinations (ABP, HOP, CHOP, BCVP, MABOP) have included either ADM or BLM or both with the intent to improve the remission rate as well as the remission duration. Two regimens (COMA, MEV) have attempted to combine an alkylating agent (CTX) with cell cycle specific drugs (MTX, VCR, ARA-C).

Table 4. Some representative cyclical drug combinations

Acronym	Regimen (mg/m²)			No. patients	CR (%)	Histol. types	Median duration (months)	Authors
COP	CTX	800	(D 1)	140	45	Ly 50% Hi 39%	Ly 9 Hi 6	Luce *et al.* [27]
	VCR	1·4	(D 1)					
	P	100	(D 1–5)					
CVP	CTX	400	(D 1–5)	35	57	(all Ly)	81% at 1 yr.	Bagley *et al.* [28]
	VCR	1·4	(D 1)					
	P	100	(D 1–5)	27	44	Ly 50% Hi 35%	Ly 18+ Hi 7+	Milan
ABP	ADM	75	(D 1)	30	46	Ly 62% Hi 35%	Ly 8+ Hi 11+	Milan
	BLM	10	(D 1+8)					
	P	100	(D 1–5)					
MEV	CTX	800	(D 1)	30	73	Ly 56% Hi 93%	Ly 15+ Hi 14+	Lauria *et al.* [29]
	VCR	2	(after 36 hr)					
	MTX	20	(after 48 hr)					
HOP	ADM	80	(D 1)	153	61		Data not available	McKelvey *et al.* [30]
	VCR	1·4	(D 1)					
	P	100	(D 1–5)					
CHOP	CTX	750	(D 1)	142	67		Data not available	McKelvey *et al.* [30]
	ADM	50						
	VCR	1·4						
	P	100	(D 1–5)					
MOPP, C-MOPP	HN2 or	6	(D 1+8)	26	35	(all Hi)	25+	Schein *et al.* [4]
	CTX	650	(D 1+8)					
	VCR	1·4	(D 1+8)					
	PCZ	100	(D 1–14)					
	P	40	(D 1–14)*					
COPP	CTX	600	(D 1+8)	32	59	Ly 69% Hi 16%	Ly 21+ Hi 11+	Stein *et al.* [31]
	VCR	1·4	(D 1+8)					
	PCZ	100	(D 1–14)					
	P	40	(D 1–14)					
COMA	CTX	1500	(D 1)	15	60	(all Hi)	22	Levitt *et al.* [32]
	VCR	1·4	(D 1, 8, 15)					
	MTX	120 + Leukovorin						
	Ara-C	300	(D 22–71)					
BCVP	BLM	10	(D 1–4)	48	61	Ly 65% Hi 60%	Data not available	Luce *et al.* [33]
	CTX	125	(D 1–14)					
	VCR	1·4	(D 1+8)					
	P	60	(D 1–5)					
MABOP	HN2	6	(D 1+8)	58	55	Ly 56% Hi 55%	Ly 20 Hi 9	Milan
	ADM	25	(D 1+8)					
	BLM	30	(D 1+8)†					
	VCR	1·2	(D 1+8)					
	P	40	(D 1–14)†					
BACOP	BLM	4 × 2/wk		22	73		4+	Skarin *et al.* [34]
	ADM	45 q. 4 wks						
	CTX	600 q. 4 wks						
	VCR	1·2/wk						
	P	40/day × 5 wks						
BACOP	CTX	650	(D 1+8)	21	43		8+	Schein *et al.* [35]
	ADM	25	(D 1+8)					
	VCR	1·4	(D 1+8)					
	BLM	5	(D 15+22)					
	P	60	(D 15+28)					

D: day; Ly: lymphocytic; Hi: histiocytic
*In cycles 1 and 4.
†In cycles 1, 3 and 5.

The current Memorial Sloan–Kettering Center protocol superimposes a large i.v. dose of CTX upon a polydrug regimen originally developed for the therapy of acute lymphoblastic leukemia. The remission induction is accomplished by CTX, VCR, P and daunomycin. When the tumor mass has been markedly reduced, consolidation is attempted with several courses of the cell cycle-specific agents (Ara-C and 6-thioguanine). The maintenance phase is carried out by rotating different drugs with 5-day cycles. Local radiation was given to initial massively involved areas either at the beginning or during the course of therapy. The recent data of this intensive treatment [37] failed to provide in adults the same brilliant results achieved in children. In fact, CR was obtained only in 10 of 34 patients (29%).

Though the difficulties in making correlations on the base of retrospective analysis do remain also for recent series of patients, it is interesting to try to analyze what was the impact of the various combinations on the different histologic types and patterns. Unfortunately, not all published series report their results according to the Rappaport classification. According to the data reported in Table 4 in only 4 combinations (COP, COPP, CVP, ABP) lymphocytic lymphoma appears more responsive than histiocytic lymphoma. With the exception of ABP, the higher response rate was translated into a longer median length of remission. The MEV regimen yielded an impressively high percent of complete response (93%) in histiocytic lymphoma, while COMA, BCVP and MABOP induced CR in histiocytic lymphoma ranging from 55–60%.

As far as the response to chemotherapy in relation to the histologic pattern is concerned, nodular lymphomas achieved in most series a higher incidence of CR when compared to diffuse lymphomas (Table 5). This fact was almost always associated to a longer median length of remission as well as to a longer survival.

It is well known that survival remains the most reliable measurable indication of the efficacy of any given treatment, and is related to histology, initial response and prior therapy. In general, with all types of combination chemotherapy median survival of patients with lymphocytic lymphoma and with nodular pattern was better than that of patients with histiocytic lymphoma and with diffuse pattern, respectively [22]. The most complete analysis of survival in relation to histologic subgroups remains, at the present moment, that of Schein *et al.* [4]. In a series of 80 patients treated with three basic treatment programs (CVP for lymphocytic lymphoma, MOPP and C-MOPP for histiocytic and mixed type lymphoma) the authors noticed that well differentiated and nodular histology were positive determinants for survival. In particular, at 40 months after the start of chemotherapy all 4 patients with differentiated lymphocytic lymphoma were alive. The median survival of NLPD was in excess of 30 months, that of NM of 34 months. In contrast, patients with DLPD had a median survival of 14 months while none of those with DU lived longer than 4 months. DH showed a median survival of 6 months. However, of 41% (11 of 27) of patients who achieved CR after 6 months of treatment only one has had a recurrence of tumor. All other patients have remained free of disease without maintenance therapy from 26 to 105 months. De Vita *et al.* [38] concluded that in diffuse histiocytic lymphoma a survival free of disease beyond 2 years from the end of chemotherapy may be considered, as for radiation therapy, tantamount to cure. In contrast, patients with DLPD and well differentiated lymphocytic lymphoma demonstrate a pattern of continuous

Table 5. *Incidence of complete remission in relation to nodular and diffuse pattern*

Combination	Nodular		Diffuse		Authors
	No.	CR (%)	No.	CR (%)	
CVP	4	75	23	39	Milan
ABP	5	80	25	40	Milan
CVP, MOPP, C-MOPP	37	62	43	30	Schein *et al.* [4]
HOP and CHOP	Data not available	71	Data not available	60	McKelvey *et al.* [30]
COPP	17	71	11	36	Stein *et al.* [31]
BCVP	8	50	40	62	Luce *et al.* [33]
BACOP	6	67	13	62	Skarin *et al.* [34]
MABOP	17	71	28	68	Milan

late recurrence in lymph nodes and bone marrow which were involved prior to treatment [5]. For the sake of orientative comparison, it can be noted that in the series of Stein *et al.* [31] where COPP is a combination similar to CVP and C-MOPP, the median survival related to histology was as follows: NM 2 months, NLPD 28, DLPD 28, DH 10·5. As can be seen, the similarity with Schein's data appears restricted only to NLPD and DH.

Controlled trials

The above reported data indicate that, although an appreciable progress has been made during the past few years in terms of overall incidence of CR and improvement of median survival compared to the results achieved in the past, it is clear that the control and especially the cure rate of advanced non-Hodgkin's lymphomas is only obtained in a limited fraction of patients. During the past decade the therapeutic tools have been sharpened, and clinical trials should now be designed to answer specific questions. They concern, for example, the usefulness of combined treatment modalities, the appropriate treatment for each histologic subgroup, the response rate of non-cross resistant combinations, the value of maintenance therapy, etc.

At the present moment, the results of a limited number of prospective controlled studies is available from the literature. The first deals with combined chemotherapy and radiotherapy in pathologic stage III disease [20]. Since radiation therapy alone can cure only a small fraction of patients with stage III extent, the scope of the study was to explore the best therapeutic sequence, i.e., whether 6 cycles of CVP might facilitate radiotherapy control or, vice versa, whether in patients achieving CR after the irradiation program (involved fields) 6 cycles of adjuvant chemotherapy could decrease the relapse rate. Our preliminary updated results (Table 6) fail to indicate that after the combined modality approach the incidence of CR, the median duration of complete response and the relapse rate were significantly affected by either sequential program. The first relapse occurring during RT was mostly represented by nodal extension, indicating that total lymphoid irradiation would probably be preferable to field radiotherapy. Conversely, relapses occurring during or after chemotherapy occurred in about half of patients in bone marrow and/or viscera suggesting that a more effective combination than CVP at least for DH lymphomas should be considered.

The appropriate selection of treatment in relation to histology was recently attempted by the Stanford Group [21]. Sixty-three patients with pathological stage IV and favourable histology (NLWD, NLPD, NM, DLWD) were randomized to CVP, CVP plus total lymphoid irradiation and to single agent chemotherapy (CTX or CLB). Although there was a difference in the incidence of CR among the treated groups (61%, 50% and 25%, respectively), survival at 30 months was 100% for CTX or CLB, 91% for CVP, and 68% for CVP plus radiotherapy. These data indicate first that single agent chemotherapy with alkylating agents appears as effective in terms of survival as the more aggressive forms of treatment. Furthermore, it seems that in patients with favourable histology the incidence of CR is not necessarily translated into an improved survival. The Stanford data were substantiated by the findings observed by Kennedy *et al.* [39] in 45 patients with stage III and IV lymphocytic lymphomas. Patients were randomly treated with combination chemotherapy (COP) or with the same agents used successively in maximal doses (C–O–P). The survival from chemotherapy was not different. The response rate for either therapy was similar for patients with favourable histology (NLPD) while single agent chemotherapy was less effective in those with unfavourable histology (DLPD). In con-

Table 6. *Incidence and duration of complete remission in stage III–III$_S$ after combined treatment modality*

Sequence	Evaluable patients	CR	Median duration (months)	Relapse		
				During RT	During CVP	After combined therapy
RT → CVP	15	9 (60%)	9+ (2–33)	5	1	4/9
CVP → RT	17	9 (53%)	6+ (1–14)	2	5	1/9

trast with the above reported findings, those of Stein *et al.* [31] showed that the median survival after intensive combination chemotherapy (COPP) was superior to that observed after VCR plus prednisone (VP) in both groups with favourable (33 vs 14 months) and with unfavourable (9 vs 2 months) histology. In a randomized study COPP was also superior to VP, in the rates of induction of CR (58% vs 14%).

The initial results of a randomized trial comparing in 65 patients with advanced lymphocytic lymphoma CVP and total body irradiation (TBI) were recently reported by Canellos *et al.* [40]. The overall response rate and the incidence of CR were not statistically different between the two regimens. The results indicated comparable results in nodular lymphoma but with some advantages of TBI in diffuse lymphoma only in the first 18 months since the survival curves converged at 24 months. The authors conclude that the utility of both therapies suggests a role for their combined administration, particularly in those patients with diffuse histological patterns.

Since only a fraction of patients with advanced disease achieves a prolonged remission after combination chemotherapy, it is important to have an effective regimen which can be administered in primary failures as well as in patients with relapse after obtaining an initial response. For this reason, a prospective trial was started in previously untreated pathological stage IV patients by Bonadonna *et al.* [20] with the aim of exploring the therapeutic effects and the lack of cross resistance of two active combinations (CVP and ABP). The updated preliminary results are reported in Tables 4 and 5. Both treatments appear equally effective in terms of CR (44% vs 46%). No statistical differences were noticed between lymphocytic and histiocytic types as well as between nodular and diffuse patterns. Upon progression or relapse, the secondary treatment after cross over yielded complete plus partial remission in 7 of 25 treated with CVP (28%) and in 3 of 10 given ABP (33%). At the present moment, both regimens are administered in our Institute on alternating cycles in the attempt to improve the incidence and the duration of CR.

TREATMENT OF CHILDHOOD LYMPHOMAS

Non-Hodgkin's lymphomas in children differ somewhat from those of the adults. Nodular histology is exceedingly rare, and DLPD and DU are the most frequently observed types. Primary intestinal involvement as well as early dissemination to central nervous system and to bone marrow with propensity to leukemic changes are prominent findings. All these facts explain why the results of local or local plus regional treatment in apparently localized disease were poor for a number of years. Retrospective analyses showed that the overall survival rate was lesser than one year [41–43].

The modern aggressive approach is best exemplified by the protocol (LSA_2-L_2) of Memorial Sloan–Kettering Center applied to all stages of the disease [43, 44]. This complex protocol is similar to that successfully utilized by the same institution in the treatment of acute leukemias. In short, it consists of an induction phase (30–32 days) with CTX plus radiotherapy delivered to one or more involved sites, P, VCR and daunomycin as well as intrathecal MTX. This intensive therapy is justified by the observation that if a complete regression of all measurable evidence of disease is not achieved within one month from starting therapy, the chances of treatment failure are very high [44]. The consolidation phase includes therapy with Ara-C plus thioguanine followed by L-Asparaginase, intrathecal MTX and BCNU. Induction plus consolidation phases have an average duration of 120–125 days. The maintenance phase starts within one week with five-day cycles of different drugs (thioguanine plus CTX, hydroxyurea plus daunomycin, MTX plus BCNU, Ara-C plus VCR and MTX intrathecal for 2 doses two to three days apart). After a 7–10 day rest period, the whole five cycles are restarted. One course of five cycles lasts an average of 55–65days. For stage I, II and III, treatment continues for two years and for stage IV three years, provided there is no relapse.

The initial results were brilliant. Of 43 patients entered into the study, 80% were alive free of disease at 40 months with a median observation time of 16 months for the entire group. The median survival time for children treated earlier by the same institution with CTX (40 mg/kg for 6 months) plus radiotherapy or with no specific protocol (various agents plus radiation) was 12 and 7 months respectively. The authors concluded that the main prognostic factor in the survival of children with non-Hodgkin's lymphoma is therapy, and it must be early, aggressive, prolonged, and should utilize all three modalities (chemotherapy, irradiation and surgery), whenever

feasible [44]. There are other critical prognostic factors such as the initial amount of bulky disease, failure to achieve a CR within the first 6 weeks from the start of treatment, as well as relapses within the first 12 months of diagnosis.

The above mentioned conclusions are shared by most investigators at least for patients with stage II, III and IV. For children adequately investigated and with pathologic stage I malignant lymphoma of peripheral lymph nodes or with stage I–II and primary disease in the Waldeyer's ring, the prognosis is fairly good [41, 42], and local irradiation combined with few cycles of combination chemotherapy may be sufficient. This is important, in order not to overtreat early stage patients.

CONCLUSIONS

At the time of present knowledge, firm conclusions are difficult and may be premature. However, the large body of recently available clinical data allows to outline some practical therapeutic considerations.

Despite the contemporary enthusiasm aroused for the undoubted results achieved by chemotherapy in the field of malignant lymphomas, the effective contribution which can be derived by the appropriate use of radiation therapy should not be neglected or minimized. Aside from cytological subgroups, in pathological stage I–II patients there is recent evidence that the irradiation program alone can produce a 60–75% two-year disease free survival [20, 21]. Since most of initial relapses, especially in patients with diffuse histology, seem to occur within the first 24 months after completion of therapy, it is reasonable to think that radiotherapy can cure about two thirds of adult lymphomas presenting with early disease. The irradiation program should be adequate in terms of dosage, particularly in presence of extranodal lymphoma and/or diffuse pattern. The extent of radiation fields to be employed in early pathological stages probably deserves a controlled trial. Nevertheless, there are sufficient data indicating that for patients with infradiaphragmatic disease the whole abdomen must be included within the portal fields (abdominal bath), and that for those with supradiaphragmatic extent the irradiation of mediastinum can be spared because of its infrequent involvement. Furthermore, the patterns relapse would indicate that an extensive irradiation (probably total lymphoid) could be useful in patients with nodular lymphoma primary in the lymph nodes and that regional prophylactic radiotherapy is indicated in primary lymphomas of the Waldeyer's ring [15].

The vascular invasion, which is often associated with unfavourable histology [14, 45] is probably the most responsible factor, with inadequate staging approach, for treatment failure. In this situation, a combined treatment approach is indicated, and there is a preliminary evidence for the usefulness of adjuvant chemotherapy. Since most, if not all, relapsing patients did so within 2–5 months from beginning of their irradiation [20, 21], probably a "split course" chemotherapy and the use of combinations, including new effective agents such as adriamycin and bleomycin, could gain a more satisfactory systematic control of the disease.

In patients with stage III and IV chemotherapy represents the treatment of choice. With modern aggressive diagnostic approach, true (pathologic) stage III lymphoma appears to be an uncommon finding, both in adults and children. A split course chemotherapy combined with extensive radiotherapy appears theoretically to be the most logical approach, although results of ongoing trials [21] are not yet available. Our study trying to control the disease by a sequential combined approach failed to indicate a superiority of either treatment modality especially because of new manifestations occurring during the course of both radiotherapy and chemotherapy [20]. In presence of histiocytic or mixed type lymphomas the combination should include adriamycin and bleomycin.

The appropriate selection of treatment for patients with stage IV disease presents difficult problems. Many effective multiple drug treatments are now available, although none can presently be recognized as the treatment of choice for all cytologic subgroups. However, it is important to remember, as shown by Portlock *et al.* [46] that patients with nodular and well differentiated histology can also be controlled for long periods of time with single agent chemotherapy. In general, lymphocytic lymphomas respond favourably to a combination including CTX, VCR and P, while to improve the incidence of complete remission in diffuse histiocytic and mixed type lymphomas the addition of ADM and BLM or of Ara-C and MTX seems almost essential in most patients. It remains to be evaluated also in stage IV the appropriate use of radiotherapy either in the reduction of major bulky sites of disease or as consolidation treatment after

achieving a complete response with chemotherapy. Childhood lymphomas were shown to be controlled by the use of aggressive and combined treatment protocols which were similar to those successfully employed in the management of acute lymphoblastic leukemia.

Recent results reported in diffuse histiocytic and in childhood lymphoma would indicate that aggressive chemotherapy is able to cure an appreciable fraction of patients with these neoplasms. In fact, patients who are alive at 2 years after the end of treatment and are free of all evidence of disease, almost never relapse, as evidenced by the flat survival curves [38, 43, 44]. This definition of cure, previously used only for radiotherapy, seems now applicable also to those patients who remain in complete remission beyond the high-risk period of relapse. In patients with lymphocytic lymphoma or nodular histology the concept of cure appears more difficult to define, despite their significantly better median survival compared to diffuse histiocytic lymphoma, because of the pattern of continuous late relapse beyond the second year after completion of therapy. On the basis of this knowledge, patients with this type of lymphoma may benefit from the use of maintenance chemotherapy.

In conclusion, despite the present difficulties surrounding the subject of non-Hodgkin's lymphomas new effective therapies and new guidelines for treatment have been brought to light in recent years. It is hoped that an international and interdisciplinary approach to this group of neoplasms would also facilitate an improved therapeutic strategy, although the progress is expected to be slow and difficult.

REFERENCES

1. Symposium on non-Hodgkin's lymphomata, *Brit. J. Cancer* **31,** (Suppl. II) 1, (1975).
2. Leading Article, Classification of lymphomata. *Brit. med. J.* **2,** 4 (1975).
3. Z. Fuks, E. Glatstein, H. S. Kaplan, Patterns of presentation and relapse in the non-Hodgkin's lymphomata. *Brit. J. Cancer* **31,** 286 (1975).
4. P. S. Schein, B. A. Chabner, G. P. Canellos, R. C. Young, C. Berard and V. T. De Vita, Potential for prolonged disease free survival following combination chemotherapy of non-Hodgkin's lymphoma. *Blood* **43,** 181 (1974).
5. P. S. Schein, B. A. Chabner, G. P. Canellos, R. C. Young and V. T. De Vita Jr., Non-Hodgkin's lymphoma: patterns of relapse from complete remission after combination chemotherapy. *Cancer (Philad.)* **35,** 354 (1975).
6. L. M. Fuller, F. L. Banker, J. J. Butler, J. F. Gamble and M. P. Sullivan, The natural history of non-Hodgkin's lymphomata. Stage I and II. *Brit. J. Cancer* **31** (Suppl. II), 270 (1975).
7. M. Tubiana, P. Pouillart, M. Hayat, M. Schlienger, R. Gerard-Marchant, J. Schlumberger, J. Brugere, J. L. Amiel and G. Mathé, Results of radiotherapy in stage I and II of non-Hodgkin's lymphoma. *Brit. J. Cancer* **31,** (Suppl. II), 402 (1975).
8. J. A. M. Van Unnik, K. Breur, J. M. V. Burgers, F. Cleton, A. A. M. Hart, W. F. Stenfert Kroese, R. Somers and J. M. M. P. M. van Turnhout, Non-Hodgkin's lymphomata: clinical features in relation to histology. *Brit. J. Cancer* **31,** (Suppl. II), 201 (1975).
9. M. J. Peckham, J. P. Guay, M. E. Hamlin and R. J. Lukes, Survival in localized nodal and extranodal non-Hodgkin's lymphomata. *Brit. J. Cancer* **31,** 413 (1975).
10. B. U. R. Werf-Messing, Reticulum cell sarcoma and lymphosarcoma. A retrospective study of potential survival in loco-regional disease. *Europ. J. Cancer* **4,** 549 (1968).
11. B. Hoerni, J. Chauvergne, M. Durand, G. Hoerni-Simon, J. Touchard, C. Meugé, C. Lagarde, Evolution des réticulosarcomes et lymphosarcomes. Analyse d'une série des 268 malades. *Bull. Cancer* **61,** 61 (1974).
12. K. Musshoff and H. Schmidt-Vollmer, Prognostic significance of primary site after radiotherapy in non-Hodgkin's lymphomata. *Brit. J. Cancer* **31,** 425 (1975).
13. J. Dumont, C. Duffillot, G. Flandrin, N. Chelloul, H. Tristant and J. Bernard, Non-Hodgkin's lymphomata: clinical and immunological data in relation to histology. *Brit. J. Cancer* **31** (Suppl. II), 187 (1975).
14. A. S. Patchefsky, H. S. Brodovsky, H. Menduke, M. Southard, J. Brooks, D. Nicklas and W. S. Hoch, Non-Hodgkin's lymphomas: a clinicopathologic study of 293 cases. *Cancer (Philad.)* **34,** 1173 (1974).

15. A. Lattuada, A. Banfi, S. Basso Ricci, F. Milani, C. Uslenghi, F. Volterrani and R. Zucali, Non-Hodgkin's lymphoma results after treatment in stage I and II in 250 cases. *Proc. XIth Int. Cancer Cong., Florence* 20–26 October 1974. (Edited by P. Bucalossi, U. Veronesi and M. Cascinelli). Vol. 4, p. 808. Excerpta Medica, Amsterdam (1975).
16. D. S. Wong, L. M. Fuller, J. J. Butler and C. C. Shullenberg, Extranodal non-Hodgkin's lymphomas of the head and neck. *Amer. J. Roentgenol.* **123,** 471 (1975).
17. E. Cecchetti, La radioterapia delle neoplasie mesenchimali maligne della faringe. *Radiobiol. Radioter. Fis. med.* **25,** 146 (1970).
18. J. Brugere, M. Schlienger, R. Gerard-Marchant, M. Tubiana, P. Pouillart and Y. Cachin, Non-Hodgkin's malignant lymphomata of upper digestive and respiratory tract: natural history and results of radiotherapy. *Brit. J. Cancer* **31** (Suppl. II), 435 (1975).
19. G. Freeman, J. W. Berg, S. J. Cutler, Occurrence and prognosis of extranodal lymphomas. *Cancer (Philad.)* **29,** 252 (1972).
20. G. Bonadonna, M. De Lena, A. Lattuada, F. Milani, S. Monfardini and G. Beretta, Combination chemotherapy and radiotherapy in non-Hodgkin's lymphomata. *Brit. J. Cancer* **31,** (Suppl. II), 481 (1975).
21. S. A. Rosenberg and H. S. Kaplan, Clinical trials in the non-Hodgkin's lymphomata at Standford University: Experimental design and preliminary results. *Brit. J. Cancer* **31** (Suppl. II), 456 (1975).
22. G. Bonadonna and S. Monfardini, Chemotherapy of non-Hodgkin's lymphomas. *Cancer Treat. Rev.* **1,** 167 (1974).
23. S. E. Jones, S. A. Rosenberg, H. S. Kaplan, M. E. Kadin and R. F. Dorfman, Non-Hodgkin's lymphomas II. Single agent chemotherapy. *Cancer (Philad.)* **30,** 31 (1972).
24. S. Monfardini, G. Tancini, M. Gasparini, F. Pizzetti, M. De Lena and G. Bonadonna, Single agent sequential chemotherapy in non-Hodgkin's lymphomas. *Tumori* **59,** 219 (1973).
25. V. T. De Vita, A. A. Serpick and P. P. Carbone, Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Ann. int. Med.* **73,** 881 (1970).
26. B. Hoogstraten, A. H. Owens, R. E. Benhard *et al.*, Combination chemotherapy in lymphosarcoma and reticulum cell sarcoma. *Blood* **33**, 70 (1969).
27. J. Luce, J. F. Gamble, J. F. Wilson *et al.*, Combined cyclophosphamide, vincristine and prednisone therapy of malignant lymphoma. *Cancer (Philad.)* **28,** 306 (1971).
28. C. Bagley, V. T. De Vita, C. W. Berard and C. P. Canellos, Intensive cyclical chemotherapy with cyclophosphamide, vincristine and prednisone. *Ann. int. Med.* **76,** 227 (1972).
29. F. Lauria, M. Baccarani, M. Fiacchini, M. Gobbi and S. Tura, Methotrexate cyclophosphamide and vincristine (MEV regimen) for non-Hodgkin's lymphomas. *Europ. J. Cancer* **11,** 343 (1975).
30. E. M. McKelvey, J. A. Gottlieb, A. Haut and M. Lane, Hydroxyldaunomycin (Adriamycin) combination chemotherapy in non-Hodgkin's lymphoma. *Proc. Amer. Ass. Cancer Res.* **16,** 233 (1975).
31. R. S. Stein, E. M. Moran, R. K. Desser, J. B. Miller, H. M. Golomb and J. E. Ultmann, Combination chemotherapy of lymphomas other than Hodgkin's disease. *Ann. int. Med.* **81,** 601 (1974).
32. M. Levitt, J. C. Marsh, R. C. De Conti *et al.*, Combination sequential chemotherapy in advanced reticulum cell sarcoma. *Cancer (Philad.)* **29,** 630 (1972).
33. J. K. Luce, F. C. De Laney and E. A. Gehan, Remission induction chemotherapy of disseminated malignant lymphoma with combination bleomycin, cyclophosphamide, vincristine and prednisone. *Proc. Amer. Ass. Cancer Res.* **14,** 66 (1973).
34. A. T. Skarin, E. Frei III, W. C. Moloney and J. U. Gutterman, New agents and combination chemotherapy of non-Hodgkin's lymphomata. *Brit. J. Cancer* **31**, (Suppl. II), 497 (1975).
35. P. S. Schein, V. De Vita, G. Canellos, B. Chabner and R. Young, A new combination chemotherapy program for diffuse histiocytic (DHL) and mixed (DML) non-Hodgkin's lymphoma. *Proc. Amer. Ass. Cancer Res.* **16,** 248 (1975).
36. G. Bonadonna, C. Uslenghi and R. Zucali, Recent trends in the medical treatment of Hodgkin's disease. *Europ. J. Cancer* **11,** 251 (1975).

37. T. Gee, B. J. Lee, C. W. Young, M. Dowling, L. J. F. Little and B. Clarkson, Treatment of non-Hodgkin's lymphomas in adults with a multiple drug regimen and radiation therapy (Cytoxan L-2). *Proc. Amer. Ass. Cancer Res.* **16,** 264 (1975).
38. V. T. De Vita, G. P. Canellos, B. C. Chabner, P. Schein, S. P. Hubbard and R. C. Young, Advanced histiocytic lymphoma, a potentially curable disease. Results with combination chemotherapy. *Lancet* **i,** 248 (1975).
39. B. J. Kennedy, J. Hill, C. Bloomfield, D. Kiang, I. Fortuny and A. Teologides, Combination (COP) versus successive single agent chemotherapy (C–O–P) in lymphocytic lymphoma. *Proc. Amer. Ass. Cancer Res.* **16,** 142 (1975).
40. G. P. Canellos, V. T. De Vita, R. C. Young, B. A. Chabner, P. S. Schein and R. E. Johnson, Therapy of advanced lymphocytic lymphoma. A preliminary report of a randomized trial between combination chemotherapy (CVP) and intensive radiotherapy. *Brit. J. Cancer* **31,** (Suppl. II), 474 (1975).
41. D. Pinkel, W. Johnson and R. J. A. Aur, Non-Hodgkin's lymphoma in children. *Brit. J. Cancer* **31,** (Suppl. II), 298 (1975).
42. M. Lemerle, R. Gerard-Marchant, H. Sancho and O. Schweisguth, Natural history of non-Hodgkin's lymphomata in children. A retrospective study of 109 cases. *Brit. J. Cancer* **31** (Suppl. II), 324 (1975).
43. N. Wollner, P. Lieberman, P. Exelby, G. D'Angio, J. Burchenal, S. Fang and M. L. Murphy, Non-Hodgkin's lymphoma in children: Results of treatment with LSA_2–L_2 protocol. *Brit. J. Cancer* **31** (Suppl. II), 337 (1975).
44. N. Wollner, J. H. Burchenal, P. Exelby, P. H. Lieberman, G. D'Angio and M. L. Murphy, Non-Hodgkin's lymphomas in children. *Med. ped. Oncol.*, in press.
45. E. M. Moran and H. Rappaport, Prognostic value of vascular invasion (V.I.) in histiocytic lymphoma (HLY). *Proc. Amer. Ass. Cancer Res.* **16,** 249 (1975).
46. C. S. Portlock, S. A. Rosenberg, E. Glatstein and H. S. Kaplan, Stage IV non-Hodgkin's lymphoma: a prospective trial comparing CVP (Cyclophosphamide, vincristine, prednisone) alone vs. split course CVP with total lymphoid irradiation (CVP–RAD) VS. Continuous single alkylating agent (SA). *Proc. Amer. Ass. Cancer Res.* **16,** 266 (1975).

Europ. J. Cancer Vol. 12, pp. 675–677. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Tolerance of CNS to Total Body Irradiation Combined with Chemotherapy Applied for the Treatment of Leukemia

A. J. VAN DER KOGEL, D. W. VAN BEKKUM and G. W. BARENDSEN
Radiobiological Institute TNO, Rijswijk, The Netherlands

TOTAL BODY irradiation with large doses combined with intensive chemotherapy is increasingly being employed in the treatment of leukemic patients who fail to respond to chemotherapy alone. The objective of this combined treatment, i.e., complete eradication of the leukemic cell population, can only be achieved, however, by the application of doses which cause such severe damage to the normal haemopoietic system that replacement by transplantation is required. Thus far, the main source for this replacement has been bone marrow from HLA identical, MLC negative sibling donors, which source is only available for roughly 1/3 of the patients. Presently, the feasibility of employing autologous bone marrow collected during complete haematological remission and stored at low temperature, is being explored in several centers. Due to these developments the number of patients to be subjected to high dose whole body irradiation is expected to increase.

The consecutive intensive treatments with chemotherapy and radiation involved in these applications have introduced a new risk factor, namely late damage to normal tissues. Among the latter, the CNS should be of major concern since many leukemic patients—especially children—now routinely receive prophylactic irradiation of the brain and intrathecal methotrexate treatment in conjunction with remission induction therapy. Among the survivors of this group of patients, the candidates for whole body irradiation eventually occur.

The aim of this communication is to discuss the scarce data on late damage of the brain and spinal cord in this context.

Tolerance of CNS to irradiation

Experimental as well as clinical data indicate that the induction of late damage to brain and spinal cord by fractionated irradiation depends more strongly on the number of fractions and to lesser extent on the overall time of treatment than damage to other tissues, such as skin or intestine which have a relatively high rate of cellular turnover. Without presupposing a specific mechanism of action, empirically developed formulae have been derived in order to enable a comparison of total doses administered in a given fractionated scheme with other total doses and modes of application. For this comparison each treatment is expressed in a single value denoted equivalent dose (ED).

Experimental and clinical data indicate that, within the limits of uncertainty involved, total doses associated with a specific incidence of severe late damage can be converted to an equivalent dose, using a formula similar to that proposed first by Ellis [1]:

Equivalent dose (ED rad) = total dose $\times N^{-0.38} \times T^{-0.05}$ where N is the number of fractions and T is the overall time of treatment. The exponents in this formula have been based on experimental data obtained for rats [2]. Wara *et al.* have proposed closely similar

Accepted 22 March 1976.

exponent values for late damage to lung, namely $N^{-0.377}$ and $T^{-0.058}$, and their analysis of clinical data on late damage after thoracic cord irradiation suggested good agreement with this formula [3]. For skin damage Ellis has proposed factors of $N^{-0.24}$ and $T^{-0.11}$ [1].

Tolerance doses of brain or spinal cord are commonly derived as equivalent to a dose level at which late complications occur in less than 5% of the cases. For irradiation of a small volume of the brain, a tolerance level of 7000 rad of γ-rays in 7 weeks has been proposed [4]. On the basis of our formula this is equivalent to 1495 ED rad. For larger volumes, however, the tolerance level is approximately 20% lower, 5400 in 6 weeks, equivalent to 1233 ED rad. In young children the CNS is assumed to be more sensitive as compared to adults. For small volumes a dose of 5400 rad in 6 weeks (1233 ED rad) is considered as tolerance dose and for large volumes 20% less, i.e., 4000 rad in 5 weeks (988 ED rad) is assumed. At what age the sensitivity of CNS in children decreases to that of CNS in adults is not well known.

Information from the literature about the tolerance of CNS to combined treatments with radiation and chemotherapeutic agents are not adequate to derive quantitative data on dose reduction factors. Considerations of many radiobiological data on other tissues can lead to the conclusion that tolerance to irradiation might be reduced by factors ranging from 1·0 to 1·5. For the purpose of further discussions we will assume a dose-reduction factor of 1·2. This implies that for large irradiated volumes in patients who are in addition subjected to high dose chemotherapy, tolerance doses would be equivalent to 1028 ED rad for adults and to 823 ED rad for children.

For an evaluation of the risk of late CNS damage to be expected from total body irradiation combined with intensive chemotherapy, the total dose and the dose rate must be considered. The combination which thus far has been used most frequently in attempts to eradicate leukemia prior to bone marrow transplantation is a combination of high dose cyclophosphamide and irradiation of the whole body with a dose of 1000 rad of γ-radiation commonly given in several hours, i.e., at a dose rate of about 5 rad/min [5]. In order to convert this treatment to "equivalent dose" values, the only data which can be used are those obtained for rat spinal cord. These show that at 6·5 rad/min, total doses of about 2500 rad are less effective by a factor of 1·3 as compared with single doses applied at 300 rad/min [6]. If this factor is applicable to CNS in man, it follows that the dose of 1000 rad of whole body γ-radiation is equivalent to 769 rad equivalent dose. For the combined treatment with cyclophosphamide it can be deduced that this is equivalent to 769/1028 = 0·75 of the tolerance level in adults and to 769/823 = 0·93 of the tolerence level in children. It should be emphasized that these are only rough estimates pertaining to a risk of less than 5% of occurrence of late damage to the CNS. For a risk of 10% it can be derived from Wara *et al.* [3] that the dose level is approximately 10% larger and for a risk of 20% this level is about 20% larger.

Latent damage from preceding treatments in relation to eradicative treatment

In prophylactic treatments of children aimed at preventing meningeal leukemia, the most commonly employed scheme consists of total brain irradiation with a dose of 2400 rad in 2·5 weeks and a total of 5 doses of 12·5 mg/m^2 of intrathecal methotrexate given twice a week during the same period (referred to as St. Jude's scheme [7]). According to the formula used, this radiation treatment would be equivalent to 812 ED rad. This dose would be equal to a fraction 812/823 = 0·99 of the tolerance level in young children. Thus far no data have been reported which can be used to test the results of these calculations. The results published by Simone *et al.* [7] with 9 cases of leukoencephalopathy observed in 150 patients are most relevant to this issue. However, most cases in which CNS damage was observed, were found in a group which had received maintenance therapy with high doses of methotrexate.

Data concerning the fading of latent damage to CNS with the time after treatment are very limited. Preliminary experimental data on rats for intervals of up to 30 weeks indicate that between 5 and 10% of the "equivalent dose" may fade per month [8]. If exponential fading of the damage is assumed, it can be calculated that after 12 months a fraction of between 0·54 and 0·28 of the damage is still present and after 24 months between 0·29 and 0·08 of the initial damage remains.

These data on fading of latent damage are very tentative, but if approximately 30% of the damage induced by the previous treatment is still present at the time of administration of total body irradiation, this would amount to a value of 243 ED rad, that might contribute to complications after eradicative treatment.

It follows that in young children, assuming a tolerance level of 823 ED rad, only a treatment equivalent to 823–243 = 580 ED rad can be permitted for the eradicative treatment. In adults the commonly employed eradicative treatment with 769 ED rad would, if added to a value of 243 ED rad remaining from previous prophylactic treatment, yield a total of 1012 ED rad, i.e., just about equal to the expected tolerance level of 1028 ED rad.

If young children received a treatment equivalent to 769 ED rad, while 243 ED rad remained from previous treatment, the total sum equal to 1012 ED rad would be equivalent to 1012/823 = 1·23 times the value at which the risk of late CNS damage is expected to be smaller than 5%. Thus in such cases either a lower dose should be given to young children for eradicative treatment or, if higher doses are considered necessary for eradication, a larger risk must be accepted for late CNS damage.

It is evident that in view of the various uncertainties mentioned, these estimates are tentative. They indicate, however, that the risk of CNS damage might not be negligible and that exact knowledge of preceding treatments as well as careful dosimetry and observation are required to evaluate risks as early as possible.

REFERENCES

1. F. Ellis, The relationship of biological effect to dose–time–fractionation factors in radiotherapy. *Curr. Top. Rad. Res. Quart.* **4,** 357 (1968).
4. A. J. Van der Kogel and G. W. Barendsen, Late effects of spinal cord irradiation with 300 kV X-rays and 15 MeV neutrons. *Brit. J. Radiol.* **47,** 393 (1974).
3. W. M. Wara, T. L. Phillips, G. E. Sheline and J. G. Schwade, Radiation tolerance of the spinal cord. *Cancer (Philad.)* **35,** 1558 (1975).
4. S. Kramer, M. E. Southard and C. M. Mansfield, Radiation effect and tolerance of the central nervous system. In: *Frontiers of Radiation Therapy and Oncology*, (Edited by J. M. Vaeth,) Vol. 6, 332, Karger, Basel (1972).
5. E. D. Thomas, R. Storb, R. A. Clift, A. Fefer, F. L. Johnson, P. E. Neiman, K. G. Lerner, H. Glucksberg and C. D. Buckner, Bone marrow transplantation. *New Engl. J. Med.* **292,** 832 part 1 (1975).
6. A. J. van der Kogel, Radiation tolerance of different regions of the rat spinal cord: time–dose relationships. *Radiology*, submitted for publication.
7. J. V. Simone, R. J. A. Aur, H. O. Hustu and M. Verzosa, Acute lymphocytic leukemia in children. *Cancer (Philad.)* **36,** 770 (1975).
8. A. J. van der Kogel, Tolerance of different regions of the rat spinal cord to fractionated irradiation. *Int. J. Rad. Biol.* **29,** 104 (1976) (abstract).

Europ. J. Cancer Vol. 12, pp. 679–682. Pergamon Press 1976. Printed in Great Britain

General Papers

In Vitro Effects of Prolactin upon Testosterone Metabolism by Rat Mammary Adenocarcinomata*

WILLIAM R. MILLER

Department of Clinical Surgery, University Medical School, Edinburgh EH8 9AG, Great Britain

Abstract—*The effects of* in vitro *addition of prolactin* (50 μg/ml) *upon the conversion of testosterone to* 5α *dihydrotestosterone* (5α *DHT*) *and* 5α *androstanediol by incubates of rat mammary carcinomas have been investigated. The studies have been performed on* (*a*) *primary adenocarcinomata induced by the carcinogen* 7,12-*dimethylbenzanthracene* (*DMBA*) *in female Sprague–Dawley rats, and* (*b*) *transplantable tumours obtained by serial transplantation of a further DMBA-induced tumour into neonatally thymectomized rats. In incubations of primary DMBA-induced tumours, addition of prolactin inhibited the production of both* 5α *DHT and* 5α *androstanediol, the overall* 5α *reduction being between* 27 *and* 40% *lower than that in control incubations. In contrast, prolactin failed to inhibit, and in some cases stimulated, the* 5α *reduction of testosterone in the transplantable tumour.*

INTRODUCTION

BOTH PROLACTIN and steroid hormones are known to influence the growth and development of rat mammary tumours [1–3]. In the DMBA treated rat, there appears to be a direct correlation between plasma prolactin levels and the genetically determined susceptibility to mammary cancer [4]. Elevation of plasma prolactin levels accelerates the growth of mammary tumours [5–7] and conversely lowered levels may cause tumour regression [8]. Similarly, administration of steroids of the androstane series such as 5α dihydrotestosterone may inhibit the growth of mammary tumours in the rat [9, 10].

The aim of the present study was to determine the *in vitro* effects of prolactin, upon the production of 5α androstane steroids by rat mammary tumours.

Accepted April 1, 1976.

*This work was performed with a grant from the Cancer Research Campaign, grant number SP 1256.

MATERIAL AND METHODS

Tumour tissue

(*a*) *DMBA-induced tumours.* Random bred female Sprague–Dawley rats were given 5 mg dimethylbenzanthracene (DMBA) intravenously at 50 days of age. Subsequently induced mammary tumours were allowed to grow to 2 × 2 cm in size. At this stage, three rats were sacrificed by exsanguination and the tumours incubated; three others were sequentially oophorectomised and given oestradiol-17β (1 μg) to determine tumour hormone dependence. All tumours regressed after oophorectomy but renewed growth following oestrogen administration and were therefore hormone dependent.

(*b*) *Transplanted tumours* (*TG* 3). The tumour studied was originally induced by DMBA as above, in an inbred Sprague–Dawley rat (ADRA) [4]. It was serially transplanted into further ADRA rats (neonatally thymectomized) by dorsal skin implantation and studied at its 2nd and 6th passages. Although all the tumours investigated were derived from intact rats, the

same tumour at its 6th passage in other rats, failed to regress following oophorectomy but was stimulated by oestradiol (1 μg daily). Tumour growth was therefore independent of oophorectomy but sensitive to oestradiol.

Tumour processing and incubation

All tumours were processed at 0°C until incubation was carried out (within 30 min of tissue removal). One gram of each tumour was finely sliced and Krebs Ringer phosphate buffer pH 7·4 (10 ml), an NADPH generating system (200 μ mol glucose-6-phosphate, 25 mg NADP, and 50 units glucose-6-phosphate dehydrogenase) and the radioactive precursor (45 μCi 7α-^{3}H testosterone) added. Duplicate incubations were set up with the addition of either ovine prolactin, rat prolactin or rat growth hormone (50 μg/ml). The tumour slices were then incubated by shaking at 37°C in an atmosphere of oxygen for 1 hr. The reaction was stopped by adding methanol to 80% (v/v) and the incubations stored at −10°C until processed.

Purification and characterization of metabolites

Before extraction, 500 μg of non-radioactive carrier steroids were added to monitor recovery losses. The metabolites were extracted, separated into individual steroids and purified by thin layer chromatography, as described previously [11]. The percentage of metabolism of testosterone and conversion to 5α DHT and 5α androstanediol were determined by measuring the percentage of incorporation of radioactive label into the appropriate metabolites after correction for recovery losses.

RESULTS

The effects on testosterone metabolism of *in vitro* addition of prolactin to incubations of DMBA-induced tumours are presented in Table 1. No consistent effects were produced by prolactin on the absolute level of testosterone metabolised. Prolactin, however, inhibited the conversion of testosterone to both 5α DHT and 5α androstanediol. Estimates of total 5α reduction obtained by combining the production of 5α DHT with that of 5α androstanediol showed that prolactin produced a consistent inhibition of 5α reduction (27·4–40·3% of control incubations), irrespective of the type of prolactin used, or whether the tumour was obtained from intact, or endocrine manipulated rats. Similar *in vitro* addition of rat growth hormone at the same concentration had negligible effects in 4 tumours and inhibited 5α reduction in only one tumour (to a lesser extent than prolactin).

The results of similar studies using the transplanted tumour are presented in Table 2. In these incubations, the overall level of testosterone metabolism was extremely high and relatively unaffected by the addition of prolactin. In contrast to the primary DMBA tumours, in this transplanted tumour, prolactin tended to increase the percentage of production of 5α DHT and total 5α reduction of testosterone.

DISCUSSION

The results presented in this study indicate that the 5α reduction of testosterone by rat mammary carcinomas may be influenced by the *in vitro* addition of either rat or ovine prolactin. These effects may be partially specific to prolactin as the *in vitro* addition of growth hormone at the same concentration had less or no effect on testosterone metabolism.

In primary DMBA-induced tumours, prolactin consistently inhibited the production of 5α reduced metabolites of testosterone. This is interesting in view of the inhibitory properties of the 5α androstane steroids on the growth of hormone dependent rat mammary tumours [9, 10]. Although evidence for hormone dependence was obtained for only three of the DMBA tumours studied in this series, it was our experience that the majority of DMBA tumours induced in this group of animals were hormone dependent. It would therefore be in keeping with the growth promoting effects of prolactin on hormone dependent tumours that prolactin should inhibit the production of 5α reduced steroids at tumour level.

The results from *in vitro* incubations of the transplanted tumour differ from the primary DMBA tumours in two major respects. Firstly, although the DNA content and cellularity of the two groups of tumours were similar, the overall metabolism and 5α reduction of testosterone in control incubations was, in general, higher in the transplanted tumour. Secondly, prolactin tended to stimulate 5α reduction in the transplanted tumour in diret contrast to its inhibitory effects in the primary tumours. Several factors may be implicated in these differences between transplanted and DMBA tumours. Firstly, whereas the DMBA tumours are likely to be hormone dependent, the transplanted tumour failed to regress following

Table 1. *Metabolism of 7α-^3H testosterone by DMBA induced tumours*

Rat		Per cent testosterone metabolised		Per cent conversion to 5α DHT		Per cent conversion to 5α androstanediol		Per cent 5α reduction	
A*	Control	94·80		28·50		25·40		53·90	
	+ prolactin (rat)	85·00	(−10·3)	13·20	(−53·6)	21·30	(−16·1)	34·50	(−36·0)
	+ growth hormone	90·20	(− 4·9)	24·20	(−15·1)	22·10	(−13·0)	46·30	(−14·1)
B†	Control	46·50		5·50		27·05		32·55	
	+ prolactin (rat)	35·10	(−24·5)	3·90	(−29·1)	17·00	(−37·2)	20·90	(−35·8)
	+ growth hormone	46·10	(− 0·9)	6·20	(+12·7)	26·70	(− 1·3)	32·90	(+ 1·1)
C*	Control	47·45		16·15		14·15		30·30	
	+ prolactin (rat)	43·10	(− 9·2)	8·40	(−49·0)	13·55	(− 4·2)	21·95	(−27·6)
	+ growth hormone	48·20	(+ 1·6)	13·60	(−15·8)	17·20	(+21·55)	30·80	(+ 1·7)
D†	Control	42·50		5·05		20·10		25·15	
	+ prolactin (rat)	37·10	(−12·6)	2·50	(−50·5)	13·30	(−33·8)	15·80	(−37·2)
E*	Control	33·85		5·65		12·45		18·10	
	+ prolactin (ovine)	34·75	(+ 2·6)	5·50	(− 2·7)	7·60	(−39·0)	13·10	(−27·6)
	+ growth hormone	31·60	(− 6·7)	5·00	(−11.5)	11·55	(− 7·2)	16·55	(− 8·6)
F†	Control	47·80		19·20		11·20		30·40	
	+ prolactin (ovine)	59·40	(+24·3)	9·40	(−51·1)	8·75	(−21·9)	18·15	(−40·3)
	+growth hormone	44·45	(− 7·0)	19·40	(+ 1·0)	3·95	(−64·7)	23·35	(−23·2)

Figures in parentheses represent the percentage of change caused by addition of prolactin or growth hormone (50 μg/ml).

*Intact rat.

†Rat sequentially oophorectomized and given oestradiol (1 μg daily).

Table 2. *Metabolism of 7α-^3H testosterone by transplanted tumours*

Rat		Per cent testosterone metabolised		Per cent conversion to 5α DHT		Per cent conversion to 5α androstanediol		Per cent 5α reduction	
1*	Control	88·70		19·90		31·30		51·20	
	+prolactin (ovine)	95·50	(+7.7)	26·50	(+ 33·2)	59·00	(+88·5)	85·50	(+67·0)
2*	Control	96·80		9·65		33·60		43·25	
	+ prolactin (ovine)	97·15	(+0·3)	21·90	(+127·0)	30·30	(− 9·8)	52·20	(+20·7)
3*	Control	98·70		13·30		66·15		79·45	
	+ prolactin (ovine)	95·50	(− 3·3)	16·50	(+ 24·0)	66·10	(0)	82·60	(+ 4·0)
4†	Control	94·00		9·50		39·20		48·70	
	+ prolactin (rat)	97·00	(+ 3·1)	24·50	(+158·0)	31·70	(−19·1)	56·20	(+15·4)
5†	Control	96·70		3·40		50·70		54·10	
	+ prolactin (rat)	88·05	(− 9·0)	5·25	(+ 54·4)	55·10	(+ 8·6)	60·35	(+11·6)

*Tumour at 2nd passage.

†Tumour at 6th passage.

Figures in parentheses represent % change caused by addition of prolactin.

oophorectomy; the effects of prolactin may therefore reflect differences between hormone dependent and hormone sensitive tumours. Secondly, there are different circulating prolactin levels in the two strains of rats used—the ADRA strain, in which the transplanted tumour was induced and subsequently transplanted, has a lower basal level of circulating prolactin, in comparison with the randombred variety in which the primary DMBA tumours were induced [4].

At present, it is not possible to remove the strain difference as tumour induction rate by DMBA in the ADRA rat is exceptionally low and the transplanted tumour is not accepted by randomly bred animals.

In conclusion, it is worth emphasizing that whilst prolactin has already been shown to

affect steroid metabolism in other tissues, such as the prostate [12] and testis [13], its effects in mammary tumour tissue may assume added importance in view of the properties of the 5α reduced metabolites involved to influence tumour growth.

Acknowledgements—The author thanks Professor A. P. M. Forrest for his interest and encouragement, the Cancer Research Campaign for the Grant to Professor Forrest supporting this work and Miss J. Telford for her technical assistance. Rat and ovine prolactin and rat growth hormone were gifts from the National Institutes of Health (Bethesda, Maryland).

REFERENCES

1. T. L. Dao and D. Sinha, In *Fourth Tenovus Workshop, Prolactin and Carcinogenesis—Oestrogen and Prolactin in Mammary Carcinogenesis* in vivo *and* in vitro *Studies.* (Edited by A. R. Boyns and K. Griffiths), p. 199. Alpha Omega Alpha Publishing, Cardiff, U.K. (1972).
2. J. Meites, In *Fourth Tenovus Workshop, Prolactin and Carcinogenesis—Section* 2, *Introduction.* (Edited by A. R. Boyns and K. Griffiths), p. 54. Alpha Omega Alpha Publishing, Cardiff, U.K. (1972).
3. O. H. Pearson, R. L. M. Murray, G. Mozaffarian and J. Pensky, In *Fourth Tenovus Workshop, Prolactin and Carcinogenesis—Prolactin and Experimental Breast Cancer.* (Edited by A. R. Boyns and K. Griffiths). p. 154. Alpha Omega Alpha Publishing, Cardiff, U.K. (1972).
4. A. R. Boyns, R. Buchan, E. N. Cole, A. P. M. Forrest and K. Griffiths, Basal prolactin blood levels in three strains of rat with differing incidence of 7,12-dimethylbenz(a)anthracene induced mammary tumours. *Europ. J. Cancer* **9,** 169 (1973).
5. J. A. Clemens, C. W. Welsch and J. Meites, Effects of hypothalmic lesions on incidence and growth of mammary tumours in carcinogen-treated rats. *Proc. Soc. exp. Biol.(N.Y.)* **127,** 969 (1968).
6. C. W. Welsch, J. A. Clemens and J. Meites, Effects of multiple pituitary homografts or progesterone on 7,12-dimethylbenz(a)anthracene-induced mammary tumours in rats. *J. nat. Cancer Inst.* **41,** 465 (1968).
7. C. W. Welsch and J. Meites, Effects of a norethylnodrel-mestranol combination (Enovid) on development and growth of carcinogen-induced mammary tumours in female rats. *Cancer (Philad.)* **23,** 601 (1969).
8. E. E. Cassell, J. Meites and C. W. Welsch, Effects of ergocornine and ergocryptine on growth of 7,12-dimethylbenz(a)anthracene-induced mammary tumours in rats. *Cancer Res.* **31,** 1051 (1971).
9. C. Huggins, G. Briziarelli and H. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumours. *J. exp. Med.* **109,** 25 (1959).
10. C. Huggins and K. Mainzer, Hormonal influence on mammary tumours of the rat. II. Retardation of growth of a transplanted fibroadenoma in intact female rats by steroids in the androstane series. *J. exp. Med.* **105,** 485 (1957).
11. W. R. Miller, A. P. M. Forrest and T. Hamilton, Steroid metabolism by human breast and rat mammary carcinomata. *Steroids* **23,** 379 (1974).
12. A. R. Boyns, E. N. Cole, M. P. Golder, V. Danutra, M. E. Harper, B. Brownsey, T. Cowley, G. E. Jones and K. Griffiths, In *Fourth Tenovus Workshop, Prolactin and Carcinogenesis—Prolactin studies with the prostate.* (Edited by A. R. Boyns and K. Griffiths). Alpha Omega Alpha Publishing, Cardiff, U.K. (1972).
13. A. A. Hafiez, C. W. Lloyd and W. Bartie, The role of prolactin in the regulation of testis function: the effects of prolactin and luteinizing hormone on the plasma levels of testosterone and androstenedione in hypophysectomized rats. *J. Endocr.* **52,** 327 (1972).

Europ. J. Cancer Vol. 12, pp. 683–688. Pergamon Press 1976. Printed in Great Britain

The Immunoglobulins in Hodgkin's Disease*

D. J. TH. WAGENER†, P. J. J. VAN MUNSTER‡ and C. HAANEN†
†*Division of Haematology, Department of Internal Medicine, and* ‡ *Department of Pediatrics, St. Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands*

Abstract—*Serum immunoglobulin levels IgG, IgA and IgM have been investigated in* 51 *untreated patients with Hodgkin's disease and* 102 *control persons. In the patients with Hodgkin's disease the mean IgG and IgA level was significantly raised (P:* 0·0001 *for both). The mean IgM level in males was normal and was significantly increased (P:* 0·0012) *in females. The increase in IgA level was the most pronounced among the three immunoglobulins.*

INTRODUCTION

THE STUDIES of Dubin in 1947 [1], and later of Schier *et al.* [2[in 1956, of the delayed hypersensitivity reaction in patients with Hodgkin's disease have ushered extensive research of the immunological status of Hodgkin's patients. The initial general conclusion that patients with Hodgkin's disease have an impaired cell-mediated immunity [2–10] is recently modified. The earlier studies were conducted on treated patients and it became clear that radiotherapy and chemotherapy have a depressant influence on immunity [9, 11–13]. Recent studies on newly admitted untreated patients showed that an impairment of skin test reactivity and lymphocyte function exists, but that this deficiency is a quantitative, rather than a qualitative one [14–16].

The only study to our knowledge in Hodgkin's patients regarding immunoglobulin levels [17], as indicators of the humoral immunological status, revealed a reduced IgM level in 24 out of 50 cases. The IgA levels also tended to be low; the IgG levels were normal or raised. This study was carried out in treated patients and had therefore to be reevaluated.

In this paper IgG, IgA and IgM levels in untreated patients with Hodgkin's disease are presented and correlated with staging and histology.

Accepted 26 March 1976.

*This study was supported in part by the Queen Wilhelmina Foundation, K.W.F. (The Netherlands Organisation against Cancer).

MATERIAL AND METHODS

Patients

Fifty-one consecutive, new and untreated Hodgkin's patients were examined. Twenty-four were male and 27 female; their mean age was 30 years (range 14–60 years, median 28 years). None of the patients had concomitant overt infectious disease. The histologic classification used was that of the Rye Conference [18]. Patients were classified in 4 stages according to the Ann Arbor Conference [19]. Exploratory laparotomy was performed in 39 patients. Six patients with stage IV disease and one patient with stage IIIB were only classified on clinical grounds, because of the severity of their illness. Five patients were not submitted to explorative laparotomy, because of commitment to the EORTC-trial which requires a randomization between laparotomy and no laparotomy in clinical stage I and II. When laparotomy was performed, patients were classified according to pathologic stage, otherwise on clinical criteria. Patients' data are summarized in Table 1. One hundred and two healthy donors from the Blood Transfusion Service served as normal controls (their mean age was 32 years, median 32 years, range 17–62 yr). The age distribution of the patients and the control group is shown in Table 2.

Methods

Blood was collected by venipuncture before staging-laparotomy was done. The samples were taken between 8.30 and 10.30 a.m. After 30 min the clotted blood was centrifuged. The

Table 1. *Summary of patients' data and serum immunoglobulin levels (mg/ml)*

Patient	Sex	Age	Histologic type*	Clinical staging		Pathologic staging†		Serum immunoglobulins IgG	IgA	IgM
1. M.L.–S.	F	35	NS	I	A	I	H–S–N–M–	11·0	2·22	3·75
2. G.L.–H.	F	26	MC	I	A	I	H–S–N–M–	12·4	2·18	2·03
3. H.B.	M	47	MC	I	A	I	H–S–N–M–	10·4	2·68	1·41
4. A.H.	M	26	MC	I	A	I	H–S–N–M–	9·7	1·57	1·95
5. J.D.	M	19	MC	I	A	N.D.‡	EORTC	11·9	2·03	0·86
6. B.H.–U.	F	28	LP	I	B	I	H–S–N–M–	14·7	2·37	2·07
7. E.M.	M	38	LD	I	B	I	H–S–N–M–	16·4	4·59	2·18
8. J.v.O.	M	43	LP	II	A	II	H–S–N–M–	8·4	4·67	5·43
9. J.L.–S.	F	32	NS	II	A	II	H–S–N–M–	13·7	2·45	1·76
10. M.L.–H.	F	21	NS	II	A	II	H–S–N–M–	15·7	1·84	2·23
11. J.V.	M	36	NS	II	A	N.D.	EORTC	12·1	2·88	1·01
12. A.H.–H.	F	43	MC	II	A	N.D.	EORTC	14·6	2·08	1·09
13. J.V.	F	20	LP	III	A	II	H–S–N–M–	10·8	2·95	1·72
14. M.B.–M.	F	26	NS	III	A	II	H–S–N–M–	16·7	3·52	1·70
15. M.R.–J.	F	24	MC	III	A	II	H–S–N–M–	15·1	2·68	2·71
16. M.E.–G.E.	F	39	NS	III	A	II	H–S–N–M–	14·4	3·10	0·80
17. J.S.	M	29	LP	II	A	II	H–S–N–M–	15·7	2·58	1·79
18. A.L.	F	20	NS	II	B	II	H–S–N–M–	12·8	2·24	2·26
19. E.R.	F	33	NS	II	B	II	H–S–N–M–	16·9	4·47	1·92
20. W.P.	M	22	NS	II	B	II	H–S–N–M–	8·7	2·68	0·69
21. J.T.	M	35	NS	II	B	II	H–S–N–M–	27·9	5·60	1·92
22. D.V.–S.	F	35	NS	II	B	N.D.	EORTC	11·2	1·88	1·48
23. W.T.–S.	F	47	MC	II	B	II	H–S–N–M–	18·1	1·91	4·15
24. J.d.B.	F	26	MC	II	B	II	H–S–N–M–	17·7	4·13	3·03
25. J.S.–L.	F	31	MC	II	B	N.D.	EORTC	19·1	4·90	2·39
26. N.S.	F	31	LD	II	B	II	H–S–N–M–	14·1	2·68	0·86
27. J.D.	F	17	NS	III	B	II	H–S–N–M–	15·1	3·46	1·72
28. R.D.	M	18	MC	III	B	II	H–S–N–M–	12·7	3·44	1·08
29. M.L.	F	17	LD	III_E	B	II_E	H–S–N–M–	12·1	3·66	3·12
30. W.D.	M	17	LP	III	A	III	H–S–N+M–	8·8	1·47	0·78
31. E.L.	M	60	LD	III	A	III	H–S–N+M–	11·0	3·10	1·64
32. A.K.–T.	F	35	NS	II	A	III	H–S+N–M–	14·4	3·05	2·11
33. M.U.	M	49	LP	III	A	III	H–S+N–M–	11·4	2·91	1·02
34. A.S.	F	17	NS	III	A	III	H–S+N+M–	15·3	4·01	1·56
35. A.K.	M	32	NS	III	A	III	H–S+N+M–	15·7	4·67	2·18
36. P.G.–B.	F	40	MC	III	B	III	H–S–N+M–	16·7	5·89	1·18
37. M.D.–P.	F	48	MC	III	B	III	H–S–N+M–	11·0	4·98	1·09
38. R.v.d.B.	F	18	MC	III	B	III	H–S+N–M–	13·7	3·44	2·71
39. A.v.W.	M	34	MC	III	B	III	H–S+N–M–	15·7	3·98	0·94
40. E.v.d.S.	M	20	LD	III	B	III	H–S+N+M–	7·4	1·93	0·70
41. G.D.	M	27	LD	III	B	N.D.		14·4	3·86	1·01
42. H.V.	F	14	NS	IV_{L+}	A	N.D.		19·8	5·79	1·40
43. D.R.	F	26	NS/MC	IV_{D+}	A	N.D.		8·7	2·22	2·18
44. M.D.–H.	F	51	MC	IV	B	IV	H+S+N+M–	24·7	5·84	1·64
45. P.v.D.	M	37	MC	IV	B	IV	H+S+N+M–	15·1	6·92	1·09
46. J.B.	M	26	MC	IV	B	IV	H+S+N+M–	9·4	0·92	0·70
47. G.H.	M	26	MC	IV	B	IV	H+S+N+M–	13·5	2·84	1·40
48. M.G.	M	22	NS	IV_{L+}	B	N.D.		11·2	3·66	1·20
49. A.S.	M	29	NS	IV_{M+}	B	N.D.		13·9	2·29	2·81
50. R.A.	M	26	LD	IV_{L+}	B	N.D.		17·1	2·69	2·03
51. J.J.	M	38	NS/LD	IV_{L+}	B	N.D.		15·3	6·20	2·96

*L.P.: lymphocyte predominance, N.S.: nodular sclerosing, M.C.: mixed cellularity, L.D.: lymphocyte depletion.
†H+ or H− for liver positive or negative; S+ or S− for spleen positive or negative; N+ or N− for lymph node positive or negative; M+ or M− for marrow positive or negative. L+ for lung positive; D+ for (derma) skin positive; E: extranodal.
‡N.D.: not done.

Table 2. Age distribution

Age (yr)	Hodgkin's patients (*N*)	(%)	Normal controls (*N*)	(%)
10–20	11	22	15	15
20–30	16	31	35	34
30–40	16	31	30	29
40–50	6	12	15	15
50–60	2	4	6	6
60–70			1	1
Total	51		102	

serum was decanted and stored at −70°C. Quantitative estimations of IgG, IgM and IgA in serum were performed with an automated turbidimetric immunoprecipitation method [20] and expressed in mg/ml. During the period of examination the same serum standard was used. This standard was prepared by mixing serum from 40 healthy adults and was stored after freeze drying in ampoules at −25°C. This reference standard had previously been calibrated against a highly purified polyclonal IgG, IgA and IgM preparation.

RESULTS

In 40 patients, the serum *IgG* level was normal and was increased in 11 as shown in Table 3 and Fig. 1. The mean was significantly ($P = 0{\cdot}0001$) higher than the mean of the controls. Although the number of patients is too small for reliable statistical analysis, the data obtained in this study (Fig. 1) suggest that the mean of serum IgG globulin levels in patients with signs of generalization (B symptoms) was higher than in patients without signs of generalization (A symptoms).

The *IgA* level was in the normal range in 29 patients and increased in 22. The rise of IgA was much more pronounced than IgG. The mean of IgA was very significantly ($P = 0{\cdot}0001$) increased in Hodgkin's patients compared with the controls.

The mean *IgM* level in males was normal, in females significantly ($P = 0{\cdot}0012$) increased. There were 5 male and 9 female Hodgkin's patients with an IgM level above the accepted upper limit of normal for our population. No patient had an IgM level below the lower limit of normal. The mean immunoglobulin levels in Hodgkin's patients, according to histologic type, are given in Table 4. It appeared that patients with Hodgkin's disease–lymphocytic predominance have almost normal immunoglobulin levels. It is to be noted that this subgroup consists of the smallest number of patients. The raised mean IgM level in males, in this histologic subtype, is due to the enormous rise of IgM level in one single patient (J.v.O.: IgM: 5·43 mg/ml). There were no significant differences in mean immunoglobulin levels between the four histologic subgroups.

DISCUSSION

In agreement with Goldman [17] we found a normal or increased IgG level in patients with Hodgkin's disease. However, contrary to

Table 3. Serum immunoglobulin levels in patients and controls

	IgG mg/ml	Range	IgA mg/ml	Range	IgM mg/ml	Range
	mean ± s.d.	mean ± 2 s.d.	mean ± s.d.	mean ± 2 s.d.	mean ± s.d.	mean ± 2 s.d.
Controls males	10·9 ± 2·5 ($N = 44$)	5·9–15·9	2·06 ± 0·64 ($N = 46$)	0·78–3·34	1·24 ± 0·44 ($N = 40$)	0·36–2·12
females	11·2 ± 2·6[a] ($N = 58$)	6·0–16·4	1·98 ± 0·74[b] ($N = 52$)	0·50–3·46	1·47 ± 0·36[c] ($N = 55$)	0·57–2·19
total	11·1 ± 2·6 ($N = 102$))	5·9–16·3	2·02 ± 0·69 ($N = 98$)	0·64–3·40		
Hodgkin's disease males	14·0 ± 3·8[1] ($N = 51$)		3·34 ± 1·37[2] ($N = 51$)		1·62 ± 1·04[3] ($N = 24$)	
females					2·02 ± 0·82[4] ($N = 27$)	

Significance of difference between males and females in the controls (Mann and Whitney U-test of Wilcoxon's two samples test):
[a]: $P = 0{\cdot}43$, [b]: $P = 0{\cdot}55$, [c]: $P = 0{\cdot}007$.
Significance of difference between Hodgkin's patients and controls:
[1]: $P = 0{\cdot}0001$, [2]: $P = 0{\cdot}0001$, [3]: $P = 0{\cdot}2502$, [4]: $P = 0{\cdot}0012$.

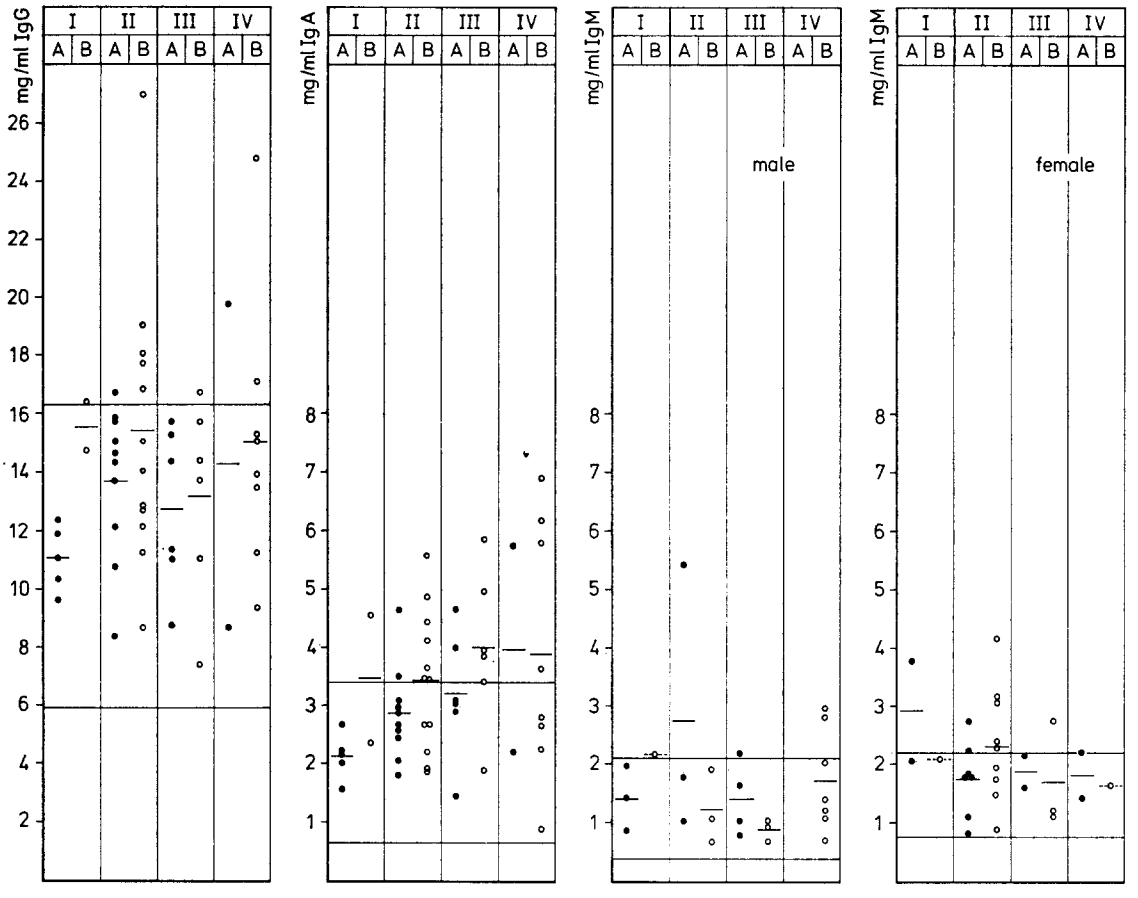


Fig. 1. *Serum immunoglobulins in patients with Hodgkin's disease according to stage. Below the figure should be typed.*
● : *patients without general symptoms.*
○ : *patients with general symptoms.*

Goldman's results, it was found, in this study, that the mean IgA level in Hodgkin's patients was significantly higher than in the controls. No patient had a decreased IgA level. In addition, it appeared that the mean IgM level in male Hodgkin's patients was normal, and that it was significantly increased in females. No patient had an IgM level below the lower limit of normal. This is in disagreement with the findings of Goldman.

Comparing immunoglobulin levels between different groups of persons is difficult. Serum immunoglobulin concentrations show a great range between individual persons [21–23], but are rather constant in the course of time for any individual person [24–26]. All immunoglobulins show a rise in the second decade to reach at that time adult levels [27–29]. In adults the serum IgG and IgA levels gradually increase slightly according to the study of Kalff [21], but is constant according to Buckley and Dorsey [30]. Kalff found no change in IgM level in elderly persons, Buckley and coworker observed, however, a fall in advancing age. The patients, in this study, were mostly young adults, and the ages were comparable with

Table 4. *Serum immunoglobulin levels in controls and in patients with Hodgkin's disease according to histologic type*

No. of patients M	No. of patients F	Histologic type	IgG mg/ml ± s.d.	IgA mg/ml ± s.d.	IgM mg/ml ± s.d. M	IgM mg/ml ± s.d. F
4	2	L.P.	11·6 ± 3·0	2·83 ± 1·05	2·29 ± 2·14	1·90
6	12	N.S.	14·8 ± 4·0	3·32 ± 1·20	1·64 ± 0·80	1·89 ± 0·71
8	11	M.C.	14·2 ± 4·0	3·40 ± 1·66	1·20 ± 0·38	2·23 ± 0·91
6	2	L.D.	13·5 ± 3·2	3·59 ± 1·34	1·72 ± 0·81	1·89
		controls	11·1 ± 2·6 (N = 102)	2·02 ± 0·69 (N = 98)	1·24 ± 0·44 (N = 40)	1·47 ± 0·36 (N = 55)

those of the controls. An alteration due to age is therefore unlikely.

According to literature [26, 31–33] there exist no differences in IgA and IgG levels between males and females. IgM levels, however, are higher in females than in males [21, 26, 31–33]. We found also in the control group a significant higher IgM level in females than in males, and no difference in IgA and IgG levels. Goldman [17] does not mention the sex neither in Hodgkin's patients, nor in the controls. The decrease in IgM level which he found could possibly be explained by a special male/female ratio.

The tendency of rise of all the three immunoglobulins could be caused by a generalized hyperactivity of the reticulo–endothelial-system as found in certain chronic infections.

The increase in IgA level is the most pronounced from the three immunoglobulins and points possibly to another explanation. The increase of IgA is not necessarily concomitant with a rise of IgG and IgM. In the 22 patients with an increased IgA, 9 had IgG levels and 8 IgM levels above normal. Raised IgA levels are found in other kinds of neoplastic diseases: breast cancer [34], hepatocellular carcinoma [35], cancers of the lung, mouth and gut [32]. It is suggested by Hughes [32] that this raised IgA level in patients with cancers associated with secreting mucous membranes is due to infection of the mucosa.

The decreased IgA level found by Goldman [17] could be explained by the fact that the patients studied were almost all treated patients.

No significant differences in immunoglobulin levels between the four histological forms of Hodgkin's disease were found.

Acknowledgements—The authors gratefully acknowledge Mrs. M. Holtman and Miss M. Mantingh for their technical assistance. They are indebted to Mr. Ph. van Elteren, M.Sc., for his statistical analysis.

REFERENCES

1. I. N. Dubin, The poverty of the immunological mechanism in patients with Hodgkin's disease. *Ann. int. Med.* **27,** 898 (1947).
2. W. W. Schier, A. Roth, G. Ostroff and M. H. Schrift, Hodgkin's disease and immunity. *Amer. J. Med.* **20,** 94 (1956).
3. A. C. Aisenberg, Immunologic status of Hodgkin's disease. *Cancer (Philad.)* **19,** 385 (1966).
4. M. W. Chase, Delayed-type hypersensitivity and the immunology of Hodgkin's disease, with a parallel examination of sarcoidosis. *Cancer Res.* **26,** 1097 (1966).
5. A. C. Aisenberg, Studies on delayed hypersensitivity in Hodgkin's disease. *J. clin. Invest.* **41,** 1964 (1962).
6. J. E. Sokal and N. Primikirios, The delayed skin test reponse in Hodgkin's disease and lymphosarcoma: effect of disease activity. *Cancer (Philad.)* **14,** 597 (1961).
7. W. D. Kelly, D. L. Lamb, R. L. Varco and R. A. Good, An investigation of Hodgkin's disease with respect to the problem of homotransplantation. *Ann. N.Y. Acad. Sci.* **87,** 187 (1960).
8. W. W. Kelly, R. A. Good and R. L. Varco, Anergy and skin homograft survival in Hodgkin's disease. *Surg. Gynec. Obstet.* **107,** 565 (1958).
9. T. Han and J. E. Sokal, Lymphocyte response to phytohemagglutinin in Hodgkin's disease. *Amer. J. Med.* **48,** 728 (1970).
10. E. M. Hersh and J. J. Oppenheim, Impaired *in vitro* lymphocyte transformation in Hodgkin's disease. *New Engl. J. Med.* **273,** 1006 (1965).
11. R. S. Schwartz, Are immunosuppressive anticancer drugs self-defeating? *Cancer Res.* **28,** 1452 (1968).
12. J. Braeman and T. J. Deeley, Radiotherapy and the immune response in cancer of the lung. *Brit. J. Radiology* **46,** 446 (1973).
13. J. H. Check, J. I. Damsker, L. W. Brady and E. A. O'Neill, Effect of radiation therapy on mumps-delayed type hypersensitivity reaction in lymphoma and carcinoma patients. *Cancer (Philad.)* **32,** 580 (1973).
14. R. C. Young, M. P. Corder, C. W. Berard and V. T. DeVita, Immune alterations in Hodgkin's disease: effect of delayed hypersensitivity and lymphocyte transformation on course and survival. *Arch. intern. Med.* **131,** 446 (1973).
15. J. R. Eltringham and H. S. Kaplan, Impaired delayed hypersensitivity responses in 154 patients with untreated Hodgkin's disease. *Nat. Cancer Inst. Monogr.* **36,** 107 (1973).

16. R. Levy and H. S. Kaplan, Impaired lymphocyte function in untreated Hodgkin's disease. *New Engl. J. Med.* **290,** 181 (1974).
17. J. M. Goldman and J. R. Hobbs, The immunoglobulins in Hodgkin's disease. *Immunology* **13,** 421 (1967).
18. R. J. Lukes, L. F. Craver, T. C. Hall, H. Rappaport and P. Rubin, Report of the nomenclature committee. *Cancer Res.* **26,** 1311 (1966).
19. P. P. Carbone, H. S. Kaplan, K. Musshoff, D. W. Smithers and M. Tubiana, Report of the committee on Hodgkin's disease staging classification. *Cancer Res.* **31,** 1860 (1971).
20. P. van Munster and T. Hoelen, An automated turbidimetric immunoprecipitation method for serum proteins. Submitted for publication.
21. M. W. Kalff, *Genetic and Environmental Influences on Serum Immunoglobulin Levels in Man.* Thesis (1969), Leiden.
22. H. H. Fudenberg, J. F. Heremans and E. C. Franklin, A hypothesis for the genetic control of synthesis of the gamma-globulins. *Ann. Inst. Pasteur* **104,** 155 (1963).
23. M. Allansmith, B. McClellan and M. Butterworth, The influence of heredity and environment on human immunoglobulin levels. *J. Immunol.* **102,** 1504 (1969).
24. M. Allansmith, B. McClellan and M. Butterworth, Stability of human immunoglobulin levels. *Proc. Soc. exp. Biol.* **125,** 404 (1967).
25. W. Mondorf and M. Kollmar, Das quantitieve Verhalten von IgG, IgA und IgM, sowie des Transferrins, Ceruloplasmins, α_1-Antitrypsins und des β_1A-Globulins bei Normalpersonen im Verlaufe von 5 Monate. *Klin. Wschr.* **47,** 1055 (1969).
26. J. H. S. M. Nadorp, *Immuunglobuline A en immuunglobuline A deficientie.* Thesis, Nijmegen (1975).
27. E. R. Stiehm and H. H. Fudenberg, Serum levels of immune globulins in health and disease. A survey. *Pediatrics* **37,** 715 (1966).
28. M. Allansmith, B. McClellan, M. Butterworth and J. R. Malloney, The development of immunoglobulin levels in man. *J. Pediat.* **72,** 276 (1968).
29. J. W. Stoop, B. J. M. Zegers, P. C. Sanders and R. E. Ballieux, Serum immunoglobulin levels in healthy children and adults. *Clin. exp. Immunol.* **4,** 101 (1969).
30. C. E. Buckley and F. C. Dorsey, Serum immunoglobulin levels throughout the life-span of healthy man. *Ann. int. Med.* **75,** 673 (1971).
31. M. Butterworth, B. McClellan and M. Allansmith, Influence of sex on immunoglobulin levels. *Nature (Lond.)* **214,** 1224 (1967).
32. N. R. Hughes, Serum concentrations of IgG, IgA and IgM immunoglobulins in patients with carcinoma, melanoma and sarcoma. *J. nat. Cancer Inst.* **46,** 1015 (1971).
33. S. G. Johansson, C. F. Hogman and J. Killander, Quantitative immunoglobulin determination. *Acta path. microbiol. scand.* **74,** 519 (1968).
34. M. M. Roberts, E. M. Bathgate and A. Stevenson, Serum immunoglobulin levels in patients with breast cancer. *Cancer (Philad.)* **36,** 271 (1975).
35. K. Akdamer, A. C. Epps, L. T. Maumus and R. D. Sparks, Immunoglobulin changes in liver disease. *Ann. N.Y. Acad. Sci.* **197,** 101 (1972).

Europ. J. Cancer Vol. 12, pp. 689–694. Pergamon Press 1976. Printed in Great Britain

Inhibition of Mitogen-Induced Lymphocyte Blastogenesis by Serum from Tumor-Bearing Animals: Changes with Tumor Growth*

D. E. SCHUMM†, D. F. BILLMIRE† and H. P. MORRIS‡

†*Department of Physiological Chemistry, Ohio State University College of Medicine, Columbus Ohio* 43210 *and* ‡*Department of Biochemistry, Howard University School of Medicine, Washington, D.C.* 20001, *U.S.A.*

Abstract—*Serum from rats bearing hepatoma* 5123*D was found to be inhibitory to the mitogen-induced blastogenic response of normal rat peripheral blood lymphocytes, and this inhibition increased with increasing tumor size. Tumor-bearer serum inhibited the phytohemagglutinin and concanavalin A responses at an earlier stage of tumor growth than the response to dextran sulfate. The concentration of phytohemagglutinin eliciting maximal incorporation of tritiated thymidine was* 5 *μl/ml for lymphocytes cultured in normal serum and* 15 *μl/ml for tumor-bearer serum. For concanavalin A the best response was at* 2·5 *μg/ml in both sera. For dextran sulfate* 40 *μg/ml elicited the maximal incorporation in normal serum while* 20 *μg/ml was found for tumor-bearer serum. In all cases, the maximal response at all mitogen levels was significantly less when tumor-bearer as opposed to normal serum was used. The inhibitory component(s) in the tumor-bearer serum was heat stable, nondializable and could be removed from the serum by exposure to normal lymphocytes.*

INTRODUCTION

THERE IS accumulating evidence showing a correlation between neoplasia and alterations in cell-mediated immune responses when measured by a variety of tests both *in vivo* and *in vitro*. In human patients treatment with immunosuppressive drugs is associated with an increased risk of cancer [1]. Many, but by no means all, cancer patients have been shown to have a decreased lymphocyte response to mitogens, such as phytohemagglutinin (PHA) and concanavalin A (ConA), especially when cells are cultured in autologous serum [2, 3]. In many of these cases, it is not possible to determine which was the precipitating event, an immunological depression or neoplastic transformation. Thus studies were undertaken using intact rats injected with hepatoma 5123D in order to follow the effect of tumor growth on the immune response.

Earlier work from this laboratory [4] has shown that serum from rats or mice bearing a variety of transplantable tumors inhibited both early biochemical events occurring during mitogen-induced blastogenesis as well as the later incorporation of tritiated thymidine into DNA. Since serum for these experiments was always taken from animals with large well-developed tumors and PHA was the only mitogen used, it was of interest to determine both the time after tumor cell inoculation at which this serum inhibition first occurred, and whether the response to mitogens other than PHA was similarly affected. Furthermore, the characteristics of the serum-inhibitory component when compared to other known inhibitors of cell division needed to be determined.

Accepted 23 March 1976.

*This work was supported by a grant from the American Cancer Society and by grant CA 12 411 from the NCI, Bethesda Md.

MATERIAL AND METHODS

(*a*) *Lymphocyte preparation*

Heparinized peripheral blood obtained by

venus puncture of male rats of the Buffalo strain was allowed to settle for 1 hr at 37°C. The lymphocyte-rich upper layer was removed and separated from contaminating red blood cells and platelets by differential centrifugation. The cells were then washed in 0·9% saline and resuspended at 1–2 $\times 10^6$ cells/ml in RPMI 1640 media (Swartz-Mann, Orangeburg, N.Y.) containing glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and 10% serum, either normal or tumor-bearing rat serum as indicated. All operations were performed at room temperature unless otherwise indicated. Nucleated cells were counted under a light microscope using 3% acetic acid as the diluting fluid. All attempts to prepare lymphocytes by purification on a Ficoll–Hypaque density gradient resulted in cells with a significantly decreased response to mitogen stimulation.

thymidine (^{3}HTdR) (New England Nuclear, Boston, Mass.) was added, and incubation continued for an additional 24 hr. Labelling was terminated by the addition of 3 ml ice cold 0·9% saline. Cells were poured on to wetted glass fiber filters (Whatman GF/C), and washed with 10 ml 0·9% saline, 10 ml ice cold 5% trichloroacetic acid, and 5 ml 95% ethanol. Filters were dried and counted in liquid scintillant. Cell cultures were done in triplicate and each experiment performed at least twice.

RESULTS

Previous experiments [4] have shown that serum from animals bearing large transplantable tumors inhibited the blastogenic response of normal rat lymphocytes to the mitogen phytohemagglutinin (PHA). In the experiments presented in Table 1, lymphocytes from normal rats were cultured with serum from rats 0, 2, 3, and 4 weeks after injection of 5123D tumor cells. The mitogens used were PHA and ConA, both of which are recognized as mitogens for T or thymus-dependent cells [5], and dextran sulfate, a mitogen specific for B or thymus-independent cells [6]. Prior to tumor cell injection, all three mitogens cause a large increase in DNA synthesis in the presence of 10% serum. However, serum taken from rats 13 days after tumor cell inoculation causes the response to PHA to be reduced to 13% of normal, ConA to 50%, while dextran sulfate remains at 97%. At this time, the tumors were approximately 0·5 cm in diameter and each tumor weighed 0·4 g. Eighteen days after tumor cell injection the tumors were 1·0 cm dia and weighed 1·6 g. Culture of normal lymphocytes in serum from these rats caused a complete inhibition in the response to

Table 1. *Mitogen response of normal rat lymphocytes cultured in serum from tumor-bearing rats*

Mitogen (μg/ml)	Counts/min ^{3}HTdR incorporated/10^6 nucleated cells Serum from rats with tumors for 0 days	13 days	18 days	26 days
None	2007	2271	1957	2172
PHA-P (5 μg/ml)	95,311	12,082	2685	2167
ConA (5 μg/ml)	124,869	62,427	8193	2078
Dextran Sulfate (40 μg/ml)	29,796	28,573	23,762	8324

Results are the average of triplicate cultures for one experiment. Replicate cultures within an experiment generally agreed within 10%, duplicate experiments agreed within 15%.

(*b*) *Serum preparation*

For sera, blood was drawn from either normal rats or from rats bearing the Morris Hepatoma 5123D. Tumor cells were prepared by removing a large tumor, dissecting the cells free of surrounding tissue, taking them up in a sterile syringe fitted with an 18 gauge needle and injecting 0·5 ml bilaterally into the thigh muscle. Blood was drawn for serum preparation at various times after tumor cells inoculation, allowed to clot for 30 min at room temperature and 2 hr at 4°C. The resulting serum was separated from the clot by centrifugation. Sera, thus prepared, was frozen until use.

(*c*) *DNA synthesis assay*

In the standard assay, 1 ml cultures were incubated with no mitogen, PHA-P (Difco Laboratores, Detroit, Mich.), ConA or dextran sulfate (Sigma Chemical Co., St. Louis, Mo.) for 48 hr at 37°C after which 1 μCi of tritiated

PHA. Response to ConA was only 6% of the control, while response to dextran sulfate remained nearly normal at 81% of the control value. By 26 days after tumor injection, the tumors were 2 cm or larger in diameter and weighed 4–7 g. Serum taken at this stage caused almost complete inhibition of ^{3}HTdR incorporation in response to T-cell mitogens, the response to B-cell mitogens being reduced to 25% of control.

Since there is no apparent mitogenic response when the culture conditions used are those which were found to be optimal for rat lymphocytes cultured in normal rat serum, it was of interest to determine the optimal conditions for culture in the presence of serum from an animal bearing a large transplantable tumor. In these experiments, normal lymphocytes were cultured in 10% serum taken from a rat 26 days after tumor inoculation, and the ^{3}HTdR incorporation determined at various mitogen doses. Fig. 1 shows the curve for PHA.

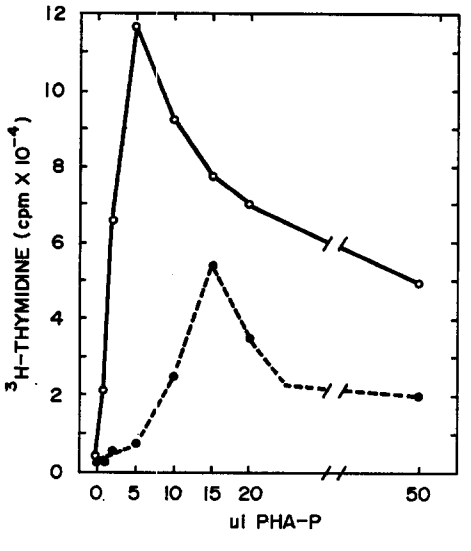


Fig. 1. Dose–response curve of ^{3}HTdR incorporation into normal rat lymphocytes in response to various concentrations of PHA-P. ○——● *culture in* 10% *normal rat serum,* ●– – –● *culture in* 10% 26 *day tumor-bearer serum.*

In 10% serum from a non-tumor bearing rat, the optimal response occurs at 5 μl of PHA-P/ml of culture. When 10% serum from a rat bearing a 26 day tumor is used, the optimum response occurs at 15 μl/ml. At all mitogen concentrations tested, response in the presence of tumor-bearer serum is significantly less than in the presence of normal serum. When ConA is the mitogen being tested, the optimal concentration is 2·5 μg/ml for culture in either normal or tumor-bearing serum (Fig. 2). The ^{3}HTdR incorporation at this mitogen dose is

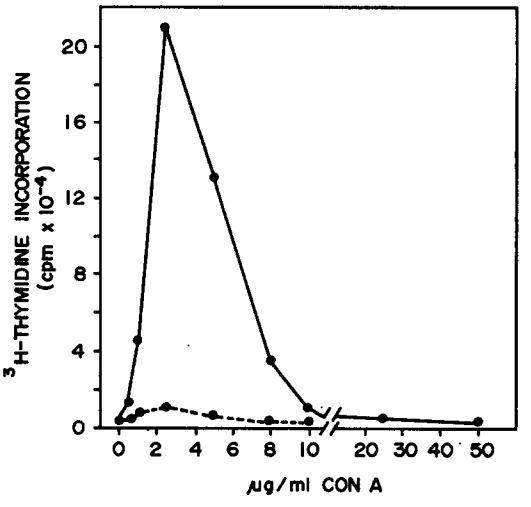


Fig. 2. Dose–response curve of ^{3}HTdR incorporation into normal rat lymphocytes in response to various concentrations of ConA. ●——● *culture in* 10% *normal rat serum,* ●– –● *culture in* 10% 26 *day tumor-bearer serum.*

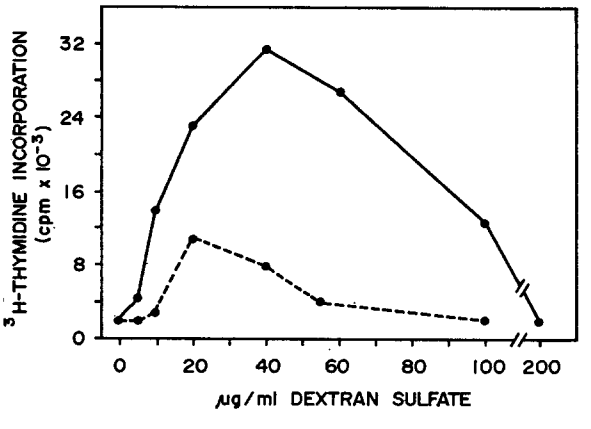


Fig. 3. Dose–response curve of ^{3}HTdR incorporation into normal rat lymphocytes in response to various concentrations of dextran sulfate. ●——● *culture in* 10% *normal rat serum,* ●– –● *culture in* 10% 26 *day tumor-bearer serum.*

only 3% of the control when tumor-bearer as opposed to normal serum is used. Fig. 3 gives the dose response curve for dextran sulfate. The maximal ^{3}HTdR incorporation occurs at 40 μg/ml when culture is performed in the presence of 10% normal serum and 20 μg/ml when tumor-bearer serum is used. At the latter dose, response in the presence of tumor serum is 45% of that found with normal serum, while at 40 μg/ml, it is reduced to 25%. For all mitogens and both sera tested, reducing the serum concentration to 5% or increasing it to 20% resulted in an overall decrease in ^{3}HTdR incorporation without altering the optimal mitogen dose (results not shown).

Culture of normal lymphocytes in 10% serum from an animal bearing a tumor for

26 days virtually eliminates the blastogenic response to PHA when both mitogen and serum are present together in the entire culture period. However, shorter contact times between lymphocytes and tumor serum, in the absence of mitogen, may give a time course of inhibition as well as reveal some insight into the mechanism of this inhibition. In the experiments summarized in Table 2, normal lymphocytes were exposed to 10% serum from a tumor-bearing rat for 0, 1, 2, 4 and 24 hr. They were then removed from the culture media by centrifugation, washed with saline and resuspended in fresh media containing 10% normal serum with either no mitogen, 5 μl/mg of PHA-P or 5 μg/ml of ConA. As short a contact as 2 hr with tumor-bearer serum causes a 90% inhibition of the ability of normal lymphocytes to respond to either PHA or ConA. A similar treatment with normal serum does not impair the ability of lymphocytes to incorporate ^{3}HTdR into DNA when exposed to mitogens (results not shown).

Table 2. Time course of inhibition of normal lymphocytes upon exposure to tumor-bearer serum

Hours with tumor-bearer serum	Mitogen	Counts/min per 10^6 cells
0	none	2025
	PHA	93,532
	ConA	112,837
1	none	2179
	PHA	46,743
	ConA	55,268
2	none	1890
	PHA	4919
	ConA	5782
4	none	2167
	PHA	2577
	ConA	2423
24	none	2101
	PHA	2405
	ConA	2437

Error is as given in Table 1.

Since normal lymphocytes can be inhibited by brief contact with tumor-bearer serum, can the inhibitory component(s) then be absorbed by these cells thus restoring the ability of tumor-bearer serum to support mitogen induced ^{3}HTdR incorporation? Table 3 gives the result of such an absorption experiment. Here normal lymphocytes were exposed for various times to media containing 10% tumor-bearer serum without the addition of mitogens. After the indicated time, cells were removed by centrifugation and fresh cells were added. PHA was placed in half of the cultures and a DNA synthesis assay performed in the usual manner. After 1 hr of absorption, much of the inhibitory factor appears to be removed since mitogen-induced blastogenesis now occurs in the presence of treated-serum. The maximum amount of ^{3}HTdR incorporation occurs after only a two hour absorption and remains constant up to 24 hr. Thus the kinetics of removal of the inhibitory factor(s) are the same as those for the inactivation of normal lymphocytes by the serum. The maximum amount of ^{3}HTdR incorporation is only about 30% of that found with an equivalent concentration of normal serum. This may be due to the fact that there were not sufficient lymphocytes present during the absorption period to remove all of the inhibitory component. Similar absorption of media containing normal serum does not alter its characteristics toward mitogen induced ^{3}HTdR incorporation.

Table 3. Absorption of inhibitory component by normal lymphocytes

Hours of absorption	Mitogen	Counts/min per 10^6 cells
0	none	2512
	PHA	3916
1	none	2852
	PHA	18,783
2	none	2897
	PHA	30,742
4	none	3008
	PHA	31,619
24	none	2935
	PHA	32,070

Error is as given in Table 1.

Several attempts were made to determine other properties of the inhibitory components(s) of the tumor-bearer serum. It is not removed by dialysis for up to 24 hr against 0·9% saline nor is it inactivated by heating for 30 min at 56°C. Neither of these procedures produced any observable changes in normal serum.

DISCUSSION

The experiments presented here indicate that the presence of a tumor in normal rats, untreated with immunosuppressive drugs, causes the production of a factor or factors which inhibit the mitogen induced blastogenesis of normal rat lymphocytes. The factor which affects T-cell response, that induced by PHA or ConA, appears to occur earlier in

tumor development or is effective at a lower concentration than the factor which affects B cell or dextran sulfate responsive lymphocytes. Tumors must be quite large before the serum is inhibitory for dextran sulfate induced ^{3}HTdR incorporation into normal rat lymphocytes, while serum from rats with very small tumors is significantly inhibitory to the PHA response. This work is in substantial agreement with earlier work from this laboratory [4] and with that of Whitney and Levy [7] who showed that serum from mice bearing several chemically induced tumors was inhibitory to the mitogen response of spleen cells from normal animals. Although no time course of the appearance of the inhibitory component was made in this study, it did show that the phenomena of tumor bearer serum inhibition of mitogen induced blastogenesis is relatively common at least for chemically induced tumors, such as the one used here.

Many serum factors have been reported to cause an inhibition in PHA-induced lymphocyte transformation; among these are placental steroids [8], chalones [9], antigen–antibody complexes [10], pregnancy zone proteins [11], α-globulins [12] and α-fetoprotein [13]. Since the factor or factors present in the three week tumor-bearer serum is not dialyzable and is stable to heating at 56°C for 30 min, it is unlikely to be either a steroid or complement. Also, since the tumor is moderately well-differentiated and was induced and carried in male rats, it is unlikely to be one of the pregnancy zone proteins. The irreversibility of its binding to normal lymphocytes rules out the inhibitory factor being either a chalone or an antigen–antibody complex since the effects of both of these are generally found to be reversible. Likewise, the α-globulin found in mouse serum [12] was also reversible in its inhibitory effect on the immune response of mouse spleen cells to sheep erythrocytes. Because the tissue and species specificity was not investigated in this study, it is not known whether the inhibitor is similar to the human inhibitory factor studied by Hersh and Drewinko [14]. The characteristics of α-fetoprotein (AFP) appear to be consistent with those of the tumor-bearing rat serum inhibitor although further purification of the rat factor will be necessary before identity can be proved. AFP is often found in patients with primary liver cancer, and the tumor being carried in these rats is a hepatoma. A preparation containing AFP inhibits the *in vitro* primary antibody response of mouse spleen cells to sheep erythrocytes with a time course similar to that found with the rat factor—the first noticeable effect being observed at 2 hr of incubation of spleen cells in media containing the factor; complete inhibition occurs after 8 hr of exposure. Furthermore, the inhibitor was not removed by washing the cells [15]. Also, similar to results obtained here, both B cell and T cell responses to mitogens are inhibited by purified AFP [13].

The binding of the inhibitory factor to normal lymphocytes appears to be very tight, if not irreversible, since transfer of the cells to media containing normal serum plus mitogen does not result in stimulation. This experiment also demonstrated that the factor is not binding the mitogen thus preventing its interaction with the cells, since tumor serum and mitogen were never present at the same time, but added sequentially. Also, the factor and the mitogen do not appear to be competing for the same binding sites on the lymphocytes plasma membrane. This was shown by the reduction in ^{3}HTdR incorporation when the serum concentration was reduced to 5% or raised to 20% with no change observed in the shape of the curve or the optimal mitogen dose. If competition were occurring one would expect to find a decrease in the optimal dose at low serum concentrations and an increase at higher concentrations. However, experimentally this is not observed.

The absorbability of the inhibitory factor from tumor-bearer serum by normal lymphocytes follows the same kinetics as the reverse experiment, the inactivation of lymphocytes by tumor serum. The fact that the component can be removed suggests that some of the physical properties of the factor can be ascertained by the difference between tumor serum absorbed and nonabsorbed by normal lymphocytes as compared to normal serum treated in the same manner. Experiments are under way exploring this possibility.

The alteration in the shape of the dose-response curves when different mitogens and different sera are used leads to speculation that these changes may be due to the existence of various subpopulations of lymphocytes responding optimally to different mitogen concentrations. If some of these populations were preferentially inhibited by the serum factor the alterations in the curves could be explained. The optimal dose for response to ConA is the same in both normal and tumor-bearer serum, although the extent of ^{3}HTdR incorporation is markedly reduced in the latter case. The range of concentrations of Con A eliciting significant responses is quite narrow suggesting that only a single cell population may be react-

ing. On the other hand, the range of concentrations of PHA producing a ^{3}HTdR incorporation response is much broader, suggesting that more than one population of cells may be responding—one at a dose of 5 μl/ml, and at least one at a higher dose. When tumor, as opposed to normal, serum is used, the population of cells responding to the low dose of PHA is inhibited while those responding to higher doses, 15 μl/ml, are relatively active, producing ^{3}HTdR incorporation 2/3 of that found for normal serum at this PHA dose. A similar situation is found for dextran sulfate, in which a wide optimum is observed with normal serum with a peak at 40 μg/ml. In the presence of tumor serum, the populations of cells responding at high dextran sulfate concentrations are inhibited while those responding to 20 μg/ml are still responsive. In light of these results, it would be of interest to examine separated populations of lymphocytes as well as a variety of other mitogens, such as pokeweed and *E. coli* lipopolysaccharide, for the dose response curves in the presence of normal and tumor-bearer serum.

The mitogen induced responses of the lymphocytes from these tumor-bearing animals has been investigated and will be presented elsewhere (D. E. Schumm and D. F. Billmire, manuscript in preparation).

Acknowledgment—The authors wish to thank Joseph Baggott for technical assistance and Dr. Thomas Webb for many helpful discussions.

REFERENCES

1. R. A. Good, Relations between immunity and malignancy. *Proc. nat. Acad. Sci. (Wash.)* **69,** 1026 (1972).
2. W. J. Catalona, W. F. Sample and P. B. Chretien, Lymphocyte reactivity in cancer patients: correlation with tumor histology and clinical stage. *Cancer (Philad.)* **31,** 65 (1973).
3. N. Suiciu-Foca, J. Buda, J. McManus, T. Thiem and K. Reemstsma, Impaired responsiveness of lymphocytes and serum-inhibitory factors in patients with cancer. *Cancer Res.* **33,** 2373 (1973).
4. D. E. Schumm, H. P. Morris and T. E. Webb, Early biochemical changes in phytohemaglutinin-stimulated peripheral blood lymphocytes from normal and tumor-bearing animals. *Europ. J. Cancer* **10,** 107 (1974).
5. R. A. Good, Structure-function relations in the lymphoid system. *Clin. Immunol.* **1,** 1 (1972).
6. R. Dörries, A. Schimpl and E. Wecker, Action of dextran sulfate as a direct and general B cell mitogen. *Europ. J. Immunol.* **4,** 230 (1974).
7. R. B. Whitney and J. G. Levy, Effects of sera from tumor-bearing mice on mitogen and allogeneic cell stimulation of normal lymphoid cells. *J. nat. Cancer Inst.* **54,** 733 (1975).
8. R. Rembrisa, W. Ptak and M. Bubak, The immunosuppressive effects of mouse placental steroids. *Experientia* **30,** 82 (1974).
9. N. Kiger, I. Florentin, E. Goreia-Giralt and G. Mathe, Lymphocyte inhibitory factors (chalones) extracted from lymphoid organs. Extraction, partial purification and immunosuppressive properties. *Transplantation Proc.* **5,** 531 (1972).
10. R. A. Robins and R. W. Baldwin, Tumor-specific antibody neutralization of factors in rat hepatoma-bearer serum which abrogate lymph-node-cell cytotoxicity. *Int. J. Cancer* **14,** 589 (1974).
11. B. von Schoultz, T. Stigbrand and A. Tärnvik, Inhibition of PHA-induced lymphocyte stimulation by the pregnancy zone protein. *FEBS Lett.* **38,** 23 (1973).
12. B. Veit and J. G. Michael, Characterization of an immunosuppressive factor present in mouse serum. *J. Immunol.* **111,** 341 (1973).
13. R. A. Murgita and T. B. Tomasi, Jr., Suppression of the immune response by α-fetoprotein. II. The effect of mouse α-fetoprotein on mixed lymphocyte reactivity and mitogen-induced lymphocyte transformation. *J. exp. Med.* **141,** 440 (1975).
14. E. M. Hersh and B. Drewinko, Specific inhibition of lymphocyte blastogenic responses to mitogens by a factor produced by cultured human malignant lymphoma cells. *Cancer Res.* **34,** 215 (1974).
15. R. A. Murgita and T. B. Tomasi, Jr., Suppression of the immune response by α-fetoprotein. I. The effect of mouse α-fetoprotein the primary and secondary antibody response. *J. exp. Med.* **141,** 269 (1975).

Europ. J. Cancer Vol. 12, pp. 695–700. Pergamon Press 1976. Printed in Great Britain

Isoelectric Focusing of Estradiol Receptor Protein from Human Mammary Carcinoma—a Comparison to Sucrose Gradient Analysis*

ÖRJAN WRANGE†, BO NORDENSKJÖLD‡, CLAES SILFVERSWÄRD§, PER OLA GRANBERG and JAN-ÅKE GUSTAFSSON†

†*Department of Chemistry, Karolinska Institutet,* ‡*Radiumhemmet, Karolinska Sjukhuset, S-*104 01 *Stockholm* 60, *Sweden,* §*Department of Pathology, Södersjukhuset, S-*100 64 *Stockholm and Department of Surgery, Karolinska Sjukhuset, S-*104 01 *Stockholm* 60, *Sweden*

Abstract—*Sucrose density gradient centrifugation and isoelectric focusing were compared as methods for measurement of estrogen receptor protein in human mammary carcinoma. Twenty-six carcinomas were investigated. It is concluded that the micromethod used for isoelectric focusing may be used as a routine tool for estimation of estradiol receptor levels in human mammary carcinoma. With sucrose gradient analysis, control runs are required in which the specific binding sites are displayed by excess of unlabeled ligand. These type of controls were found to be unnecessary with isoelectric focusing. We therefore suggest that isoelectric focusing may be a more specific technique for the detection of estrogen receptor in human mammary carcinoma. Final evaluation of which of these methods is preferable in predictive tests for therapy of breast cancer, however, awaits correlation between measured receptor levels and response to endocrine therapy.*

THE OCCURRENCE of estradiol receptor protein in human mammary carcinoma has been shown to correlate with response to endocrine therapy [1]. However, all "receptor-positive" tumors do not respond to endocrine therapy and the frequency of "false-positive" tumors has been estimated to be approximately 40% of the total number of receptor-positive tumors.

Estradiol receptor in tumor tissue is usually measured by sucrose density gradient centrifugation analysis [2] or by quantitation of saturable high-affinity estradiol binding sites after removal of unbound estradiol with dextran-coated charcoal treatment [2, 3]. The data are often plotted according to Scatchard to obtain the quantitative and kinetic characteristics of the binding [4]. Part of the difficulties with false positive tumors referred to above may be related to insufficient specificity of the methods currently used to measure estradiol receptor protein. Since a few years we have used a micromodification of electrofocusing, originally described by Katsumata and Goldman [5], and have found it to be a versatile and specific method for analysis of steroid receptor proteins. We therefore compared sucrose gradient centrifugation and electrofocusing analysis of estradiol receptor protein from twenty-six mammary carcinomas. We conclude that isoelectric focusing has potential value as a method for screening of receptor proteins in breast tumors.

Accepted 19 March 1976.

*This work was supported by grants from Cancerföreningen i Stockholm, from Lotten Bohmans fond (to J.-Å. G.) and from the Swedish Cancer Society (to B. N.).

MATERIAL AND METHODS

Radioactive steroid

[2,4,6,7-^{3}H] estradiol (specific radioactivity, 80 Ci per mmole) was purchased from Radiochemical Centre, Amersham, England.

Chemicals

Unlabeled estradiol and Tris were bought from Sigma Chemical Co., St. Louis, Mo., USA.

Tissue material

Twenty-six primary or locally recurrent mammary carcinomas from 26 patients were cut out immediately after operation, and parallel pieces were fixed for histology or transferred to ice-cold, isotonic Tris–phosphate buffer (0·14 M NaCl–5 mM KCl–0·6 mM Na_2HPO_4–0·7 mM $CaCl_2$–0·5 mM $MgCl_2$–25 mM Tris–HCl, pH 7·5) for receptor analyses. In some cases stated below tumor tissue was frozen at −70 C in dry state.

Our technique for staining and handling of primary tumors was described elsewhere [6]. The tumors were classified as ductal, lobular or colloid as described by McDivitt *et al.* [7]. They were then grouped into low, medium and high degree of differentiation and parameters were considered that have previously been analyzed in a review of five hundred breast tumors [6].

Labeling of receptor with radioactivity

Tumor tissue, either quickly thawed after storage in the freezer or kept at 0 C for 1–2 hr after operation, was minced and divided into two equal portions. Each portion was suspended in 1 ml of ET-buffer (0·4 M Tris–HCl, pH 7·4–1·5 mM Na_2 EDTA) containing 2×10^{-9} M [^{3}H]estradiol or 2×10^{-9} M [^{3}H]estradiol plus 10^{-6} M unlabeled estradiol, respectively. After incubation for 30 min at 0 C the tissue was homogenized using a polytrone (Kinematica, Luzern, Switzerland).

After homogenization, the tissue was incubated a second time for 45 min at 0 C and then centrifuged at 20,000 × *g* for 10 min. The supernatant was shaken with 0·1 ml of a suspension of 4 mg dextran-coated charcoal (dextran:charcoal, 1:10 (w/w)) to remove most of the unbound steroid [3] and centrifuged at 800 × *g* for 10 min. Fifty microliters of each supernatant was analyzed for radioactivity and the rest reserved for sucrose gradient centrifugation and isoelectric focusing. Sediments after centrifugation of tumor homogenate contained the cell nuclei and were taken for measurement of DNA according to Burton [8]. These DNA measurements were used for the correlation of estradiol binding to the amount of DNA in the tissue (see below).

Sucrose gradient centrifugation

An aliquot, 0·2 ml, of the labeled tumor supernatant was layered on top of a 5 ml linear 10 to 30% (w/v) sucrose density gradient in ET-buffer. The tubes were centrifuged at 50,000 rev/min for 16 hr at +2 C in an SW 56 Beckman–Spinco rotor. After centrifugation the bottom of the tube was punctured and 0·2 ml fractions were collected and measured for radioactivity. Yeast phenylalanin transfer-RNA was used as sedimentation marker and assayed for absorbance at 260 nm.

Isoelectric focusing

Isoelectric focusing was performed essentially as described by Katsumata and Goldman [5]. An aliquot of the labeled tumor supernatant, with a protein amount of less than 2 O.D. units (measured as the absorbance at 280 nm minus the absorbance at 310 nm), was diluted to 2·6 ml with a solution containing 10% (w/v) glycerol and 1 mM Na_2 EDTA and with a pH adjusted to 7·0 with 1 M KOH. The resulting mixture was distributed in a discontinuous 60–10% glycerol gradient with a total volume of 5·2 ml, containing 1% (v/v) Ampholine®, pH range 3·5–10 (LKB-Produkter, Stockholm, Sweden) that was layered in a 10 ml column and focused at increasing voltage to 1200 V during 12–15 hr. The gradient was fractionated in 3-drop fractions into plastic counting vials and pH was measured at ambient temperature using a surface electrode (type 403-30, Ingold, Zürich, Switzerland). Contaminating hemoglobin was used as internal pH standard and had a reproducible pI of 7·6. Fractions where precipitates were collected were marked in the electrofocusing diagram. Radioactivity was measured using 5 ml of Instagel® (Packard Instrument Co., Inc., Warrenville, Downers Grove, Ill., USA) as scintillator liquid.

Quantitation of high-affinity, low-capacity ("specific") binding sites for [^{3}H]estradiol

Quantitation of specific binding of [^{3}H] estradiol (i.e. [^{3}H] estradiol bound to receptor) with sucrose gradient centrifugation analysis was performed by calculating the amount of [^{3}H]estradiol displaced from the 4S and 8S complexes in incubations with 500-fold excess of unlabeled estradiol. A value of more than 0·05 fmoles of estradiol bound per μg of DNA was considered positive.

With isoelectric focusing analysis the [^{3}H] estradiol-protein complex focusing around

pH 6·6 was considered specific since the radioactivity in this region was always completely displaced in incubations with excess of unlabeled estradiol, indicating the absence of nonspecific, nonsaturable estradiol-binding proteins with a pI of about 6·6. Consequently, with electro-focusing, the amount of specifically bound radioactivity after incubation with [^{3}H]estradiol may be calculated using the base line in the radioactivity diagram as background.

RESULTS AND DISCUSSION

Table 1 compares sucrose gradient centrifugation with isoelectric focusing analysis of estrogen receptor protein from twenty-six mammary carcinomas.

Nine of the 14 sucrose-gradient-analysis-positive ("SG-positive") tumors demonstrated a single 4S complex whereas five of the SG-positive tumors gave rise to both a 4S and an 8S complex (Fig. 1). The 8S complex was always found to be saturable with excess of unlabeled estradiol whereas the 4S complex contained variable amounts of nonsaturable binding sites. These findings are in agreement with the results reported by e.g., McGuire [2].

Isoelectric focusing analysis showed that 12 of the 36 tumor specimens contained estradiol receptor protein focusing at around pH 6·6 ± 0·3 ("IF-positive" specimens). The radioactive peak sometimes appeared as a double peak

Table 1. *Sucrose gradient centrifugation and isoelectric focusing analysis of estradiol receptor from twenty-six mammary carcinomas, classified with respect to histological type and degree of differentiation.* Explanations: primary, the primary tumor was analyzed; metastasis, a locally recurrent carcinoma was analyzed; frozen, the tumor tissue was stored at −70C prior to analysis (in three cases, patients Nos. 3, 16 and 17, the tumor was analyzed both in fresh state and after storage at −70C)

Patient no.	Material analyzed	Sucrose gradient (fmoles of receptor per μg of DNA) 4S	8S	Total	Isoelectric focusing (fmoles of receptor per μg of DNA)	Pathology Type of tumor	Degree of differentiation
1	primary	0·56	0·69	1·25	0·25	ductal	low
2	primary	0·24	0·72	0·96	0·24	ductal	medium
3	primary	0·27	0·38	0·65	0·23	lobular	medium
3	primary (frozen)	0·13	0·53	0·66	0·02	lobular	medium
4	primary	0·38	0·20	0·58	0·24	ductal	low
5	primary	0·56	0	0·56	0·12	ductal	medium
6	primary (frozen)	0·56	0	0·56	0·20	ductal	medium
7	metastasis	0·25	0	0·25	0·13	ductal	low
8	metastasis	0·24	0	0·24	0·03	ductal	low
9	primary	0·17	0	0·17	0	ductal	medium
10	primary	0·12	0	0·12	0·10	ductal	low
11	metastasis	0·10	0	0·10	0·06	lobular	low
12	primary	0·09	0	0·09	0	lobular	low
13	primary	0·06	0·02	0·08	0·01	ductal	low
14	metastasis	0·06	0	0·06	0	ductal	low
15	metastasis	0·05	0	0·05	0	ductal	low
16	primary	0	0	0	0·03	colloid	medium
16	primary (frozen)	0·04	0·01	0·05	0·03	colloid	medium
17	primary	0	0·02	0·02	0	not classified	not classified
17	primary (frozen)	0	0·02	0·02	0	not classified	not classified
18	metastasis	0	0	0	0	ductal	high
19	metastasis	0	0	0	0	ductal	medium
20	primary (frozen)	0	0	0	0	ductal	low
21	metastasis	0	0	0	0	ductal	low
22	primary	0	0	0	0	ductal	low
23	primary	0	0	0	0	ductal	low
24	metastasis	0	0	0	0	un-differentiated	low
25	primary	0	0	0	0	lobular	low
26	primary (frozen)	0	0	0	0	not analyzed	not analyzed

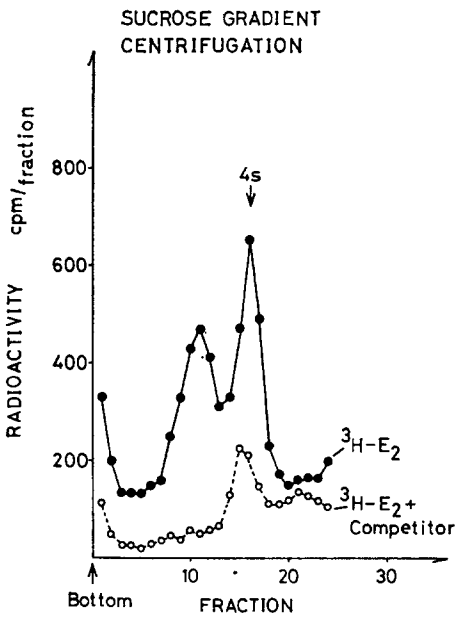


Fig. 1. Sucrose gradient centrifugation. *Fresh tissue from patient No.* 3 (*see Table* 1) *was analyzed as described in Material and Methods. Explanations:* ●——●, *incubation with* [^{3}*H*]*estradiol;* ○ – – – ○, *incubation with* [^{3}*H*]*estradiol in the presence of* 500*-fold excess of unlabeled estradiol. The arrow indicating* 4*S corresponds to the mobility of yeast phenylalanin transfer-RNA.*

with a maximum at pH 6·65 (Fig. 2). The pI 6·6 complex was always found to be saturable with unlabeled estradiol. It was found that focusing of larger amounts of protein than that corresponding to 2 O.D. units (280–310 nm) sometimes resulted in the formation of heavy protein precipitates that could interfere with quantitation of receptor-bound radioactivity with pI about 6·6. However, if a maximum amount of 2 O.D. units (280–310 nm) of protein was electrofocused, precipitates were only formed below pH 5·5 and consequently did not interfere with receptor quantitation. The protein precipitates usually contained non-specific estradiol binding sites but in some cases they also bound material that was displaced to some extent by unlabeled estradiol. Experiments with mouse uterus estradiol receptor protein (not illustrated) showed that the same relative distribution of [^{3}H]estradiol between the pI 6·6 complex and the pH 4·0–5·5 precipitates was observed when varying amounts of protein (0·1–2·0 O.D. units) were electrofocused suggesting that the pI 6·6 complex is not precipitated as a result of too much protein being present during the focusing. Only the pI 6·6 complex was regarded as a measure

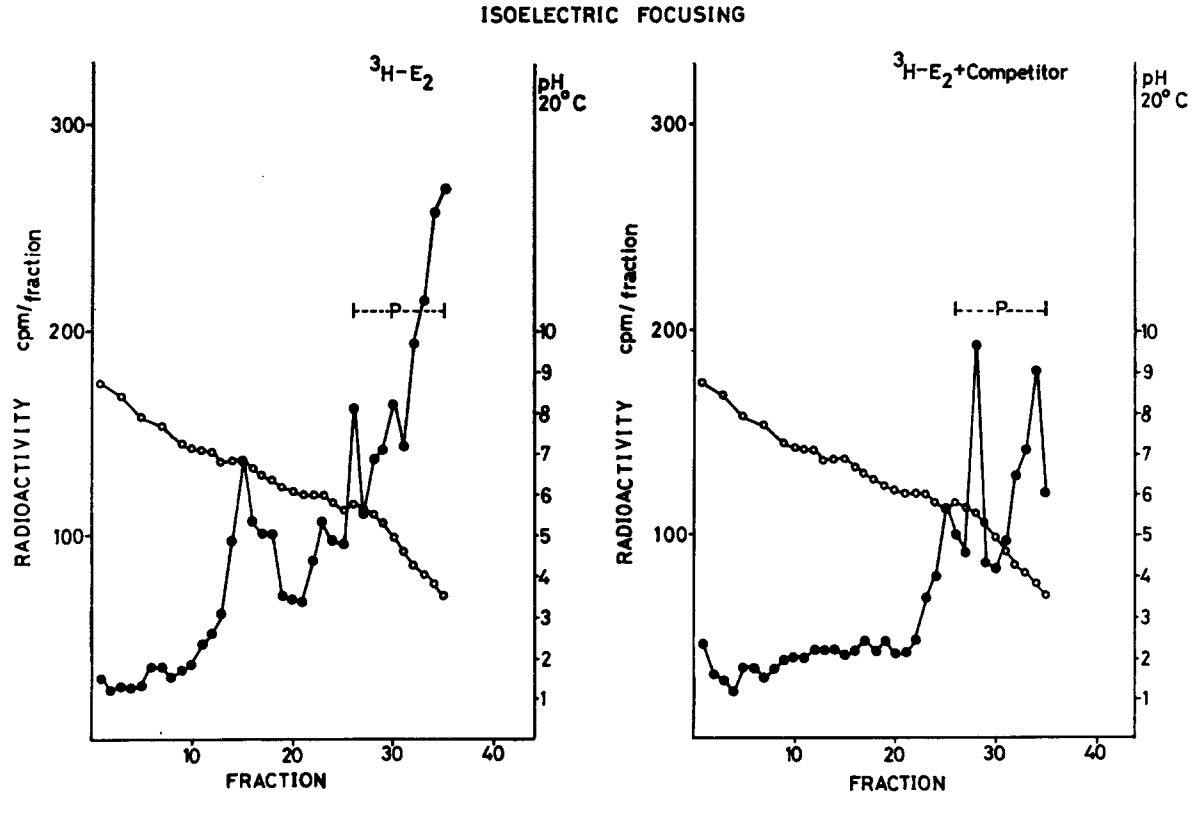


Fig. 2. Isoelectric focusing analysis. *Fresh tissue from patient No.* 1 (*see Table I*) *was analyzed as described in Material and Methods. The left diagram shows incubation with* [^{3}*H*]*estradiol and the right diagram shows incubation with* [^{3}*H*]*estradiol in the presence of* 500*-fold excess of unlabeled estradiol. Explanations:* ●——●, *radioactivity;* ○——○, *pH. The section marked with a P indicates localization of protein precipitate.*

of the content of estradiol receptor protein in the tumor, since the protein precipitates formed during analysis mostly contained non-saturable binding sites.

Table 1 illustrates several cases in which sucrose gradient centrifugation and isoelectric focusing analysis did not give similar results. In one case (patient No. 16) the tumor was classified as IF-positive but SG-negative. Patients Nos. 9, 12 and 14 were SG-positive but IF-negative. These specimens only showed 4S peaks that were partially displaced by unlabeled estradiol. On the other hand, six specimens that were IF-positive (patients Nos. 5, 6, 7, 8, 10 and 11) also only showed a 4S peak in sucrose gradients. However, in all cases where saturable binding occurred as both 4S and 8S peaks in sucrose gradients, the specimens were classified as IF-positive (patients Nos. 1, 2, 3, 4, 13 and 16). These findings indicate a positive correlation between sucrose gradient centrifugation and electrofocusing analysis in cases where tumors show both 4S and 8S binding and a less positive correlation in cases where only 4S binding occurs. A possible explanation for this is that the 4S region of sucrose gradients may contain not only saturable receptor and unsaturable non-receptor binding sites for estradiol but also saturable, non-receptor binding sites. If this is the case, the 4S SG-positive, IF-negative tumor specimens Nos. 9, 12 and 14 may not have contained estradiol receptor protein but some kind of non-receptor protein(s) that bind(s) estradiol with a relatively limited number of binding sites. We interpret our data to mean that isoelectric focusing is a more specific method to estimate estradiol receptor protein. However, final evaluation of isoelectric focusing as a technique for measurement of estradiol receptors in mammary carcinoma must await correlation with results of endocrine therapy. Such studies are underway.

In most cases microscopic slides from the primary tumors were reexamined and classified according to tumor type and differentiation. The data show that tumors of all three types of morphology (ductal, lobular and colloid) may contain estrogen receptor as judged both by sucrose gradient centrifugation and isoelectric focusing analysis.

Previous reports [1] demonstrate no clear correlation between degree of differentiation and receptor content. Our data show an increased frequency of receptor positive tumors among differentiated carcinomas. If only carcinomas with more than 0·1 fmoles of receptor per μg DNA in isoelectric focusing analysis or more than 0·1 fmoles of 8S receptor per μg of DNA are considered, this correlation becomes more marked. Further analysis of more tumors are planned to investigate the reproducibility of this phenomenon.

The amount of specific estradiol binding was generally less when calculated from electrofocusing results than when calculated from sucrose gradient centrifugation results. Part of the reason for the difference may be that sucrose gradient centrifugation analysis also measures some saturable, non-receptor binding sites, as discussed above. It is also probable that some receptor is aggregated or denatured during electrofocusing. Experiments should be carried out with partially purified receptor preparations to investigate this possibility. De Sombre *et al.* [9] have shown that estradiol receptor protein from calf uterus sedimenting at 4S and 8S in low ionic strength have pI values of 6·4 and 5·8, respectively. Similar findings have been described by Puca *et al.* [10]. De Sombre *et al.* [9] also reported that the 8S form of the receptor had a considerably greater tendency to aggregate than the 4S form. Therefore, the pI 6·6 complex formed in our experiments may represent only the 4S form of the estradiol receptor. A part of the saturable binding sites sometimes present in the pH 4–5·5 protein precipitates could then represent aggregated 8S estradiol receptor. Experiments aimed at investigating these possibilities are in progress in our laboratory. Preliminary results from model studies on the estradiol receptor in rat uterus indicate that the 4S form indeed is focused at pI 6·6 and that the 8S form is precipitated under the conditions used. We conclude that the sensitivity of isoelectric focusing of estradiol receptor may be improved if preanalytical transformation of the 8S form of the receptor into the 4S form is possible to perform. We are currently testing this possibility.

Acknowledgement—The skilful technical assistance of Mrs. Solveig Humla and Miss Inga-Lill Ramberg is gratefully acknowledged.

REFERENCES

1. In *Estrogen Receptors in Human Breast Cancer* (Edited by W. L. McGuire, P. P. Carbone and E. P. Vollmer), Raven Press, New York (1975).

2. W. L. McGuire, Quantitation of estrogen receptor in mammary carcinoma. In *Methods in Enzymology* (Edited by W. O'Malley and J. G. Hardman), Vol. XXXVI Part A, p. 248, Academic Press, New York (1975).
3. M. Beato and P. Feigelson, Glucocorticoid-binding proteins of rat liver cytosol. Separation and identification of the binding proteins. *J. biol. Chem.* **247,** 7890 (1972).
4. G. Scatchard, The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).
5. M. Katsumata and A. S. Goldman, Separation of multiple dihydrotestosterone receptors in rat ventral prostrate by a novel micromethod of electrofocusing. Blocking action of cyproterone acetate and uptake by nuclear chromatin. *Biochim. biophys. Acta* **359,** 112 (1974).
6. A. Wallgren and C. Silfverswärd, Clinical and histological factors of prognostic importance in breast cancer. *Int. J. Radiat. Oncol. Biol. Phys.* In press (1976).
7. R. W. McDivitt, F. W. Stewart and J. W. Berg, Tumours of the breast. In *Atlas of Tumor Pathology* 2nd series, Fasc. 2, 18. Armed Forces Institute of Pathology, Washington, D.C. (1969).
8. K. Burton, Determination of DNA concentration with diphenylamine. In *Methods in Enzymology* (Edited by L. Grossman and K. Moldave), Vol. XII Parb B, 163. Academic Press, New York (1968).
9. E. R. DeSombre, G. A. Puca and E. V. Jensen, Purification of an estrophilic protein from calf uterus. *Proc. nat. Acad. Sci.* **64,** 148 (1969).
10. G. A. Puca, E. Nola, V. Sica and F. Bresciani, Estrogen-binding proteins of calf uterus. Partial purification and preliminary characterization of two cytoplasmic proteins. *Biochemistry* **10,** 3769 (1971).

Europ. J. Cancer Vol. 12, pp. 701–704. Pergamon Press 1976. Printed in Great Britain

Age at Menarche as a Prognostic Factor in Human Breast Cancer

P. JURET, J. E. COUETTE, A. M. MANDARD, A. CARRÉ, T. DELOZIER, D. BRUNE and J. C. VERNHES

Centre Régional François-Baclesse, Route de Lion/Mer, 14018 *Caen, France*

Abstract—*An analysis of a series of* 926 *female patients, of whom* 755 *were followed for* 3 *years and* 647 *for* 5 *years after mastectomy, revealed age at menarche to be a valuable prognosticator. Survival rates were the lowest for women who had very early menarche (≦ 11 years of age) but prognosis improved progressively with increase in menarchial age. Optimal menarchial age was found to be at age* 15; *onset of menses beyond this age was once again associated with an unfavorable outcome. The possible relationship between age at menarche and axillary node involvement was then examined from two points of view in an effort to establish whether early menarche would be associated with extensive nodal involvement, or whether it would retain its unfavorable prognostic significance independently of the extent of this involvement. Both of these hypotheses were found to be plausible in our series. It is stressed that further research on larger study populations is required before definitive conclusions can be drawn.*

INTRODUCTION

THE RELATIONSHIP between breast cancer risk and age at menarche has been the subject of numerous studies. In most cases, an association of early menarche with increased breast cancer risk was reported [1–8], although in some the differences between case and control groups were small, and in a few no association was found [9, 10]. However, the direction of the difference, when found, is consistent enough to provide strong evidence for the existence of a relationship.

There are two other epidemiologic aspects of age at menarche which have been paid less attention. These are the relationship between age at menarche and age at cancer onset, and the prognostic significance of age at menarche.

In a previous study on the possible relationship between age at menarche and age at clinical onset of breast cancer, we found that the disease appeared at an average age of 55·1 yr in women who had had menarche before age 14 and 57·6 yr in those who had first menstruated at age 14 or older [11]. The results were statistically significant, but possible bias rendered their validity questionable. It has been established that menarche has been occurring at steadily earlier ages over the past 100 years [12], and it seems possible that our results may merely have been a reflection of this phenomenon. This hypothesis is supported by Schwartz's study of the breast cancer patients at the Institut Gustave Roussy, Villejuif. Although his results were in agreement with our own, and the early appearance of breast cancer was observed in women with early menarche, further analysis of his data revealed a tendency to earlier menarche in the younger women; this phenomenon was found to an identical extent in the case and the non-cancerous control groups [13].

The second aspect, the possible prognostic significance of age at menarche, is the subject of the present study.

Accepted 8 March 1976.

MATERIAL AND METHODS

A retrospective study was made of the records of patients followed post-mastectomy at the "Centre François Baclesse" during the years 1958–1974. The study was limited to women under 70 yr of age at the time of surgery, which in all cases was radical mastectomy (conventional or modified) for potentially curable breast cancer. The T.N.M. classification for all the patients considered here was T_{1-2-3}, N_{0-1}, M_0. Two hundred and eighty-six records where the essential informa-

tion was missing were excluded from the analysis: in 182 cases it had been difficult to record age at menarche due to the state of the patient on admission (loss of recollection, mental disorders, deafness, etc.) and in another 104, the examining doctor had omitted to note the information. After exclusions, 926 cases with more than one year's follow-up remained for analysis. Of these, 755 had been followed for more than 3 yr and 647 for 5 yr.

In all cases, age at menarche was supplied by the patient herself. When it was given as falling between two birthdays, the age at birthday preceding menarche was retained (for example, menarche given as occurring at 13 years 6 months, or "between 13 and 14" was recorded as age 13, etc.).

RESULTS

The results of this study are summarized in Fig. 1, 2 and 3.

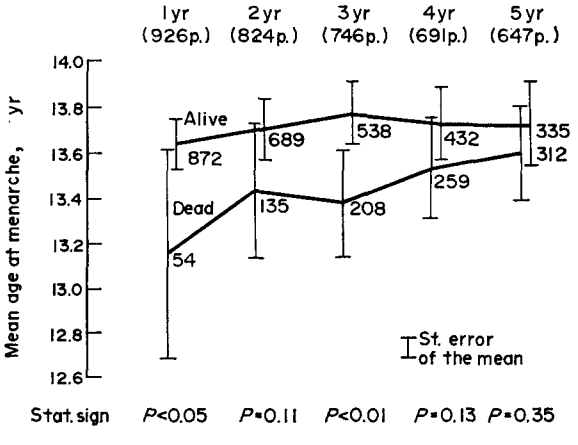


Fig. 1. Actuarial survival after mastectomy with respect to mean age at menarche.

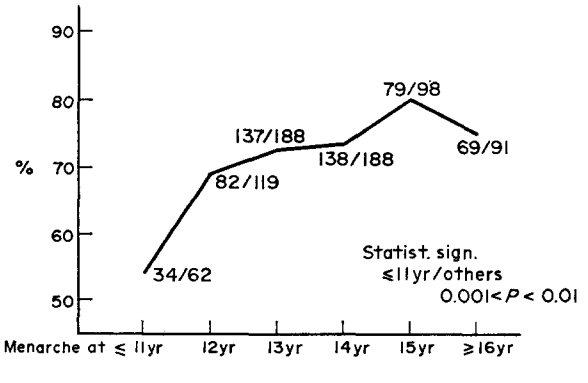


Fig. 2. Three-year survival rate of 746 women under 70 years of age at time of surgery irrespective of axillary node status in relation to age at menarche.

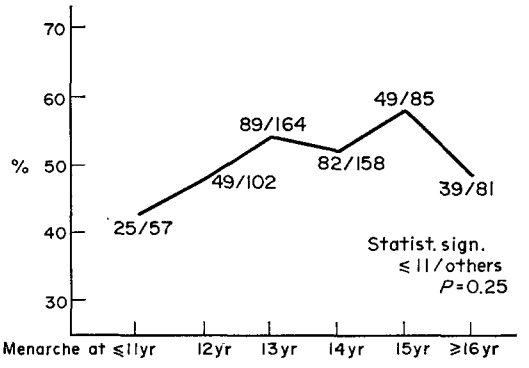


Fig. 3. Five-year survival rate of 647 women under 70 years of age at time of surgery irrespective of axillary node status in relation to age at menarche.

Fig. 1 shows that the mean age at menarche of the women who died within the first 5 post-operative years was lower than the mean menarchial age of those who survived.

Fig. 2 and 3 give survival at 3 and 5 yr respectively for patients grouped according to their age at menarche. It can be seen that the survival rates were significantly lower for women who had menarche at or before age 11 (statistical significance at 3 years, $P < 0.01$). Prognosis was found to improve progressively with increase in menarchial age, and reached an optimum for the group with menarche at age 15. The outcome was poorer again with late menarche at age 16 or older, but this reversal in the trend was not statistically significant in our series.

DISCUSSION

As our results strongly suggested that a relationship between bad prognosis and early menarche exists, we found it of interest to investigate the possible relationship between early menarche, and that other, universally recognized major prognosticator, axillary node involvement. Two possibilities were envisaged, and we sought to establish whether the mean menarchial age would be lower among patients with extensive nodal involvement, or whether early menarche would retain its unfavorable prognostic significance independently of the extent of this involvement.

(a) Was the mean menarchial age lower for patients with extensive nodal involvement? We eliminated from our study those patients whose nodal status was unknown and those where nodal involvement was confirmed but the extent not given in sufficient detail. Six-hundred and fifty-one cases (of the original

926) were retained for analysis, and classified into 3 categories: N negative with no metastatic nodes, N positive with 1 or 2 nodes involved, and N positive with 3 or more nodes involved. Our choice of these two levels of nodal involvement, with the distinction between slight involvement, with 1 or 2 nodes affected, and extensive involvement, when 3 or more nodes were affected, was based on a study conducted at the Institut Gustave-Roussy by Contesso *et al.* These authors reported a marked deterioration in prognosis in their series once 3 lymph nodes were involved [14].

Table 1. *Mean age at menarche in relation to node involvement in* 651 *patients*

	Node involvement	Number of patients	Mean age at menarche	Standard deviation
A	N—	238	13·65 y.	1·73
B	N+1 or 2 nodes	138	13·59 y.	1·61
C	N+ ⩾ 3 nodes	275	13·32 y.	1·59

Statist. signif. A/B not significant
A+B/C $0{\cdot}01 < p < 0{\cdot}02$

Table 1 shows the results of our analysis of the mean age at menarche in relation to axillary node involvement. No significant difference in mean menarchial age was found between the groups with no or only slight nodal involvement, but there was a significant difference when the group with $N^+ \geqslant 3$ nodes was compared to the two other groups combined (N^- or N^+ 1 or 2 nodes). These results thus only provide partial support for our hypothesis.

(b) Did age at menarche retain its prognostic significance for patients with equal node involvement? We used as material for this second part of our analysis the patients with $N^+ \geqslant 3$ nodes, as this group, with its low survival rate, lent itself best to this particular study. Our findings are given in Fig. 4.

Due to the limited number of patients who had had menarche before age 11, it was difficult to carry out a statistical analysis of the results. But the tendency expressed in Fig. 4 shows that this hypothesis is indeed a plausible one.

Thus the findings of this study do not provide a clear answer to the question of a possible relationship between axillary node involvement and age at menarche, although neither of our hypotheses can be excluded. There is need for further study on larger study populations for more reliable conclusions to be drawn.

We will not go into the physiopathological considerations that these results suggest, but would just like to draw attention to the positive relationship that has been found between the early onset of menses and decreased carbohydrate tolerance in pregnant women [15]. Early menarche, grave breast cancer prognosis, disturbed carbohydrate metabolism: do all these imply the existence of a common biological mechanism?

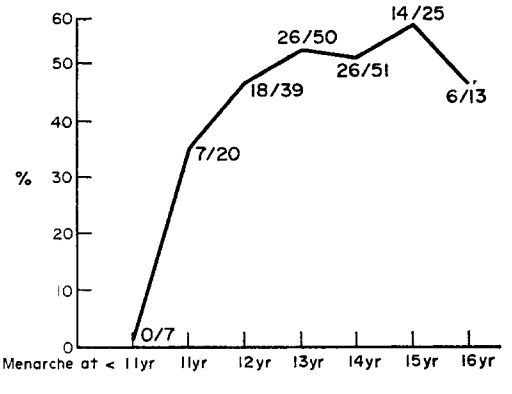


Fig. 4. *Three-year survival rate of* 205 *patients with* 3 *or more axillary nodes involved in relation to age at menarche.*

Finally, we would like to emphasize the parallel effects of age at menarche on morbidity and mortality, in contrast to the contradictory effects of obstetric history on risk and survival that we reported in a previous study. In the latter case, nulliparity, although a generally accepted high risk factor, was found to have a favorable effect on prognosis [16].

REFERENCES

1. V. G. Valaoras, B. MacMahon, D. Trichopoulos and A. Polychronopoulou, Lactation and reproductive histories of breast cancer patients in Greater Athens, 1965–67, *Int. J. Cancer* **4,** 350 (1969).
2. E. J. Salber, D. Trichopoulos and B. MacMahon, Lactation and reproductive histories of breast cancer patients in Boston, 1965–66, *J. nat. Cancer Inst.* **43,** 1013 (1969).
3. S. Yuasa and B. MacMahon, Lactation and reproductive histories of breast cancer patients in Tokyo, Japan, *Bull. WHO* **42,** 192 (1970).
4. T. M. Lin, K. P. Chen and B. MacMahon, Epidemiologic characteristics of breast cancer in Taiwan, *Cancer (Philad.)* **27,** 1497 (1970).
5. B. Ravnihar, B. MacMahon and J. Lindtner, Epidemiologic features of breast cancer in Slovenia, 1965–67, *Europ. J. Cancer* **7,** 295 (1971).
6. S. Shapiro, P. Strax and L. Venet, The search for risk factors in breast cancer, *Amer. J. Public Health* **58,** 820 (1968).
7. M. Segi, I. Fukushima and S. Fujisaku, An epidemiological study on cancer in Japan, *Gann* **48,** suppl. I (1957).
8. Straszewski, Age at menarche and breast cancer. *J. nat. Cancer Inst.* **47,** 935 (1972).
9. A. P. Mirra, P. Cole and B. MacMahon, Breast cancer in an area of high parity: Sao Paulo, Brazil. *Cancer Res.* **31,** 77 (1971).
10. E. L. Wynder, I. J. Bross and Hirayama, A study of the epidemiology of cancer of the breast. *Cancer (Philad.)* **13,** 559 (1960).
11. P. Juret, J. Robillard, J. E. Couette, D. Brune and J. C. Vernhes, Données épidémiologiques apparemment contradictoires en matière de cancer du sein. *Sem. Hôp. Paris (Sem. méd. prof. méd-soc.)* **50,** 43 (1974).
12. J. M. Tanner, *Growth at Adolescence,* 1st, Blackwell Scientific, Oxford, (1955).
13. D. Schwartz, personal communication.
14. G. Contesso, J. Rouëssé and J. L. Caen, Facteurs anatomo-pathologiques du pronostic des cancers du sein. *Ann. Anat. path.* **18,** 373 (1973).
15. F. D. Loffer, Decreased carbohydrate tolerance in pregnant patients with an early menarche. *Amer. J. Obstet. Gynecol.* **123,** 180 (1975).
16. P. Juret, J. E. Couette, D. Brune and J. C. Vernhes, L'âge à la première naissance: une variable à la signification simple, double ou triple en cancérologie mammaire. *Bull. Cancer* **62,** 165 (1975).

Europ. J. Cancer Vol. 12, pp. 705–709. Pergamon Press 1975. Printed in Great Britain

The Relationship of DNA Polymerase Activity to Cell Cycle Stage*

R. D. BARR*, P. SARIN†, G. SARNA* and S. PERRY‡

*Office of the Associate Director for Program and †the Laboratory of Tumor Cell Biology, Division of Cancer Treatment, National Cancer Institute, and the ‡Office of the Director, National Institutes of Health, Bethesda, Maryland 20014

Abstract—*Cytoplasmic* (α) *and nuclear* (β) *DNA polymerase activities were estimated, at each cell cycle stage, in single cell suspensions of thymuses from leukemic AKR mice, separated by velocity sedimentation at unit gravity. DNA polymerase* α *activity was essentially absent in small* G_0 *cells, but markedly elevated in cells progressing through* G_1 *to S phase. This finding has therapeutic as well as basic biological implications.*

INTRODUCTION

IN ORDER to make a clear distinction between true G_0 cells and those in an active pre-DNA synthesizing phase (G_1) of the proliferative cycle, it has been proposed [1] that a biochemical difference between these two populations would require to be demonstrated.

DNA replication is an essential part of the normal cycle of cell growth and division, and preparation for DNA synthesis begins in the post-mitotic rest phase [2]. In this report we describe the activity of DNA polymerizing enzymes at each stage of the proliferative cycle, in an effort to define a biochemical discriminant between G_1 cells and those post-mitotic cells which are in a dormant state (G_0).

MATERIAL AND METHODS

AKR mice with spontaneous leukemia were obtained from Microbiological Associates, Bethesda, Maryland 20014. Single cell suspensions of 5 thymuses were prepared by the method of Omine and Perry [3] and were individually subjected, in duplicate, to velocity sedimentation at unit gravity in a sucrose gradient [4]. Utilizing this technique, cells are separated more on the basis of differences in size than of differences in density [5]. Separated suspensions were harvested in 30 × 50 ml aliquots from each sedimentation chamber, after an initial discard of 150 ml of acellular fluid, and corresponding fractions from the two chambers were pooled at the end of each experiment. A correlation between fraction number and cell cycle phase has previously been outlined in a report from this laboratory [6]. Cell counts were performed with a Coulter Counter Model B, low values being confirmed manually using a hemocytometer. Cell viability was assessed by trypan blue exclusion [7]. Pooled fractions were pelleted and frozen for assay of enzyme activities.

Cell extractions

Cell extractions were carried out at 0–4°C. Cells from corresponding pools were combined, suspended in 5 vol of buffer A (10 mM Tris–HCl, pH 7·4, 10 mM KCl and 1·5 mM Mg^{2+}) and allowed to swell for 10 min. The cells were manually disrupted in a tight fitting stainless steel homogenizer and mixed with an equal volume of buffer B (50 mM Tris–HCl, pH 7·5, 5 mM DTT, 1 M KCL, 1% triton X100 and 20% glycerol) and stirred in ice for 3 hr. The preparation was then centrifuged at 100,000 *g* for 2 hr. The soluble extract was removed, dialyzed against buffer C (50 mM Tris–HCl, pH 7·5, 0·05% triton X100, 5 mM DTT and 20% glycerol) containing 0·5 M KCl and subsequently against buffer C alone. The dialyzed extract was used for the isolation of DNA polymerases.

Enzyme purification

Step 1. A DEAE cellulose (Whatman, DE52) column (6 × 1·5 cm) was washed with 4 M

Accepted 4 May 1976

*Reprint requests should be sent to Dr. S. Perry, National Institutes of Health, Bldg. 1, Room 111, Bethesda, Md., 20014, U.S.A.

NaCl and then equilibrated with buffer C containing 0·3 M KCl. The dialyzed cell extract was made 0·3 M in KCl and applied to the column in order to remove nucleic acids. The column was washed with buffer C containing 0·3 M KCl. Fractions (1 ml volume) were collected and assayed for DNA polymerase activity. Fractions containing the enzyme activity were pooled and dialyzed against buffer C.

Step 2. The dialyzed solution was then applied to a second DEAE cellulose column (6 × 1·5 cm) equilibrated with buffer C. The column was washed with 50 ml buffer C containing 0·05 M KCl followed by 50 ml buffer C containing 0·3 M KCl. Fractions (1 ml) were collected and 5 μl aliquots assayed for DNA polymerase activity. Under these conditions DNA polymerase β is eluted with 0·05 M KCl and DNA polymerase α is eluted with 0·3 M KCl [8, 9]. The pooled enzymes from the 0·05 M KCl wash and the 0·3 M KCl wash were used for the calculation of nuclear (β) and cytoplasmic (α) DNA polymerase activities, respectively. Subsequent chromatography on phosphocellulose columns revealed that the enzyme eluting with 0·05 M KCl was mainly DNA polymerase β, and the enzyme eluting with 0·3 M KCl was mainly DNA polymerase α.

Enzyme assays

DNA polymerase activity was assayed at 37°C for 1 hr as described earlier [10], in a standard reaction mixture (0·05 ml) containing 50 mM Tris–HCl (pH 7·5), 50 mM KCl, 0·5 mM $MnCl_2$, 5 mM dithiothreitol, 100 μM of the labelled deoxyribonucleoside triphosphate, 2–5 μg of $(dT)_{15}.(dA)_n$ as the primer-template and 5 μl of the enzyme fraction. The specific activity of (^{3}H)dTTP used was 2200 cpm/pmole and was obtained from Schwarz–Mann, Orangeburg, New York. $(dT)_{15}.(dA)_n$ was obtained from P. L. Biochemicals, Milwaukee, Wisconsin.

RESULTS

In the series of 5 experiments, the optimum number of cells ($1{\cdot}4 \times 10^8$) was applied to each chamber, resulting in a median recovery of 89% (range 85–100%). There being minimal variation, a representative size distribution profile, showing the characteristic reproducibility between duplicates, is shown in Fig. 1. Because of the initial discard of 150 ml (equivalent to 3 × 50 ml. fractions), the first fraction collected is designated number 4, to correspond with enumeration in experiments previously reported from this laboratory, in which no discard was made. Fractions 4–8 consist predominantly of inviable debris. As assessed by light microscopy, debris was not apparent in fractions 9–30, in which there was no significant variation in viability (greater than 80% trypan blue exclusion).

The distribution of cytoplasmic DNA polymerase (α) and nuclear DNA polymerase (β) activities in the various fractions is shown in Fig. 2. As can be seen, there is an approximately 12-fold increase in cytoplasmic DNA polymerase (α) activity, in fractions known to

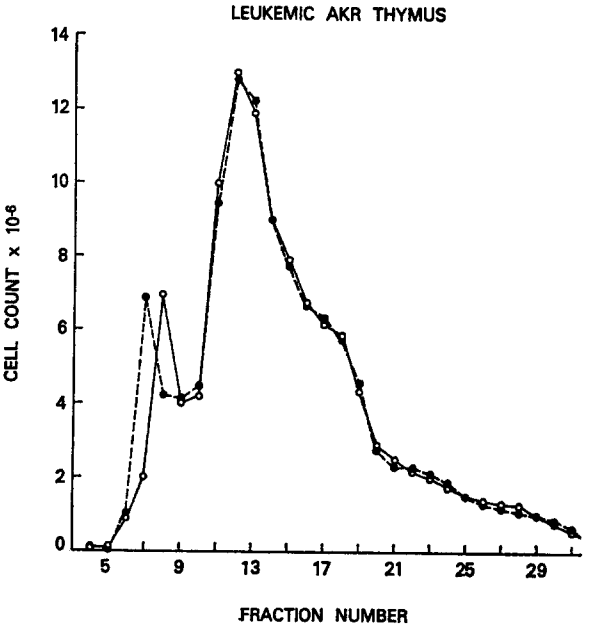


Fig. 1. Cell size distribution profile of a single cell suspension of leukemic AKR thymus separated by velocity sedimentation at unit gravity. Fractions 4–8 *contain cell debris,* 9 *predominantly* G_0 *cells,* 10 G_0 *plus* G_1 *cells,* 11–18 G_1 *and S phase cells, and* 19–30 *cells in late S,* G_2 *and M phases.*

contain predominantly S-phase cells [6], compared to activity determined in the smallest and purest population of G_0 cells (fraction 9). There is a marked rise in enzyme activity in later fractions, with the progression of cells from $G_1 \rightarrow G_1 \rightarrow S$, while a lesser (4-fold) increase in nuclear DNA polymerase (β) activity occurs with the advance of cell cycle phase.

DISCUSSION

The concept of a state of "no cell cycle" (state of dormancy, true resting stage in respect of growth), originally outlined by Lajtha and Oliver [11] in a steady state system, was later designated G_0, and has received considerable support [12]. In a new concept of G_0 the term

is expanded to include all post-mitotic cells, except those in which the metabolic events leading to DNA synthesis have actually begun [13].

Aside from theoretical considerations, the issue has major practical implications, notably in relation to the growth fraction of animal and human tumors, and their corresponding susceptibility to chemotherapy [14–18].

We have previously reported the use of a velocity sedimentation system to separate cell suspensions of thymus from leukemic AKR mice

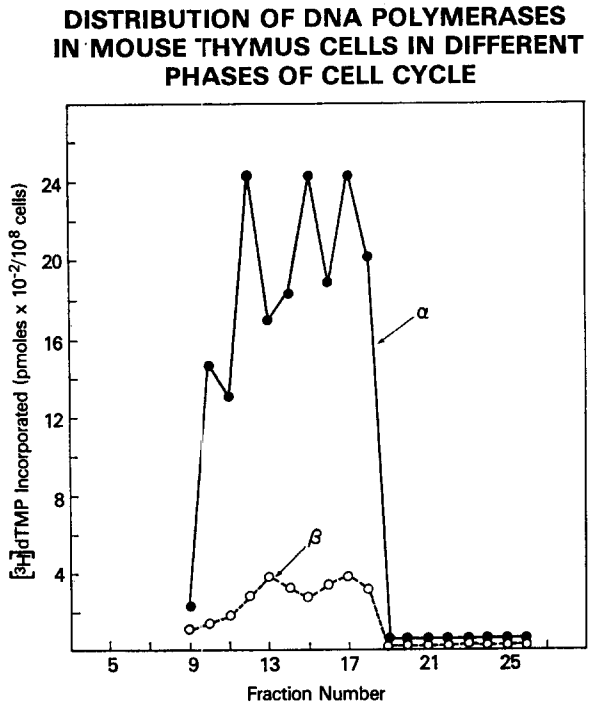


Fig. 2. Cytoplasmic (α) and nuclear (β) DNA polymerase activities in leukemic AKR thymus cells separated by cell cycle phase. Fractions 26–30 *have been pooled with fraction* 25.

[3, 6, 19, 20] and the probable existence of a distinct G_0 or "very long G_1" population, smaller than "active" G_1 cells, was suggested. This population of "G_0" cells has 2C DNA content, good viability by trypan blue exclusion and leukemic clonogenicity by transplantation assay [19]. It includes the 2% of cells which are unlabeled after 65·5 hr of multiple ^{3}H-TdR labeling [21]. This value of 2% is numerically close to that recorded by Skipper for the fraction surviving treatment with cytosine arabinoside administered during a 24-hr period [22]. Cell separation experiments have shown that those cells surviving 3 days of Palm O–Ara–C therapy are in fact such small cells [3].

A variety of enzymes involved in DNA synthesis have been studied in relation to cell cycle phase [23–26]. The most frequently investigated have been the DNA polymerases. Three separate DNA polymerase activities are demonstrable in human cells [27, 28] and it has been shown that the major activity in mammalian cells is in the cytoplasm and not in the nucleus [29]. This is supported by the finding of the cytoplasmic enzyme in reticulocytes [30]. Hence the use of techniques which involve cytoplasmic stripping [31], to evaluate the relationship of DNA polymerase activity to replicate status, are of dubious validity.

Unstimulated lymphocytes, widely regarded as being out of cycle, in G_0 [32], contain very little DNA polymerase activity [33–35], but when cultured with phytohemagglutinin a marked increment in enzyme activity has uniformly been recorded [10, 36–41]. Our results, in leukemic murine thymocytes, correspond closely to the maximum order of change (12-fold) observed by Chang *et al.* [42] in mouse L cells.

Although enzyme levels are also very low in G_2 cells (Fig. 2), it is unlikely that these will be confused with cells in G_0, for the former are readily distinguishable on the basis of relative cell size and DNA content. In a study on rat thymus, fractionated on a discontinuous albumin gradient, Coleman, *et al.* [43] reported low levels of activity in large cells and higher levels in the small lymphoid cells. It is entirely possible that the former were largely in G_2 and the latter in G_1 and early S phases of their proliferative cycle.

DNA polymerase appears to be a target for a number of drugs currently employed in the treatment of numerous forms of cancer [44–46] and the demonstration of the virtual absence of the enzyme from non-proliferating (G_0) cells therefore has important therapeutic as well as basic biological implications.

Acknowledgements—We thank Ada Brooks and Carole Williams for expert technical assistance.

REFERENCES

1. R. Baserga, Biochemistry of the cell cycle: A review. *Cell Tiss. Kinet.* **1**, 167 (1968).

2. R. A. Tobey, L. R. Gurney, C. E. Hildebrand, R. L. Ratcliff and R. A. Walters, Sequential biochemical events in preparation for DNA replication and mitosis. In: *Control of Proliferation in Animal Cells.* (Edited by Bayard Clarkson and Renato Baserga). Cold Spring Harbor Conference on Cell Proliferation **1**, p. 665 (1974).
3. M. Omine and S. Perry, Perturbation of leukemic cell populations in AKR mice due to chemotherapy. *Cancer Res.* **33,** 2596 (1973).
4. L. H. Brubaker and W. H. Evans, Separation of granulocytes, monocytes, lymphocytes, erythrocytes and platelets from human blood and relative tagging with di-isopropyl fluoro phosphate (DFP). *J. lab. clin. Med.* **73,** 1036 (1969).
5. R. G. Miller and R. A. Phillips, Separation of cells by velocity sedimentation. *J. cell Physiol.* **73,** 191 (1969).
6. M. Omine, G. P. Sarna and S. Perry, Composition of leukemic cell populations in AKR leukemia and effects of chemotherapy. *Europ. J. Cancer* **9,** 557 (1973).
7. A. Boyum, Separation of leucocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* **21,** Supplement 97 (1968).
8. D. Gillespie, W. C. Saxinger and R. C. Gallo, Information transfer in cells infected by RNA tumor viruses and extension to human neoplasia. *Progr. nucl. Acids Res. mol. Biol.* **15,** 1 (1975).
9. B. J. Lewis, J. W. Abrell, R. G. Smith and R. C. Gallo, DNA polymerases in human lymphoblastoid celll infected with simian sarcoma virus. *Biochim. biophys. Acta* **349,** 148 (1974).
10. R. G. Smith and R. C. Gallo, DNA dependent DNA polymerases I and II from normal human blood lymphocytes. *Proc. nat. Acad. Sci.* **69,** 2879 (1972).
11. L. G. Lajtha and R. Oliver, Cell population kinetics following different regimens of irradiation. *Brit. J. Radiol.* **3,** 131 (1962).
12. G. G. Steel, The kinetics of cell proliferation in tumors. In: Time and dose relationship in radiation biology as applied to radiotherapy. Proceedings of an NCI–AE Conference, Carmel, 1969. Brookhaven Report BNL–50203, p. 130, 1970.
13. M. L. Greenberg, A. D. Chanana, E. P. Cronkite, G. Giacomelli, K. R. Rai, L. M. Schiffer, P. A. Struyckmans and P. C. Vincent, The generation time of human leukemic myeloblasts. *Lab. Invest.* **26,** 245 (1972).
14. B. D. Clarkson, The survival value of the dormant state in neoplastic and normal cell populations. In: *Control of Proliferation in Animal Cells* (Edited by Bayard Clarkson and Renato Baserga). Cold Spring Harbor Conferences on Cell Proliferation **1**, 945 (1974).
15. J. Denekamp, The cellular proliferation kinetics of animal tumours. *Cancer Res.* **30,** 393 (1970).
16. M. L. Mendelsohn, Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. *J. nat. Cancer Inst.* **28,** 1015 (1962).
17. G. Sarna, The resting cell: a chemotherapeutic problem, Parts I and II. *Biomedicine* **20,** 322 and 384 (1974).
18. G. G. Steel, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer* **3,** 381 (1967).
19. P. J. Rosen, S. Perry and F. M. Schabel, Proliferative capacity of leukemic cells in AKR leukemia. *J. nat. Cancer Inst.* **45,** 1169 (1970).
20. O. Heby, G. P. Sarna, J. M. Laurence, M. Omine, S. Perry and D. H. Russell, Polyamine content of AKR leukemic cells in relation to the cell cycle. *Cancer Res.* **33,** 2959 (1973).
21. M. Omine and S. Perry, Use of cell separation at 1 g for cytokinetic studies in spontaneous AKR leukemia. *J. nat. Cancer Inst.* **48,** 697 (1972).
22. L. F. Lamerton and N. M. Blackett, A comparison of proliferative characteristics of bone marrow and tumors and response to cytotoxic agents. In: *Control of Proliferation in Animal Cells.* (Edited by Bayard Clarkson and Renato Baserga). Cold Spring Harbor Conference on Cell Proliferation **1**, 973 (1974).
23. R. Adler and B. R. McAuslan, Expression of thymidine kinase variants as a function of the replicative state of cells. *Cell* **2,** 113 (1974).
24. G. Bray and T. P. Brent, Deoxyribonucleoside 5′ triphosphate pool fluctuations during the mammalian-cell cycle. *Biochim. biophys. Acta.* **269,** 184 (1972).

25. T. P. Brent, Periodicity of DNA synthetic enzymes during the HeLa life cycle. *Cell Tiss. Kinet.* **4,** 297 (1971).
26. T. P. Brent, J. A. V. Butler and A. R. Crathorn, Variations in phosphokinase activities during the cell cycle in synchronous populations of HeLa cells. *Nature (Lond.)* **207,** 176 (1965).
27. W. D. Sedwick, T. S-F. Wang and D. Korn, Purification and properties of nucleic acid cytoplasmic deoxyribonucleic acid polymerases from human KB cells. *J. biol. Chem.* **247,** 5026 (1972).
28. A. Weissbach, A. Schlabach, R. Fridlender and A. Bolden, DNA polymerases from human cells. *Nature New Biol.* **231,** 167 (1971).
29. R. M. S. Smellie and R. Eason, Studies on the intracellular location of deoxyribonucleic acid polymerase. *Biochem. J.* **80,** 39P (1961).
30. J. J. Byrnes, K. M. Downey and A. G. So, Metabolic regulation of cytoplasmic DNA synthesis. *Proc. nat. Acad. Sci.* **71,** 205 (1974).
31. L. M. Schiffer, A. M. Markoe, A. Winkelstein, J. S. R. Nelson and J. M. Mikulla, Cycling characteristics of human lymphocytes I*n vitro*. *Blood* **44,** 99 (1974).
32. L. M. van Putten, G_0, a useful term? *Biomedicine* **20,** 5 (1974).
33. L. A. Loeb, B. J. Poiesz, R. O. Williams and C. Springgate, On multiple DNA polymerases after stimulation of normal and leukemic lymphocytes. In: *Proceedings of the VII Leukocyte Culture Conference*, p. 151. Fritz Daguillard, Quebec, Canada, New York, Academic Press (1973).
34. R. J. Mayer, R. G. Smith, M. G. Sarngadharan and R. C. Gallo, Induction of DNA metabolizing enzymes in normal human blood lymphocytes following stimulation with phytohemagglutinin (PHA). *Blood* **44,** 921 (1974).
35. A. M. Pedrini, F. Nuzzo, G. Ciarocchi, L. Dalpra and A. Falaschi, Induction of polynucleotide ligase in human lymphocytes stimulated by phytohemagglutinin. *Biochem. biophys. Res. Commun.* **47,** 1221 (1972).
36. B. R. Fridlender, E. Medrano and J. Mordoh, Synthesis of DNA in human lymphocytes: possible control mechanism. *Proc. nat. Acad. Sci. (Wash.)* **71,** 1128 (1974).
37. L. A. Loeb, S. S. Agarwal and A. M. Woodside, Induction of DNA polymerase in human lymphocytes by phytohemagglutinin. *Proc. nat. Acad. Sci. (Wash.)* **61,** 827 (1968).
38. G. C. F. P. Noy, L. Dalpra, A. M. Pedrini, G. Ciarocchi, E. Guilotto, F. Nuzzo and A. Falaschi, Evidence for two waves of induction of DNA enzymes in stimulated human lymphocytes. *Nucl. Acids Res.* **1,** 1183 (1974).
39. Y. Rabinowitz, I. S. McCluskey, P. Wong, B. A. Wilhite, DNA polymerase activity of cultured normal and leukemic lymphocytes. Responses to phytohemagglutinin. *Exper. Cell. Res.* **57,** 257 (1969).
40. B. I. S. Srivastava, Deoxynucleotide polymerizing enzymes in normal and malignant human cells. *Cancer Res.* **34,** 1015 (1974).
41. G. Trysted, B. Munch-Peterson and L. Cloos, DNA polymerase activity in stimulated and non-stimulated human lymphocytes. *Exper. Cell Res.* **77,** 415 (1973).
42. L. M. S. Chang, McK. Brown and F. J. Bollum, Induction of DNA polymerase in mouse L cells. *J. mol. Biol.* **74,** 1 (1973).
43. M. S. Coleman, H. J. Hutton and F. J. Bollum, Terminal deoxynucleotidyl transferase and DNA polymerase in classes of cells from rat thymus. *Biochem. biophys. Res. Commun.* **58,** 1104 (1974).
44. G. Hartmann, H. Goller, K. Koschel, W. Kersten and H. Kersten, Hemmung der DNA abhangigen RNA und DNA Synthese durch Antibiotica. *Biochem. Z.* **341,** 126 (1964).
45. J. G. Lindsay, S. Berryman and R. L. P. Adams, Characteristics of deoxyribonucleic acid polymerase activity in nuclear and supernatant fractions of cultured mouse cells. *Biochem. J.* **119,** 839 (1970).
46. G. P. Wheeler and B. J. Bowdon, Effects of 1,3-bis (2-chloroethyl)-1-nitrosourea and related compounds upon the synthesis of DNA by cell free systems. *Cancer Res.* **28,** 52 (1968).

Europ. J. Cancer Vol. 12, pp. 711–717. Pergamon Press 1976. Printed in Great Britain

Effects of Oestradiol–17β and Tamoxifen on Total and Accessible Cytoplasmic Oestradiol–17β Receptors in DMBA-Induced Rat Mammary Tumours

R. I. NICHOLSON, M. P. GOLDER, P. DAVIES and K. GRIFFITHS
Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, Great Britain

Abstract—*An exchange assay for the measurement of total cytoplasmic oestradiol-17β binding sites in DMBA-induced rat mammary tumours has been established. The assay was used to examine the* in vivo *interactions of tamoxifen and oestradiol-17β with cytoplasmic receptor proteins for oestradiol-17β. Although certain quantitative differences were observed between the actions of tamoxifen and oestradiol-17β, the compounds show an initial similarity of response, each ligand promoting an early elevation in receptor levels. Tamoxifen, however, was unable to sustain the replenishment of the cytoplasmic receptor.*

INTRODUCTION

THE ASSOCIATION of the oestradiol-17β molecule with a specific cytoplasmic binding protein and the subsequent translocation of the complex to the nucleus are thought to be fundamental in the action of oestradiol-17β [1]. Compounds which interfere with these events prevent oestrogens from exerting their full influence on target tissues. Such compounds are of particular interest in the management of metastatic breast cancer since approximately 30% of patients shown an objective remission of tumour growth following ablative or additive endocrine therapy.

This paper describes the influence of oestradiol-17β and the anti-oestrogen, tamoxifen (I.C.I. 46474: trans 1-(p-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene) on the levels of total and accessible cytoplasmic oestradiol-17β binding sites in rat mammary tumours induced by 7,12-dimethylbenz(a)-anthracene (DMBA). Such studies can provide information on the mechanisms by which anti-oestrogens influence tumour growth. A preliminary report of this work has been presented [2].

Accepted 31 March 1976.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50±2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and fed diet and water *ad libitum*. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in the study.

Preparation of cytosol fractions and assay for specific oestradiol-17β binding sites

(*a*) *Accessible sites.* Animals were killed by cervical dislocation, the tumours removed and dissected free of fat, washed with 0·25 M sucrose (adjusted to pH 7·4 with $NaHCO_3$),

cut into small pieces and passed through a stainless steel press (pore diameter, 1·5 mm). The brei was homogenized in 0·25 M sucrose using a Potter–Elvehjem glass-on-teflon homogeniser with a clearance of 14–16 μm (6 strokes, speed 2500 rev/min). This procedure results in maximum breakdown of tumour cell membranes with minimum damage to cell organelles [3]. A high-speed supernatant (cytosol) preparation was obtained by centrifugation of the homogenate in a Beckman L2-65B ultracentrifuge (T50 rotor, rav. 5·9 cm) for 45 min at 4°C. Aliquots (200 μl) of cytosol (approx. 20 mg protein/ml) were incubated (16 hr at 4°C) with equal volumes of 50 mM-Tris–HCl buffer, pH 7·4, containing 5 mM-EDTA and 1 mM-dithiothreitol (Tris–HCl buffer) and also containing various concentrations of [^{3}H]oestradiol-17β (0.1–10 nmol/l) in the presence and absence of a 1000-fold higher concentration of unlabelled oestradiol-17β. After incubation, tubes were maintained at 4°C and an equal volume (400 μl) of charcoal suspension (0·5% (w/v) Norit-A, 0·05% (w/v) Dextran T-70, 0·1% (w/v) gelatin) in Tris–HCl buffer added. The resultant mixture was shaken for 60 min. at 4°C and charcoal removed by centrifugation at 800 *g* for 10 min. Aliquots (200 μl) of the supernatant containing protein-bound [^{3}H]-oestradiol-17β were added to 6 ml scintillation fluid (Aquasol, N.E.N. Chemicals GmBh) and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

The protein content of cytosol fractions was estimated using the method of Lowry, Rosebrough, Farr and Randell [4].

(*b*) *Total.* Cytosol fractions were preincubated at 4°C with varying concentrations of either oestradiol-17β or tamoxifen (0–5 nmol/l). After incubation, tubes were maintained at 4°C and excess ligand removed by charcoal adsorption. Aliquots (200 μl) of the charcoal free supernatant were incubated at either 4, 10, 15, 25 or 37°C for periods up to 24 hr with an equal volume of Tris–HCl buffer containing a saturating concentration of [^{3}H]oestradiol-17β (5 nmol/l) in the presence or absence of a 1000-fold higher concentration of unlabelled oestradiol-17β. The remaining experimental procedure was identical to that described earlier for the determination of accessible sites.

In other experiments cytosol fractions were preincubated with a saturating concentration of [^{3}H]oestradiol-17β (5 nmol/l) and reincubated with unlabelled oestradiol-17β or tamoxifen (5 nmol/l).

Determination of total and accessible cytoplasmic oestradiol-17β binding sites following in vivo *administration of either oestradiol-17β or tamoxifen.*

Two experiments were undertaken:

(*a*) Ten rats bearing 15 tumours were divided randomly into three groups (5 tumours per group). Each group received either oestradiol-17β (5 μg) or tamoxifen (free base) (100 μg) in 0·1 ml vehicle (10% (v/v) ethanol in 0·15 M NaCl) by i.v. injection. Control animals received vehicle alone. Tumour biopsy samples (approx. 200 mg of tissue) were removed aseptically from animals (anaesthetized with nembutal) at time 0 (prior to injection) and at 10, 20, 30 and 60 min. after injection. Aliquots of cytosol preparations were incubated at either 4°C for 16 hr (accessible sites) or 15°C for 2 hr (total sites) with a saturating concentration of [^{3}H]oestradiol-17β or [^{3}H]oestradiol-17β plus a 1000-fold excess of oestradiol-17β.

(*b*) Fifteen animals were divided randomly into three groups (5 tumours per group). The experimental design was as described in (*a*) except that tumour tissue was biopsied at time 0 and at 0·5, 1, 2, 4, 24 and 48 hr after injection.

RESULTS

Measurement of total and accessible cytoplasmic oestradiol-17β binding sites

The establishment of conditions for the exchange of [^{3}H]oestradiol-17β with oestradiol-17β or tamoxifen bound to a specific cytosol receptor protein involved (*a*) reduction of the interference of non-specific binding components and (*b*) determination of the optimum temperature to ensure the greatest rate of exchange with the minimum rate of degradation of specific binding sites.

(*a*) *Allowance for non-specific oestradiol-17β binding sites.* The binding of oestradiol-17β by non-specific components was a linear function of the [^{3}H]oestradiol-17β concentration (Fig. 1a). Subtraction of this non-specific binding from the value obtained in the presence of [^{3}H]oestradiol-17β alone, produces a curve demonstrating saturability of binding sites at approx. 2 nmol/l. These are termed specific sites. Analysis of the corrected values by the method of Scatchard [5] gives an apparent dissociation constant, K_D, of 230 pmol/l (Fig. 1b).

(*b*) *Temperature optimisation of incubation conditions.* Figure 2 illustrates (*a*) the rate of uptake of labelled oestradiol-17β by specific receptor proteins and (*b*) the thermostability of the

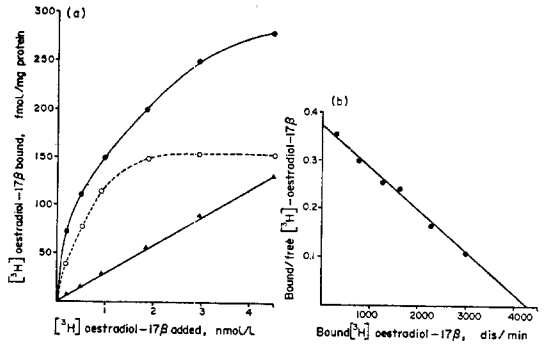


Fig. 1. Determination of the number of specific cytoplasmic binding sites for oestradiol-17β in rat mammary tumour tissue.
(a) Cytosol fractions were incubated for 16 hr at 4°C with various concentrations of (1) [3H]oestradiol-17β (●——●) or (2) [3H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β (▲——▲). Specific oestradiol-17β binding was obtained by subtraction of (2) from (1) (○ - - ○).
(b) Scatchard analysis of the corrected values.

ligand–receptor complex formed. Rapid uptake of [3H]oestradiol-17β was observed at all experimental temperatures. Determination of the number of binding sites at 10, 15 or 25°C produced levels of binding which were increased relative to the value obtained at 4°C. At these elevated temperatures, however, thermal degradation of the ligand receptor complex occurred. This was especially evident at 25°C and 37°C.

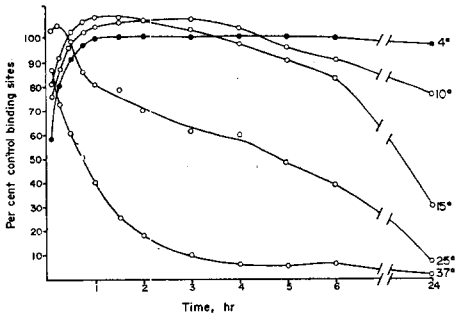


Fig. 2. Determination of binding sites—effect of temperature.
Cytosol preparations were incubated at 4, 10, 15, 25 and 37°C for periods up to 24 hr with either (1) a saturating concentration of [3H]oestradiol-17β (5·0 nmol/l) or (2) [3H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β. Specific oestradiol-17β binding was obtained by subtraction of (2) from (1). Each result is expressed as a percentage of the maximum value obtained during the incubation of cytosol with labelled steroid at 4°C.

In order to establish whether incubations at temperatures above 4°C released oestradiol-17β previously bound to receptor, cytosols were preincubated at 4°C with a saturating concentration of cold oestradiol-17β and then

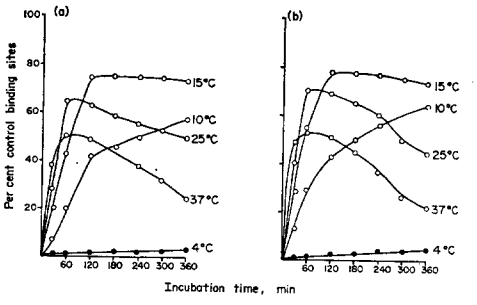


Fig. 3. Effect of temperature on the rate of exchange of bound oestradiol-17β or tamoxifen.
Cytosol preparations were preincubated for 90 min at 4°C with a saturating concentration (5·0 nmol/l) of either (a) oestradiol-17β or (b) tamoxifen. Excess ligand was removed by charcoal adsorption and the ligand-receptor complex reincubated for periods up to 360 min at 4, 10, 15, 25 or 37°C with either (1) a saturating concentration of [3H]oestradiol-17β (5·0 nmol/l) or (2) [3H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β. Specific oestradiol-17β binding was obtained by subtraction of (2) from (1). Each result is expressed as a percentage of the value obtained by incubation of cytosol (untreated) with labelled steroid at 4°C.

reassayed in the presence of [3H]oestradiol-17β. Incubations performed at temperatures above 4°C resulted in the binding of [3H]oestradiol-17β to otherwise occupied sites (Fig. 3a). The exchange was maximal at 15°C reaching 75% after a 2 hr incubation period. Similar results were obtained when cytosols were preincubated with tamoxifen (Fig. 3b). The exchange process is partially dependent upon

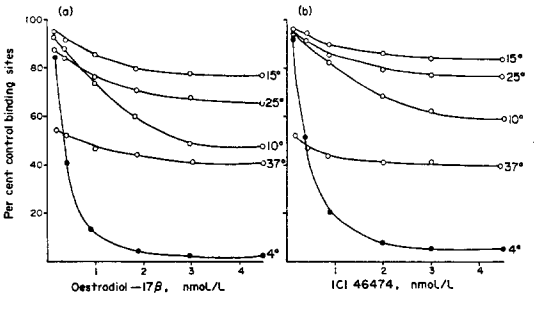


Fig. 4. Effect of temperature on the exchange of specifically bound oestradiol-17β or tamoxifen.
Cytosol preparations were preincubated for 90 min at 4°C with various concentrations of either (a) oestradiol-17β or (b) tamoxifen. Excess ligand was removed by charcoal adsorption and the ligand receptor complex reincubated for 2 hr at 4, 10, 15, 25 or 37°C with either (1) a saturating concentration of [3H]oestradiol-17β (5·0 nmol/l) or (2) [3H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β. Specific oestradiol-17β binding was obtained by subtraction of (2) from (1). Each result is expressed as a percentage of the value obtained following the incubation of cytosol (untreated) with labelled steroid at 4°C.

the concentration of bound ligand present within the incubate (Fig. 4). Exchange values approached 100% in those cytosol fractions

previously incubated with non-saturating concentrations of either tamoxifen or oestradiol-17β. Incubations carried out at 37°C gave lower levels of [^{3}H]oestradiol-17β binding than those observed at 10, 15 and 25°C.

Preincubation of cytosol preparations with a saturating concentration of [^{3}H]oestradiol-17β (Fig. 5) at 4°C and reincubation of the [^{3}H]-ligand–receptor complex with cold oestradiol-17β or tamoxifen at 15°C shows the events to be

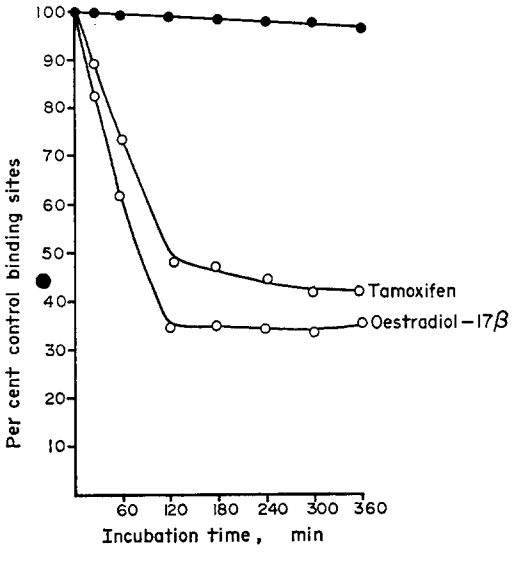


Fig. 5. Rate of exchange of bound [^{3}H]oestradiol-17β.

Cytosol preparations were preincubated for 90 min at 4°C with a saturating concentration of [^{3}H]oestradiol-17β (5·0 nmol/l). Excess [^{3}H]oestradiol-17β was removed by charcoal adsorption and the ligand receptor complex reincubated for periods up to 360 min at 4°C (●——●) or 15°C (○——○) with either oestradiol-17β (5·0 nmol/l) or tamoxifen (5·0 nmol/l). Each result is expressed as a percentage of the total number of accessible oestradiol-17β binding sites determined in the absence of the preincubation step.

a true exchange process. Under the conditions employed, approximately 60% loss of bound [^{3}H]oestradiol-17β was noted. In view of the results it seems likely that an incubation performed for 2 hr at 15°C provides a good estimation of the total number of binding sites for oestradiol-17β, while a similar incubation, performed at 4°C for 16 hr, measures only those sites which are accessible at the time of assay. These conditions of assay were used in the determination of total and accessible cytoplasmic oestradiol-17β binding sites in biopsy samples of DMBA-induced mammary tumour exposed, *in vivo*, to oestradiol-17β or tamoxifen.

Within 30 min following *in vivo* administration of oestradiol-17β (5 μg), very little binding, total or accessible, of [^{3}H]oestradiol-17β to specific binding proteins was observed (Fig. 6a). Tamoxifen, like oestradiol-17β, produced an initial decrease in receptor levels, although the effect was less pronounced (Fig. 6b). Injection of saline vehicle had no appreciable effect on either total or accessible binding sites (Fig. 6c). A secondary period of elevated oestradiol-17β binding was observed after administration of either tamoxifen or oestradiol-17β (Fig. 7). The results indicate that accessible receptor levels are considerably increased during the

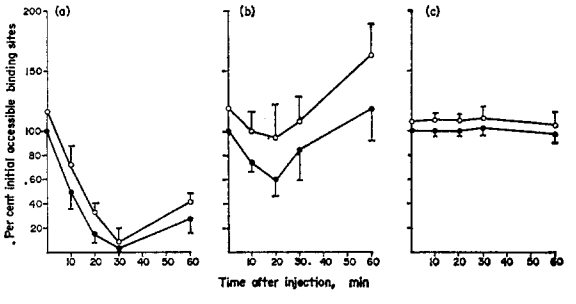


Fig. 6. Effect of in vivo *administration of oestradiol-17β and tamoxifen on the cytoplasmic levels of total and accessible oestradiol-17β binding sites.*

Rats were injected with either (a) 5 μg oestradiol-17β (b) 100 μg tamoxifen or (c) vehicle alone. Tumour biopsy samples were removed at time 0 and after 10, 20, 30 and 60 min. Cytosol preparations from the biopsies were incubated at either 4°C (accessible sites) (●) or 15°C (total sites) (○) for 120 min with a saturating concentration of [^{3}H]oestradiol-17β (5·0 nmol/l) or [^{3}H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β. Each result is expressed as a percentage of the value obtained by incubation of cytosol with labelled steroid at 4°C (accessible binding sites at time 0) and is the mean ± SEM of 5 separate tumours.

2–24 hr period following injection of oestradiol-17β, and during the 2–4 hr period following treatment with tamoxifen. Total and accessible sites behave similarly, demonstrating the presence of specifically bound oestradiol-17β or tamoxifen within the cytosol. Tamoxifen, unlike oestradiol-17β, was unable to maintain elevated receptor levels, which decreased considerably in concentration during the 24–48 hr experimental period.

DISCUSSION

This report describes the development and use of a method to determine the total number of cytoplasmic oestradiol-17β binding sites present in DMBA-induced rat mammary tumours, regardless of whether the sites are occupied by oestradiol-17β or tamoxifen. The

criteria of minimum loss of binding sites with relatively high rates of exchange (approximately 75% exchange of specifically bound ligand with [^{3}H]oestradiol-17β) were met using an assay temperature of 15°C and an incubation period of 2 hr. Incubations at temperatures above 15°C resulted in considerable degradation of the ligand-receptor complex. It is evident, therefore, that although elevated temperatures increase the rate of dissociation of the ligand from the cytoplasmic oestradiol-17β binding protein [6, 7] they have limited application to a mammary tumour assay system. Accessible binding sites for oestradiol-17β or tamoxifen were determined at 4°C. At this temperature little dissociation of ligand from the cytoplasmic receptor occurs, rates of entry of these compounds into mammary tumour cells [10, 11]. The depletion of cytoplasmic binding sites correlates with an increase in nuclear uptake of oestradiol-17β and tamoxifen [12] a step which is deemed essential for the action of oestradiol-17β [13]. Tamoxifen elicited further oestrogen-like actions resulting in increased cytosol receptor concentrations subsequent to the observed initial decrease in receptor levels. This oestrogenicity of tamoxifen probably arises as a consequence of its entry into the tumour cell nuclei. We can therefore envisage a similar mechanism by which oestradiol-17β and tamoxifen act. The primary association of either ligand with the receptor protein causes translocation of the complex to the nucleus, thus

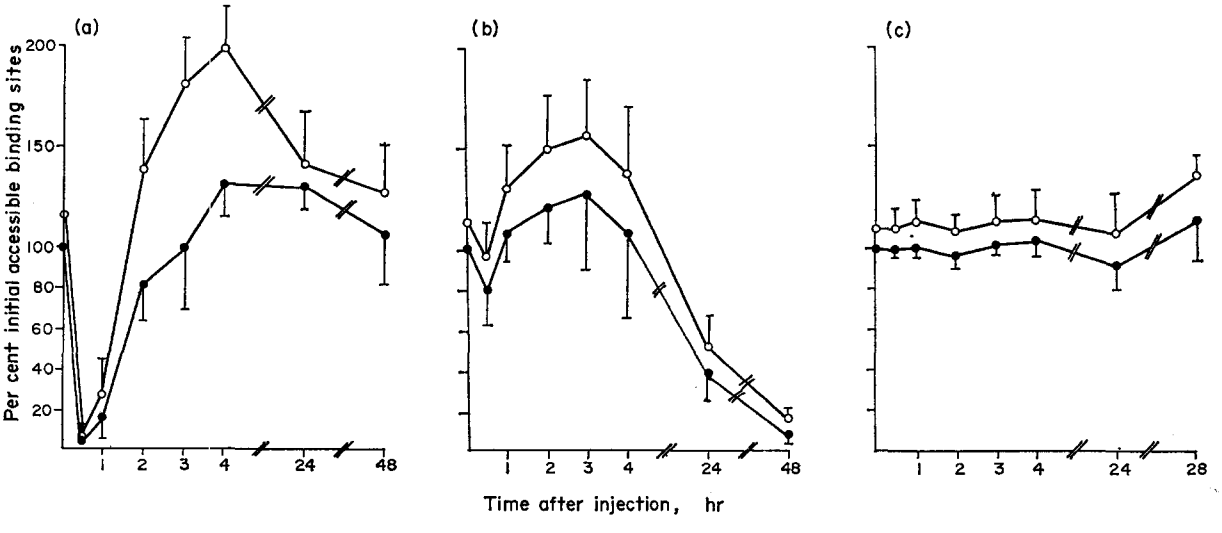


Fig. 7. Effect of in vivo *administration of oestradiol-17β and tamoxifen on the cytoplasmic levels of total and accessible oestradiol-17β binding sites.*

The experimental design was as described in the legend to Fig. 6. except that tumour tissue was biopsied at time 0 and after 0·5, 1, 2, 4, 24 and 48 hr.

a situation similar to that shown in rat uterine fractions [6–8].

Previously, it has been reported that the administration of tamoxifen and oestradiol-17β for 6 days to intact animals reduced the capacity of the DMBA-induced rat mammary tumour to bind [^{3}H]oestradiol-17β *in vitro* [9]. It was then not possible to decide whether this was due to a loss of binding protein or to a saturation of the available binding sites by the ligands. The present study extends the initial observations to include the early actions of oestradiol-17β and tamoxifen on receptor protein concentrations. Tamoxifen, like oestradiol-17β, caused an initial decrease in total and accessible cytoplasmic binding sites for [^{3}H]oestradiol-17β. The effect was more pronounced with oestradiol-17β and may reflect the relative depleting cytoplasmic receptor concentrations. A further association of the complex with acceptor sites in chromatin could increase transcription of the modified genome, resulting in the production of messenger RNA species coding for specific proteins, including the receptor for oestradiol-17β. The administration *in vivo* of either substance brings about increased activity of RNA polymerase enzyme B in mammary tumour nuclei [12], correlating with the nuclear uptake of the ligand–receptor complex. Any quantitative changes in response to oestradiol-17β and tamoxifen may be a reflection of the relative ability of the substances to bring about transformation of the receptor to an active form, translocation to nuclei and association with specific nuclear sites.

The present studies, utilizing the total oestradiol-17β binding site assay, demonstrates the presence of considerable quantities of specifically bound ligand in cytosol fractions following the administration of either tamoxifen or oestradiol-17β. Noteboom and Gorski [14] and King and Gordon [15] have described a dual intracellular incorporation of labelled oestrogens in rat uteri following oestrogen treatment, observing that although most of the hormone was found in the nuclear fraction, a considerable portion (20–30%) was present in the high-speed supernatant or cytosol fraction. No further characterization into specific or non-specifically bound oestradiol-17β was undertaken by these authors. The biological significance of the specifically bound ligand present in cytosol fractions of DMBA-induced rat mammary tumours is not clear. It may represent a pool of ligand capable of eliciting gene expression if translocation of the ligand receptor complex to the nucleus occurs. Alternatively, it may be defective ligand bound receptor present in either a pre- or post-nuclear form. Sucrose density gradient centrifugation of the bound material together with an examination of the characteristics of the ligand receptor complex in a reconstituted cell free system, such as that described by Davies and Griffiths [16] for rat ventral prostate, may resolve the significance of this material to the cell.

Our observations suggest that although certain quantitative differences are noted between the early actions of tamoxifen and oestradiol-17β, the compounds show an initial similarity of response. This similarity is not prolonged. Tamoxifen, unlike oestradiol-17β, is unable to maintain elevated receptor concentrations during the remaining experimental period, as reported also by Clark, Anderson and Peck, [17] and Rochefort and Capony [18] for nafoxidine and for CI628 by Katzenellenbogen and Furguson [8] in rat uteri. In addition the latter authors observed that CI628 induced oestrogen-like actions which included the synthesis of a specific uterine protein and elevated uterine weights. No elevation in accessible receptor concentrations was, however, noted. It is apparent that the anti-oestrogen, tamoxifen, like nafoxidine and CI628 in rat uteri, is unable to sustain the replenishment of the cytoplasmic receptor in DMBA-induced rat mammary tumours. Although the reasons for this are not readily obvious, such actions would clearly induce tumour regression and is therefore consistent with our previous findings [9].

Research is now under way to investigate the action of tamoxifen in both nuclear and cytoplasmic compartments. Such data may elucidate the oestrogen-like and oestrogen antagonistic actions of tamoxifen.

Acknowledgements—The authors wish to thank the Tenovus Organization and the M.R.C. for their generous financial support, also Dr. D. A. Walpole and Dr. D. N. Richardson (I.C.I. Ltd.) for the gift of tamoxifen.

REFERENCES

1. E. V. Jensen, H. I. Jacobson, S. Smith, P. W. Jungblut and E. R. DeSombre, The use of estrogen antagonists in hormone receptor studies. *Gynec. Invest.* **3,** 108 (1972).
2. R. I. Nicholson, M. P. Golder, P. Davies and K. Griffiths, Effects of oestradiol-17β and tamoxifen on total and accessible cytoplasmic oestradiol-17β receptors in DMBA-induced rat mammary tumours. *J. Endocr.* (In press).
3. R. I. Nicholson and M. Davies, Distribution and some properties of acid phosphatase in the 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinoma. *Europ. J. Biochem.* **44,** 25 (1974).
4. D. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randell, Protein measurements with the Folin–phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
5. G. Scatchard, The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).
6. J. Mester and D. M. Robertson, The determination of the dissociation velocity constants of rabbit uterine cytosol receptor-oestrogen complexes . *Biochim. biophys. Acta,* **230,** 543 (1971).
7. B. M. Sanborn, B. B. Rao and S. G. Korenman, Interaction of oestradiol-17β and its specific uterine receptor. Evidence for complex kinetic and equilibrium behavior. *Biochemistry* **10,** 4955 (1971).
8. B. S. Katzenellenbogen and E. R. Furguson, Antioestrogen action in the uterus: Biological ineffectiveness of nuclear bound oestradiol-17β after anti-oestrogen. *Endocrinology* **97,** 1 (1975).

9. R. I. NICHOLSON and M. P. GOLDER, The effect of synthetic antioestrogens on the growth and biochemistry of rat mammary tumours. *Europ. J. Cancer* **11,** 571 (1975).
10. W. POWELL-JONES, P. DAVIES and K. GRIFFITHS, Influence of antioestrogens on specific binding of [^{3}H]oestradiol-17β *in vitro* by nuclei from rat mammary tumours. *J. Endocr.* **66**· 437 (1975).
11. W. POWELL-JONES, D. A. JENNER, R. W. BLAMEY, P. DAVIES and K. GRIFFITHS, Influence of antioestrogens on the specific binding *in vitro* of [^{3}H]oestradiol-17β by cytosols of rat mammary tumours and human breast carcinomata. *Biochem. J.* **150,** 71 (1975).
12. P. DAVIES, R. I. NICHOLSON and K. GRIFFITHS, (Submitted for publication) The effects of oestradiol-17β and tamoxifen on RNA polymerase activities of DMBA-induced mammary tumor nuclei.
13. E. V. JENSEN and E. R. DESOMBRE, Estrogen–receptor interaction. *Science* **182,** 126 (1973).
14. W. D. NOTEBOOM and J. GORSKI, Stereospecific binding of oestrogens in rat uterus. *Arch. Biochem. Biophys.* **111,** 559 (1965).
15. R. J. B. KING and J. GORDON, The localization of [6,7,-^{3}H]-oestradiol-17β in rat uterus. *J. Endocr.* **34,** 431 (1966).
16. P. DAVIES and K. GRIFFITHS, Further studies on the stimulation of prostatic ribonucleic acid polymerase by 5α-dihydrotestosterone–receptor complexes. *J. Endocr.* **62,** 385 (1974).
17. J. H. CLARK, J. N. ANDERSON and E. J. PECK, Estrogen receptor. Anti-estrogen complex: atypical binding by uterine nuclei and effects on uterine growth. *Steroids* **22,** 707 (1973).
18. A. H. ROCHEFORT and F. CAPONY, Etude comparée du comportement d'un anti-oestrogène et de l'oestradiol dans les cellules utérines. *C.R. Acad. Sci. Paris,* **276,** 2321 (1973).

Europ. J. Cancer Vol. 12, pp. 719–723. Pergamon Press 1976. Printed in Great Britain

Plasma Hormones in Patients with Advanced Breast Cancer Treated with Tamoxifen*

MERIEL P. GOLDER†, M. ELIZABETH A. PHILLIPS†, D. R. FAHMY†, P. E. PREECE‡, V. JONES§, J. M. HENK§ and K. GRIFFITHS†

† *Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff*, CF4 4XX, *Wales*

Abstract—*Plasma concentrations of prolactin, FSH, LH and oestradiol-17β were measured in patients with advanced breast cancer being treated with tamoxifen. Plasma FSH levels dropped during the first month of treatment in patients who subsequently responded to therapy and in those who did not. After prolonged therapy plasma FSH in the responders returned to pre-treatment levels whereas those in the non-responders remained lower than the pre-treatment value. LH levels were lowered during tamoxifen therapy but there was no significant difference between the two group of patients. Plasma prolactin and oestradiol-17β concentrations were unaffected by treatment.*

INTRODUCTION

ALTHOUGH it is well recognized that a large proportion of patients with recurrent breast cancer require palliative treatment, only approximately one third of these respond to hormone therapy, whether additive or ablative. The selection of the type of treatment given is largely empirical and a more scientific basis is required.

Little is known regarding the biochemical effects of the hormonal therapy regimes which are used although it is accepted that these may involve a suppression of pituitary hormone secretion, a direct action on the tumour cells or an enhancement of the immune competence of the patient. Although a number of studies have been undertaken in an attempt to provide a scientific basis for the selection of therapy [1, 2] little is known about the endocrine changes that occur during the treatment of patients with various hormone regimes or of the plasma hormone concentrations of those who respond to therapy compared to those who do not.

In this study, plasma concentrations of prolactin, follicle stimulating hormone (FSH), luteinizing hormone (LH) and oestradiol-17β have been studied in patients with advanced breast cancer treated with the anti-oestrogen tamoxifen, (I.C.I. 46474). This compound has been used extensively in the treatment of the disease in recent years by other groups [3, 4]. There are however no reports of studies of plasma hormone changes in treated patients.

MATERIAL AND METHODS

Patients

Studies were carried out on patients attending the Combined Breast Clinic of the Departments of Surgery and Radiotherapy. All patients included in the study had either local or general carcinoma of the breast. They were either post-menopausal (in all cases more than two years since the last menstrual period) or had undergone oophorectomy. Thirty patients were treated with tamoxifen and of these 24 had sufficient blood samples taken for analysis. None had previously received any hormone treatment nor had undergone ablative endocrine surgery which would require maintenance therapy. None had received cytotoxic drugs.

Blood samples were taken from the patient on the morning before the commencement of treatment. Subsequent samples were taken at 7 and 14 days and at one, two and three months later.

Accepted 22 March 1976.
*Supported by the Tenovus Organization.
‡Department of Surgery, University Hospital of Wales.
§Velindre Hospital, Whitchurch, Cardiff, United Kingdom.

Treatment

Patients were given tamoxifen (trans- (p-β-dimethylamino-ethoxyphenyl) - 1, 2 - diphenyl-but-1-ene, Nolvadex, I.C.I. Ltd., Macclesfield, Cheshire) 20 mg twice a day for a minimum of one month.

Hormone assays

Blood was collected into lithium sequestrene tubes, centrifuged at 4°C, separated, and the plasma stored at −20°C until assayed.

Protein hormones

Prolactin was assayed by a double antibody radioimmunoassay using the method described by Cole & Boyns [5]. Results are expressed in terms of MRC Standard 71/222 and 1 mU is equal to 50 ng in this assay system. LH and FSH were assayed using methods described previously [6], LH is expressed in terms of MRC Standard 68/40 and FSH in terms of 2nd 1RP–HMG where 1 mIU is equivalent to 230 ng.

Plasma oestradiol-17β

Oestradiol-17β was determined by a RIA procedure, using an antiserum raised against an oestradiol-6-[O-carboxymethyl]-oxime bovine serum albumin conjugate. Steroids were extracted with diethyl ether (the recoveries ranged from 95–105%) and no purification procedures were incorporated in the assay since results, with and without chromatography, showed no significant difference. Free and bound steroid were separated using dextran-coated charcoal and the free steroid in a 500 μl aliquot dissolved in 5 ml Aquasol (NEN Chemicals) and counted in a liquid scintillation counter.

Data analysis

The clinical response to tamoxifen was assessed three months after beginning therapy according to the criteria described by Forrest [7], and patients were grouped as responders or non-responders. Their pretreatment plasma hormone values were compared and are presented as median and range because the distributions for prolactin and oestradiol-17β were markedly skewed. Within subjects, however, hormone concentrations approximated to a Gaussian distribution so that transformation of the data was not warranted. In order to minimize between subject variation hormone concentrations were first normalized by expressing each concentration as a fraction of the average for an individual. For both groups of patients at each time interval mean ± S.E.M. was calculated and the figures then scaled to make the pretreatment value 100.

RESULTS

Three months after the start of treatment all patients were assessed clinically and classified as responders, non-responders or those with an equivocal response. For the purpose of analysis the two patients assessed as equivocal responders were grouped with the non-responders. The distribution of sites of the disease with which the patient presented was similar between the two groups of patients (Table 1). There were no differences between the ages, years post-menopausal and cancer free intervals in all the patients studied (Table 2).

Table 1. *Distribution of disease type in patients treated with tamoxifen*

Disease type	Number of patients	
	Responders	Non-responders
Local	5	5
General	1	2
Local and General	4	7
Total	10 (42%)	14 (58%)

Patients were assessed 3 months after start of treatment and classed as responders or non-responders.

Table 3 shows the pre-treatment plasma hormone concentrations of the patients treated with tamoxifen. The results indicate that the nature of the response of the patients to the anti-oestrogen treatment is not determined by differences in the hormonal status of the patient before commencement of hormone therapy.

Plasma prolactin levels in the patients were unaffected by tamoxifen treatment (Fig. 1a). Although there was a wide range of concentrations before the start of therapy in both responders and non-responders, some patients having high prolactin levels and others very low levels, the values in the individual patients did not alter significantly during the treatment time and there was no difference between the two groups of patients. Plasma oestradiol-17β concentrations were also unaffected by tamoxifen treatment in either group of patients (Fig. 1b).

Gonadotrophin levels during treatment are shown in Fig. 2. Plasma FSH decreased in the

Table 2. Distribution of patients treated with tamoxifen

	N	Age (yr)	Time since L.M.P. (yr)	Cancer free Interval (yr)
Responders	10	64·5 (56–80)	14 (2–40)	2·0 (0–12)
Non-responders	14	61·5 (54–82)	14 (3–29)	1·5 (0–6)

Patients were assessed 3 months after starting treatment and classed as responders or non-responders.
Values are expressed as the median and range.
N = number of patients in each sub-group.
There are no differences between responders or non-responders.
LMP = Last Menstrual period.
0 = Patients who presented with advanced disease.

Table 3. Plasma Hormone concentrations before starting treatment with tamoxifen

		Plasma hormones			
	N	Prolactin (mU/ml)	Oestradiol-17β (pg/ml)	LH (mIU/ml)	FSH (mIU/ml)
Responders	10	0·13 (0·04–0·98)	27 (16–52)	25 (15–48)	72 (33–112)
Non-responders	14	0·21 (0·04–0·41)	25 (16–52)	36 (17–50)	80 (28–124)

Results are expressed as Median and range.
There are no differences between responders and non-responders.

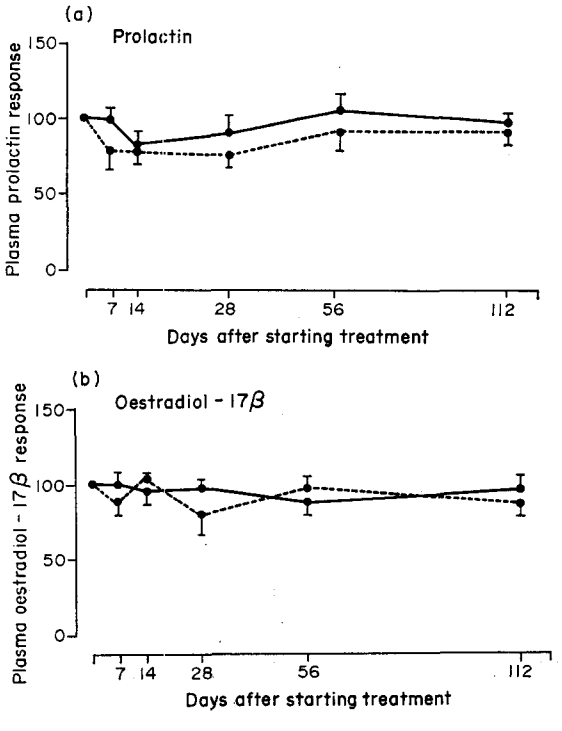


Fig. 1. Response of plasma prolactin and oestradiol-17β to tamoxifen therapy in responding and non-responding breast cancer patients.
Values are expressed as mean ± S.E.M. of normalised data. (see text for details of analysis).
● – – – ● *responders* ●——● *non-responders*

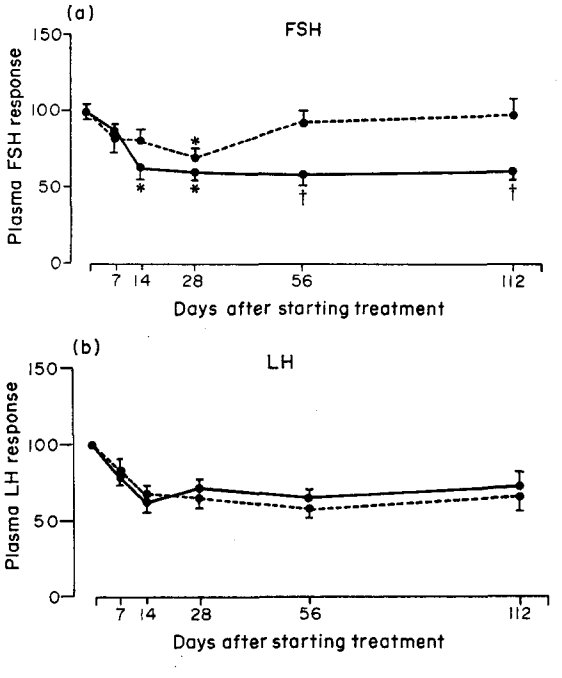


Fig. 2. Response of plasma FSH and LH to tamoxifen therapy in responding and non-responding breast cancer patients.
Values are expressed as mean ± S.E.M. of normalised data. (see text for details of analysis).
● – – – ● *responders* ●——● *non-responders*
* *V FSH pretreatment value* $P < 0.05$
† *Non-responders v. responders* $P < 0.05$

first week of tamoxifen therapy and at one month was significantly lower ($P < 0{\cdot}05$) than the pretreatment level in both responders and non-responders. However, within 2 and 3 months of the start of treatment, plasma FSH concentration in patients responding to tamoxifen had returned to the value found before tamoxifen treatment started, whereas in the non-responders, hormone concentrations were significantly lower ($P < 0{\cdot}05$) than the basal level throughout the course of therapy. There was a significant difference ($P < 0{\cdot}05$) between the FSH levels in the responders and non-responders in the second and third month of treatment.

By contrast, there was no difference between LH concentrations in the two groups of patients (Fig. 2b). After 1 week of treatment, plasma LH had decreased in both groups and remained significantly lower than the pre-treatment value over the three-month period.

DISCUSSION

The remission rate of approximately 40% which was induced by tamoxifen treatment in this series of patients is similar to that obtained by other groups who have also used this agent in the treatment of advanced breast cancer [3, 4]. This proportion of patients showing an objective response is comparable with the results of other studies from this group using other single hormone agents [8]. Using clomiphene, a compound closely related chemically to tamoxifen, a response rate of approximately 40% has also been observed [9].

Evidence from animal studies suggests that the rat mammary tumour, induced by the administration of dimethylbenz-anthracene is dependent on the pituitary hormone prolactin [10, 11]. Although Murray [12] demonstrated elevated plasma prolactin levels in post-menopausal women with advanced breast cancer, other studies from the Tenovus Institute have, however, failed to demonstrate such a difference or any relationship between plasma prolactin concentration and breast disease [13, 14].

In this study, neither prolactin concentrations nor oestradiol-17β levels altered during the course of tamoxifen therapy. The plasma prolactin concentration before treatment ranged from low normal through to high normal values but in each patient levels were not affected by treatment. This result appears to be in contrast to the action of tamoxifen in the pre-menopausal woman, when tamoxifen administration caused a decrease in plasma prolactin with a subsequent increase in oestradiol-17β concentration [15].

Plasma FSH concentrations were lowered during the first week of treatment with tamoxifen in patients who subsequently responded to therapy and in those who did not. Significantly reduced levels were maintained during the first month of treatment but after prolonged therapy, plasma FSH in the responders returned to pre-treatment levels whereas those in the non-responders remained lower than the pretreatment value. At two and three months after the start of tamoxifen administration, FSH concentrations, in the group of non-responders remained significantly lower than in the responders.

Plasma FSH levels are also affected in a similar way when patients with advanced breast cancer were treated with clomiphene [9]. Both these compounds are generally regarded to be anti-oestrogens although the mechanism of action of neither is fully understood. Although tamoxifen is known to affect the binding of oestradiol-17β to the cytoplasmic receptor protein in both the rat DMBA-tumour [16] and in human breast carcinoma [17], it would also seem to have some action on the hypothalamic–pituitary axis. The response of the rat to antioestrogen treatment appears to be dependent on dose since a large amount of tamoxifen administered over a long period of time can be oestrogenic and cause an elevation of prolactin levels (R. Nicholson & M. P. Golder, unpublished observations) whereas lower doses are anti-oestrogenic [18, 19].

In view of the use of tamoxifen in pituitary–gonodal function tests in the pre-menopausal woman it will be interesting to study in greater depth pituitary function in post-menopausal women.

Acknowledgements—We thank Miss K. Baker for expert technical assistance.

We are grateful to Professor L. E. Hughes, Mr. M. Baum, and Dr. T. Priestman for allowing us to study patients under their care.

REFERENCES

1. R. D. Bulbrook and J. L. Hayward, Abnormal urinary steroid excretion and subsequent breast cancer. *Lancet* **i**, 519 (1967).

2. E. R. DE SOMBRE, S. SMITH, G. E. BLOCK, D. J. FERGUSON and E. V. JENSEN, Prediction of breast cancer response to endocrine therapy. *Cancer Chemother. Rep.* p. 39, (1974).
3. M. P. COLE, C. T. A. JONES and I. D. H. TODD, A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of I.C.I. 46474. *Brit. J. Cancer* **25,** 270 (1971).
4. H. W. C. WARD, Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Brit. med. J.* **1,** 13 (1973).
5. E. N. COLE and A. R. BOYNS, Radioimmunoassay for human pituitary prolactin using antiserum against an extract of human amniotic fluid. *Horm. Res.* **4,** 261 (1973).
6. G. V. GROOM, M. A. GROOM, I. D. COOKE and A. R. BOYNS, The secretion of immunoreactive luteinizing hormone and follicle stimulating hormone by the human foetal pituitary in organ culture. *J. Endocr.* **49,** 335 (1971).
7. A. P. M. FORREST, In: *The Clinical Management of Advanced Breast Cancer*, p. 88, (Edited by C. A. F. JOSLIN and E. N. GLEAVE). Alpha Omega Alpha, Cardiff (1970).
8. V. JONES, A prospective trial of oestrogens, androgens and progestogens for the treatment of advanced carcinoma of the breast. In: *The Clinical Management of Advanced Breast Cancer*, p. 24, (Edited by C. A. F. JOSLIN and E. N. GLEAVE). Alpha Omega Alpha, Cardiff (1970).
9. M. P. GOLDER, M. E. A. PHILLIPS, P. E. PREECE, V. JONES, C. A. F. JOSLIN and K. GRIFFITHS, Submitted for publication.
10. O. H. PEARSON, R. L. MURRAY, G. MOZAFFERIAN and J. PENSKY, Prolactin and experimental breast cancer. In: *Prolactin and Carcinogenesis*, p. 154, (Edited by A. R. BOYNS and K. GRIFFITHS). Alpha Omega Alpha, Cardiff (1972).
11. A. R. BOYNS, R. BUCHAN, E. N. COLE, A. P. M. FORREST and K. GRIFFITHS, Basal prolactin blood levels in three strains of rats with differing incidence of 7,12,dimethylbenz(a)anthracene-induced mammary tumours. *Europ. J. Cancer* **9,** 169 (1973).
12. R. M. L. MURRAY, G. MOZAFFERIAN and O. H. PEARSON, Prolactin levels with L-Dopa treatment in metastatic breast carcinoma. In: *Prolactin and Carcinogenesis*, p. 158. (Edited by A. R. BOYNS and K. GRIFFITHS). Alpha Omega Alpha, Cardiff (1972).
13. A. R. BOYNS, E. N. COLE, K. GRIFFITHS, M. M. ROBERTS, R. BUCHAN, R. G. WILSON and A. P. M. FORREST, Plasma prolactin in breast cancer. *Europ. J. Cancer* **9,** 99 (1973).
14. R. G. WILSON, R. BUCHAN, M. M. ROBERTS, A. P. M. FORREST, A. R. BOYNS, E. N. COLE and K. GRIFFITHS, Plasma prolactin and breast cancer. *Cancer (Philad.)* **33,** 1325 (1974).
15. G. V. GROOM and K. GRIFFITHS, *J. Endocr.* (1976). In press.
16. W. POWELL-JONES, D. A. JENNER, R. W. BLAMEY, P. DAVIES and K. GRIFFITHS, Influence of anti-oestrogens on the specific binding in vitro of [^{3}H]oestradiol by cytosol of rat mammary tumours and human breast carcinomas. *Biochem. J.* **150,** 71 (1975).
17. V. C. JORDON and S. KOERNER, Tamoxifen (I.C.I. 46474) and the human carcinoma 8S oestrogen receptor. *Europ. J. Cancer* **11,** 205 (1975).
18. V. C. JORDON, S. KOERNER and C. ROBISON, Inhibition of oestrogen stimulated prolactin release by anti-oestrogens. *J. Endocr.* **65,** 151 (1975).
19. R. I. NICHOLSON and M. P. GOLDER, The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Europ. J. Cancer* **11,** 571 (1975).

Europ. J. Cancer Vol. 12, pp. 725–735. Pergamon Press 1976. Printed in Great Britain

Breast Cancer in Britain and Japan: Plasma Oestradiol-17β, Oestrone and Progesterone, and Their Urinary Metabolites in Normal British and Japanese Women*

R. D. BULBROOK,† MARGARET C. SWAIN,† D. Y. WANG,† J. L. HAYWARD,‡ S. KUMAOKA,§ O. TAKATANI,§ O. ABE¶ and J. UTSUNOMIYA‖

†*Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX, U.K.*, ‡*ICRF Breast Cancer Unit, Guy's Hospital, London, SE1 9RT, U.K.*, §*National Cancer Center, Tokyo, Japan*, ¶*Keio University, Tokyo, Japan*, ‖*Tokyo Medical and Dental University, Tokyo, Japan*

Abstract—*The plasma concentrations of oestrone, oestradiol-17β and progesterone, and the urinary excretion of oestrone, oestradiol-17β, oestriol and pregnanediol have been determined in normal Japanese and British adolescent, pre-menopausal, menopausal and post-menopausal women.*

The amounts of plasma oestrogen in British and Japanese women were not significantly different except in the adolescent group. In this latter category the mean level of plasma oestrone and oestradiol-17β in the luteal phase was significantly higher in Japanese compared with British women. The mean excretion of urinary oestrogens in the two races was similar for all age groups studied, as was plasma progesterone and urinary pregnanediol.

The mean ratio of oestriol to oestrone and oestradiol-17β for Japanese was higher than that for British women in the two pre-menopausal groups, although the difference was not statistically significant. The difference in the incidence rates of breast cancer in Britain and Japan does not appear to be related to differences in plasma levels of oestrogens or progesterone.

INTRODUCTION

THE INCIDENCE of breast cancer and the death rate from the disease is lower in Japan than in Western countries, particularly in post-menopausal women [1, 2]. Some aspects of the histology of the tumour and the clinical course of the disease in Japan also appear to be different from those found in the United States of America or Britain [3–5]. The reasons for these differences are unknown but it has been suggested that they are associated with differences in endocrine function [6].

In 1968, a collaborative study was established between the Imperial Cancer Research Fund in London and the National Cancer Center in Tokyo with the aim of making a systematic comparison of breast cancer in Britain and Japan. The main themes in this study were, first, a detailed examination of endocrine function in normal women of both races and in patients with early or advanced breast cancer; second, a comparison of the clinical features of the disease in Japanese and British patients in terms of antecedent history, presentation of the tumour, survival after treatment of the early disease and response to hormone therapy, and third, a detailed comparison of the histology of the tumour.

This paper describes the results of a comparative study of the peripheral blood levels of the female sex hormones in normal Japanese and

Accepted 19 March 1976.

*This study was supported in part by a grant for Cancer Research from the Ministry of Health and Welfare, Japan.

British women and the amounts of the principal metabolites of these hormones in the urine.

MATERIAL AND METHODS

1. *Normal volunteers*

Two groups of normal pre-menopausal women were investigated in each country. They comprised 23 British and 26 Japanese girls aged from 16 yr to less than 19 yr old and 28 Japanese and 30 British women aged 35 yr or more. All volunteers for the study claimed that they were menstruating regularly.

Medical and Dental University Hospital or were housewives living in suburban areas of Tokyo. The British volunteers were staff members of the Imperial Cancer Research Fund, or lived on the Island of Guernsey. All were approached by personal contact.

Due to the vagaries of laboratory organisation, various endocrine assays were not always carried out on the total group. The numbers of assays performed in any sub-set are given in the text.

Some of the characteristics of the study populations are shown in Table 1.

Table 1. *Characteristics of British and Japanese study populations*

Population	No.	Age (yr)	Height (cm)	Weight (kg)	Stated cycle length	Day of cycle blood taken	Days to next cycle	No. in luteal phase
British pre-menopausal	30	42 ± 4	161 ± 6	60 ± 9	28 (24–38)	18 (15–22)	10 (2–20)	25
Japanese pre-menopausal	28	43 ± 5	151 ± 5	53 ± 6	29 (21–40)	21 (15–36)*	9 (1–17)	17
British adolescent girls	23	17 ± 0·6	162 ± 7	55 ± 6	28 (19–35)	18 (16–21)	—	6
Japanese adolescent girls	26	17·9 ± 0·4	159 ± 6	53 ± 7	29 (25–35)	23 (19–30)	8 (3–18)	11
British menopausal	20	51.5 ± 4	160 ± 8	64 ± 9	—	—	—	—
Japanese menopausal	19	53 ± 4	151 ± 5	51 ± 8	—	—	—	—
British post-menopausal	30	59 ± 5	160 ± 5	63 ± 6	—	—	—	—
Japanese post-menopausal	35	60 ± 6	148 ± 5	53 ± 8	—	—	—	—

Standard deviations are shown for age, height and weight.

*Two women in this group reported that they were 4 m and 2 m after their last periods. The mean range shown refers to the remaining 26 women.

Menopausal women of both races were defined as those whose last menstrual period (LMP) was more than six months but not more than 5½ yr before the date of entry to the study. Post-menopausal women were defined as women whose LMP was more than 5½ yr from entry. There were 20 menopausal British and 19 menopausal Japanese women, and 30 post-menopausal British and 35 post-menopausal Japanese volunteers.

Women with previous or current endocrine or cardiovascular disease or who were using contraceptive steroids (currently or within the preceding year) were excluded. The Japanese volunteers were staff members of the Tokyo

There are no significant differences in the mean ages except for the adolescent girls where the Japanese group are almost 1 yr older than the British. Their mean weights and heights are not different from the British adolescent girls but in all other groups, the Japanese women are significantly shorter and lighter than the British. Note also that the Japanese adolescents are some 8–11 cm taller than the other Japanese groups: the difference is significant.

2. *Collection of urine and blood specimens*

According to the protocol, blood and urine specimens were supposed to be collected

between the 16th and 24th day of the menstrual cycle, in the presumptive luteal phase.

On the first day of the collection (i.e. the 16th to 20th day of the cycle) a 50 ml specimen of blood was taken into a heparinised vacutainer, between 11.00 and 17.00 hours. The red cells were removed by centrifugation and the plasma stored at −20°C. On days 2–5, three consecutive 24 hr urine specimens were collected and stored at +4°C. When the collection of the third specimen was completed, the urines were pooled, taken to pH 6·5–6·8 with 5 N HCl and stored at −20°C until analysed. The Japanese plasma and urine specimens were packed in dry ice and sent to London by air for analysis. On day 6, after an overnight fast, the pre-menopausal group (aged > 35 yr) were given an oral dose of L-tryptophan (0·03 g/k of body weight) and an 8 hr specimen of urine collected for determination of tryptophan metabolites. The results of this study will be described subsequently.

Pre-menopausal volunteers were asked to supply details of the dates of their menstruation subsequent to the collection of blood and urine so that there was a second check on the timing of the collection within the cycle. For the menopausal and post-menopausal women a blood specimen was taken followed by three 24 hr urine specimens and the tryptophan test.

3. *Analytical methods*

Plasma oestradiol-17β and oestrone were measured by the method of Korenman, Perrin and McCallum [7] as modified by Wang and Swain [8]; progesterone by the method of Swain [9] and urinary pregnanediol by the method of Moore and Cooper [10]. Urinary oestrogens were measured by the method of Salokangas and Bulbrook [11] except that tritiated standards were added to the urine after hydrolysis to measure recovery and all results were corrected to 100% recovery.

While the results in the tables are given as arithmetical means ± standard deviation to facilitate comparisons with other workers' values (which are usually given in this form) the logarithms of the steroid values were taken for all statistical analyses.

RESULTS

1. *Plasma and urinary oestrogens in normal British and Japanese pre-menopausal women aged over 35 yr.*

(*a*) *Plasma.* The mean plasma level of oestradiol-17β was 109 pg/ml in British women and 103 pg/ml in Japanese women. The corresponding mean plasma oestrone levels were 72 pg/ml and 61 pg/ml, respectively (Table 2). The individual values are shown in Fig. 1. The variation of values between subjects is very large and the small differences between the means are clearly not significant.

A second comparison was made in which only women with evidence of normal luteal function (i.e., plasma progesterone levels > 3 ng/ml) were considered. Twenty-five of the 30 British women and 17 of the 26 Japanese women were in this category. The mean plasma oestradiol-17β and oestrone levels in these women were similar in the two races and did not differ significantly (Table 2).

(*b*) *Urine.* The amounts of oestrone, oestradiol-17β and oestriol excreted in the urine by British and Japanese women are shown in

Table 2. Plasma oestradiol-17β and oestrone, and urinary oestrone, oestradiol-17β and oestriol in normal British and Japanese pre-menopausal women aged more than 35 years

	All subjects		Definitive luteal phase	
Hormone	British	Japanese	British	Japanese
Plasma oestradiol	109 ± 56 (N = 29)	103 ± 91 (N = 24)	105 ± 45 (N = 24)	130 ±97 (N = 15)
Plasma oestrone	71 ± 27 (N = 29)	61 ± 48 (N = 14)	68 ± 21 (N = 24)	72 ± 56 (N = 8)
Urinary oestrone	9·1 ± 3·5 (N = 30)	8·8 ± 4·7 (N = 27)	9·4 ± 3·5 (N = 25)	9·1 ± 4·2 (N = 17)
Urinary oestradiol	3·3 ± 1·5 (N = 30)	2·8 ± 1·9 (N = 27)	3·5 ± 1·5 (N = 25)	3·2 ± 2·0 (N = 17)
Urinary oestriol	11·4 ± 7·5 (N = 30)	12·7 ± 9·6 (N = 27)	12·0 ± 8·0 (N = 25)	14·0 ± 7·3 (N = 17)

Plasma oestrogens expressed in pg/ml. Urinary oestrogens in μg/24 hr. The arithmetic means ± standard deviations are shown and the number of subjects are given in parenthesis.

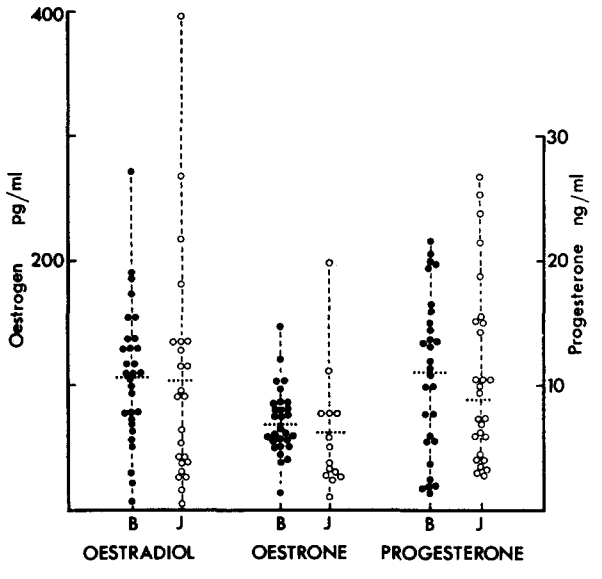


Fig. 1. *Plasma oestrone, oestradiol, and progesterone in normal British (B) and Japanese (J) pre-menopausal women. The dotted horizontal lines show the arithmetic means.*

Fig. 2. The most striking feature of these results is the close similarity between the amounts of oestrogens excreted by the two races. The mean amounts do not differ significantly. If women who were not in the luteal phase of the cycle are excluded, the mean amounts of urinary oestrone, oestradiol-17β and oestriol are almost identical in the two groups (Table 2).

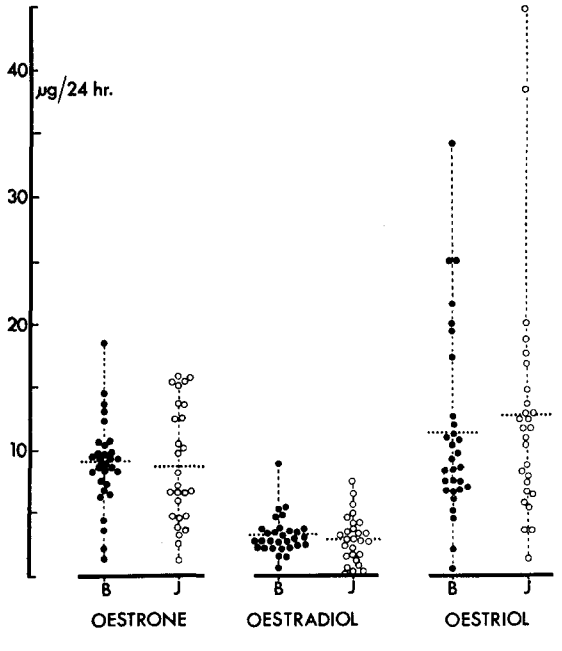


Fig. 2. *Urinary oestrone, oestradiol, and oestriol excretion of normal British (B) and Japanese (J) pre-menopausal women. The dotted horizontal lines show the arithmetic means.*

2. *Plasma and urinary oestrogens in normal British and Japanese girls aged* 16 to 18 *yr*

(*a*) *Plasma.* The mean plasma oestradiol-17β levels in British and Japanese girls were 140 and 133 pg/ml respectively, and the mean levels of plasma oestrone differed only by 9 pg/ml (76 pg/ml for British and 85 pg/ml for Japanese girls). The individual values are shown in Fig. 3. The variability is large and the small differences between the means are not significant (Table 3).

Only 6 of the 23 British volunteers had plasma progesterone levels in excess of 3 ng/ml, compared with 11 of the 26 Japanese girls. The mean plasma oestrogen level in the Japanese adolescents with evidence of normal luteal function was significantly higher than in the British group (Table 3).

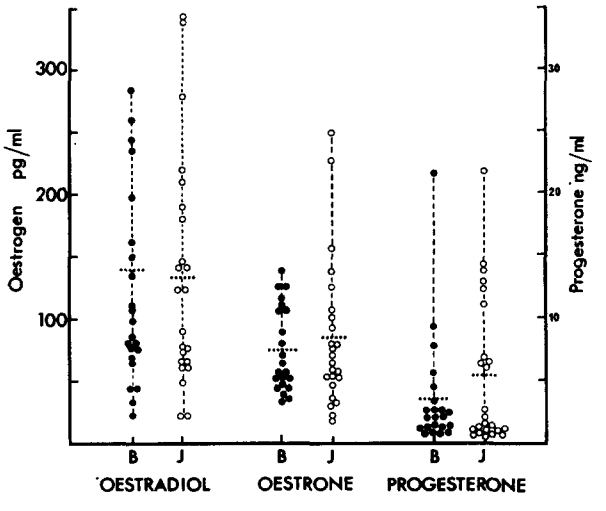


Fig. 3. *Plasma oestradiol, oestrone, and progesterone in normal British (B) and Japanese (J) adolescent girls. The dotted horizontal lines show the arithmetic means.*

(*b*) *Urine.* The amounts of urinary oestrone and oestriol excreted by British and Japanese girls are shown in Fig. 4. They are clearly very similar and the mean amounts do not differ significantly. When the amounts of urinary metabolites excreted by girls who were definitely in the luteal phase are compared there are no significant differences in the means (Table 3) This is in contrast to the significant differences found in the plasma oestrogens but it should be remembered that the urine was collected for 3 days after the blood specimen was taken.

3. *Plasma progesterone and urinary pregnanediol in normal British and Japanese women aged more than* 35 *years*

The plasma progesterone values in the 2 groups are shown in Fig. 1. The mean value for the British women (11 ng/ml) is slightly

Table 3. *Plasma oestradiol-17β and oestrone, and urinary oestrone, oestradiol-17β and oestriol in normal British and Japanese girls aged* 16–18 *years*

Hormone	All subjects		Definitive luteal phase	
	British	Japanese	British	Japanese
Plasma oestradiol	140 ± 82 ($N = 23$)	133 ± 91 ($N = 24$)	77 ± 33 ($N = 6$)	134 ± 69 ($N = 11$)
Plasma oestrone	76 ± 33 ($N = 23$)	85 ± 59 ($N = 24$)	52 ± 10 ($N = 6$)	92 ± 55 ($N = 11$)
Urinary oestrone	10·7 ± 6·7 ($N = 21$)	9·7 ± 9·4 ($N = 19$)	9·8 ± 3·2 ($N = 6$)	9·0 ± 6·3 ($N = 8$)
Urinary oestradiol	3·2 ± 2·0 ($N = 21$)	2·5 ± 3·1 ($N = 19$)	3·2 ± 1·5 ($N = 6$)	2·3 ± 2·0 ($N = 8$)
Urinary oestriol	10·1 ± 6·7 ($N = 21$)	11·4 ± 9·2 ($N = 19$)	7·1 ± 4·3 ($N = 6$)	12·5 ± 10·3 ($N = 8$)

Plasma oestrogens expressed in pg/ml. Urinary oestrogens in μg/24 hr.
The arithmetic means ± standard deviations are shown and the number of subjects are given in parenthesis.

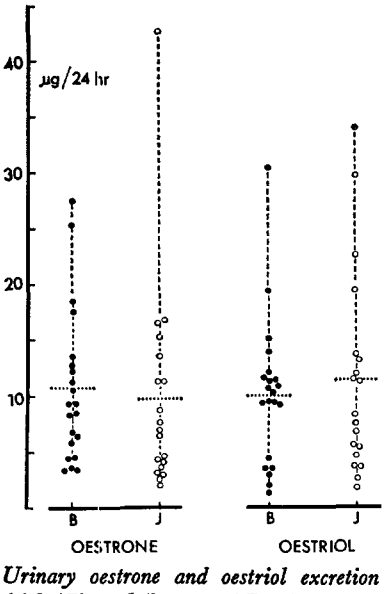


Fig. 4. *Urinary oestrone and oestriol excretion of normal British (B) and Japanese (J) adolescent girls. The dotted horizontal lines show the arithmetic means.*

higher than that for the Japanese women but the difference is not significant. If women with levels below 3 ng/ml are omitted, the mean value for plasma progesterone in British women is 12·8 ng/ml compared with 12·3 ng/ml in Japanese women (Table 4).

The urinary pregnanediol values show exactly the same picture. The mean urinary pregnanediol for British women is 2·3 mg/24 hr and that for Japanese 1·7 mg/24 hr, and the means are still very similar after the omission of women not in the luteal phase of the cycle (Table 4).

4. *Plasma progesterone and urinary pregnanediol in normal British and Japanese girls aged* 16 *to* 18 *years*

The mean plasma progesterone values for British and Japanese girls are 3·6 and 5·2 ng/ml respectively. They do not differ significantly.

Table 4. Plasma progesterone and urinary pregnanediol in normal British and Japanese women and in adolescent girls

Hormone	All subjects		Definitive luteal phase	
	British	Japanese	British	Japanese
Aged > 35 *years*				
Plasma progesterone	11·0 ± 6·2 ($N = 30$)	8·8 ± 7·2 ($N = 27$)	12·8 ± 5·1 ($N = 25$)	12·3 ± 6·9 ($N = 17$)
Urinary pregnanediol	2·3 ± 1·4 ($N = 30$)	1·7 ± 1·3 ($N = 25$)	2·6 ± 1·3 ($N = 25$)	2·1 ± 1·3 ($N = 17$)
Aged 16–18 *years*				
Plasma progesterone	3·6 ± 4·5 ($N = 23$)	5·2 ± 5·7 ($N = 26$)	8·8 ± 6·6 ($N = 6$)	10·5 ± 5·4 ($N = 10$)
Urinary pregnanediol	1·0 ± 0·7 ($N = 21$)	1·2 ± 1·0 ($N = 26$)	1·2 ± 0·7 ($N = 6$)	1·6 ± 0·9 ($N = 11$)

Plasma progesterone expressed in ng/ml. Urinary pregnanediol in mg/24 hr. The arithmetic means ± standard deviations are shown and the number of subjects are given in parenthesis.

The individual values are shown in Fig. 3 and it can be seen that a substantial proportion of the values lie below the limit thought to be compatible with normal luteal function (3 ng/ml). The mean plasma progesterone values do not differ significantly if subjects who are not in the luteal phase of the cycle are omitted (Table 4). The mean pregnanediol values are 1·0 mg/24 hr for the British and 1·2 mg/24 hr for the Japanese girls.

5. *Plasma and urinary oestrogens in normal British and Japanese menopausal and post-menopausal women*

The mean plasma oestrone and oestradiol-17β levels in British and Japanese menopausal and post-menopausal women are shown in Table 5 and in Fig. 5. In five cases, very high plasma oestrogen levels were found (57–243 pg/ml). It is assumed that these levels result from residual ovarian activity. Whether these values are included or not there are no significant differences between the means.

The plasma oestrogen levels are marginally lower in the post-menopausal women compared with the menopausal group but the differences are not significant.

The mean amounts of urinary oestrogens do not differ in the British and Japanese women (Table 5).

6. *Plasma progesterone and urinary pregnanediol in normal British and Japanese menopausal and post-menopausal women*

The mean plasma progesterone levels and mean amounts of pregnanediol excreted in the urine are shown in Table 6. There are no significant differences between the means for the two populations.

7. *The oestriol quotient*

It has been reported [12, 13] that the amount of urinary oestriol relative to that of oestrone and oestradiol-17β is higher in Asian women than in Caucasian women. The oestriol quotient ($E_3/E_1 + E_2$) for both British and Japanese girls,

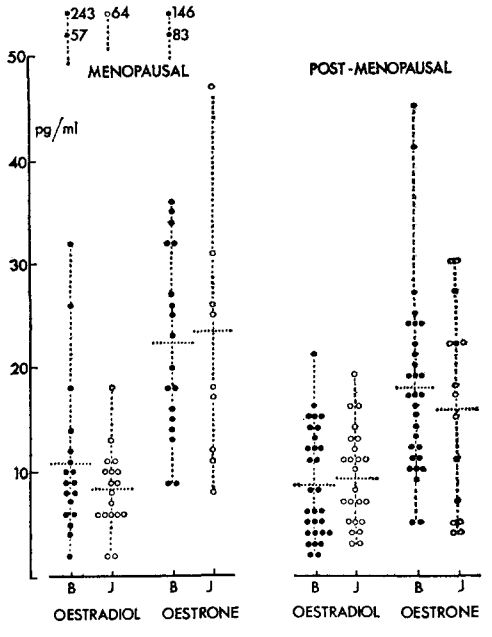


Fig. 5. Plasma oestradiol and oestrone in normal British (B) and Japanese (J) menopausal and post-menopausal women. The dotted horizontal lines show the arithmetic means.

and pre-menopausal, menopausal and post-menopausal women is shown in Table 7. The mean quotient is some 30% higher in both pre-menopausal Japanese groups compared with the British groups but the differences are not significant. In the menopausal women, the quotient is higher in British than in Japanese

Table 5. Plasma oestradiol-17β and oestrone, and urinary oestrone, oestradiol and oestriol in normal British and Japanese menopausal and post-menopausal women

Hormone	Menopausal		Post-menopausal	
	British	Japanese	British	Japanese
Plasma oestradiol	13 ± 13 ($N = 19$)	11 ± 13 ($N = 19$)	9 ± 5 ($N = 30$)	9 ± 4 ($N = 29$)
Plasma oestrone	25 ± 16* ($N = 19$)	22 ± 12 ($N = 9$)	18 ± 9 ($N = 30$)	18 ± 10 ($N = 20$)
Urinary oestrone	1·7 ± 0·5 ($N = 12$)	2·4 ± 1·3 ($N = 16$)	1·7 ± 0·6 ($N = 20$)	2·4 ± 1·9 ($N = 19$)
Urinary oestradiol	1·0 ± 1·2 ($N = 12$)	1·4 ± 1·0 ($N = 12$)	0·9 ± 0·6 ($N = 20$)	1·1 ± 1·1 ($N = 19$)
Urinary oestriol	2·1 ± 1·4 ($N = 12$)	1·4 ± 1·9 ($N = 16$)	1·5 ± 0·9 ($N = 20$)	2·2 ± 2·6 ($N = 19$)

*One subject omitted from calculation: her plasma E_2 was 243 and E_1 146 pg/ml. Plasma oestrogens in pg/ml: urinary oestrogens in μg/24 hr. The arithmetic means ± standard deviations are shown and the number of subjects are given in parenthesis.

Table 6. *Plasma progesterone and urinary pregnanediol in normal menopausal and post-menopausal British and Japanese women*

Hormone	Menopausal		Post-menopausal	
	British	Japanese	British	Japanese
Plasma progesterone (ng/ml)	0·61 ± 0·25 (N = 17)	0·90 ± 0·29 (N = 15)	0·80 ± 0·27 (N = 22)	0·72 ± 0·29 (N = 25)
Urinary pregnanediol (mg/24 hr)	0·24 ± 0·12 (N = 12)	0·23 ± 0·09 (N = 18)	0·29 ± 0·12 (N = 20)	0·22 ± 0·17 (N = 19)

The arithmetic means ± standard deviations are shown and the number of subjects are given in parenthesis.

Table 7. *Oestriol quotient in British and Japanese women*

	British	Japanese
Adolescents	0·97 ± 1·26 (N = 21)	1·24 ± 0·86 (N = 19)
Pre-menopausal	0·96 ± 0·60 (N = 30)	1·20 ± 0·6 (N = 27)
Menopausal	0·88 ± 0·41 (N = 12)	0·46 ± 0·69 (N = 16)
Post-menopausal	0·64 ± 0·46 (N = 20)	0·65 ± 0·50 (N = 19)

The arithmetic means ± standard deviations are shown and the number of subjects are in parenthesis.

women but not significantly so. The quotients are almost identical in post-menopausal women.

8. *Correlations between variables*

The numbers of subjects in each group are small and only the strongest correlations might be expected to show up. Within pre-menopausal women who were judged to be in the luteal phase of the menstrual cycle on the basis of plasma progesterone values, there were no significant associations between the plasma and urinary oestrogens, although there was a general trend for high plasma values to be reflected in high urinary metabolite titres. There was, however, a significant correlation between plasma progesterone and urinary pregnanediol (r = 0·46, P< 0·01).

DISCUSSION

There is a long-standing assumption that oestrogens may be carcinogenic in man as they are in some species of laboratory animals [14, 15]. One of the reasons for the present study was to see whether the low incidence of breast cancer in Japan, compared with that in most Western countries, could be explained on the basis of underlying endocrinological differences between the races.

The main conclusion to emerge from the study is the striking similarity between the plasma levels of oestradiol-17β, oestrone and progesterone in Japanese and British women of all ages. There appears to be no correlation between plasma oestrogen and progesterone levels and incidence rates in Japan and Britain. To emphasise this point the mean plasma oestradiol levels in the four age groups studied

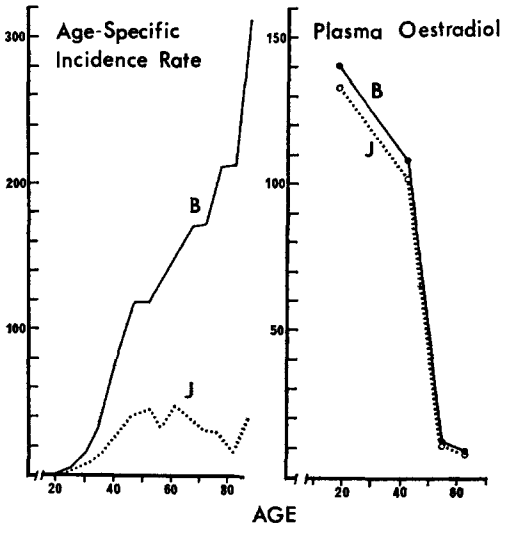


Fig. 6. *Age-specific incidence rates of breast cancer and plasma oestradiol levels in British and Japanese women. B and J represent British and Japanese women.*

are shown with incidence data from Britain and Japan in Fig. 6. These results are somewhat unexpected. MacMahon, Cole and Brown [16] have reviewed the evidence for the involvement of the ovaries in the aetiology of human breast cancer and have made a persuasive case for their importance. Yet the plasma levels of the major oestrogens and progesterone are virtually identical in two races with marked differences in incidence. Indeed, the only significant difference found in the entire study was that

young Japanese girls in the luteal phase of their menstrual cycle had *greater* amounts of oestrone and oestradiol in their plasma than did British adolescents. This finding is probably an artefact since the amounts of oestrogen metabolites found in urine collected over the following three days were identical in both groups.

The young girls are obviously a difficult group to study. Döring [17] measured the luteal rise in basal body temperature in over 3000 cycles. In the age group 12–14 only 10% of cycles were complete, the remainder being anovulatory or with a short luteal phase. At 15–17 yr, 16% of cycles were normal and at 18–20 yr, 36% were normal. These findings may explain the menstrual cycle chaos encountered in the young girls in the present study. Winter and Faiman [18] studying plasma oestradiol and progesterone in much younger girls (12–13 yr) comment that it is not clear how soon regular ovulation occurs after menarche and whether the relative infertility of adolescents is the result of frequent anovulatory cycles. They also noted a corpus luteum dysfunction as suggested by short luteal phase in one subject.

In pre-menopausal women aged 35–50 yr, Döring [17] found 6% of 982 women had anovulatory cycles and a further 18% had short luteal phases. Swain, Bulbrook and Hayward [19] measured plasma progesterone in 341 women and found 64 had levels less than 3 ng/ml, which Israel *et al.* [20] have shown is related to anovulatory cycles. These results are consistent with the present findings in the pre-menopausal groups—the majority showed normal luteal function at the expected time in the cycle.

Considerations of these findings illustrate the difficulties and drawbacks of an analytical epidemiological study of the type we have carried out. In both Britain and Japan, the study populations were small and were drawn from limited geographical areas. The women were highly selected, having been persuaded by personal contact to supply blood and urine specimens and were made up of a disproportionate number of the higher social classes. A further bias is introduced by the nature of the protocol; women with irregular menstrual cycles were supposed to be excluded, as were those with a variety of previous diseases and those using steroidal contraceptives. As for the measurement of hormonal status, a single blood specimen and three urine collections in what was hoped to be the luteal phase of the menstrual cycle can only give an approximate guide to ovarian function. In the case of the young girls in both nationalities, a majority had not established a regular adult pattern.

Nevertheless, the findings in the present study are in general agreement with those found by other workers. Kanbegawa [21] found a mean luteal phase excretion of urinary oestrone, oestradiol-17β and oestriol of 9, 5 and 15 μg/24 hr respectively, in remarkable agreement with the luteal phase means shown in Table 2 (9, 3 and 14 μg/24 hr), and a luteal pregnanediol of 1·4 mg/24 hr. Makino [22] found a mean plasma progesterone level of $5{\cdot}1 \pm 2{\cdot}5$ ng/ml (n = 6) and oestrone and oestradiol-17β levels of 46 ± 20 pg/ml (n = 8) and 85 ± 71 pg/ml which are within the range of values found in the present study.

The hormone levels found in the British women are in general agreement with those found for Western women by other workers [23–32].

If hormone levels early in life govern the subsequent life-long risk of breast cancer then a possible reason for our failure to find convincing differences in plasma oestrogen and progesterone levels may be that the study was carried out too late. The low incidence of breast cancer in Japan, predominantly in post-menopausal women, may be related to a particular endocrine status that existed 20–40 yr ago and which has now changed under the influence of the westernisation of Japan during this period (see De Waard [33]). The corollary to this argument is that, if ovarian steroids play a key role in the genesis of human breast cancer, and if the level of these steroids found in British women are those required for a high incidence of the disease, then it might be expected that incidence in Japan will rise rapidly in the next decade or two. On the basis of a projection of incidence rates in Japan from past data, Hirayama (personal communication) believes that incidence may be about to rise rapidly in Japan. Certainly, large changes in incidence can occur over relatively short periods of time since it has been shown recently that there has been a 10-fold increase in breast cancer in Iceland for the cohort born in 1940–1949, compared with 1840–1849 [34].

The urinary metabolites in the Japanese and British women in this study follow the pattern of their plasma precursors. The possibility still remains that other plasma oestrogens (such as oestriol or oestrone sulphate) are at different levels in the two populations, and that abnormalities exist in the amounts of metabolites not accounted for by measurement of the three classical urinary metabolites.

Cole and MacMahon [15] have suggested

that oestriol may be of great importance in man in that it might modify the carcinogenic action of oestradiol and oestrone. In support of their hypothesis, they have shown that the oestriol quotient (the ratio of urinary oestriol to oestrone and oestradiol) is higher in Asians than in Caucasian women, and other workers have found high oestriol quotients in low risk populations (in Africans [35] and in Yemenite Jews [36]). Recently the oestriol quotient has been compared in Japanese, Hawaiian Japanese and Caucasians, and in women from the American mainland [12]. The quotient was high in Japanese, intermediate in value in Hawaiian Japanese and low in Caucasians in both Hawaii and the USA. Although our results show that the mean oestriol quotient in the younger Japanese women is some 30% higher than that in the British women, the variance in both groups is very large and the difference is not significant. What is perhaps more important is that the oestriol ratio does not appear to be closely related to plasma levels of precursor steroids. Longcope [37] has found similar production and plasma levels of oestriol in Caucasians with high or low oestriol ratios. Examination of the results of MacMahon and his colleagues [12, 13] shows that the high ratio that they have found stems not so much from an increased excretion of oestriol but from a diminished excretion of oestrone and oestradiol. Our present results show that the plasma levels of these two steroids are the same in British and Japanese women.

There are two further pieces of evidence that do not fit well with the Cole–MacMahon hypothesis. The first is that oestriol has now been found to be carcinogenic in mice [38] and the second is that there was no evidence that oestriol inhibited the uptake of tritiated oestradiol in the tumours of patients with breast cancer [39].

In summary, the most reasonable interpretation of our data is that differences in the incidence of breast cancer in Britain and Japan are not related to differences in the plasma levels of oestrone, oestradiol or progesterone.

Acknowledgements—We are very much indebted to the volunteers who took part in this study and to Mr. J. W. Moore and his staff and Miss Marenka Herian for performing the steroid assays. Professor T. Hirayama has given us much advice and encouragement.

REFERENCES

1. M. Segi, M. Kurihara and T. Matsuyama, *Cancer mortality for selected sites in 24 countries. No. 5:* (1964–1965), Sendai, Tohoku University School of Medicine (1969).
2. R. Doll, C. Muir and J. Waterhouse, *Cancer Incidence in Five Continents, II.* Springer-Verlag, (UICC Publication), Berlin, Heidelberg and New York (1970).
3. A. B. Chabon, S. Takeuchi and S. C. Sommers, Histologic differences in breast carcinoma of Japanese and American women. *Cancer (Philad.)* **33,** 1577 (1974).
4. G. H. Friedell, E. A. Soto, S. Kumaoka, O. Abe, J. L. Hayward and R. D. Bulbrook, Sinus histiocytosis in British and Japanese patients with breast cancer. *Lancet* **ii,** 1228 (1974).
5. E. L. Wynder, J. C. Lucas and J. Farrow, A comparison of survival rates between American and Japanese patients with breast cancer. *Surg. Gynec. Obstet.* **117,** 196 (1963).
6. E. L. Wynder, I. J. Bross and T. Hirayama, A study of the epidemiology of cancer of the breast. *Cancer* **13,** 559 (1960).
7. S. G. Korenman, L. E. Perrin and T. P. McCallum, A radio-ligand binding assay system for estradiol measurement in human plasma. *J. clin. Endocr.* **29,** 879 (1969).
8. D. Y. Wang and M. C. Swain, Hormones and breast cancer. In *Biochemistry and Women: Methods for Clinical Investigation,* p. 191. (Edited by A. S. Currey and J. V. Hewitt). C.R.C. Press, Cleveland, Ohio (1974).
9. M. C. Swain, The assay of plasma progesterone in women by a simple competitive protein binding method using guinea-pig serum. *Clin. chim. Acta* **39,** 455 (1972).
10. J. W. Moore and W. Cooper, A rapid method for the measurement of pregnanediol in non-pregnancy urine. *Clin. chim. Acta* **56,** 215 (1974).

11. R. A. A. Salokangas and R. D. Bulbrook, The determination of small quantities of urinary oestrone, oestradiol-17β and oestriol using Ittrich's extraction method. *J. Endocrinol.* **22,** 47 (1961).
12. L. E. Dickinson, B. MacMahon, P. Cole and J. B. Brown, Estrogen profiles of Oriental and Caucasian women in Hawaii. *New Engl. J. med.* **292,** 1211 (1974).
13. B. MacMahon, P. Cole, J. B. Brown, K. Aoki, T. M. Lin, R. W. Morgan and N.-C. Woo, Oestrogen profiles of Asian and North American women. *Lancet* **ii,** 900 (1971).
14. R. Hertz, Experimental and clinical aspects of the carcinogenic potential of steroid contraceptives. *Int. J. Fert.* **13,** 273 (1968).
15. P. Cole and B. MacMahon, Oestrogen fractions during early reproductive life in the aetiology of breast cancer. *Lancet* **i,** 604 (1969).
16. M. MacMahon, P. Cole and J. B. Brown, Etiology of human breast cancer. *J. nat. Cancer Inst.* **50,** 21 (1973).
17. G. K. Doring, The incidence of anovular cycles in women. *J. Reprod. Fert.* Suppl. **6,** 77 (1969).
18. J. S. D. Winter and C. Faiman, The development of cyclic pituitary-gonadal function in adolescent females. *J. clin. Endocrinol.* **37,** 714 (1973).
19. M. C. Swain, R. D. Bulbrook and J. L. Hayward, Ovulatory failure in a normal population and in patients with breast cancer. *J. Obstet. Gynaec. Br. Cwlth.* **81,** 640 (1974).
20. R. Israel, D. R. Mishell, S. Stone, I. H. Thorneycroft and D. L. Moyer, Single luteal phase serum progesterone assay as an indicator of ovulation. *Amer. J. Obstet. Gynec.* **112,** 1043 (1972).
21. A. Kanbegawa, Determination of sex hormones. *Sogo Rinsho* **16,** 935 (1967).
22. T. Makino, Radioimmunoassay of plasma sex steroids. *Endocr. jap.* **49,** 629 (1973).
23. J. B. Brown, Determination and significance of the natural oestrogens. In *Advances in Clinical Chemistry.* (Edited by H. Sobotka and C. P. Stewart), Vol. 3, p. 157. Academic Press, New York & London (1960).
24. S. Furuyama and C. A. Nugent, A radioimmunoassay for plasma progesterone. *Steroids* **17,** 663 (1971).
25. N.-S. Jiang, R. J. Ryan and A. Albert, Radioimmunoassay of serum estrogen. *Clin. Chem.* **19,** 740 (1973).
26. D. Mayes and C. A. Nugent, Plasma estradiol determined with a competitive protein binding method. *Steroids* **15,** 389 (1970).
27. G. Mikhail, C. H. Wu, M. Ferin and R. L. Vande Weile, Radioimmunoassay of plasma estrone and estradiol. *Steroids* **15,** 333 (1970).
28. D. R. Mishell, R. M. Nakamura, P. G. Crosignani, S. Stone, K. Kharma, Y. Nagata and I. H. Thorneycroft, Serum gonadotrophin and steroid patterns during the normal menstrual cycle. *Amer. J. Obstet. Gynec.* **111,** 60 (1971).
29. N. Nagain and C. Longcope, Estradiol-17β and estrone: Studies on their binding to rabbit uterine cytosol and their concentration in plasma. *Steroids* **17,** 631 (1971).
30. J. S. Rankin, Hormonal patterns and interactions in the normal menstrual cycle. *Clin. Obstet. Gynec.* **12,** 741 (1969).
31. B. M. Sherman and S. G. Korenman, Hormonal characteristics of the human menstrual cycle throughout reproductive life. *J. clin. Invest.* **55,** 699 (1975).
32. D. Tukchinsky and D. G. Korenman, A radio-ligand assay for plasma estrone; normal values and variations during the menstrual cycle. *J. clin. Endocr.* **31,** 76 (1970).
33. F. De Waard, The epidemiology of breast cancer: Review and prospects. *Int. J. Cancer* **4,** 577 (1969).
34. O. Bjarnason, N. Day, G. Snaedal and H. Tulinius, The effect of year of birth on the breast cancer age-incidence curve in Iceland. *Int. J. Cancer* **13,** 689 (1974).
35. M. Briggs, Ethnic differences in urinary oestrogens. *Lancet* **i,** 324 (1972).
36. J. Gross, Hormone profiles in Israelis of differing susceptibility to breast cancer. In *Proc. 1st Breast Cancer Task Force Working Conference.* Williamsburg, Va. (1969).

37. C. LONGCOPE, Breast cancer and estriol dynamics (abstr.). *Program of the Breast Cancer Task Force*, p. 148 (1975).
38. G. RUDALI, F. APIOU and B. MUEL, Mammary cancer produced in mice with oestriol. *Europ. J. Cancer* **11,** 39 (1975).
39. N. DESHPANDE, P. CARSON and J. HORNER, Estriol in human breast tumours. *J. Steroid Biochem.* **7,** 11 (1976).

Europ. J. Cancer Vol. 12, pp. 737–741. Pergamon Press 1976. Printed in Great Britain

The Target Cell in the Chemical Induction of Carcinomas in Mouse Submandibular Gland*

CAROLINE B. WIGLEY†‡ and A. W. CARBONELL
Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Field, London WC2A 3PX, Great Britain

Abstract—*An emulsion containing 2 mg of the potent hydrocarbon carcinogen dimethylbenz(a)anthracene was injected into the submandibular glands of young adult male C57/BL/Icrf a' mice. Subsequent preneoplastic changes, including duct hyperplasia and squamous metaplasia, are described. Squamous cell carcinomas and fibrosarcomas arose with approximately equal frequency after 12–20 weeks. About 75% of surviving animals developed one or both types of tumour; the specific cell type from which squamous carcinomas developed was investigated. Ultrastructural characteristics and histochemical "markers" of differentiated function were used as criteria of cell identity and added to histological information. It was concluded that the main target cell in carcinogen-induced tumorigenesis was derived from the granular tubule. These cells continued to secrete characteristic proteases during the early preneoplastic phase but this function was lost in advanced squamous metaplasia and carcinoma.*

INTRODUCTION

Early studies of experimental chemical carcinogenesis in laboratory animals led to the observation that while many organs developed only sarcomas when pellets of carcinogen were implanted, methylcholanthrene induced epidermoid or squamous carcinomas in salivary glands of rats and mice [1]. Rusch *et al.* [2] obtained similar results with 3,4 benzpyrene and 1, 2, 5, 6 dibenzanthracene injected into various tissues. Most parenchymatous tumours obtained were squamous cell carcinomas of the submandibular gland. The susceptibility of this gland to tumour induction by aromatic polycyclic hydrocarbons bears no relationship to the spontaneous incidence of tumours which is very low in rodents [3, 4].

More recently, experimental dimethylbenz(*a*)anthracene (DMBA) carcinogenesis in the submandibular gland has been studied in hamsters [5], rats [6] and mice [7]. In hamsters only fibrosarcomas developed although squamous metaplasia of epithelial elements was reported. Other authors described the sequence of histological changes following carcinogen treatment; atrophy, inflammation, degeneration and later regeneration of ductal elements. These often formed large, stratified, cyst-like structures of "dedifferentiated" epithelium from which squamous carcinomas arose, with a peak incidence at about 100–140 days. The cell type of origin of squamous carcinomas was debated, but evidence was based on histological studies only.

The mouse submandibular gland is a complex, hormonally-dependent, tubuloacinar structure with four main epithelial cell components. These are the mucus secreting acinar cell, the connecting intercalated duct cell, the granular tubule cell which secretes serous components of saliva, protease, amylase and nerve growth factor, and the striated duct cell which is involved in ion regulation and possesses 11-β hydroxysteroid dehydrogenase (11β-HSD) activity. The structure and function of this gland in C57BL mice has been described elsewhere [8].

Accepted 13 April 1976.

*This work was carried out during the tenure by one of us (C.B.W.) of a bursary award from the Imperial Cancer Research Fund.

†Present address: Chemical Carcinogenesis Division, Institute of Cancer Research, Pollards Wood Research Station, Nightingales Lane, Chalfont St. Giles, Buckinghamshire, Great Britain.

‡To whom reprint requests should be sent.

The present study was carried out in order to characterize more clearly the cell type or types involved in submandibular gland carcinogenesis, using ultrastructure and histochemical demonstration of differentiated function as criteria of cell identity.

MATERIAL AND METHODS

Tumour induction

A technique was developed to inject an emulsion of dimethylbenz(*a*)anthracene (DMBA) directly into submandibular gland tissue. This was a modification of previously used methods [5–7]. DMBA was dispersed finely in a minimum volume of acetone (final concentration of 10%) and an emulsion prepared in distilled water and KY water soluble jelly (Johnson and Johnson, Slough, Bucks., England) in equal volumes. This emulsion could be injected through a size 23 syringe needle but was viscous enough to remain *in situ* after injection. 0·05 ml (2 mg) of a 40·0 mg/ml DMBA emulsion was injected through the skin into the submandibular glands of 5 month old male C57/BL/Icrf a^t mice anaesthetised with Nembutal. A total of 65 mice were used in 3 experiments. In one experiment, mice were allowed to develop tumours unless skin ulceration necessitated killing earlier. In another experiment, pairs of mice were killed at regular intervals during the latent period of tumour development to study histological changes. The third experiment was concerned with obtaining tissue for study of early histochemical and ultrastructural changes. 10 additional control mice were injected intraglandularly with carrier emulsion only or subcutaneously in the flank with 2 mg DMBA emulsion.

Tumour transplantation

One to two cubic millimetre pieces of tumour tissue from a DMBA-induced squamous cell carcinoma were used to initiate a transplant line in syngeneic male mice. The line was discontinued at the 10th passage when a frozen stock in liquid nitrogen had been established and tested for viability.

Histology

Tissue was fixed in formol calcium for 24 hr, dehydrated in graded ethanols, cleared with chloroform and embedded in paraffin wax. Sections were routinely stained with Ehrlich's haematoxylin and eosin (H & E).

Histochemistry

Mucins in preneoplastic, tumour and normal tissues were demonstrated by the Alcian blue periodic acid Schiff AB/PAS stain [9], proteases using a gelatin substrate film method [10] and 11β-hydroxysteroid dehydrogenase (11β-HSD) using reduced formazan to localize the enzyme [11]. These last two histochemical techniques were performed with cryostat sections of frozen unfixed tissue. All three methods have been described previously for explant cultures of submandibular tissue [8] and were selected as "markers" of differentiated function in three major epithelial cell types in the gland. These are acinar, granular tubule and striated duct cells respectively.

Electron microscopy

One cubic millimetre pieces of tissue were fixed for 24 hr in 2·5% glutaraldehyde (Taab Laboratories, Reading, Berks., England) in Sorensen's buffer at pH 7·3, washed for 24 hr and post-fixed in 1% osmium tetroxide for 30 min. Tissue was then dehydrated through graded acetones, embedded in Araldite and polymerized at 70°C for 24 hr. Three μm sections were cut with a glass knife on a Sorvall MT 2 ultramicrotome, expanded with heat and stained with toluidine blue at 60°C. Ultrathin sections of areas selected by light microscopy were then cut with a diamond knife, double-stained on copper grids with uranyl acetate and lead citrate and coated with carbon. Sections were viewed on Hitachi HS-7S or Siemens Elmiskop 1A electron microscopes.

RESULTS

Tumour development

Two types of tumour developed with about equal frequency after a latent period of 12–20 weeks following DMBA injection. These were squamous cell carcinomas and fibrosarcomas. Occasionally both types of tumour developed in the same animal but were considered to arise independently.

Some animals were killed before the first tumour was seen at 12 weeks and are not considered at risk. Of 37 mice surviving for more than 12 weeks, 9 had squamous carcinomas, 6 fibrosarcomas and 12 had both types of tumour. No evidence of malignancy is available for 10 mice, some of which were not subjected to post-mortem examination. Both types of tumour were locally invasive but no distant metastases were found.

Animals receiving the carrier emulsion intraglandularly showed no dermal oedema, ulceration or alteration of submandibular gland histology during a six month observation period. Control mice receiving DMBA emulsion subcutaneously in the flank all showed some dermal hyperplasia as was seen in the neck region. One of the five animals developed a fibrosarcoma after four months.

Early morphological changes

Histological changes similar to those reported elsewhere [5–7] were observed in male mouse submandibular gland tissue after injection of DMBA. During the first few weeks, a necrotic zone developed around residual carcinogen emulsion. Between this and surrounding histologically normal gland, an intermediate area of young granulation tissue became apparent in which the following changes were observed. Acinar cells degenerated rapidly and the space became filled with fibrotic tissue, infiltrated by polymorphonuclear leucocytes. Granular tubules lost their secretory granules and the cells became cuboidal. Striated ducts showed little initial change but could be distinguished from atrophic granular tubules by their basal striations and more apically situated nuclei. Intercalated duct cells were more difficult to identify histologically.

After about a week, hyperplastic changes were seen in a few ducts near the necrotic zone. Multiple layers of cuboidal cells were irregularly arranged around the lumen. These ducts resembled granular tubules rather than striated ducts in their size and distribution. From two to three weeks onwards, squamous metaplasia was seen in some hyperplastic ducts. This is shown in Fig. 1.

At this stage, although the general histological pattern of ducts was preserved, changes could be observed at the cellular level. Selected areas were studied in more detail under the electron microscope. Some cells possessed a few secretory granules which stained with toluidine blue in 3 μm Araldite sections. These closely resembled those found in mature granular tubule cells. This is shown in Fig. 2, where in addition, the cells show infolding of their basal membranes and have numerous associated mitochondria. This is also a feature of atrophic granular tubule cells (see discussion).

Ultrastructure of "intermediate" and carcinomatous cells

The cells shown in Fig. 2 were in direct contact within the basement membrane with cells that had some features in common with neoplastic squamous cells. These were an irregularly shaped nucleus containing large blocks of electron-dense chromatin, increased intercellular space filled with microvilli and more prominent desmosomes and associated tonofilaments. The ground cytoplasm was generally more electron-dense, giving the cells a dark appearance. The two cells on the right of Fig. 3 show these characteristics and form part of a hyperplastic duct. The cell on the left is more normal in appearance, except for increased numbers of tonofilaments. Intermediate cells with ultrastructural characteristics of both normal and neoplastic cells might be termed preneoplastic, although at the light microscope level such changes could not be detected at this stage.

An example of a carcinomatous cell is shown in Fig. 4. Here, cell polarisation is lost and desmosomes are found around the whole cell periphery at points of contact with neighbouring cells. In mature, keratinizing squamous cells, tonofilaments were the most prominent cytoplasmic feature.

Histochemical identification of the cell type involved in carcinoma development

(*a*) *AB/PAS Orange G. stain.* Paraffin wax sections demonstrating the early stages of duct hyperplasia and squamous metaplasia and sections of tumour-tissue were stained by this method to demonstrate the presence of acid mucins which might identify acinar cells. No secretory acinar cells containing blue staining material were seen in association with carcinogen-induced histological changes. Some extracellular material did show positive blue staining but this was thought to be either of mesenchymal origin or to have derived from degenerated acinar cells. Hyperplastic ducts contained no obvious secretory material, but their cytoplasm stained with Orange G counterstain. This is characteristic of ductal rather than acinar cells in the submandibular gland [12]. Tumour tissue also stained with Orange G, but not Alcian blue.

(*b*) *11β-HSD.* Blue formazan granule deposition was never found in association with hyperplastic or metaplastic cells in cryostat sections showing preneoplastic changes. Striated ducts in adjacent "normal" regions of tissue reacted positively, however, indicating presence of the steroid dehydrogenase enzyme specific to these cells. Tumour tissue showed no specific enzyme reaction.

(*c*) "*Protease*". Figures 5 and 6 show a stained cryostat section demonstrating early hyperplasia and squamous metaplasia after

DMBA injection and the adjacent section which had been exposed to developed photographic emulsion as described previously [8]. The histologically normal granular tubules at the top of the section show secretory protease activity comparable to that in an untreated gland. This is indicated by clear areas in the dark emulsion, where gelatin has been digested and developed silver grains released from the film. The hyperplastic tubules in the intermediate zone between the normal gland and the degenerate area, although they have lost their typical secretory appearance, still possess proteolytic activity. One tubule which shows histological signs of squamous metaplasia (arrow) also still retains its proteolytic function. Cells demonstrating more advanced squamous changes, and squamous carcinoma cells, had no detectable protease function under the conditions of incubation used here.

DISCUSSION

Squamous carcinomas have been induced in the submandibular gland of male C57/BL/Icrf a^t mice. The injection technique combined the simplicity of Glucksmann and Cherry's acetone solution method [6] with the more accurately controlled positioning and dosage of Matsumura's implanted pellet technique [7]. The high yield of squamous carcinomas and short latent period of tumour development make this a useful system for the study of experimental hydrocarbon carcinogenesis. Fibrosarcomas also developed with approximately equal frequency.

No adenocarcinomas were found, though these as well as adenoacanthomas and mixed carcinomas with columnar and squamous strains have been reported by Bauer and Byrne [13], Glucksmann and Cherry [6], Heiman and Meisel [14] and Steiner [15].

The early degenerative response of the submandibular gland after carcinogen treatment was similar to the response of this gland to other trauma such as partial excision [16], intraperitoneal isografting [12], organ or explant culture [17, 8]. In all these cases, regenerative proliferation of duct cells followed, but this proliferation did not lead to the development of tumours. In the case of submandibular gland explants cultured *in vitro*, it has been shown that regenerating ducts derived from granular tubule cells [8].

In the experiments reported here, a number of different lines of evidence strongly suggest that granular tubule cells are the major target cell type in DMBA-induced carcinogenesis. The histochemical evidence showed that atrophic, hyperplastic and early metaplastic tubule cells still possessed secretory protease function, typical of granular tubule cells, but not 11β-HSD activity, characteristic of striated duct cells. Some hyperplasia of intercalated duct cells could not be ruled out but acinar cell involvement seems unlikely.

Although hyperplastic duct cells did not appear to contain secretory granules in routine H & E stained sections, toluidine blue stained 3 μm araldite sections demonstrated clearly the presence of a few residual deep blue granules. These closely resembled those found in normal granular tubules. This was confirmed ultrastructurally. No other epithelial cell type in the male mouse gland possesses secretory granules, although intercalated duct cells in the female mouse contain a few small granules of uncertain composition [18].

Some hyperplastic tubules possessed both secretory granules, suggesting a granular tubule origin and basal striations, typical of striated or excretory duct cells. Granular tubules differentiate from specialised striated ducts during development and the reappearance of basal striations may therefore reflect a reversal of this process in response to trauma. A similar finding was reported by Chretien [19] for atrophic granular tubules in submandibular glands of castrated male mice. In the absence of sex hormones there was a loss of secretory function and appearance of basal membrane infolding. These results are not therefore inconsistent with the proposed granular tubule origin of hyperplastic and neoplastic cells.

The experiments reported here put forward histological, ultrastructural and histochemical evidence that granular tubule cells are the primary target cell type in the chemical induction of squamous carcinoma in the mouse submandibular gland.

Acknowledgements—The authors would like to thank Dr. L. M. Franks for his help with the interpretation of pathological sections and for helpful criticism of this manuscript. We would also like to thank Mr. M. U. Sheriff and Miss Joan Payne of this department and Mr. G. Leach and the staff of the photographic department.

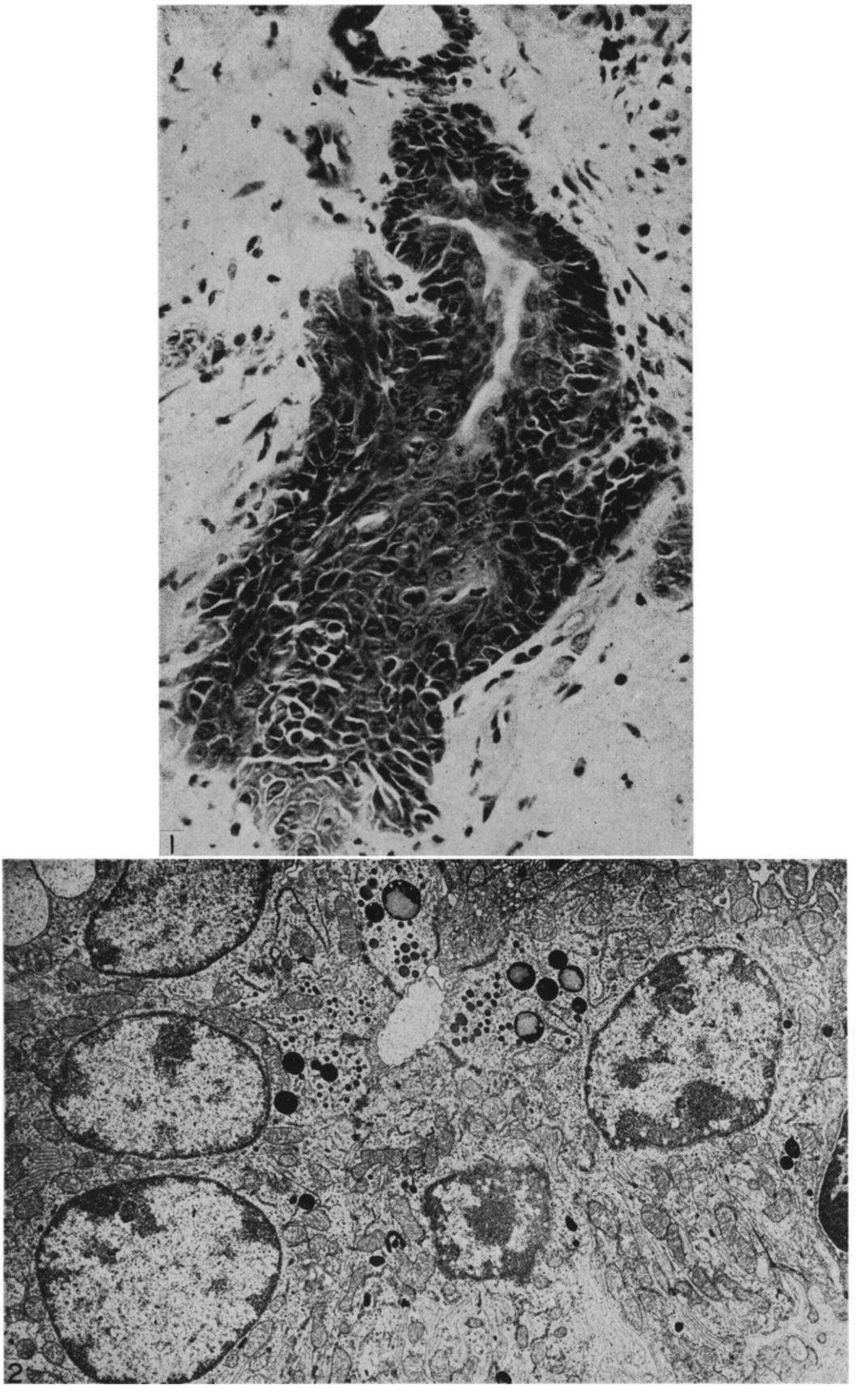


Fig. 1. *Hyperplastic duct from adult male mouse submandibular gland, 4 weeks after DMBA injection, with an island of metaplastic squamous cells arising from it (top).* (*H & E*) × 400

Fig. 2. *Electron micrograph of adult male mouse submandibular gland 15 weeks after carcinogen injection, in a region adjacent to a squamous carcinoma. An atrophic duct is shown with residual secretory granules and basal membrane striations. These cells were in direct contact with overtly squamous cells.* × 8000

(to face page 740)

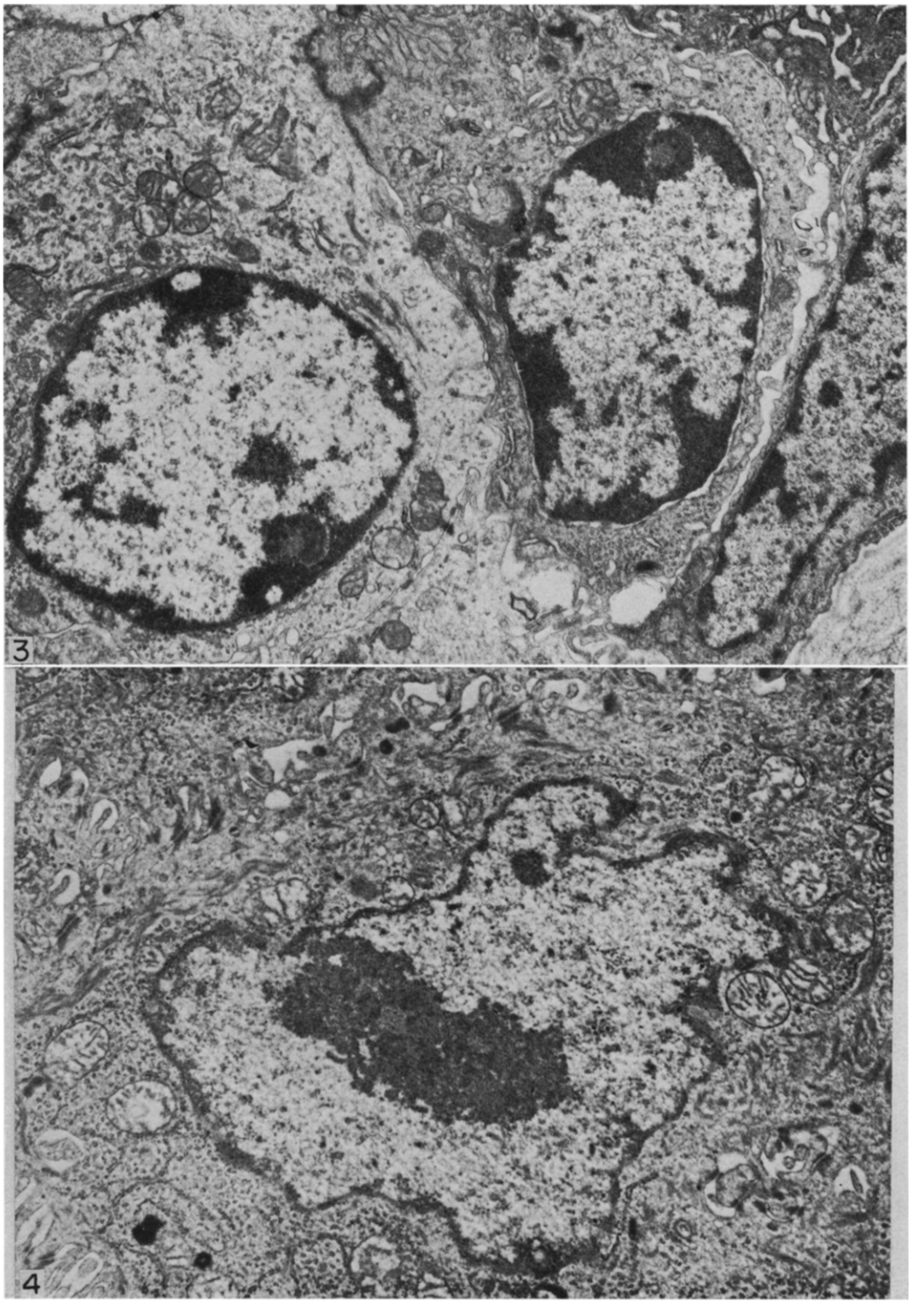


Fig. 3. Electron micrograph of "intermediate" cells in a hyperplastic duct. Features typical of squamous carcinoma cells are present; increased intercellular space, dense cytoplasm with prominent desmosomes and tonofilaments and early signs of loss of polarity. (× 14,000)

Fig. 4. Electron micrograph of cells of a squamous carcinoma of the submandibular gland, induced by DMBA. (× 15,000)

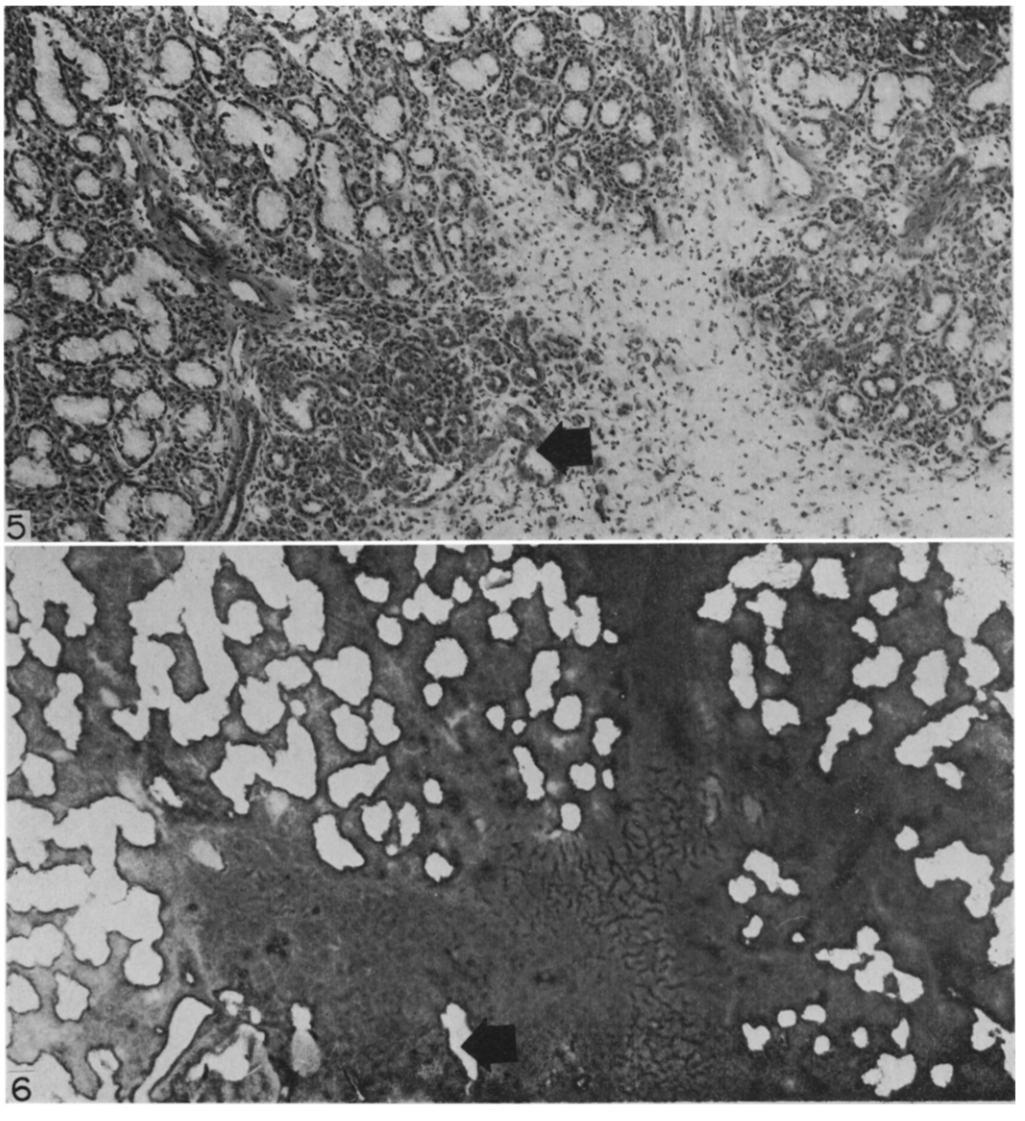


Fig. 5. *Adult male mouse submandibular gland* 4 *weeks after carcinogen injection. Stained cryostat section of frozen unfixed tissue showing an area of duct hyperplasia and early squamous metaplasia.* (*H & E*) × 100

Fig. 6. *The adjacent serial cryostat section, exposed to developed photographic emulsion to demonstrate proteolytic activity. Hyperplastic ducts and one duct showing squamous changes* (*arrowed*) *possess protease enzyme activity, characteristic of granular tubule cells.* × 100

REFERENCES

1. C. C. Franseen, J. C. Aub and C. L. Simpson, Experimental tumours in lymph nodes and in endocrine and salivary glands. *Cancer Res.* **1,** 489 (1941).
2. H. P. Rusch, C. A. Baumann and G. L. Maison, Production of internal tumours with chemical carcinogens. *Arch. Path.* **29,** 8 (1942).
3. A. Glucksmann and C. P. Cherry, Tumours of the salivary glands. In *Pathology of Tumours in Laboratory Animals.* vol. 1. Part 1. Tumours of the rat. p. 75. International Agency for Research on Cancer Scientific Publications 5. Lyon, IARC (1973).
4. E. D. Murphy, Characteristic tumours. In *Biology of the Laboratory Mouse.* (Edited by E. L. Green.), p. 521. McGraw-Hill Book Co., N.Y. (1966).
5. E. Cataldo and G. Shklar, Chemical carcinogenesis in the hamster submaxillary gland. *J. dent. Res.* **43,** 568 (1964).
6. A. Glucksmann and C. P. Cherry, Sex difference and carcinogenic dosage in the induction of neoplasms in salivary glands of rats. *Brit. J. Cancer* **25,** 212 (1971).
7. T. Matsumura, Enzyme histochemistry of experimentally induced tumors in the mouse submaxillary and sublingual glands during carcinogenesis. *Gann* **57,** 251 (1966).
8. C. B. Wigley and L. M. Franks, Salivary epithelial cells in primary culture: characterisation of their growth and functional properties. *J. Cell Sci.* **20,** 149 (1976).
9. R. Lev and S. S. Spicer, A histochemical comparison of human epithelial mucins in normal and in hypersecretory states including pancreatic cystic fibrosis. *Amer. J. Path.* **46,** 23 (1965).
10. R. Daoust, Histochemical localisation of enzyme activities by substrate film methods: ribonucleases: ribonucleases, deoxyribonucleases, proteases, amylase and hyaluronidase. *Int. Rev. Cytol.* **18,** 191 (1965).
11. A. H. Baillie, M. M. Ferguson and D. McK. Hart, *Developments in Steroid Histochemistry.* Academic Press, New York (1966).
12. K. Hoshino and C. D. Lin, Induction of hyperplasia in mouse salivary gland isografts. *Europ. J. Cancer* **7,** 373 (1971).
13. W. H. Bauer and J. J. Byrne, Induced tumors of the parotid gland. *Cancer Res.* **10,** 755 (1950).
14. J. Heiman and D. Meisel, Tumors of the salivary and parathyroid glands in rats fed with 2-acetylaminofluorene. *Cancer Res.* **6,** 617 (1946).
15. P. E. Steiner, Comparative pathology of induced tumors of the salivary glands. *Arch. Path.* **34,** 613 (1942).
16. C. T. Hanks and A. P. Chaudhry, Regeneration of rat submandibular gland following partial extirpation. A light and electron microscopic study. *Amer. J. Anat.* **130,** 195 (1971).
17. R. L. Tapp, An attempt to maintain cultures from the submandibular gland of the adult rat *in vitro Exp. Cell Res.* **47,** 536 (1967).
18. F. Caramia, Ultrastructure of mouse submaxillary gland. (i) Sexual differences. *J. Ultrastruct. Res.* **16,** 505 (1966).
19. M. Chretien, Action de la testosterone sur la structure fine d'un effecteur: la glande sous-maxillaire de la souris male. (i) Morphologie des tubes secreteurs avant et apres castration. *J. Microscop.* **14,** 35 (1972).

Europ. J. Cancer Vol. 12, pp. 743–745. Pergamon Press 1976. Printed in Great Britain

Letters to the Editor

The Nature of Benzo[*a*]pyrene Binding to DNA in an Epithelial Cell Culture System*

C. B. WIGLEY†, M. H. THOMPSON‡ and P. BROOKES
Chemical Carcinogenesis Division, Institute of Cancer Research, Pollards Wood Research Station, Nightingales Lane, Chalfont St. Giles, Buckinghamshire HP8 4SP, Gt. Britain
‡Present address: *Bacterial Metabolism Research Laboratory, Colindale Hospital, Colindale Avenue, London NW9 5DX*

WHEN benzo[*a*]pyrene (B[*a*]P), a potent hydrocarbon carcinogen, is painted on to mouse skin or added to mouse embryo fibroblasts in primary culture, it is metabolised to a reactive derivative which binds covalently to DNA [1, 2]. Recently Sims *et al.* [3] proposed that the intermediate which binds to DNA in cellular (*in vivo*) systems is a diolepoxide, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene 9,10-oxide. It is important to know whether this B[*a*]P derivative also binds to DNA in an epithelial cell system where the parent hydrocarbon is known to induce carcinomas. Mouse skin is an approximation to this ideal, but has the disadvantage that DNA is isolated from a mixture of epidermal (epithelial) and dermal (mesenchymal) cell types. The experiments described here demonstrate that tritiated B[*a*]P is metabolised by salivary gland epithelial cells in primary explant culture and subsequently binds to epithelial cell DNA. The nature of this binding was investigated using established chromatographic methods [4].

Squamous carcinomas are readily induced in the male mouse submandibular gland by a number of polycyclic hydrocarbon carcinogens [5,6]. It has been shown that in 7, 12-dimethylbenz[*a*]anthracene (DMBA)-treated glands, these epithelial tumours derive from the granular tubule cell [7], which secretes protein components of saliva. These include a characteristic proteolytic enzyme which can be demonstrated histochemically and used *in situ* as a specific marker of cell identity.

Mouse submandibular gland tissue can be grown in long-term primary explant culture for several months. The cellular outgrowth consists predominantly of a single epithelial cell type, also derived from granular tubules [8]. Explants were treated with 0·1 μg/ml DMBA or 1·0 μg/ml B[*a*]P or dibenz[*a*, *h*]-anthracene for 24 hr at the time of maximum epithelial cell proliferation (day 3–4). Approximately 80 days later, foci of hyperplastic epithelium appeared in carcinogen-treated cultures, but not in control solvent-treated cultures or those treated with the non-carcinogenic analogues benzo[*e*]pyrene, benz[*a*]-anthracene and dibenz[*a*, *c*]anthracene (Wigley, unpublished results).

When foci of hyperplastic epithelial cells were isolated and grown in serially-passaged culture for reimplantation into syngeneic animals, carcinomas developed at the site of injection after about eight weeks (M. Knowles, personal communication). It has proved difficult to subculture epithelium from control experiments where solvent-treated cells have a limited ability to proliferate. Experiments to test the tumorigenicity of these cells are therefore still in progress.

The submandibular gland explant system was adapted for large-scale experiments so that purified DNA could be isolated from cultured salivary epithelial cells after treatment with tritiated B[*a*]P. Explants were prepared from the glands of groups of 10 young adult male C57BL mice, and distributed between four 175 cm^2 tissue culture flasks (Nunclon, Nunc, Denmark). Each flask contained 50 ml of growth medium (Dulbecco's modified Eagle's

Accepted 12 April 1976.

*The work was supported by N.I.H. (U.S.A.) contract No. NO1-CP-33367 and in part by grants to the Institute of Cancer Research from the Medical Research Council and the Cancer Research Campaign.

†To whom reprint requests should be sent.

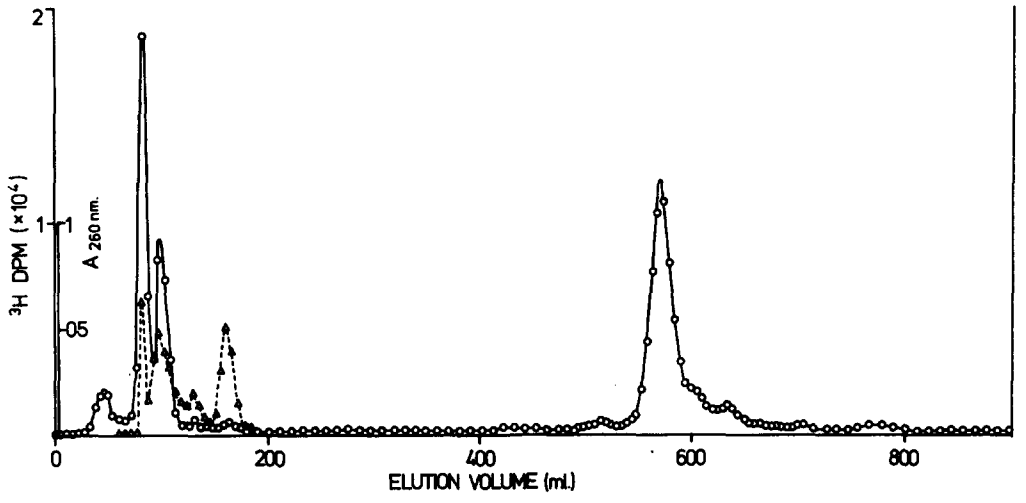


Fig. 1. Sephadex LH 20 column chromatography of an enzyme digest of 0·8 mg of B[a]P-DNA (10·8 μmole [^{3}H]-B[a]P/M DNA phosphorus) derived from a 48 hr incubation from day 3 to day 5 of mouse submandibular gland explants in tissue culture. Addition of the digest to an 80 × 1·5 cm column of LH 20 was followed by elution with a gradient of 500 ml 3:7 methanol/water against 500 ml methanol [4]. 5·5 ml fractions were collected and their tritium content determined by counting total samples in 10 ml of Triton X100 phosphor.

○——○——○ *total d.p.m./fraction*

△ – – △ – – △ $O.D._{260}$.

MEM + 10% foetal calf serum—Gibco: Bio-Cult Ltd., Scotland) supplemented with 1·0 IU/ml insulin (Burroughs Wellcome & Co., London). Totals of 40–80 mice were used to obtain sufficient numbers of cells for each DNA isolation. An estimate was made of the average number of epithelial cells present in each explant at the time of treatment with B[*a*]P. This estimate was based on average explant size, average cell diameter and the assumption that by day 5, one third of the explant volume is cellular. From these figures, each explant contained 4×10^4 cells and each flask (approximately 250 explants) contained therefore about 10^7 cells. A negligible number of mesenchymal cells was present at this early stage of culture [8].

After three days *in vitro*, tritiated B[*a*]P. (Generally tritiated, Radiochemical Centre, Amersham) was added to 50 ml of fresh growth medium in 0·25 ml of dimethylsulphoxide to give a final hydrocarbon concentration of 1·0 μg/ml (specific activity = 10 mCi/mg). 1·0 ml samples of medium were removed at zero time and after 48 hr to measure metabolism of B[*a*]P to water-soluble derivatives [2]. Approximately 30% (15μg/flask) of the hydrocarbon was metabolized in 48 hr. This increased to 40% (20 μg/flask) when cultures were treated on the seventh day *in vitro*, when each flask contained approximately 2×10^7 cells.

At the end of 48 hr explants were harvested, washed and the DNA isolated by the phenol method described previously [4]. Tissue was first ground to powder in liquid nitrogen to ensure adequate cell lysis. The covalent binding of hydrocarbon derivatives to DNA was measured and the sample then enzymatically degraded to deoxyribonucleosides for analysis on an LH 20 Sephadex column. DNA isolated from salivary gland epithelium on day 5 of culture had a binding figure of 11 μmole B[*a*]P/mole DNA phosphorus. At day 9, DNA treated for the previous 48 hr showed a binding figure of 27 μmole/mole DNA phosphorus. At the earlier time, most cells were dividing, which would tend to dilute out bound tritium, and could explain the difference between the two observed binding figures. There was no difference however in the pattern of hydrocarbon products bound to DNA at the two treatment times. This is shown in Fig. 1. The single product species eluting at 570 ml was the same as that described previously in mouse embryo fibroblasts [9] and BHK 21/13 cells [10]. This has been identified as the diolepoxide derivative of B[*a*]P bound to purine deoxyribonucleosides [10]. The nature of the radioactivity eluting in the early region has been discussed previously [11].

These results confirm that the same derivative of B[*a*]P binds to DNA in epithelial as well as fibroblastic cell systems. The epithelial cell type used in these experiments is the target cell in hydrocarbon carcinogenesis both *in vivo* and *in vitro* under identical experimental conditions to those used here. These findings therefore support the hypothesis that binding of this

nature is relevant in studies of the mechanism of hydrocarbon carcinogenesis.

Acknowledgements—The authors wish to thank Jacqueline Amos for excellent technical assistance.

REFERENCES

1. P. Brookes and P. D. Lawley, Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin; Relation between carcinogenic power of hydrocarbons and their binding to DNA. *Nature (Lond)*. **202,** 781 (1964).
2. M. Duncan, P. Brookes and A. Dipple, Metabolism and binding to cellular macromolecules of a series of hydrocarbons by mouse embryo cells in culture. *Int. J. Cancer* **4,** 813 (1969).
3. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, Metabolic activation of benzo[*a*]pyrene proceeds by a diol epoxide. *Nature (Lond.)* **252,** 326 (1974).
4. W. M. Baird and P. Brookes, Isolation of the hydrocarbon deoxyribonucleoside product from the DNA of mouse embryo cells treated in culture with 7-methylbenz[*a*]anthracene-^{3}H. *Cancer Res.* **33,** 2378 (1973).
5. H. P. Rusch, C. A. Baumann and G. L. Maison, Production of internal tumours with chemical carcinogens. *Arch. Path.* **29,** 8 (1942).
6. T. Matsumura, Enzyme histochemistry of experimentally induced tumours in the mouse submaxillary and sublingual glands during carcinogenesis. *Gann* **57,** 251 (1966).
7. C. B. Wigley and A. W. Carbonell, The target cell in the chemical induction of carcinomas in mouse submandibular gland. *Europ. J. Cancer* **12,** 737–741 (1976).
8. C. B. Wigley and L. M. Franks, Salivary epithelial cells in primary culture: Characterization of their growth and functional properties. *J. Cell Sci.* **20,** 149 (1976).
9. W. M. Baird, R. G. Harvey and P. Brookes, Comparison of the cellular DNA-bound products of benzo[*a*]pyrene with the products formed by the reaction of benzo[*a*]pyrene-4,5-oxide with DNA. *Cancer Res.* **35,** 54 (1975).
10. M. R. Osborne, M. H. Thompson, E. M. Tarmy, F. A. Beland, R. G. Harvey and P. Brookes, The reaction of 7,8-dihydro-7,8-dihydroxybenzo[*a*]-pyrene-9,10-oxide with DNA in relation to the benzo[*a*]pyrene-DNA products isolated from cells. *Chem. biol. Interact.* **13,** 343 (1976).
11. M. R. Osborne, M. H. Thompson, H. W. S. King and P. Brookes, Retention of tritium during the binding of tritiated benzo[*a*]pyrene to DNA. *Int. J. Cancer* **16,** 659 (1975).

Europ. J. Cancer Vol. 12, pp. 747–748. Pergamon Press 1976. Printed in Great Britain

Cell Proliferation in the Mammary Glands of the Mouse During Prolonged Unilateral Lactation

G. H. ZEILMAKER and C. M. P. M. VERHAMME
Department of Endocrinology, Growth and Reproduction, Erasmus University, Rotterdam, The Netherlands

IT HAS been shown that lactation has a profound influence on mammary tumorigenesis in mice [1, 2]. In unilaterally lactating mice, in which both the lactating and the thelectomized glands are exposed to the same hormones, tumours invariably occur first in the thelectomized glands. This influence on tumorigenesis is attributed to a protective effect of regular milk removal on preneoplastic nodule formation. The transformation of nodules into tumours is not affected by lactation in intact glands [3, 4].

In order to investigate whether nodule genesis in the thelectomized glands is the result of an increase in cell proliferation induced by thelectomy, an autoradiographic study was initiated in pituitary-graft-bearing mice subjected to prolonged unilateral lactation.

Eight to 12 weeks old nulliparous ($C_3H \times 020$) F1 hybrid mice were allowed to become pregnant. On pregnancy days 15–19 two isologous pituitary glands were grafted under the left kidney capsule and the nipples were excised unilaterally. Following parturition prolonged lactation was induced [3, 5]. After 5 weeks of continuous milk production 21 lactating mice were injected twice daily during 3 consecutive days with 25 μCi (Methyl-H3) thymidine (Amersham, 15–30 C/mMol). One day later the animals were sacrificed and the 4th lactating and thelectomized mammary glands were fixed separately in Bouin solution. Deparaffinized sections (5 μm) were prepared for autoradiography by the dipping technique (Ilford K2 emulsion). The exposure time was 5 weeks.

In the lactating glands very little of the interstitial fat-tissue was present and the structure of the alveoli was normal. Depending on the degree of distention of the alveoli the epithelial cells and their nuclei were more or less flattened. The degree of labelling in the lactating glands was very low. This was quantitated by determining the percentage of labelled nuclei in 4–10 samples of 100 epithelial cells per mammary gland [average 1·3%, median 0·5%, range 0–8%, 18 animals (Fig. 2)]. A low degree of labelling in lactating glands during normal lactation in mice [6] has been described earlier.

In the thelectomized glands of the same mice, intact small and distended alveoli as well as degenerated and disorganized alveoli were present. Labelling was intense in small intact alveoli and in disorganized alveoli, in which the nuclei were no longer situated in the periphery [average percentage of labelled nuclei in 4–10 randomly chosen samples of epithelial cells: 13%, median 9·5%, range 1–60%, 18 animals (Fig. 1)].

In 3 animals the histology of the lactating and thelectomized glands both showed the presence of many degenerated alveoli and labelled alveolar nuclei, probably as a result of inadequate milk production. These animals were not included in the data.

The present observations are in line with the hypothesis [3] that in thelectomized glands, exposed to a strong hormonal stimulus, the distended alveoli degenerate as a result of the stagnation of secretion products.

The adipose tissue, present only in the thelectomized glands as a result of this degeneration process, provides space for the occurrence of epithelial cell proliferation, leading to the formation of new distended alveoli. These alveoli may originate by reorganization of the remnants of degenerated alveoli into small tubular alveoli [7]. Alveolar degeneration occurring after cessation of milk removal following thelectomy or suppression of young has been observed earlier [8, 9].

The results of this study indicate that in the mammary gland of susceptible mice an increase in cell proliferation correlates with the formation of preneoplastic and neoplastic lesions.

Accepted 14 April 1976.

REFERENCES

1. H. J. Bagg, Experimental studies concerning the functional activity of the breast in relation to mammary carcinoma in mice. *J. Cancer Res.* **9,** 498 (1925).
2. O. Mühlbock and W. van Ebbenhorst Tengbergen, Functional components in the genesis of mammary cancer in mice, pregnancy and lactation. *Acta Un. int. Cancr.* **17,** 88 (1961).
3. G. H. Zeilmaker, Prolonged lactation in mice and its effect on mammary tumorigenesis. *Int. J. Cancer* **3,** 291 (1968).
4. G. H. Zeilmaker, Prolonged lactation in nodule bearing mice and its effect on mammary tumor genesis. *Europ. J. Cancer* **4,** 447 (1968).
5. G. H. Zeilmaker, Milk yield during prolonged lactation in mice; effects of ovariectomy. *J. Reprod. Fertil.* **19,** 361 (1969).
6. H. H. Traurig, Cell proliferation in the mammary gland during late pregnancy and lactation. *Anat. Rec.* **157,** 489 (1967).
7. H. A. Cole, The mammary gland of the mouse, during the oestrous cycle, pregnancy and lactation. *Proc. Roy. Soc. ser. B* **114,** 136 (1934).
8. W. L. Williams, The effect of non-suckling and the non-removal of milk upon individual mammary glands in the lactating mouse. *Yale J. Biol. Med.* **14,** 201 (1941).
9. S. R. Wellings and K. B. DeOme, Electron microscopy of milk secretion in the mammary gland of the C_3H/Crgl mouse. III Cytomorphology of the involuting gland. *J. nat. Cancer Inst.* **30,** 241 (1963).

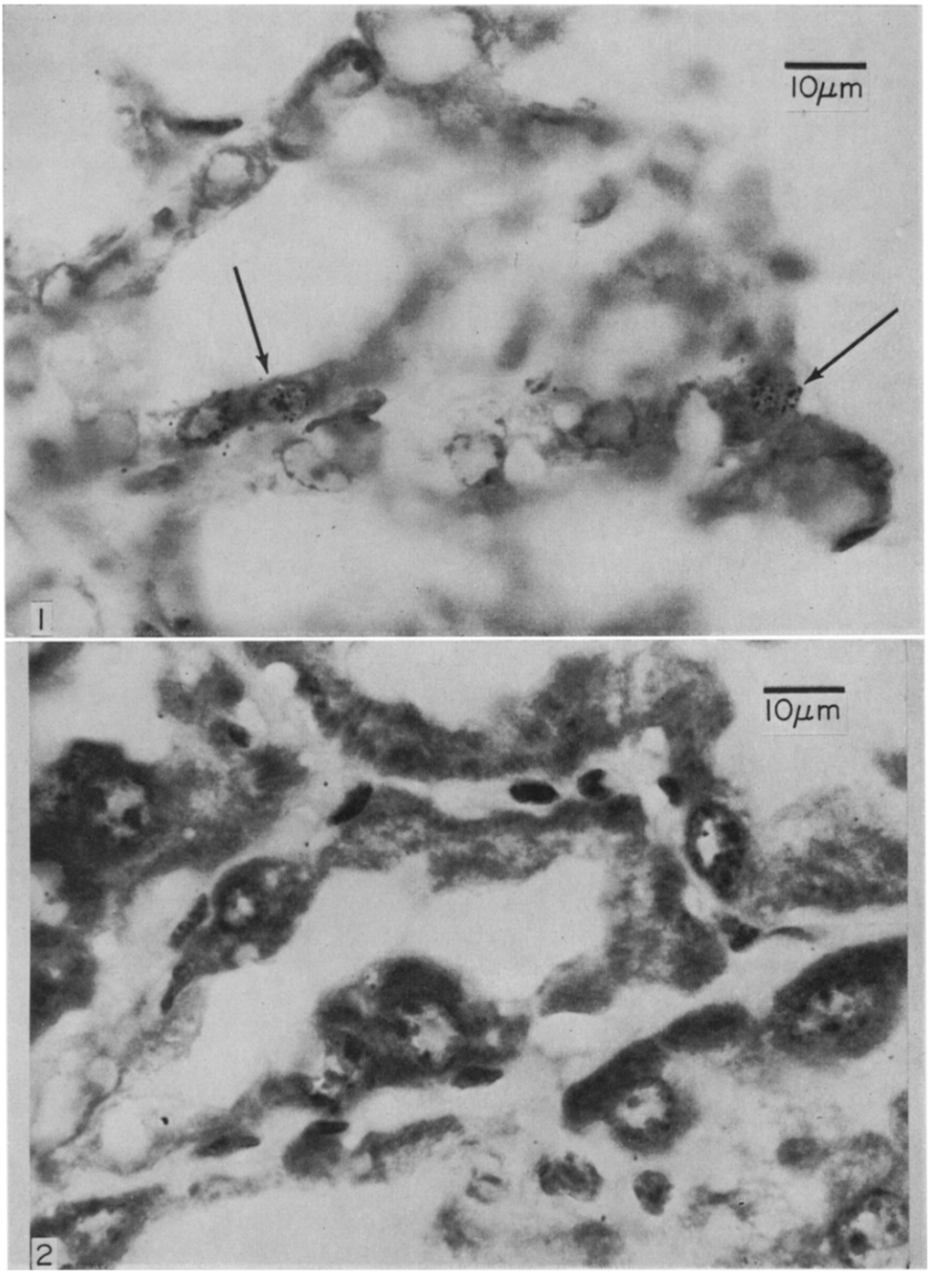


Fig. 1. *Autoradiograph of a thelectomized mammary gland of a mouse, bearing* 2 *isologous pituitary grafts under the kidney capsule and lactating continuously for* 5 *weeks. Labelled epithelial cells after* 6×25 *μC* ^{3}*H-thymidine in* 72 *hr* (*arrows*).

Fig. 2. *Section of contralateral lactating gland of the same animal subjected to the same autoradiographic treatment. Note the absence of labelling.*

(to face p. 748)

Europ. J. Cancer Vol. 12, pp. 749–751. Pergamon Press 1976. Printed in Great Britain

Control of an EL4 Lymphoma in Nude Mice by Passively Administered Antibody

G. J. O'NEILL
Searle Research Laboratories, Lane End Road, High Wycombe, England

THE NUDE mouse mutant (nu/nu) is at present attracting considerable attention as an experimental model for a variety of purposes. This animal is congenitally athymic and is deficient in T cell function [1, 2]. Consequently the nude will accept allografts and xenografts of both normal and malignant tissue and is being used increasingly as a carrier of human tumours [3–6]. In recent papers from our laboratories we have described the control of the EL4 lymphoma in C57BL/6 mice by the use of the globulin fraction of a rabbit anti-EL4 serum given in conjunction with or chemically linked to cytotoxic drugs [7–10]. There is an obvious attraction in using the nude mouse a a means of extending this work to human tumours, but before doing so it would seem appropriate to explore our ability to control the development of EL4 in the nude by this approach. The paper summarizes the results of experiments in which anti-tumour globulin was administered 24 hours after subcutaneous injection of live EL4 cells.

The preparation of the antiserum has been described previously [7]. Briefly, rabbits were given three i.v. injections of live EL4 cells at 10 day intervals and were bled out 7 days after the third injection. The heat inactivated serum was absorbed with packed mouse spleen cells until it showed no reactivity in a C'-dependent lymphocytotoxic assay when tested against C57BL/6 lymphocytes. The absorbed serum was fractionated with ammonium sulphate and the total Ig fraction was used without any further purification. The nude mice were obtained from Carworth Europe (Huntingdon, England) and were fed on a conventional diet. They were kept separate from other mice and were housed initially in normal boxes with a sealed-on filter top and subsequently in a small animal isolator with a filtered air supply (Vickers Ltd). Under these conditions we have been able to maintain the mice for over 100 days with few losses.

The EL4 tumour used in these experiments was maintained as an ascites in C57BL/6 mice. The nude mice were injected s.c. in the groin with live EL4 cells then intraperitoneally 24, 48, 72 and 96 hr later with either saline or anti-tumour globulin. The results of 4 separate experiments are shown in Table 1 which gives the survival times in days of the individual mice in the experiments. The significance of the differences was determined by a non-parametic ranking method based on X^2 [11]. As can be seen from experiments N-1, N-3 and N-4, administration of anti-EL4 globulin conferred a significant degree of protection on mice previously challenged with 5×10^4 or 1×10^5 EL4 cells. The protective effect was lost when the number of EL4 cells was increased to 5×10^5 or 10^6 (Expt. N-2). An inhibitory effect was not obtained with Ig from an absorbed antiserum against C57BL/6 lymphocytes (Expt. N-4) indicating that the effectiveness of the anti-EL4 Ig is due to the presence of tumour-specific antibodies, not to other antibodies undetected in the C'-dependent lymphocytotoxicity assay used to monitor the absorption of the antisera. Any such antibodies should presumably have been present in the antiserum against normal C57BL/6 lymphocytes. The ineffectiveness of this Ig also suggests that the non tumour-directed antigen–antibody complexes which are undoubtedly present in such extensively absorbed antisera are not responsible. These results with EL4 are in contrast with those of Fink *et al.* [12] who reported stimulation of the growth of a mouse fibroblast tumour in thymectomised, irradiated, reconstituted (Tx-B) and nude mice by certain levels of rabbit anti-tumour cell serum. The discrepancy may be a reflection of the different tumours used but it should be noted that in their experiments the antiserum treatment began before injection of the tumour cells whereas in the experiments reported here the

Accepted 2 April 1976.

Table 1

Experiment	No. of EL4 cells	Ig used	Survival time (days) of mice treated with Saline	Ig	
N-1*	5×10^4	1mg R165/68	26, 26, 28, 29, 30, 60, 60	60, 60, 60, 60, 60, 60, 60	$P<0{\cdot}025$
N-2	5×10^5	1mg R165/68	19, 21, 22, 22, 22, 23, 24, 25	20, 20, 22, 22, 22, 24, 33	N.S.
	5×10^6	1mg R165/68	14, 17, 19, 19, 21, 21, 21, 22	17, 17, 19, 20, 22, 22, 22	N.S.
N-3	1×10^5	2·8 mg R197/203	20, 22, 23, 23, 23	68, 86, 100, 107, 117	$P<0{\cdot}025$
N-4†	1×10^5	1mg R197/203	16, 21, 25, 25, 30, 33	26, 29, 155, 155, 155, 155	$P<0{\cdot}025$
		1mg R105/107‡		23, 26, 27, 28, 28, 32	N.S.

*In this experiment the survivors were killed at Day 60.
†This experiment was terminated at 155 days.
‡R105/107 is the Ig fraction of a rabbit antiserum against C57BL/6 lymphocytes, absorbed and fractionated in the same way as the rabbit anti-EL4 sera R165/68 and R197/203.

mice received the tumour cells 24 hr before anti-tumour globulin. The mechanism of the protective effect has not been clarified. The anti-EL4 globulin will lyse EL4 cells *in vitro* in the presence of guinea-pig or rabbit complement but it is not clear if this can take place *in vivo* with the mouse's own complement. Elimination of antibody coated tumour cells by macrophages is a possibility which will be explored by repeating the experiments in macrophage-depleted animals.

The experiments reported here are limited and have to be extended to investigate the importance of the time interval between tumour challenge and administration of antiserum and also the effect of varying the amount of antiserum used. However, the fact that under any circumstances passively administered tumour-specific antibody can inhibit tumour growth in the absence of normal T cell function is of considerable theoretical interest, especially since a depressed cellular immunity is a common feature in human cancer.

Acknowledgements—The excellent technical assistance of Mr. A. J. Manstone, Mrs. S. Buckham and Miss N. Clinch is gratefully acknowledged, and I wish to thank Mr. J. R. Shelton for the statistical analysis.

REFERENCES

1. E. M. Pantelouris, Athymic development in the mouse. *Differentiation* **1,** 437 (1973).
2. H. H. Wortis, Immunological responses of "Nude" mice. *Clin. exp. Immunol.* **8,** 305 (1971).
3. C. D. Povlsen and J. Rygaard, Heterotransplantation of human adenocarcinoma of the colon and rectum to the mouse mutant *Nude*. A study of nine consecutive transplantations. *Acta pathol. microbiol. scand.* Section A **79,** 159 (1971).
4. C. D. Povlsen and J. Rygaard, Heterotransplantation of human epidermoid carcinomas to the mouse mutant *Nude*. *Acta pathol. microbiol. scand.* **80,** 713 (1972).
5. P. C. Giovanelia, J. S. Stehlin and L. J. Williams, Heterotransplantation of human malignant tumours in "Nude" thymusless mice. II. Malignant tumours induced by injection of cell cultures derived from human solid tumours. *J. nat. Cancer Inst.* **52,** 921 (1974).
6. L. Ozzello, B. Sordat, C. Mesanda, S. Carrel, J. Hurlimann and J. P. Mach, Transplantation of a human mammary carcinoma cell line (BT 20) into Nude mice. *J. nat. Cancer Inst.* **52,** 1669 (1974).
7. D. A. L. Davies and G. J. O'Neill, *In vivo* and *in vitro* effects of tumour specific antibodies with chlorambucil. *Brit. J. Cancer* **28,** Suppl. I, 285 (1973).
8. D. A. L. Davies, S. Buckham and A. J. Manstone, Protection of mice against syngeneic lymphomata. II. Collaboration between drugs and antibodies. *Brit. J. Cancer* **30,** 305 (1974).

9. D. A. L. DAVIES and G. J. O'NEILL, Methods of cancer immuno-chemotherapy, (DRAB and DRAC) using antisera against tumour specific cell-membrane antigens. *VII Int. Cancer Cong.* p. 218. Excerpta Medica, Amsterdam (1975).
10. G. F. ROWLAND, G. J. O'NEILL and D. A. L. DAVIES, Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage. *Nature(Lond.)* **255,** 487 (1975).
11. R. PETO and M. C. PIKE, Conservatism of the approximation $\Sigma (O—E)^2/E$ in the logrank test for survival data or tumour incidence data. *Biometrics* **29,** 579 (1973).
12. M. P. FINK, C. W. PARKER and W. T. SHEARER, Antibody stimulation of tumour growth in T-cell depleted mice. *Nature (Lond.)* **225,** 404 (1975).

Europ. J. Cancer Vol. 12, pp. 753–754. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *July, 1976*

Special Reports

E. Hecker: Definitions and terminology in cancer (tumor) etiology—an analysis aiming at proposals for a current internationally standardized terminology.

L. M. Shabad: Centennial of experimental cancer research.

Human Cancer

P. L. Grover, A. Hewer, K. Pal and P. Sims: The involvement of a diol-epoxide in the metabolic activation of benzo(a)pyrene in human bronchial mucosa and in mouse skin.

T. Katsuki and Y. Hinuma: Susceptibility of human leukocytes to transformation by Epstein-Barr virus: Quantitative analyses.

J. Dumont, A. Liabeuf, W. Henle, N. Feingold and F. M. Kourilsky: Anti-EBV antibody titers in non Hodgkin lymphomas.

N. Grosser, J. H. Marti, J. W. Proctor and D. M. P. Thomson: Tube leukocyte adherence inhibition assay for the detection of anti-tumour immunity. I. Monocyte is the reactive cell.

J. H. Marti, N. Grosser and D. M. P. Thomson: Tube leukocyte adherence inhibition assay for the detection of anti-tumour immunity. II. Monocyte reacts with tumour antigen via cytophilic anti-tumour antibody.

N. Grosser and D. M. P. Thomson: Tube leukocyte (monocyte) adherence inhibition assay for the detection of anti-tumour immunity. III. "Blockade" of monocyte reactivity by excess free antigen and immune complexes in advanced cancer patients.

F. Mitelman, P. G. Nilsson, G. Levan and L. Brandt: Nonrandom chromosome changes in acute myeloid leukemia. Chromosome banding examination of 30 cases at diagnosis.

F. Mitelman, G. Levan, P. G. Nilsson and L. Brandt: Nonrandom karyotypic evolution in chronic myeloid leukemia.

I. Miyoshi, I. Kubonishi, H. Uchida, S. Hiraki, Y. Matsuda, T. Tanaka, H. Masuji and K. Hiraki: Production of lymphoid tumors in hamsters by direct implantation of normal human peripheral and umbilical cord leukocytes.

Experimental Cancer

E. Podmaniczky, B. Szende, K. Lapis and G. Ferenc: Particular cell surface changes observed in MC-29 virus infected chicken-embryo fibroblast (CEC) cultures.

E. Huberman, H. Yamasaki and L. Sachs: Independent regulation of two types of aryl hydrocarbon (benzo(a)pyrene) hydroxylase in mammalian cells.

I. P. Witz, N. Lee and G. Klein: Serologically detectable specific and cross-reactive antigens on the membrane of a polyoma virus-induced murine tumor.

A. D. Bershadsky, V. I. Gelfand, V. I. Guelstein, J. M. Vasiliev and I. M. Gelfand: Serum dependence of the expression of transformed phenotype: Experiments with subline of mouse L fibroblasts adapted to growth in serum-free medium.

S. Haba, T. Hamaoka, K. Takatsu and M. Kitagawa: Selective suppression of T cell activity in tumor-bearing mice and its improvement by lentinan, a potent anti-tumor polysaccharide.

R. Michalides, G. Vlahakis and J. Schlom: A biochemical approach to the study of the transmission of mouse mammary tumor viruses in the mouse strains RIII and C3H.

G. Braslawsky, M. Ran and I. P. Witz: Tumor bound immunoglobulins: The relationship between the *in vivo* coating of tumor cells by potentially cytotoxic anti-tumor antibodies, and the expression of immune complex receptors.

British Journal of Cancer *September, 1976*

D. S. Nelson and R. Kearney: Macrophages and lymphoid tissues in mice with concomitant tumour immunity

D. Munjal and D. M. Goldenberg: A study of carcinoembryonic antigen and glucose phosphate isomerase in human colonic cancer and in the GW-39 human tumour model.

Mirella Giovarelli, P. M. Comoglio and G. Forni: Induction of resistance or enhancement to a transplantable murine plasmacytoma (MOPC-460) by transfer of non-immune leukocytes.

Ilse Lasnitzki: Reversal of methylcholanthrene-induced changes in mouse prostates *in vitro* by retinoic acid and its analogues.

N. Prasad and R. Prasad: Altered enzyme expression in "differentiated" murine neuroblastoma cells.

W. B. Looney: Solid tumour models for the assessment of different treatment modalities: IV. The combined effects of radiation and 5-fluorouracil.

J. A. Dickson and B. E. Oswald: The sensitivity of a malignant cell line to hyperthermia (42°C) at low intracellular pH.

V. J. Pickthall: Detailed cytogenetic study of a metastatic bronchial carcinoma.

F. Gielen, J. Dequeker, A. Drochmans, J. Wildiers and M. Merlevede: Critical evaluation of hydroxyproline excretion in patients with breast cancer: Relation with bone metastases as revealed by radiology and scintygraphy.

A. Jacobs, B. Jones, C. Ricketts, J. L. Hayward, R. D. Bulbrook and D. Y. Wang: Serum ferritin concentration in early breast cancer.

Short Communications

Tessa E. Sadler and J. E. Castro: Abrogation of the anti-metastatic activity of corynebacterium parvum by antilymphocyte serum.

W. R. Miller: Hormonal status and testosterone metabolism of DMBA induced rat mammary carcinomas.

Raman Gokal, Jill Durrant, J. David Baum and M. J. Bennett: Successful pregnancy in acute monocytic leukaemia.

Europ. J. Cancer Vol. 12, pp. 755–762. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Anticoagulants in the Treatment of Cancer

P. HILGARD*† and R. D. THORNES‡

**Innere Klinik and Poliklinik (Tumorforschung), Universitätsklinikum, Essen, Germany*

‡Department of Experimental Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland

INTRODUCTION

A PATHOGENIC role of blood coagulation in the haematogenous spread of cancer was suggested by the microcinematographic investigations of S. Wood Jr. using the Hopkins rabbit ear chamber [1]. In subsequent studies tumour cell suspensions were injected intravenously into animals and the fate of these cells in the capillary bed was observed. If these animals are allowed to survive tumour colonies develop in various organs dependent upon the number of cells injected. After sacrifice of the animals these colonies can be evaluated with respect to their number, size and organ distribution. This experimental design provided most of our knowledge concerning the morphology of fibrin formation and platelet aggregation in connection with tumour cell lodgement [2–4]. Similar experimental models were used to investigate the effects of altered blood coagulability on haematogenous metastasis formation, detailed presentations can be found in the review articles of Hagmar [5] and Wood [6]. Most of the results, which are summarized in Fig. 1. seem to support the evidence for the pathophysiological importance of blood coagulation in the earliest stages of metastasis formation.

The above colony technique was highly artificial because tumour cell suspensions were used which were removed from their original milieu and treated in various ways. These cell suspensions were then injected into healthy animals which lacked all the changes in the plasma protein pattern induced by the primary tumour. Furthermore, most of these experiments were carried out with allogeneic tumours,

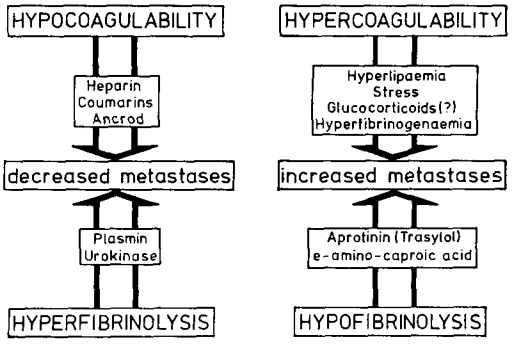


Fig. 1. Diagrammatic presentation of the effect of altered blood coagulability on the development of tumour colonies in various animal systems. (Detailed informations are given in the review articles cited in the text.)

therefore, especially in long term studies, uncontrolled immunological factors might have influenced the results. Consequently, the present paper will focus mainly on more recent experimental results which were obtained in spontaneously metastasizing, syngeneic tumours and the sparse clinical results available. From this viewpoint a broader concept of the pharmacology of anticoagulants in the treatment of cancer should emerge.

The blood clotting system can be influenced by drugs at different stages of the enzymatic process involving plasma factors and blood

Accepted 7 May 1976.

†The author's experimental work described in this article was supported by grants from the "Deutsche Forschungsgemeinschaft" Bad Godesberg, Germany (Hi 213/1-2).

platelets. Most of the drugs have been used in tumour dissemination studies, they are summarized in Fig. 2.

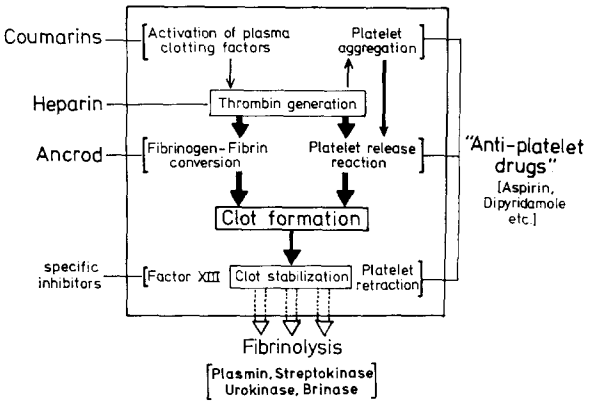


Fig. 2. Simplified coagulation scheme indicating the mode of action of various antithrombotic drugs which have been used to prevent experimental metastases.

HEPARIN

Historically heparin was the first anticoagulant shown to have antimetastatic properties. In 1952 Terranova and Chiossone [7] demonstrated that heparinization of the animals prior to the intravenous injection of tumour cells resulted in a significant reduction of tumour colonies in the lungs. Subsequently, this observation was confirmed by numerous authors [8].

Retik and co-workers [9] investigated the influence of continuous anticoagulation with heparin on spontaneous metastasis formation from the murine sarcoma DBA 49 and sarcoma T241. In the DBA 49 system no antimetastatic effect was found whereas in the T241 system metastasis formation increased significantly. No influence on the growth of the primary tumour was demonstrated. Later Hagmar [10, 11] confirmed Retik's findings. He reported an increase of spontaneous metastasis formation after continuous heparin therapy and he also demonstrated that the intravenous tumour cell injection into heparinized animals resulted in a slight decrease of lung colonies but with an increase of extra pulmonary tumour colonies. Dicumarol did not show this effect in the same tumour host system. It was, therefore, suggested that the drugs act independently of blood coagulation.

Wilkins [12] found that polystyrene latex particles injected intravenously into rats were arrested in certain organs corresponding to the surface charge of the particles. Negatively charged particles passed through the lung and were mainly held up in liver and spleen. In contrast, positively charged particles were arrested primarily in the lung. Heparinization of the animals reversed the distribution of the positively charged particles which then behaved like negatively charged particles. The coumarin type anticoagulant Warfarin did not influence particle distribution. This phenomenon was therefore interpreted as being due to changes in the surface charge through heparinization.

It was demonstrated by cell electrophoresis that heparin indeed alters the surface charge of tumour cells [13]. The migration of Walker tumour cells through the interstitial space could be changed through heparinization of the animals, yet the clearance of these cells from the circulating blood was not influenced by heparin. This finding cannot be explained solely via the mechanism of anticoagulation [14].

The biological activities of heparin and other polyanions have a broad spectrum; cellular functions may be influenced and changes in the host's defence mechanisms may occur. Two synthetic heparinoids—polyethylene sulfate and pyrancopolymere—are even used as cytostatic agents [15]. The chemical combination of alkylating agents with heparin has increased their cytotoxic action in experimental animals [16]. The elucidation of the role of heparin in cancer therapy is, however, further complicated by the experimental findings of synergism between unspecific immunostimulation and heparin therapy [17].

Clinical experience seems to confirm the beneficial action of heparin therapy. Ludwig [18] has reported longer recurrence free intervals in patients treated with anticoagulants, mainly heparin, during irradiation of gynaecological cancers. Elias used heparin as adjuvant therapy to combination chemotherapy of inoperable lung cancer and claimed enhanced therapeutic effects in a comparative trial [19]. We are presently investigating the possible enhancing effect of short term heparin therapy on cytotoxic therapy. The mode of action of heparin, however, remains unclear, its influence on the clotting mechanism appears to be only one of several other therapeutical aspects in the treatment of established autochthonous tumours.

COUMARIN-DERIVATIVES

(Warfarin, Phenprocoumon etc.)

A significant antimetastatic effect of Phenprocoumon in a syngeneic, methylcholanthrene induced rhabdomyosarcoma of the mouse was described by Hagmar [20]. In the same tumour

system heparin was ineffective although controlled anticoagulation was maintained over an equal period. A similar finding was reported by Berkarda and coworkers [21] using the spontaneously metastasizing Lewis lung carcinoma. Heparin anticoagulation was without any significant influence upon pulmonary metastases in this system, in contrast, Warfarin anticoagulation decreased the number of lung metastases to 8% of the control group. Similar results were reported by Ryan and coworkers [22] using two spontaneously metastasizing carcinomas and more recently Brown [23] confirmed these findings. The antimetastatic effect of Warfarin was also demonstrated in autochthonous, carcinogen induced murine tumours [24]. Surgical treatment of the sarcoma T241 could only cure the animals in 8%, whereas adjuvant anticoagulation increased the cure rate to 55% [25].

Most investigators agree that coumarin derivatives are potent antimetastatic drugs and in some tumour systems even a significant inhibition of primary tumour growth was found. The latter effect may point to the possibility that these drugs act directly on tumour cells. Lisnell and Mellgren [26] demonstrated a cytotoxic effect of dicumarol on human culture cells which was dependent upon the concentration of dicumarol in the culture medium. However, the investigations of Chang and Hall [27] showed that only very high doses of Warfarin led to a significant inhibition of thymidine and uridine uptake into the DNA and RNA of L1210 leukaemia cells. The survival rate of mice bearing the leukaemia L1210 was not altered by various dose schedules of Warfarin. No direct cytotoxic effect of short term Warfarin therapy was observed on primary or metastatic L1210 leukaemia and adenocarcinoma 755 [28]. On the other hand human malignant glial cells in culture appear sensitive to very low concentrations of Warfarin. Concentrations of 10^{-10} M of l-sodium Warfarin suspend cell replication over a 48-hr period [29].

In time lapse cinematography of cell cultures Warfarin concentrations of 10^{-6} M were shown to inhibit cell motility and mitotic activity [29]. This observation is interesting in the light of the *in vivo* effect of Warfarin anticoagulation on the motility of V2 carcinoma cells in rabbits [30]. Tumour cells were placed in Hopkins rabbit ear chambers in control and Warfarin treated animals. Specific inhibition of tumour cell locomotion was observed after 4 days of anticoagulant therapy sufficient to produce a doubling of the animals' prothrombin time. Leucocyte locomotion was not inhibited for polymorphnuclear infiltration of the tumours was more rapid and intense in the treated animals. Cessation of Warfarin therapy and administration of vitamin K resulted in a return of motility to the remaining tumour cells. The reinduction of Warfarin therapy again inhibited locomotion and prolonged therapy resulted in necrosis of tumour cells and their removal by macrophages. These latter cells appeared earlier and in greater numbers in the Warfarin treated animals which may be important in view of the postulated role of macrophages in immunodefense.

In terms of synergism with cytotoxic agents Warfarin was shown by Kirsch and coworkers [29] to alter the tissue distribution and plasma clearance of 5-fluorouracil. In the spontaneously metastasizing Lewis lung carcinoma of mice we found that carefully monitored oral anticoagulation with Phenprocoumon throughout the period of tumour growth produced a significant reduction in lung metastases but short term anticoagulation was ineffective. The primary tumour growth rate was significantly reduced and combination of cyclophosphamide and 5-fluorouracil with Phenprocoumon gave additive effects against the tumour. In contrast, combination of oral anticoagulation and bleomycin resulted in a highly significant tumour inhibition compared to bleomycin alone, suggesting some kind of drug synergy between bleomycin and the anticoagulant [31].

Oral anticoagulants were first used deliberately to observe their effects on clinical cancer from 1961 to 1967. There was no evidence that anticoagulants alone in advanced cases produced regression of tumour but once regression was obtained together with cytotoxic drugs, anticoagulants appeared to be adequate maintenance therapy [32]. A controlled trial with 128 patients [33] using Warfarin as adjuvant therapy to chemotherapy gave a two year survival for the Warfarin group of 40·6% compared to cytotoxic therapy alone of 17·7%. In a small group of patients on maintenance therapy for lymphoma or chronic leukaemia sensitivity to cytotoxic drugs was restored by addition of Warfarin oral anticoagulation in 21 patients out of 30 [34]. At present there is an ongoing trial of Warfarin as an adjuvant to cyclical combined chemotherapy of lymphoma and breast cancer. The pilot study for this showed that Warfarin slows down the regrowth of tumours between courses of chemotherapy. It has, therefore, been possible to increase the

intervals between courses and thus decrease toxicity [35].

In patients on long term anticoagulation for diseases other than cancer Michaels [36] has shown that the incidence of cancer in this group is as expected for the general population but that the death rate from cancer is only one eighth of the expected rate. Short term Warfarin therapy is being used in an uncontrolled trial to prevent metastases postoperatively in a series of patients with osteogenic sarcoma and the results appear to be promising [37].

From the foregoing it can be concluded that several actions may be ascribed to oral anticoagulants of the coumarin type. In short courses of therapy anticoagulation may play a role in preventing the haematogenous spread of cancer. In long term therapy the direct effect of these oral anticoagulants on cancer cells would appear to be more important. The experimental and clinical evidence that oral anticoagulants have the ability to slow down tumour growth rate may be their most important contribution to the therapy of malignant diseases.

DEFIBRINATING VENOMS (Ancrod)

Another anticoagulant with a completely different mode of action is of some, at least theoretical, interest. Ancrod, a defibrinating pit viper venom is a clinically and experimentally well documented antithrombotic drug. Up to now only a few investigations into the influence of this drug on tumour growth and tumour dissemination are available. The lack of experimental data is mainly due to the difficulty of inducing adequate therapy in rats and mice. However, Austin and Glaser succeeded in maintaining continuous defibrination in mice by 8-hourly intraperitoneal injections [38]. This treatment was accompanied by a significant inhibition of tumour growth of the allogeneic sarcoma 180. If the same dose of Ancrod was given in 16–24 hr intervals and only partial defibrination was achieved, no influence on tumour growth was detectable. Hagmar [39] investigated the influence of experimental defibrination on the development of lung colonies after intravenous tumour cell injections and did not find any significant effect in two murine systems. The therapeutically effective dose of Ancrod in mice is approximately 25 times higher than in rabbits, therefore, we tried to check Hagmar's results using rabbits and the V2 carcinoma [40]. A significant decrease of lung colonies and of extrapulmonary colonies after i.v. tumour cell injection into defibrinated animals was found. These findings were recently confirmed in rats which were rendered afibrinogenaemic with Defibrase, another thrombin-like enzyme from the venom of Bothrops atrox [41]. A variety of proteases derived from viper venoms have shown tumour inhibitory capacities [42] and it remains obscure which mechanism is responsible for the action of defibrinating agents.

Clinically we found Ancrod too antigenic for long term treatment of patients with cancer. However, in a small phase-I like study with 15 patients with various malignancies Rieche [43] claimed a potentiation of the cytostatic effects of conventional chemotherapy by concomitant defibrination of the patients. In these cases Ancrod therapy was well tolerated, provided adequate laboratory control was available.

FACTOR XIII—INHIBITORS

Laki has recently propagated the importance of fibrin stabilizing factor (factor XIII) in tumour growth and tumour spread [44]. Yancey and Laki [45] found a significant inhibition of experimental tumour growth by pharmacological inhibition of factor XIII. However, these results have not yet been confirmed by other authors. In contrast, the *in vivo* inhibition of factor XIII by monodansylcadaverine in rabbits resulted in a considerable increase of colonies to the lung and other organs after intravenous injection of V2 carcinoma cells [46]. The experimental results currently available do not necessarily support the importance of fibrin stabilization for tumour growth and tumour dissemination.

FIBRINOLYSIS

Since Johnson and Wood [47] were able to show in the rabbit ear chamber that the thrombotic material around tumour cell emboli could be lysed by injections of fibrinolytic agents, it was shown under other experimental conditions that fibrinolytic agents had antimetastatic effects, while inhibitors of fibrinolysis increased metastasis formation [48]. The only study in which the influence of plasmin on spontaneously metastasizing tumours was investigated [49] confirmed the antimetastatic effects of induced fibrinolysis, yet no increasing effect of the inhibitors e-amino-caproic acid

and aprotinin was found. In the light of our present knowledge the influence of fibrinolytic agents or inhibitors of fibrinolysis on metastasis formation and primary tumour growth seem to involve more complex mechanisms. Proteolytic enzymes, especially plasmin, may be cytotoxic [50, 51]. On the other hand, they seem to have some importance for the maintenance of malignant characteristics such as contact inhibition [52]. Reich and coworkers [53] found that malignant transformation was associated with the appearance of an intracellular plasminogen activator. Conversely, plasmin has been found to labilize the lysosomal hydrolases in rat Yoshida ascites sarcoma cells and to enhance the effect of cytotoxic agents [54]. The multiplicity of the interactions between endogenous proteolytic enzymes and cancer cells complicate the interpretation of experimental results concerning the role of fibrinolysis in the spread of tumours.

Therapeutical fibrinolysis with urokinase and streptokinase was first used to treat cancer clinically in 1961 [55] following the observation that human plasmin was cytotoxic *in vitro* [50]. In 1964 Larsen and coworkers [56] used porcine plasmin to treat eleven patients with advanced cancer and they reported some beneficial effects of this therapy. In 1966, 31 patients with cancer were treated with fibrinolytic agents by Cliffton [57]. Our further studies in patients with malignant diseases produced many initially unexplained phenomena; leukaemia cells were removed from the circulation [58], complement dependent autocytotoxicity was produced [59] and tests for delayed hypersensitivity were enhanced [60]. It was found that the fibrinolytic agents used—Brinase and streptokinase—are potent antianergic drugs which unblock the cellular immune mechanisms [61].

The interaction of plasmin with the activation of the complement system is well known [62]. It is thus an additional difficulty to separate fibrinolytic effects from immunological effects on cancer cells. The only known randomized controlled clinical trial of a fibrinolytic agent is streptokinase, given immediately after resection of tumour [61, 63]. The trial was based on the work of Grossi and coworkers [64] in rats and intended to show that lysis of fibrin would prevent entrapment of tumour cells released during operation. The favourable trends in this study may be due more to immune mechanisms than lysis of fibrin. An ongoing study using postoperative urokinase therapy seems to confirm the beneficial effects of induced fibrinolysis in the management of tumours [65].

INHIBITION OF PLATELET AGGREGATION

Among the drugs with antithrombotic capacities some interest has focussed on inhibitors of platelet aggregation. Ultrastructural investigations suggested that circulating tumour cells may have a close contact with blood platelets [66]. On the other hand tumour cells attached to the vascular endothelium are rapidly surrounded by platelet aggregates [5, 6, 67]. Gasic and coworkers [68] reported that neuraminidase induced thrombocytopenia resulted in a significant reduction of lung colonies after intravenous injection of tumour cells and that this antimetastatic effect could be reversed by platelet transfusions. The rate of fatal tumour cell emboli after intravenous tumour cell injection in rats could be reduced by various inhibitors of platelet aggregation [69]. These observations led to the assumption that there may be an interrelation between blood platelets and intravascular tumour cells in the pathogenesis of metastasis formation. Again Gasic and coworkers [70] showed in various mouse tumours that acetyl-salicylic acid was an antimetastatic drug. This was interpreted as being due to the inhibition of platelet functions. Although laboratory tests of platelet functions showed distinct alterations by Aspirin pretreatment, we could not find any significant differences in the distribution of tumour colonies in control and treated rabbits after intravenous injection of V2 carcinoma cells [71]. Recently we investigated the effect of various inhibitors of platelet aggregation on the growth and spread of the syngeneic Lewis lung carcinoma in mice [72]. The lung colony assay after intravenous tumour cell injection was used and simultaneously the effect of long term treatment on spontaneous metastasis formation was investigated. Surprisingly, in the lung colony assay all drugs provoked an increase of colonies whereas no significant influence on spontaneous metastasis formation was found. Presently these different results are difficult to interpret since only few and partially heterogenous investigations are available concerning the pathophysiological importance of blood platelets in haematogenous metastasis formation [67].

CONCLUSION

There is sufficient experimental evidence to suggest that the coagulation mechanism is involved in the spread of cancer. The pathogenic significance of fibrin formation and platelet aggregation around embolic tumour cells,

however, is not yet fully understood. Antithrombotic drugs are, generally, also antimetastatic drugs and influence to some degree primary tumour growth as well. The multiplicity and often diverse pharmacological actions of these agents render interpretations of experimental results difficult. Of interest to the treatment of established human cancer are the long term effects of some anticoagulants. Both heparin and coumarin derivatives appear to have differing effects independent of their influence on blood coagulation. Heparin may potentiate cytotoxic drugs whereas in addition coumarin derivatives inhibit or slow down tumour cell growth. Fibrinolysis would appear to have a major influence on the cellular immune mechanism which is the host's main defence against malignant growth. More recent approaches to alter blood coagulability by defibrinating viper venoms, inhibitors of fibrin stabilizing factor or decreased platelet aggregability, have not yet come to conclusive results.

The hazards of anticoagulant therapy are relatively few and the little information about the effects of this therapy on the clinical course of cancer which is currently available, strongly calls for further basic and clinical research.

REFERENCES

1. S. Wood Jr., Mechanisms of establishment of tumor metastases. In *Pathobiology Annual*, (Edited by H. L. Ioachim, Columbia University Press, New York (1971).
2. D. S. Jones, A. C. Wallace and E. E. Fraser, Sequence of events in experimental metastases of Walker 256 tumor: Light, immunofluorescent, and electron microscopic observations. *J. nat. Cancer Inst.* **46,** 493 (1971).
3. W. F. Sindelar, T. S. Tralka and A. S. Ketcham, Electron microscopic observations on formation of pulmonary metastases. *J. surg. Res.* **18,** 137 (1975).
4. P. Hilgard and E. C. Gordon-Smith, Microangiopathic haemolytic anaemia and experimental tumour-cell emboli. *Brit. J. Haemat.* **26,** 651 (1974).
5. B. Hagmar, Experimental tumour metastases and blood coagulability. *Acta path. microbiol. scand.* **78,** *Suppl.* 211 (1970).
6. S. Wood Jr., Experimental studies on the spread of cancer with special reference to fibrinolytic agents and anticoagulants. *J. Med.* **5,** 7 (1974).
7. T. Terranova and F. Chiossone, Il fattore coagulazione nell'attecchimento delle cellule neoplastiche immesse in circulo. *Boll. Soc. ital. Biol. sper.* **28,** 1224 (1952).
8. H. Gastpar, Metastasierung und Blutgerinnung. *Thrombos. Diathes. haemorrh. Suppl.* **28,** 119 (1968).
9. A. B. Retik, M. S. Arons, A. S. Ketcham and N. Mantel, The effect of heparin on primary tumors and metastases. *J. surg. Res.* **2,** 49 (1962).
10. B. Hagmar and B. Boeryd, Effect of heparin, e-amino-caproic acid and coumarin on tumour growth and spontaneous metastasis formation. *Path. europ.* **3,** 509 (1968).
11. B. Hagmar, Tumour growth and spontaneous metastasis spread in two syngeneic systems. Influence of heparin, coumarin and e-aminocaproic acid (EACA), *Acta path. microbiol. scand.* **78,** 131 (1970).
12. D. J. Wilkins, Interaction of charged colloids with the RES. *Advanc. exp. Med. Biol.* **1,** 25 (1967).
13. K. Suemasu and S. Ishikawa, Inhibitive effect of heparin and dextran sulfate on experimental pulmonary metastases. *Gann* **61,** 125 (1970).
14. P. Hilgard, L. Beyerle, R. Hohage, V. Hiemeyer and M. Kübler, The effect of heparin on the initial phase of metastasis formation. *Europ. J. Cancer* **8,** 347 (1972).
15. W. Regelson, The antimitotic activity of polyanions: Heparin and heparinoids. *J. Med.* **5,** 50 (1974).
16. G. Csaba, C. Korosa, K. Horvath and T. Mold, Effect of heparin-bound alkylating agents and enzyme inhibitors on neoplastic growth. *Neoplasma (Bratislava)* **11,** 137 (1964).
17. I. Kiricuta, C. Todorutiu, T. Muresian and R. Risca, Prophylaxis of metastases formation by unspecific immunologic stimulation associated with heparin therapy. *Cancer (Philad.)* **31,** 1392 (1973).
18. H. Ludwig, Antikoagulantien beim fortgeschrittenen Carcinom. *Gynäkologe* **7,** 1 (1974).

19. E. G. Elias, Heparin as an adjuvant to chemotherapy in lung carcinoma. *Proc. Amer. Ass. Cancer Res.* **14,** 26 (1973).
20. B. Hagmar, Effect of heparin, e-aminocaproic acid and coumarin on tumour growth and spontaneous metastasis formation. *Path. europ.* **3,** 622 (1968).
21. F. B. Berkarda, J. P. d'Souza and R. F. Bakemeier, The effect of anticoagulants on tumor growth and spread in mice. *Proc. Amer. Ass. Cancer Res.* **15,** 99 (1974).
22. J. J. Ryan, A. S. Ketcham and H. Wexler, Reduced incidence of spontaneous metastases with long term coumadin therapy. *Ann. Surg.* **168,** 163 (1968).
23. J. M. Brown, A study of the mechanism by which anticoagulation with warfarin inhibits blood-borne metastases. *Cancer Res.* **33,** 1217 (1973).
24. J. J. Ryan, A. S. Ketcham and H. Wexler, Warfarin treatment of mice bearing autochthonous tumors: Effect on spontaneous metastases. *Science* **162,** 1493 (1968).
26. A. Lisnell and J. Mellgren, Effect of heparin, protamine dicoumarol, streptokinase and epsilon-amino-M-caproic acid on the growth of human cells *in vitro*. *Acta path. microbiol. scand.* **57,** 145 (1963).
27. J. C. Chang and T. C. Hall, *In vitro* effect of sodium warfarin on DNA and RNA synthesis of mouse L1210 leucemic cells and Walker tumor cells. *Oncology* **28,** 232 (1973).
28. H. Higashi and C. Heidelberger, Lack of effect of warfarin (NSC-59813) alone or in combination with 5-fluorouracil (NSC-19893) on primary and metastatic L1210 leukemia and adenocarcinoma 755. *Cancer Chemother. Rep, Part* 1, **55,** 29 (1971).
29. W. M. Kirsch, D. Schulz, J. J. van Buskirk and H. E. Young, Effects of sodium warfarin and other carcinostatic agents on malignant cells: A study of drug synergy. *J. Med.* **5,** 69 (1974).
30. R. D. Thornes, D. W. Edlow and S. Wood Jr., Inhibition of locomotion of cancer cells *in vivo* by anticoagulant therapy. I. Effects of sodium warfarin on V2 cancer cells, granulocytes, lymphocytes and macrophages in rabbits. *John Hopk. med. J.* **123,** 305 (1968).
31. P. Hilgard, H. Schulte, G. Wetzig, G. Schmitt and C. G. Schmidt, Oral anticoagulation in the treatment of a spontaneously metastasising murine tumor (3LL). (In press).
32. R. D. Thornes, Anticoagulant therapy in patients with cancer. *J. Irish med. Ass.* **62,** 426 (1969).
33. R. D. Thornes, Warfarin as maintenance therapy for cancer. *J. Irish Coll. Phys. Surg.* **2,** 41 (1972).
34. R. D. Thornes, Fibrin and cancer. *Brit. med. J.* **1,** 110 (1972).
35. R. D. Thornes, unpublished.
36. L. Michaels, Cancer incidence and mortality in patients having anticoagulant therapy. *Lancet* **ii,** 832 (1964).
37. A. S. Ketcham, personal communication.
38. J. P. Austin and E. M. Glaser, Inhibition of experimental tumours by defibrinogenation. *Clin. Sci.* **37,** 878 (1969).
39. B. Hagmar, Defibrination and metastasis formation: Effects of Arvin on experimental metastases in mice. *Europ. J. Cancer* **8,** 17 (1972).
40. S. Wood Jr., and P. Hilgard, Arvin-induced hypofibrinogenmia and metastasis formation from blood-borne cancer cells. *John Hopk. med. J.* **133,** 207 (1973).
41. L. Ivarsson, Pulmonary metastasis formation after intravenous tumour cell injection in defibrinogenated rats. *Z. Krebsforsch.* **85,** 83 (1976).
42. A. T. Tu and J. B. Giltner, Cytotoxic effects of snake venoms on KB and Yoshida sarcoma cells. *Res. Comm. chem. Path. Pharm.* **9,** 783 (1974).
43. K. Rieche, Combination of anticoagulants and antineoplastic drugs in cancer chemotherapy. *9th Int. Congr. Chemotherapy*, London 1975 (In press).
44. K. Laki, Fibrinogen and metastases. *J. Med.* **5,** 32 (1974).
45. S. T. Yancey and K. Laki, Evaluation of monodansylcadaverine for effects on tumor growth. *J. nat. Cancer Inst.* **52,** 733 (1974).
46. L. Beyerle, Fibrin stabilization and metastasis. (In press).
47. J. H. Johnson and S. Wood Jr., An *in vivo* study of fibrinolytic agents on V2 carcinoma cells and intravascular thrombi in rabbits. *Bull. John Hopk. Hosp.* **113,** 335 (1963).

48. E. E. Cliffton, Effect of fibrinolysin on spread of cancer. *Fed. Proc.* **25,** 89 (1966).
49. N. Back, R. R. Shields and J. L. Ambrus, Role of the fibrinolysin system in the metastatic distribution of spontaneously metastasising and intravenously injected rodent tumor cells. *Proc. Amer. Ass. Cancer Res.* **7,** 4 (1966).
50. R. D. Thornes and W. T. Martin, The cytopathic effect of fibrinolytic agents and human placental fractions on HeLa cells. *Irish J. med. Sci.* **431,** 487 (1961).
51. P. Hilgard and V. Hiemeyer, The cytotoxic effect of plasmin on Yoshida sarcoma cells. *Rev. franç. Etud. clin. biol.* **14,** 902 (1969).
52. I. E. Goetz, C. Weinstein and E. Roberts, Effects of protease inhibitors on growth of hamster tumor cells in culture. *Cancer Res.* **32,** 2469 (1972).
53. E. Reich, Tumor-associated fibrinolysis. *Fed. Proc.* **32,** 2174 (1973).
54. H. Shimoyama, H. Niitani, T. Taniguchi, J. Inagaki and K. Kimura, The role of lysosomes in cancer chemotherapy. III. Influence of plasmin on the lysosomes of tumour cells and on the cytocidal effect of mitomycin-C. *Gann* **60,** 33 (1969).
55. R. D. Thornes, Fibrin and fibrinolysis in cancer. Ph.D. Thesis, Dublin University, 1961.
56. V. B. Larsen, B. Mogensen, C. J. Amris and O. Storm, Fibrinolytic enzyme in the treatment of patients with cancer. *Danish med. Bull.* **11,** 137 (1964).
57. E. E. Cliffton, Fibrinolytic therapy for thromboembolic disease. Principles and practice. *J. La med. Soc.* **118,** 309 (1966).
58. R. D. Thornes, Inhibition of antiplasmin and effect of protease 1 in patients with leukaemia. *Lancet* **ii,** 1220 (1968).
59. R. D. Thornes, P. F. Deasy, R. Carroll, D. J. Reen and J. D. MacDonell, The use of the proteolytic enzyme brinase to produce autocytotoxicity in patients with acute leukemia and its possible role in immunotherapy. *Cancer Res.* **32,** 280 (1972).
60. R. D. Thornes, H. Smyth, O. Browne and P. D. J. Holland, B.C.G. plus protease 1 in malignant melanoma. *Lancet* **i,** 1386 (1973).
61. A. P. Clery, B. L. Hogan, P. D. J. Holland, J. D. H. Widdess, M. Ryan, J. S. Doyle, G. J. Burke and R. D. Thornes, Early experience in a controlled clinical trial using streptokinase induced fibrinolysis during resections for colon and rectal carcinomas in an attempt to prevent haematogenous metastasis. *J. Irish Coll. Phys. Surg.* **1,** 91 (1972).
62. D. L. Brown, Complement and coagulation. *Brit. J. Haemat.* **30,** 377 (1975).
63. R. D. Thornes, Adjuvant therapy of cancer via the cellular immune mechanism of fibrin by induced fibrinolysis and oral anticoagulants. *Cancer* **35,** 91 (1975).
64. C. E. Grossi, D. Agostino and E. E. Cliffton, The effect of human fibrinolysin on pulmonary metastasis of Walker 256 carcinoma. *Cancer Res.* **20,** 605 (1960).
65. A. J. Salsbury, personal communication.
66. B. A. Warren, Tumor metastasis and thrombosis. *Thrombos. Diathes. haemorrh. Suppl.* **59,** 139 (1974).
67. P. Hilgard, The role of blood platelets in experimental metastases. *Brit. J. Cancer* **28,** 429 (1973).
68. G. Gasic, T. Gasic and C. C. Stewart, Antimetastatic effects associated with platelet reduction. *Proc. nat. Acad. Sci.* **61,** 46 (1968).
69. H. Gastpar, Stickiness of platelets and tumor cells influenced by drugs. *Thrombos. Diathes. haemorrh. Suppl.* **42,** 291 (1970).
70. G. Gasic, T. Gasic, N. Galanti, T. Johnson and S. Murphy, Platelet–tumor-cell interactions in mice. The role of platelets in the spread of malignant disease. *Int. J. Cancer* **11,** 704 (1973).
71. S. Wood Jr. and P. Hilgard, Aspirin and tumour metastasis. *Lancet* **ii,** 1416 (1972).
72. P. Hilgard, H. Heller and C. G. Schmidt, The influence of platelet aggregation inhibitors on metastasis formation in mice (3LL). *Z. Krebforsch.* (in press).

Europ. J. Cancer Vol. 12, pp. 763–766. Pergamon Press 1976. Printed in Great Britain

General Papers

Rapid Blastic Transformation and Early Ectopic Proliferation of Hyperdiploid Myeloblasts in Chronic Myeloid Leukemia

SVEN-AAGE KILLMANN*, P. PHILIP* and M. BACCARANI†

**Division of Hematology, Department of Medicine A, Rigshospitalet, University Hospital of Copenhagen DK-2100 Copenhagen, Denmark,*

†*Ospedale Policlinico S. Orsola, University of Bologna, 40138, Bologna, Italy*

Abstract—*A case of CML with some peculiar features is reported. The time from the initial diagnosis (chronic phase stage) until death in blastic crisis was three months. At a time when blood and bone marrow still were compatible with early CML (cytology and karyotypes), nodules consisting of 100% hyperdiploid myeloblasts were found surrounding an area which had contained a large hematoma. The case supports (1) the notion that highly malignant myeloblasts are present from the beginning of CML and (2) illustrates the profound influence which the microenvironment exerts on leukemic cells.*

INTRODUCTION

DURING the past two decades, impressive therapeutic improvements have been reached in acute lymphoblastic leukemia (ALL), particularly in the childhood variety [1]. In acute myeloid leukemia (AML) the therapeutical results have been much less encouraging. In chronic myeloid leukemia (CML) no real advance has been made during the last 25 years, *i.e.*, since the introduction of busulfan [2]. In fact, the median survival time of CML now is equal to, or less than the median survival time of optimally treated childhood ALL.

The major threat of CML is the development of blastic transformation, *i.e.*, a condition akin to AML. This is the cause of death in about two thirds of CML patients [2]. Provocatively, it has been stated [3] that CML is essentially only a highly characteristic preleukemic condition which carries a very high risk of developing into AML. Therefore, a major aim in the treatment of CML must be to postpone or prevent the development of blastic transformation since once this condition is established, it is very resistant to chemotherapy.

Hence, observations of the behavior of myeloblasts in CML are of considerable importance. The present study is concerned with a single case of CML which, however, displayed two interesting features: (1) an unusual brief course from diagnosis to death in blastic transformation (3 months). (2) Early ectopic proliferation of a pure myeloblastic population with highly abnormal karyotypes at the site of a former large hematoma of the femur at a time when the cytology of blood and bone marrow still corresponded to that of early CML.

Accepted 28 June 1976.

Address correspondance to: Sven-Aage Killmann, Professor of Medicine, Division of Hematology, Department of Medicine A, Rigshospitalet, 9, Blegdamsvej, DK-2100 Copenhagen, Denmark.

MATERIAL AND METHODS

Case report

The patient was a 28 year old male. The

previous history was negative. On June 4, 1971, he had a severe blunt trauma of the left femur. At the local hospital a large hematoma of the femur was diagnosed and bed rest at home advised. Three weeks later, the hematoma was found to persist. On incision, 600–700 ml of hematoma fluid was removed. At this time the patient was found to have hepatosplenomegaly and a leukocyte count of 258,000/μl. A blood smear revealed a predominance of granulocytic cells and their precursors. CML was diagnosed and the patient transferred to this department for further treatment. On admission here the physical examination revealed a moderate hepatomegaly and a huge splenomegaly. Besides, the left femur displayed a 20 cm long incision wound; at its middle the distance between its edges was approximately 8 cm. Some infection was present (staphylococcus aureus and E. coli).

Laboratory findings. Hemoglobin Fe-concentration: 7·8 mmol/l (hemoglobin-Fe normal: 7·9–10·5), platelet count 1,104,000/μl, leukocyte count: 203,000/μl. Differential count (in fractions): 0·10 segmented neutrophils; 0·29 stabs; 0·12 metamyelocytes; 0·24 myelocytes; 0·03 promyelocytes; 0·03 myeloblasts; 0·2 eosinophils; 0·08 basophils; 0·01 nucleated erythroid precursors.

For investigative reasons, 2·9 mg of vincristine was given on July 3, 1971. On July 5, busulfan in a dose of 4 mg/day was started and increased to 6 mg/day on July 13, and maintained until September 1, 1971. The results of the therapy in terms of blood counts are shown in Fig. 1. In spite of a marked reduction of the leukocyte and platelet count, the spleen size was not reduced but rather increased somewhat during the period of busulfan treatment.

In the middle of August, several nodules, rapidly approaching 3–4 cm in diameter, appeared along the edges of the incision wound of the left femur. They were first thought to be abscesses since the main wound was still infected at that time in spite of conservative surgical treatment including drainage and treatment with chloramine locally. However, cultures from an incised tumor were negative. A soft X-ray picture revealed no calcifications. A biopsy from the incised tumor showed tissue infiltrated with leukemic cells. On August 24, a fine-needle biopsy was done from a minor tumor and from a major tumor at the distal end of the incision wound. Imprints from these biopsies showed a pure population of myeloblasts. Differential counts performed on this date are shown in Table 1. The table illustrates the remarkable difference of the

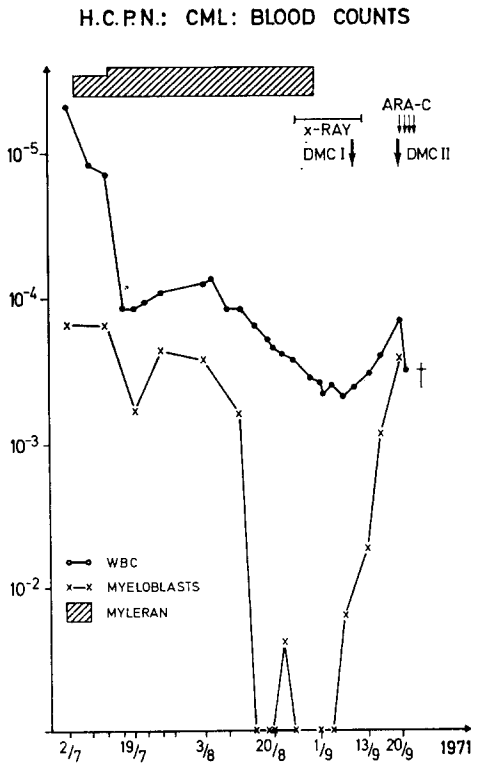


Fig. 1. Leukocyte count and total myeloblast count during the disease.

Table 1. Counts on August 24, 1971

	Blood (WBC 41,600/μl)	Marrow	Tumor
Myeloblasts	0·4%	3·4%	100%
Promyelocytes	1·4%	1·6%	0
Myelocytes	14·0%	12·3%	0
Other cells	84·2%	82·7%	0

frequency of myeloblasts in the various anatomical sites.

Since the infiltration of the femur was growing rapidly, radiotherapy of the area was initiated on August 27, 1971. Between this day and September 7, a total dose of 2200 rad was given to this area resulting in a marked decrease in the size of the infiltrates. At the same time the incision wound was healing rapidly. Figures 2 and 3 depict the wound and nodules on August 17 and September 10, respectively. On September 9, a catheter was inserted into the left external iliac artery and 100 mg daunomycin injected.

In spite of further vigorous therapy, the disease progressed. The patient died on September 21 from thrombocytopenic hemorrhages in the lungs.

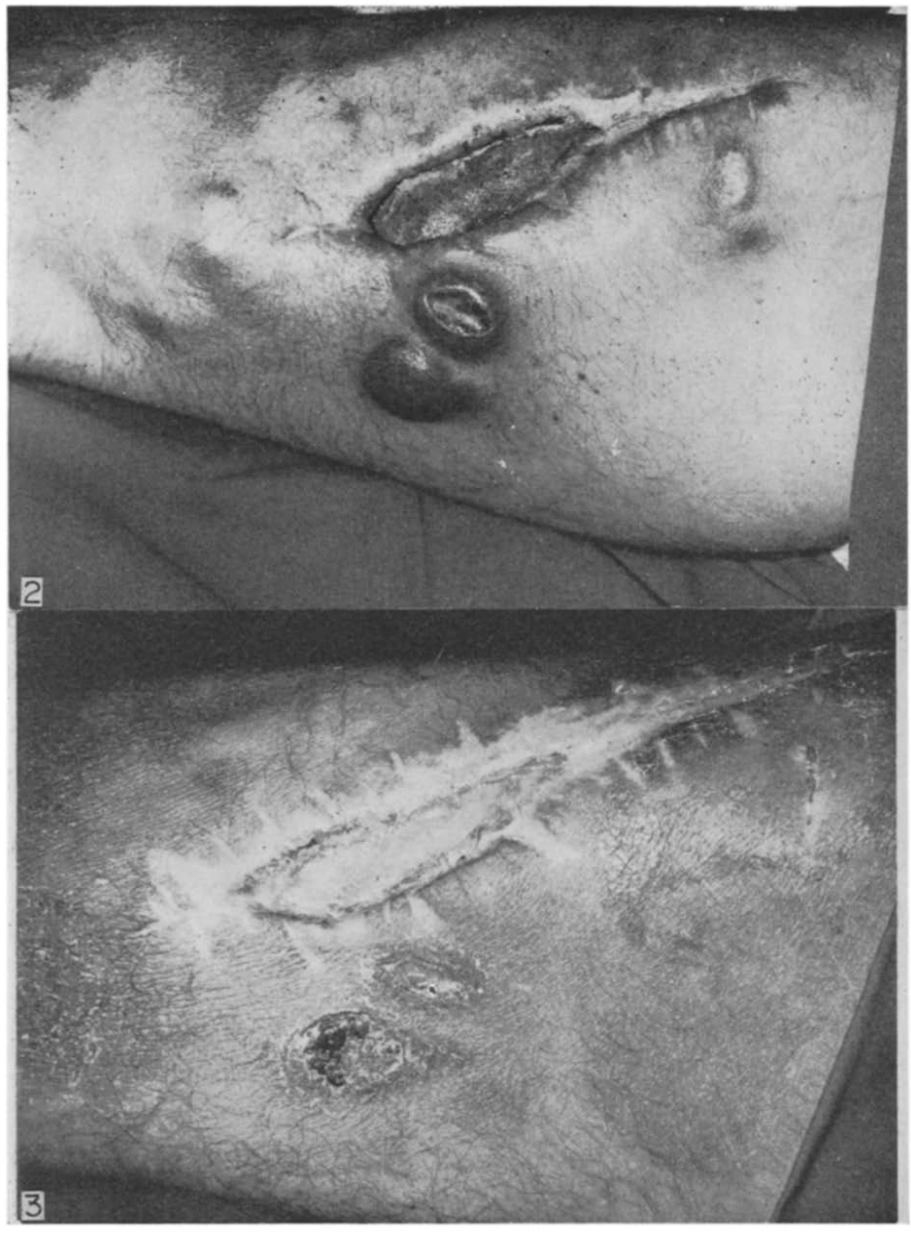


Fig. 2. *Wound with surrounding tumor nodules on August* 17, 1971.

Fig. 3. *Same nodules as in Fig.* 2 *after X-irradiation on September* 9, 1971.

(to face page 764)

METHODS

Blood, bone marrow and tumor cells were incubated with ^{3}H-TDR (specific activity 1·9 Ci/mM) for 1·5 hr, final concentration about 1 μC. Autoradiograms were prepared, using Kodak NTB-2, 1 week's exposure at 4°C, and Giemsa stained. Mitotic counts were performed on slides made immediately after removal of the tissue.

Chromosome analyses were performed on bone marrow aspirates according to the direct method described by Lam-Po-Tang [4]. Excised tumor tissue was treated likewise after having been minced with knives.

RESULTS

Results of special studies

Karyotype studies of the cells from the tumor and cells from the bone marrow, taken at the same time on August 24, showed that the cells of the tumor were highly abnormal, exhibiting various degrees of hyperdiploidy whereas the cells from the bone marrow with a single exception showed the normal 46, XY, Ph^1 pattern of initial CML (Table 2). On September 15, the marrow now showed highly atypical karyotypes (Table 2) and the smear was dominated by myeloblasts.

Table 2. *Chromosome constitution of bone marrow and tumor cells. All cells were Ph^1-positive*

Sample	Chromosome number													Total cells counted
	44	45	46	47	48	49	50	51	52	53	54	55	56	
Marrow, Aug. 24	2	2	43											47
Tumor, Aug. 24	1					2	1		5	11	9	2	1	32
Marrow, Sept. 15		1	1	1		4	9	5	1	2	6	2	1	33

Table 3

	Blood	Marrow	Tumor
^{3}H-thymidine labeling index of myeloblasts	6·5% (N=400)	25·4% (N=800)	6·7% (N=1000)
Mitotic index of myeloblasts	no mitoses	1·00% (N = 1000)	0·33% (N = 7669)

N = number of cells counted.

The ^{3}H-TDR labeling index and the mitotic index of the myeloblasts are shown in Table 3. The labeling index was about 4 times higher in the marrow than in the blood and the tumor. As usual in CML, no mitoses were found in the blood. In the marrow, the ratio labeled/mitotic myeloblasts was about 25:1, in the tumor this ratio was very similar, about 20:1.

Autopsy showed leukemic infiltrates (myeloblasts) in lymphnodes, kidney, liver, spleen and in the bone marrow.

DISCUSSION

The present case is interesting from several points of view.

First, the very rapid development from what appeared to be an ordinary case of CML to blastic crisis is striking. The whole course of disease lasted for three months only. It is intriguing to speculate that this highly malignant evolution was in some way connected to the hematoma of the left femur that lead to the detection of the hematologic disease which until then had passed unnoticed. When first examined, the areas surrounding the wound was filled with a pure population of myeloblasts with highly abnormal karyotypes while at the same time the bone marrow and the blood still had the appearance of early chronic myeloid leukemia. This shows that highly malignant myeloblasts of a type which are responsible for the fatal outcome of the disease in blastic transformation were indeed present already from the clinical beginning of the disease. In the present case there is good reason to speculate that the organized hematoma around the site of the blunt trauma provided a microenvironment which allowed the abnormal myeloblasts to accumulate with much less restraint than in other anatomical sites. It is to be noted that the thymidine labeling index of the tumor myeloblasts was low (6·7%), but the biopsy was taken only *after* the nodules had grown quickly which of course was the expression of a severely disturbed balance between local cell production and local cell death and/or emigration. Attempts were made to demonstrate a bone marrow-like environment in the left femur by doing a soft X-ray exposure but

no calcifications were apparent. In this context it may be worthy of note that recently, arguments have been presented that already at the beginning of CML, myeloblasts are present which in several respects behave as the myeloblasts of acute myeloid leukemia: the myeloblastic cells of early (untreated) CML have a lower labeling index with ^{3}H-TDR than normal myeoblasts and moreover, the labeling index of these myeloblasts in the blood is lower by a factor of 2 or 3 than in the bone marrow [5]. A similar pattern was seen in this patient (Table 3).

At the present time it is not understood why in some cases blastic crisis ensues very quickly, whereas other patients may live for many years without evidence of blastic transformation. In the present case, one may suspect that the unique environment provided by the hematoma of the left leg contributed to facilitate the development of the malignant myeloblastic clones. It cannot be overlooked, however, that the spleen did not respond as well to therapy as it usually does. In fact, it increased at a time when the hematological parameters approached normality. It is tempting to speculate that malignant myeloblasts were exported from the femoral nodules to the spleen, cf. the identical labeling index of tumor and blood myeloblasts. This, however, cannot be answered from a single case. Conceivably, any locus minoris resistentiae, e.g., hematoma, edema, infarction, etc. might set up the framework for the growth of particularly malignant cells which perhaps otherwise would have been kept in restraint by less neoplastic, differentiating cells. One reason for publishing this case is to provoke publication of similar cases which at present maybe are in hospital files.

The present case also illustrates that karyotypically normal (except for the Ph^{1}-chromosome) cells may coexist with highly abnormal cells in various anatomical sites at the same time. This is one reason that has made us believe that truly malignant myeloblasts may be present in CML from the clinical onset of the disease [5]. Therefore, therapeutic protocols aiming at applying courses of anti AML treatment which are now being tested in several centers appear well justified although they obviously may "backfire" by eradicating differentiating leukemic cells and thus perhaps open the way for the blastic crisis. In principle, however, it has rather recently been demonstrated that anti-AML therapy in full blastic crisis may be of benefit, increasing the proliferation of functionally useful cells together with a decrease of highly abnormal karyotypes [6].

REFERENCES

1. A. S. Spiers, Management of the different forms of leukemia, p. 655. In: Leukemia III. (Edited by F. Gunz and A. G. Baikie) Grune & Stratton, New York, London (1974).
2. S. Tura and M. Baccarani (Eds.): *Chronic Myeloid Leukemia. Proceedings of the International Symposium*, Bologna, April 15–16 1972.
3. S.-Aa. Killmann, Chronic myelogenous leukemia: preleukemia or leukemia? In: *Proceedings of the International Symposium on Chronic Myeloid Leukemia* (Edited by S. Tura and M. Baccarani), p. 45. Bologna, April 15–16, (1972).
4. P. R. L. C. Lam-Po-Tang, An improved method of processing bone marrow for chromosomes. *Scand. J. Haemat.* **5,** 158 (1968).
5. M. Baccarani and S.-Aa. Killmann, Cytokinetic studies in chronic myeloid leukemia: evidence for early presence of abnormal myeloblasts. *Scand. J. Haemat.* **9,** 283 (1972).
6. B. Pedersen and S.-Aa. Killmann, Leukaemic subclones in chronic myelogenous leukaemia: differences in drug sensitivity and in differentiation capacity. *Acta med. Scand.* **190,** 61 (1971).

Europ. J. Cancer Vol 12, pp. 767–774. Pergamon Press 1976. Printed in Great Britain

Plasma Prolactin, Thyroid-Stimulating Hormone, Follicle-Stimulating Hormone and Luteinizing Hormone in Normal British and Japanese Women*

S. KUMAOKA†, O. TAKATANI†, O. ABE‡, J. UTSUNOMIYA§, D. Y. WANG¶
R. D. BULBROOK¶, J. L. HAYWARD‖ and F. C. GREENWOOD**

†*National Cancer Center, Tokyo,* ‡*Keio University, Tokyo,* §*Tokyo Medical & Dental University, Tokyo,*
¶*Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX,* ‖*ICRF Breast Cancer Unit, Guy's Hospital, London SE1 9RT and*
***School of Medicine, University of Hawaii at Manoa, Honolulu*

Abstract—*The concentrations of plasma prolactin, thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured in normal Japanese and British adolescent, pre-menopausal, menopausal and post-menopausal women.*

The mean levels of prolactin and TSH were similar in both races for all age categories studied. The concentrations of plasma LH were also similar for both races except for pre-menopausal women where the average level in Japanese was higher than in British women ($P = 0{\cdot}05$). The concentrations of plasma FSH were also similar for both races except for the post-menopausal group, where the British women had a higher average level than Japanese women ($P = 0{\cdot}02$).

These results do not support the concept that the low incidence of breast cancer in Japan compared with that in Western countries is due to basic endocrinological differences.

INTRODUCTION

IT HAS been known for many years than an enhanced prolactin stimulus is associated with an increase in the incidence of breast cancer in some strains of mice [1], and that the DMBA-induced tumour in the rat is prolactin-dependent [2]. The introduction of reliable radioimmunoassays for human prolactin has led to several investigations of the role of this hormone in patients with breast cancer but current results are more confusing than informative.

It has been suggested that the biological action of prolactin may be modified at the breast cell level by thyroid hormone and that TSH levels are significantly raised in post-menopausal women with breast cancer [3–5]. Sub-clinical hypothyroidism might therefore be an important factor in the aetiology of the disease.

We have so far shown that plasma oestrone, oestradiol-17β and progesterone levels are virtually identical in adult normal British and Japanese women [6], and in view of these results described above, the next logical step in attempting to explain differences in incidence of breast cancer in Britain and Japan in endocrinological terms was to measure plasma prolactin and TSH levels in the two races. As another parameter of pituitary function, and as a check on the validity of the finding of similar oestrogen levels in the two populations, plasma FSH and LH were also measured.

MATERIAL AND METHODS

The normal adolescent girls, pre-menopausal, menopausal and post-menopausal British and Japanese women have been described previously [6].

Plasma LH and FSH were measured by a

Accepted 12 May 1976.

*This study was supported in part by a grant for Cancer Research from the Ministry of Health & Welfare, Japan.

Reprint requests to DYW, London.

double-antibody radioimmunoassay method. The antiserum to FSH was kindly supplied by Dr. W. R. Butt and the antiserum to LH by the National Institutes of Health, Bethesda.

LH (MRC 71/53) and FSH (MRC 69/104) were supplied by the Medical Research Council and iodination of these compounds carried out by the method of Greenwood, Hunter and Glover [7]. The iodinated hormones were isolated from the reaction mixture by chromatography on a Sephadex G25 column (10 × 1 cm). The iodinated LH was purified on CF11 cellulose immediately before use. All samples were assayed at three concentrations to check parallelism with the standard curve. The concentrations were expressed in terms of the MRC standards for LH (MRC 68/40. 77 I.U./Ampoule) and FSH (MRC 68/39. 32·8 I.U./Ampoule) [8].

Plasma prolactin and TSH were measured by conventional double antibody radioimmunoassays using the reagents and protocols kindly supplied by the National Pituitary Agency, N.I.H. Bethesda.

RESULTS

1. *Prolactin*

The plasma prolactin levels in adolescent, pre-menopausal, menopausal and post-menopausal women are shown in Fig. 1. The means of the arithmetical values for the four groups of women are given in Table 1. There appear to be no real differences between the amounts of this hormone in the plasma in British and Japanese women in any of the four groups. For a statistical analysis, the logarithms of the prolactin values were taken: no significant differences were found. In both races, prolactin levels were lower in menopausal and post-menopausal women than in the pre-menopausal groups but the differences are not significant if the log prolactin values are compared.

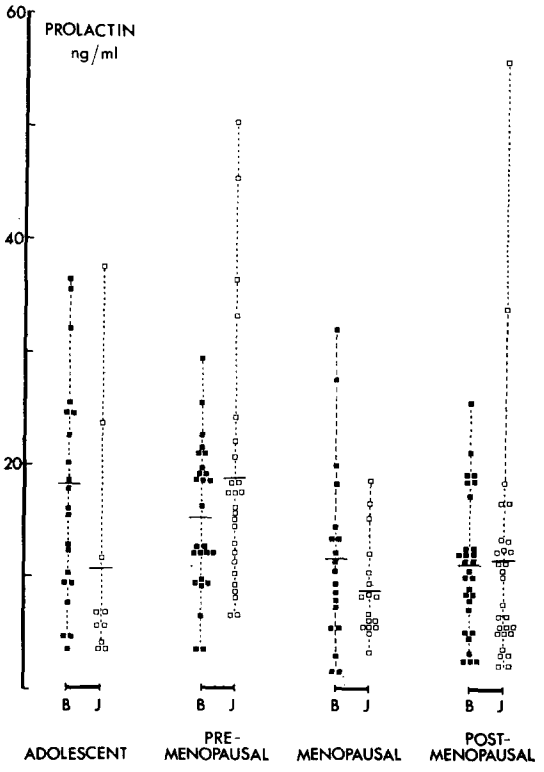


Fig. 1. *Plasma prolactin levels in normal British (B) and Japanese (J) women.*
The horizontal lines show the arithmetic means.

Table 1. *Mean plasma prolactin and TSH in normal British and Japanese women*

Menopausal status	Prolactin (ng/ml) B	Prolactin (ng/ml) J	TSH (ng/ml) B	TSH (ng/ml) J
Adolescents	17·3 ± 9·8 (N = 21)	10·8 ± 11·1 (N = 10)	1·4 ± 1·6 (N = 21)	0·7 ± 0·3 (N = 10)
Pre-menopausal	15·3 ± 6·6 (N = 26)	19·0 ± 11·2 (N = 25)	1·4 ± 0·9 (N = 27)	1·2 ± 0·7 (N = 27)
Menopausal	11·6 ± 8·3 (N = 19)	9·0 ± 4·3 (N = 17)	3·1 ± 6·0 (N = 19)	1·0 ± 0·5 (N = 17)
Post-menopausal	11·4 ± 6·1 (N = 29)	11·1 ± 11·0 (N = 29)	1·8 ± 2·8 (N = 29)	1·2 ± 0·2 (N = 29)

B = British J = Japanese.

The arithmetical means are given with standard deviations. Numbers of women studied are shown in parenthesis.

2. *TSH*

The plasma TSH values in the four groups of British and Japanese women are shown in Fig. 2 and the means and standard deviations are given in Table 1. The British mean values are consistently higher than those of the Japanese but are not significantly different in any of the four groups. When all the values for each population are pooled, the mean British TSH level is still not significantly greater than that of the Japanese population.

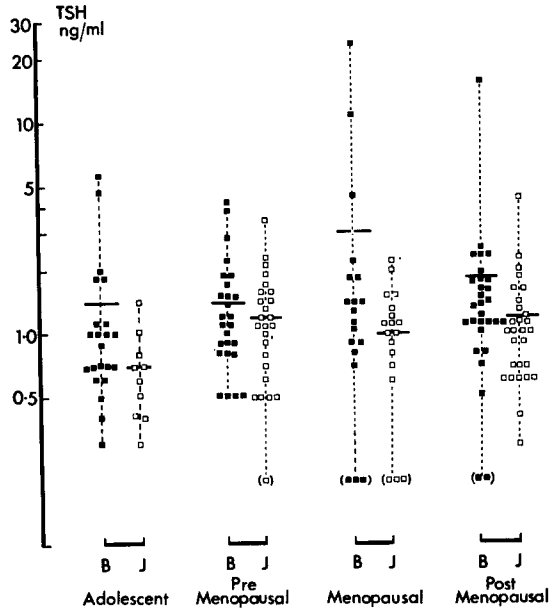


Fig. 2. Plasma TSH levels in normal British (B) and Japanese (J) women.
The horizontal lines show the arithmetic means.

3. *LH and FSH*

The results of the assays for plasma LH and FSH are shown in Figs. 3 and 4 and Table 2. The geometric mean level of LH or FSH tends to be marginally higher in British compared to Japanese women in all the groups studied (Table 3) except in the case of plasma LH in the pre-menopausal category. In this last group the mean level of LH in Japanese women was significantly higher than that found in British subjects ($P < 0{\cdot}02$). However, when women definitely known to be in the luteal phase (based on progesterone results) were considered, this difference became marginally significant ($P = 0{\cdot}05$; Table 3).

The only other instance in which the difference between the races was significant was for plasma FSH in post-menopausal women; here the mean concentration of plasma FSH was higher in British women ($P = 0{\cdot}02$; Table 3). In all other groups the inter-racial differences in plasma gonadotrophins were not statistically significant.

DISCUSSION

The plasma levels of prolactin are remarkably similar in normal British and Japanese women aged 16–70 yr. This finding indicates that differences in incidence of breast cancer in the two populations are unlikely to be related to differences in basal prolactin levels.

There is little other epidemiological evidence concerning the possible role of prolactin in the

Table 2. Mean plasma LH and FSH levels in normal British and Japanese women

Menopausal status	LH (mIU/ml) B	LH (mIU/ml) J	FSH (mIU/ml) B	FSH (mIU/ml) J
Adolescents	11·5 ± 11·1 ($N = 23$)	10·5 ± 13·9 ($N = 25$)	2·6 ± 1·6 ($N = 19$)	2·7 ± 1·8 ($N = 17$)
Pre-menopausal	6·7 ± 6·4 ($N = 28$)	13·2 ± 10·5 ($N = 18$)	4·0 ± 3·0 ($N = 26$)	4·1 ± 5·0 ($N = 21$)
Menopausal	38·1 ± 17·2 ($N = 17$)	32·2 ± 9·2 ($N = 14$)	38·4 ± 19·6 ($N = 19$)	32·5 ± 11·4 ($N = 17$)
Post-menopausal	31·4 ± 11·9 ($N = 30$)	30·1 ± 10·1 ($N = 19$)	23·1 ± 7·0 ($N = 20$)	18·5 ± 6·4 ($N = 19$)

B = British J = Japanese

The arithmetical means are given with standard deviations. Numbers of women studied are shown in parenthesis.

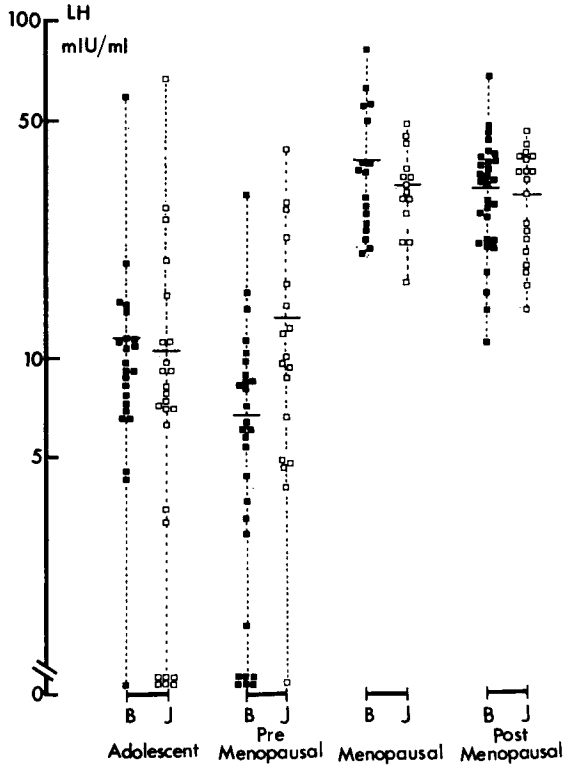


Fig. 3. Plasma LH levels in normal British (B) and Japanese (J) women.
The horizontal lines show the arithmetic means.

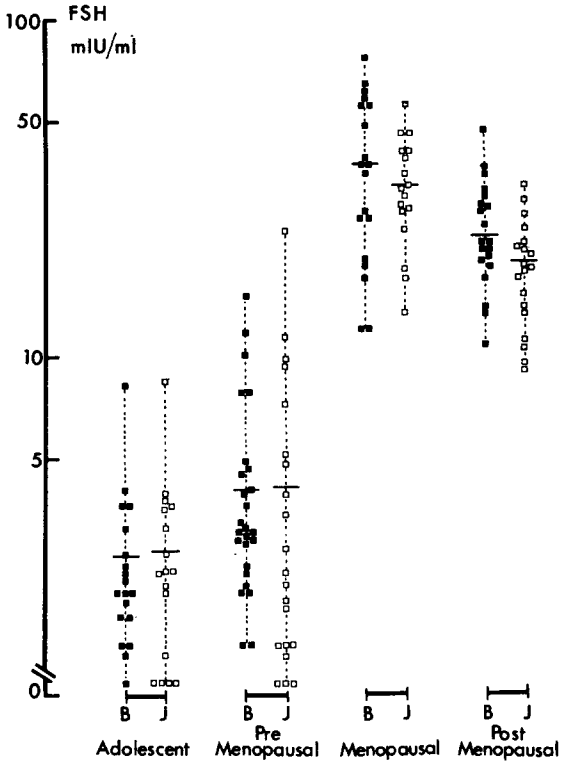


Fig. 4. Plasma FSH levels in normal British (B) and Japanese (J) women.
The horizontal lines show the arithmetic means.

Table 3. Logarithmic mean plasma LH and FSH levels in normal British and Japanese women

Menopausal status	LH (log mIU/ml) B	LH (log mIU/ml) J	FSH (log mIU/ml) B	FSH (log mIU/ml) J
Adolescent	0·91 ± 0·26 (N = 23)	0·77 ± 0·52 (N = 25)	0·35 ± 0·21 (N = 19)	0·35 ± 0·26 (N = 17)
	t = −1·23		t = −0·09	
Adolescent Luteal phase only	0·69 ± 0·38 (N = 6)	0·46 ± 0·42 (N = 10)	0·19 ± 0·15 (N = 5)	0·14 ± 0·19 (N = 5)
	t = −1·08		t = −0·09	
Pre-menopausal	0·67 ± 0·43 (N = 28)	0·99 ± 0·38 (N = 18)	0·52 ± 0·27 (N = 26)	0·48 ± 0·40 (N = 21)
	t = 2·6, P < 0·02		t = −0·4	
Pre-menopausal Luteal phase only	0·63 ± 0·42 (N = 25)	0·94 ± 0·43 (N = 12)	0·49 ± 0·26 (N = 22)	0·31 ± 0·31 (N = 14)
	t = 2·09, P = 0·05		t = −1·94	
Menopausal	1·54 ± 0·18 (N = 17)	1·49 ± 0·13 (N = 14)	1·52 ± 0·25 (N = 18)	1·48 ± 0·16 (N = 17)
	t = −0·94		t = −0·57	
Post-menopausal	1·47 ± 0·17 (N = 30)	1·45 ± 0·16 (N = 19)	1·36 ± 0·16 (N = 20)	1·24 ± 0·16 (N = 19)
	t = −0·28		t = −2·44, P = 0·02	

B = British J = Japanese

The geometric means, standard deviations and number of women studied are given with the corresponding value of t.

aetiology of human breast cancer. The fact that the incidence of the disease is diminished in women who have their first child at an early age [9] appears to conflict with the simple concept that high plasma prolactin levels are related to increased risk since the concentration of this hormone rises markedly during pregnancy and lactation [10, 11].

It has been suggested that tranquillizing drugs that increase prolactin secretion might lead to an increase in the incidence of breast cancer, and Ettigi *et al.* [12] found that twice as many of their patients had received phenothiazine treatment in a mental hospital before diagnosis than controls, but they point out that other workers [13] found no abnormalities in the mortality rates from breast cancer in New York mental hospitals during a ten year period. Recently, three independent groups found a three-fold excess of breast cancer (in addition to excess cancers at other sites) in patients with hypertension treated with reserpine [14–16]. No association was found, however, with the limited data available for phenathiazine or methyldopa. The findings have been criticized on methodological grounds [17, 18] and have not been confirmed by Mack *et al.* [19].

Turning from the possible role of prolactin in the induction of breast cancer to a consideration of its function in the established disease, some workers have claimed that patients with breast cancer have elevated prolactin levels [20, 21]. The main weight of the current evidence now indicates that prolactin levels are normal [5, 22–25] or even subnormal in the patients in the earlier stages of the disease [26]. There are two other curious findings that may be relevant in this context. The first is that patients with advanced breast cancer treated by stalk-section may have very high levels of plasma prolactin but may nevertheless have remissions of their disease [27–29]: and the second is that high plasma levels of prolactin after transphenoidal or transfrontal hypophysectomy may also be found in patients with objective response to treatment [30].

Work with animal models has also yielded contradictory results. Early findings showed that mammary tumours could be induced in certain strains of mice by isologous transplantation of pituitary glands [1] and this work has been amply confirmed [31, 32]. The amounts of prolactin produced by such grafts may be so large that it is questionable whether the results are relevant to man. However, it has been shown recently that progesterone synergizes with prolactin in inducing mammary cancer in mice [33] and that under conditions of continuous stimulation amounts of oestrogen, progesterone and prolactin within or below the normal physiological range can lead to a considerable increase in incidence in (C57BL × CBA)F1 mice [34]. Röpcke [32] has recently concluded that "mammary tumours can be induced by physiological doses of oestrogen, prolactin and progesterone given in a suitable combination, disproving the basic concept that high-dose hormone treatment is a pre-requisite in all types of hormonal carcinogenesis. The continuity of stimulation may well be essential". This model still does not fit with our observations on British and Japanese women. Oestrogen–progesterone–prolactin status appears to be identical in the two races and there is no evidence in our data that the British population is preferentially exposed to a more *continuous* stimulation than the Japanese, although the earlier age at menarche may mean that they are exposed to a longer stimulus.

The pituitary content of prolactin is higher in C3H/St mice over their life span than that in the low-tumour strain C57BL/St mice but the plasma prolactin levels of the low incidence cross are consistently higher than in the high incidence animals [35]. In the rat, there is little doubt that the DMBA-induced tumour in the Sprague–Dawley strain is prolactin dependent [2] but growth of the transplantable R35 MT tumour in this animal is inhibited by administration of perphenazine, in spite of increased plasma prolactin levels which appear to induce mammary gland growth [36]. This effect was also noted by Hilf *et al.* [37] with the R 3032 AC tumour in the Fischer rat. However, growth of the 13762 MT in this strain was inhibited by perphenazine administration [36]. It would appear that a judicious choice of animal would provide evidence to support any theory concerning the role of prolactin in human breast cancer.

The role of the thyroid in the disease is also a matter of some controversy. The incidence of breast cancer does not appear to be abnormal in patients with antecedent thyroid disorders although the latter may have marked effects on the clinical course of breast cancer [38]. Of great interest in the context of our own investigations is the recent claim that thyroid hormone inhibits the action of prolactin at the breast cell level in the rat and that while postmenopausal women with breast cancer have normal prolactin levels and a normal prolactin response to TRF, they have raised basal and stimulated TSH levels, implying a degree of hypothyroidism [3, 4]. We found no significant

difference between basal TSH levels in any of the British or Japanese groups. Nevertheless, the mean TSH values were consistently higher in British women and the number of high values of TSH (2·0 and above) was twice as great (17/95 compared with 8/81). Also,the ratio of androsterone to aetiocholanolone (the 5α/5β ratio) was found to be lower in British women than in Japanese women, which might again indicate a higher degree of thyroid function in the latter race [39, 40]. A more detailed examination of thyroid function in the two races would appear to be warranted.

There has been some controversy concerning gonadotrophin excretion in patients with breast cancer. Two groups of workers have found that their patients excreted more of these hormones than controls but a recent study in which specific radioimmunoassays for FSH and LH were used, no significant differences were found [8]. Similarly in the present study, no consistent differences in plasma gonadotrophin levels were found in British and Japanese women. A significant difference was noted between LH levels in pre-menopausal British and Japanese women but the main bulk of the values were similar in both races and the difference derives from several unexpectedly high values in the Japanese women. We place little reliance on the finding since all the high values were associated with plasma progesterone values at the lower end of the range associated with ovulation and with low urinary pregnanediol concentrations. This implies that the high values have been obtained because the blood was taken near the ovulatory peak of LH. The raised levels of FSH in post-menopausal British women is at the borderline of significance and again should be regarded with some caution. The only way to settle these points would be to analyze daily plasma samples throughout the cycle or over several days in post-menopausal women.

The object of the present series of investigations was to see whether the differences in the incidence of breast cancer in Britain and Japan can be explained in terms of basic endocrinological differences between the two races. Our results make such an explanation unlikely. The breast tissues of normal British and Japanese women appear to receive the same stimuli from the three hormones that are important in mammary cancer in rodents, that is, the oestrogens, progesterone and prolactin. The only convincing endocrinological difference between Japanese and British women has been the finding that plasma androstenedione and dehydroepiandrosterone sulphate levels (and their urinary metabolites) are lower in Japanese than in British women [40]. On present evidence [41], it might be expected that such findings would be associated with a higher rather than a lower incidence of breast cancer in Japan.

While it is quite possible that subtle differences in endocrine function, which could not be detected in a study of the type we have carried out, may be important in the aetiology of breast cancer, or that very early endocrine influences are of paramount importance, it might be advisable to seek to explain differences in incidence among non-endocrine factors. The finding that there was a marked difference in the degree of sinus histiocytosis in the lymph nodes of British and Japanese patients with breast cancer [42] is a possible indication that immunological factors should be considered.

REFERENCES

1. L. Loeb and M. Moskop Kirtz, The effect of transplants of anterior lobes of the hypophysis on the growth of mammary gland carcinoma in various strains of mice. *Amer. J. Cancer* **36,** 56 (1939).
2. O. H. Pearson, O. Llerena, L. Llerena, A. Molina and T. Butler, Prolactin-dependent rat mammary cancer: a model for man? *Trans. Ass. Amer. Phys.* **82,** 225 (1969).
3. I. Mittra, Mammotropic effect of prolactin enhanced by thyroidectomy. *Nature (Lond.)* **248,** 525 (1974).
4. I. Mittra and J. L. Hayward, Hypothalamic–pituitary–thyroid axis in breast cancer. *Lancet* **i,** 885 (1974).
5. I. Mittra, J. L. Hayward and A. S. McNeilly, Hypothalamic–pituitary–prolactin axis in breast cancer. *Lancet* **i,** 889 (1974).
6. R. D. Bulbrook, M. C. Swain, D. Y. Wang, J. L. Hayward, S. Kumaoka, O. Abe and J. Utsunomiya, Plasma oestradiol 17β-oestrone and progesterone, and their urinary metabolites in normal British and Japanese women. *Europ. J. Cancer* **12,** (1976). In press.

7. F. C. Greenwood, W. M. Hunter and J. S. Glover, The preparation of I^{131}-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89,** 114 (1963).
8. D. Y. Wang, P. R. Goodwin, R. D. Bulbrook and J. L. Hayward, Plasma FSH and LH in post-menopausal women with breast cancer. *Europ. J. Cancer* **12,** 305 (1976).
9. B. MacMahon, P. Cole, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, V. G. Valaoras and S. Yuasa, Age at first birth and breast cancer risk. *Bull. Wld. Hlth. Org.* **43,** 209 (1970).
10. K. M. Gautvik, B. D. Weintraub, C. T. Graeber, F. Maloof, J. E. Zuckerman and A. H. Tashjian, Serum prolactin and TSH: effects of nursing and pyroGly-His-ProNH_2 administration in postpartum women. *J. clin. Endocr.* **36,** 135 (1973).
11. P. Hwang, H. Guyda and H. Friesen. A radioimmunoassay for human prolactin. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1902 (1971).
12. P. Ettigi, S. Lal and H. G. Friesen, Prolactin, phenothiazines, admission to mental hospital and carcinoma of the breast. *Lancet* **ii,** 266 (1973).
13. J. Katz, S. Kunofsky, R. E. Patton and N. C. Allaway, Cancer mortality among patients in New York mental hospitals. *Cancer (Philad.)* **20,** 2194 (1967).
14. B. Armstrong, N. Stevens and R. Doll, Retrospective study of the association between use of Rauwolfia derivatives and breast cancer in English women. *Lancet* **ii,** 672 (1974).
15. Boston Collaborative Drug Surveillance Program, Reserpine and breast cancer. *Lancet* **ii,** 669 (1974).
16. O. P. Heinonen, S. Shapiro, L. Tuominen and M. I. Turuen, Reserpine use in relation to breast cancer. *Lancet* **ii,** 675 (1974).
17. R. D. Mann, W. M. Troetel and J. Nadelmann, Rauwolfia derivatives and breast cancer. *Lancet* **ii,** 966 (1974).
18. E. A. Saxen, Rauwolfia derivatives and breast cancer. *Lancet* **ii,** 833 (1974).
19. T. M. Mack, B. E. Henderson, V. R. Gerkins, M. Arthur, J. Baptista and M. C. Pike, Reserpine and breast cancer in a retirement community. *New Engl. J. Med.* **292,** 1366 (1975).
20. R. M. L. Murray, G. Mozaffarian and O. H. Pearson, Prolactin levels with L-dopa treatment in metastatic breast cancer. In *Prolactin and Carcinogenesis.* (Edited by A. R. Boyns and K. Griffiths) p. 158. Alpha Omega Alpha, Cardiff (1972).
21. E. Roland, T. Barreca, P. Masturzo, A. Polleri, F. Indiveri and A. Barabino, Plasma-prolactin in breast cancer. *Lancet* **ii,** 845 (1974).
22. A. R. Boyns, E. Cole, K. Griffiths, R. Buchan, M. M. Roberts, R. G. Wilson and A. P. M. Forrest, Plasma prolactin in breast cancer. *Europ. J. Cancer* **9,** 99 (1973).
23. S. Franks, D. N. L. Ralphs, V. Seagroatt and H. S. Jacobs, Prolactin concentrations in patients with breast cancer. *Brit. med. J.* **4,** 320 (1974).
24. H. G. Kwa, M. De Jong-Bakker, E. Engelsman and F. J. Cleton, Plasma prolactin in human breast cancer. *Lancet* **i,** 433 (1974).
25. R. G. Wilson, R. Buchan, M. M. Roberts, A. P. M. Forrest, A. R. Boyns, E. Cole and K. Griffiths, Prolactin and breast cancer. *Proc. roy. Soc. Med.* **66,** 865 (1973).
26. L. Francis, Studies on Human Prolactin. Ph.D. Thesis, Reading University (1975).
27. C. Bowers, H. Friesen, P. Hwang, H. Guyda and K. Folkers, Prolactin and thyrotropin release in man by synthetic pyroglutamyl-histidyl-prolinamide. *Biochem. biophys. Res. Comm.* **45,** 1033 (1971).
28. G. Ehni and N. E. Eccles, Interruption of the pituitary stalk in patients with breast cancer. *J. Neurosurg.* **16,** 628 (1959).
29. R. W. Turkington, L. E. Underwood and J. J. Van Wyck, Elevated serum prolactin levels after pituitary-stalk sections in man. *New Engl. J. Med.* **285,** 707 (1971).
30. T. Bates, R. D. Rubens, R. D. Bulbrook, P. R. Goodwin, D. Y. Wang, M. A. Falconer, L. F. W. Salmon, P. H. Schurr, O. H. Shaheen, R. K. Knight and J. L. Hayward, Comparison of pituitary function and clinical response after transphenoidal and transfrontal hypophysectomy for advanced breast cancer. (Abstract) *Brit. J. Surg.* **62,** 654 (1975).

31. L. M. Boot, Prolactin and mammary gland carcinogenesis. The problem of human prolactin. *Int. J. Cancer* **5,** 167 (1970).
32. G. Röpcke, Interaction of hypophyseal isografts and ovarian hormones in mammary tumour development in mice. *Academisch Proefschrift*, Amsterdam (1975).
33. G. Röpcke and L. M. Boot, Prolactin and the ovarian hormones in carcinoma of the mammary gland in mice. *Proc. 4th Int. Congress of Endocrinol.* p. 1232, Excerpta Medica, Amsterdam (1972).
34. L. M. Boot, H. G. Kwa and G. Röpcke, Radioimmunoassay of mouse prolactin. Prolactin levels in isograft-bearing orchidectomized mice. *Europ. J. Cancer* **9,** 185 (1973).
35. Y. N. Sinha, F. W. Selby and W. P. Vanderlaan, The natural history of prolactin and GH secretion in mice and high and low incidence of mammary tumours. *Endocrinology* **94,** 757 (1974).
36. A. E. Bogden, J. Taylor, E. Y. H. Kuo, M. M. Mason and A. Speropoulos, The effect of perphenazine-induced serum prolactin response on estrogen-primed mammary tumour-host systems 13762 and R-35 mammary adenocarcinoma. *Cancer Res.* **34,** 3018 (1974).
37. R. Hilf, C. Bell, H. Goldenberg and I. Michel, Effect of fluphenazine HCl on R3230 AC mammary carcinoma and mammary glands of the rat. *Cancer Res.* **31,** 1111 (1971).
38. A. R. Moosa, D. A. Price Evans and A. C. Brewer, Thyroid status and breast cancer. Re-appraisal of an old relationship. *Ann. roy. Coll. Surg. Engl.* **53,** 178 (1973).
39. R. D. Bulbrook, B. S. Thomas, J. Utsunomiya and E. Hamaguchi, The urinary excretion of 11-deoxy-17-oxosteroids and 17-hydroxycorticosteroids by normal Japanese and British women. *J. Endocrinol.* **38,** 401 (1967).
40. D. Y. Wang, R. D. Bulbrook, J. L. Hayward, S. Kumaoka, O. Takatani, O. Abe and J. Utsunomiya, Plasma dehydroepiandrosterone and androsterone sulphates, androstenedione and urinary androgen metabolites in normal British and Japanese women. *Europ. J. Cancer* (in press).
41. R. D. Bulbrook, J. L. Hayward and C. C. Spicer, Relation between urinary androgen and corticoid excretion and subsequent breast cancer. *Lancet* **ii,** 395 (1971).
42. G. H. Friedell, E. A. Soto, S. Kumaoka, O. Abe, J. L. Hayward and R. D. Bulbrook, Sinus histiocytosis in British and Japanese patients with breast cancer. *Lancet* **ii,** 1228 (1974).

Europ. J. Cancer Vol. 12, pp. 775–782. Pergamon Press 1976. Printed in Great Britain

Comparison of Pituitary Function and Clinical Response after Transphenoidal and Transfrontal Hypophysectomy for Advanced Breast Cancer

T. BATES*†, R. D. RUBENS*, R. D. BULBROOK‡, P. R. GOODWIN‡, D. Y. WANG‡, R. K. KNIGHT* and J. L. HAYWARD*

**I.C.R.F. Breast Cancer Unit, Guy's Hospital, London, SE1 9RT*

‡Dept. of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX, Great Britain.

†Present address: Ashford Hospital, Kings Avenue, Ashford, Kent, Great Britain.

Abstract—*Plasma levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), prolactin and the ketosteroids, dehydroepiandrosterone sulphate and androsterone sulphate were measured before and after transphenoidal and transfrontal hypophysectomy. To increase the possibility of detecting residual pituitary tissue, the same hormones were also measured 20, 30 and 60 min after pituitary stimulation with luteinizing hormone-releasing hormone (LH-RH) and thyrotropin releasing hormone (TRH).*

The results indicated that hypophysectomy by the transfrontal route was significantly superior to the transphenoidal route in reducing basal or stimulated LH or FSH levels and TSH after TRH stimulation. The majority of women treated by either operation had basal levels of prolactin which were within the normal range.

In the transphenoidal patients, but not in the transfrontal group, prolactin was appreciably stimulated by TRH.

Of the ten patients treated by either operation one in each group had an objective remission. However, both women had evidence of substantial residual pituitary function. There was no correlation between the quality of remission and the completeness of hypophysectomy.

INTRODUCTION

IT HAS been suggested that complete ablation of the pituitary is necessary to bring about clinical remission of advanced breast cancer [1, 2]. Evidence to support this view has come from studies in which I^{131} uptake or plasma steroid levels have been measured after operation [3, 4]. On the other hand, other workers have not found a correlation between remission after hypophysectomy and pituitary, adrenal and thyroid function [5–7].

Since there is uncertainty on this point the relationship between clinical response and pituitary function was investigated by measuring luteinising hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), prolactin, and plasma androgen sulphates before and after transphenoidal or transfrontal hypophysectomy. The hormones were measured both in the resting state and after stimulation with luteinizing hormone releasing hormone (LH–RH) and thyrotropin releasing hormone (TRH).

Accepted 15 April 1976.

Proofs to; and requests for reprints should be addressed to Dr. D. Y. Wang.

METHODS

1. *Patients*

Twenty women (under the age of 65) with advanced breast cancer were alternately treated by either transfrontal (TF) or transphenoidal (TS) hypophysectomy. The clinical characteristics of the two groups are shown in Table 1.

2. *Hypophysectomy*

The transfrontal hypophysectomies [8] were carried out at the Guy's–Maudsley Neuro-

Table 1. *Clinical characteristics of the patients*

	Transfrontal group	Transphenoidal group
No. of patients	10	10
Mean age	56	48
Age range	48–64	37–61
Sites of metastases:		
Osseous	5	7
Soft tissue (non-visceral)	8	6
Visceral	6	2
Previous treatment:		
Oophorectomy	2	5
Oestrogen therapy	4	1
Androgen therapy	5	5
Recurrence-free interval of greater than 2 years	7	2

surgical Unit and the transphenoidal operations [9] were done at Guy's Hospital.

3. *Pituitary stimulation tests*

Pituitary stimulation tests were carried out in the fasting state between 7.00 and 9.00 a.m. at about 10 days pre-operatively and at 10 days and 6 weeks post-operatively. An i.v. cannula was placed in a forearm vein and after 30 min a basal blood sample was taken. LH–RH (200 μg: Hoechst Pharmaceuticals) and TRH (400 μg: Roche Laboratories) were then injected intravenously and further blood samples were taken at 20, 30 and 60 min after injection.

4. *Hormone estimations*

The concentration of plasma LH and FSH was determined as previously described [10] using a radioimmunoassay double-antibody method. The levels were expressed in terms of MRC standards for LH (MRC 68/40, 77 IU/ampoule) and FSH (68/39; 32·8 IU/ampoule). Plasma TSH and prolactin were also assayed in a similar manner using MRC standards for TSH (68/38; 136 mU/ampoule) and prolactin (71/222). The amounts of dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) present in plasma were measured using gas–liquid chromatography as outlined previously [11].

RESULTS

1. *Plasma FSH and LH levels*

The mean basal levels of FSH and LH are shown in Tables 2 and 3 and were higher before operation in the TS group of patients (40·0 and 43·4 mIU/ml) than in the TF group (19·5 and 29·7 mIU/ml). The difference is significant for FSH ($P < 0{\cdot}02$). This is probably due to the fact that 5 of the 10 patients in the TS group had previously been treated by oophorectomy compared with only two of the TF patients, and all these patients had high plasma levels of gonadotrophins.

Transfrontal hypophysectomy led to a considerable reduction in the mean basal FSH and LH levels (1·2 and 1·8 mIU/ml) 10 days after operation. The effects were less marked in the TS group (FSH 14·8, LH 17·8 mIU/ml). The FSH and LH values were significantly lower in the TF group than in the TS group (See Tables 2 and 3).

Similar results were obtained six weeks after

Table 2. *Plasma FSH in women before and after hypophysectomy*

TF and TS refer to transfrontal and transphenoidal hypophysectomy, respectively. The concentration (mIU/ml) of hormone is expressed as mean ± S.E. The numbers in parentheses refer to the number of patients. The levels of statistical significance, using the *t* test, between the TF and corresponding TS operations are indicated as follows: A = $P < 0{\cdot}05$; B = $P < 0{\cdot}02$; C = $P < 0{\cdot}01$; D = $P < 0{\cdot}005$ and E = $P < 0{\cdot}001$.

Time after injection of LH–RH		Basal	20 min	30 min	60 min
Pre-operative	TF	19·5 ± 3·1 (9) B	31·9 ± 5·8 (9) A	36·3 ± 7·7 (8) A	41·2 ± 8·9 (8) A
	TS	40·0 ± 6·5 (10)	55·3 ± 7·6 (10)	63·5 ± 8·6 (10)	69·1 ± 9·3 (10)
10 days post-operative	TF	1·2 ± 0·8 (9) D	1·6 ± 0·9 (9) E	1·8 ± 0·9 (9) E	2·2 ± 1·1 (9) E
	TS	14·8 ± 3·5 (10)	22·3 ± 4·2 (10)	21·0 ± 3·6 (10)	22·6 ± 3·9 (10)
6 weeks post-operative	TF	1·1 ± 0·7 (6) D	1·7 ± 0·8 (6) D	1·4 ± 0·7 (6) D	1·6 ± 0·8 (6) D
	TS	17·9 ± 4·0 (8)	22·3 ± 4·2 (8)	23·1 ± 4·3 (8)	24·3 ± 4·7 (8)

Table 3. Plasma LH in women before and after hypophysectomy

Legend as for Table 2.

Time after injection of LH–RH		Basal	20 min	30 min	60 min
Pre-operative	TF	29·7 ± 5·4 (10)	81·9 ± 16·0 (9)	86·4 ± 13·2 (10)	97·8 ± 15·6 (9) A
	TS	43·4 ± 4·8 (10)	102·8 ± 14·4 (10)	127·3 ± 15·4 (10)	146·2 ± 13·7 (9)
10 days post-operative	TF	1·8 ± 0·9 (9) D	8·8 ± 3·1 (9) D	10·3 ± 4·6 (10) D	9·8 ± 4·1 (10) E
	TS	17·8 ± 4·5 (10)	33·4 ± 6·6 (10)	39·5 ± 7·2 (10)	45·3 ± 7·7 (10)
6 weeks post-operative	TF	2·2 ± 1·1 (8) D	2·2 ± 1·1 (8) E	1·7 ± 1·2 (8) E	2·4 ± 1·2 (8) D
	TS	24·3 ± 5·8 (8)	35·3 ± 7·1 (8)	45·3 ± 9·3 (8)	61·8 ± 17·0 (7)

operation. Basal FSH levels were reduced to 6 % of the pre-operative values in the TF group but to only 45 % in the TS group. LH values were reduced to 7 % in the TF group and 56 % in the TS patients.

Before hypophysectomy, both groups of patients responded to stimulation by an approximate doubling of their plasma FSH levels and a tripling of their LH levels. At 10 days or 6 weeks after operation there was little evidence of stimulation of FSH in the TF group but the levels in the TS group rose by about 50 % after stimulation. These results are presented graphically in Fig. 1.

LH was marginally stimulated at 10 days but not at six weeks in the TF group, but was doubled by the releasing factor in the TS patients.

2. *Plasma TSH levels*

The mean basal level of this hormone was similar before and after operation for both the TF and TS groups of patients (Table 4). However, 10 days after endocrine ablation, there was a significantly higher level of TSH in the TS group compared to the TF group 20 min after administration of TRH (5·4 and 1·9 μU/ml, respectively; $P < 0{\cdot}01$).

At 6 weeks after hypophysectomy, 5 patients in the TF group and 2 in the TS group showed some return of TSH response to TRH stimulation. This resulted in a similar mean stimulated level of TSH in the two groups (Table 4).

Seven of the TF and 2 of the TS group required and were given thyroid replacement therapy. There was no obvious correlation between TSH response to TRH stimulation and clinical hypothyroidism.

3. *Plasma prolactin levels*

The mean basal levels of plasma prolactin, 10 days post-hypophysectomy were in the high normal range for both groups (Table 5). Although these mean basal levels were similar, stimulation with TRH resulted in a signifi-

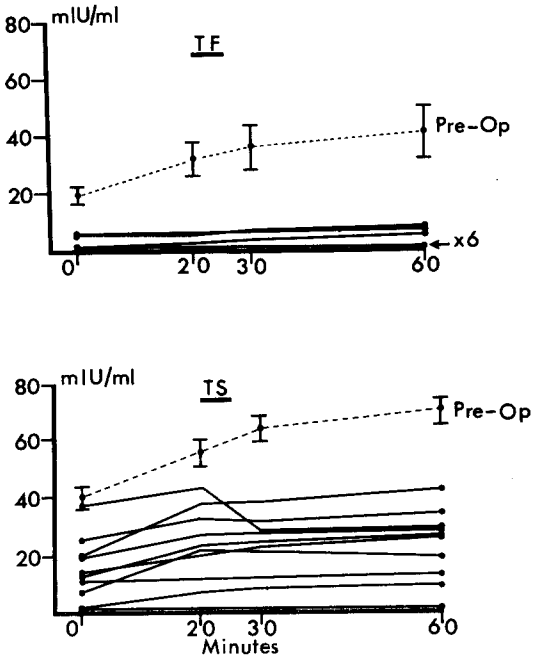


Fig. 1. Effect of transfrontal and transphenoidal hypophysectomy on basal and LH–RH stimulated FSH levels.

The upper figure shows the effect of transfrontal hypophysectomy (TF) on 9 patients and the lower figure, the effect of transphenoidal hypophysectomy (TS) on 10 patients. The pre-operative mean (± S.E.) basal and stimulated levels of FSH are shown as I– – –I *. The levels of FSH for individual patients 10 days after hypophysectomy are shown as* ●——●. *In the upper figure for the TF operation, 6 patients showed minimal stimulation and this is indicated by X6.*

Table 4.

Legend as for Table 2 except the concentration of TSH is expressed as μU/ml.

Time after injection of TRH		Basal	20 min	30 min	60 min
Pre-operative	TF	4·3 ± 1·0 (10) A	21·2 ± 5·2 (9)	19·9 ± 5·1 (9)	15·2 ± 3·4 (9)
	TS	1·9 ± 0·4 (10)	14·5 ± 3·8 (10)	21·7 ± 5·6 (9)	12·9 ± 3·1 (9)
10 days post-operative	TF	1·5 ± 0·5 (10)	1·9 ± 0·7 (10) C	2·2 ± 0·9 (10) A	2·4 ± 1·0 (10) A
	TS	2·0 ± 0·4 (10)	5·4 ± 0·9 (10)	5·9 ± 1·3 (10)	5·5 ± 0·9 (10)
6 weeks post-operative	TF	1·2 ± 0·3 (8)	2·7 ± 0·9 (8)	2·3 ± 0·9 (8)	5·4 ± 2·0 (8)
	TS	2·1 ± 0·8 (8)	8·1 ± 3·3 (8)	10·9 ± 4·6 (8)	8·4 ± 3·9 (8)

Table 5. *Plasma prolactin in women before and after hypophysectomy*

Legend as for Table 2 except the concentration of prolactin is expressed as ng/ml.

Time after injection of TRH		Basal	20 min	30 min	60 min
Pre-operative	TF	19·9 ± 3·5 (10)	54·9 ± 3·9 (9)	58·0 ± 2·7 (9)	51·2 ± 5·9 (9)
	TS	30·8 ± 6·9 (9)	62·7* (10)	63·0* (9)	54·9 ± 4·3 (9)
10 days post-operative	TF	16·7 ± 3·4 (10)	22·2 ± 6·5 (10) B	22·5 ± 6·0 (10) B	22·5 ± 6·2 (10)
	TS	16·9 ± 4·2 (9)	47·0 ± 6·6 (10)	45·4 ± 7·2 (10)	39·2 ± 6·6 (10)
6 weeks post-operative	TF	19·3 ± 4·8 (8)	24·8 ± 6·6 (8) A	24·6 ± 6·2 (8) A	21·5 ± 6·4 (8) A
	TS	13·0 ± 1·7 (8)	49·3 ± 9·3 (8)	48·4 ± 9·0 (8)	49·3 ± 9·3 (8)

*No standard errors are available because the majority of results were beyond the upper limit of the assay.

cantly higher mean level in the TS group compared to the TF group (47·0 and 22·2 ng/ml, respectively $P < 0{\cdot}02$). A significant difference was also observed at 6 weeks after pituitary ablation (Table 5).

Only 3 patients in the transfrontal group and one in the transphenoidal group had very low basal prolactin levels after hypophysectomy and showed no response to stimulation.

In view of the high basal prolactin levels found before and after operation, careful search was made for all drugs that might have been given which are known to raise the serum prolactin. Full drug records were available for the pre-operative and 10-day post-operative tests but it is possible at 6 weeks post-operatively that some patients were receiving drugs from their general practitioners at the time of the test. All patients in the TF group were receiving phenobarbitone 30 mg b.d. from the time of operation.

Two patients in the TS group had received perphenazine shortly before the six-week post-operative test. In one patient the basal prolactin level was high but rose on stimulation and in the other the basal level was low and remained so on stimulation.

4. *Plasma androgen sulphates*

Both types of hypophysectomy led to a pronounced fall in the basal plasma levels of the adrenal steroids, DS and AS. The mean levels at 10 days and 6 weeks after operation were approximately 20% of the pre-operative levels.

While the mean DS and AS values after

operation were lower in the TF than in the TS group, both at 10 days and at 6 weeks, the differences were not significant (Table 6).

5. *Relation between endocrine function and remission*

Only one patient in each group had a remission classified as successful by the British Breast Group criteria [12]. There were six intermediate responses and three failures in the TF group, and four intermediates and five failures in the TS group.

The intermediate and successful responders have been pooled for the purposes of this analysis and their endocrine function compared with that of the failure group. The data are given in Tables 7 and 8.

There is a tendency for responders to have lower levels of FSH, LH and TSH than the unresponsive group but the differences are not significant. Similarly, the higher mean prolactin level in responders is not significantly different from that of the failure group.

Table 6. *Plasma DS and AS in women before and after hypophysectomy*

Legend as for Table 2 except the concentrations of DS and AS are expressed as μg/100 ml, and none of the differences between TF and TS means were significant.

		Basal DS	Basal AS
Pre-operative	TF	44·1 ± 9·4 (10)	12·9 ± 4·5 (10)
	TS	53·0 ± 13·7 (10)	16·6 ± 9·3 (10)
10 days post-operative	TF	6·8 ± 1·9 (10)	2·9 ± 0·7 (10)
	TS	15·8 ± 4·7 (10)	5·3 ± 2·9 (10)
6 weeks post-operative	TF	4·1 ± 1·0 (8)	2·1 ± 0·5 (8)
	TS	13·1 ± 5·0 (8)	4·3 ± 2·0 (8)

Table 7. *FSH and LH levels and clinical response to hypophysectomy*

Responders refer to patients who had a successful or intermediate remission and Non-responders to patients who did not undergo remission after hypophysectomy. The concentration of FSH (mIU/ml) and LH (mIU/ml) are expressed as means ± S.E. The numbers in parentheses refer to the number of patients. There was no significant difference between the mean values for Responders and the corresponding Non-responder group.

Time after injection of LH–RH	FSH			
	Basal	20 min	30 min	60 min
Pre-operative (Responders)	25·8 ± 5·3 (11)	39·5 ± 7·3 (11)	43·9 ± 8·2 (11)	49·9 ± 8·7 (11)
Pre-operative (Non-responders)	19·9 ± 7·0 (8)	50·4 ± 8·0 (8)	63·3 ± 10·0 (7)	67·3 ± 11·8 (7)
10 days post-operative (Responders)	6·4 ± 2·7 (11)	9·8 ± 3·9 (11)	9·8 ± 3·9 (11)	10·4 ± 4·1 (11)
10 days post-operative (Non-responders)	10·9 ± 4·5 (8)	16·1 ± 5·8 (8)	14·9 ± 4·7 (8)	16·4 ± 5·1 (8)
6 weeks post-operative (Responders)	8·8 ± 3·8 (9)	12·7 ± 5·1 (9)	13·6 ± 5·5 (9)	13·6 ± 5·7 (9)
6 weeks post-operative (Non-responders)	13·8 ± 6·1 (5)	15·0 ± 5·5 (5)	14·0 ± 4·9 (5)	16·2 ± 5·7 (5)

Time after injection of LH–RH	LH			
	Basal	20 min	30 min	60 min
Pre-operative (Responders)	38·7 ± 4·8 (12)	92·0 ± 16·8 (12)	104·5 ± 18·1 (12)	113·7 ± 17·1 (12)
Pre-operative (Non-responders)	33·1 ± 6·4 (8)	87·3 ± 13·8 (7)	102·4 ± 13·9 (8)	126·4 ± 21·1 (6)
10 days post-operative (Responders)	9·5 ± 3·5 (11)	18·6 ± 6·7 (12)	18·8 ± 6·9 (12)	21·7 ± 7·7 (12)
10 days post-operative (Non-responders)	11·2 ± 5·6 (8)	29·9 ± 5·8 (7)	34·2 ± 7·8 (8)	36·4 ± 8·9 (8)
6 weeks post-operative (Responders)	11·5 ± 4·4 (11)	17·3 ± 7·0 (11)	22·9 ± 9·7 (11)	32·2 ± 14·6 (11)
6 weeks post-operative (Non-responders)	17·1 ± 9·2 (5)	14·7 ± 6·2 (5)	24·7 ± 10·5 (5)	24·2 ± 12·1 (4)

Table 8. TSH and prolactin levels and clinical response to hypophysectomy

Legend as for Table 7 except the concentrations of TSH and prolactin are expressed as μU/ml and ng/ml, respectively.

Time after injection of TRH	TSH			
	Basal	20 min	30 min	60 min
Pre-operative (Responders)	3·3 ± 0·9 (12)	15·2 ± 3·3 (11)	20·3 ± 4·4 (12)	13·7 ± 2·8 (12)
Pre-operative (Non-responders)	3·0 ± 0·8 (8)	20·9 ± 6·1 (8)	21·7 ± 7·2 (6)	14·7 ± 4·1 (6)
10 days post-operative (Responders)	1·5 ± 0·4 (12)	3·2 ± 1·1 (12)	3·7 ± 1·4 (12)	3·4 ± 1·0 (12)
10 days post-operative (Non-responders)	2·1 ± 0·4 (8)	4·3 ± 0·7 (8)	4·5 ± 0·9 (8)	4·8 ± 1·0 (8)
6 weeks post-operative (Responders)	1·2 ± 0·3 (11)	4·6 ± 1·3 (11)	5·3 ± 2·0 (11)	5·0 ± 1·3 (10)
6 weeks post-operative (Non-responders)	2·6 ± 1·1 (5)	7·3 ± 5·3 (5)	9·4 ± 7·2 (5)	9·0 ± 6·4 (5)
Time after injection of TRH	Prolactin			
	Basal	20 min	30 min	60 min
Pre-operative (Responders)	22·8 ± 3·4 (12)	56·5 ± 3·3 (11)	59·8 ± 2·1 (12)	50·8 ± 4·6 (12)
Pre-operative (Non-responders)	28·9 ± 8·9 (7)	62·5 ± 0·5 (8)	62·0 ± 1·0 (6)	57·3 ± 5·7 (6)
10 days post-operative (Responders)	12·7 ± 1·5 (11)	27·9 ± 6·6 (12)	28·8 ± 6·8 (12)	26·6 ± 6·0 (12)
10 days post-operative (Non-responders)	22·4 ± 5·4 (8)	44·6 ± 8·2 (8)	41·6 ± 8·0 (8)	37·3 ± 7·7 (8)
6 weeks post-operative (Responders)	15·4 ± 2·2 (11)	35·0 ± 7·2 (11)	33·1 ± 6·7 (11)	29·3 ± 6·6 (11)
6 weeks post-operative (Non-responders)	17·8 ± 7·3 (5)	35·8 ± 10·5 (5)	38·4 ± 10·2 (5)	37·2 ± 10·1 (5)

The mean level of plasma DS of 9·8 μg % for non-responders was not significantly different from the 8·1 μg % for responders.

Two illustrative cases are shown in Figs. 2 and 3. In case 1, data in the figure show clearly that FSH, LH, TSH and prolactin levels are reduced and cannot be stimulated, yet this patient failed to respond. In case 2, there was an objective remission of the disease in spite of the fact that hypophysectomy had led to little reduction in the plasma levels of the four pituitary hormones and that some degree of stimulation was obtainable for FSH, LH and TSH while stimulated prolactin levels after operation were as high as those beforehand. In the second patient who had a successful response, plasma prolactin levels remained above 20 ng/ml at all times after operation but were not increased by stimulation.

DISCUSSION

Both operations reduce the basal and stimulated plasma levels of FSH and LH but the transfrontal operation causes a greater reduction than the transphenoidal approach (to approximately 10% of pre-operation levels compared with 50% for the transphenoidal operation). While the basal TSH levels were similar after either operation, the TRH-stimulated levels were significantly higher after TS hypophysectomy compared to the TF route.

Neither operation led to a marked reduction of the basal prolactin levels. Whereas TRH led to a significant stimulation of prolactin secretion in the TS group it was virtually ineffective in the TF patients.

The most likely explanation for all the endocrine findings is that the TF operation leads to the severing of the pituitary stalk and the removal of the bulk of pituitary tissue. The few remaining cells would not be stimulated by positive trophic factors to secrete LH, FSH and TSH but would be free from the inhibitory effects of prolactin inhibiting factor (PIF) (see [13]) and would therefore secrete prolactin at a maximum rate. By contrast, it could be postulated that after the TS operation many more cells remain,

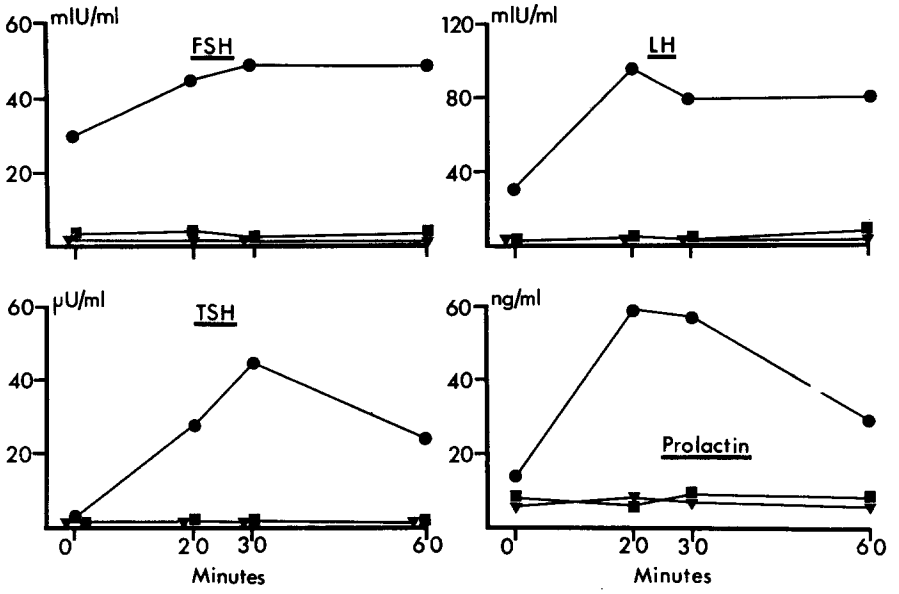


Fig. 2. Effect of hypophysectomy on FSH, LH, TSH and prolactin levels.
Patient BC (aged 64) who failed to respond to transfrontal hypophysectomy. Time 0 refers to the time at which LH-RH (for LH and FSH stimulation) and TRH (for TSH and prolactin stimulation) were injected.
The pre-operative levels are shown by ●——● and ▼——▼ and ■——■ are levels at 10 days and 6 weeks respectively, after hypophysectomy.

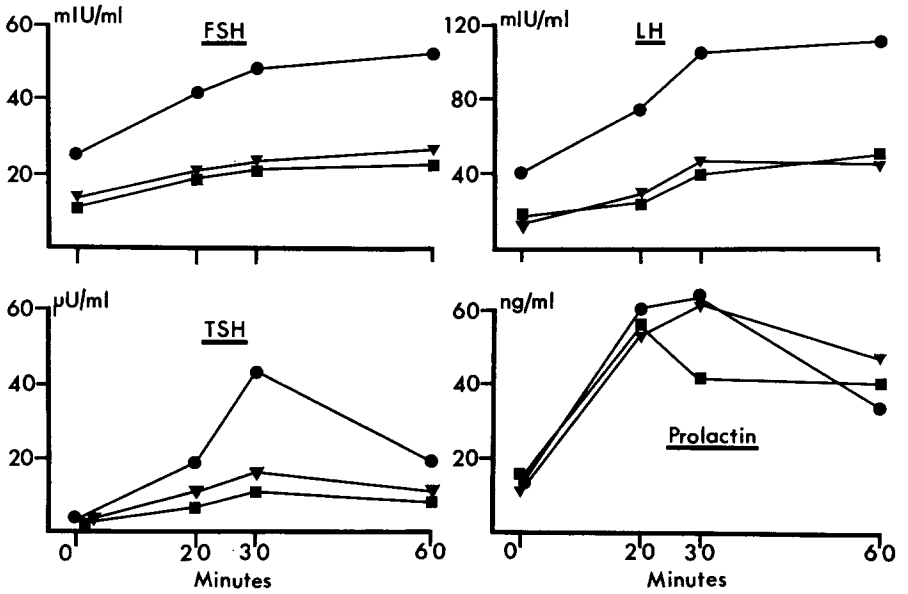


Fig. 3. Effect of hypophysectomy on FSH, LH, TSH and prolactin levels.
Patient EH (aged 51) who had a successful remission after transphenoidal hypophysectomy Time 0 refers to the time at which LH-RH (for LH and FSH stimulation) and TRH (for TSH and prolactin stimulation) were injected.
The pre-operative levels are shown by ●——● and ▼——▼ and ■——■ are levels at 10 days and 6 weeks respectively, after hypophysectomy.

and that the pituitary stalk would not necessarily be severed. The result of this would be some continuing degree of stimulation of LH, FSH and TSH, accounting for the levels of these hormones found after operation. In the TS group, a continued secretion of PIF would allow a larger number of pituitary cells to produce no more prolactin than a lesser number of cells in the TF group. This would also account for the fact that TRH is an effective stimulator of prolactin in the TS patients but is not so in the TF group.

Complete abolition of pituitary hormone secretion was uncommon after hypophysectomy but this does not compromize the therapeutic effect of either operation. There was no correla-

tion between remaining endocrine function and remission. The 2 patients who had successful remissions both had substantial pituitary function after hypophysectomy. These findings are in agreement with other workers' results [5, 6, 14–16].

It has never been clear how endocrine ablation leads to remission. Recent findings show that the proliferation rate in many human breast tumours is only marginally greater than the cell death rate [17, 18]. If the proliferation rate were wholly or partially dependent on endocrine stimuli, then even a minor alteration in these stimuli might mean that the balance between cell growth and death favours the latter.

Acknowledgements—We wish to thank Mr. M. A. Falconer and Mr. P. H. Schurr of the Guy's–Maudsley Neurosurgical Unit and Mr. L. F. W. Salmon and Mr. O. H. Shaheen of the Ear, Nose and Throat Dept. of Guy's Hospital for their co-operation in this study. We are also grateful to Hoechst Pharmaceuticals for the gift of LH–RH.

REFERENCES

1. M. M. Roberts, A comparison of transethmoidal hypophysectomy, Yttrium 90 implant and adrenalectomy. In *The Clinical Management of Advanced Breast Cancer*. 2nd Tenovus Workshop, p. 54, Alpha Omega Alpha Publishing, Cardiff (1970).
2. A. P. M. Forrest, Discussion. In *The Clinical Management of Advanced Breast Cancer*. 2nd Tenovus Workshop, p. 63, Alpha Omega Alpha Publishing, Cardiff (1970).
3. E. Boesen, E. J. R. Smith and D. N. Baron, Further experiences with hypophysectomy in advanced breast cancer. *Brit. med. J.* **2,** 790 (1961).
4. P. Juret and M. Mayem, In *Mammary Cancer and Neuroendocrine Therapy*. (Edited by B. A. Stoll), p. 283, Butterworth, London (1974).
5. F. C. Peck and K. B. Olson, The treatment of advanced breast cancer by hypophysectomy. *New York State J. Med.* **63,** 2191 (1963).
6. J. C. Beck, A. J. Blair, M. M. Griffiths, M. W. Rosenfeld and E. E. McGarry, In search of hormonal factor as an aid in predicting the outcome of breast carcinoma. Proceedings of the 6th Canadian Cancer Research Conference. p. 3, Pergamon Press (1964).
7. B. A. Stoll, Completeness of hypophyseal ablation. In *Hormonal Management in Breast Cancer*, p. 92, Pitman, London (1969).
8. P. H. Schurr, Techniques and effects of hypophysectomy, pituitary stalk section and pituitary transplantation in man. In *The Pituitary Gland*. (Edited by G. E. Harris and B. T. Donovan) Vol. 2, p. 22, Butterworth, London (1966).
9. J. A. James, Hypophysectomy in general medicine and surgery. *J. roy. Coll. Surg. Edinb.* **1,** 83 (1964).
10. D. Y. Wang, R. D. Bulbrook, Marenka Herian and J. L. Hayward, Studies on the sulphate esters of dehydroepiandrosterone and androsterone in the blood of women with breast cancer. *Europ. J. Cancer* **10,** 477 (1974).
11. D. Y. Wang, P. R. Goodwin, R. D. Bulbrook and J. L. Hayward, Plasma FSH and LH in post-menopausal women with breast cancer. *Europ. J. Cancer* **12,** 305 (1976).
12. British Breast Group, Assessment of response to treatment in advanced breast cancer. *Lancet* **ii,** 790 (1974).
13. F. G. Sulman, Hypothalamic control of lactation. In *Monographs on Endocrinology*. (Edited by F. Gross, A. Labhart, T. Mann). L. T. Samuels and J. Zander, Vol. 3, p. 4 (1970).
14. O. H. Pearson and B. Ray, Hypophysectomy in the treatment of metastatic mammary cancer. *Amer. J. Surg.* **99,** 544 (1960).
15. J. F. Newsome, R. L. Timmons, J. Van Wyk and G. S. Dugger, Pituitary stalk section for metastatic carcinoma of the breast. *Ann. Surg.* **174,** 769 (1971).
16. R. W. Turkington, L. E. Underwood and J. J. Van Wyk, Elevated serum prolactin levels after pituitary stalk section in man. *New Engl. J. Med.* **285,** 707 (1971).
17. R. Silvestrini, O. Sanfilippo and G. Tedesco, Kinetics of human mammary carcinomas and their correlation with the cancer and the host characteristics. *Cancer (Philad.)* **34,** 1252 (1974).
18. British Medical Journal Editorial, Cell life and death in human tumours. *Brit. med. J.* **1,** 177 (1976).

Europ. J. Cancer Vol. 12, pp. 783–792. Pergamon Press 1976. Printed in Great Britain

Effect of Leucocyte Washings on Cellular Immunity to Human Renal Carcinoma

MOGENS KJAER

Division of Clinical Immunology, Medical Department TA, and Surgical Department C, Rigshospitalet, University Hospital of Copenhagen, Denmark

Abstract—*The leucocyte migration capillary tube technique (LMCT) was used to detect tumour-directed, cell-mediated hypersensitivity (TCMH) employing allogeneic hypernephroma and corresponding normal renal tissue extracts in* 16 *patients with renal carcinoma and in* 89 *controls. In parallel series* 3, 6, *and* 10 *leucocyte washings were used before incubation of the capillary tubes in the migration chambers.*

The proportion of detectable TCMH in the LMCT was significantly increased after extensive cell washings in vitro. *Also the proportion of patients with renal carcinoma showing a high degree of TCMH could be significantly increased by washing of leucocytes. The effect of washing was especially manifest in patients with disseminated disease, and appeared to be specifically confined to reactivity towards allogeneic hypernephroma tissue extracts in patients with renal carcinoma. The findings suggest that a factor is removed by extensive cell washing, from the migrating cells, supporting the hypothesis of antigenic inhibition of TCMH in patients with renal carcinoma, the factor involved most probably being circulating tumour antigen.*

INTRODUCTION

IN PATIENTS with cancer coating of tumour specific transplantation antigens (TSTA) on the surface of tumour cells, presumably by antigen–antibody complexes [1] was shown by Hellström and Hellström in 1969 [2]. This type of efferent enhancement of tumour-directed, cell-mediated hypersensitivity (TCMH) was termed "blocking", and has been found in numerous other studies [3–5].

In 1972 Currie and Basham demonstrated significantly greater cytotoxicity of cancer patients' lymphocytes after extensive washing *in vitro*, as compared to lymphocytes washed only twice [6]. They suggested the term "antigenic inhibition" for this type of efferent enhancement presumably due to coating of lymphocytes by exfoliated TSTA [6, 7].

Two types of studies have confirmed the hypothesis of antigenic inhibition in man:

1. Studies on the effect of lymphocyte washing on *in vitro* detection of TCMH [8, 9].
2. Studies on the inhibitory effect on lymphocyte cytotoxicity *in vitro* after addition of solubilized TSTA to the test system [10, 11].

The above mentioned studies have been carried out using cytotoxicity assays.

Accepted 13 April 1976.

In recent studies by Kjaer [12–18] with the leucocyte migration capillary tube technique (LMCT) and soluble extracts of tumour tissue in patients with renal carcinoma it has been shown,

—that at the time of initial treatment, 50% of the patients have detectable TCMH against extracts of the autologous tumour and allogeneic hypernephroma [12–14].
—that a dose-related effect of tumour extracts on the migration of leucocytes is evident at extract concentrations of 100, 300 and 400 μg of protein per ml [13–16],
—that patients without distant metastases significantly more often have evidence of TCMH than patients with metastases [12–16],
—that in the LMCT serum (autologous or allogeneic) from patients with renal carcinoma can specifically abolish reactivity of renal carcinoma leucocytes [17].

The present study on the effect of leucocyte washing in the LMCT was undertaken to investigate if the lack of detectable TCMH in renal carcinoma patients with disseminated disease might be attributed to a factor coating the effector cells and removable by extensive cell washing *in vitro*.

MATERIAL AND METHODS

A total of 105 persons were investigated. They were divided into the following groups:

1. Sixteen consecutive patients with renal carcinoma: 10 male, and 6 female patients, age 46–72 years, mean 60 years. Fourteen patients had the histological diagnosis of hypernephroma, one of adenopapillary carcinoma, and one of transitional cell carcinoma of the renal pelvis with macroscopic invasion in the kidney parenchyma.

Eleven patients had demonstrable distant metastases at the time of diagnosis, and 5 had not. The present material thus is representative for renal carcinoma materials with respect to sex, age, and dissemination of the disease [18]. The clinical and immunological data of the 16 patients are given in table 1. One patient (no. 4, Table 1) did not have the tumour removed during operation because of local invasion. The diagnosis was confirmed by biopsy during surgery.

2. Thirty-nine patients with non-neoplastic kidney disease or solid carcinoma of non-kidney origin. Twenty-six male, and 13 female patients, age 24–78 years, mean 62 years.

The diagnoses of the 17 patients with non-neoplastic kidney disease were the following: Solitary renal cyst (7), hypertensive nephropathy (3), chronic glomerulonephritis (2), polycystic renal disease (1), renal adenoma (1), renal tuberculosis (1), hydronephrosis (1), and nephrolithiasis (1 patient).

The diagnoses of the 22 patients with solid carcinoma of non-kidney origin were all histologically confirmed: Gastric carcinoma (9), colonic carcinoma (4), pancreatic carcinoma (3), bladder carcinoma (2), mammary carcinoma (2), prostatic carcinoma (1), pulmonary carcinoma (1 patient). This group of patients was tested at only one occasion—preoperatively. All patients were without detectable distant metastases at the time of testing.

3. Fifty healthy blood donors from the blood donor group of Rigshospitalet, Copenhagen. Thirty-six male, and 14 female persons, age 31–63 years, mean 51 years.

Persons above the age of 65 are not permitted to act as blood donors. Therefore, the mean age of this control group was somewhat lower than that of the renal carcinoma group.

Preparation of tissue extract and the LMCT

A histological evaluation of the renal carcinomas was performed according to criteria earlier described in detail [16]. The preparation of tissue extracts from renal carcinomas and corresponding normal renal parenchyma has earlier been described in detail [12–14].

The LMCT was performed according to Bendixen and Søborg [19], micromodified according to Maini *et al.* [20], as earlier described in detail [12–15].

In essence, venous blood was collected in heparin and Dextran. After sedimentation the leucocytes were washed in Hanks' solution (see below), and collected in 20 μl glass capillary tubes. Each of these was placed in a 0·5 ml disposable culture chamber containing TC 199 with 10% horse serum. To the test cultures the adequate amount of tissue extract was added. All cultures were set up at least in triplicate. After 24 hours the migration area of leucocytes around the opening of the capillary tube was measured in a projection microscope. The average area of extract-containing (M_x) cultures, and control (M_0) cultures determines the migration index MI: $MI = M_x/M_0$.

TEST PROTOCOL

The patients with renal carcinoma were admitted consecutively to the investigation irrespective of the clinical stage at the time of initial treatment. The diagnosis was made on the basis of renal arteriography.

Beginning preoperatively 3–8 tests were performed in each patient. In each test at least two renal carcinoma extracts and two corresponding renal tissue extracts were employed at protein concentrations of 10, 50, 100, 300, 400 and 600 μg per ml. In all tests only highly antigenic non-pooled renal carcinoma tissue extracts with a migration inhibition score of more than 20% [16] were used.

The leucocyte suspension was divided into three parts and washed three, six, and ten times before incubation respectively. The leucocytes were counted, and resuspended in TC 199 + 10% horse serum. The cells were drawn into 20 μl glass capillary tubes, sealed over a flame and centrifuged giving approx. 3×10^6 leucocytes packed in each capillary tube. Thus in each set up three capillary tubes at each antigen concentration were run at 3, 6, and 10 leucocyte washings.

In each patient 3–8 different antigens were used over the total test period. Titration curves were drawn for each patient at each leucocyte washing.

For the statistical evaluation a numerical grading of hypersensitivity was performed.

The grading was based on the minimal tumour extract concentration giving significant

leucocyte migration inhibition or stimulation. Significant migration inhibition and stimulation has earlier been defined [12, 15] as MI's beyond the mean value ± 2. s.d. of the migrations in the control groups at the maximal nontoxic dose, i.e., 400 μg of protein per ml. Cut off MI=greater than 1·19 and smaller than 0·79.

The following criteria for grading were used as exemplified in Fig. 1.

Grade 0: MI > 0·79, MI < 1·19 at all tissue extract concentrations employed except 600 μg/ml which has been shown to inhibit migration of leucocytes from patients as well as controls [13, 15].

Grade 1: MI < 0·79 or MI > 1·19 at 400 μg ml, but not at lower concentrations.

Grade 2: MI < 0·79 or MI > 1·19 at 400 and 300 μg/ml, but not at lower concentrations.

Grade 3: MI < 0·79 or MI > 1·19 at 400, 300 and 100 μg/ml, but not at lower concentrations.

Grade 4: MI < 0·79 or MI > 1·19 at 400, 300, 100 and 50, or 10 μg/ml.

STATISTICAL EVALUATION

For evaluation of the results a computerized Wilcoxon Mann–Whitney test and Fisher's exact probability test were used [21]. Level of significance = 5 %.

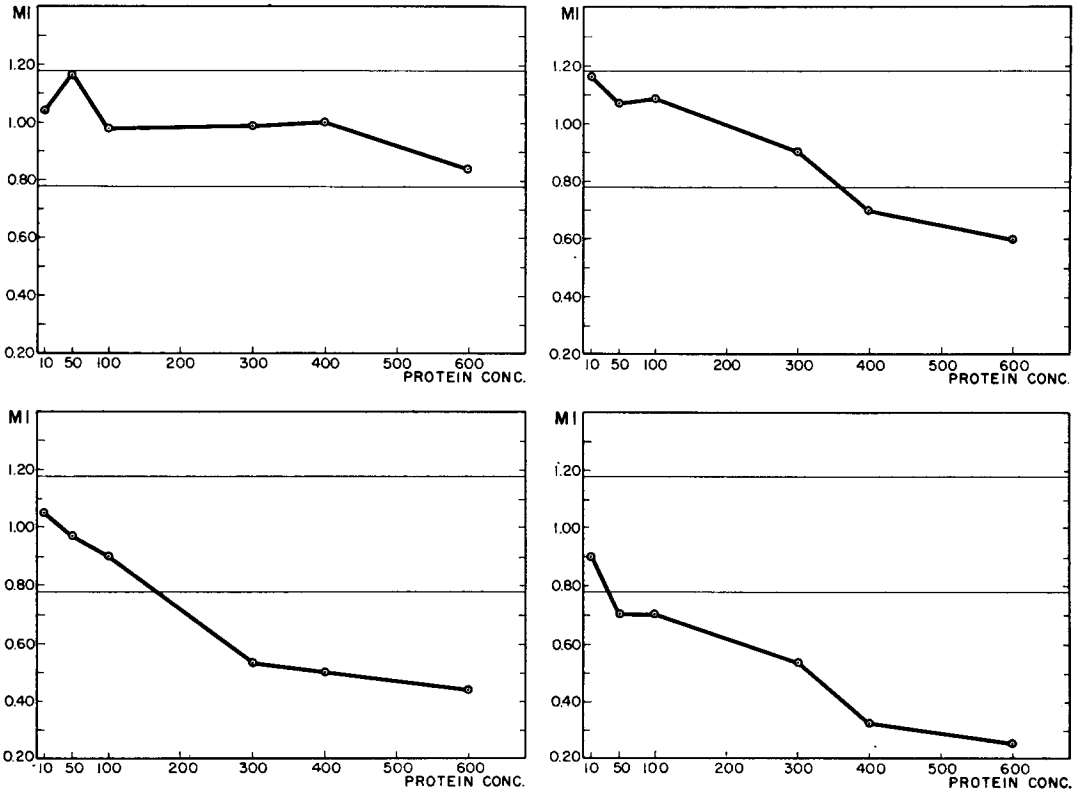


Fig. 1. *Titration curves from* 4 *patients with hypernephroma using allogeneic hypernephroma tissue extracts as antigen. Normal range, MI* = 0·79–1·19. *Three leucocyte washings.*

(*a*) *Top left: Titration curve from a patient with extensive skeletal and pulmonary metastases. Grade* 0.
(*b*) *Top right: Titration curve from a patient without distant metastases. Grade* 1.
(*c*) *Bottom left: Titration curve from a patient without distant metastases. Grade* 2.
(*d*) *Bottom right: Titration curve from a patient without distant metastases. Grade* 4.

RESULTS

The results of the investigation are shown in the Fig. 2–5 and in the Tables 1–5.

SPECIFICITY OF THE WASHING EFFECT

In the Fig. 2–4 are shown the leucocyte reactivity of the patients with renal carcinoma, of patients with benign renal disease or carcinomas of non-kidney origin, and of blood donors using 3, 6 and 10 leucocyte washings at tissue extract concentrations of 10, 50, 100, 300, 400 and 600 μg/ml.

In the groups of benign renal disease, carcinomas of non-kidney origin and blood donors (Fig. 3–4) a significant inhibitory effect of both renal carcinoma and normal renal tissue extracts was evident at all washings using 600 μg of protein/ml. This is consistent with earlier reports [13, 15]. Apart from this no significant inhibitory effect was seen either of renal carcinoma nor of normal renal tissue extracts at any concentration employed, regardless of the number of leucocyte washings. In the renal carcinoma group no effect of leucocyte washing was seen in the presence of allogeneic normal renal tissue except at 600 μg/ml (Fig. 2). However, using allogeneic hypernephroma tissue extracts significant reactivity was demonstrated at 400 μg of protein per ml using 3 leucocyte washings. At 3 leucocyte washings we have earlier reported a dose-related effect of allogeneic hypernephroma at extract concentrations of 100, 300 and 400 μg of protein/ml [13, 15]. This could not be demonstrated in the present series as the number of patients without distant metastases was too small to reach significant levels [13, 15]. Using 6 and 10 leucocyte washings a highly significant dose-related reactivity was detectable in the renal carcinoma group with allogeneic hypernephroma tissue extracts at concentrations of 50, 100, 300, 400 and 600 μg of protein/ml. (Fig. 2). The most

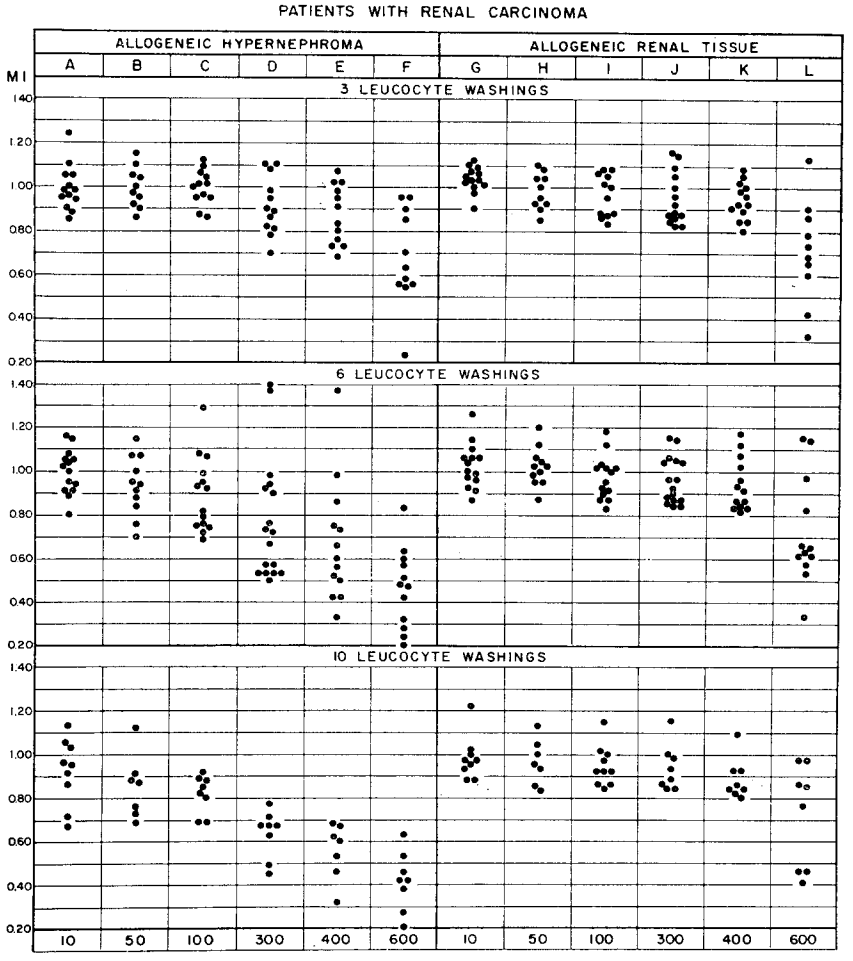


Fig. 2. Migration indices of 16 *patients with renal carcinoma after* 3, 6 *and* 10 *leucocyte washings using allogeneic hypernephroma and normal renal tissue extracts as antigen. Abscissa indicates protein concentration, μg per ml. Wilcoxon Mann–Whitney test:* 3 *leucocyte washings: A–E, significant, A–F significant.* 6 *leucocyte washings: A–C, significant, A–D, significant, A–E, significant, A–F, significant.* 10 *leucocyte washings: A–D, significant, A–E, significant, A–F, significant.*

Comparison between 3 *and* 6 *leucocyte washings: significant differences at* 100, 300, 400 *and* 600 *μg. Comparisons between* 3 *and* 10 *leucocyte washings: significant differences at* 50, 100, 300, 400 *and* 600 *μg/ml. Comparisons between* 6 *and* 10 *leucocyte washings: not significant.*

Table 1. *The effect of leucocyte washings on the* in vitro *detection of tumour-directed, cell-mediated hypersensitivity in* 16 *patients with renal carcinoma. Clinical data and TCMH*

Patient No.	Sex	Age	Metastases		Invasion		Degree of differentiation	Tumour type	TCMH grade		
			Local	Distant	Macroscopic	Microscopic			3 wash.	6 wash.	10 wash.
1	male	63	+	+	+	+	II	hypern.	0	2	n.i.
2	male	58	+	+	0	0	I	hypern.	0	2	n.i.
3	male	72	0	+	+	+	II	adeno.	0	2	n.i.
4	male	70	n.i.	+	n.i.	n.i.	n.i.	m.r.t.	0	2	n.i.
5	male	58	+	+	+	n.i.	n.i.	hypern.	0	0	2
6	female	61	0	+	+	+	II	hypern.	0	0	n.i.
7	female	59	0	0	+	0	I	hypern.	2	2	2
8	female	46	0	+	+	+	II	hypern.	0	4	n.i.
9	male	58	+	+	+	+	n.i.	t.c.c.	0	4	2
10	male	64	+	+	+	+	II	hypern.	0	3	4
11	female	53	0	0	0	+	I	hypern.	2	2	2
12	female	64	0	0	+	+	II	hypern.	0	1	n.i.
13	male	58	0	+	+	+	I	hypern.	0	1	4
14	female	55	+	0	0	+	I	hypern.	1	3	3
15	male	62	+	+	+	+	I	hypern.	0	2	2
16	male	59	+	0	+	+	II	hypern.	1	3	2

n.i. = not investigated. hypern. = hypernephroma. m.r.t. = malignant renal tumour. t.c.c. = transitional cell carcinoma of the renal pelvis. adeno. = adenopapillary carcinoma.

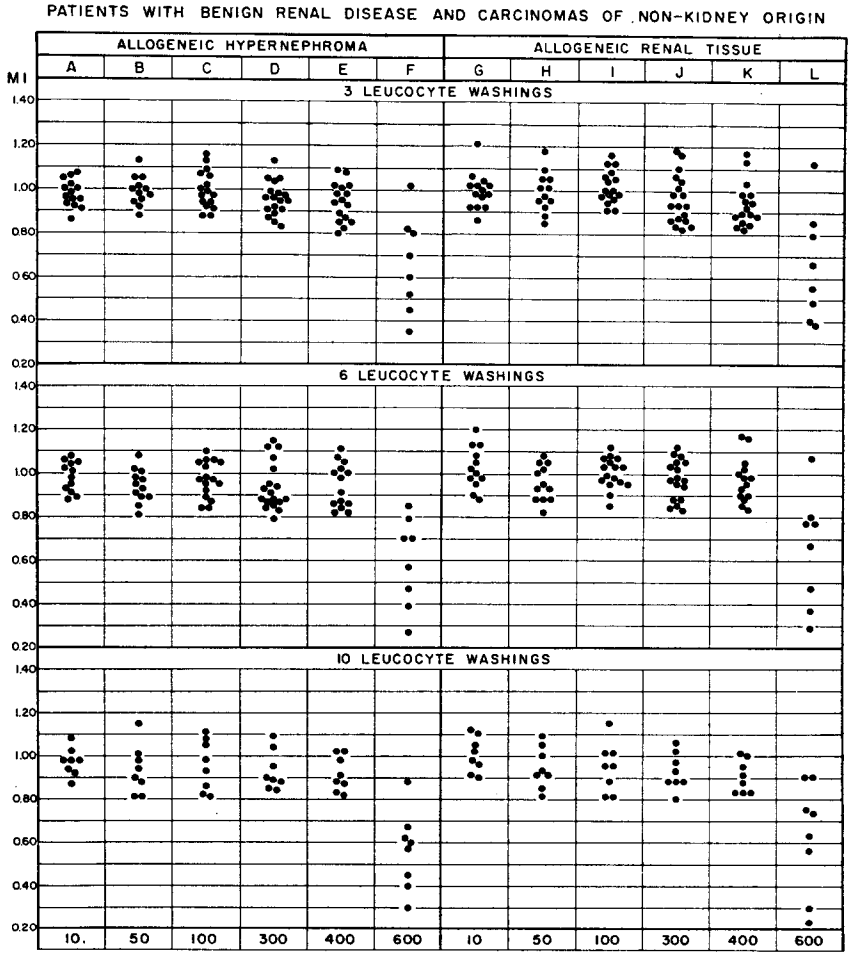


Fig. 3. *Migration indices of* 39 *patients with benign renal disease and carcinomas of non-kidney origin after* 3, 6 *and* 10 *leucocyte washings using allogeneic hypernephroma and normal renal tissue extracts as antigen. Abscissa indicates protein concentration, µg/ml. Significant migration inhibition was found at* 600 *µg/ml at all leucocyte washings. All other comparisons within the group gave non significant results.*

significant differences in reactivity were found after 6 leucocyte washings. It is worth noticing that using 6 leucocyte washings some significantly stimulated MIs were found at 100, 300 and 400 μg/ml whereas no stimulation could be demonstrated, after 10 leucocyte washings. The significance of leucocyte migration stimulation has earlier been discussed [15]. The same level of significance was reached when the data from the renal carcinoma patients were compared to those of patients with benign renal disease, carcinomas of non-kidney origin, and of blood donors.

Using criteria for grading of TCMH as described above it was shown (Table 2) when 3 and 6 leucocyte washings were compared that the number of patients showing reactivity (grade 1–4) increased (4/16 patients at 3 washings, 14/16 at 6 washings). Also the proportion of patients showing high degrees of TCMH (grade 3–4) increased significantly (0/16 patients at 3 washings, 6/16 at 6 washings).

Table 2. Relation between leucocyte washing in vitro *and TCMH-grading in* 16 *patients with renal carcinoma*

	TCMH grade			
	0	1–2	3–4	Total
3 leucocyte washings	12	4	0	16
6 leucocyte washings	2	8	6	16
10 leucocyte	0	6	3	9

Significance: 3 washings/6 washings, $P < 0{\cdot}0001$.
6 washings/10 washings, $P > 0{\cdot}05$.
P-value calculated according to J. H. Edwards [21].

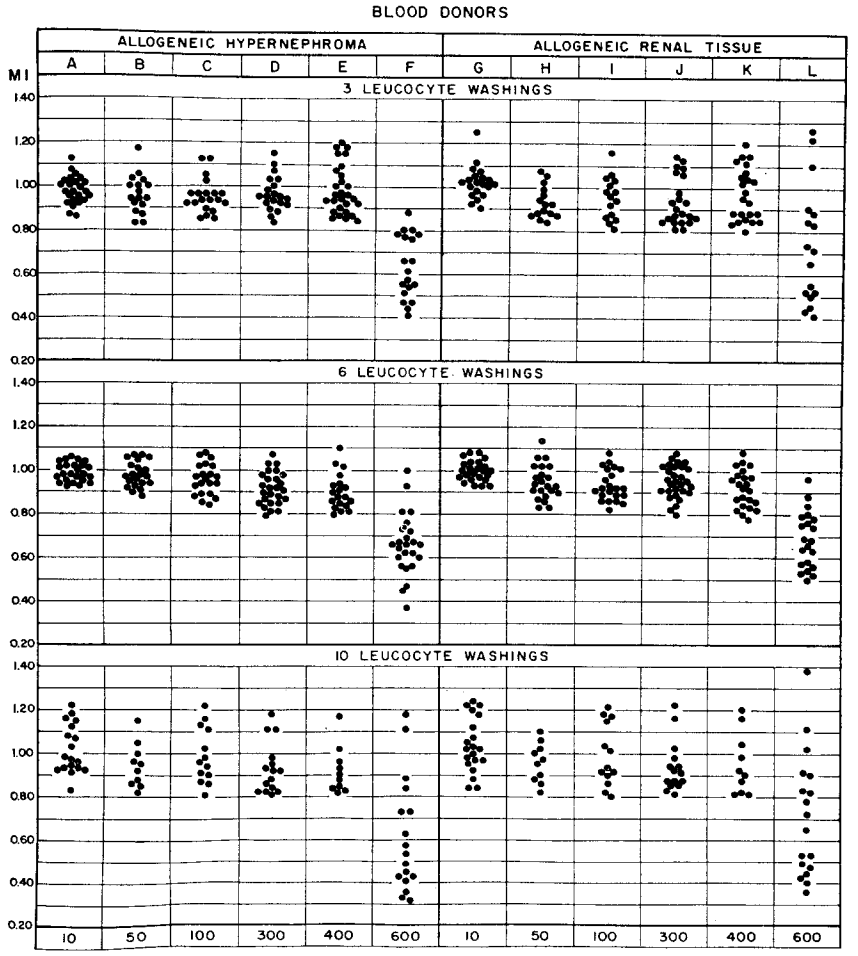


Fig. 4. Migration indices of 50 *blood donors after* 3, 6 *and* 10 *leucocyte washings using allogeneic hypernephroma and normal renal tissue extracts as antigen. Abscissa indicates protein concentration, μg/ml.*

Significant migration inhibition was found at 600 *μg/ml at all leucocyte washings. All other comparisons within the group gave non significant results.*

CORRELATION BETWEEN WASHING EFFECT AND CLINICAL DATA

No significant correlation was observed between the effect of leucocyte washings in renal carcinoma patients and any of the following: age, sex; blood type; previous history of transfusions; erythrocyte sedimentation rate; hemoglobin concentration; local macroscopic or microscopic tumour invasion into the renal vein, renal pelvis, lymph nodes, fibrous capsule, fatty capsule, hilus vessels, tumour diameter; degree of differentiation [16], necrosis, bleeding, or lymphocyte infiltration in the stroma or tumour tissue.

As shown in Table 3, however, a significant relation was found between the absence of distant metastases and detectable TCMH (grade 1–4) at 3 leucocyte washings. Using 6 and 10 leucocyte washings no such relation could be demonstrated as almost all patients had demonstrable TCMH regardless of the clinical stage. As an overall result it appears that the frequency of detectable TCMH in the LMCT is significantly increased after extensive leucocyte washings *in vitro*. This effect is most marked in patients with disseminated disease. The effect of leucocyte washing appears to be confined to reactivity towards allogeneic hypernephroma tissue extracts in patients with renal carcinoma.

INDIVIDUAL TITRATIONS

In Fig. 5 are shown representative examples of individual titration curves from 4 of the renal carcinoma patients at 3, 6 and 10 leucocyte washings. Very different patterns of leucocyte reactivity are evident, related to the clinical stage of the patients. The use of the minimal concentration of tumour extracts giving significant leucocyte migration inhibition as the basis for grading of TCMH (grade 0–4) has the advantage of discriminating those patients with a high degree of TCMH (grade 3–4) from those with a low degree (grade 1–2). The present material did not allow analysis of

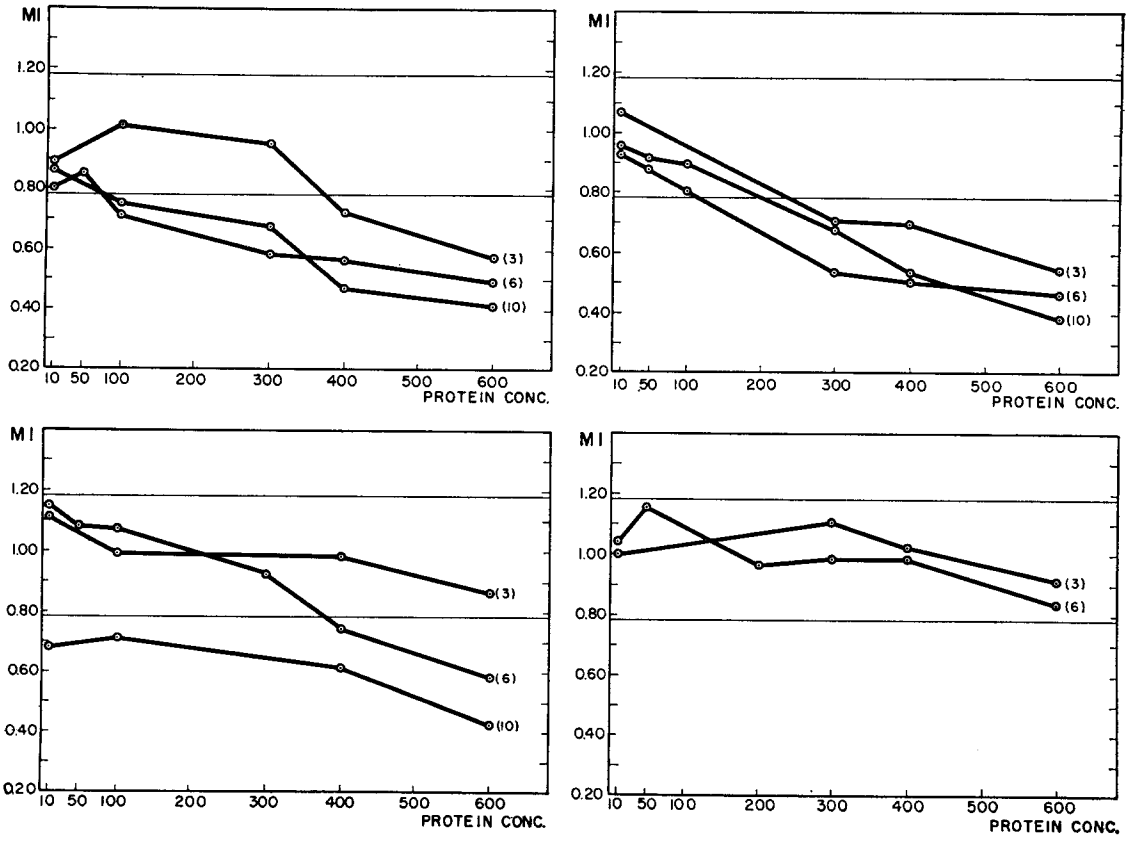


Fig. 5. Titration curves from four patients with renal carcinoma (no. 14 top left, no. 7 top right, no. 13 bottom left, and no. 6 bottom right, Table 1) showing the reactivity towards allogeneic hypernephroma at 3, 6 and 10 leucocyte washings. Normal range, MI = 0·79–1·19.

Table 3. *Relation between leucocyte washing* in vitro *and clinical stage in* 16 *patients with renal carcinoma*

	3 leucocyte washings TCMH grade 0	1–4	
Patients without distant metastases	1	4	
Patients with distant metastases	11	0	$P < 0.05$
	6 leucocyte washings TCMH grade 0	**1–4**	
Patients without distant metastases	0	5	
Patients with distant metastases	2	9	$P > 0.05$
	10 leucocyte washings TCMH grade 0	**1–4**	
Patients without distant metastases	0	4	
Patients with distant metastases	0	5	$P > 0.05$

the relation between TCMH-grading and prognosis. The use of this grading at different numbers of leucocyte washings for monitoring TCMH during the clinical course of the patients has been discussed in a preliminary report [14]. The present material is part of a prospective study of TCMH-grading at different numbers of leucocyte washings as indicator of clinical stage and prognosis in patients with renal carcinoma.

NATURE OF THE EFFECTOR CELLS

In order to elucidate if the washing effect on migration inhibition might be due to selective loss of cell populations during washing a few experiments on the composition of the migrating cell population were performed. One representative example is shown in Table 4. T and B cells were evaluated as earlier described in detail [22]. A loss of leucocytes of approximately 25 % is evident after 6 washings compared to whole blood, but no significant changes in the proportions of T and B cells were found.

MIGRATION INHIBITION WITH MONONUCLEAR CELLS

In a few experiments (patients no. 3, 5, and 10 Table 1) washings of leucocytes were per-

Table 4. *The effect of cell washing on the composition of the migrating cell population*

	Leucocytes/ ml*	Neutrophils (%)	Lymphocytes (%)	Lymphocyte conc./ml*	T cells (%)	B cells (%)
Whole blood	$5{\cdot}6 \times 10^6$	69	28	$1{\cdot}6 \times 10^6$	63	18
Pooled plasma	$5{\cdot}7 \times 10^6$	68	29	$1{\cdot}7 \times 10^6$	55	18
After 3 cell washings	$5{\cdot}1 \times 10^6$	70	27	$1{\cdot}4 \times 10^6$	n.i.	n.i.
After 6 cell washings	$4{\cdot}0 \times 10^6$	73	22	$0{\cdot}9 \times 10^6$	68	15

*Cells resuspended in constant volume of medium.

Table 5. In vitro *migration of leucocytes after* 3, 6, *and* 10 *washings and* in vitro *migration of Ficoll-separated mononuclear cells using extract of allogeneic hypernephroma in a patient* (*no.* 5 *Table* 1) *with extensive local and distant metastases*

	MI Protein concentration, μg per ml				
	10	100	300	400	600
3 leucocyte washings	0·94	0·98	1·12	1·08	0·87
6 leucocyte washings	1·05	1·01	0·96	0·89	n.i.
10 leucocyte washings	0·91	0·79	0·74	0·70	n.i.
Mononuclear cells	0·79	0·63	0·58	0·55	0·44

formed 3, 6 and 10 times as described above. Besides in parallel series using the same tissue extracts at the same concentrations, migration tests were performed with Ficoll-separated mononuclear cells [22]. The preparations contained 92–95% lymphocytes. At the concentrations tested (10–600 μg/ml) no migration inhibitory effect was seen on mononuclear cells from blood donors except at 600 μg/ml in a few cases. In 5/5 experiments (1 of which is shown in Table 5) employing leucocytes and mononuclear cells from renal carcinoma patients a clear inhibitory effect was seen with leucocytes after 6 or 10 washings. In all cases lymphocytes responded to allogeneic hypernephroma tissue extract at all concentrations used except 10 μg/ml.

DISCUSSION

The main finding of the present investigation is that in patients with renal carcinoma the proportion showing TCMH by the LMT is significantly increased after extensive washing of the leucocytes employed in the test. This is consistent with the concept that coating of the migrating cells by a factor specifically inhibits their reactivity to tumour extracts. The reversible non-reactivity was significantly related to the tumour burden of the patients, and thus seems to be a significant cause of immunological non-reactivity in renal carcinoma patients with distant metastases.

It is not possible from the present study to draw any conclusions concerning the nature of the factor removed, or the cell type susceptible to the washing effect. The concept of removing a factor from the cells was supported, in a few experiments, by addition of the supernatants after cell washing to the test system thereby restoring non-reactivity. These findings are consistent with the hypothesis of antigenic inhibition, i.e., that the factor removed is tumour antigen [7, 9]. Moreover, it has earlier been shown that a comparable effect can be obtained by the addition of serum from patients with renal carcinoma [17]. In assays for cell-mediated hypersensitivity in cancer patients where purified lymphocyte preparations have been used, such as cytotoxicity tests, correlation between reactivity and the clinical stage or course of the patients has not been a general finding [23]. However, stage-relation of TCMH has been reported by several authors in LMT-studies with peripheral leucocytes as migrating cells [24–27]. Recently Lurie *et al.* have shown that the results of a direct migration inhibition assay employing purified lymphocyte preparations did not correlate to clinical stage or course in colonic cancer patients as all patients reacted [28].

In the present study cell populations consisting of both lymphocytes and granulocytes were equally as able to react after extensive washing *in vitro* as cell populations consisting exclusively of purified lymphocytes. It does not seem then that the cell composition was of significant importance, but that removing of a factor either by cell washing or by Ficoll-separation [28] was essential.

REFERENCES

1. H. O. Sjögren, I. Hellström, S. C. Bansal and K. E. Hellström, Suggestive evidence that the "blocking" antibodies of tumor-bearing individuals may be antigen–antibody complexes. *Proc. nat. Acad. Sci. U.S.A.* **68,** 1372 (1971).
2. I. Hellström, K. E. Hellström, C. A. Evans, G. H. Heppner, G. E. Pierce and P. S. Yang, Serum-mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor-specific antigens. *Proc. nat. Acad. Sci. (Wash.)* **62,** 362 (1969).
3. I. Hellström, H. O. Sjögren, G. Warner and K. E. Hellström, Blocking of cell-mediated tumor immunity by sera from patients with growing neoplasms. *Int. J. Cancer* **7,** 226 (1971).
4. F. J. Cummings, G. H. Heppner, L. Stolbach and P. Calabresi, Demonstration of cell-mediated and blocking immune responses to tumor antigens in cancer patients with the colony-inhibition-in-gel test. *Isr. J. Med. Sci.* **9,** 308 (1973).
5. A. M. Cohen, A. S. Ketcham and D. L. Morton, Specific inhibition of sarcoma-specific cellular immunity by sera from patients with growing sarcomas. *Int. J. Cancer* **11,** 273 (1973).
6. G. A. Currie and C. Basham, Serum mediated inhibition of immunological reactions of the patient to his own tumour: A possible role for circulating antigen. *Brit. J. Cancer* **26,** 427 (1972).

7. G. CURRIE, The role of circulating antigen as an inhibitor of tumour immunity in man. *Brit. J. Cancer* **28,** Suppl. 1, 153 (1973).
8. G. A. CURRIE, Effect of active immunization with irradiated tumour cells on specific serum inhibitors of cell-mediated immunity in patients with disseminated cancer. *Brit. J. Cancer* **28,** 25 (1973).
9. P. ALEXANDER, Escape from immune destruction by the host through shedding of surface antigens: Is this a characteristic shared by malignant and embryonic cells? *Cancer Res.* **34,** 2077 (1974).
10. R. W. BALDWIN, M. J. EMBLETON and M. R. PRICE, Inhibition of lymphocyte cytotoxicity for human colon carcinoma by treatment with solubilized tumour membrane fractions. *Int. J. Cancer* **12,** 84 (1973).
11. M. J. EMBLETON, Significance of tumour associated antigens on human colonic carcinomata. *Brit. J. Cancer* **28,** Suppl. 1, 142 (1973).
12. M. KJAER, *In vitro* demonstration of cellular hypersensitivity to tumour antigens by means of the leucocyte migration technique in patients with renal carcinoma. *Europ. J. Cancer* **10,** 523–531 (1974).
13. M. KJAER and G. BENDIXEN, Tumour-directed, cellular hypersensitivity detected by means of the leucocyte migration technique in patients with renal carcinoma. *Ann. N.Y. Acad. Sci.* 1976 (in press).
14. M. KJAER and G. BENDIXEN, Clinical detection and monitoring of tumour-specific cell-mediated hypersensitivity (TCMH) in man: Examinations of hypernephroma with leucocyte migration technique. In In vitro *Methods in Cell-Mediated Immunity.* (Edited by B. BLOOM and J. R. DAVID) 1976 (in press).
15. M. KJAER, The dose-related effect of tumour extract on the *in vitro* migration of leucocytes from patients with renal carcinoma. *Europ. J. Cancer* **11,** 281–289 (1975).
16. M. KJAER and N. CHRISTENSEN, Ability of renal carcinoma tissue extracts to induce leucocyte migration inhibition in patients with non-metastatic renal carcinoma: Correlation with clinical and histopathological findings. *Cancer Immunol. Immunother.* 1976 (In press).
17. M. KJAER, The capillary tube leucocyte migration technique (LMT) as a method for detection of serum inhibiting activity of tumour-directed, cellular hypersensitivity in patients with renal carcinomas. *Acta. path. microbiol. scand.* Sect. B., **82,** 294–296 (1974).
18. M. KJAER, Prognostic value of tumour-directed, cellular hypersensitivity detected by means of the leucocyte migration technique in patients with renal carcinoma. *Europ. J. Cancer* 1976 (In press).
19. G. BENDIXEN and M. SØBORG, A leucocyte migration technique for *in vitro* detection of cellular (delayed type) hypersensitivity in man. *Dan. med. Bull.* **16,** 1–6 (1969).
20. R. N. MAINI, L. M. ROFFE, I. T. MAGRATH, D. C. DUMONDE, Standardization of the leucocyte migration test. *Int. Arch. Allergy.* **45,** 308–321 (1973).
21. J. H. EDWARDS, A note on the interpretation of $n \times 2$ tables. *Brit. J. prev. soc. Med.* **12,** 141 (1958).
22. M. KJAER and M. THOMSEN, General immunocompetence and tumour-directed, cell-mediated hypersensitivity *in vitro* in patients with renal carcinoma. *Acta. path. microbiol. scand. Sect.* C., 1976 (In press).
23. R. B. HERBERMAN, Cell-mediated immunity to tumor cells. *Adv. Cancer Res.* **19,** 207 (1974).
24. A. J. COCHRAN, R. M. MACHIE, C. E. THOMAS, R. M. GRANT, D. E. CAMERON-MOWAT and W. G. S. SPILG, Cellular immunity to breast carcinoma and malignant melanoma. *Brit. J. Cancer* **28,** Suppl. 1, 77 (1973).
25. M. M. BLACK, H. P. LEIS, B. SHORE and R. E. ZACHRAU, Cellular hypersensitivity breast cancer. Assessment by a leucocyte migration procedure. *Cancer* **33,** 952 (1974).
26. E. G. ELIAS and L. L. ELIAS, Autogenous leucocyte migration in human malignancies. *Cancer* **36,** 1393 (1975).
27. C. E. ROSS, A. J. COCHRAN, D. E. HOYLE, R. M. GRANT and R. M. MACKIE, Formalinized tumour cells in the leucocyte migration inhibition test. *Clin. exp. Immunol.* **22,** 126 (1975).
28. B. B. LURIE, D. M. BULL, N. ZAMCHECK, A. M. STEWARD and R. A. HELMS, Diagnosis and prognosis in colon cancer based on a profile of immune reactivity *J. Natl. Cancer Inst.* **54,** 319 (1975).

Europ. J. Cancer Vol. 12, pp. 793–796. Pergamon Press 1976. Printed in Great Britain

Reproductive Survival *In Vitro* of Recently Explanted Human Tumour Cells Following Exposure to Vinblastine. A Suggestion for Improved Clinical Dose Schedules‡

J. WELLS*, R. J. BERRY† and A. H. LAING
Churchill Hospital Research Institute, Oxford, U.K.

Abstract—*Colony forming-ability* in vitro *was studied in cells of* 6 *recently-explanted human tumours after exposure to various concentrations of vinblastine sulphate (Velbe) in the growth medium for* 45 *hr. The maximum drug concentration used* (1 *MBC*) *was equivalent to that likely to be achieved by distributing the dose usually used in clinical practice* (15 *mg*) *in* 50 *l of body fluid* (1 *MBC* = 0·3 *μg/ml*).

For all 6 *cell lines,* maximum *cell-killing was achieved at dose levels* circa 100*-fold* lower *than those used in clinical practice, and tumour cell survival* increased *at higher dose levels. As the limiting toxic effects of the* Vinca *alkaloids in man appear to be related mainly to the maximum drug concentration achieved, these results suggest that the therapeutic ratio may be increased by prolonged infusion of these drugs at low concentration rather than by single intravenous injection as is now used.*

INTRODUCTION

THE ABILITY to measure, by their colony-forming ability, survival of the reproductive capacity of mammalian cells after exposure to the cytotoxic drugs used in cancer chemotherapy either *in vitro* (e.g., [1]) or *in vivo* (e.g., [2]) is now over a decade old. However, only very few studies have been reported in which this same biological end-point has been used for cells of *human* tumours which have been recently explanted. The experiments reported in this paper were undertaken as part of a larger study of the chemosensitivity of freshly-explanted and serially cultured human tumours [3, 4] in which reproductive survival *in vitro* was compared with other, technically simpler biological end-points which it was hoped might prove more amenable for use as routine screening tests for the selection of appropriate anti-cancer chemotherapy. The shape of the dose-survival curves obtained after exposure of cells of 6 recently explanted human tumours to vinblastine, however, suggested possible improvements in the dose–time schedules by which this agent is administered in clinical practice.

Accepted 8 March 1976.

*Present address: C.E.G.B., Berkeley Nuclear Laboratories, Berkeley, Glos.
†Present address: M.R.C., Radiobiology Unit, Harwell, Didcot, Oxon.

‡During the period in which these studies were carried out, J. W. was the recipient of a grant-in-aid from Messrs. Eli Lilly and Co., Ltd., and R. J. B. was a member of the Medical Research Council External Scientific Staff at the Churchill Hospital, Oxford. A.H.L. was aided by a grant from the United Oxford Hospitals.

MATERIAL AND METHODS

(a) *Culture of explanted cells*

Tumour cells were obtained from freshly-excised biopsies of solid human tumours and from ascitic and pleural fluids freshly-taken. The methods of establishing primary cultures of these cells *in vitro*, albeit with only infrequent success, have been described previously [3]. The sites of origin and presentation of the 6 tumours which are the subject of this report are detailed in Table 1.

(b) *Survival of cell reproductive capacity following exposure to vinblastine*

A suspension of tumour cells was obtained from either a primary culture or from its subsequent subcultures by gentle trypsinization (0·1 % w/v, Difco 1:250). The cells were centrifuged at 30–40 ***g*** for 5 min and resuspended in a complete growth medium which consisted of medium 199 (BDH) supplemented with 15 % human type AB serum, L-glutamine, sodium bicarbonate and antibiotics. The cells were counted electronically using a Coulter Model D cell counter with a 140 μm orifice; this counter had been calibrated previously against haemacytometer counting of cells of comparable size. Appropriate dilutions were made and 10^5 cells contained in 1 ml of culture medium were inoculated into each of eight 50 mm Nunclon TC plastic Petri dishes each containing 3 ml of culture medium. These were gassed with 5 % CO_2 in air, sealed in brass gas-tight boxes and incubated for 5 hr at (36 ± 2)°C. Any remaining cells that had been obtained from the original culture were centrifuged and resuspended in 4 ml of culture medium and irradiated with 250 kVp X-rays to a total dose of 7·2 krad so that they could be used as reproductively non-viable "feeder" cells.* As this dose was delivered in 20 min, cells could be removed from the Petri dish without the need of trypsin by gently pipetting the culture medium to and from the Petri dish. Aliquots of culture medium containing $5{\cdot}10^4$ of these irradiated cells were added to each of 8 Petri dishes so that the final volume of culture medium in each Petri dish was 3 ml. The Petri dishes were then placed in a gas-tight box which was gassed with 5 % CO_2 in air and incubated at (36 ± 2)°C.

After the 5-hr period, 7 of the 8 Petri dishes containing unirradiated cells had varying concentrations of vinblastine sulphate added to them. The vinblastine sulphate (15 mg) was initially dissolved in 10 ml isotonic saline and then serially diluted in culture medium. The highest concentration used was chosen to approximate to the maximum concentration likely to be found in the body fluids of a patient undergoing "standard" anti-cancer chemotherapy. This approximate "maximum body concentration" (MBC) was defined for vinblastine as 0·3 μg/ml (15 mg in 50 l). The Petri dishes containing the cells were then replaced in the gas-tight box, gassed with 5 % CO_2 in air, sealed, and incubated for either 20 or 45 hr at (36 ± 2)°C, after which the contents of each Petri dish were poured into a corresponding "Universal" bottle. The cells remaining in the Petri dishes were then washed with culture medium which was then poured into the corresponding "Universal" bottle. The cells that remained attached to the Petri dishes were removed with 0·1 % (w/v) trypsin solution and gently pipetted until a suspension of single cells was obtained. This cell suspension was poured into the corresponding "Universal" bottle and further culture medium added after having first been used to wash out the appropriate Petri dish. The resulting cell suspensions were then centrifuged at 30–40 ***g*** for 5 min. The supernatant was poured away and the cells in each bottle resuspended in 10 ml of fresh culture medium, a 1 ml aliquot of which was further diluted by a factor of 10. One millilitre of the diluted cell suspension was then pipetted into a corresponding Petri dish containing the irradiated "feeder" cells. This dilution procedure was sufficient to prevent a significant "carry-over" of vinblastine sulphate into the 14/18 day cultures (i.e., the concentration of vinblastine sulphate in final culture < 0·0001 MBC) although the point corresponding to 0·1 MBC for the Σ 19 cells may indicate some effect in that one case. The Petri dishes were then placed in a gas-tight box, gassed with 5 % CO_2 in air and incubated at (36 ± 2)°C for 14 to 18 days depending on cell growth rate.

Table 1. *Explants of human tumours for which survival curves were obtained* in vitro

Code	Tumour	Presentation
B	Carcinoma of bronchus	Solid tumour biopsy (lung)
Σ	Carcinoma of bronchus	Solid tumour biopsy (lung)
L	Carcinoma of breast	Pleural fluid
S	Carcinoma of stomach	Solid tumour biopsy
K	Melanoma of skin	Solid tumour biopsy
Q	Seminoma (testis)	Pleural fluid

After the incubation period, the medium was carefully poured from the Petri dishes and the colonies of cells stained for 3–5 min with a saturated solution of Nile Blue in isotonic saline. The Petri dishes were then washed with

*"Feeder" cells were considered important not only in increasing the plating efficiency of the test cell population but also in helping to ensure that the survival of the drug treated test cell population was independent of the number of "dead" cells produced in the test cell population.

isotonic saline and the colonies counted while the dishes were still wet, so that any overlapping colonies could be identified readily from their three-dimensional characteristics with the aid of a dissecting microscope. The smallest colonies counted as survivors contained more than 50 cells, except in the case of the seminoma (Q) cells which were exceedingly slow-growing and for which a minimum colony size of 20 cells was accepted after an incubation period of 18 days.

RESULTS

The dose–survival curves for 45-hr exposure to vinblastine of the six human tumour cell lines are shown in Fig. 1. For all except the seminoma (Q) cells, duplicate experiments were carried out at different subcultures of the

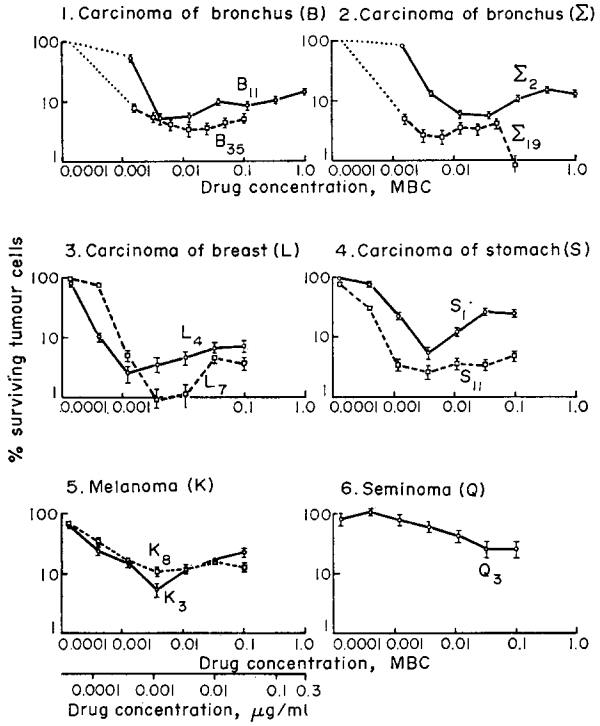


Fig. 1. Reproductive survival of explanted human tumour cells following 45-hr exposure to various concentrations of vinblastine sulphate. The individual tumour cell lines are detailed in Table 1, and the number following the symbol indicates the subculture in vitro from which the tumour cells were taken for testing.

tumour. One experiment was performed with the earliest subculture of the tumour *in vitro* which yielded good plating efficiency (i.e., $\gtrsim 30\%$ except in the case of the "Q" cells) and the other after further serial subculture. Even with the differences among the cell lines and even within one cell line between different serial subcultures, it is clear that the maximum reduction in tumour cell reproductive capacity is achieved with between 1/10 and 1/1000 of the drug concentration which is currently in clinical use in cancer chemotherapy. Over a wide range of drug concentrations, the level of tumour cell survival is little altered and, if anything, there is a strong tendency for the survival level to *increase* as the concentration of vinblastine is increased towards the levels in current clinical use. Some dose–survival curves corresponding to 20-hr exposure to vinblastine are shown in Fig. 2 and indicate vinblastine

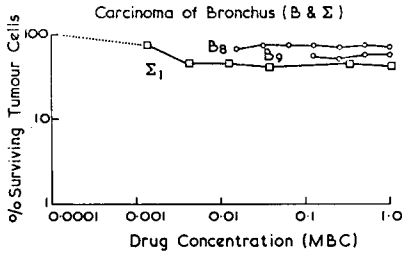


Fig. 2. Reproductive survival of explanted human tumour cells following 20-hr exposure to various concentrations of vinblastine sulphate.

to be significantly less effective for this shorter period of exposure.

DISCUSSION

Vinblastine is classed as a cycle-dependent chemotherapeutic agent because its major cytotoxic action is exerted only upon cells attempting to undergo mitosis in its presence (e.g., [2]). If this was its only action, the dose–survival curves produced by exposure to vinblastine would have a steep initial region followed by a completely flat plateau where survival was unaffected by increasing doses of the drug. The survival level at which the plateau was reached then represents the proportion of cells which failed to attempt mitosis during the limited period for which the drug was present. Studying haemopoietic colony-forming-units (CFU) in the femora of mice exposed for 48 hr to vinblastine and vincristine, Bruce, *et al.* described just such survival curves [2]. When examined in detail, however, their own data show a tendency for CFU survival to *increase* at the highest drug doses used, exactly as has been seen in our present data for survival of human tumour cells. This increase in survival can be explained in terms of the dual mode of action of vinblastine at high doses. It is known that vinblastine can inhibit the production of DNA (and RNA to a lesser extent) as well as

prevent mitosis [5]. Therefore, at higher doses, it is probable that cells spend an extended period in S-phase in order to produce the quantity of DNA necessary for cell division to take place, thus decreasing the number of cells at risk of the lethal action of this mitotic poison during a limited period of incubation. Vinblastine sulphate at high doses may therefore be regarded as self-protecting in terms of its principal mode of action.

In the experience of this laboratory only one other cancer chemotherapeutic agent, vincristine sulphate, has maintained its maximal effect *in vitro* at concentrations significantly less than its defined MBC. However, the end-point used in this case was not true cell reproductive survival but increase in cell number after 7 days incubation; this procedure has been described previously [3]. Because the clinical toxicity of vincristine appears to be related to the maximum concentration employed, these early findings gave impetus towards initiating a clinical trial in which the "standard" dose has been infused into patients over a period of 24 hr rather than being given as a single shot [6]. This has resulted in a remarkable absence of neuro-toxicity and appears so far to be clinically effective. This method of administering vincristine has been used in a clinical trial of treatment of carcinoma of the ovary, which has been underway for over a year.

As the mitotic effects of vinblastine sulphate are more pronounced at concentrations orders of magnitude less than the defined MBC and a they also appear to be somewhat more reversible *in vitro* than those of vincristine sulphate [5] it is probable that vinblastine sulphate may also be more efficiently employed clinically as an infusion. Clearly there will be an optimum infusion *time* and *concentration* if one is to maximize the principal anti-tumoural property of this agent. Only a carefully planned trial in which particular attention is paid to the nature of normal tissue toxicity will allow an optimum treatment regime to be established.

Acknowledgement—We thank Miss J. Paxton for her technical assistance.

REFERENCES

1. R. J. Berry, A comparison of effects of some chemotherapeutic agents and those of X-rays on the reproductive capacity of mammalian cells. *Nature (Lond.)* **203,** 1150 (1964).
2. W. R. Bruce, B. E. Meeker, W. E. Powers and F. A. Valeriote, Comparison of the dose- and time-survival curves for normal haematopoietic and lymphoma colony-forming cells exposed to vinblastine, vincristine, arabinosylcytosine and amethopterin. *J. nat. Cancer Inst.* **42,** 1015 (1969).
3. R. J. Berry, A. H. Laing and J. Wells, Fresh explant cultures of human tumours *in vitro* and the assessment of sensitivity to cytotoxic chemotherapy. *Brit. J. Cancer* **31,** 218 (1975).
4. J. Wells, R. J. Berry and A. H. Laing, The chemosensitivity of freshly explanted tumour cells of various origins as determined by clonal assay and six-day growth *in vitro*, and variation in chemosensitivity with subsequent subculturing. In *Short-Term Culture of Human Tumours—Techniques and Clinical Applications* (Edited by P. P. Dendy), Academic Press, London (in press).
5. A. C. Sartorelli and W. A. Creasey, Cancer chemotherapy. *Ann. Rev. Pharmacol.* **9,** 51 (1969).
6. A. H. Laing, personal communication.

Europ. J. Cancer Vol. 12, pp. 797–805. Pergamon Press 1976. Printed in Great Britain

Antimetastasis Effect of Two Derivatives of Epipodophyllotoxin in Mice

A. CATTAN, C. POURNY, Y. CARPENTIER, E. CATTAN and M. BAROUH

Institut Jean-Godinot 45, *rue Cognacq Jay BP* 171 51056 *REIMS Cedex*

Abstract—*The effectiveness of two derivatives of epipodophyllotoxin, VM* 26 *and VP* 16213, *on* 3 *LL was tested. Both remarkably reduced the number and volume of pulmonary metastases as well as the number of animals bearing metastases, but not always the volume of the primary tumor. The mechanism of this effect is not elucidated yet.*

INTRODUCTION

TWO DERIVATIVES of epipodophyllotoxin, 4-demethyl-β-epipodophyllotoxin-D thenylidene glucoside (VM 26) and 4-demethyl-β-epipodophyllotoxin-D-ethylidene glucoside (VP 16213) are active in various experimental tumors in the mouse such as Erhlich's tumor, Walker's carcinosarcoma, leukemias P 1534 and L1210 [1, 2], but are reputed to have little or no effect in Lewis's lung carcinoma (LLC or 3 LL). In fact, a definite effectiveness of these two drugs on LLC has been detected by modifying the modalities of administration. This effectiveness seems to be even greater on metastatic spread than on the primary tumor.

MATERIAL AND METHODS

The tumor used was LLC (Lewis's lung carcinoma) maintained on C 57 Bl/6 and transplanted on to BDF_1 (C 57 Bl/6 × DBA/2) for the experiment. The tumors were dissected and their cells isolated by mechanical means. 10^6 cells were inoculated into BDF_1 by the intramuscular route into the thigh. Under these conditions, the tumor became palpable around the 6th day, it weighed 2,740 mg ± 200 on the 10th day. Mean survival of the mice was 30 days ± 3. On autopsy, 100% of the mice had pulmonary metastases. Ablation of the tumor by amputation of the thigh at a regular interval, showed that in the animals which had been operated and sacrificed on the 23rd day pulmonary metastases were observed in 10% of the mice operated on the 4th day, and 90% of the mice which had been operated on the 8th day.

Accepted 7 May 1976.

The two products were injected once per day by the intraperitoneal route. The control mice receiving physiological serum by the same route for the same duration of time.

In a first series of experiments, the mice received chemotherapy alone and for 5 days: VM 26 4 mg/kg/day and VP 16213 8 mg/kg/day i.e., about LD 10, 6 days for each product [1–3]. This administration was carried out early from the first to 5th day after inoculation or late on the 10th to 14th day after inoculation. The mice were than separated into two groups: in the first, they were sacrificed on the 23rd day (mean survival less 2 s.d.), the tumors were dissected and weighed, the number and weight of pulmonary metastases were noted, as well as the number of mice with metastases. The second group were followed up until spontaneous death of the mice. After each death a complete autopsy was carried out and the same criteria were noted as previously.

In a second series of experiments, chemotherapy with these two products was associated with amputation of the thigh in such a way as to eradicate the primary tumor. This combination was effected under 3 different conditions: (*a*) chemotherapy from the first to the 5th day followed by amputation on the 8th day; (*b*) chemotherapy on the 8th and 9th days followed by amputation on the 10th day; (*c*) amputation on the 10th day followed by chemotherapy on the 11th and 12th days. As above the mice were separated into two groups, in the first, they were sacrificed and an autopsy was carried out. In the second group, they were followed up until spontaneous death.

In all of the cases, chemotherapy was carried out at the same daily doses: VM 26 4 mg/kg/day, VP 16213 8 mg/kg/day.

In a last series of experiments the effectiveness of VM 26 and VP 16213 was compared to that of their solvents. The system was exactly the same except that a group of solvent treated mice was added each time.*

*The solvent for VM 26 was a mixture of benzyl alcohol, NN'dimethylacetamide, polyoxyethylen-castor oil, maleic acid, ethanol .The solvent for VP 16213 was a mixture of citric acid, benzyl alcohol, polysorbate 80, polyethylen glycol 300 and ethanol.

RESULTS

1. *Chemotherapy alone, early treatment*

Tables 1 and 2 show that VM 26 was very significantly effective on the tumor, on the number and weight of metastases but did not improve survival. VP 16213 did not modify the volume of the primary tumor, but reduced the number and weight of metastases in a very

Table 1. *Effect of Treatment with VM* 26 *and VP* 16 213 *of mice suffering from LLC on the* 1*st to the* 5*th day after inoculation. The mice were sacrificed on day* 23

Group	Weight of the tumors* (mg)	Autopsy following sacrifice (D 23) Pulmonary metastases per mouse Number*†	Weight*† (mg)	SM / S	Mice with metastases Number*‡	Weight*‡
Control	11,195 ± 571	8·40 ± 2·07	48·96 ± 35·29	10/10	8·40 ± 2·07	48·96 ± 35·29
VM 26	8993** ± 513	0·50** ± 0·54	0·26‖ 0·28	3††/10	1·66§	0·87§
VP 16 213	10,434 ± 764	2·30** ± 1·43	4·02‖ 3·74	6/10	3·83‖ ± 1·20	6·70 ± 5·27

*: mean ± 2 SE
†: all of the mice are included
‡: only those mice with at least one pulmonary metastasis are included
§: mean of 3 values: no statistical study possible
t (Student): ¶$P < 0{\cdot}05$
‖ $P < 0{\cdot}01$
** $P < 0{\cdot}001$
††: χ^2 test $P < 0{\cdot}001$
SM: number of mice with pulmonary metastases on autopsy
S: total number of mice
0: not significant
D: day

Table 2. *Effect of the treatment with VM* 26 *and VP* 16 213 *of mice suffering from LLC on days* 1 *to* 5. *The autopsies were carried out after spontaneous death of the mice*

Group	Survival	Autopsy on spontaneous death Weight of the tumours* (mg)	Pulmonary metastases per mouse Number*	Weight* (mg)	SM / M
Control	32·40 ± 2·19	17,460 ± 1204	18·10 ± 2·00	483·59 ± 145·98	10/10
VM 26	35·70 ± 2·70	± 19,272 ± 2135	5·10‖ ± 2·29	36·09†† ± 33·52	9/10
VP 16 213	38·10‖ 2·22	18,736 ± 1489	14·40 3·07	190·76†† ± 68·37	10/10

For symbols and statistics see Table 1.

Table 3. *Effect of treatment with VM* 26 *and VP* 16 213 *of mice suffering from LLC,* 10 *days after inoculation. The mice were sacrificed on day* 23

Group	Weight of the tumor (mg)*	Pulmonary metastases on D 23 number*	Weight* (mg)	SM/M
Control	12,707 ± 700	16·80 ± 3·30	121·48 ± 50·36	10/10
VM 26	7395 ± 1181††	0·70 ± 0·60††	0·36††	5/10
VP 16 213	11,639 ± 737	3·00 ± 1·04	4·12 ± 2·26††	9/10

For symbols and statistics see Table 1.

Table 4. *Effect of late treatment with VM* 26 *and VP* 16 213 *of mice suffering from LLC,* 10 *days after inoculation. The mice are followed up until spontaneous death*

Group	Survival time (*d*)	Weight of the tumors (mg)*	Pulmonary metastases Number*	Weight* (mg)	SM/S
Control	29·40 ± 2·46	13,587 ± 1639	29·60 ± 6·22	480·11 ± 189·62	10/10
VM 26	29·70 ± 3·98	8/10 mouse tumors were necrotic	± 1·20 ± 0·78**	± 3·45 ± 3·16**	7/10
VP 16 213	31·30 ± 0·94	15,867 ± 914¶	8·60 ± 2·14**	60·31 ± 30·00**	10/10

For symbols and statistics see Table 1.

Table 5. *Effect of the combination of chemotherapy with VM* 26 *or VP* 16 213, *on days* 1 *to* 5, *followed by amputation on day* 8, *in mice suffering from LLC. The mice were sacrificed on day* 28

Group	Weight of the tumors on amputation on D 8 (mg)	Pulmonary metastases on sacrifice (D 28) Number*	Weight (mg)*
Control	821 ± 85	6·30 ± 3·36	87·89 ± 45·04
VM 26	0††	0††	0††
VP 16 213	30** ± 12	0††	0††

For symbols and statistics see Table 1.

significant manner at the 23rd day (but only their weight at spontaneous death) and prolonged survival of the treated mice.

2. *Chemotherapy alone, late treatment*

Tables 3 and 4 show that VM 26 decreased the weight of the primary tumor, the number and volume of metastases in a very significant manner. Certain mice had no pulmonary metastases.

VP 16213 hardly modified the weight of the tumor on the 23rd day and even less on spontaneous death, but diminished the number and weight of pulmonary metastases in a significant manner.

The two products did not significantly modify survival.

3. *Combination of chemotherapy with surgery*

(*a*) Early chemotherapy on the first to 5th day after inoculation followed by amputation on the 8th day led to apparent recovery of the treated mice. None of the mice sacrificed on the 28th day had pulmonary metastases (Table 5). Survival of the non-sacrificed mice was extremely prolonged (Fig. 1).

Three out of 10 mice treated with VM 26 died from late toxicity, all of the others recovered; 8 out of the 10 mice treated with VP 16213 recovered.

(*b*) Late treatment 2 days before or after amputation carried out on the 10th day did not significantly decrease the number of relapses. On the other hand, the combination of chemotherapy by VM 26 followed or immediately

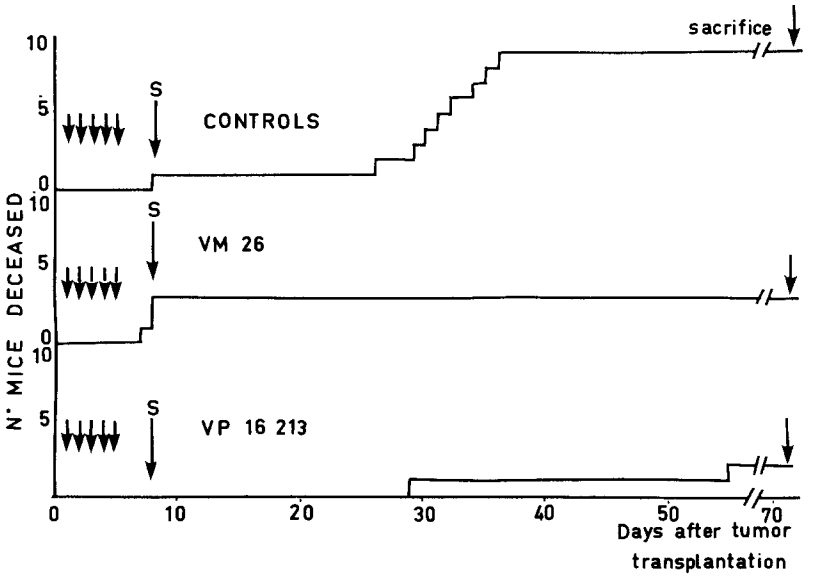


Fig. 1.

Table 6. *Effect of the combination of chemotherapy with VM* 26 *or VP* 16 213 (*D* 8 *and* 9), *followed by surgery* (*amputation*) *in mice suffering from LLC. Sacrificed on the* 23*rd day after inoculation*

Group	Weight of the tumors on amputation on D 10 (mg)*	SSR / S	SM / SSR	Sacrifice D 23 pulmonary metastases Number*	Weight (mg)*
Control	2633 ± 144	9/10	9/9	7·88 ± 1·92	104·23 ± 44·01
VM 26	2433 ± 234	8/9	7/8	2·12** ± 0·88	13·14‖ ± 7·30
VP 16 213	2602 ± 301	6/9	6/6	5·33 ± 2·76	48·56 ± 43·67

For symbols and statistics see Table 1.
SSR: mice with no recurrences
SM: mice with metastases
S: total number of mice

preceded by amputation of the thigh led to a significant reduction in the number and weight of pulmonary metastases. The combination of VP 16213 followed or preceded by amputation reduced the same parameters in a lesser and non-significant manner (Tables 6 and 7, Fig. 2 and 3). The overall results are summarized in Tables 8 and 9.

The comparison of the effectiveness of the drugs and their solvents showed no difference (Tables 10 and 11).

DISCUSSION

It therefore seems that the 2 derivatives of epipodophyllotoxin studied have an evident activity on LLC in the mouse *in vivo*, but does not significantly prolong survival.

Analysis of the results shows that administered early, in the exponential growth phase of the tumor, or, administrated late, in the declining phase of Gompertz' graph, VM 26 only leads to a reduction in volume

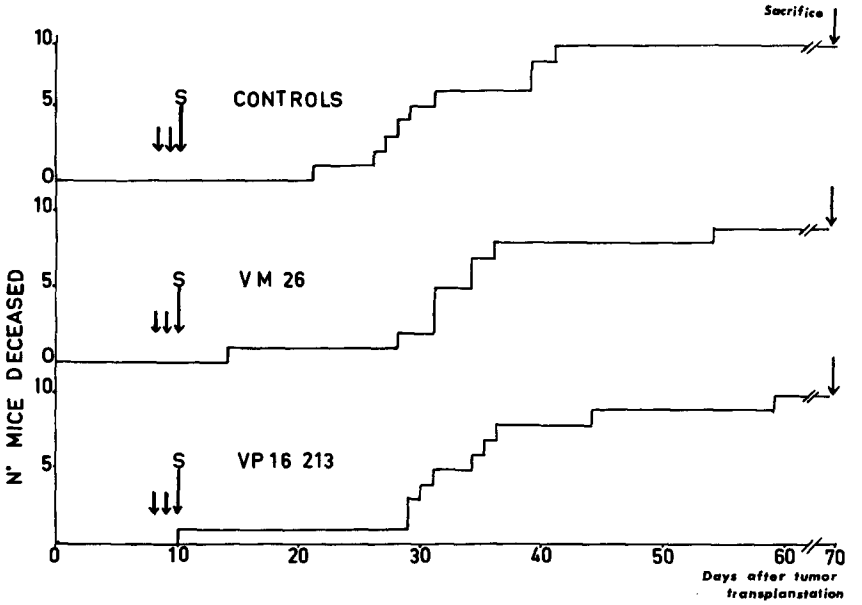


Fig. 2.

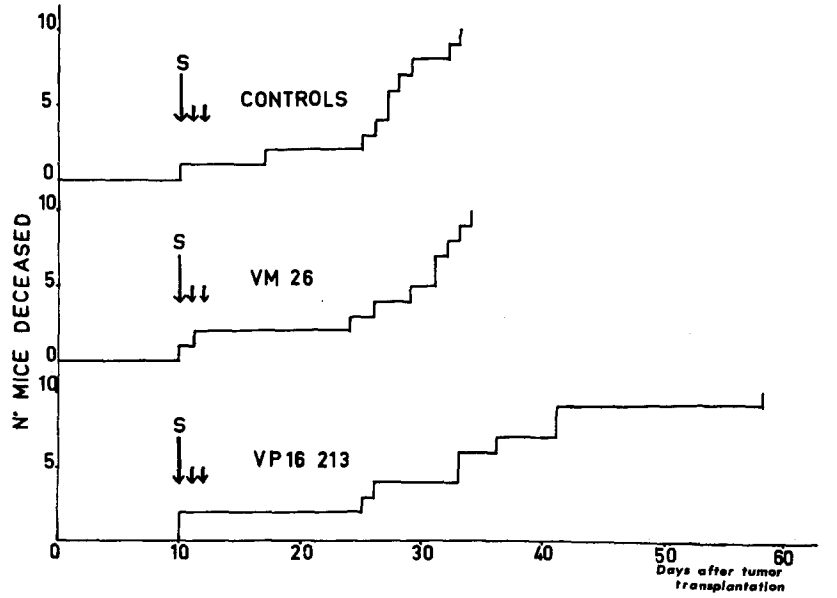


Fig. 3.

Table 7. Effect of the combination of surgery (amputation) on day 10 followed by chemotherapy with VM 26 or VP 16 213 (11 and 12) in mice suffering from LLC. Sacrificed on the 23rd day after inoculation

Group	SSR / S	Sacrifice (D 23) Pulmonary metastases Number*	Weight* (mg)	SM / SSR
Control	6/7	9·83 ± 2·38	101·76 ± 37·14	6/6
VM 26	7/7	4·43‖ ± 2·53	31·98¶ ± 32·07	7/7
VP 16 213	6/7	10·66 ± 1·76	105·76 ± 65·47	6/6

For symbols and statistics see Table 1.

Table 8. Effect of treatment with VM 26 and VP 16 213 on mice suffering from LLC. (Statistical significance of the difference between control and treated groups)

Treatment	Modality	Effect on Tumor	Number	Weight	Survival
VM 26	D 1–5	‖	**	‖	0
	D 10–14	**	**	**	0
		**	**	**	0
VP 16 213	D 1–5	0	**	‖	‖
	D 10–14	0	**	**	0
		‖	**	**	0

Every symbol represents an experiment.

Table 9. Effect of the combination of surgical exeresis of the primary tumor and VM 26 and VP 16 213 chemotherapy. (The group which had only undergone exeresis and that treated with the combination)

Treatment	Modality	Weight	Effect on Tumor Recurrence after amputation	Metastases Number	Metastases Weight	Survival
VM 26	CH. 1–5	**		**	**	‖
+	S. D 8	**		**	**	
Amputation	CH. 8–9	0	0	0	0	0
	S. D 10	0	0	**	‖	
			0			
	S. D 10		0	0	0	0
	CH. 11–12		0	‖	0	
			0			
VP 16 213	CH. 1–5	**		**	**	¶
+	S. D 8					
Amputation	CH. 8–9	0	0	0	0	0
	S. D 10	0	0	0	0	
			0			
	S. D 10		0	0	0	0
	CH. 11–12		0	0	0	
			0			

CH: Chemotherapy: day.
S: Surgery: day.
Each symbol represents an experiment.
For symbols and statistics see Table 1.

of the primary tumor, but always to almost complete disappearance of metastases, and to decrease of the percentage of animals bearing metastases; on the contrary, VP 16213, hardly modifies the volume of the primary tumor, at the doses used but remarkably reduces the number and volume of pulmonary metastases, as well as the number of animals with these metastases.

This preferential effect on metastases has been found with other drugs and especially, ICRF 159 [4–8], Triton WR 1339 [9–11], drugs which nevertheless are directly active on neoplastic cells.

Various mechanisms can be put forward:

(*a*) the drugs may modify cohesion and mobility of the tumor cells: this mechanism partially explains the effectiveness of Triton

Table 10. *Comparison of the effects of VM* 26, *its solvent and control on Lewis Lung Carcinoma in mice (summary)*

Treatment / Effect on	Day 1–5	Day 10–14	Day 1–5 + Amputation Day 8	Day 8 and 9 + Amputation Day 10	Amputation Day 10 VM 26 Day 11 and 12
Primary tumor	xxx	xxx	xxx	0	0
	ooo	ooo	ooo	0	0
	0	0	x	0	0
No. metastases	xxx	xxx	xxx	xxx	0
	ooo	ooo	ooo	oo	0
	0	xxx	0	0	0
Weight metastases	X	XX	XX	XX	
	o	o	oo	o	
	0	0	0	0	
Survival	ND	ND		ND	ND

Statistical significance (*t* of student) of the comparison.

drug-control	X	$P < 0{\cdot}05$	XX	$P \leqslant 0{\cdot}01$	XXX	$P \leqslant 0{\cdot}001$
drug-solvent	o	$P < 0{\cdot}05$	oo	$P \leqslant 0{\cdot}01$	ooo	$P \leqslant 0{\cdot}001$
solvant-control	x	$P < 0{\cdot}05$	xx	$P \leqslant 0{\cdot}01$	xxx	$P \leqslant 0{\cdot}001$
not significant	0					
not done	ND					

Table 11. *Comparison of the effect of VP* 16 213, *its solvent and control on Lewis Lung Carcinoma in mice (summary)*

Treatment / Effect on	Day 1–5	Day 10–14	Day 1–5 + Amputation: Day 8
Primary tumor	XX	XX	XXX
	ooo	oo	ooo
	0	0	0
No. metastases	XXX	XXX	XXX
	ooo	ooo	ooo
	0	0	0
Weight metastases	X	XX	XXX
	o	oo	ooo
	0	0	0
Survival (days)	X	ND	
	oo		
	0		

Statistical significance (*t* of student) of the comparison.

drug-control	X	$P < 0{\cdot}05$	XX	$P \leqslant 0{\cdot}01$	XXX	$P \leqslant 0{\cdot}001$
drug-solvant	o	$P < 0{\cdot}05$	oo	$P \leqslant 0{\cdot}01$	ooo	$P \leqslant 0{\cdot}001$
solvant-control	x	$P < 0{\cdot}05$	xx	$P \leqslant 0{\cdot}01$	xxx	$P \leqslant 0{\cdot}001$
not significant	0					
not done	ND					

WR 1339 [9, 10]. But no data enables confirmation of this hypothesis for VM 26 and VP 16213.

(*b*) they may alter tumor vessels and/or capillary permeability and therefore modify the passage of cells into the blood stream or their implantation into the lungs as ICRF 159 does [6–8]; but no histological change in the vascularization of tumors and metastases has been observed in mice treated with VM 26 or VP 16213.

(*c*) these drugs may have a particular trophism for the lung; in fact no evidence is available that it is the case.

(*d*) their preferential action on metastases may only be the consequence of a chemotherapeutic law: the smaller the volume of a tumor (and therefore the number of cells of which it is composed) the easier it is eradicated [12, 13]. With regard to VM 26, the number of 10^6 cells is too high to be eradicated such is also in the case of VP 16213.

The combination of chemotherapy with these drugs and amputation of the thigh with exeresis of the primary tumor gives variable results depending on the modalities of treatment: early chemotherapy followed by routine amputation on the 8th day leads to recovery in all of the mice treated with VM 26, and most of the mice treated with VP 16213. All the treated mice had no metastases on the 28th day. Thus, amputation improves the results. On the other hand, the addition of immediate pre- or post-operative short-term chemotherapy to amputation at a time when the metastases are formed does not modify either the frequency or volume of recurrences, or the number and volume of metastases or survival.

In summary, and in the particular case of VM 26 and VP 16213, their preferential action therefore seems to depend above all on the number of tumor cells in the various sites. Experiments are at present taking place in order to determine the part played by this mechanism and to detect other possible mechanisms.

REFERENCES

1. H. Stahelin, 4'-demethyl-epipodophyllotoxin thenylidene glucoside (VM 26), a podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6,** 303 (1970).
2. H. Stahelin, Activity of a new glycosidic lignan derivative (VP 16 213) related to podophyllotoxin in experimental tumors. *Europ. J. Cancer* **9,** 215 (1973).
3. T. L. Avery, De Wayne and R. A. Price, Delayed toxicity of 4'-Demethyl-epipodophyllotoxin 9-(4,6-0-2-thenylidene-β-D-glucopyranoside (NSC 122819; VM 26) in mice. *Cancer Chemother. Rep.* **57,** 165 (1973).
4. K. Hellmann and K. Burrage, Control of malignant metastases by ICRF 159, *Nature (Lond.)* **224,** 273 (1969).
5. A. J. Salsbury, K. Burrage and K. Hellmann, Histological analysis of the antimetastatic effect of (±)-1,2-bis(3,5-dioxopiperazin-1-yl) propane. *Cancer Res.* **34,** 843 (1974).
6. S. E. James and A. J. Salsbury, Effect of (±)-1,2-bis (3,5-dioxopiperazin-1-yl) propane on tumor blood vessels and its relationship to the antimetastatic effect in the Lewis Lung carcinoma. *Cancer Res.* **34,** 839 (1974).
7. A. Atherton, The effect of (±) 1,2-bis (3,5-dioxopiperazin-1-yl) propane (ICRF 159) on liver metastases from a hamster lymphoma. *Europ. J. Cancer* **11,** 383 (1975).
8. A. Atherton, D. Busfield and K. Hellmann, The effects of an antimetastatic agent, (±)-1,2-bis (3,5-Dioxopiperazin-1-yl) propane (ICRF 159), on platelet behavior. *Cancer Res.* **35,** 953 (1975).
9. G. Franchi, L. Morasca, I. Reyers-Degli-Innocenti and S. Garattini, Triton WR 1339 (TWR), an inhibitor of cancer dissemination and metastases. *Europ. J. Cancer* **7,** 533 (1971).
10. R. Rosso, M. G. Donelli, G. Franchi and S. Garattini, Effect of some drugs on tumor dissemination and metastases. *Cancer Chemother. Rep.* **54,** 79 (1970).
11. K. Hellmann, Metastasis and triton WR 1339, *Europ. J. Cancer* **9,** 153 (1973).
12. W. R. Laster, J. G. Mayo, L. Simpson Herren, D. P. Griswold, H. H. Lloyd, F. M. Schabel and H. E. Skipper, Success and failure in the treatment of solid tumours: II. kinetic parameters and "cell cure" of moderately advanced carcinoma 755. *Cancer Chemother. Rep.* **53,** 169 (1969).

13. J. G. MAYO, W. R. LASTER, C. M. ANDREWS and F. M. SCHABEL, Success and failure in the treatment of solid tumors: III "Cure" of metastatic Lewis Lung Carcinoma with Methyl-CCNU (NSC 95441) and surgery–chemotherapy. *Cancer Chemother. Rep.* **56,** 183 (1972).

Europ. J. Cancer Vol. 12, pp. 807–813. Pergamon Press 1976. Printed in Great Britain

Treatment of a Solid Tumor by Local Simultaneous Hyperthermia and Ionizing Radiation: Dependence on Temperature and Dose*

AHARON YERUSHALMI

Radiation Unit, The Weizmann Institute of Science, Rehovot, Israel

Abstract—*Studies in this laboratory have shown that the application of local combined simultaneous heat and X-rays significantly increased local tumor control and cure in mice bearing an SV-40 fibrosarcoma in the limb and in humans bearing metastatic or locally recurrent tumors.*

The present studies were carried out in order to investigate the relations involved in the radiosensitization of tumor tissues with different local heating temperatures and different doses of ionizing radiation.

(BALB/C × C57BL/6)F1 mice, bearing an SV-40 fibrosarcoma in the limb, were exposed to (a) local X-rays alone (500–800 rads); (b) local hyperthermia alone (hot air temperatures 45°C, 50°C, 55°C); duration of heat treatment 15, 30 and 45 min), and (c) simultaneous heat and radiation in various combinations of (a) and (b). Hyperthermia was achieved by means of hot air. Heating and X-irradiation were administered separately or simultaneously once, or twice after a 7-day interval.

The results confirm our previous conclusions that although the SV-40 tumor is both poorly thermosensitive and radioresistant, the synergistic effect of heat and irradiation is striking. The tumor response to the combined simultaneous treatment indicates that the radiation enhancement effect depends upon the local heating temperature and duration, and upon the X-ray dose. It is apparent from these data that the combined simultaneous treatment has a synergistic effect on neoplastic tissues and has important implications in radiation therapy of human cancer.

INTRODUCTION

THERE exists substantial experimental evidence to indicate that hyperthermia (temperatures above 40°C) affects malignant tissue in a more deletory way than normal tissue [1–4]. Studies on mammals and mammalian cells have shown clearly that radiation sensitivity is modified by elevated temperatures [5–7].

Hyperthermal enhancement of ionizing radiation sensitivity of cancer cells has been proved by experimental evidence covering a wide spectrum, from human tumors [8, 9–13] through experimental implanted tumors [6, 14–18] and cells in culture [1, 2, 5].

Hyperthermia was achieved by whole body [12, 19, 20] or local heating [10, 14, 17, 18, 21, 22]. However, information regarding the heating temperature, heating time, and ionizing radiation dose relations which may determine such an effect *in vivo* is insufficient. The present studies were carried out on (BALB/C × C57BL/6)F1 mice bearing an SV-40 fibrosarcoma in the limb, in order to investigate the relations involved in the radiosensitization of tumor tissues with different local heating temperatures (hot air temperature 45°C, 50°C and 55°C), different heating times (15, 30 and 45 min), and different doses of ionizing radiation (500–800 R).

MATERIAL AND METHODS

$(BALB/C \times C57BL)F_1$ hybrid mice were used throughout the experiments. The tumor was an SV-40 fibrosarcoma. The tumor was

Accepted 5 May 1976.

*This work was supported by a grant from the Gulton Foundation, Englewood, New Jersey, USA.

maintained by repeated passages in (BALB/C × C57BL/6)F_1 mice. The tumor is positively detected 5–7 days after transplantation and causes 100% death within 30–50 days in untreated animals. Spontaneous cures and metastases were not observed during the experimental period in untreated animals. Tumors from two to four mice were aseptically excised and pooled in Tyrode's solution. After removal of grossly necrotic tissue, the tumor was minced finely with scissors, washed by low speed centrifugation and treated with 0·25% trypsin solution to produce a disaggregated cell suspension. The suspension was filtered through a fine stainless steel mesh. Tumor cells were then centrifuged, washed twice in Eagle's medium, and viable cells were estimated by exclusion of trypan blue counted in a hemocytometer. 1×10^6 viable tumor cells in 0·5 ml were injected i.m. into the left hind leg. Ten to 11 days after transplantation, animals with tumors of the same volume (1000–1100 mm^3) were mixed together, randomly selected, and separated into the following groups of treatment: (*a*) local X-rays alone (500–800 rads); (*b*) local hyperthermia alone (hot air temperature 45°C, 50°C and 55°C; duration of heat treatment 15, 30 and 45 min); (*c*) simultaneous heat and radiation in various combinations of (*a*) and (*b*); and (*d*) controls.

Heating system

The heating system consisted of (*a*) a hot air blower; (*b*) a heat insulated cylinder (diameter 10·5 cm, length 24 cm) and (*c*) a temperature control unit. For local heating the tumor carrying limbs of 4 mice were inserted into the four holes drilled in the upper part of the cylinder. Hot air was blown in through an insulated pipe connecting the blower with the cylinder (Fig. 1). The hot air atmosphere was achieved by passing the hot air through an attenuator to reduce turbulence. Homogeneity was achieved and confirmed by a system of valves and thermocouples installed for temperature stability and control. Simultaneous temperature measurements were made in the cylinder, in the tumor (to specify intratumor temperature) and in the ear of the mouse (to record skin temperature). The temperature measurements were taken by copper constantan thermocouples 1·8 mm thick. The leads from the installed thermocouples were connected to an electronic control unit which included a continuous digital temperature read-out and a X–Y recorder. Intratumor temperature profiles are given in Fig. 2. Local heating of the tumor-bearing leg at 45°C hot air temperature for 15 min raised intratumor temperature to 38·7 ± 0·2°C. Heating for 30 and 45 min resulted in intratumor temperatures of 39·7 ± 0·2°C. Local heating at 50°C for 15 min raised intratumor temperature to 41·4 ± 0·2°C, while 30 and 45 min of heating at this temperature resulted in 42·3 ± 0·2°C and 42·7 ± 0·2°C intratumor temperature. Local heating for 15 min at 55°C hot air temperature elevated intratumor temperature to 42·7 ± 0·2°C; 30 min resulted in 43·6 ± 0·2°C and 45 min of heating resulted in 44·5 ± 0·2°C in intratumor temperature. The local heat treatment at the highest heating temperature, namely 55°C hot air temperature, 45 min, did raise the animals' ear temperature to 38·5°C, which was well tolerated by the treated animals. The X-ray machine used was a Picker Vanguard, 250 kV, 15 mA, HVL 1·35 mm Cu, F.S.D. 34 cm, with an exposure rate of 100 R/min. Except for the tumor-bearing limbs, the irradiated animals were shielded by half-cylinders of lead 6·5 mm thick.

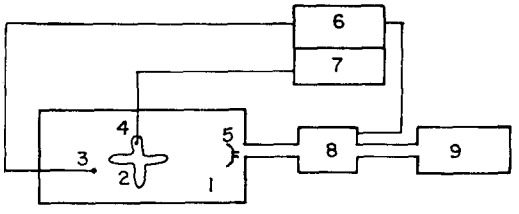


Fig. 1. *Heating system.*

(1) *Cylinder;* (2) *Maltese cross;* (3) *thermocouple for hot air temperature measurement;* (4) *thermocouple for intratumor temperature;* (5) *attenuator;* (6) *hot air temperature control unit;* (7) *intratumor temperature readout and recording system;* (8) *valve system; and* (9) *hot air blower.*

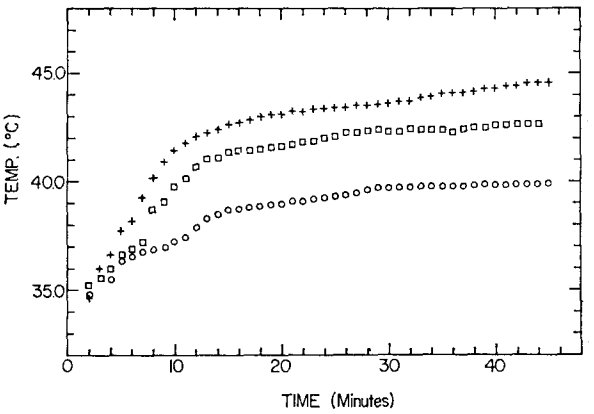


Fig. 2. *Intratumor temperature buildup.*

○ *Hot air temperature* 45°C
□ *Hot air temperature* 50°C
\+ *Hot air temperature* 55°C

RESULTS

In normal and tumor-bearing animals, locally heated, and irradiated, we had no leg losses and no external thermal injury was seen except for hair discoloration. No deaths were caused by the heat treatment. Although there was a small delay in tumor growth caused by heat treatment alone, the death rate was nearly the same in mice given one or two heat treatments as in untreated controls. The mean survival time of control animals and of animals given one or two heat treatments was 35–47 days. For purposes of comparison it was found useful to calculate the median survival time (MST) ratios obtained for the groups only irradiated and for the groups which were simultaneously heated and irradiated (X+H). Each value in Tables 1, 2 and 3 is the MST value of the X+H treated groups divided by the MST of control tumor-bearing animals (controls), namely the enhanced MST ratio (EMSTR). The results indicate that, at each dose of X-irradiation given alone once or twice at a 7-day interval, the MST ratio of animals locally irradiated alone is equal to or greater than that of the untreated tumor-bearing controls (controls). The EMSTR of X+H groups treated once or twice are greater than those of only irradiated.

Table 1.
M.S.T. of treated animals* / *M.S.T. of untreated animals*
Hot air temp 45°C

Dose (R)	X† / Control	X+H 15‡ / Control	X+H 30§ / Control	X+H 45¶ / Control
Single treatment				
800	1·10	1·30	1·43	1·49
700	1·19	1·30	1·3	1·34
600	1·01	1·12	1·15	1·15
500	1·0	1·14	1·2	1·14
Double treatment‖				
800	1·49	1·5	1·52	1·50
700	1·41	1·35	1·48	1·40
600	1·05	1·17	1·27	1·4
500	1·0	1·2	1·2	1·2

*(M.S.T.) median survival time, days.
†M.S.T. of animals locally irradiated.
‡M.S.T. of animals locally heated 15 min and irradiated, simultaneously.
§M.S.T. of animals locally heated 30 min and irradiated, simultaneously.
¶M.S.T. of animals locally heated 45 min and irradiated, simultaneously.
‖Two treatments at a seven-day interval.

Table 2.
M.S.T. of treated animals* / *M.S.T. of untreated animals*
Hot air temp 50°C

Dose (R)	X† / Control	X+H 15‡ / Control	X+H 30§ / Control	X+H 45¶ / Control
Single treatment				
800	1·10	1·32	1·48	1·59
700	1·19	1·36	1·8	1·5
600	1·01	1·23	1·12	1·41
500	1·0	1·12	1	1
Double treatment				
800	1·49	1·57	1·81	1·51
700	1·41	1·62	1·78	2·47
600	1·05	1·18	1·29	1·47
500	1·0		1·17	1·18

*See footnotes to Table 1.

Table 3.
M.S.T. of treated animals* / *M.S.T. of untreated animals*
Hot air temp 55°C

Dose (R)	X† / Control	X+H 15‡ / Control	X+H 30§ / Control	X+H 45¶ / Control
Single treatment				
800	1·1	1·43	1·57	2·42
700	1·19	1·30	1·46	1·70
600	1·01	1·16	1·36	1·49
500	1·0	1·21	1·12	1·31
Double treatment‖				
800	1·49	1·64	1·78	↑
700	1·41	1·57	1·65	2·1
600	1·05	1·21	1·56	1·69
500	1·0	1·31	1·31	1·31

*See footnote to Table 1.

45°C *hot air temperature*

Starting at X+H treatment of 500 rads, once or twice, there was no significant increase in EMSTR and the enhancement of heating at the 3 time durations is low. In the groups treated X+H with 600 rads once, the EMSTR is again low and is similar to that of the 500 rad X+H treated groups. However, single X+H treatment of 700 and 800 rads given once, and 600, 700, 800 rads given twice show an increase in the EMSTR values. However, while in the single 700 and 800 rad X+H treatment there is an increase in EMSTR with time of

heating, in the double X+H treatments at these doses it begins to show a maximal value at the 30 min treatment.

50°C *hot air temperature*

Here again X+H treatments of 500 rads show no significant increase in EMSTR. In the groups of 600 rad X+H treatment, single treatment of X+H 45 min of simultaneous heating and radiation starts to show significant increase in EMSTR and in the 700 rads X+H treatments the maximum value is of the 30 min treatment, while in the 800 rad X+H treatment the increase in EMSTR depends upon the heating time.

In the double treatments of 600 rads X+H 45 min of heating, we reach EMSTR values near that of the maximum obtained by the 45°C heating double treatments. These values increase with a maximum value at 700 rads X+H, 45 min treatment. Here again at 800 rad X+H double treatments repeat itself the maximum value at 30 min heat treatment as in the 45°C hot air group.

55°C *hot air temperature*

At this hot air temperature, single treatment 500 rads X+H groups start to show a significant increase in EMSTR at 45 min heat treatment and from 600 rads X+H treatment the EMSTR depends upon dose and heating duration. In the double X+H treatments, the increase in EMSTR is clearly dose and heating time dependent up to the maximal value of EMSTR in the 800 rads X+H treatment. At this value the cure rate of treated animals is between 80 and 90% [17, 18] and cured animals live longer than one and a half years without a palpable tumor [18]. Moreover, the dependence is clearly manifested by checking Table 3. Checking the data in the tables shows that while EMSTR values of the 500 rads X+H treatment show no significant differences between single and double treatment at 45°C, 50°C and the single treatment groups of 55°C; the same holds for single and double treatment of the 600 rads X+H groups of 45°C and 50°C.

From Table 3 the 55°C hot air treatment groups, in the single-treatment groups, the EMSTR of 600 rads X+H 30 min group is 1·36, while that of the 700 rad X+H 15 min is 1·30. EMSTR of 600 rads X+H 45 min is 1·49, that of 700 rads X+H 30 min is 1·46, and that of the 800 rads X+H 15 min group is 1·43.

EMSTR of 700 rads X+H 45 min is 1·7, while that of the 800 rads X+H 30 min group is 1·57. In the double treatment groups, EMSTR of 600 rads X+H 30 min is 1·56, while that of 700 rads X+H 15 min is 1·57. The EMSTR of 600 rads X+H 45 min is 1·69, that of the 700 rads X+H 30 min group is 1·65, and that of the 800 rads X+H 15 min is 1·64.

Table 4 represents the number of cured animals 13–21 months after treatment. There were no cures in the 500 rads X+H single and double treatment groups. Table 5 represents the average day of death of treated animals.

Single treatment groups

Six hundred rads X+H treatment resulted in cure only at the highest heating, namely 55°C. In the 45°C X+H series, one treatment of 700 rads X+H 45 min resulted in 1/15 cure, and nearly the same value was obtained for the X+H 30 and X+H 45 in the 50°C hot air heat series, while 1/14 and 2/14 were obtained in the 55°C heating series. Three out of 16 and 3/17 cures resulted from the 800 rads X+H 30 and X+H 45 min in the 50°C series. The maximum cure 4/16 was obtained in the X+H 45 min, 55°C hot air treatment groups.

Double treatment groups

In this series of treatments, the curative effect commences in the 45°C hot air, 600 rads X+H 45 treatment with a cure of 1/12, 1/15 in the 700 rads X+H 45, and a constant cure percentage of 10–12% in the 800 rads X+H groups.

In the 50°C hot air groups, the 600 and 700 rad X+H 30 and X+H 45 treatments show approximately the same cure percentage, 13–16%, which increases to 20–22% in the 800 rad X+H 30 and X+H 45 groups.

In the 55°C series, while the 600 rad X+H 30 and X+H 45 resulted in a cure of 1/17 and 1/13, which is about half that of the same treatment in the 50°C group, X+H 30 of the 700 rads 55°C hot air temperature resulted in a cure percentage approximately the same for the 50°C treatment. However, a significant increase in cure was obtained at the 700 rads X+H 45 treatment, namely 5/15. In the 800 rads X+H treatment, the cure reached 23/30, in full agreement with our previous results [17, 18].

DISCUSSION

Although there exists evidence of cure of inoculated tumors in animals [6, 14–18] and in humans [8, 9–13], there is a lack of information regarding the heating temperature, heating time, and ionizing radiation dose involved,

which may determine the effect of the combined treatment of hyperthermia and radiation. The test for the existence of any synergism between heat and radiation would be to show that the combined heat and radiation treatment with doses of both components which separately would give no cures or only a few cures may result in a definite increase in the curative effect [23]. This was the strategy of a series of investigations carried out in this

Table 4. *Fraction of cured animals* (*Number in parentheses = months after treatment, when animals were sacrificed for histology examination*)

Dose (R)	X irradiation only	Hot air temperature 45°C			Hot air temperature 50°C			Hot air temperature 55°C		
		X+H 15*	X+H 30†	X+H 45‡	X+H 15	X+H 30	X+H 45	X+H 15	X+H 30	X+H 45
					Single treatment					
800	1/48	0/20	0/19	5/22 (18)	0/18	3/16 (13)	3/17 (13)	1/27 (17)	2/29 (17)	4/16 (20)
700	0/20	0/17	0/17	1/15 (18)	0/15	1/16 (18)	1/18 (18)	0/19	1/14 (17)	2/14 (21)
600	0/20	0/8	0/8	0/8	0/10	0/8	0/8	0/18	1/14 (20)	1/16 (20)
500	0/10	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/7
					Double treatment§					
800	2/24	3/16 (18)	2/19 (18)	5/24 (18)	1/20 (13)	3/15 (13)	4/18 (13)	4/29 (17)	14/35 (20)	23/30 (20)
700	0/18	0/17	0/15	1/15 (19)	0/15	2/14 (18)	2/15 (18)	0/15	1/16 (21)	5/15 (21)
600	0/18	0/8	0/8	1/12 (18)	0/11	2/12 (18)	2/12 (18)	0/19	1/17 (20)	1/13 (20)
500	0/10	0/8	0/11	0/10	—	0/12	0/10	0/8	0/4	0/10

*Local heating 15 min and simultaneous irradiation. †Local heating 30 min and simultaneous irradiation.
‡Local heating 45 min and simultaneous irradiation. §Two treatments at an interval of 7 days.

Table 5. *Average day of death of treated animals* (*Number in parentheses = fraction of uncured animals*)

Dose (R)	X irradiation only	Hot air temperature 45°C			Hot air temperature 50°C			Hot air temperature 55°C		
		X+H 15*	X+H 30†	X+H 45‡	H+H 15	H+H 30	X+H 45	X+H 15	X+H 30	X+H 45
					Single treatment					
800	45·8 (47/48)	52·8 (20/20)	58·3 (19/19)	59 (17/22)	52 (18/18)	58·1 (13/16)	62·3 (14/17)	52 (26/27)	57·1 (27/29)	81·6 (12/16)
700	43·9 (20/20)	54 (17/17)	52·3 (17/17)	53·5 (14/15)	52·3 (15/15)	57 (15/16)	60 (17/18)	53·5 (19/19)	65·6 (13/14)	67 (12/14)
600	42·9 (20/20)	49 (8/8)	52 (8/8)	53 (8/8)	47·4 (10/10)	47·6 (8/8)	52 (8/8)	54·5 (18/18)	63·5 (13/14)	70·5 (15/16)
500	35·5 (10/10)	47 (8/8)	48·5 (8/8)	48·1 (8/8)	42·1 (8/8)	35·7 (8/8)	42·6 (8/8)	49·5 (8/8)	46·2 (8/8)	54·2 (7/7)
					Double treatment§					
800	57 (22/24)	63 (13/16)	61 (17/19)	65 (19/24)	67·5 (19/20)	67·5 (12/15)	89·5 (14/18)	65·3 (25/29)	152·7 (21/35)	186·7 (7/30)
700	51·5 (18/18)	55·5 (17/17)	59·5 (15/15)	56 (14/15)	54·2 (15/15)	57·3 (12/14)	67·2 (13/15)	61 (15/15)	70·5 (15/16)	86 (10/15)
600	43 (18/18)	50·6 (8/8)	55 (8/8)	57 (11/12)	51·3 (11/11)	50 (10/12)	60·8 (10/12)	58 (19/19)	65 (16/17)	73·1 (12/13)
500	37·5 (10/10)	46·3 (8/8)	54 (11/11)	48 (10/10)	—	46·2 (12/12)	45·8 (10/10)	54·2 (8/8)	57·7 (4/4)	58·3 (10/10)

Footnotes as in Table 4.

laboratory [17, 18]. The existing experimental evidence presented clearly demonstrate the existence of the synergism between heat and radiation. In the present system, the SV-40 induced tumor was shown to be poorly thermosensitive, and except for a minor delay in tumor growth which resulted from local heat treatment alone once or twice, there were no cures, and the death rate was nearly the same as for untreated controls [18]. The cure one year after radiotherapy alone was ca 2% for a single treatment of 800 rads and ca 8% for two 800 rads treatments, reaching 10% for two treatments of 2000 rads [18].

The results of the present investigation show that the EMSTR values do not simply reflect the cures achieved by the different X+H treatments, and any explanation of the heat–radiation interaction leading to cure is far from simple. The results indicate that EMSTR values generally increase with the radiation dose, heating temperature and time of heating. In all the treatment groups, it is clearly shown that an increase in life span compared with untreated controls increases with the increase of heating time, temperature and radiation dose, and that the increase in life span in the double treatment groups is greater than that of the single-treatment groups. Moreover, the EMSTR values and percentage cure in groups which resulted in cure show that all the three factors, namely heating temperature, heating time and radiation dose, are interrelated. Elevating the heating temperature at the same radiation dose, and time of heating, or increasing the radiation dose at the same heating time and heating temperature, or increasing the heating time at the same radiation dose and heating temperature all lead generally to an increase in EMSTR value and in the percentage of cure in groups in which the combined treatment resulted in cure.

Though the significance of the quantitative relations involved in the X+H interaction is not sufficiently clarified, its importance in treatment of neoplastic tissues is self-evident.

A study of the literature available on the use of heat and radiation in the treatment of malignancy results in a wide spectrum of proposed mechanisms which shall not be dealt with here. However, the results of the combined heat and radiation clinical studies [9–13] by different methods are encouraging and justify further investigations in order to understand the relationships involved in the heat–radiation interaction with neoplastic tissues.

REFERENCES

1. T. T. Chen and C. Heidelberger, Quantitative studies on malignant transformation of mouse prostate cells by carcinogenic hydrocarbons *in vitro*. *Int. J. Cancer* **4,** 166 (1969).
2. E. M. Levine and E. B. Robbins, Differential sensitivity of normal and cancer cells in culture. *J. cell. Comp. Physiol.* **76,** 373 (1970).
3. J. H. Kim, S. H. Kim and E. W. Hahn, Thermal enhancement of the radiosensitivity, using cultured normal and neoplastic cells. *Amer. J. Roentgenol.* **121,** 860 (1974).
4. D. S. Muckle and J. A. Dickson, The selective inhibitory effect of hyperthermia on the metabolism and growth of malignant cells. *Brit. J. Cancer* **25,** 771 (1971).
5. E. Ben-Hur, M. M. Elkind and B. V. Bronk, Thermally enhanced radioresponse of cultured Chinese hamster cells: Inhibition of repair of sub-lethal damage and enhancement of lethal damage. *Radiat. Res.* **58,** 38 (1974).
6. J. R. Robinson, M. J. Wizenberg and W. A. McCready, Radiation and hyperthermal response of normal tissue in situ. *Radiology* **113,** 195 (1974).
7. J. E. Robinson, M. J. Wizenberg and W. A. McCready. In: *Abstracts of Contributed Papers, IV International Congress of Biophysics, Moscow,* August 7–14, Section I–IV, p. 289 (1972).
8. G. Crile, Selective destruction of cancers after exposure to heat. *Ann. Surg.* **156,** 404 (1962).
9. O. S. Selawry, J. C. Carlson and G. E. Moore, Tumor response to ionizing rays at elevated temperatures. *Amer. J. Roentgenol.* **80,** 833 (1958).
10. A. T. K. Cockett, M. Kazim, R. Nakamura, A. Fingerhut and J. J. Stein, Enhancement of regional bladder megavoltage irradiation in bladder cancer using local bladder hyperthermia. *J. Urol. (Baltimore)* **97,** 1034 (1967).
11. H. S. Shoulders, E. L. Turner, L. D. Scott and W. H. Grant, Effect of combined fever and X-ray therapy on far advanced malignant growths. *Radiology* **39,** 184 (1942).

12. S. L. WARREN, Preliminary study of effect of artificial fever upon hopeless tumor cases. *Amer. J. Roentgenol.* **33,** 75 (1935).
13. H. J. BRENNER and A. YERUSHALMI, Combined local hyperthermia and X-irradiation in the treatment of metastatic tumors. *Brit. J. Cancer* **33,** 91 (1975).
14. G. CRILE, The effect of heat and radiation on cancers implanted on the feet of mice. *Cancer Res.* **23,** 372 (1963).
15. K. OVERGAARD and J. OVERGAARD, Investigations on the possibility of a thermic tumour therapy. II. Action of combined heat roentgen treatment on a transplanted mouse mammary carcinoma. *Europ. J. Cancer* **8,** 573 (1972).
16. E. HAHN, A. A. ALFIERI and J. M. KIM, Increased cures using fractionated exposures of X-irradiation and hyperthermia in the local treatment of the Ridgeway Osteogenic Sarcoma in mice. *Radiology* **113,** 199 (1974).
17. A. YERUSHALMI and I. HAR-KEDAR, Enhancement of radiation effects by heating of the tumor. *Israel J. med. Sci.* **10,** 772 (1974).
18. A. YERUSHALMI, Cure of a solid tumor by simultaneous administration of microwaves and X-ray irradiation. *Rad. Res.* **64,** 602 (1975).
19. R. T. PETTIGREW, J. M. GALT, C. M. LUDGATE and A. N. SMITH, Clinical effects of whole body hyperthermia in advanced malignancy. *Brit. med. J.* **4,** 679 (1974).
20. M. VON ARDENNE, *Theoretische und experimentelle Grundlagen der Krebs Mehrschrift therapie.* 2nd ed. VEB Verlag Volk und Gesundheit, Berlin (1971).
21. K. WOEBER, The effect of ultrasound in the treatment of cancer. In: *The Ultrasonic Energy* (Edited by E. KELLYS), p. 138, Chicago University Press, Chicago (1965).
22. A. YERUSHALMI, Influence on metastatic spread of whole-body or local tumor by hyperthermia. *Europ. J. Cancer* **12,** 455 (1976).
23. K. OVERGAARD and J. OVERGAARD, Radiation sensitizing effect of heat. *Acta Radiol.* **13,** 501 (1974).

Europ. J. Cancer Vol 12, pp. 815–821. Pergamon Press 1976. Printed in Great Britain

Leukocyte Migration Studies in Chronic Myeloid Leukemia (CML). Specificity of Reactions

B. P. GOTHOSKAR*, H. D'SILVA*, H. GHARPURE† and S. H. ADVANI‡

**Biophysics Division, †Chemotherapy Division, Cancer Research Institute, and ‡Tata Memorial Hospital, Tata Memorial Centre, Bombay 400 012, India*

Abstract—*The leukocyte migration inhibition (LMI) technique was used to investigate the status of cellular sensitization in human patients suffering from chronic myeloid leukemia (CML). Peripheral leukocytes from CML patients in remission (remission-leukocytes) and from normal donors (normal-leukocytes) were examined for their susceptibility to extracts prepared from myeloid type of leukemic cells. In further experiments to define the specificity of the reactions observed, both normal-leukocytes and remission-leukocytes were also examined in the presence of similar extracts prepared from (a) normal leukocytes, (b) lymphocytic leukemia cells, and (c) 9 biopsies of solid tumors of different types (2 melanomas, 2 sarcomas, and 5 carcinomas).*

Extracts of myeloid leukemic cells prepared by hypotonic sodium chloride (NaCl) and hypertonic potassium chloride (KCl) extraction procedures showed significant inhibitory activity preferentially directed against the migration of remission-leukocytes. The KCl-extracts showed greater inhibitory activity and exerted it in a dose-related manner. The activity persisted in extracts of leukemic cells obtained serially from CML patients before and during treatment, and during subsequent relapses.

Similar extracts of normal leukocytes and of 9 biopsies of solid tumours failed to show such discriminatory behavior against the two types of leukocytes tested. However, the susceptibility of the remission-leukocytes was not uniquely directed towards the extracts of myeloid leukemic cells. Their migration was also inhibited in a considerable fraction of the tests carried out with the extracts of lymphocytic leukemic cells.

Inhibition of migration of remission-leukocytes by extracts of "allogeneic" leukemic cells indicated common antigenic component(s) shared by leukemic cells.

INTRODUCTION

LEUKOCYTE migration and its inhibition (LMI) is a simple *in vitro* technique, now accepted as a reliable correlate of Cell-Mediated Immunity (CMI) in man. A number of workers have used this technique to detect tumor-directed CMI in patients suffering from a variety of neoplastic diseases [1–10]. It has also been used for sequential monitoring of cellular responses to autologous cancer tissue [11], and for observing blocking effects of sera from tumor-bearing animal hosts [12, 13].

We have used this technique to assess the status of cellular sensitization in human patients suffering from chronic myeloid leukemia (CML). Preliminary studies indicated that the leukocytes obtained from CML patients in remission (remission-leukocytes) showed inhibition of migration when exposed to extracts of autologous or allogeneic leukemic cells [14]. The present experiments were designed to investigate the specificity of reactions shown by remission-leukocytes and the manner in which they differed from the leukocytes of control healthy donors (normal-leukocytes). In particular, they dealt with the following points; (*a*) antigenicity of hypotonic- and hypertonic-salt extracts of leukemic cells in LMI, (*b*) detection of antigenicity in leukemic extracts prepared during serial relapses, (*c*) differential responses of remission-leukocytes and normal-leukocytes against extracts of normal leukocytes, leukemic cells, and solid tumor biopsies. The results are presented in this communication.

Accepted 5 May 1976.

MATERIAL AND METHODS

Antigens

Leukemic cells were obtained from the peripheral blood of 38 leukemic patients when they were first admitted to the Tata Memorial Hospital. Clinical evidence and bone-marrow studies led to the diagnosis of CML in 30 of these patients, chronic lymphocytic leukemia (CLL) in 4, and acute lymphocytic leukemia (ALL) in the remaining 4 patients. The white cell counts of these patients ranged from 52,000 to 250,000 per mm^3. In 4 of the CML patients, additional leukemic cell samples were obtained 2/3 weeks after the start of chemotherapeutic treatment and during relapses occurring subsequent to therapy. The leukemic cells were washed thrice with normal saline and subjected to extraction procedures. The first 10 CML samples were divided into 2 equal aliquots and processed through (i) hypotonic sodium chloride (NaCl) extraction procedure [15], and (ii) hypertonic potassium chloride (KCl) extraction procedure [16] with slight modifications. After extractions, both NaCl- and KCl-extracts were first centrifuged in the cold at 500 $\times g$ for 10 min, dialyzed overnight against cold phosphate-buffered saline (PBS) at pH 7·2, and the dialysates were centrifuged in the cold at 40,000 $\times g$ for 60 min. Without further purifications, the supernatants were concentrated to 1/10 volume, sterilized through Millipore filters (0·45 μm) and used as "leukemic" extracts. All the rest of the leukemic cell samples were subjected to the KCl-extraction procedure only.

Nine biopsies of solid tumors of different types (2 melanomas, 2 sarcomas and 5 carcinomas) were obtained, washed free of blood, minced and sieved to obtain cell suspensions, which were then subjected to the KCl-extraction procedure.

Normal leukocytes separated from 250 ml blood samples collected from 12 healthy donors were processed similarly to yield "normal leukocyte" extracts.

In all there were 77 extracts tested in these investigations. The protein concentrations of all the extracts were determined with the use of Folin–Phenol reagent [17], and all the extracts were stored in small aliquots at −20°C till use.

Leukocyte samples

Leukocytes were obtained from 15–20 ml peripheral blood samples collected from 68 normal donors and 66 CML patients in remission. The remission patients had not received any blood transfusions and were tested between 2 and 24 months of remission. The peripheral white cell counts of these patients ranged from 6000 to 14,000 per mm^3 at the time of testing. Red cell sedimentation was achieved using 3% dextran solution and the leukocytes were washed thrice with Eagle's minimal essential medium (MEM) before being used.

The Leukocyte migration inhibition (LMI) tests

The procedure used for the LMI tests was that described by Cochran *et al.* [18], and the medium used was MEM supplemented with 10% (inactivated) human AB serum. All the extracts used as "antigens" in these tests were employed at a concentration of 100 μg protein/ml. In experiments for dose-relation studies,

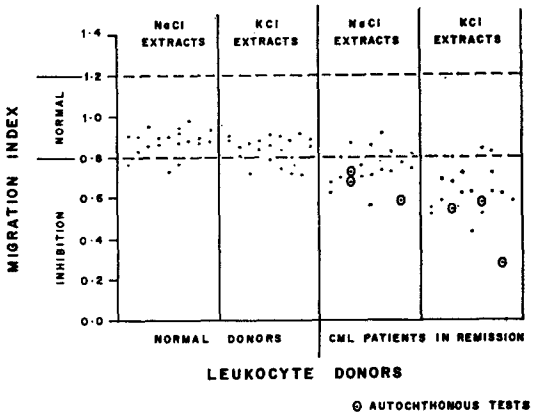


Fig. 1.

the extracts were tested in the range 50–300 μg protein per ml. Each sample was tested in quadruplicate and the migration index (M.I.) was calculated as described by Federlin *et al.* [19]. Migration indices ranging from 0·8 to 1·2 were considered as within the normal range [19, 20]. Tests with M.I. less than 0·8 were categorized as "inhibitions" and those with M.I. above 1·2 as "enhancements". The statistical significance of the results was assessed by Chi square analysis.

RESULTS

Figure 1 presents the migration indices of 10 samples of normal leukocytes and 10 samples of remission-leukocytes in the presence of either NaCl- or KCl-extracts of leukemic cells collected from 10 different CML patients.

Each extract was tested against 2 samples of each type of the leukocytes. Accepting 0·8–1·2 M.I. as the "cut-off" limits for the range of "normal reactivity" of leukocytes in such a migration system [19 20], it is seen that the migration of normal leukocytes was not inhibited in 17 out of 20 tests with the NaCl-extracts and in 16 out of 20 tests with the KCl-extracts. In contrast, the migration of remission-leukocytes was inhibited in 14 out of 20 tests with the NaCl-extracts and in 17 out of 20 tests with the KCl-extracts. In addition, the degree of migration inhibition was found to be greater

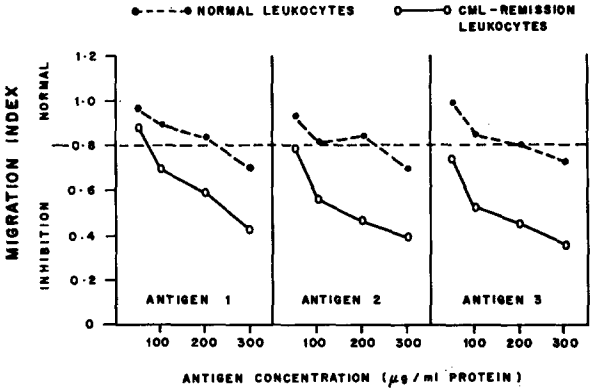


Fig. 2.

in tests with the KCl-extracts than in tests with the NaCl-extracts. It was possible to test 3 samples of the remission-leukocytes against autologous extracts of the 2 types. Even in these autochthonous combinations the KCl-extracts caused greater inhibitions than the corresponding NaCl-extracts.

That the migration-inhibitory activity of the KCl-extracts is dependent on the dose (in terms of protein concentration) is seen from the results shown in Fig. 2. Three different KCl-extracts of myeloid leukemic cells were examined, in the concentration range 50–300 µg protein per ml, for their activity against both the normal-leukocytes and remission-leukocytes The differential susceptibility of the two types of leukocytes was quite evident at 100 and 200 µg protein/ml concentrations, which brought down the migration indices of remission-leukocytes only below the 0·8 level. At higher concentration tested, a marginal inhibitory effect on normal leukocytes was noticeable.

Figure 3 compares the inhibitory activity of the KCl-extracts of leukemic cells obtained serially from 4 CML patients. In each case,

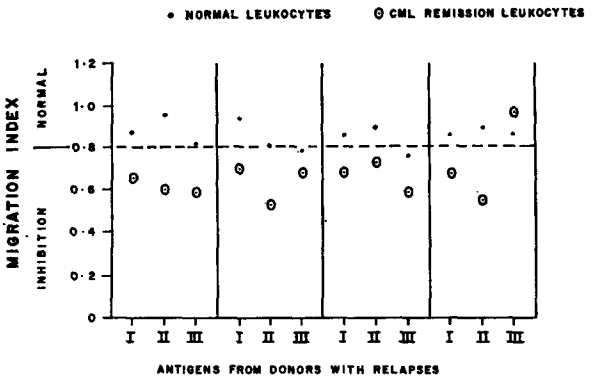


Fig. 3.

antigen I was an extract of leukemic cells obtained from the patient before any treatment was started. Antigen II was a similar extract of leukemic cells collected from the patient after 2/3 weeks of chemotherapeutic treatment. Antigen III was prepared from leukemic cells obtained when the same patient went into a relapse between 4–10 months subsequent to the therapy. All the extracts, except extract III from one patient, showed continued presence of the migration-inhibitory activity against remission-leukocytes. None of them affected the migration of normal leukocytes.

Table 1. *Migration of leukocytes from normal donors and CML* patients in remission in the presence of hypertonic KCl-extracts of leukemic† cells*

Leukocyte donors and No.	Type of extracts and No.	Number of tests	Migration pattern		
			Inhibition	Normal	Enhancement
Normal donors (42)	Leuk. extracts (30)	126	33 (26%)	83 (65·8%)	10 (7·9%)
CML patients in remission (36)	Leuk. extracts (30)	159	105 (66%)‡	45 (28·3%)	9 (5·6%)
CML patients in remission (30)	Autologous Leuk. extracts (30)	30	22 (73%)	6 (20%)	2 (6·6%)

*Chronic myeloid leukemia.
†Peripheral leukemic cells from patients with CML.
‡Statistically significant ($\chi^2 = 44·3$, 1° freedom, $P < 0·005$).

Table 2. Migration of leukocytes from normal donors and CML patients in remission in the presence of hypertonic KCl-extracts of normal leukocytes*

Leukocyte donors and No.	Number of extracts	Number of tests	Migration pattern		
			Inhibition	Normal	Enhancement
Normal donors (14)	12	58	16 (27·6%)	37 (63·7%)	5 (8·6%)
CML patients in remission (18)	12	64	20 (31·2%)	38 (59·3%)	6 (9·3%)

*Chronic myeloid leukemia.

Table 3. Migration of leukocytes from normal donors and CML patients in remission in the presence of hypertonic KCl-extracts of different types of malignancies*

Leukocyte donors and number	Number of extracts	Number of tests	Migration pattern		
			Inhibition	Normal	Enhancement
Normal donors (10)	9†	52	12 (23%)	34 (65%)	6 (11·5%)
CML patients in remission (12)	9†	56	16 (28·5%)	34 (60·7%)	6 (10·7%)

*Chronic myeloid leukemia.
†Extracts of 2 melanomas, 2 sarcomas and 5 carcinomas.

Table 4. Migration of leukocytes from normal donors and CML patients in remission in the presence of hypertonic KCl-extracts of other types of leukemic cells†*

Leukocyte donors and No.	Number of extracts	Number of tests	Migration pattern		
			Inhibition	Normal	Enhancement
Normal donors (12)	8	59	12 (20·3%)	39 (66·1%)	8 (13·5%)
CML patients in remission (14)	8	48	25 (52%)‡	19 (39·5%)	4 (8·3%)

*Chronic myeloid leukemia.
†Chronic and acute lymphocytic leukemia (4 CLL, 4 ALL).
‡Statistically significant ($\chi^2 = 10{\cdot}1$, 1° of freedom, $P < 0{\cdot}005$).

Table 5. Proportion of positive reactions shown by normal leukocytes and remission leukocytes exposed to KCl-extracts of different types of cells

Source of antigenic extracts	% positive* reactions in tests with	
	Normal leukocytes	Remission leukocytes
Normal leukocytes	36·2	40·5
Myeloid leukemic cells	33·9	71·6†
Solid tumor biopsies	34·5	39·2
Lymphocytic leukemic cells	33·8	60·3†

$$\text{*}\frac{\text{Number of tests showing inhibitions and enhancements}}{\text{Total number of tests performed}} \times 100$$

†Significant at level of $P < 0{\cdot}05$.

Table 1 presents the summary of the results obtained in the tests carried out with the KCl-extracts of 30 samples of myeloid leukemic cells. Leukocytes for these tests were obtained from 42 normal donors and 36 CML patients in remission. Thirty of the remission-leukocyte samples were tested against extracts of both autologous and allogeneic leukemic cells. The distribution of the results of 126 tests on normal leukocytes was: 33 inhibitions (26%), 83 normals (65·8%) and 10 enhancements (7·9%). The corresponding distribution for 159 tests on remission-leukocytes was: 105 inhibitions (66%), 45 normals (28·3%) and 9 enhancements (5·6%). The proportion of inhibitions was significantly greater in tests on remission-leukocytes than in tests on normal leukocytes (χ^2=44·3, 1° of freedom, $P < 0·005$). The proportion of such inhibitions was still higher (22 out of 30 tests, 73%) in tests with autologous leukemic cell extracts.

Some tests with both normal leukocytes and remission leukocytes showed enhancement. These fractions were similarly infrequent in normal and leukemic donors. On the other hand, considering enhancement as a manifestation of "weak sensitization" [19–21], the proportion of positive reactions (inhibitions + enhancements) in the tests with remission-leukocytes still remains significantly high (χ^2= 38·7, 1° of freedom, $P < 0·005$).

Table 2 gives the data on tests with KCl-extracts of 12 samples of normal leukocytes. The distribution of results in 58 tests with normal leukocytes was not found to differ from that seen in 64 tests with remission-leukocytes. Moreover, the proportion of inhibitions in these tests was similar to that seen in tests with normal leukocytes exposed to KCl-extracts of myeloid leukemic cells.

Results of the migration patterns of normal leukocytes (52 tests) and remission-leukocytes (56 tests) exposed to KCl-extracts of 9 solid tumor biopsies are given in Table 3. Here again the distribution of results for the two types of leukocytes tested was similar and did not show any preponderance of inhibitions.

Table 4 gives the data on tests carried out with KCl-extracts of lymphocytic leukemic cells obtained from 4 CLL and 4 ALL patients. While 12 out of 59 (20·3%) tests with normal leukocytes showed inhibitions, as many as 25 out of 48 (52%) tests with remission-leukocytes showed inhibitions. This preferential inhibition of migration of remission leukocytes was significant in this case too (χ^2=11·4, 1° of freedom, $P < 0·005$).

DISCUSSION

The present investigations were carried out to explore the specificity of reactions shown by remission leukocytes from CML patients against extracts of a variety of malignant cells. The results bring out the following salient features:

(i) Both in autologous and in allogeneic combinations, the KCl-extracts of leukemic cells showed better migration–inhibitory activity than the corresponding NaCl-extracts. This might be due to better extraction and/or preservation of the antigenic material in the KCl-extracts. This suggestion gains support in the light of results reported on physical injury to leukemic cells [22], wherein the myeloblastic leukemia cells were shown to be resistant to hypotoxicity as against lymphocytic leukemic cells which were susceptible to hypotonicity.

(ii) The preferential activity of KCl-extracts of leukemic cells against remission-leukocytes was statistically significant and expressed in a dose-dependent manner, pointing to its specificity [19, 20, 23]. Since the majority of the tests with remission-leukocytes and leukemic cell extracts were allogeneic, the results also indicate the presence of common antigenic component(s) in extracts of leukemic cells obtained from different patients.

(iii) The antigenic character of leukemic cells was persistent since the extracts of leukemic cells, obtained before or during treatment or during subsequent relapse showed qualitatively similar effects on the migration of remission-leukocytes.

(iv) Similar extracts of normal leukocytes and 9 biopsies of solid tumors failed to show such preferential activity against remission leukocytes. On the other hand, extracts of lymphocytic leukemic cells exerted a significant inhibitory effect on the migration of remission leukocytes, though the proportion of inhibitions in such tests was less than that seen in the tests with extracts of myeloid leukemic cells.

It is to be noted that both normal leukocytes and remission leukocytes showed a spectrum of effects on exposure to different types of extracts. Since migration–inhibition and migration–enhancement are reported to be two

facets of interactions between an antigen and specifically sensitized leukocytes dependent upon the degree of sensitization [19–21], it is worthwhile to look at the proportion of total positive reactions (inhibitions + enhancements) encountered in these experiments. Table 5 summarizes such data and stresses the point that the baseline reactivity (beyond the cutoff limits) of even the normal leukocytes against such extracts cannot be ignored. Other investigators have also reported similar results [24]. In addition to *leukemia-associated* reactivity, the present results also suggest involvement of non-related/nonspecific components in such interactions between cellular extracts and sensitized cells. Since both hypotonic- and hypertonic-salt extraction procedures have been reported to release other types of surface associated antigens also [25, 26], the baseline reactivity seen in different types of controls in the present investigations could be due to such alloantigens not associated with leukemia. Using lymphocyte blastogenesis as a parameter, Dean *et al.* [27] have reported a high reactivity of normal leukocytes towards KCl-extracts of allogeneic cells of normal as well as malignant origin. It would, thus, be necessary to selectively fractionate cellular extracts for obtaining "*disease-specific*" components that are capable of discriminating between *normal* and *sensitized* leukocytes with a larger margin and lesser overlap, in such *in vitro* test systems.

REFERENCES

1. V. Andersen, G. Bendixen and T. Schiodt, An *in vitro* demonstration of cellular immunity against autologous mammary carcinoma in man. *Acta med. scand.* **186,** 101 (1969).
2. A. J. Cochran, R. M. Mackie, C. E. Thomas, R. M. Grant, D. E. Cameron-Mowat and W. G. S. Spilg, Cellular immunity to breast cancer and malignant melanoma. *Brit. J. Cancer* **28,** suppl. 1, 77 (1973).
3. M. M. Black, D. H. Moore, B. Shore, R. E. Zachrau and H. P. Leis, Jr., Effect of murine milk samples and human breast tissue on human leukocyte migration indices. *Cancer Res.* **34,** 1054 (1974).
4. R. M. Mackie, W. G. S. Spilg, C. E. Thomas and A. J. Cochran, Cell mediated immunity in patients with malignant melanoma. *Brit. J. Derm.* **87,** 523 (1972).
5. A. J. Cochran, U. W. Jehn and B. P. Gothoskar, Cell mediated immunity to malignant melanoma. *Lancet* **i,** 1340 (1972).
6. M. S. Goldstein, B. Shore and S. B. Gusberg, Cellular immunity as a host response to a squamous carcinoma of the cervix. *Amer. J. Obst. Gynec.* **111,** 751 (1971).
7. M. Braun, L. Sen, A. E. Bachmann and A. Pavlovski, Cell migration inhibition in human lymphomas using lymph node and cell line antigens. *Blood* **39,** 368 (1972).
8. A. Segall, O. Weiler, J. Genin, J. Lacour and F. Lacour, *In vitro* study of cellular immunity against autochthonous human cancer. *Int. J. Cancer* **9,** 417 (1972).
9. M. Kjaer, *In vitro* demonstration of cellular hypersensitivity to tumor antigens by means of leukocyte migration technique in patients with renal carcinoma. *Europ. J. Cancer* **10,** 523 (1974).
10. J. L. McCoy, L. F. Jerome, J. H. Dean, E. Perlin, R. K. Oldham, D. H. Char, M. H. Cohen, E. L. Felix and R. B. Herberman, Inhibition of leukocyte migration by tumor associated antigens in soluble extracts of human malignant melanoma. *J. nat. Cancer Inst.* **55,** 19 (1975).
11. M. M. Black and H. P. Leis, Jr., Cellular responses to autologous breast cancer tissue: sequential observations. *Cancer (Philad.)* **32,** 384 (1973).
12. W. J. Halliday, Blocking effect of serum from tumor bearing animals on macrophage migration inhibition with tumor antigens. *J. immunol.* **106,** 855 (1971).
13. M. F. Poupan, G. Lespinats and J. P. Kolb, Blocking effect of the migration inhibition reaction by sera from immunized syngeneic mice and by sera from plasmacytoma bearing BALB/c mice. Detection of free circulating antigen. *J. nat. Cancer Inst.* **52,** 1127 (1974).
14. S. G. Gangal, B. P. Gothoskar, C. S. Joshi and S. H. Advani, Demonstration of cellular immunity in chronic myeloid leukemia using leukocyte migration inhibition assay. *Brit. J. Cancer* **33,** 267 (1976).

15. M. E. OREN and R. B. HERBERMAN, Delayed cutaneous hypersensitivity reactions to membrane extracts of human tumor cells. *Clin. exp. Immunol.* **8,** 001 (1971).
16. M. S. MELTZER, E. J. LEONARD, H. J. RAPP and T. BORSOS, Tumor specific antigen solubilized by hypertonic Potassium Chloride. *J. Natl. Cancer Inst.* **47,** 703 (1971).
17. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with Folin Phenol reagent. *J. Biol. Chem.* **193,** 265 (1951).
18. A. J. COCHRAN, G. KLEIN, R. KIESSLING and P. GUNVEN, Migration inhibiting effect of sera from patients with Burkitt's Lymphoma. *J. Natl. Cancer Inst.* **51,** 1431 (1973).
19. K. FEDERLIN, R. N. MAINI, A. S. RUSSELL and D. C. DUMONDE, A micro-method for peripheral leukocyte migration in tuberculin sensitivity. *J. Clin. Path.* **24,** 533 (1971).
20. M. KJAER, The dose related effect of tumor extract on the *in vitro* migration of leukocytes from patients with renal carcinoma. *Europ. J. Cancer* **11,** 281 (1975).
21. C. G. MITCHELL, M. G. M. SMITH, P. L. GOLDING, A. L. W. F. EDDLESTON and R. WILLIAMS, Evaluation of the leukocyte migration tests as a measure of delayed hypersensitivity in man. *Clin. exp. Immunol.* **11,** 535 (1972).
22. M. MIURA, K. KAWASHIMA, H. NISHIWAKI, M. KOBAYASHI, A. MORITA, R. OHNO, H. KAKIZAWA, T. UETANI, M. HIRANO and K. YAMADA, A study of leukemic cell injury by physical agents. *Cancer Res.* **31,** 1451 (1971).
23. R. N. MAINI, L. M. ROFFE, J. J. MAGRATH and D. C. DUMONDE, Standardization of the leukocyte migration test. *Int. Arch. Allergy* **45,** 308 (1973).
24. A. J. COCHRAN, R. M. GRANT, W. G. SPILG, R. M. MACKIE, C. E. ROSS, D. E. HOYLE and J. M. RUSSELL, Sensitization to tumor associated antigens in human breast carcinoma. *Int. J. Cancer (Philad.)* **14,** 19 (1974).
25. D. A. L. DAVIES, Mouse histocompatibility antigens derived from normal and from tumor cells. *Immunology* **11,** 115 (1966).
26. R. A. REISFELD, M. A. PELLEGRINE and B. D. KAHAN, Salt extraction of soluble HL-A antigens. *Science* **172,** 1134 (1971).
27. J. H. DEAN, J. S. SILVA, J. L. MCCOY, C. M. LEONARD, M. MIDDLETON, G. B. CANNON and R. B. HERBERMAN, Lymphocyte blastogenesis induced by potassium chloride extracts of allogeneic breast carcinoma and lymphoid cells. *J. nat. Cancer Inst.* **54,** 1295 (1975).

Europ. J. Cancer Vol. 12. pp. 823–825. Pergamon Press 1976. Printed in Great Britain

Characterization of Human Fibroblasts from Cancer Patients: Loss of Fibrin Clot Retractile Activity after '*In Vitro*' Spontaneous Transformation*

E. DOLFINI†, B. AZZARONE‡, D. PEDULLÀ‡, E. OTTAVIANO†, G. DE GAETANO†, M. B. DONATI† and L. MORASCA†

†*Mario Negri Institute for Pharmacological Research, Milano, Italy and*
‡*Institute for Medical Microbiology, University of Genova, Italy*

Abstract—*Human fibroblast-like cells cultured from skin and stromal tissue of lung carcinomas, are able to induce fibrin clot retraction similarly to fibroblasts from the skin of normal individuals. Following* in vitro *spontaneous transformation, these cells loose their retractile property; fibrin clot retraction could be a rather specific parameter to identify fibroblastic cells and to monitor their* in vitro *oncogenic transformation.*

INTRODUCTION

HUMAN fibroblast-like cells deriving from either the stromal tissue of bronchial carcinomas or the skin of the same patients have recently been reported to undergo spontaneous *in vitro* transformation [1, 2]. Caryological analysis performed in the early phases of the cultures showed varying degrees of aneuploidy in these cells, despite their normal morphology and biological behaviour [1, 2].

Transformed cells are characterized by deep modifications in their membrane properties and function [3, 4]; normal human fibroblasts have been recently shown to induce fibrin clot retraction [5], an activity which is considered as a reliable parameter of the membrane function of some cells [6–8]; it was therefore considered of interest to study the retractile activity of fibroblast-like cells before and after their *in vitro* spontaneous transformation.

MATERIAL AND METHODS

This study was performed on fibroblast-like cells deriving from the skin of 4 apparently normal individuals, from the skin of 4 patients with bronchial carcinoma and from the stromal portion of the tumour in the same patients. The same cells were studied for spontaneous transformation [1, 2].

Cells were reconstituted in culture from liquid nitrogen and were harvested, cultured and studied for transformation as previously described [1, 2].

Fibrin clot retraction induced by fibroblast suspensions was measured as previously described for platelets [9] and BA-1112 cultured cells [7].

RESULTS

As indicated in Table 1, fibroblasts from the skin of normal subjects and fibroblast-like cells from both the skin and the stromal portion of bronchial carcinomas were able to induce fibrin clot retraction. Following *in vitro* spontaneous transformation, the cells deriving from both the skin and the lung of patients affected

Table 1. Fibrin clot retraction induced by fibroblasts (for each group mean and standard error of determinations performed on 4 samples are reported)

Origin of cells	% clot retraction
Blank	10·4 ± 1·6
Healthy subjects: skin	62·4 ± 5·2
Cancer patients: skin before trans.	64·6 ± 4·9
after transf.	11·3 ± 0·8
lung before transf.	62·2 ± 4·3
after transf.	13·2 ± 1·4

Accepted 14 April 1976.

*This work was partially supported by Grant NIH-PHRB-1RO1-CA 12764-01.

by bronchial carcinoma, lost their ability to induce fibrin clot retraction. In contrast, fibroblasts derived from normal subjects did not loose their ability to retract a fibrin clot, during a comparable number of passages in culture.

DISCUSSION

This paper confirms the data of Niewiarowski *et al.* [5] indicating that normal human fibroblasts are able to provoke fibrin clot retraction. These observations offer indirect support to the hypothesis that the fibroblast-like cells deriving from the stromal tissue of bronchial carcinomas or from the skin of the same patients are identical with fibroblasts. *In vitro* transformation of both skin and lung fibroblast-like cells was accompanied by loss of the ability to induce fibrin clot retraction. Decrease in this activity has also been observed by Niewiarowski *et al.* [10] during ageing of normal human fibroblasts: the phenomenon we have observed, however, cannot be ascribed to ageing of fibroblast-like cells, since the fibroblasts of normal subjects, treated in the same way, did not loose retractile activity after a comparable number of passages in culture. Ageing of cells is accompanied by caryological modifications [11–13] which may have some characteristics in common with those described during oncogenic transformation [1, 12, 13]: the capacity to interact with fibrin seems therefore to be a functional parameter of fibroblasts which can be modified in association with various types of cellular transformation.

The ability to retract fibrin clot is considered to be linked, in platelets, to the presence of a contractile protein which is very similar to muscle actomyosin [14].

A decrease in the actin content of the cell membrane or a modification in the distribution pattern of intracellular actin and myosin have been very recently described in virally transformed fibroblasts [15, 16].

In human surgical specimens from skin and mammary carcinomas, however, an increase in contractile proteins has been recently demonstrated with immunofluorescent techniques using specific antibodies against various fractions of the smooth muscle actomyosin protein [17]. These immunohistological data on tissue specimens do not necessarily disagree with our findings which describe a functional disturbance of cultivated cells; loss of the retractile activity could indeed occur in the presence of a normal or even increased content of contractile proteins. The activity we have observed in human fibroblasts could also be linked to some of the recently described trypsin-resistant filamentous structures located in the cytoskeleton of the cell surface [18–20].

REFERENCES

1. B. Azzarone and D. Pedullà, Spontaneous transformation of human fibroblast cultures derived from bronchial carcinomata. *Europ. J. Cancer* **12,** 1976, in press.
2. B. Azzarone and D. Pedullà, Spontaneous transformation of stromal fibroblasts derived from human lung cancers. In *Proc. 6th Int. Symposium on the Biological Characterization of Human Tumours,* pp. 150–155. Excerpta Medica, Amsterdam, 1976.
3. M. Abercrombie and E. J. Ambrose, The surface properties of cancer cells. A review. *Cancer Res.* **22,** 525 (1962).
4. M. M. Burger, A difference in the architecture of the surface membrane of normal and virally transformed cells. *Proc. nat. Acad. Sci. (Wash.)* **62,** 994 (1969).
5. S. Niewiarowski, E. Regoeczi and J. F. Mustard, Adhesion of fibroblasts to polymerizing fibrin and retraction of fibrin induced by fibroblasts. *Proc. Soc. exp. Biol. (N.Y.)* **140,** 199 (1972).
6. F. De Clerck, M. Borgers, G. de Gaetano and J. Vermylen, Dissociation of clot retraction from platelet granule fusion and degranulation: an ultrastructural study of reptilase–human platelet-rich plasma clots. *Brit. J. Haemat.* **29,** 341 (1975).
7. M. B. Donati, E. Dolfini, L. Morasca and G. de Gaetano, Fibrin clot retraction by a rat rhabdomyosarcoma cell line. *Thrombos. Res.* **8,** 707 (1976).
8. S. Niewiarowski and S. Goldstein, Fibrin clot retraction by human skin fibroblasts. Effects of ADP and thrombin. *Proc. nat. Acad. Sci. (Wash.)* 1976, in press.

9. G. DE GAETANO, D. BOTTECCHIA and J. VERMYLEN, Retraction of reptilase-clots in the presence of agents inducing or inhibiting the platelet adhesion–aggregation reaction. *Thrombos. Res.* **2,** 71 (1973).
10. S. NIEWIAROWSKI and S. GOLDSTEIN, Interaction of cultured human fibroblasts with fibrin: modification by drugs and ageing *in vitro*. *J. Lab. clin. Med.* **82,** 605 (1973).
11. L. HAYFLICK, The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37,** 614 (1965).
12. Z. K. KADANKA, J. D. SPARKES and H. G. MACMORINE, A study of the cytogenetics of the human cell strain WI–38. *In Vitro* **8,** 353 (1973).
13. M. TERZI and T. S. C. HAWKINS, Chromosomal variation and the establishment of somatic cell lines *in vitro*. *Nature* (*Lond.*) **253,** 361 (1975).
14. M. BETTEX-GALLAND and E. F. LUSCHER, Thrombosthenin: a contractile protein from thrombocytes. Its extraction from human blood platelets and some of its properties. *Biochim. biophys. Acta.* **49,** 536 (1961).
15. G. WICKUS, E. GRUENSTEIN, P. W. ROBBINS and A. RICH, Decrease in membrane-associated actin of fibroblasts after transformation by rous sarcoma virus. *Proc. nat. Acad. Sci.* (*Wash.*) **72,** 746 (1975).
16. R. POLLACK, M. OSBORN and K. WEBER, Patterns of organization of actin and myosin in normal and transformed cultured cells. *Proc. nat. Acad. Sci.* (*Wash.*) **72,** 994 (1975).
17. G. GABBIANI, P. TRENCHEV and E. J. HOLBOROW, Increase of contractile proteins in human cancer cells. *Lancet* **ii,** 796 (1975).
18. E. ROUSLAHTI and A. VAHERI, Novel human serum protein from fibroblast plasma membrane. *Nature* (*Lond.*) **248,** 789 (1974).
19. K. WANG, J. F. ASH and S. J. SINGER, Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. nat. Acad. Sci.* (*Wash.*) **72,** 4483 (1975).
20. B. R. BRINKLEY, G. M. FULLER and D. P. HIGHFIELD, Cytoplasmic microtubules in normal and transformed cells in culture: analysis by tubulin antibody immunofluorescence. *Proc. nat. Acad. Sci.* (*Wash.*) **72,** 4981 (1975).

Europ. J. Cancer Vol. 12, pp. 827–831. Pergamon Press 1976. Printed in Great Britain

Cytokinetic Studies on the Effect of Hyperthermia on Chinese Hamster Lung Cells

HELMUT SCHLAG and CHRISTINE LÜCKE-HUHLE
Kernforschungszentrum Karlsruhe, Institut für Strahlenbiologie, Postfach 3640, 7500 *Karlsruhe, Federal Republic of Germany*

Abstract—*Hyperthermic killing of V* 79 *cells is dependent on the state of growth: while survival of plateau phase cells decreases to* 0·45, *only* 0·03 *of the exposed exponentially growing cells survive* 4 *hr at* 42°*C. Flow-microfluorometric analysis during and after heating yields the fraction of cells in the individual phases of cell cycle and allows conclusions about changes in the proliferation kinetics of the exposed cell population.*

Mild heat treatment (1 *hr at* 42°*C*) *leads to a synchronisation effect caused by a block in S and* $G_2 + M$, *while after a stronger heat exposure* (4 *hr at* 42°*C*) *cell death in S-phase is predominant.*

INTRODUCTION

PUBLICATIONS concerning the use of elevated temperatures in therapy date back until the middle of the last century (review: 1). More recently, there is renewed interest in hyperthermia for treatment of cancer, especially in combination with radiation therapy. Such a combined treatment might possibly offer an alternative to high LET radiations, since the respective dose–effect curves show quantitative and qualitative similarities. It is not yet clear whether the increased efficiency for cell killing by the heat-X-ray therapy is of additive or synergistic kind, but cytokinetic studies on cells after hyperthermic treatment should reveal some of the underlying mechanism. Though it is known that the response of cells to hyperthermia depends on their stage in the cell cycle [2, 3] cell survival curves of several cell lines tested either in the logarithmic or plateau phase of growth show contradictory results [4–6].

The present study was undertaken to examine the influence of the state of growth on thermal sensitivity using V79 cells and to investigate the effect of heat on cell kinetics during and after exposure to a temperature (42°C) within acceptable limits for potential clinical treatment.

Accepted 14 April 1976.

It is hoped that the results might contribute to selecting optimum sequential application of heat–X-ray or heat–chemotherapy.

MATERIAL AND METHODS

Cell culture

Chinese hamster lung cells (line V 79, obtained from Dr. R. B. Painter, San Francisco) were grown as monolayer cultures on plastic tissues culture dishes in Eagle's basal medium supplemented with 15% foetal calf serum (Gibco Biocult, BCL 005a) and neomycin sulfate (0·1 g/l). Exponentially growing cultures were obtained by plating cells at a density of 1×10^4 cells/cm^2 and letting them proliferate to about 4×10^4 cells/cm^2. For the studies on plateau phase cultures cells were incubated at 37°C without exchanging medium until they attained inhibition of growth at approximately 3×10^5 cells/cm^2 with at least 65% of the cells being in a G_1-like phase of the cell cycle [7].

Experimental procedures

Cell monolayers were exposed to an elevated temperature of 42°C by placing the culture flask into a waterbath temperature of which was kept constant within ± 0·1°C. Immediately before heat exposure fresh medium was applied to all cultures to avoid differences in the thermal

sensitivity caused by depletion of nutritional factors [4]. After different periods of heat treatment (up to 4 hr) cells were either immediately trypsinized using 0·05% trypsin or the culture flasks were returned to 37°C for various periods and trypsinized afterwards. Both clonogenity and the number of progeny cells were determined from the obtained single cell suspensions as described in full detail elsewhere [7].

To determine the effect of heat on cell population kinetics during and after exposure to 42°C the changes in the DNA-distribution of the cell population were analysed by flow-microfluorometry with the "Cytofluorograf" (Model 4801; Bio/Physics Instr.) coupled to a multichannel analyzer. For that purpose single cell suspensions were washed with tris buffer, fixed in ethanol (70%) for 30 min and treated with RNase (0·1% in tris buffer) for 1 hr at 37°C. After washing once more with tris buffer cells were stained with ethidium bromide (0·01 mg/ml). Under these conditions, the amount of dye is proportional to the DNA content of the cells [8]. The distribution of fluorescence intensity of the nuclear DNA (usually of $2–5 \times 10^4$ cells) was displayed as conventional histograms (cell number versus fluorescence intensity). The percentage of cells in the individual phases of the cell cycle (G_1, S, G_2+M) was calculated from the area under the DNA histograms assuming a rectangular distribution of the S-cells between the G_1 and G_2-peak [9]. The reproducibility of independent experiments was better than ± 3%.

RESULTS

Figure 1 demonstrates a marked difference for survival of exponentially growing cells compared to plateau phase cells after the same exposure to 42°C. While survival of plateau phase cells decreases to 45%, only 3% of the exposed exponentially growing cells survive 4 hr of heat treatment. Figure 2 compares growth curves of exponentially growing cell cultures either untreated (control) or previously exposed to 42°C for 30 and 240 min, respectively. Shorter hyperthermic treatment (30 min) leads, after a mitotic lag period of 4 hr to a synchronisation effect on cell growth, clearly demonstrated by the stepwise increase of cell number; 240 min at 42°C results in an extension of the mitotic lag to about 24 hr; 48 hr later, however, the growth rate is normalized. Figure 3 shows the results of a cytofluorometric analysis on cells during and after heating.

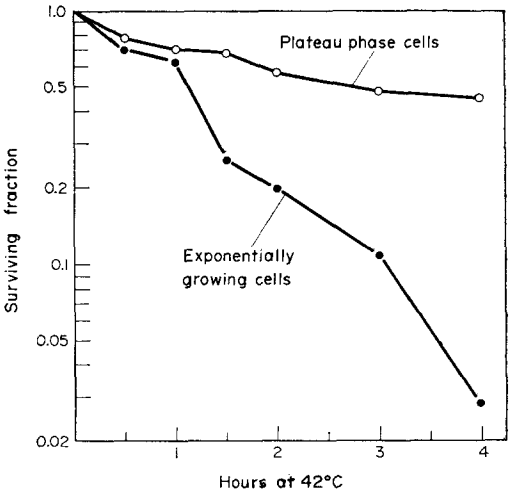


Fig. 1. Survival of exponentially growing and unfed plateau phase V 79 cell, exposed for various times to 42°C.

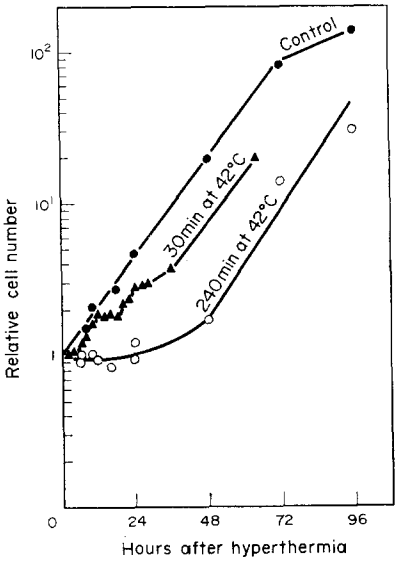


Fig. 2. Growth curves of exponentially growing V 79 cells after hyperthermic treatment.
● *control cells*
▲ *cells exposed for 30 min to 42°C*
○ *cells exposed for 240 min to 42°C*

The percentages of cells in the individual phases (G_1, S, G_2+M) are plotted against the time of treatment. During heat exposure we find a depletion of G_1-cells indicating a block in G_2+M. (see Fig. 4, second cytofluorogram) The accumulation of cells in G_2+M is only moderate because cells are arrested already in S-phase as evident from the strongly increasing level of S- phase cells. Since these cell samples also show a pronounced loss of clonogenity (see Fig. 1) we may conclude that cells die during S-phase. This conclusion presupposes, of course, that the staining is sufficiently gently not to prevent the dead cells from being

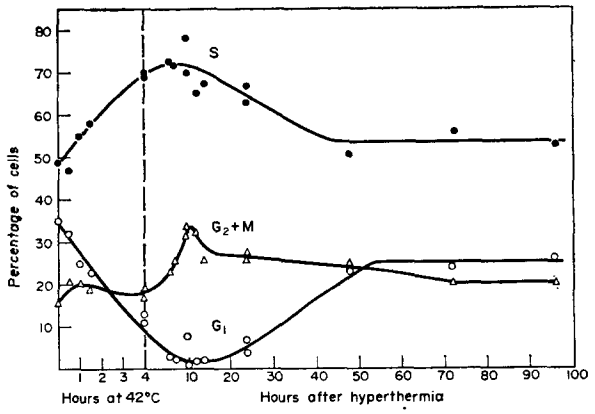


Fig. 3. Variation of the DNA-distribution with time during and after heat exposure (4 hours at 42°C)
● *Cells in S-phase*
○ *cells in G_1-phase*
△ *cells in G_2+M-phase*

recorded by cytofluorometry [10]. Cell cultures returned to 37°C show 10 hr after heating a pronounced increase of cells in the G_2+M-phase and reach normal DNA-distribution values about 48 hr later, a time at which dead cells degrade and are no longer recorded within the normal distribution (cf. Lücke-Huhle and Dertinger, manuscript in preparation).

Though treatment of 4 hr at 42°C gives satisfactory results for cell kinetical examinations *during* heat exposure, the survival rate of 3% is too low for studying the proliferation kinetics *following* heat treatment. We therefore used a lower exposure time of 1 hr (at 42°C) also which led to not more than 35% cell killing. A selection of cytofluorograms (analog print-outs from the multichannel analyzer) illustrating the changes on proliferation kinetics with the time after heating is presented in Fig. 4. The DNA-distributions exhibit a partial synchronisation of cell growth due to a block in S and G_2+M. Figure 5 shows the results in more detail: The frequencies of the individual phases of the cell cycle are plotted against the time after hyperthermia. Four hours after the return to 37°C the block in S is released and cells enter the G_2+M phase causing a steady increase in that fraction of cells for the next 10 hr reaching maximal values of 61%. This accumulation of cells is accompanied by a decrease in G_1 and S populations an indication for the block in G_2+M. The release of that block at 10 hr after heating generates a wave of synchronous cells which repopulates the G_1-compartment to a first maximum at 14 hr and a second peak at 30 hr after hyperthermia.

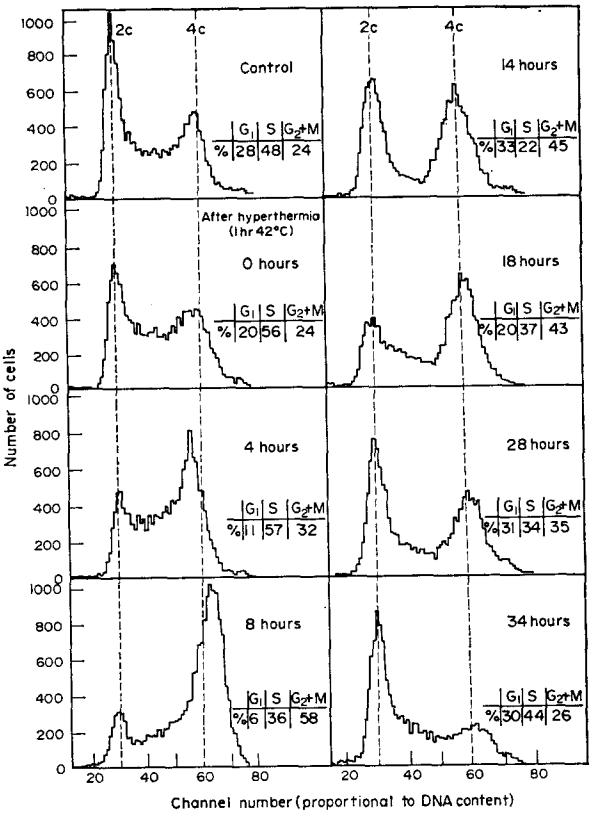


Fig. 4. DNA-histograms of cells after different periods of time following hyperthermic treatment (1 hour at 42°C).

The time interval between the two G_1-peaks gives us the generation time for the second division after hyperthermic treatment, amounting to 16 hr instead of 11 hr for the control cells. The wave of synchronization is not equally clear cut in the S and G_2+M compartments possibly due to dead cells contaminating results. Beyond 36 hr DNA-distributions approximate that of the control.

DISCUSSION

The results presented in this paper demonstrate that heat treatment causes a block in S and G_2+M; but, while the block in G_2+M is transient (10 hr after hyperthermia cells proceed into G_1) most of the cells blocked in S die in that phase. This conclusion is supported by the finding that the accumulation of cells in S-phase during 4 hr at 42°C (cf. Fig. 3) corresponds to a drastic loss in clonogenity as demonstrated in Fig. 1. After a mild heat treatment (1 hr at 42°C: 70% survivors) the release of the G_2+M block leads to a partial synchronization of cell growth (Fig. 5). Evidently, exact knowledge of its amount for a tumor cell population would greatly facilitate

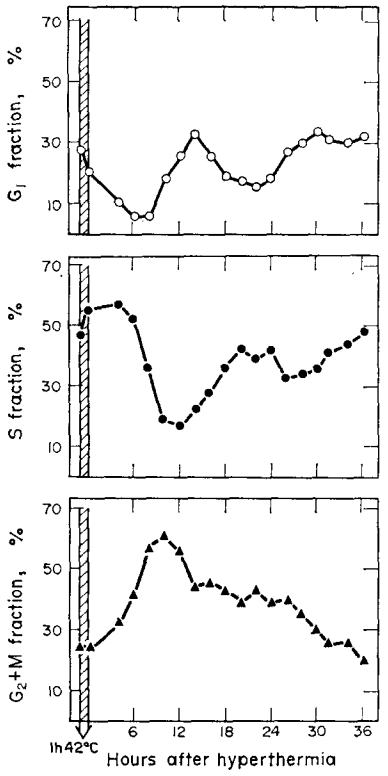


Fig. 5. Percentages of V 79 cells in G1, S and G_2+M phases as a function of time after hyperthermic treatment (1 hr at 42°C).

● *cells in S-phase*
○ *cells in G_1-phase*
△ *cells in G_2+M-phase*

the design of optimum schedules for combined treatment. This synchronizing effect of hyperthermia is certainly one of the synergistic factors which influence a following X-ray therapy.

After a more rigorous treatment (4 hr at 42°C) cell killing in S-phase is the major effect (survival 0·03). The unchanged kinetics of G_1-cells during heating and after the return to 37°C confirms the relative thermal resistance of that cell fraction which also seems to be responsible for the higher survival rate of heat exposed plateau phase cultures. Figure 1 clearly illustrates that the effect of hyperthermic treatment exhibits a strong dependency on the state of growth in which the cell cultures were exposed to heating: The survival of exponentially growing cells is much lower than that for plateau phase cells. This agrees well with the findings of Palzer and Heidelberger [2] and Westra and Dewey [3], that S-phase cells are the most sensitive cell fraction to heat shock. Thus we may conclude that in our experiments mainly S-phase cells are killed and that the apparent differences in sensitivity between plateau phase cells and exponentially growing cells is due to the difference in the frequency of S-cells.

Similar dose–effect curves have been reported by Kase and Hahn [6] for Wi 38 cells. In contrast to the just mentioned results, however, are the findings of Schulman and Hall [5] and Hahn [4] who found V 79 cells and HA1 cells, respectively, exposed to 43°C to be more sensitive in the plateau phase of growth than in an exponentially growing culture. This discrepancy could result from differences in the oxygen supply of the plateau phase cultures. Therefore, special care was taken in our experiments to provide a sufficient supply of oxygen and nutrients during hyperthermia (see material and methods), since the greater thermal sensitivity of hypoxic cells has been described [4, 5].

The results of our cytofluorometrical analysis of V 79 monolayer cells are in good agreement not only with the findings of Kal *et al.* [11] who describes a similar kinetical response to hyperthermia for murine sarcoma cells (EMT 6) but also with own experiments on V 79 spheroids (Lücke-Huhle and Dertinger, manuscript in preparation). The exponentially growing cells of the outer shell of such spheroids show the same changes in proliferation kinetics after hyperthermic treatment as found for the monolayer cultures in spite of an important difference: A much higher thermo-resistance caused by the three-dimensional contact.

In conclusion, the cell kinetics following heating suggest, that hyperthermia may be a useful adjunct to radiotherapy since the cells most resistant to conventional X-ray therapy (S-phase cells) are sensitive to heat and the synchronization effect might probably be used to hit the cycling tumour cells in their most radiosensitive phase. For the same reason combined treatment by heat and conventional X- or γ-rays might eventually compete successfully with therapy by high LET-radiations such as neutrons or π^--mesons.

Acknowledgements—The authors wish to thank Prof. K. G. Zimmer for critically reading the manuscript and Mrs. Katharina Roth for skillful technical assistance.

REFERENCES

1. F. Dietzel, *Tumor und Temperatur.* Urban & Schwarzenberg, München–Berlin–Wien 1975.
2. R. J. Palzer and Ch. Heidelberger, Influence of drugs and synchrony on the hyperthermic killing of HeLa cells. *Cancer Res.* **33,** 422 (1973).
3. A. Westra and W. C. Dewey, Variation in sensitivity to heat shock during the cell-cycle of Chinese hamster cells *in vitro*. *Int. J. Rad. Biol.* **19,** 467 (1971).
4. G. M. Hahn, Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment. *Cancer Res.* **34,** 3117 (1974).
5. N. Schulman and E. J. Hall, Hyperthermia: its effect on proliferative and plateau phase cell cultures. *Radiology* **113,** 209 (1974).
6. K. Kase and G. M. Hahn, Differential heat response of normal and transformed human cells in tissue culture. *Nature* (*Lond.*) **225,** 228 (1975).
7. H. Dertinger and Ch. Lücke-Huhle, A comparative study of post-irradiation growth kinetics of spheroids and monolayers. *Int. J. Rad. Biol.* **28,** 255 (1975).
8. J.-B. Le Pecq and C. Paoletti, A fluorescent complex between ethidium bromide and nucleic acids. *J. mol. Biol.* **27,** 87 (1967).
9. H. Baisch, W. Göhde and W. A. Linden, Analysis of PCP-data to determine the fraction of cells in the various phases of cell cycle. *Rad. and environm. Biophys.* **12,** 31 (1975).
10. Ch. Lücke-Huhle, Proliferation-dependent cytotoxicity of diethylenetriaminepentaacetate (DTPA) *in vitro*. *Health Physics* in press (1976).
11. H. B. Kal, M. Hatfield and G. M. Hahn, Cell cycle progression of murine sarcoma cells after X-irradiation or heat shock. *Radiology* **117,** 215 (1975).

Europ. J. Cancer Vol. 12, pp. 833–837. Pergamon Press 1976. Printed in Great Britain

Hepatic and Tumor Proteins Reactive with Tyrosine Transaminase—Specific Rabbit Antiserum*

GEORGE P. TRYFIATES and FRANK L. SAUS
Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, *U.S.A.*

Abstract—*Availability of dietary pyridoxine has been shown to affect tumor growth as well as expression of enzymatic activity. Whether pyridoxine influences the expression of hepatic and tumor tyrosine transaminase (TT) was investigated in the present study by a combination of electrophoretic and immunological procedures. Buffalo strain female weanling rats were fed ad libitum a pyridoxine free diet or pair-fed the same diet with pyridoxine added. After* 21 *days they were inoculated with No.* 7777 *Morris hepatoma cells and sacrificed* 14 *days later* 4 *hr after the i.p. administration of hydrocortisone hemisuccinate. High speed liver and tumor supernatants were electrophoresed on polyacrylamide gels and TT bands were subsequently immunoprecipitated in agar-gel using rabbit antiserum specific to highly purified rat liver tyrosine transaminase. In addition, Ouchterlony tests were also performed using the same preparations. The results obtained were as follows: Four and* 5 *gel protein bands were immunoprecipitated from liver supernatants of hormonally induced animals fed the supplemented and pyridoxine free diets, respectively. Hepatomas grown in animals fed the vitamin free and supplemented diets developed* 3 *and* 4 *precipitation bands, respectively. The immunoprecipitation pattern of the hepatoma was also distinctly different than that of normal liver. Additional analyses by the technique of double diffusion in agar gel (Ouchterlony) showed three precipitation lines developed with samples from either deficient or control animals. Hepatoma samples from control animals (B_6 plus) showed three precipitation lines while those from deficient animals showed an altered precipitation pattern and only two bands. Our results show that (a) in hydrocortisone treated animals, lack of dietary pyridoxine induced in liver an extra protein species reactive with transaminase antiserum while the appearance of an antitransaminase reactive protein was inhibited in the hepatoma, and (b) an immunoprecipitation pattern peculiar to this tumor tissue.*

INTRODUCTION

IT HAS been reported that soluble cytoplasmic liver transaminase (TT) (L-tyrosine: 2-oxoglutarate aminotransferase, E.C. 2.6.1.5) consists of three or four isozyme proteins [1–4]. In addition, the response of this pyridoxal phosphate requiring enzyme following the administration of hydrocortisone to normal animals is altered in the absence of dietary pyridoxine [5, 6]. Further, the hormonal enzyme induction pattern in the presence of growing hepatoma No. 7777 is highly altered and after seven days of tumor growth the rise in activity is considerably diminished [7]. These observations suggested synthesis of altered transaminase proteins and/or alteration(s) of these after synthesis both during the absence of dietary pyridoxine or the presence of the developing hepatoma. In this study soluble liver and tumor proteins were tested for immunoreactivity using antiserum to highly purified hepatic tyrosine transaminase in attempts to decipher differences in antigenic reactivity brought about by lack of vitamin B_6 or by the growing hepatoma.

Accepted 13 April 1976.

*This investigation was supported by USPHS grant CA 13759 from the National Cancer Institute.

MATERIAL AND METHODS

Buffalo strain female rats weighing approximately 50–60 g were purchased from Simonsen Laboratories, Gilroy, California and handled

as reported previously [6]. Animals were either fed a pyridoxine free diet ad libitum or pair-fed the same diet with pyridoxine added [6, 8]. Following a 21 day feeding period [9], animals were inoculated intramuscularly with Morris hepatoma No. 7777 cells [6–9] and were sacrificed three weeks later prior to and 4 hr after the administration of hydrocortisone [10]. Average tumor weights were as reported previously [8]. Livers and tumors were removed, homogenized and high speed supernatants obtained by centrifugation at $105{,}000 \times g$ for 1 hr and 4°C. Liver tyrosine transaminase was purified according to the procedure of Valeriote *et al.* [3]. The final enzyme preparation was $3000x$ fold purified. The purified enzyme was electrophoresed on polyacrylamide gels [11], protein bands were stained with Amido Schwartz and after destaining in 7% acetic acid gels were scanned at 600 nm in a Gilford Model 2410 Linear Transport System. Figure 1 shows the gel scan of the purified transaminase. Transaminase-specific rabbit antiserum was prepared as described previously [12] using this purified enzyme preparation.

Gel electrophoresis and immunoprecipitation

High speed liver and tumor supernatants were made 2×10^{-4} M, 1×10^{-3} M and 1×10^{-3} M with pyridoxal-5-phosphate, β-mercaptoethanol and EDTA, respectively, to protect the transaminase from inactivation [1, 3]. These were electrophoresed on polyacrylamide gels by the method of Davis [11]. Gels were cast in 0·5 cm in diameter glass tubes. The running gel was 7·5% acrylamide, 4·5 cm long and the spacer gel 2·5% and 1 cm long. Samples of 100 μg of Lowry protein were electrophoresed at 2°C for 3 hr and 2 ma per gel in a Biorad Model 150 electrophoresis cell. The electrophoresis buffer was 5 mM Tris–HCl, 38 mM glycine, pH 8·4. Immunodiffusion plates were prepared as follows: Clean glass plates 105 mm × 85 mm × 3 mm were precoated with agar by immersing in 0·1% hot Noble agar solution, quickly removed and allowed to dry at 25°C. The dry precoated plates were placed on a level surface and unto each were pipetted 16 ml of a solution consisting of 2% Noble agar, water (dist) and 63 mM barbital buffer, pH 8·2 in a 2:1:1 (v/v/v) ratio, respectively. Plates were stored in a cool, wet chamber and used within 24 hr. Following electrophoresis, spacer gels were removed and each running gel was carefully placed inside a precut trough on the plate with approximately the dimensions of the running gel. Gels were sealed into position using cool (45°C) 0·1% agar solution. Plates with positioned gels were placed in a cold (4°C) moist chamber overnight to allow sufficient time for diffusion of electrophoresed protein species outward from the gel into the agar. Antiserum (antitransaminase) was afterwards placed into a trough running parallel positioned gels. Immunoprecipitation occurred within 24 hr at 4°C in the moist chamber. Results reported here are from plates developed for 48–55 hr.

Analyses by the technique of double diffusion in agar gel were performed as described previously [12].

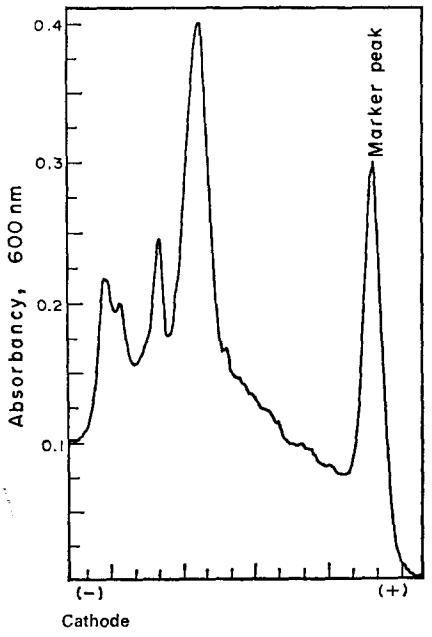


Fig. 1. *Gel electrophoretic pattern of highly purified soluble rat liver tyrosine transaminase. Approximately* 30 μg *protein were electrophoresed (see Material and Methods section). This pattern was repeated several times.*

RESULTS

Figure 1 shows a gel scan pattern of highly purified soluble rat liver tyrosine transaminase following electrophoresis in polyacrylamide gel. Four distinct protein peaks were repeatedly resolved.

Figure 2 shows the immunoprecipitation lines developed with tyrosine transaminase-specific rabbit antiserum and reacting soluble liver protein species following their separation by gel electrophoresis. Patterns of cytosol proteins from pyridoxine deficient and control animals after induction with hydrocortisone are shown. Five and 4 precipitation lines were developed by samples derived from the deficient and control animals respectively.

Figure 3 shows precipitation patterns ob-

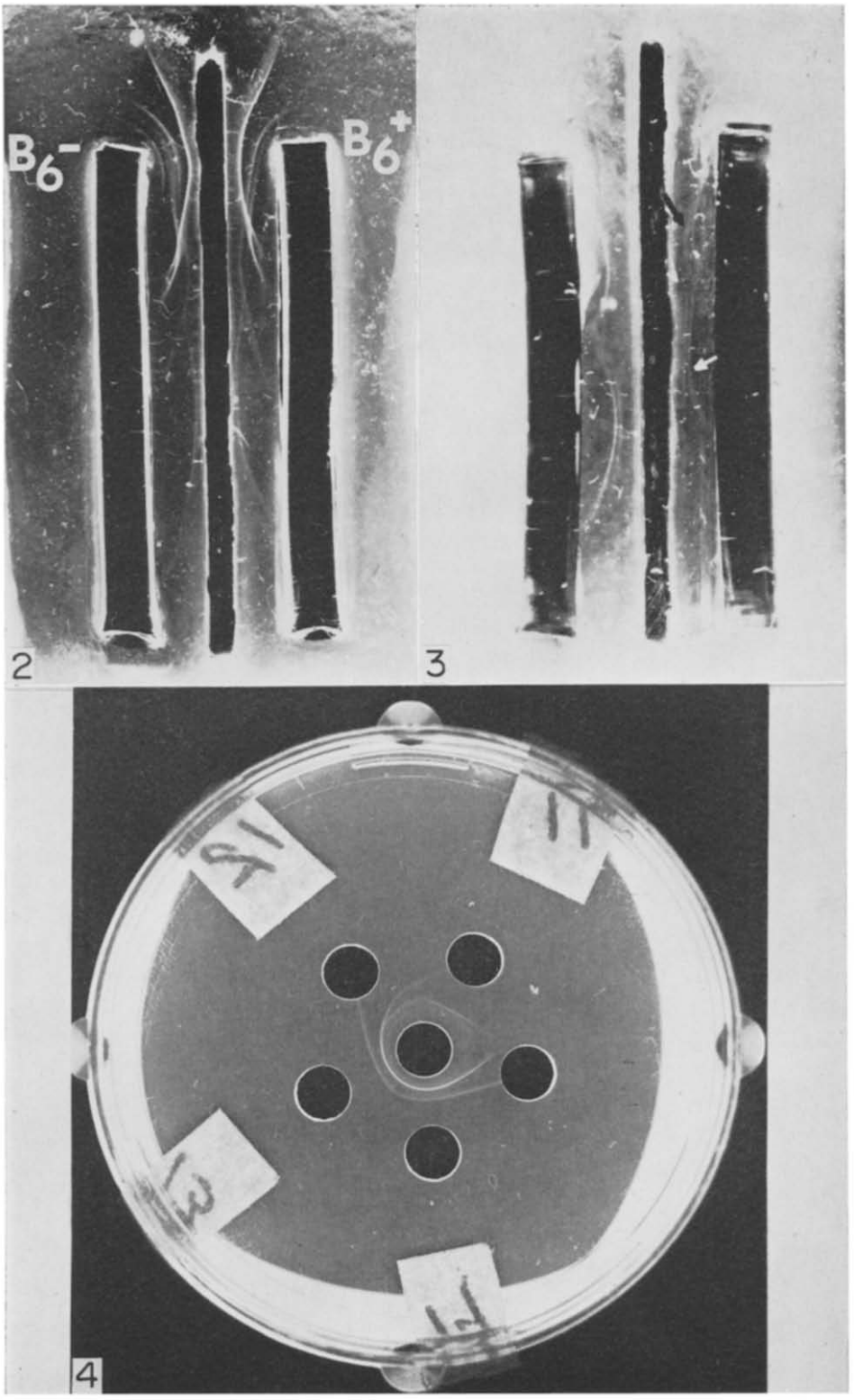


Fig. 2. *Photograph showing immunoprecipitation patterns developed with tyrosine transaminase-specific rabbit antiserum and gel electrophoresed soluble, reacting, liver protein species. Other details in Material and Methods section.*

Fig. 3. *Photograph showing immunoprecipitation patterns developed with tyrosine transaminase-specific rabbit antiserum and gel electrophoresed soluble, reacting, hepatoma proteins. Patterns on the left and right were developed by tumors grown in deficient and control animals, respectively. Other details as in figure* 2.

Fig. 4. *Photograph of a double-diffusion analysis test (Ouchterlony). The immunoprecipitation patterns between tyrosine transaminase-specific rabbit antiserum and soluble protein species from liver and hepatoma from control and vitamin* B_6 *deficient animals are shown. Antiserum was placed in the center well. Wells numbered* 11–14 *each contained* 200 *μg of Lowry protein* [13]. *Number* 11 *is hepatoma No.* 7777 *grown in vitamin* B_6 *deficient animals; Number* 12, *same as No.* 11 *but from control animals;* 13 *and* 14 *are liver samples from deficient and control animals, respectively.*

(to face page 834)

tained with soluble Morris hepatoma No. 7777 protein species from pyridoxine deficient and control, hydrocortisone-treated animals. Three and 4 immunoprecipitation lines were developed by hepatoma samples from vitamin B_6 deficient and control (non-deficient, see arrows) animals, respectively.

Figure 4 is a photograph of a double-diffusion analysis test (Ouchterlony). The immunoprecipitation patterns between tyrosine transaminase-specific rabbit antiserum and soluble protein species from liver and hepatoma grown in control and vitamin B_6 deficient animals are shown. Antiserum was placed in the center well. Wells numbered 11–14 each contained approximately 200 μg of Lowry protein [13]. Number 11 is hepatoma No. 7777 grown in vitamin B_6 deficient animals; No. 12, same as No. 11 but from control animals; 13 and 14 are liver samples from deficient and control animals, respectively. Only three and two immunoprecipitation lines were developed by liver and tumor samples, and from tumor samples grown in vitamin B_6 deficient animals, respectively.

DISCUSSION

Absence of pyridoxine from the diet of rats is known to affect the specific activity levels of liver tyrosine transaminase and serine dehydratase of hydrocortisone treated or untreated animals [5, 6, 10, 12, 14]. The transaminase hormonal induction pattern is also characterized by the absence of a lag period [12] and by a steady rise in activity with progressive vitamin deficiency [6]. The presence of growing hepatoma No. 7777 on the other hand, results in a completely altered time–course induction pattern in liver which is similar to that seen in the tumor [7]. The exercise of a regulatory control over the expression of enzymatic activity by vitamin B_6 was suggested previously [6, 12, 14]. The results presented here support this conclusion. Lack of availability of dietary pyridoxine resulted in the appearance of an additional protein reactive with tyrosine transaminase-specific antiserum in liver cytoplasm. Whether this anti-transaminase reactive protein species is peculiar to deficiency or is also formed in control rats but is immunologically unreactive is not known. Polyacrylamide gel electrophoresis tests of partially purified hepatic tyrosine transaminase from pyridoxine deficient rats showed additional resolution of the enzyme (results not shown here). Similar tests using preparations from control animals, however, showed only four protein bands (Fig. 1). Valeriote *et al.* [3] found 4 or possibly 5 pyridoxal PO_4 moles bound per molecule of purified, native hepatic tyrosine transaminase of which one was not removed by exhaustive dialysis of the enzyme against a buffer system previously reported to completely resolve the coenzyme from the protein [1]. It is possible that the extra mole of the tightly bound cofactor plays a key role in holding the transaminase isozymes together in the native state and/or an extra protein which may be needed for this purpose. During cofactor depletion (i.e., deficiency), however, the tightly bound cofactor may be rendered labile resulting in further resolution of the native enzyme. To our knowledge other reports on the facilitation of enzyme (protein) resolution by lack of dietary vitamin B_6 in liver have not appeared.

Attempts to decipher differences between cytoplasmic liver and tumor proteins reactive with transaminase antiserum showed that absence of dietary pyridoxine resulted in the appearance of only three tumor immunoreactive proteins. It appears that the influence of lack of dietary pyridoxine in this hepatoma is opposite to that seen in liver. How pyridoxine deficiency produces these effects is presently not known. Other results from this laboratory showed, in addition, inhibition of the growth of this and other Morris hepatomas by lack of dietary pyridoxine [8, 9]. It is possible that inhibition of tumor growth may result from alteration(s) in protein structure and/or expression of enzymatic activity [5, 6, 9, 14] requiring pyridoxal phosphate for catalysis.

Results of Ouchterlony tests support those obtained by gel electrophoresis–immunoprecipitation analyses. An immunoprecipitation pattern peculiar to the tumor was obtained by these methods. However, only three and two immunoprecipitation lines were developed by liver and tumor samples, and from tumor samples grown in vitamin B_6 deficient animals, respectively. Protein resolution is limited in these tests which employ only passive diffusion in agar-gel. The high degree of resolution obtained by prior electrophoresis of proteins in polyacrylamide gels is not comparable to that seen by passive protein diffusion. It is also possible that the high protein content of the tested samples may be responsible for some immunoreactive protein species to run together. Albeit this, Ouchterlony tests show only two bands and a peculiar tumor pattern developed by tumors from deficient animals. Boctor and

Grossman [15] as well as ourselves [12] have reported the development in agar-gel (Ouchterlony) of only one precipitation line by the interaction of high speed liver supernatant and rabbit antiserum to highly purified tyrosine transaminase. However, in our hands, a reduced number of immunoprecipitation lines is developed when tested supernatants do not contain enough protein (i.e. diluted) or tests are not performed in excess of antibody. Kenney [16] has also reported the development of several immunoprecipitation lines by interaction of liver supernatant and antiserum to highly purified transaminase. Further, Johnson *et al.* [17] reported a transaminase purification method using a CM-Sephadex column which results in the isolation of three highly purified enzyme forms (isozymes). When tested against transaminase antiserum one continuous precipitation line was developed by each enzyme form [17]. It follows then that separation by gel electrophoresis of transaminase forms followed by immunoprecipitation (this report) will show as many precipitation lines as the separated reactive protein species. It should also be mentioned that several attempts in our laboratory to reproduce the purification chromatographic procedure of Johnson *et al.* [17] failed repeatedly. Instead of the reported three activity peaks (forms) [17] several were eluted with maximal purification 10 × less than the one reported by these workers [17].

Acknowledgements—Thanks are due to Sister Ernestine Davis for typing the manuscript. We also wish to thank Dr. H. P. Morris for supplying the donor animal and continued interest in this work.

REFERENCES

1. S. Hayaski, D. K. Granner and G. M. Tomkins, Tyrosine aminotransferase. Purification and characterization. *J. biol. Chem.* **244,** 3998 (1967).
2. G. P. Tryfiates, On the synthesis of tyrosine transaminase *in vitro*. *Biochim. biophys. Acta.* **174,** 779 (1969).
3. F. A. Valeriote, F. Auricchio, G. M. Tomkins and D. Riley, Purification and properties of rat liver tyrosine aminotransferase. *J. biol. Chem.* **244,** 3618 (1969).
4. H. C. Pitot, Y. Iwasaki, H. Inone, C. Kasper and H. Mohrenweiser, Regulation of the levels of multiple forms of serine dehydratase and tyrosine aminotransferase in rat tissues. *Gann* **13,** 191 (1972).
5. G. P. Tryfiates, Hormonal and cofactor induction of tyrosine transaminase in vitamin B_6 deficiency. *Life Sci.* **10,** 1147 (1971).
6. G. P. Tryfiates and F. L. Saus, Hormonal induction patterns of tyrosine aminotransferase in normal and cofactor depleted animals. *Cancer Biochem. Biophys.* **1,** 63 (1975).
7. G. P. Tryfiates, F. L. Saus and H. P. Morris, Hormonal induction of tyrosine aminotransferase activity in host liver and hepatoma No. 7777 of normal and cofactor depleted animals. *J. nat. Cancer Inst.* **55,** 839 (1975).
8. G. P. Tryfiates and H. P. Morris, Effect of pyridoxine deficiency on tyrosine transaminase activity and growth of four Morris hepatomas. *J. nat. Cancer Inst.* **52,** 1259 (1974).
9. G. P. Tryfiates, J. K. Shuller, M. H. Hefner and H. P. Morris, Effect of B_6 deficiency on hepatoma 7794A growth rate: Activities of tyrosine transaminase and serine dehydratase before and after induction by hydrocortisone. *Europ. J. Cancer* **10,** 147 (1974).
10. G. P. Tryfiates, J. K. Shuller and H. P. Morris, Enzyme activities in Vitamin B_6 deficient, normal and tumor-bearing animals: Effect of hydrocortisone. *Proc. Soc. exp. Biol. (N.Y.)* **145,** 1363 (1974).
11. B. J. Davis, Disk electrophoresis: II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).
12. T. Puskar and G. P. Tryfiates, Induction of tyrosine transaminase by hydrocortisone in vitamin B_6 deficient rats. *J. Nutr.* **104,** 1407 (1974).
13. O. H. Lowry, N. H. Rosebrough, A. L. Far and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
14. G. P. Tryfiates, Effect of pyridoxine availability on the activity of serine dehydratase of normal liver, host liver and three Morris hepatomas. *J. nat. Cancer Inst.* **54,** 171 (1975).
15. A. Boctor and A. Grossman, Alterations of tyrosine aminotransferase turnover in rat liver following glucocorticoid administration. *J. biol. Chem.* **245,** 6337 (1970).

16. F. T. KENNEY, Induction of tyrosine-α-ketoglutarate transaminase. *J. biol. Chem.* **237,** 1610 (1962).
17. R. W. JOHNSON, L. E. ROBERSON and F. T. KENNEY, Regulation of tyrosine aminotransferase in rat liver. *J. biol. Chem.* **218,** 4521 (1973).

Europ. J. Cancer Vol. 12, 839–845. Pergamon Press 1976. Printed in Great Britain

Effects of Methotrexate on Deoxyribonucleotide Pools and Nucleic Acid Synthesis in Human Osteosarcoma Cells*

LAMBERT SKOOG, BO NORDENSKJÖLD, SOLVEIG HUMLA and TORSTEN HÄGERSTRÖM

Departments of Biochemistry and Radiumhemmet, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Abstract—*Six-hr intravenous infusion of 14 g of methotrexate to patients with osteosarcoma resulted in 0·9 and 6 mM concentration of the drug in serum and urine, respectively. After discontinuation of the infusion the serum concentration decayed with an initial half life of two hours. A second longer half life of the drugs was also observed and between day three and day seventeen the serum concentration fell from 100 to 20 nM.*

Two concentrations (3 and 100 nM) were selected for an investigation of the effect of methotrexate on deoxyribonucleoside triphosphate pools and nucleic acid biosynthesis in cultured human osteosarcoma cells.

Incubation in 3 nM of the drug resulted in a transient decrease of the pools of dTTP and dGTP and in a delayed biosynthesis of DNA. A one hundred nM concentration of the drug resulted in low levels of the dTTP and dGTP pools and completely inhibited DNA and RNA synthesis.

During incubation in 3 nM and 100 nM drug the intracellular concentration of drug was higher than that contained in medium indicating an active transport of the drug.

We also compared the effect of Methotrexate on ^{3}H-deoxyuridine incorporation into osteosarcoma cell DNA and into DNA from bone marrow aspirates. Both 3 and 100 nM drug inhibited isotope incorporation into osteosarcoma cultures while 100 nM drug was required to inhibit incorporation into bone marrow aspirates.

INTRODUCTION

ANTIMETABOLITES specifically interfering with DNA replication are very schedule-dependent and are best applied at intervals with a dose which is high enough to kill the cells replicating DNA [1]. Methylaminopterin (methotrexate) interferes with *de novo* purine synthesis and also with thymidine monophosphate synthesis [2]. As a consequence, methotrexate inhibits both RNA and DNA synthesis. In spite of this, methotrexate has recently been infused in high dose (grams) at fixed intervals [3]. Using such a schedule remarkable results were reported on the treatment of human osteosarcoma [4].

We have measured serum levels of methotrexate in patients treated for metastazied osteosarcoma and have performed an *in vitro* investigation of the mechanism of action of methotrexate. We also compared the methotrexate sensitivity of bone marrow cells and osteosarcoma cells.

MATERIAL AND METHODS

Patients

A man age 32 and a boy age 16 with lung metastases from osteosarcoma were treated with 6 hr infusions of 200 mg of methotrexate per kg and a third patient age 67 with mammary

Accepted 5 April 1976.

*This work was supported by the Swedish Cancer Society, Magnus Bergvalls stiftelse and Karolinska Institutet.

carcinoma received a total of 1 g during 24 hr as described in the legend of Fig. 1. Serum samples were obtained at indicated intervals from the arm not used for infusions of methotrexate.

Folinic acid (Leucovorin) (18 mg) was given every six hours starting 2 hr after the end of infusion and continued for 2–7 days.

Assay of methotrexate

Methotrexate contained in serum or cell homogenates was measured enzymatically by monitoring the inhibition by methotrexate of dihydrofolate reductase activity [5]. Dihydrofolate reductase was purified from calf thymus as described by Bertino [6]. Dihydrofolate was synthesized by the method of Futterman [7].

Osteosarcoma cells

An established culture of human osteosarcoma cells (2 OS) [8] was obtained from Dr. Jan Pontén and kept in Eagles MEM with 10% dialyzed fetal bovine serum. Confluent cells were subcultured at a density of 1×10^6 cells in 5 cm plastic Petri dishes (NUNC, Denmark) in 4% CO_2 in air at 37°C. Cultures were free from mycoplasma as judged from absence of extranuclear ^{3}H-thymidine incorporation.

Nucleic acid synthesis

The amount of DNA in each culture was measured according to Burton as previously described [9]. RNA was measured colorimetrically with orcinol [10].

The synthesis of nucleic acid was measured by the incorporation of ^{3}H-deoxyuridine (1 μM; 15·0 Ci/mmole) or ^{3}H-thymidine (1 μM; 6·7 Ci/mmole) into acid insoluble material as previously described [11]. When ^{3}H-deoxyuridine incorporation was used the acid insoluble residue was dissolved in 0·3 M KOH and kept overnight at 37°C to hydrolyze RNA. After hydrolysis, the solution was chilled in ice and perchloric acid was added (0·5 M final concentration). The radioactivity in the precipitate (DNA) could then be separated from the radioactivity in the hydrolyzed RNA contained in the supernatant solution.

Bone marrow cells

Sternal bone marrow aspirates were diluted to a concentration of 5×10^5 cells/ml in MEM in Earles salts in 10% nondialyzed serum. The cells were warmed to 37°C for a few hours prior to the start of the experiments. In some experiments stated below red blood cells were lysed by addition to the marrow aspirates of 10% (v/v) of a 0·16 M solution of ammonium chloride. After 10 min of incubation at 0°C the aspirates were washed by centrifugation and treated as described above.

Deoxyribonucleoside triphosphate pools

Deoxyribonucleoside triphosphates were assayed enzymatically as previously described [12, 13].

Chemicals

Methotrexate and Leucovorin were obtained from Lederle and Dihydrofolate was obtained from Sigma. ^{3}H-thymidine and ^{3}H-deoxyuridine were purchased from Amersham.

RESULTS

Serum and urine levels of methotrexate

We measured the methotrexate levels during and after infusion in order to define the serum concentration interval that is reached *in vivo*. Furthermore, to estimate the time period needed for post infusion Leucovorin therapy, we needed information about the period during which potentially toxic levels of methotrexate were present in serum.

Figure 1 demonstrates very high methotrexate levels in both serum and urine during and immediately after infusion of the drug. Between the first and sixth hours of infusion of 200 mg/kg the serum levels increased from 120 to 850 μM. This rate of infusion was thus higher than the rate of excretion. In contrast, during a 24 hr infusion of 1 g of methotrexate no accumulation of drug was observed (Fig. 1).

In accordance with the known renal excretion of methotrexate [14], high drug levels were observed in the urine and a maximum of 6 mM was observed 9 hr after the beginning of infusion.

After infusion the initial half life of methotrexate in serum was 2 hr. Later, the rate of decay decreased and a level of 100 mM was reached about three days after high dose methotrexate infusion and a low level of approximately 10 nM was observed during the third week (not shown).

For the experiments described below we selected a high methotrexate level (100 nM) that the cells were exposed to for a few days *in vivo* and compared it with a low (3 nM) level which was contained in serum for three weeks after the infusion was discontinued.

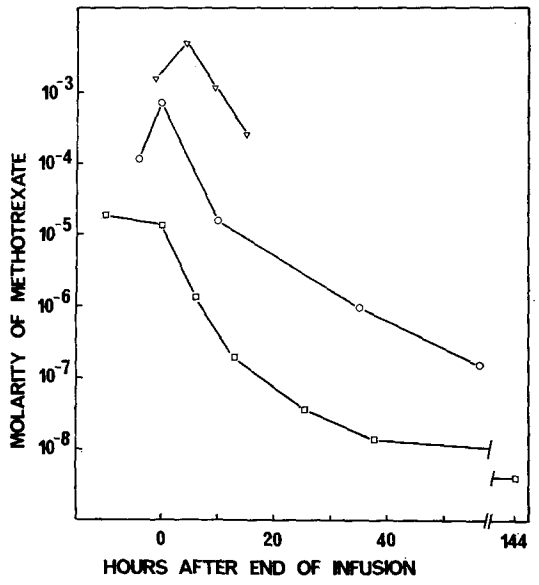


Fig. 1. Serum and urine levels of methotrexate.
A man age 16 received 200 mg of methotrexate per/kg (a total of 14 g) during a 6-hr infusion. Serum (○) and urine (▽) levels were measured.
A woman age 67 with mammary carcinoma received a total of 1 g of methotrexate during a 24 hr intravenous infusion. Serum (□) levels were recorded.

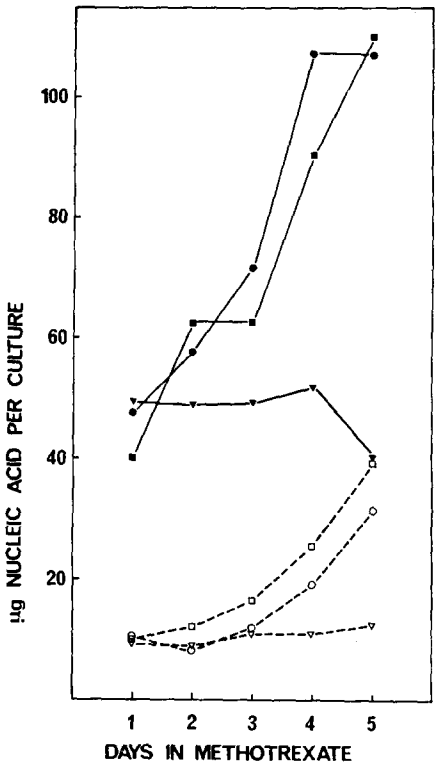


Fig. 2. RNA and DNA synthesis in osteosarcoma cells.
The amount of RNA (solid lines) and DNA (broken lines) was measured in osteosarcoma cultures after incubation in 100 nM (triangles) or 3 nM (circles) methotrexate or after incubation in medium without methotrexate (squares).

Effects of methotrexate on growth of osteosarcoma cultures

The growth of osteosarcoma cultures both in the presence and absence of methotrexate was followed for five days and monitored by measurements of the accumulation of DNA and RNA. Figure 2 demonstrates that 100 nM methotrexate markedly inhibited both DNA and RNA accumulation while 3 nM methotrexate did not affect RNA synthesis and only incompletely inhibited DNA synthesis.

Effects on ^{3}H-deoxyuridine incorporation

In cells incubated in methotrexate *de novo* synthesis of thymidine nucleotides is blocked [15, 16]. ^{3}H-thymidine circumvents this block and may therefore not be used for the measurement of DNA synthesis during incubation in methotrexate. ^{3}H-deoxyuridine can be used as an alternative isotope for incorporation into DNA [16] and Fig. 3 shows a very marked

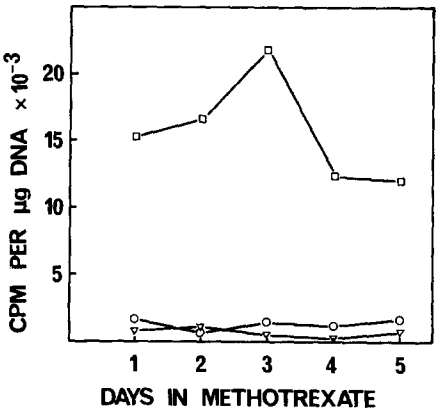


Fig. 3. ^{3}H-deoxyuridine incorporation into DNA.
Cells were incubated in 3 nM (○) or 100 nM (▽) or without methotrexate (□) and ^{3}H-deoxyuridine incorporation was measured during 30 min pulse periods.

inhibition by methotrexate on the incorporation of ^{3}H-deoxyuridine into DNA. The isotope incorporation was decreased approximately 15- and 30-fold at drug concentrations of 3 nM and 100 nM, respectively.

Effects of methotrexate on deoxyribonucleotide pools

Conflicting data were reported on the effect of 50–100 nM methotrexate on HeLa cells [18] and lymphoid cells [15, 16]. In an attempt to resolve this conflict we measured deoxyribonucleoside triphosphate pools in osteosarcoma cells incubated in absence and presence of two concentrations of methotrexate. Figure 4 shows

that with the exception of deoxyadenosine triphosphate (dATP) the deoxyribonucleoside triphosphate pool levels in osteosarcoma cells were similar to those we have defined in other mammalian cells [11, 19, 20]. In agreement with previous data the pool of deoxyguanosine triphosphate (dGTP) was the smallest of the triphosphate pools. The dATP pool was unusually large while the pools of deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) were of intermediate size.

Addition of methotrexate to the cells markedly decreased both dGTP and dTTP pools (Fig. 4). Twenty-four hours after addition

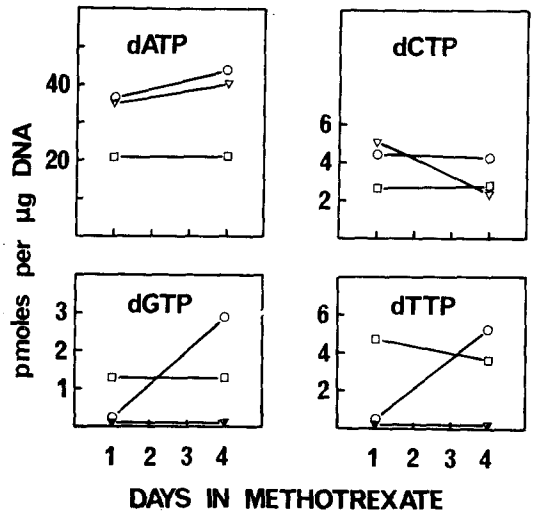


Fig. 4. Deoxyribonucleoside triphosphate pools.
Deoxyribonucleoside triphosphate pools were measured after one and four days of incubation in 3 nM (○) or 100 nM (▽) methotrexate or without drug (□).

of both 3 nM and 100 nM methotrexate the dGTP and dTTP pools were reduced to 10% of their levels in control cells. Prolonged incubation in 3 nM methotrexate resulted in an unexpected expansion of both pools. However, during continued incubation in 100 nM methotrexate dGTP and dTTP levels remained low. This decreased pool of dGTP probably reflects the inhibition of the two tetrahydrofolate requiring steps in the biosynthesis of purines [2].

The pool of dATP doubled during incubation in both concentrations of methotrexate. The dCTP pool initially expanded during incubation in 100 nM methotrexate.

Cellular methotrexate levels

We measured the intracellular concentrations of methotrexate in order to correlate these to the effects observed on nucleic acid synthesis. The results in Fig. 5 indicate no significant variations in intracellular methotrexate concentration during 5 days of incubation in 3 nM drug. Assuming a cell volume of 1500 cu µm and a DNA content of 10 pg per cell the intracellular methotrexate level was calculated to be approximately 700 nM. This intracellular level indicates an active transport of methotrexate into osteosarcoma cells during incubation in 3 nM drug. During incubation in 100 nM methotrexate the cells gradually accumulated

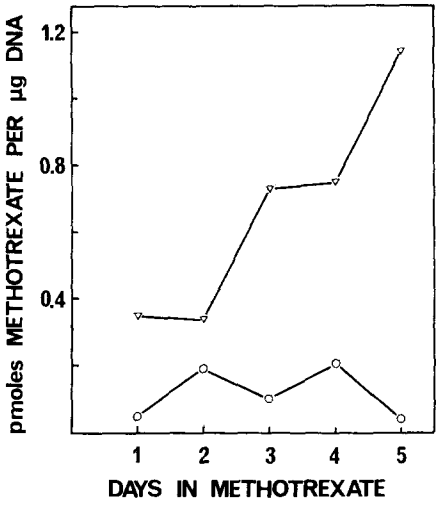


Fig. 5. Intracellular methotrexate.
The amount of cell associated methotrexate was measured at intervals during incubation in 3 nM (○) or 100 nM (▽) methotrexate.

methotrexate and after five days the intracellular drug concentration was approximately 7000 nM indicating the presence of an active transport.

Effects of methotrexate on isotope incorporation into bone marrow cell DNA

Gastrointestinal and bone marrow toxicity limit the use of methotrexate [14]. To compare the effects of the drug on osteosarcoma cells with those on bone marrow cells we measured ^{3}H-deoxynucleoside incorporation into bone marrow aspirates kept in the presence or absence of methotrexate. We used this method rather than measurement of DNA accumulation since bone marrow aspirates contain many non-replicating cells which do contain DNA. DNA measurements are therefore an insensitive method for measuring DNA synthesis in such aspirates.

Figure 6 demonstrates that both ^{3}H-thymidine and ^{3}H-deoxyuridine incorporation proceeds proportional to time during the first 40–80 min after addition of either deoxy-

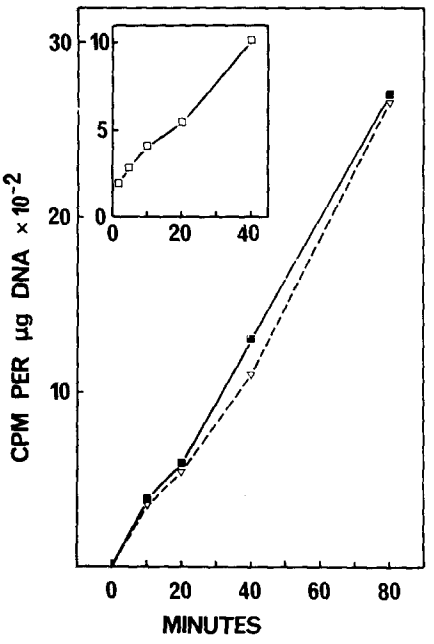


Fig. 6. Isotope incorporation into bone marrow aspirates. We measured ^{3}H-deoxythymidine incorporation (inserted □) and ^{3}H-deoxyuridine incorporation (■) into bone marrow cells. Triangles indicate cultures that received 100 nM methotrexate plus 1 µM ^{3}H-deoxyuridine at time 0.

nucleoside. Moreover, 100 nM of methotrexate added at the same time as ^{3}H-deoxyuridine did not affect isotope incorporation during an 80 min incubation period.

Figure 7 compares ^{3}H-deoxyuridine incorporation 2·5 hr and 24 after addition of 10–1000 nM methotrexate to bone marrow aspirates. Isotope incorporation had decreased after 2·5 hr of incubation in 1000 nM methotrexate and after 24 hr of incubation in 100 nM of the drug.

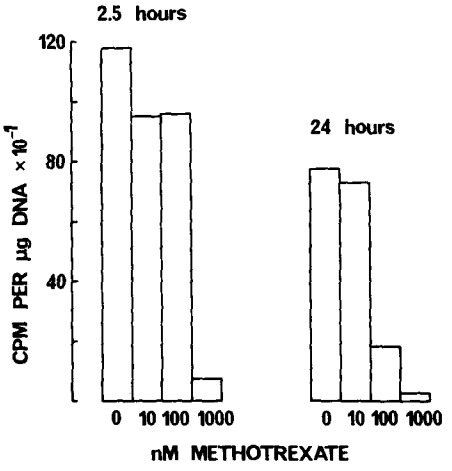


Fig. 7. ^{3}H-deoxyuridine incorporation into bone marrow cultures. ^{3}H-deoxyuridine was added after 2·5 hr or 24 hr of incubation in medium containing 0–1000 nM methotrexate.

DISCUSSION

There is an apparent discrepancy between the low (3 nM) level of methotrexate required to inhibit ^{3}H-deoxyuridine incorporation into DNA in osteosarcoma cells and the high level (100 nM) required to completely inhibit net DNA synthesis. Measurement of ^{3}H-deoxyuridine incorporation thus appears to overestimate the effects of methotrexate on DNA synthesis. Tattersall *et al.* [17] have demonstrated an expanded dUMP pool in methotrexate treated mouse lymphoma cells. dTTP regulates dCMP deaminase which catalyzes the formation of dUMP. During incubation in methotrexate, the dTTP pool depletion activates the enzyme and consequently the endogenous pool of dUMP is expanded. The decreased incorporation of ^{3}H-deoxyuridine during incubation in methotrexate may therefore partly be explained by the dilution of radioactivity into an expanded pool of dUMP formed by *de novo* synthesis.

The success of high dose methotrexate infusions during limited time periods initially suggested to us that methotrexate acted on osteosarcoma cells as a S-phase specific agent [1]. However, our experiments reject such a mechanism. The data on DNA precursor pool levels demonstrate both a decreased dGTP and a decreased dTTP level during incubation in methotrexate. Methotrexate thus decreases both the purine DNA precursor as well as dTTP formation.

The effect of methotrexate on purine synthesis occurs prior to the separation, at the level of ribonucleotide reduction, of the two pathways leading to RNA and DNA, respectively [21]. The antipurine effect of methotrexate indicated by the decay of the dGTP pool probably also explains the effect of methotrexate on RNA synthesis.

In contrast to the decay of dGTP, the pool of dATP increased during incubation in methotrexate. This was also observed by Fridland [16] but only as a transient increase. The expanded dATP pool probably reflects the large pools of adenosine phosphates which the cell can reduce and which accumulate as deoxyadenosine phosphates in the absence of a normal drain via DNA synthesis.

The decay of the dGTP and dTTP pools confirm data obtained with lymphoid cells [15, 16] while the increased pool of dCTP correlates well with the increased dCTP pool observed in mouse lymphoma cells [15]. The decay of dCTP observed by Fridland during incubation in methotrexate [16] has not been

confirmed and may be unique to the lymphoblasts analysed in that study.

The need for high dose treatment of oesteosarcoma with methotrexate suggested to us the possibility that osteosarcoma cells lack an active transport of methotrexate and thus would not respond to lower levels of the drug. Our findings do not support this hypothesis. On the contrary, they demonstrate a cellular accumulation of methotrexate. Furthermore, as judged by ^{3}H-deoxyuridine incorporation into DNA, osteosarcoma cells show an increased sensitivity to methotrexate as compared to cells aspirated from bone marrow. Data obtained with ^{3}H-deoxyuridine incorporation into DNA must, however, be interpreted with caution (see above) and at present we are unable to decide if the differences in deoxyuridine incorporation reflect differences in the size of dUMP pools or if they are indeed due to differences in the rate of DNA replication.

We finally stress the limitation that our data represent results with one single cultured line of osteosarcoma cells which had been kept for approximately 60 transplant generations *in vitro*. Although this line was shown to have a morphology [8] and interferon sensitivity [22] typical for cultured osteosarcoma cells, we think that further studies should be encouraged and that these should include other lines of osteosarcoma.

Acknowledgements—We thank Dr Hans Strander for fruitful discussions and for allowing us to analyze serum from two of his patients.

REFERENCES

1. H. E. Skipper, The cell cycle and chemotherapy of cancer. In *The Cell Cycle and Cancer*, p. 358 (Edited by R. Baserga). Marcel Dekker, New York (1971).
2. J. R. Bertino and D. G. Johns, Folate antagonists. *Ann. Rev. Med.* **18,** 27 (1974).
3. J. H. Goldie, L. A. Price and K. R. Harrap, Methotrexate toxicity: Correlation with duration of administration plasma levels, dose and excretion pattern. *Europ. J. Cancer* **8,** 409 (1972).
4. N. Jaffe, E. Frei, D. Traggis and Y. Bishop, Adjuvant methotrexate and citrivorum-factor treatment of osteogenic sarcoma. *New Engl. Med.* **291,** 994 (1974).
5. J. R. Bertino, B. A. Both, A. Cashmore, A. L. Bieber and A. C. Sartorelli, Studies of the inhibition of dihydrofolate reductase by folate antagonists. *J. biol. Chem.* **239,** 479 (1964).
6. J. R. Bertino, A. R. Cashmore and B. L. Hillcoat, "Induction" of dihydrofolate reductase: Purification and properties of the induced human erythrocyte and leukocyte enzyme and normal bone marrow enzyme. *Cancer Res.* **30,** 2372 (1970).
7. S. Futterman, Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. *J. biol. Chem.* **288,** 1031 (1971).
8. J. Pontén and E. Saksela, Two established *in vitro* cell lines from human mesenchymal tumours. *Int. J. Cancer* **2,** 434 (1967).
9. K. A. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
10. W. C. Schneider, Determination of nucleic acids in tissues by pentose analysis. In *Methods of Enzymology*, Vol. III, p. 68. (Edited by S. P. Colowick and N. O. Kaplan). Academic Press, New York (1957).
11. L. Skoog and B. Nordenskjöld, Effects of hydroxyurea and 1βD-arabinofuranosylcytosine on deoxyribonucleotide pools in mouse embryo cells. *Europ. J. Biochem.* **19,** 81 (1971).
12. U. Lindberg and L. Skoog, A method for the determination of dATP and dTTP in picomole amounts. *Anal. Biochem.* **34,** 152 (1970).
13. L. Skoog, An enzymatic method for the determination of dCTP and dGTP in picomole amounts. *Europ. J. Biochem.* **17,** 202 (1970).
14. B. A. Chabner, C. F. Myers, C. N. Coleman and D. G. Johns, The clinical pharmacology of antineoplastic agents. *New Engl. J. Med.* **292,** 1107 (1975).
15. M. H. N. Tattersall and K. R. Harrap, Changes in the deoxyribonucleoside triphosphate pools of mouse 5178Y lymphoma cells following exposure to methotrexate or 5-fluorouracil. *Cancer Res.* **33,** 3086 (1973).

16. A. FRIDLAND, Effects of methotrexate on deoxyribonucleotide pools and DNA synthesis in human lymphocytic cells. *Cancer Res.* **34,** 1883 (1974).
17. M. H. N. TATTERSALL, R. C. JACKSON, T. A. CONNORS and K. R. HARRAP, Combination chemotherapy: the interaction of methotrexate and 5-fluorouracil. *Europ. J. Cancer* **9,** 733 (1973).
18. C. R. BAUMUNK and D. L. FRIEDMAN, Absence of an effect of amethopterin and 5-fluorodeoxyuridine upon levels of thymidine triphosphates in HeLa cells. *Cancer Res.* **31,** 1930 (1971).
19. K. L. SKOOG, B. A. NORDENSKJÖLD and K. G. BJURSELL, Deoxyribonucleoside triphosphate pools and DNA synthesis in synchronized hamster cells. *Europ. J. Biochem.* **33,** 428 (1973).
20. K. L. SKOOG, K. G. BJURSELL and B. A. NORDENSKJÖLD, Cellular deoxyribonucleoside triphosphate pool levels and DNA synthesis. *Advanc. Enzyme Regul.* **12,** 345 (1974).
21. P. REICHARD, The biosynthesis of deoxyribonucleotides. *Eur. J. Biochem.* **3,** 259 (1968).
22. H. STRANDER and K. CANTELL, Studies on antiviral and antitumor effects on human leukocyte interferon *in vitro* and *in vivo*. In: *The Production and Use of Interferon for the Treatment and Prevention of Human Virus Infections.* Proceedings of a Tissue Culture Association Workshop, p. 49. Tissue Culture Association, Monograph No. 3, Rockville, Maryland (1974).

Europ. J. Cancer Vol. 12, pp. 847–849. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *September, 1976*

Human Cancer

A. S. Morrison, C. R. Lowe, B. MacMahon, B. Ravnihar and S. Yuasa: Some international differences in treatment and survival in breast cancer.

M. Inbar and H. Ben-Bassat: Fluidity difference in the surface membrane lipid core of human lymphoblastoid and lymphoma cell lines.

A. J. Cochran, R. M. Mackie, R. M. Grant, C. E. Ross, M. D. Connell, G. Sandilands, K. Whaley, D. E. Hoyle and A. M. Jackson: An examination of the immunology of cancer patients.

E. Klein, H. Ben-Bassat, H. Neumann, P. Ralph, J. Zeuthen, A. Polliack and F. Vánky: Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia.

Experimental Cancer

R. Yanai and H. Nagasawa: Importance of progesterone in DNA synthesis of pregnancy-dependent mammary tumors in mice.

S. W. Russell, W. F. Doe, R. G. Hoskins and C. G. Cochrane: Inflammatory cells in solid murine neoplasms. I. Tumor disaggregation and identification of constituent inflammatory cells.

S. W. Russell, G. Y. Gillespie, C. B. Hansen and C. G. Cochrane: Inflammatory cells in solid murine neoplasms. II. Types found throughout the course of Moloney sarcoma regression or progression.

H. W. S. King, M. H. Thompson and P. Brookes: The role of 9-hydroxybenzo(a)pyrene in the microsome mediated binding of benzo(a)pyrene to DNA.

S. M. Thorpe: Increased uptake of iodide by hormone-responsive compared to hormone-independent mammary tumors in GR mice.

I. Hellström and K. E. Hellström: Demonstration of tumor associated immunity with a leukocyte adherence inhibition (LAI) assay.

J. W. Flesher, R. G. Harvey and K. L. Sydnor: Oncogenicity of K-region epoxides of benzo(a)pyrene and 7,12-dimethylbenz(a) anthracene.

M. R. Osborne, F. A. Beland, R. G. Harvey and P. Brookes: The reaction of (±)-7α, 8β-dihydroxy-9β, 10β-epoxy-7, 8, 9, 10-tetrahydrobenzo (a)pyrene with DNA.

M. Hosokawa, M. Imamura, T. Oikawa, M. Nakayama, E. Gotohda, F. Sendo, T. Kodama and H. Kobayashi: Inhibition of growth of syngeneic tumor in rats immunized with allogeneic skin grafts.

E. Ruoslahti and H. O. Sjögren: Specific anti-fibroblast cytotoxicity of antibodies to fibroblast surface antigen.

J-C. Salomon, A. Galinha, V. Lascaux, J. Prin, F. Puvion and N. Lynch: Intra-lesional injection of immunostimulants in bilateral rat tumors.

International Journal of Cancer *October, 1976*

Human Cancer

I. T. Magrath and R. M. Simon: Immunosuppression in Burkitt's lymphoma. II. Peripheral blood lymphocyte populations related to clinical status.

A. P. Lanier, T. R. Bender, W. J. Blot, J. F. Fraumeni and W. B. Hurlburt: Cancer incidence in Alaska natives.

E. Klucis, L. Jackson and P. H. Parsons: Survey of human lymphoblastoid cell lines and primary cultures of normal and leukaemic leucocytes for oncorna-virus production.

Experimental Cancer

R. S. Kerbel and H. F. Pross: Fc receptor-bearing cells as a reliable marker for quantitation of host lymphoreticular infiltration of progressively growing solid tumors.

N. M. Hogg: Specificity of serum from rodents immune to Moloney C-type virus-induced tumors.

F. Oesch, P. Bentley and H. R. Glatt: Prevention of benzo(a)pyrene-induced mutagenicity by homogeneous epoxide hydratase.

T. Matsuo, S. Nishi, H. Hirai and T. Osato: Studies on Epstein–Barr virus-related antigens. I. Indirect single radial immunidiffusion as a useful method for detection and assay of soluble antigen.

R. S. Epstein, D. M. Lopez, G. Ortiz-Muniz and M. M. Sigel: Emergence of a subpopulation of lymphocytes bearing θ antigen and complement receptor during tumor growth.

L. A. Smets, W. P. van Beek and H. van Rooij: Surface glycoproteins and Concanavalin A mediated agglutinability of clonal variants and tumour cells derived from SV40-virus transformed mouse 3T3 cells.

G. Daroi and K. Munk: Neoplastic transformation of rat embryo cells with herpes simplex virus.

H. M. Hayes, Jr. and T. W. Pendergrass: Canine testicular tumors: Epidemiologic features of 410 dogs.

M. A. Kall, I. Hellström and K. E. Hellström: *In vitro* generation of primary and secondary cytotoxic cell-mediated immune responses to chemically induced mouse sarcomas.

B. A. Voyles and C. M. McGrath: Markers to distinguish normal and neoplastic mammary epithelial cells *in vitro*: Comparison of saturation density, morphology and Concanavalin A reactivity

H. H. Fiebig, W. J. Zeller and D. Schmähl: An experimental model for meningeal leukemia in rats (L5222). Effect of treatment with BCNU and cyclophosphamide.

L. N. Payne and M. Rennie: Sequential changes in the numbers of B and T lymphocytes and other leukocytes in the blood in Marek's disease.

R. Revoltella, L. Bertolini and L. Diamond: *In vitro* stimulation of mouse lymphoid cells by C1300 neuroblastoma cells or tumor membrane extracts.

S. V. Bhide, R. D. D'Souza, M. M. Sawai and K. J. Ranadive: Lung tumour incidence in mice treated with hydrazine sulphate.

M. Bistrović, Ž. Mariĉić and K. Kolarić: Interaction of bleomycin and radiation in combined treatment on mouse L cells.

British Journal of Cancer *October, 1976*

E. Engvall, M. Vuento and E. Ruoslajti: A monkey antigen crossreactive with carcinoembryonic antigen, CEA.

J. C. Ridway, G. M. Taylor, C. B. Freeman and R. Harris: Receptors for human immunoglobulin on acute myeloid leukaemic leucocytes.

Ole P. Børmer: Purification of a cytotoxic factor from human and rat tissues.

M. V. Pimm, D. G. Hopper and R. W. Baldwin: BCG treatment of malignant pleural effusions in the rat.

G. R. Betton and L. N. Owen: The allogenic transplantation of spontaneous canine melanoma in neonatal immunosuppressed dogs.

T. M. Saba and T. G. Antikatzides: Decreased resistance to intravenous tumour cell challenge during period of reticuloendothelial depression following surgery.

N. Wedderburn, R. L. Carter and M. H. Salaman: Growth of a transplantable lymphoma and its modification in recipients infected with the inducing virus.

P. W. Sheldon and J. F. Fowler: The effect of low dose pre-operative X-irradiation of implanted mouse mammary carcinomas on local recurrence and the development of metastases.

G. D. Zanelli and P. B. Lucas: Effects of X-radiation on vascular function in transplanted tumours and normal tissues in the mouse.

O. Fodstad, S. Olsnes and A. Pihl: Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice.

Y. Ishimaru, K. Kudo, H. Ishihara and H. Hayashi: The significance of a glycoprotein of rat ascites hepatoma cell surface in tumour cell adhesiveness, with special reference to development of intercellular junctions.

R. Parkin and S. Neale: The effect of isoprenaline sulphate on induction of tumours by N-methyl-N-nitrosourea in the salivary and mammary glands of female Wistar rats.

E. G. Knox: Ages and frequencies for cervical cancer screening.

Brief Communication

M. V. Pimm and R. W. Baldwin: *C. parvum* suppression of rat tumours in athymic nude mice.

Europ. J. Cancer Vol. 12, p. 850. Pergamon Press 1976. Printed in Great Britain

Papers to be Published

C. S. B. GALASKO and D. W. HAYNES
Survival of VX2 carcinoma cells *in vitro*.

MOGENS KJAER
Prognostic value of tumour-directed, cell-mediated hypersensitivity detected by means of the leucocyte migration technique in patients with renal carcinoma.

LISE THIRY
Herpes simplex virus and carcinoma of the cervix.

JOAN THILLY HARMON and RUSSELL HILF
Effect of insulin on glucose transport in DMBA-induced mammary tumors.

S. LEVY, D. PAPADOPOULO, S. NOCENTINI, L. CHAMAILLARD, O. BEESAU, M. HUBERT-HABART and P. MARKOVITS
Transformation of hamster embryo cells by 7-methyl benz(a)anthracene *in vitro*: tumorigenicity, chromosome pattern, and spheroid formation.

H. STÄHELIN
Delayed toxicity of epipodophyllotoxin derivatives (MV 26 and VP 16–213), due to a local effect.

RENÉ VERLOES, LOUIS KANAREK and GHANEM ATASSI
Antitumour immunoprotection by an immunobacterial lectin approach.

M. G. DONELLI, A. MARTINI, T. COLOMBO, A. BOSSI and S. GARATTINI
Heart levels of adriamycin in normal and tumor-bearing mice.

L. GONÇALVES SOBRIHNO, L. FARO, E. F. HENRIQUES, L. S. BOTELHO and M. F. FONTES DE SOUSA
The urinary excretion of pregnanetriol and pregnenetriol in patients with breast cancer. A postulation on the meaning of Bulbrook's discriminant.

HIROSHI MAEDA, NAOKI YAMAMOTO and AKIRA YAMASHITA
Fate and distribution of [;;C] succinyl neocarzinostatin in rats.

H. C. VAN PROOIJEN, G. VIERWINDEN, J. VAN EGMOND, J. M. C. WESSELS and C. HAANEN
A sensitive bio-assay for pharmacokinetic studies of cytosine arabinoside in man.

SYAMAL K. DATTA and ROBERT S. SCHWARTZ
Susceptibility to lymphomas and expression of C-type RNA viruses during the graft versus host reaction.

ALLAN FENSELAU and CEDRIC LONG
In vitro inhibition of cell growth by aldose phosphates.

HOROSHI NAGASAWA, REIKO YANAI and HISASHI TANIGUCHI
Reduction by pituitary grafts of mammary tumor age: Its variability in a high mammary tumor strain of mice; effects on mammary DNA synthesis.

R. B. RAIKOW, L. L. SCHENKEN and S. LESHER
Urinary excretion of 5-hydroxyindoles as an indicator of *in vivo* growth of P815X1 mastocytoma.

Europ. J. Cancer Vol. 12, pp. 851–858. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Herpes Simplex Virus and Carcinoma of the Cervix

LISE THIRY
Institut Pasteur du Brabant, Bruxelles, Belgium

VIRUSES may be related to cancer in several ways.

1. If herpes simplex type 2 virus (HSV_2) was the one and only cause of carcinoma of the cervix, then eradication of genital herpetic infection would also eradicate this cancer. The incidence of cervical carcinoma is very low among nuns, but this may be due to other features of their mode of life; prevention of this cancer by means of a specific vaccine would be the final proof.
2. If HSV_2 was one of the several causes of cervical carcinoma, then an efficacious vaccine would significantly decrease the number of cases of this cancer, but not suppress them. In most virus-induced tumours, viral genes are integrated in the chromosomes. Thus, if either 1. or 2. were true, part or whole of the HSV-2 genome should be found always or often in the squamous cells of cervical carcinoma; HSV-2 genes might or might not be expressed and lead to the production of HSV-2 proteins.
3. Alternatively, HSV-2 might be unable to cause cancer by itself, but might act as a cofactor. It might, for instance, play the part of a virus helper for an oncornavirus, triggering the induction of an endogenous virus, or helping an exogenous oncornavirus to spread from one cell to another.
4. There are several other possibilities: HSV-2 may play some part a posteriori, after cancer transformation has occurred. It may interact specifically with the cancer cells, leading to:
   a) latency with frequent reactivations of the complete virions,
   b) continuous replication of part or whole of the complete HSV-2 genome, with partial expression of the genome, leading to the presence of some HSV-2 proteins in the cancer cells; these proteins might influence the growth or spread of the cancer cells and thus be of prognosis value; if not, they may represent at least some protein markers of the cancer cells and represent an additional diagnosis tool.
   c) finally there may be no correlation at all between cervical cancer cells and HSV-2 infection.

Among the above hypotheses we want to show that only (*a*) and (*c*) are certainly wrong: i.e., HSV-2 and carcinoma of the cervix are not randomly associated; frequent reactivations of mature HSV-2 virions do not occur in the cancer cells, since there is general agreement that infectious viruses cannot be isolated directly from these tissues.

This review will deal with the various other hypotheses and will not be presented in an historical order. However, it should be pointed out that the first statistical evidence for an association between precancerous or cancerous lesions of the cervix and herpes genitalis infection appeared in 1969 [1]: 56,418 women had been followed and anaplastic lesions were found more frequently in women who also showed, at one moment, evidence of herpes genitalis infection. The peak for the histological detection of genital HSV infection was ten years earlier than that for carcinoma *in situ*, indicating that expression of HSV-2 does not follow cancer transformation.

EXPERIMENTAL STUDIES

Does HSV-2 cause cancer in animals?

Nine new born hamsters inoculated with small doses of HSV-2 developed sarcomas at or

near the site of inoculation, while one hamster developed a distant fibrosarcoma after inoculation of a tissue culture medium without cells [2]. No HSV markers were found in the sarcomas, while oncornaviruses were demonstrated in some of them. A large trial with cebus monkeys has attempted to mimic human situations [3] and has now been conducted for 3 years. Of 129 females who were inoculated with HSV-2 into the cervix 13 developed very persistent atypia and/or dysplasia, which appeared 14–50 months after inoculation.

No such lesions were found in animals inoculated with the control material. Interestingly enough, the animals could be successfully reinfected 6 months after the primary inoculation indicating that women can probably also be infected with HSV-2 more than one time in their life, even in the face of high titers of neutralising antibodies. Vaccines which induce these antibodies may thus be unable to prevent local genital infection.

Does HSV-2 induce cell transformation in vitro*?*

In a broad sense, cells are considered to be transformed by a virus when the action of this virus triggers the appearance of a new specific hereditary property; viral genes are considered to be integrated in the cell genome when the new cell characteristic is related to the presence of a virus coded protein. These conditions are fulfilled in experiments showing that thymidine-kinase deficient mouse L cells can be transformed with ultraviolet-irradiated herpes simplex viruses into cell lines that produce a thymidine-kinase with the antigenic specificity of the herpes simplex virus-kinase [4,6]. Cell transformation is often used with a more restricted meaning, implying that the transformed cells have acquired oncogenic properties. To characterize these properties tumourigenicity *in vivo* is the most stringent criterium, while altered morphology and growth characteristics are often ill-defined and not necessarily linked with *in vivo* cancerous effects. For this reason the results dealing with some transformation of human cells with herpes simplex virus [7] bear no convincing relevancy to the oncogenic properties of this virus in man. On the other hand, it has been widely confirmed that herpes simplex viruses have oncogenic properties for hamster and mouse cells, since the pioneer works which showed that hamster embryo fibroblasts treated with HSV-2 inactivated by ultraviolet light [8, 9] or by photodynamic effects of dyes [10, 11] become tumourigenic for the hamsters. These findings have raised questions about the innocuousness of killed herpes simplex virus vaccines which are available in some countries and used in humans with the hope of decreasing the severity of recurrent herpetic lesions. The same objection holds for the treatment of these lesions by application of dyes followed by exposure to visible light; a first report [12] had shown that this treatment caused symptomatic improvement as compared to therapy used previously. However, placebo controled studies [13, 14] demonstrated an absence of efficacy of such treatments. The oncogenic effects of viruses vary with the host cells and it has been argued that oncogenic potentialities of herpes simplex virus for man cannot be derived from studies with hamster cells which are very susceptible to oncogenic action of various agents. However, a mouse cell line which is very susceptible to contact inhibition has been shown to be transformed by herpes simplex viruses with even a greater efficacy than in the hamster systems [15].

Is there an oncogenic gene in herpes simplex virus?

It has been shown that the genome site necessary for virus infectivity is more susceptible to u.v. inactivation than is the site for cellular transformation, indicating that only part of the viral information is used during the transforming event. A better definition of the gene(s) involved is difficult with u.v. or dye inactivated viruses, since these agents hit the viral DNA at random and produce populations which are mixtures of viruses with defects at random loci. Using a given u.v. inactivated preparation of herpes simplex virus type 2, two morphological types of foci were obtained [15]; after cloning, the foci gave rise to fibroblastoid and epitheloid cell lines. This indicates that the host genome may be differently modified, either because a given viral gene can be inserted at various sites of the cell genome, or because different viral genes are able to be inserted into this genome. To distinguish between these two possibilities, it will be useful to use cloned viral spontaneous mutants which may have arisen from a single mutational event. This will probably be feasible since temperature sensitive mutants have been selected [16] and only some of these mutants induced transformation of hamster cells [17]. Since the transformation assays were only done once, it is still not clear whether given mutants would be repeatedly unable to cause transformation. The importance of this field is great, since there is a wide array of herpes virus strains, whose prevalence may vary from one country to another [18].

Convincing results have not yet been pub-

lished showing that some of these strains are less oncogenic than the others. The identification of strains lacking oncogenic genes would re-open the possibility of vaccine preparation.

Which properties of the transformed cells are necessary for tumourigenicity?

During the first passages after the transformation event, the oncogenic cells possess herpes simplex virus antigens which are shared by the serotypes 1 and 2; these antigens were demonstrated in the cytoplasm by immunofluorescent techniques which did not detect nuclear antigens similar to the T antigens present in cells transformed by other viruses [10]. Some of these cell lines transformed with u.v. inactivated HSV-2 were retested more than one year later and the viral antigens were no more detected, [19] although the cells were still tumourigenic. Other HSV-1 transformed cells kept the viral antigen(s) for at least 96 passages, while cells transformed by dye-photo inactivated HSV had no detectable antigens. It is not yet clear whether virus strains, or the mode of inactivation of these strains, bear a necessary relationship with the persistent expression of virus proteins, but many laboratories now agree that tumourigenic properties of the cells do not depend on the expression of viral proteins at detectable levels. Do they then depend on the presence of herpes simplex virus genes? Viral RNA was transcribed in early passages of hamster cells transformed with HSV-2 and represented only 10–13% of the viral sequences transcribed during productive infection [20]. Similar experiments with the same cells after numerous passages have not been published. Viral DNA could only be detected in the transformed cell lines when the technique involved hybridization of denatured excess cellular DNA with trace amounts of labelled viral DNA with a high specific activity [21]; different cell lines transformed with HSV-2 contained from 5% to 40% of the viral genome. The amount of the viral genome was not a fixed characteristic of a line since these amounts were different in sublines of an original line. One may then expect a whole array of situations in tumours growing *in vivo*. A search for herpes simplex virus genetic information in transformed cells was also performed with an elegant biological approach [22]. Thermosensitive mutants were grown at non-permissive temperature in transformed and in non-transformed cells, and only some of the mutants were helped by the information present in the transformed cells. Those mutants which were helped were defective in late viral functions, indicating that the information present in the two transformed cells studied was not random but more specifically localized in the genes of the late functions [45]. This was an unexpected finding since in other virus-transformed cells the viral genes which are conserved are those which trigger early events of the virus replication cycle, such as the production of T antigen. These results, however, fit well with the fact that no T antigens were found in HSV-transformed cells.

Immunological responses of tumour-bearing hamsters

Subcutaneous inoculation of HSV transformed cells into hamsters produces local tumours with lung metastases; neutralizing antibodies which cross react with both serotypes of HSV appear only in those hamsters which were inoculated with cell lines containing HSV antigens [19]. In that case lymphocytes from the tumour-bearing animals were specifically cytotoxic to cells infected with HSV, or transformed by this virus, with no effect on cells transformed with adenovirus [24] or with cytomegalovirus [25]. In the presence of complement sera from these animals were cytotoxic to hamster cells infected with HSV but not to the transformed cells [26]; sera contained a blocking factor which inhibited lymphocytotoxicity [25]. An obvious step was to try to decrease the *in vivo* growth and spread of HSV transformed cells by vaccination procedures. Preimmunization of the hamsters with inactivated HSV did not inhibit tumour growth and enhanced the metastases [27]; this effect may have been due to IgG antibodies since weanling hamsters inoculated with HSV transformed cells showed increased tumour growth when they were born to mothers immunized with the virions [26]. Growth of HSV-transformed cells was also enhanced in hamsters previously vaccinated with killed cells, under conditions which afforded some protection when the whole experiment was performed with adenovirus transformed cells [26]. With both types of cells the vaccination procedures induced the appearance of cytotoxic lymphocytes, while cytotoxic antibodies were only induced with the adenovirus transformed cells [26]. Finally, treatment of the hamsters with the immunopotentiator levamisole delayed the appearance of tumours caused by herpes simplex transformed cells [26] and inhibited metastases [27]. The drug probably triggers cellular immune responses which are distinct from the apparently inefficient lymphocytotoxicity. Non-specific cellular mechanism, together with the action of specific complement

dependent cytotoxic antibodies, may be the factors which prevent tumour growth, provided that they are not counteracted by blocking factors present in the serum; screening for efficient vaccines should take these data into account.

HUMAN STUDIES

Is there HSV information in cells from carcinoma of the cervix?

During primary infection of the genital tract HSV-2 might interact with the cells chromosomes and induce cell transformation, without insertion of viral genes into the chromosomes. If this were the case one would not detect any viral information in the tumour, although HSV-2 was the primary initiating event. *In vitro* studies do not support this hypothesis, since no chromosome lesions were recorded when hamster cells were treated with inactivated HSV, but rather the transformed cells progressively developed a pattern of chromosal aberrations [28], suggesting that these aberrations were not the initial event. If insertion of viral genes in the transformed cells do occur, their experimental detection within an established carcinoma will depend on the separation of cancer cells from the others, on the number of viral gene copies per cancer cell and on sensitive biochemical methods able to detect one copy per cell of a nucleic acid piece amounting to a few per cent of the total viral genome; this information would be sufficient to code for "transforming products". Negative results have been obtained with human cervical carcinoma using a technique which would have succeeded only if more than one complete viral genome per cancer cell had been present [29]. Using more refined techniques, and the largest tumour available (70 g), HSV DNA representing 39 % of the viral genome was demonstrated in one cervical carcinoma [30].

Other positive results have not been published, and it is not known how many negative results have been registered. As compared to the above data, would the search for viral antigens prove a more sensitive tool? One viral gene can of course induce repeated transcriptions of the corresponding protein, but it has been calculated that hundreds of these proteins should concentrate in a cell in order to be visualized by the current techniques [21]. It is not clear whether these calculations are valid for the most sensitive radioimmunoassay techniques. On the other hand, a low concentration of viral gene products is sufficient to maintain a transformed state. When dealing with tumours grown *in vivo*, viral proteins may be masked by antibodies adsorbed on the tumour cells. The amount of viral proteins in these cells may also be decreased *in vivo*, due to regulation mechanisms, one of which has recently been described [31]. Specific antibodies adsorbed on measles-virus infected cells actively repressed the synthesis of the viral proteins. If confirmed with virus transformed cells, this effect may play an important role in tumour antigenicity.

In fact, no HSV-2 antigens have been demonstrated in biopsies of cervical carcinoma when antisera with neutralizing antibodies to the virions were used. These negative results have been emphasized [32] and contrasted with the fact that these tissue specimens initiated the synthesis of viral protein when grown *in vitro* under conditions of stress [33]; specific HSV antigens were also detected by immunofluorescence in the necrotic cells which exfoliate *in vivo* from the carcinoma [34]. These data fit well with the hypothesis of HSV latent genes present in cancer cells and only expressed when the cells tend to die. Cervical carcinoma cells could even yield complete infective virions [35], although the frequency of such results is not documented. However, there is now evidence that at least one HSV specific gene is expressed in healthy cervical carcinoma cells, a gene which is also specifically expressed early during infection of normal cells with HSV. The protein has been named Ag 4 and was found in 5 of 6 biopsies of squamous cervical carcinoma and not in one from an adenocarcinoma [35]. It was first suggested that Ag 4 was a non-structural protein present in infected cells, but not in the purified virions [36]. However, recent results demonstrated the presence of Ag 4 as a minor component of the virion [37], indicating that it is a virus coded protein.

Since HSV induces a virus specific thymidine-kinase in the infected cells, the presence of this enzyme in cervical carcinoma was explored; no enzyme with the virus specific antigenicity was found [38].

Specific immune responses to HSV are found in patients with carcinoma of the cervix

One of the earlier clues to the association of genital herpetic infections with carcinoma of the cervix was the finding that neutralizing antibodies to HSV-2 were present in 83 % of the patients with this tumour, as compared to 0–20 % in carefully matched control groups [39] In order to obtain such a clear demonstration, technical difficulties must be overcome, since

the serotypes 1 and 2 of HSV share many antigens; it follows that patients with labial herpetic lesions produce antibodies with specificities not very different from those of antibodies produced after genital herpetic infections. To discriminate between the two situations, the first study [39] used kinetics of neutralization, based on the fact that antibodies to HSV-2 react more quickly with the homologous HSV-2 than with HSV-1. The results obtained were confirmed with similar techniques in the United States [40, 41], in Belgium [42, 43], in Great Britain [44]. High levels of antibodies to HSV-2 in patients with carcinoma of the cervix were also found in France [45] by using indirect hemagglutination for the tests. Attempts were then made to increase the discrimination between patients and controls groups by using tests with isolated HSV proteins instead of whole virions, with the hope of demonstrating antibodies to HSV "tumour antigen". The most promising technique used the protein Ag 4 as antigen in a refined very sophisticated complement fixation test [36, 46]; a positive reaction was found in 90% of patients with invasive carcinoma, and in 10% of the matched controls; reactions were negative in successfully treated patients and positive in recurrent neoplasia, indicating that the test might be of diagnostic value. If not simplified, it will probably not be applicable as a routine procedure. Another protein, VP 134 also extracted from cells early after infection with HSV, was used in another sophisticated test with indirect radioimmune precipitation techniques [47]. A higher reactivity to VP 134 was found in sera from cervical cancer patients than in sera from other groups, but differences between the groups were not so impressive as with Ag 4. The chemical relationship of Ag 4 with VP 134 is not known.

Antibodies to cell membrane antigens are important factors in tumour immunology; reactivity of the patients' sera to cells infected with HSV was studied by means of a complement dependent cytotoxicity assay [48], as well as by a mixed haemadsorption method [49]; in both studies, lower activities of the sera were found in severe rather than in early stages of cervical carcinoma, and the antibody activities increased as the tumour was treated, indicating the possibility that these antibodies may bind *in vivo* to HSV-specific antigens present on the tumour, or solubilized from it.

Interesting information may also be derived from the type of antibody molecules which the cancerous host produces against HSV antigens. Complement fixing antibodies to Ag 4 were characterized as IgM [37] while complement fixing antibodies to HSV virions were IgG. It has also been found, however, that IgM and/or IgA neutralizing antibodies to HSV were present in cervical carcinoma patients. These antibodies were demonstrated after adsorption of the sera with *Staphylococci Cowan A*, which combine most IgG molecules, except IgG 3. Positive reactions with HSV were found in 75% of the adsorbed sera, in the cancer group, as compared to 11% in control and treated groups [50]. The presence of specific IgM antibodies in cancer patients is not yet well documented, and it is not known whether these antibodies help to reject the tumour, or have enhancing functions. In other words, are high titers of specific IgM antibodies of good or of bad prognosis? The same question will hold for cell-mediated responses. Specific stimulation of lymphocytes by HSV-2 transformed cells were found in 36% of the cervical carcinoma patients and in none of the controls [50, 51]. Differences between the two groups were not so clear cut when lymphocytes were stimulated by HSV virions. The antigen of the transformed cells, which stimulates lymphocytes of the cancer group, is ill-defined, but is not present on SV 40 transformed cells.

Is HSV uniquely related to carcinoma of the cervix?

It has already been stated that herpes genitalis and herpes labialis viruses share many antigens in common. One of these antigens has been extracted from cells infected with HSV-1 or HSV-2, as well as from carcinoma of the lip and of the cervix [52] the antigen reacts in a CF test with the sera of patients with squamous carcinoma of the head, the neck or the cervix. It also inhibits migration of the lymphocytes of these patients, and produces, *in vivo*, delayed hypersensitivity skin reactions [53]. These studies launch the problem of the role of herpes labialis in cancer.

Is carcinoma of the cervix uniquely related to HSV?

It has been argued that, since 1969, most of the surveys were prejudiced because they were conceived to prove or disprove the association between HSV and cervical carcinoma. In one survey however, the incidence of antibodies to HSV was compared to that of antibodies to cytomegalovirus and Epstein–Barr virus, and all three types of antibodies were found more frequently in women with cervical cancer than in the healthy controls, while incidence of antibodies to adenovirus were similar in both groups [54]. Another survey confirmed the

high incidence of antibodies to cytomegalovirus in the cancer cases [55]. It is now the task of these authors to demonstrate Epstein–Barr or cytomegalovirus information in the cancer cells, as well as the evolution of specific immune responses to these viruses during follow-up of the patients. Finally, it is more than certain that future works will—and should—be devoted to the search for oncornavirus expression in squamous cancer cells from the cervix, as well as to the interaction of HSV and oncornavirus infections, in tissue cultures and in animals.

REFERENCES

1. Z. Naib, A. J. Nahmias, W. E. Josey and J. H. Kramer, Genital herpetic infection. Association with cervical dysplasia and carcinoma. *Cancer* **23,** 940 (1969).
2. A. J. Nahmias, Z. M. Naib, W. E. Josey, F. Murphy and C. Luce, Sarcomas after inoculation of newborn hamsters with herpes virus hominis type 2 strains. *Proc. Soc. exp. Biol. (N.Y.)* **134,** 1065 (1970).
3. A. Palmer, W. London, A. Nahmias, Z. Naib, J. Tunca, D. Fucillo, J. Ellenberg and J. Sever, A preliminary report on investigation of oncogenic potential of herpes simplex virus type 2 in cebus monkeys. *Cancer Res.* **36,** 807 (1976).
4. W. Munyon, E. Kraiselbard, D. Davis and J. Mann, Transfer of thymidine kinase to thymidine kinaseless L cells by infection with ultraviolet irradiated herpes simplex virus. *J. Virol.* **7,** 813 (1971.
5. R. L. Davidson, S. J. Adelstein and M. N. Oxman, Herpes simplex virus as a source of thymidine kinase for thymidine kinase-deficient mouse cells: suppression and reactivation of the viral enzyme. *Proc. Soc. nat. Acad. Sci.* **70,** 1912 (1973).
6. M. Thouless, K. Chadha and W. Munyon, Serological specificity of thymidine kinase activity in herpes simplex virus-transformed L cells. *Virology* **69,** 350 (1976).
7. G. Darai and L. Munck, Human embryonic lung cells abortively infected with herpes virus hominis type 2 show some properties of cell transformation. *Nature New Biol.* **241,** 268 (1973).
8. R. Duff and F. Rapp, Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. *Nature New Biol.* **233,** 48 (1971).
9. R. Duff, J. W. Kreider, B. M. Levy, M. Katz and F. Rapp, Comparative pathology of cells transformed by herpes simplex virus type 1 or type 2. *J. nat. Cancer Inst.* **53,** 1159 (1974).
10. F. Rapp, Jui-Lien Li and M. Jerkofsky, Transformation of mammalian cells by DNA-containing viruses following photodynamic inactivation. *Virology* **55,** 339 (1973).
11. J. Li, M. Jerkofsky and F. Rapp, Demonstration of oncogenic potential of mammalian cells transformed by DNA-containing viruses following photodynamic inactivation. *Int. J. Cancer* **15,** 190 (1975).
12. T. Felber, E. Smith, J. Knox, C. Wallis and J. Melnick, Photodynamic inactivation of herpes simplex. Report of a clinical trial. *J. Amer. Medic. Assoc.* **223,** 289 (1973).
13. M. Myers, M. Oxman, J. Clark and K. Arndt, Failure of neutral red photodynamic inactivation in recurrent herpes simplex virus infections. *New Engl. J. Med.* **293,** 945 (1975).
14. A. Roome, A. Tinkler, A. Hilton, D. Montefiore and D. Waller, Neutral red with photoinactivation in the treatment of herpes genitalis. *Brit. J. vener. Dis.* **51,** 130 (1975).
15. R. Duff and F. Rapp, Quantitative assay for transformation of 3T3 cells by herpes simplex virus type 2. *J. Virol.* **15,** 490 (1975).
16. R. W. Koment and F. Rapp, Temperature sensitive host range mutants of herpes simplex virus type 2. *J. Virology* **15,** 812 (1975).
17. M. Takahashi and K. Yamanishi, Transformation of hamster embryo and human embryo cells by temperature sensitive mutants of herpes simplex type 2. *Virology* **61,** 306 (1974).
18. L. Aurelian, S. S. Merdel, I. Kessler and Z. Kulcar, Three Yugoslav herpes simplex viruses: biologic and antigenic properties and formation of giant cells *in vitro* by a cervical isolate. *J. infect. Dis.* **129,** 465 (1974).

19. C. L. Reed, G. H. Cohen and F. Rapp, Detection of a virus-specific antigen on the surface of herpes simplex virus-transformed cells. *J. Virology* **15,** 668 (1975).
20. W. Collard, H. Thornton and M. Green, Cells transformed by human herpes virus type 2 transcribe virus-specific RNA sequences shared by herpes virus types 1 and 2. *Nature New Biol.* **243,** 264 (1973).
21. B. Roizman and N. Frenkel, Does genital herpes cause cancer? A midway assessment. *Proc. roy. Soc. Med. London,* in press (1975).
22. S. Kimura, J. Esparza, M. Benyesch-Melnick and P. Schaffer, Enhanced replication of temperature-sensitive mutants of herpes simplex virus. *Intervirology,* in press.
23. M. Benyesch-Melnick, P. Schaffer, R. Courtney, J. Esparza and S. Kimura, Viral gene functions expressed and detected by temperature-sensitive mutants of herpes simplex virus. *Cold Spr. Harb. Symp. quart. Biol.* **39,** 731 (1974).
24. S. Sprecher-Goldberger, L. Thiry and P. Vandenbussche, Use of immune lymphocytes to detect expression of herpetic genome. *Nature (Lond.)* **250,** 678 (1974).
25. R. N. Lausch, C. Jones, D. Christie, K. A. Hay and F. Rapp, Spleen cell mediated cytotoxicity of hamster cells transformed by HSV. Evidence for virus specific membrane antigen. *J. Immunol.* **114,** 459 (1975).
26. L. Thiry, S. Sprecher-Goldberger, L. Tack, M. Jacques and J. Stiénon, Comparison of the immunogenicity of hamster cells transformed by adenovirus and herpes simplex virus. *Cancer Res.* **35,** 1022 (1975).
27. J. Sadowski and F. Fapp, Inhibition by levamisole of metastases by cells transformed by herpes simplex virus type 1. *Proc. Soc. Exptl. Biol. Med.* **149,** 219 (1975).
28. M. Nachtigal, R. Duff and F. Rapp, Chromosome aberrations in syrian hamster embryo cells transformed after exposure to ultraviolet-irradiated herpes simplex type 1 or 2. *J. nat. Cancer Inst.* **54,** (1975).
29. H. Zur Hausen, S. Schulte-Holfausen, H. Wolf, K. Dorries and H. Egger, Attempts to detect virus-specific DNA in human tumors. II. Nucleic acid hybridizations with complementary RNA of human herpes group viruses. *Int. J. Cancer* **13,** 657 (1974).
30. N. Frenkel, B. Roizman, E. Cassai and A. J. Nahmias, A herpes simplex 2 DNA fragment and its transcription in human cervical cancer tissue. *Proc. nat. Acad. Sci.* **69,** 3784 (1972).
31. J. S. Barry and M. B. Oldstone, Immunologic injury in measles virus infection. II. Suppression of immune injury through antigenic modulation. *J. exp. Med.* **142,** 864 (1975).
32. L. Aurelian, J. Strandberg and H. Davis, HSV-2 antigens absent from biopsied cervical tumor cells: a model consistent with latency. *Proc. Soc. exp. Biol.* **140,** 404 (1972).
33. L. Aurelian, Virions and antigens of herpes virus type 2 in cervical carcinoma. *Cancer Res.* **33,** 1539 (1973).
34. I. Royston and L. Aurelian, Immunofluorescent detection of herpes virus antigens in exfoliated cells from human cervical carcinoma. *Proc. nat. Acad. Sci.* **67,** 204 (1970).
35. L. Aurelian, J. D. Strandberg, L. V. Melendrez and L. A. Johnson, Herpes virus type 2 isolated from cervical tumor cells grown in tissue culture. *Science* **174,** 704 (1971),
36. L. Aurelian, B. Schumann, R. Margus and H. Davis, Antibody to HSV-2 induced tumor specific antigens in serums from patients with cervical carcinoma. *Science* **181,** 161 (1973).
37. L. Aurelian and B. C. Strand, Herpes virus type 2-related antigens and their relevance to humoral and cell mediated immunity in patients with cervical cancer. *Cancer Res.* **36,** 810 (1976).
38. W. Rawls, G. Cashon, E. Adam, T. Ogino, R. Duff and F. Rapp, Herpes virus type 2 induced thymidine kinase and carcinoma of the cervix. *Cancer Res.* **34,** 362 (1974).
39. W. E. Rawls, W. A. Tomkins, M. E. Figueroa and J. L. Melnick, Herpes virus type 2. Association with carcinoma of the cervix. *Science* **161,** 1255 (1968)
40. W. Rawls, W. Tompkins and J. Melnick, The association of herpes virus type 2 and carcinoma of the uterine cervix. *Amer. J. Epidem.* **89,** 547 (1969).

41. L. Aurelian, I. Royston and H. Davis, Antibody to herpes simplex virus: association with cervical atypia and carcinoma in situ. *J. nat. Canc. Inst.* **45,** 455 (1970).
42. S. Sprecher-Goldberger, L. Thiry, J. P. Cattoor, R. Hooghe and J. Pestiau, Herpes virus type 2 infection and carcinoma of the cervix. *Lancet* **i,** 266 (1970).
43. S. Sprecher-Golderbger, L. Thiry, I. Gould, Y. Fassin and C. Gompel, Increasing antibody titers to herpes simplex virus type 2 during follow-up of women with cervical dysplasia. *Amer. J. Epidem.* **97,** 103 (1973).
44. G. Skinner, M. Thouless and J. Jordan, Antibodies to type 1 and 2 herpes virus in women with abnormal cervical cytology. *J. Obstet. Gynaec. Brit. Cwlth.* **78,** 1031 (1971).
45. F. Freymuth, C. Morel, A. Longson and M. Tanguy, Antibodies to herpes simplex virus types 1 and 2: a comparative study using indirect haemagglutination in carcinoma of the cervix and in other malignant conditions. *Biomedicine* **23,** 271 (1975).
46. L. Aurelian, H. Davis and C. Julian, Herpes virus type 2 induced, tumor-specific antigen in cervical carcinoma. *Amer. J. Epidem.* **98,** 1 (1973).
47. T. Anzai, G. Dreesman, R. Courtney, E. Adam, W. Rawls and M. Benyesh-Melnick, Antibody to herpes simplex virus 2-induced non structural proteins in women with cervical cancer and in control groups. *J. nat. Cancer Inst.* **54,** 1051 (1975).
48. L. Thiry, S. Sprecher-Goldberger, Y. Fassin, I. Gould, C. Gompel, J. Pestiau and F. de Halleux, Variations of cytotoxic antibodies to cells with herpes simplex virus antigens in women with progressing or regressing lesion of the cervix. *Amer. J. Epidem.* **100,** 251 (1974).
49. B. Christenson and Å. Espmark, Long-term follow-up studies in herpes simplex antibodies in the course of cervical cancer. II. Antibodies to surface antigens of herpes simplex virus infected cells. *Int. J. Cancer* **17,** 318 (1976).
50. L. Thiry, S. Sprecher-Goldberger and E. Hannecart-Pokorni, Compared humoral and cellular immune response to herpes simplex virus antigens in women with cervical carcinoma. *3rd Int. Symp. Detection and Prevention of Cancer*, New York, April 1976, to be published.
51. S. Sprecher-Goldberger, L. Thiry, F. de Halleux, M. Bossens and I. Gould, Cell mediated response to herpes simplex virion and non-virion antigens in patients with cervical carcinoma with a depressed response to phytohemagglutinin. *Biomedicine Express* **23,** 399 (1975).
52. A. Hollinshead and G. Tarro, Soluble membrane antigens of lip and cervical carcinoma: reactivity with antibody for herpes virus non virion antigens. *Science* **179,** 698 (1973).
53. A. Hollinshead, P. Chretien, O'Bong Lee, J. Tarpley, S. Kerney, N. Silverman and J. Alexander, *In vivo* and *in vitro* measurements of the relationship of human squamous carcinomas to herpes simplex virus tumor-associated antigens. *Cancer Res.* **36,** 821 (1976).
54. B. F. Vestergaard, A. Hornsleth and S. N. Pedersen, Occurrence of herpes and adenovirus antibodies in patients with carcinoma of the cervix uteri. *Cancer (Philad.)* **30,** 68 (1972).
55. A. S. Pasca, L. Kummerländer, B. Peijtsik and K. Pali, Herpes virus antibodies and antigens in patients with cervical anaplasia and controls. *J. nat. Cancer Inst.* **55,** 775 (1975).

Europ. J. Cancer Vol. 12, pp. 859–864. Pergamon Press 1976. Printed in Great Britain

In Vitro Inhibition of Cell Growth by Aldose Phosphates*

ALLAN FENSELAU†‡ and CEDRIC LONG§

†*Department of Physiological Chemistry, The John Hopkins University School of Medicine, Baltimore, Maryland* 21205, *and* §*Flow Laboratories, Inc., Rockville, Maryland* 20852, *U.S.A.*

Abstract—*The effects of various aldehydes and phosphoaldehydes on cell growth were examined using normal 3T3 mouse fibroblasts and 3T3 mouse cells transformed with SV 40, polyoma and Kirsten sarcoma viruses.* DL-*Glyceraldehyde-3-phosphate,* D-*erythrose-4-phosphate, and* D-*ribose-5-phosphate (all at less than 1 mM) inhibited the growth of actively dividing 3T3 mouse cells more than their corresponding non-phosphorylated aldoses; only glycolaldehyde was more active than its phosphate. Addition of glutathione (0·5 mM) potentiated these effects for the phosphoaldehydes, but generally had little influence on the effects produced by the neutral aldehydes. The growth of transformed cells in the log phase was inhibited by glyceraldehyde phosphate to about the same extent: however, in a semi-resting (confluent) state, the transformed cell lines were significantly less affected by glyceraldehyde phosphate than the normal cell line. Although a mechanism for this action cannot be proposed, the conversion of glyceraldehyde phosphate into two known inhibitors of cell growth,* L-*glyceraldehyde and methylglyoxal, does not seem to account for its potency and the thiol effect.*

INTRODUCTION

VARIOUS aldehydes and ketoaldehydes can inhibit *in vivo* and *in vitro* mammalian cell growth by interfering with intermediary metabolism, protein synthesis or possibly other cellular processes [1–17]. Several of these substances, most notably kethoxal [9] and glyceraldehyde [10, 18], offer some promise as chemotherapeutic agents in the treatment of solid tumors and leukemia. The aldehydes studied to date can freely penetrate into the cell, since they mainly lack charged groups that can limit their passage through the plasma membrane [19]. On the other hand, phosphoaldehydes as such should be unable to enter cells, but their inhibitory effects have not been examined. In the following we would like to report the results of our *in vitro* studies on the effects of various phosphoaldehydes, particularly DL-glyceraldehyde-phosphate, as well as neutral aldehydes on the growth of normal and virally-transformed 3T3 mouse cells [20–22].

Accepted 9 June 1976.

*This work was supported by grants from the National Science Foundation (GB 20672) and National Cancer Institute of the USPHS (CA 11699) and by a contract with the Virus Cancer Program of the National Cancer Institute.

‡Research Career Development Awardee, USPHS, National Institute of General Medical Sciences (GM 70423).

MATERIAL AND METHODS

The cell lines employed in these studies were the random bred Swiss mouse 3T3, polyoma virus-transformed 3T3 (Py 3T3), SV 40 virus-transformed 3T3 (SV 3T3), all kindly provided by Dr. H. Green [21, 22], and Kirsten sarcoma virus-transformed Balb/c mouse embryo fibroblasts (KiSV), generously supplied by Dr. S. Aaronson [20].

The cells were grown in Eagle's minimal essential medium (EMEM) (Flow Laboratories, Rockville, Md.) supplemented with 10% calf serum or 5% heated calf serum (Colorado Serum Co. Laboratories, Denver, Colo.), glutamine, and antibiotics. The effects of various agents on cell growth were determined on cells that were actively dividing (log phase) or on cells in a semi-resting state (confluence). For most of the log phase studies, trypsinized cells ($1{\cdot}5 \times 10^5$) were plated in plastic dishes (Falcon, 6·0 cm diam.) and allowed to grow 24 hr on the usual growth medium. Then the medium was replaced with fresh, sterile medium containing the drug. The cells were observed periodically over the

next two days and were counted 72 hr after drug addition using standard procedures (Trypan blue dye exclusion). In the case of cell density dependency studies, initial concentrations of approximately 2×10^5, 5×10^5 and 10×10^5 cells were plated, 6 hr later media were changed, and after an additional 18–20 hr cell counts were obtained. For studies on cells in the semi-resting state, 10^6 cells were plated in a dish and allowed to approach confluence in EMEM with 10% calf serum (usually by over 3–4 days), after which fresh medium containing 5% heated calf serum was added. Under these latter conditions normal mouse fibroblasts became confluent and stopped dividing, while cells transformed by SV 40, polyoma or sarcoma viruses continued to divide. After 24 hr, fresh medium with the drug included was added; the plates were observed over 72 hr and the cells were counted at the end of this period. Duplicate plates were employed in all experiments. Results are expressed in terms of the ratio R of the treated-cell counts to untreated (control) cell counts. For $R = 0$ no viable cells were counted following drug treatment.

DL-Glyceraldehyde-3-phosphate (Sigma Chemical Corp., St. Louis, Mo.), dihydroxyacetone phosphate (Sigma) and glycolaldehyde phosphate (Calbiochem, La Jolla, Cal.) were prepared from the ketals of the barium salts using Dowex-50 according to the supplier's instructions. Solutions containing ethanol and $Ba(OH)_2.8H_2O$ at concentrations equivalent to those from the preparation of the organic phosphates (as free acids) were treated in a similar fashion, but failed to display any effects in cell growth. A mixture of tetrose 2,4-bisphosphates was synthesized from glycolaldehyde phosphate and purified according to the published procedure [23]. Glutathione was purchased from Schwartz–Mann (Orangeburg, N.Y.; lot no. V3705). All other chemicals were obtained from various commercial sources and were the highest purity available.

RESULTS

A survey of the effect of various substances was made by using log phase cultures of the 3T3 mouse cell line. In Table 1 may be seen the results of one such study using several phosphoaldehydes and the corresponding dephosphorylated analog. First, the observation that L-glyceraldehyde is a more effective growth inhibitor than the D-isomer has been noted previously [24] and is confirmed by our *in vitro* studies. However, the DL-phosphate is a much more potent inhibitor than L-glyceraldehyde at the same concentrations. Second, the phosphomonoesters in general are better inhibitors than the corresponding unphosphorylated derivatives. Finally, the aldose phosphates are much more active than another phosphoaldehyde, pyridoxal phosphate, which is commonly employed in preparing Schiff base derivatives with soluble proteins [25] and cell membrane components [19].

Table 1. *Effects of various aldehydes and their phosphate derivatives on the growth of 3T3 cells in the log phase*

The conditions employed in these studies are described under Material and Methods. Results are expressed in terms of the ratio (R) of treated cell counts to untreated cell counts.

	Clnc., mM	R
Glycolaldehyde	⩾ 0·2	0·0
Glycolaldehyde phosphate	0·6	0·17
	0·4	0·21
	0·2	0·67
D-Glyceraldehyde	0·6	0·67
	0·4	1·05
	0·2	1·05
L-Glyceraldehyde	0·6	0·51
	0·4	0·70
	0·2	0·86
D,L-Glyceraldehyde-3-phosphate	0·6	0·23
	0·4	0·47
	0·2	0·54
D-Erythrose	0·8	0·82
	0·4	0·94
D-Erythrose-4-phosphate	0·8	0·11
	0·4	0·72
D-Ribose	0·8	1·00
	0·4	1·06
D-Ribose-5-phosphate	0·8	0·68
	0·4	0·78
Pyridoxal	0·8	1·02
Pyridoxal phosphate	0·8	0·98

From the list of inactive compounds (Table 2) it appears that the presence of an aldehydic group alone (as in the aliphatic aldehydes) or a phosphate group alone in the test substance is insufficient for producing an inhibition of *in vitro* cell growth. Reduction or oxidation of the aldehyde in glyceraldehyde phosphate to give β-glycerophosphate or 3-phosphoglycerate, for example, eliminates activity. Isomerization of the carbonyl group to provide dihydroxyacetone phosphate also abolishes most of the activity.

The more potent drugs (Tables 1 and 2) contain an aldehydic group with an adjacent

oxygen (or sulfur) functional group on a short chain of carbon atoms. A phosphate group need not be present for the display of growth retardation or cell killing. Among the active compounds differences in inhibitory powers were noted; specifically, the glyceraldehydes, glyoxal, methylglyoxal (pyruvaldehyde), and α-mercaptoglyceraldehyde were not as effective over a concentration range of 0·2–0·8 mM in inhibiting cell growth as kethoxal, glycolaldehyde, DL-glyceraldehyde phosphate, and glycolaldehyde phosphate.

Table 2. Structural analogs of glyceraldehyde phosphate with little or no effect on the growth of 3T3 cells in the log phase

The conditions employed in these studies are described under Material and Methods. Addition of glutathione (0·5 mM) did not lead to a potentiation of an inhibition by any of the following compounds. The ratio of treated cell counts to untreated cell counts varied between 0·85 and 1·0 for the following. The highest concentration (mM) of the analog employed is given in parentheses.

Glycollate (0·4)	3-Phosphoglycerate (0·7)
Glyoxalate (0·4)	β-Glycerophosphate (0·7)
Pyruvate (0·4)	α-Glycerophosphate (0·7)
Lactate (0·4)	Acetylphosphate (0·4)
Acetaldehyde (0·4)	Phosphoenolpyruvate (0·4)
Propionaldehyde (0·6)	ATP (0·4)
Hydroxyacetone (0·7)	Dihydroxyacetone phosphate (0·6)
Dihydroxyacetone (0·7)	Fructose-1,6-bisphosphate (0·5)
2-Deoxyribose (0·6)	

The effects of added thiols, which can partially reverse the inhibitions of the neutral aldehydes [7, 13], were also examined in our *in vitro* system. With dithiothreitol, dithioerythritol, cysteine, N-acetyl-cysteine, β-mercaptoethanol, and aminoethanol (and in the absence of aldehydes), significant retardation of growth of the normal 3T3 cells was noted in all cases when calf serum was used [the ratio (R) of treated cell counts to untreated cell counts = 0·5–0·75 at 1·2 mM thiol]. However, significantly less of an effect ($R = 0·75–1·0$) resulted when fetal calf serum was substituted. Most important in this regard is that the inhibition by DL-glyceraldehyde phosphate alone is independent of serum. The addition of glutathione (0·5 mM) to media containing the various test substances (at several concentrations from 0·2–0·8 mM) had only a slight effect on the inhibition produced by neutral aldehydes. Generally the result was a small reversal of the inhibition (as shown by kethoxal, methylglyoxal and α-mercaptoglyceraldehyde in Table 3); a potentiation of the inhibition was observed, nevertheless, in the case of glyoxal (from 0·2–0·8 mM). However, all of the aldose phosphates examined exhibited enhanced inhibition in combination with glutathione (Table 3). The addition of glutathione to solutions of the corresponding aldoses did not significantly alter their inhibitory properties. The possibility that the synergistic effects with glyceraldehyde phosphate might be due to the formation of a polymeric, polyanionic inhibitor (containing thioacetal linkages between the aldehyde and thiol molecules) was tested using heparin (≤ 200 μg/ml), but no growth inhibition by the polyanion was observed.

Table 3. Effects of various aldehydes in the presence and absence of glutathione on the growth of 3T3 cells in the log phase

Conditions employed in these studies are described under Material and Methods. Results are expressed in terms of the ratio (R) of treated cell counts to untreated cell counts and are the average of two separate experiments. The concentration (mM) of the added agent is given in parentheses.

	R No glutathione added	R Glutathione (0·5 mM) added
DL-Glyceraldehyde-3-phosphate (0·4)	0·48	0·11
Glycolaldehyde phosphate (0·1)	0·68	0·18
Threose-2,4-bisphosphate (0·3)	0·70	0·41
D-Erythrose-4-phosphate (0·4)	0·68	0·34
D-Ribose-5-phosphate (0·8)	0·61	0·38
Methylglyoxal (0·6)	0·45	0·56
Glyoxal (0·4)	0·86	0·55
α-Mercaptoglyceraldehyde (0·6)	0·54	0·53
Kethoxal (0·3)	0·07	0·20

The inhibition studies were extended to virally-transformed 3T3 cells in both the growing (log phase) and semi-resting (confluent) states, using only the more readily available DL-glyceraldehyde phosphate. The four cell lines, the normal 3T3 and the Py, SV 40, and KiSV transformed derivatives, were subcultured at various initial cell population densities (8·5, 17 and 34×10^3 cells/cm^2) and were treated with varying amounts of DL-glyceraldehyde phosphate (0·45–1·35 mM at the lowest initial densities and 1–3 mM at the highest). At low initial cell densities, only the KiSV 3T3 cultures contained significant cell numbers ($R = 0·40$) when high concentrations of glyceraldehyde phosphate were used;

at lower concentrations, both KiSV 3T3 and SV 3T3 cultures had higher R values (0·70 and 0·33, respectively). This lesser sensitivity shown by two of the transformed cell lines toward glyceraldehyde phosphate inhibition was more evident at the higher initial cell densities. For example, at 34×10^3 cells/cm^2 and 1 mM glyceraldehyde phosphate essentially no growth retardation was observed with the three transformed cell lines, whereas $R = 0{\cdot}22$ for the normal 3T3 cell line; at 3 mM glyceraldehyde phosphate, though, only the KiSV 3T3 culture had a high R value (0·55).

A similar result was found when the different cell lines were grown to confluence in a medium containing 10% serum, were then adapted to a semi-resting state in a maintenance medium containing heat-inactivated serum, and finally were overlaid with a maintenance medium containing glyceraldehyde phosphate (1–3 mM). At 1 mM aldose phosphate $R = 0{\cdot}28$ for the 3T3 cells, but for the transformed cells the R-values ranged from 0·73–0·88. Growth of both Py 3T3 and KiSV 3T3 cell lines was inhibited significantly at 3 mM glyceraldehyde phosphate ($R = 0{\cdot}43$–$0{\cdot}46$); however, no cells were counted for normal 3T3 cultures that were treated identically.

DISCUSSION

The results of these studies indicate that aldose phosphates, especially glyceraldehyde phosphate and glycolaldehyde phosphate, are inhibitors of 3T3 cell growth *in vitro*. This inhibition is greater than that found *in vitro* with the corresponding neutral aldoses (with glycolaldehyde as the lone exception) and comparable to that found with several substances (L-glyceraldehyde, kethoxal and methylglyoxal), which have undergone limited testing as possible cancer chematherapeutic agents [1, 9, 18]. The potency of the phosphoaldehydes is enhanced further by the addition of a thiol compound, such as glutathione.

Inhibition by glyceraldehyde phosphate, and presumably by the other phosphoaldehydes, is greatest with sparse cultures of actively dividing cells, which can be either normal or virally-transformed. However, the resistance of the transformants to the effects of glyceraldehyde phosphate, barely noticeable with sparse cultures, becomes more evident in confluent (or serum-deprived) cultures. No explanation can be advanced at this time for these results, which certainly merit further study.

The site of action for these phosphorylated aldehydes is not at all clear. The relative impermeability of the mammalian membrane to organophosphates [19] and the greater activity *in vitro* of the charged versus the corresponding uncharged aldehydes suggest that the aldose phosphates may be acting at different or additional loci. Inhibition by the neutral glyceraldehydes occurs with the L-form at the hexokinase step in glycolysis (due to the formation of the active species sorbose phosphate by aldolase [24]) and in protein synthesis with both D- and L-forms [11, 12]. It also appears that the inhibition by the aldose phosphate involves more than formation of Schiff base derivatives with soluble serum proteins or cell membrane components, since the aldose phosphates are more active than pyridoxal phosphate.

Finally, the identity of the active species for these inhibitory effects also remains to be established. Culture media composed of amino acids in high concentration and serum containing many undefined proteins, along with the thiol, might very well chemically alter aldehydes or phosphoaldehydes. In fact the intracellular formation of thiazolidine-4-carboxylic acids from added aliphatic aldehydes (which readily enter mammalian cells) and endogenous cysteine has been invoked as an explanation for the *in vivo* inhibitory effects of the aldehydes on protein biosynthesis [13]. Furthermore, the conversion to methylglyoxal from glyceraldehyde [26] or glyceraldehyde-3-phosphate [27] can be non-enzymically catalysed by lysine, although methylglyoxal formation does not seem to be stoichiometrically related to (phospho)aldehyde loss. Our own results indicate that glyceraldehyde phosphate alone is more active against normal 3T3 cells than glyceraldehyde or methylglyoxal over a range of concentrations (0·2–0·8 mM) and that this growth inhibition by the phosphoaldehyde is augmented by the presence of added extracellular glutathione (but is neither reversed nor unaffected, as with the neutral aldehydes). More detailed study, however, is still needed in order to determine the mechanism(s) of action for the inhibitions by aldose phosphates.

Acknowledgements—We gratefully acknowledge the skilled technical assistance of Mrs. Tena Wei and Mr. Bob Baker.

REFERENCES

1. M. A. Apple and M. J. Cline, Anticancer effects of propanals: comparison between human and animal tumor systems. *Nat. Cancer Inst. Monogr.* **34,** 161 (1971).
2. M. A. Apple and D. M. Greenberg, Arrest of cancer in mice by therapy with normal metabolites. 1. 2-oxopropanal. *Cancer Chemother. Rep.* **51,** 455 (1967).
3. M. A. Apple and D. M. Greenberg, Arrest of cancer in mice by therapy with normal metabolites. II. Indefinite survivors among mice treated with mixtures of 2-oxopropanal and 2,3-dihidroxypropanal. *Cancer Chemother. Rep.* **52,** 687 (1968).
4. M. A. Apple and D. M. Greenberg, Inhibitory effect of DL-2-mercapto-3-hydroxypropanal on growth of transplantable cancers in mice. *Cancer Chemother. Rep.* **53,** 195 (1969).
5. M. A. Apple, F. C. Ludwig and D. M. Greenberg, Selective growth inhibition in mice by dihydroxypropanal without concomitant inhibition of bone marrow or other normal tissue. *Oncology* **24,** 210 (1970).
6. E. Ciaranfi, L. Loreti, A. Borghetti and G. G. Guidotti, Studies on the anti-tumor activity of aliphatic aldehydes. II. Effects on survival of Yoshida ascites hepatoma-bearing rats. *Europ. J. Cancer* **1,** 147 (1965).
7. L. G. Együd, Studies on cell division: the antagonism of α-ketoaldehydes and thiols. *Curr. Mod. Biol.* **2,** 128 (1968).
8. B. Freedlander and F. A. French, Carcinostatic action of polycarbonyl compounds and their derivatives. II. Glyoxal bis (guanylhydrazone) and derivatives. *Cancer Res.* **18,** 360 (1958).
9. F. A. French and B. L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives. I. 3-ethoxy-2-ketobutyraldehyde and related compounds. *Cancer Res.* **18,** 172 (1958).
10. B. C. Giovannella, W. A. Lohman and C. Heidelberger, Effects of elevated temperatures and drugs on the viability of L1210 leukemia cells. *Cancer Res.* **30,** 1623 (1970).
11. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
12. G. G. Guidotti, L. Loreti and E. Ciaranfi, Studies on the anti-tumor activity of aliphatic aldehydes. I. The mechanism of inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells. *Europ. J. Cancer* **1,** 23 (1965).
13. L. Loreti, M. E. Ferioli, G. C. Gazzolo and G. G. Guidotti, Studies on the anti-tumor activity of aliphatic aldehydes. III. Formation of thiazolidine-4-carboxylic acid in tissues. *Europ. J. Cancer* **7,** 281 (1971).
14. E. Mihich and C. A. Nichol, Kethoxal bis(thiosemicarbazone). I. Effects against experimental tumors. *Cancer Res.* **25,** 1410 (1965).
15. A. Sakamoto and K. N. Prasad, Effect of DL-glyceraldehyde on mouse neuroblastoma cells in culture. *Cancer Res.* **32,** 532 (1972).
16. A. C. Sartorelli and W. A. Creasey, Cancer chemotherapy. *Ann. Rev. Pharmacol.* **9,** 51 (1969).
17. A. Szent-Györgyi, L. G. Együd and J. A. McLaughlin, Keto-aldehydes and cell division. *Science* **155,** 539 (1967).
18. M. von Ardenne, Zur Berechnung der Glycerinaldehyd-Wirkdosis im Gewebe bei Applikation in den Kreislauf. *Naturwissenschaften* **51,** 217 (1964).
19. D. B. Rifkin, R. W. Compans and E. Reich, A specific labeling procedure for proteins on the outer surface of membranes. *J. biol. Chem.* **247,** 6432 (1972).
20. J. R. Stephenson and S. A. Aaronson, Antigenic properties of murine sarcoma virus-transformed BALB/3T3 non-producer cells. *J. expt. Med.* **135,** 503 (1972).
21. G. J. Todaro and H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell. Biol.* **17,** 299 (1963).
22. G. J. Todaro, H. Green and B. D. Goldberg, Transformation of properties of an established cell line by SV40 and polyoma virus. *Proc. nat. Acad. Sci. (Wash.)* **51,** 66 (1964).

23. A. L. FLUHARTY and C. E. BALLOU, D-Threose-2,4-diphosphate inhibition of D-glyceraldehyde-3-phosphate dehydrogenase. *J. biol. Chem.* **234,** 2517 (1959).
24. H. A. LARDY, V. D. VIEBELHAUS and K. M. MANN, The mechanism by which glyceraldehyde inhibits glycolysis. *J. biol. Chem.* **187,** 325 (1950).
25. G. E. MEANS and R. E. FEENEY, *Chemical Modification of Proteins* Holden-Day, Inc., San Francisco (1971).
26. A. BONSIGNORE, G. LEONCINI, A. SIRI and D. RICCI, Kinetic behaviour of glyceraldehyde-3-phosphate conversion into methylglyoxal. *Ital. J. Biochem.* **22,** 131 (1973).
27. V. RIDDLE and F. W. LORENZ, Non-enzymic, polyvalent anion-catalyzed formation of methylglyoxal as an explanation of its presence in physiological systems. *J. biol. Chem.* **243,** 2718 (1968).

Europ. J. Cancer Vol. 12, pp. 865–870. Pergamon Press 1976. Printed in Great Britain

Fate and Distribution of [^{14}C] Succinyl Neocarzinostatin in Rats*

HIROSHI MAEDA, NAOKI YAMAMOTO and AKIRA YAMASHITA†

Department of Microbiology and Department of Anatomy†
Kumamoto University Medical School, Kumamoto, Japan, 860.

Abstract—In vivo *distribution of the* [^{14}C] *succinyl derivative of the antitumor antibiotic, neocarzinostatin (NCS), was studied in rats. The nature of the derivative is almost identical to the parental NCS. The radioactivity of the derivative within* 20 *min after i.v. injection was found to have accumulated in large amounts in such organs as the kidney, bladder, ovary–uterus, bone marrow, tongue, intestine, lung, skin, stomach and muscle. The levels in the plasma and bladder, although partly due to decomposed components, were high even after* 1 *hr. Thymus, spleen, and eye were found to contain only a small amount of this derivative. No appreciable amount of radioactivity was found in the brain. The accumulation of radioactivity in the kidney was greater than* 6·9 % *of input. Within* 20 *min,* 45·5 % *of the drug was found in urine, indicating a very rapid turnover. The desuccinylation of the derivative was much milder than anticipated while degradation by proteolysis of NCS was the main mechanism of inactivation which was followed by excretion. The nature of accumulated molecules in the kidney and bladder was largely high molecular weight components (mean weight* > 6000). *NCS seems to be excreted into the urine both in the degraded forms and in almost intact forms.*

INTRODUCTION

NEOCARZINOSTATIN (NCS) is the first antibiotic of protein nature (mean weight 10,700) of known chemical structure now at the preliminary clinical stage in cancer chemotherapy [1–4]. Previously, the potential usefulness of the drug was shown experimentally in mice [5, 6] and in human [1]. Until recently NCS was known to be useful for acute myelogenous leukemia [2, 3] and stomach cancer [4]. However, the specific organs or tumors *in vivo* have been rather unclear due to the difficulties in the preparation of a radiolabelled derivative of the antibiotic NCS. Fujita *et al.* reported in a study which was based on the distribution of residual antibacterial activity of intact NCS after i.v. administration in mice and rabbits [7] where extremely rapid inactivation made detailed analysis impossible. Recently, Toriyama *et al.* reported on a similar work giving NCS orally into mice [8]. No attention was paid for the proteolytic breakdown of the drug during these experiments, which was found to be the major inactivation pathway [9].

Based on the present results, we have applied NCS for the treatment of human bladder cancer, which was found to be one of the main objectives in this study, and have obtained excellent results (S. Sakamoto, J. Ogata and H. Maeda).*

We prepared radioactive NCS by succinylation [11–13] based on its structure [14–16] as previously reported. The chemically modified NCS (bis succinyl NCS) retained most of its original properties, such as molecular size, acidic nature, amino acid sequence and composition, conformation and biological activities [12]. The succinyl derivative was almost as active as the original compound at 0·25 μg/ml against hepatoma derived cell line (He) and a lymphoblastoid cell line (P3HR-1) but slightly weak activity against human embryonic lung fibroblasts (Flow 2000). A strong *in vivo* activity was also confirmed. The succinyl derivative was found to penetrate into cytosol

Accepted 26 May 1976.

*A part of this investigation was supported by the Cancer Grant (II) for 1974 from the Ministry of Education, Science and Culture, Japan. Reprint requests should be addressed to H.M.

†Present address: Department of Anatomy, Hamamatsu University of Medicine, Hamamatsu, Japan.

*Seven out of eight patients with bladder carcinoma, treated with NCS, have been cured completely without recurrence over six months [10].

and onto/into cell-nucleus as indicated by autoradiography [13]. Its desuccinylation did not occur but proteolysis did proceed *in vitro* with serum [9, 13].

The present investigation is the first systematic study of the catabolic breakdown of protein antibiotics *in vivo*. The results will primarily shed light on which specific organs NCS can effect as a cancer chemotherapy agent in humans.

MATERIAL AND METHODS

Material

NCS and the succinylation procedure with [^{14}C] succinic anhydride were the same as previously reported [11, 13]. The succinyl NCS was more acidic (about 0·5 pH unit in pI) than NCS, but otherwise very similar to the original NCS. It has the same antigenicity as NCS. The specific radioactivity of the material used was $2{\cdot}38 \times 10^6$ dis/min per mg protein (about 24 mCi/mmole).

Animals, treatments and preparations of specimens

Female and male rats of DA strain weighing 95–140 g were used for the experiments. One mg of the drug, dissolved in 0·5 ml of 0·01 M phosphate buffered saline (0·15 M NaCl) (PBS, pH 7·0), was injected intravenously through a tail vein while the rats were under ether anesthesia. Since the original NCS has an *in vivo* half-life of less than 10 min [7], only the distribution of the drug at 20 and 60 min was studied. Twenty and sixty min after injections, each rat was anesthetized again and all of the blood was immediately replaced by 10 ml of PBS infused through the inferior vena cava and recovered from the cross sected abdominal aorta. The infusion process was repeated three times (30 ml in total) until complete replacement was ascertained by the disappearance of the color of blood in the organs such as the lung and liver. Each organ was removed immediately and weighed by a torsion balance after removal of excess liquid with soft tissue paper. The DPM counts of urine in Table 1 were obtained by placing the weighed bladder in vials with 10 ml of PBS and rinsing for about 40 min. Bone marrow specimens were obtained by applying a hydrostatic pressure to one side of femurs using a 23 gauge needle attached to a syringe filled with 1 ml of cold PBS. The content of the bone cavity (marrow) was dispersed by repeating the flushing processes into a scintillation vial. The empty bones thus obtained were used as bone specimens. The specimens of skins were obtained from back-hide and those of muscles were from rectus abdominis, free of connective tissue. Contents of stomach and small and large intestines were removed by gentle rinsing with PBS.

Table 1. Distribution of radioactivity of [^{14}C] succinyl neocarzinostatin in organs or tissues of rats

Specimens	After 20 min		After 60 min	
	Mean dis/min	S.E.	Mean dis/min	S.E.
Kidney	151,570 ±	15,661	99,514 ±	6333
Bladder	108,759 ±	28,756	133,423 ±	29,556
Ovary-uterus	14,502 ±	5878	3973§	—
Bone marrow	12,291 ±	673	5394 ±	1002
Blood plasma	10,246 ±	639	5541 ±	198
Lung	8261 ±	2664	1785 ±	390
Skin	7243 ±	767	4278 ±	353
Tongue	7076 ±	611	5158 ±	907
Stomach	5558 ±	706	2693 ±	471
Muscle	4289 ±	1302	1482 ±	206
Large intestine	4041 ±	938	1487 ±	266
Lymph nodes	3884 ±	499	1604 ±	762
Small intestine	3336 ±	1033	939 ±	77
Testicles	—	—	1350‖ ±	62
Adrenal gland	3305 ±	445	6684 ±	2469
Pancreas	2339 ±	417	1223 ±	113
Liver	2290 ±	466	1575 ±	114
Submaxillary-salivary gland	2279 ±	106	1375 ±	100
Bone	2251 ±	1115	1026 ±	79
Heart	2179 ±	246	1032 ±	206
Thymus	2072 ±	505	927 ±	139
Eye	1880 ±	317	599 ±	194
Spleen	1209 ±	126	827 ±	71
Brain	96 ±	28	108 ±	8
Cellular fraction in blood*	951 ±	129	1001 ±	195
Hypophysis†	4050 ±	680	3822 ±	689
Urine‡ (out of 1 g bladder)	5469 ±	1254	6110 ±	1230

Data are the mean of four rats at 20 min and 60 min respectively and DPM counts were expressed as per gr (wet wt.) organ. The body weight of each rat was adjusted to 100 g by calculation.

*Cellular components were washed with PBS two times by centrifugation (1500 rpm × 5 min).

†The weight of hypophysis was about 5 mg, and thus the errors of 20% was unavoidable.

‡Diffusible counts from 1 g bladder is assigned as urine arbitrary. See Table 2.

§Sample from one rat.

‖Samples from three rats.

Measurement of radioactivity

A whole or a part of the organs or tissues was oxidized by combustion with Sample Oxidizer Model 306 of Packard Inc., and [^{14}C]O_2 gas

was trapped in Carbosorb (Packard Inst. Co. Inc. Downers Grove, Ill). Oxidation of samples was carried with the aid of oxygen gas and cellulose powder, and with Combustaid (Packard) when necessary. The scintillation liquid contained both Carbosorb and Permafluor V (12:13) (Packard). Bone marrow cells, blood plasma and cellular fraction of the blood were solubilized with Soluene solubilizers and with aliquots of 30% H_2O_2 as described in the procedure of Packard Manual [17], and the radioactivity was counted.

Analysis of molecular sizes of degradation products of [^{14}C] NCS

The validity of the [^{14}C] derivative, particularly the lack of desuccinylation of the succinyl NCS is shown on Fig. 1. As soon as the blood sample was taken, it was mixed with heparin, $HgCl_2$ and di-isopropyl fluorophosphate (DFP), the latter two to minimize proteolysis. The final concentrations used were 10 units/ml for heparin and 0·1% each for the latter. Then the plasma was separated from cellular components by centrifugation. Subsequently, 0·1–0·3 ml of plasma were applied to a Sephadex G-15 column (1·5 × 46 cm) and eluted with distilled water. Elution with the latter produced a better separation of the peaks than PBS. Radioactivity was monitored on each fraction. Calibrations of the molecular weight were carried out with myoglobin (mean weight 17,000), actinomycin D (mean weight, 1,255), phenol red (mean weight 354) and acetic acid (mean weight 60).

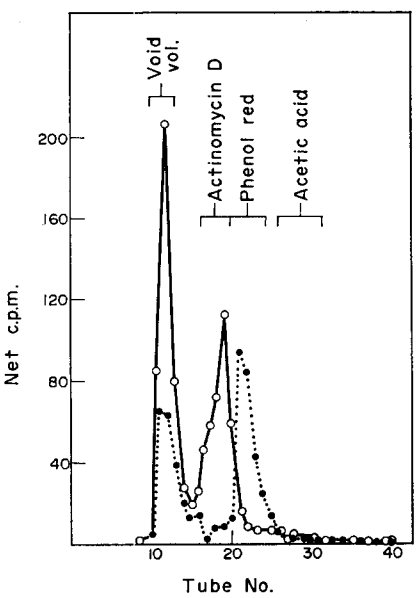


Fig. 1. Breakdown of [^{14}C] succinyl neocarzinostatin in vivo (Rat) as revealed by Sephadex G-15. The open circle-solid line and the solid circle-dotted line indicate plasma samples obtained 20 min and 60 min after injection of the drug respectively. Note the decrease of the first peak near the void volume after 60 min and the increase in amount and the size shift to the low molecular wt. region of the second peak thus indicating an extensive degradation of the protein.

The molecular sizes of [^{14}C] containing fragments obtained from homogenates of various specimens were investigated with a column (1·5 × 60 cm) of Sephadex G-25 (Fine) and eluted by PBS (2 ml per fraction). The calibrations of the column with molecular references were the same as that for G-15. The samples of each organ (about 1 g) were prepared by homogenization with a Sorval homogenizer at 4°C with 2 ml PBS containing DFP and $HgCl_2$ to give a final concentration of 0·1%, and then centrifuged at 5500 rpm for 15 min. Urine specimens were collected on filter papers and the urinal spots were cut out and extracted with about 4 ml of PBS.

RESULTS

The molecular sieve chromatography with Sephadex G-15 indicated that there was no desuccinylation of the derivative during 20 or 60 min *in vivo* treatment, however, a peptide bond cleavage of NCS which resulted in a peak of smaller molecular size was noticeable after 60 min (Fig. 1).

The results summarized on Table 1 show the distribution. The radioactivity was found to be distributed throughout the whole body within 20 min except the brain. The brain shows the least amount in the uptake even after 60 min. At the initial 20 min, the highest radioactive counts per gram of specimen was found in the kidney followed by the bladder, ovary-uterus, bone marrow, lung, tongue, stomach, muscle and skin. The high activity found in the bladder after 60 min was partly attributed to urine origin (back diffusion) since the smaller peptide fragments account for more than 60% in plasma (Fig. 1) and high counts were found in the diffusible fraction and in the urine (Tables 1 and 2).

The recovery of the drug in various parts of the body is shown in Table 2. The very rapid excretion of the drug is noteworthy. In 20 min, 45·5% of the radioactivity was found in the urine (Table 2). This radioactivity is due to large molecules and partly to small molecules (Fig. 2). The molecular sizes of the drug in various organs after 20 min were of larger molecular weight. For instance, the first peak (mol. weight > 6000) in such organs as the kidney, lung, muscle (not shown), and liver was composed of 78, 90, > 90 and 55% of the

Table 2. Recovery of [^{14}C] radioactivity of succinyl neocarzinostatin in various organs and specimens 20 min after intravenous injection

Organs or specimens	Wet weight of organs (g)*	Total dis/min per organ ($\times 10^{-3}$)	% of recovery
Kidney	1·1	166·0	6·97
Blood plasma	4·0†	41·1	1·73
Liver	5·7	13·1	0·55
Small intestine	3·5	11·7	0·49
Lung	0·9	7·36	0·31
Stomach	1·1	6·15	0·26
Large intestine	1·3	5·26	0·22
Bladder	0·045	4·89	0·21
Tongue	0·2	1·41	0·058
Pancreas	0·33	0·77	0·032
Skin	13·0†	93·7	3·94
Muscle	30†	128·8	5·4
Bone	10†	22·5	0·95
Bone marrow	2·3‡	67·0	2·81
Urine	in 20 min§	1083·4	45·5
Blood cell fraction	3·2†	3·05	0·128

*The wet weights of organs represented here are the average values of two rats about 100 g body weight.
†Estimated.
‡Estimated from G. Hudson's data in *J. Anatomy* **92,** 150 (1958).
§Excreted in the initial 20 min on filter papers followed by extraction.

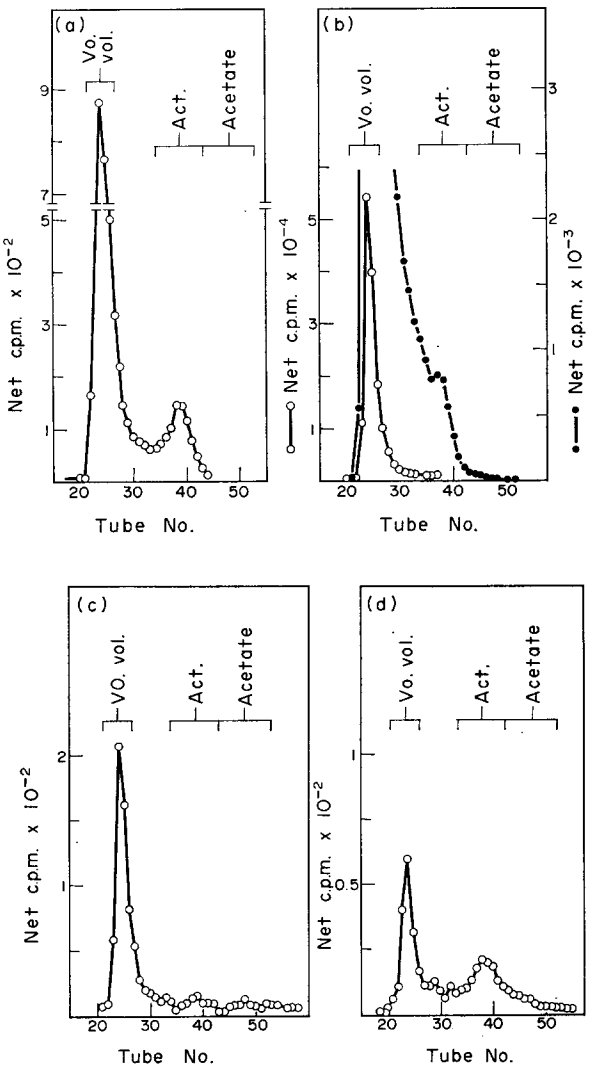


Fig. 2. Analysis of molecular size distribution in various organs of [^{14}C] succinyl neocarzinostatin 20 min after intravenous injections by Sephadex G-25 column. The preparations and fractionations were carried out as described under Material and Methods. (A), (B), (C) and (D) indicate the samples prepared from kidney, urine, lung and liver. A back ground cpm of 20 has been subtracted from each fraction. Each point represents an average of triplicate runs. In (B), two curves show the same data, but the solid circles were in expanded scale (ordinate at right).

total cpm respectively. The total recovery of radioactivity in the supernatant from the homogenates of kidney, lung, liver, and urine were 24·1, 69·2, 80·3 and 74·6% respectively after one process of homogenization and extraction (Fig. 2A-D). Only in the liver was the second peak (mol. weight ~1000) found to be at significantly higher indicating the degradation of NCS in this organ in addition to the blood.

The [^{14}C] derivatives in urine showed that NCS was excreted very rapidly, mainly as a large molecular size compound. Although smaller molecular size fragments are eluted, majority of the components are of intermediate size component when compared to the other patterns from other organs (Fig. 2B). The liver extract showed, on the contrary, more c/min in the smaller fragments (44·8%) than any of the other organs tested indicating that the degradation of this drug in the liver was one of the main pathways (Fig. 2D). In the lung, about 9% of c/min was found in the smaller fragments. Less than 10% of the total c/min in muscle was also smaller fragments (not shown).

When the radioactivities at 20-min and 60-min intervals were compared (Table 1), the pattern of excretion and retention of c/min can easily be seen. A pronounced fall in the radioactivity was observed in the ovary-uterus, lung, intestine, muscle, and eyes, On the other hand, relatively good retention or accumulation, nearly 50% or more, was observed, in the kidney, urine, bone marrow, tongue, liver, adrenal gland, blood cells and skin. The hypophysis, which is outside the blood brain barrier, had high values in contrast to the brain. The relatively high counts in the skin and muscle indicate that the absolute amount of the drug in these parts should be quite large in a whole body as shown in Table 2.

DISCUSSION

Since the prime requisite for drug action is its accessibility to particular tissues or organs, we have designed our present experiments with the use of the radioactive derivative. The drug concentration whether intact or degraded forms varied from one organ to the other 60 min after drug injection. The degradation studies clarified that more than 60% of the original NCS was distributed as molecular size of about 1000 (Fig. 1) in plasma within 60 min. The highest values found in the bladder after 60 min (Table 1) could be due to the excretion and the accumulation in urine. A back-diffusion to the bladder tissue was anticipated (details to be reported elsewhere). The radioactivities in the lung and ovary-uterus fell to about 21·8% and 27·4% to the values found at 20 min respectively. In other organs a similar or a less radioactivity than that of 20 min was found indicating relatively good retension of the drug. The low DPM counts found in the liver or spleen, both of which have well developed networks of blood vessels, could have been resulted by washing out in the flushing process partly, but nevertheless the same procedure did yield high values in lung and kidney.

These findings suggest that main areas of accumulation of this antibiotic may include the tumors in the kidney and bladder, followed by bone marrow, ovary-uterus, lung, tongue, stomach, muscle, large and small intestine, lymph nodes and skin. Despite comparatively low activity in the liver, it still may be a good area due to its vascularity. Brain and thymus seem to be poor areas perhaps due to their anatomical architectures of blood vessels, such as blood brain barrier and blood thymus barrier. The high counts in the hypophysis may be related to its lack of such a barrier.

Recent publication on toxicological studies of NCS in dogs showed renal lesions in survivors which can be rationalised by the high accumulation of the drug in these organs [18]. More than 6·9% of the drug is accumulated in the kidney in 20 min as shown in Table 2.

Previous studies of the distribution of NCS *in vivo* [7, 8] had drawbacks. These experiments were based on the residual antibacterial activity of NCS and were done without the use of inhibitors of proteolysis. The bacterial assay requires an incubation period of 1–2 hr for drug diffusion as well as an overnight for bacterial growth. Since the drug has a half life of about 10 min *in vivo*, the bioassay leads to much lower activity and makes it impossible to calculate the recovery rate. In addition, there are known inhibitory or unfavorable substances to bacterial growth *in vivo*. On the other hand, the present investigation reveals the target organs of NCS much more directly and with higher degree of sensitivity than did the bioassay method.

The study of breakdown of succinyl NCS, observed previously *in vitro* [13] with serum and cell homogenate and *in vivo* in the present study, showed, however, that desuccinylation of the derivative is not marked. Therefore, the distribution of [^{14}C] radioactivity, obtained here, is not measuring [^{14}C] succinic acid, but the fragment of succinylated polypeptide molecules of NCS within the experimental time. Thus the present results can be extended the distribution of the parental NCS molecule, in which an overall close similarity exists. Due to the well known development of penetrated capillaries in tumors *in vivo*, the accessibility of this drug to the tumors itself should be more favorable than to the normal organs.

Acknowledgements—We thank Prof. Y. Hinuma of the Department of Microbiology for his interest and encouragements, Dr. H. Ouchi of the Department of Pharmacology for discussions, Dr. Y. Koyama of Kayaku Antibiotic Res. Laboratory for his supply of NCS, Dr. Dave T. Osuga of University of California, and Dr. James Shaw of University of California Medical School, Davis, Calif., for their careful readings and suggestions during preparation of the manuscript and Mrs. M. Fujii for the typing.

REFERENCES

1. M. Takahashi, K. Toriyama, H. Maeda, M. Kikuchi, K. Kumagai and N. Ishida, Clinical trials of a new antitumor polypeptide: neocarzinostatin (NCS). *Tohoku J. exp. Med.* **98,** 273 (1969).
2. K. Hiraki, O. Kamimura, I. Takahashi, T. Nagao, K. Kitajima and S. Irino, Neocarzinostatin, une approche nouvelle dans la chimiothérapie des leucémies aiguës. *Nouv. Rev. franç. Hémat.* **13,** 445 (1973).
3. K. Hiraki, K. Kitajima, T. Nagao, I. Takahashi, H. Kinoshita, O. Kamimura, H. Hayashi, Y. Moriwaki, H. Chin and H. Sanada, Treatment of acute leukemia with neocarzinostatin. *Igaku no Ayumi* (*Progress in Medicine*) **87,** 18 (1973).

4. A. ANEHA, K. KIKUCHI and H. SUGANO, Clinical use of neocarzinostatin for tumors in advanced stage. *Proc. of the Japanese Cancer Association*, p. 103, The 33rd Annual Meeting, Oct. 1974 (Sendai).
5. W. T. BRADNER and D. J. HUTCHISON, Neocarzinostatin (NSC-69856): An antitumor antibiotic effective against ascitic leukemia L-1210 in mice. *Cancer Chemother. Rep.* **50,** 79 (1966).
6. N. ISHIDA, K. MIYAZAKI, K. KUMAGAI and M. RIKIMARU, Neocarzinostatin, an antitumor antibiotic of high molecular weight. Isolation, physicochemical properties and biological activities. *J. Antibiot. Ser. A (Tokyo)* **18,** 68 (1965).
7. H. FUJITA, H. NAKAYAMA, T. SAWABE and K. KIMURA, *In vivo* distribution and inactivation of neocarzinostatin. **Jap.** *J. Antibiot (Tokyo)* **23,** 471 (1970).
8. K. TORIYAMA, H. FUJITA and N. ISHIDA, Absorption, distribution and excretion of neocarzinostatin (NCS) in mice after oral administration. *J. Antibiotics* **28,** 64 (1975).
9. H. MAEDA and J. TAKESHITA, Degradation of neocarzinostatin by blood sera *in vitro* and its inhibition by diisopropyl fluorophosphate and N-ethylmaleimide. *Gann* **66,** 523 (1975).
10. S. SAKAMOTO, J. OGATA and H. MAEDA, Effect of neocarzinostatin on bladder carcinoma. *Proceedings of the Japanese Urological Society* (Kagoshima) April 1976.
11. H. MAEDA, Preparation of succinyl neocarzinostatin. *Antimicrob. Agents Chemother.* **5,** 354 (1974).
12. H. MAEDA, Chemical and biological characterization of succinyl neocarzinostatin. *J. Antibiotics (Tokyo)* **27,** 303 (1974).
13. H. MAEDA, S. AIKAWA and A. YAMASHITA, Subcellular fate of protein antibiotic neocarzinostatin in culture of a lymphoid cell line from Burkitt's lymphoma. *Cancer Res.* **35,** 554 (1975).
14. H. MAEDA, C. B. GLASER, J. CZOMBOS and J. MEIENHOFER, Structure of the antitumor protein neocarzinostatin. Purification, amino acid composition, disulfide reduction, isolation, composition of tryptic peptides. *Arch. biochem. Biophys.* **164,** 369 (1974).
15. H. MAEDA, C. B. GLASER, K. KUROMIZU and J. MEIENHOFER, Structure of the antitumor protein neocarzinostatin. Amino acid sequence. *Arch. biochem. Biophys.* **164,** 379 (1974).
16. J. MEIENHOFER, H. MAEDA, C. B. GLASER, J. CZOMBOS and K. KUROMIZU, Primary structure of neocarzinostatin, an antitumor protein. *Science* **178,** 875 (1972).
17. PACKARD MANUAL: Packard Inst. Co. Inc.; *Nuclear Chemicals and Supplies Catalog and Technical Data* p. T6 (1972) (Japanese Edition).
18. U. SCHAEPPI, F. MENNINGER, R. W. FLEISCHMAN, A. E. BOGDEN, P. S. SCHEIN and D. A. COONEY. Toxicity of neocarzinostatin (NSC-69856): An antitumor antibiotics with radiomimetic and antigenic characteristics. *Cancer Chemother. Rep.* Part 3, **5,** 43 (1974).

Europ. J. Cancer Vol. 12, pp. 871–876. Pergamon Press 1976. Printed in Great Britain

Transformation of Hamster Embryo Cells by 7-Methyl benz(a)anthracene *In Vitro*: Tumorigenicity, Chromosome Pattern and Spheroid Formation

S. LEVY, D. PAPADOPOULO, S. NOCENTINI, L. CHAMAILLARD, O. BEESAU, M. HUBERT-HABART and P. MARKOVITS

Fondation Curie, Institut du Radium, Section de Biologie, 26 rue d'Ulm, 75231 Paris Cedex 05, France

Abstract—*7-methyl benz(a)anthracene (7-MBA)—a mild carcinogenic compound* in vivo*—causes morphological and malignant transformation in mass cultures of hamster embryo cells. At a concentration of* 5 *μg/ml it yielded transformed cells (BA5–IR) morphologically slightly different from the controls, and possessing a low degree of transplantability:* 1×10^5 *of them are necessary to provoke tumor formation when grafted in hamsters. When cells are treated with* 10 *μg/ml of the carcinogen, we obtain a transformed cell line (BA10–IR) morphologically very different from the controls. Malignancy appears in both cases* 7 *months after treatment, but cells exposed to the higher concentration of 7-MBA become highly transformed, and cause tumors after grafting* 1×10^3 *cells. In addition, the BA10–IR cell line,* 13 *months after chemical treatment develop an interesting property, namely the ability to grow not only on the surface of the flask, but also to detach spontaneously and form spheroids in the supernatant. The cells in spheroids are distributed and behave somewhat like cells in tumors* in vivo. *The karyotype of control cells is diploid after* 18 *months, and near diploid after* 30 *months, whereas that of BA10–IR transformed cells is near triploid after* 17 *months of culture.*

INTRODUCTION

IN THIS work we study the *in vitro* transforming effects of 7-methyl benz(a)anthracene (7-MBA). This compound possesses a mild degree of carcinogenic activity *in vivo* [1]. It was proposed that methyl groups of methylated polycyclic hydrocarbons could play an important part in the interaction of these molecules with cell components [2–4]. Indeed, Daudel *et al.* [5] using a very sensitive spectrofluorimeter obtained spectra of DNA extracted from cells treated 24 hr earlier with 7-MBA, which were very close to the spectra of the original compound, rendering it most unlikely that the binding between 7-MBA and DNA involves the conjugated system.

There are several papers alluding to the fact that the methylated hydrocarbons need not be metabolized at the level of the ring system in order to act as carcinogens. For example Miller and Miller [6] found that K-region epoxide of 7-MBA is less carcinogenic *in vivo* than the original compound. Similar findings were reported by Marquardt *et al.* [4] using mouse-cells for *in vitro* transformation studies of 7-MBA and its metabolites. However, in a hamster cell system, Huberman *et al.* [7] obtained somewhat different results with the same compound, the epoxide being more active after 4-hr treatment and less active after 7 days treatment than the original compound.

The results obtained by Daudel *et al.* [8] show that microsomal fractions of hamster liver have a detoxifying effect on dimethylated derivatives of benz(a)anthracene in a hamster cell system, and that the addition of these microsomal fractions to the system do not enhance the malignant transformation of cells [9].

Accepted 24 May 1976.

Most previous studies with 7-MBA have dealt with *in vitro* morphological transformation. We were mainly concerned with demonstrating the *in vitro* carcinogenic effect of 7-MBA in mass cultures of hamster embryo cells, and ensuring the cancerous nature of the transformation by transplantation of the transformed cells. We, therefore, regularly grafted chemically treated cells into newborn hamsters and considered them cancerous whenever they caused progressively growing tumors [10].

MATERIAL AND METHODS

Primary hamster embryo cell cultures are prepared by trypsinization of 12–14 day old embryos. After 5 to 6 days of incubation, secondary cultures are made in 75 cm^2 Falcon plastic flasks. A modified Minimum Essential Medium (Eagle) complemented with 10% of a mixture of newborn and embryonic calf serums is used [11]. Two days later, carcinogen is added in acetone solution; 3 concentrations (2, 5 and 10 μg/ml) of 7-MBA are used. Parallely, the control cells are fed with a medium containing 0·5% acetone. After 24–72 hr, the controls and treated cells are grown in the usual growth medium [11].

After observing treated and untreated secondary cultures for about 4 weeks, subcultures are prepared by trypsinization. We have thus been able to isolate cell lines originating from both kinds of cultures.

We systematically checked the morphology of the cells and also regularly inoculated them subcutaneously into newborn Syrian hamsters. For grafting, cells are suspended in 0·05 ml of medium. The hamsters are kept under observation for 5–8 months [12].

To establish growth curves, 5×10^4 cells are seeded and cell counts are performed regularly using the trypan blue exclusion test to check cell viability.

Chromosomal analysis is done during the exponential growth. Cells seeded 24–28 hr earlier, are treated for 4 hr with colchicine (8 μg/10 ml of medium). After trypsinization, the cells are left for 7 min in 0·075 M potassium chloride at 37°C. Then fixative (1 part of glacial acetic acid: 3 parts absolute ethanol) is added, then removed by centrifugation (800–1000 rev/min, 5 min). The cells are again gently suspended in the fixative for 20 min and removed by centrifugation. This treatment is repeated twice. Then a concentrated cell suspension in fixative is dropped on cold, wet slides and left to dry. The slides are stained with a Giesma solution (diluted 1 : 50 in phosphate buffer solution at pH 6, 8).

RESULTS

1. *Toxic effects*

7-MBA is toxic to secondary hamster cells at all concentrations used (2, 5 and 10 μg/ml); 50–80% of cells are killed. The surviving fractions form colonies which sometimes show altered growth characteristics. Figure 1 shows a colony with abnormal cell growth in a culture treated 34 days earlier with 2 μg/ml of 7-MBA. Similar changes can be observed in all cases during the first 5–6 weeks after the treatment. Later, these toxic effects disappear and the cells begin to grow more regularly.

2. *Morphological and malignant transformation*

In the BA10–IR cell cultures, at about 4 months after the treatment, enhanced growth and stable morphological changes are observed; the large polygonal cells of the earlier periods are replaced by spindle-shaped cells which show a criss-cross pattern of growth and form several layers. At this time these morphologically transformed cells do not provoke tumor growth when grafted into hamsters (Table 1). In the BA5–IR cells, this morpho-

Table 1. *Secondary and* in vitro *passage cells originating from a hamster embryo culture initially exposed to 7-methyl benz(a)anthracene* (10 μg/ml) *during* 24 *hours were grafted into newborn hamsters.* (*Generally,* 0·05 *ml aliquot of cells in suspension was inoculated subcutaneously*)

Cells	Subculture	Days in culture	No. of cells grafted/animal	Average time of tumor appearance (days)	No. of tumor bearing animals/ no. of animals receiving graft	Days of observation after grafting
BA_{10}–IR*	6–7	135	3×10^5	—	0/11	180
	9–11	225	6×10^5	39	6/6	60
	13–14	270	1×10^5	26	7/7	60

*Morphological transformation: starting from the fourth subculture.

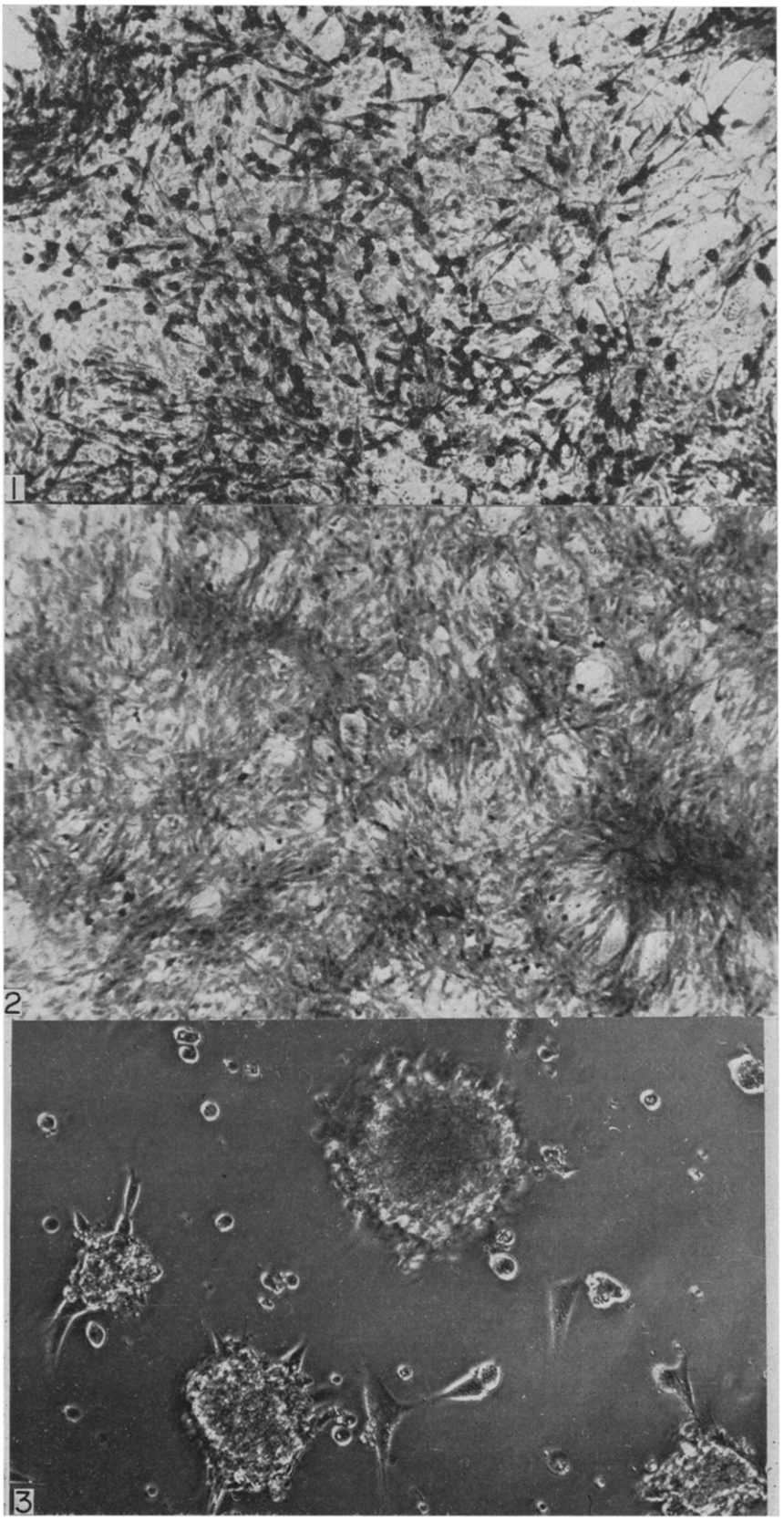


Fig. 1. *Toxic effects of* 7*-methyl benz*(*a*)*anthracene* (2 *μg*/*ml*) *on secondary hamster embryo cells* (34 *days in culture, May–Grünwald–Giemsa staining*). *Abnormal growth of cells with hyperchromatic nuclei in a thick colony.*

Fig. 2. *BA*10*-IR transformed cells* 14 *months after the chemical treatment. Spindle shaped cells, and criss-cross pattern of growth. Centers of piled up cells are seen.*

Fig. 3. *BA*10*-IR spheroids* 24 *hr after their transfer in a new flask. They attach to the bottom of the flask and some fusiform cells are migrating from them.*

(to face p. 872)

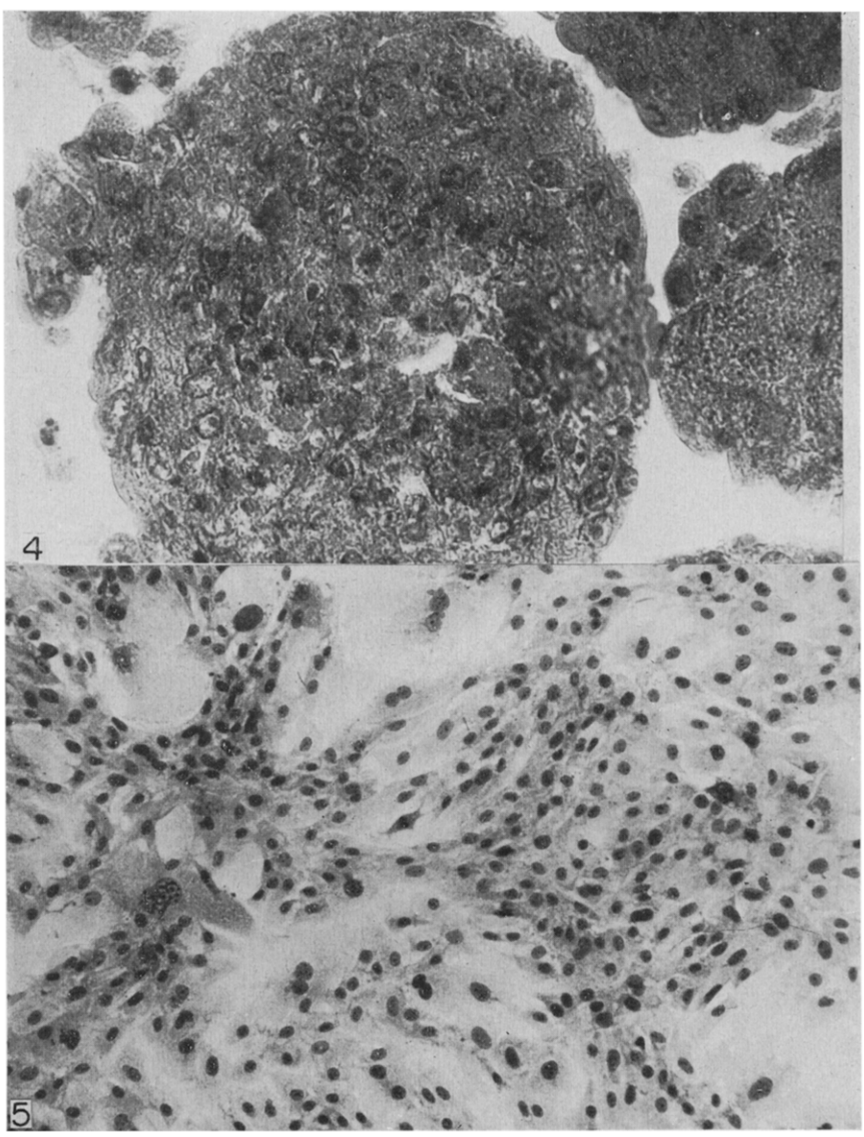


Fig. 4. *Histological section of a BA*10*–IR spheroid. As in a tumor* in vivo, *we can observe a necrotic centre, surrounded by living cells.*

Fig. 5. *T*14*–IR control cells* (18 *months in culture—*21*st subculture* in vitro). *Contact inhibited growth on the surface of the plastic flask.*

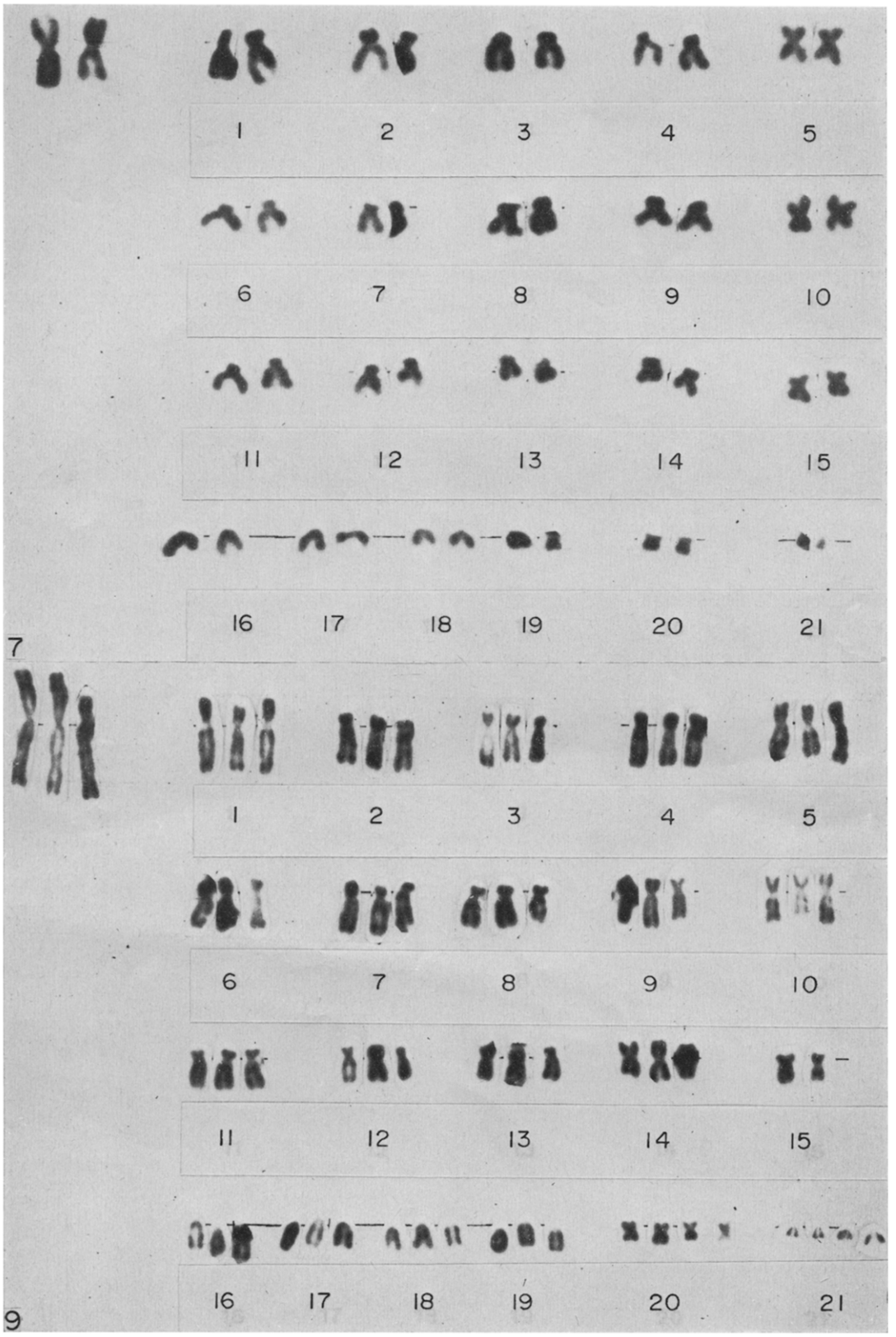


Fig. 7. Karyotype of a normal diploid cell from the 15th passage of T55–IR cells (42 autosomes + 2 X sex chromosomes).

Fig. 9. Abnormal near triploid cell from the 31st passage of BA10–IR in vitro *transformed, malignant cell line (64 autosomes + 2 X + 1 Y sex chromosomes).*

logical transformation occurs a little later, but again these cells do not provoke tumor growth 4 months after chemical treatment (Table 2).

Both BA5–IR and BA10–IR cells become malignant and produce tumors when grafted in hamsters only 7 months after the chemical treatment (Fig. 2) (Tables 1 and 2).

3. *Formation of spheroids*

Up to the 13th month after the chemical treatment, the BA10–IR cells grow only on the surface of the plastic flasks. During this period, there is a gradual increase of the growth rate and piling up of the cells. From the 13th month on, several centers of piled up cells detach from the surface of the flask and form spheroids in the medium. Cells growing on the flasks' surface are fusiform, while those of the spheroids are isodiametric. It is possible to subculture BA10–IR cells either by trypsinization of the attached layer or by simple transfer of spheroids from one flask to another.

Figure 3 shows some spheroids, 24 hr after their transfer to a new flask. They attach to the bottom of the flask and some fusiform cells are seen to spread out, grow on the flasks' surface. Eight days later, these cells begin to form piled up centers. Twenty-eight days after seeding, all the surface is covered with fusiform cells and numerous spheroids are floating in the supernatant. With the age of the culture the number of cells of these spheroids varies from 1000 cells to 50,000 cells.

Figure 4 shows a section of a spheroid. As in a tumor *in vivo*, we can observe a necrotic center surrounded with living cells. In spheroids the average proportion of living cells determined by a trypan blue exclusion test is 76%, while in cells fixed on the flasks' surface, it is 91%.

4. *Transplantability of BA10–IR cells growing on the surface of the flasks or in suspension*

The results summarized in Table 3 show that 10,000 BA10–IR cells or 10 intact spheroids (equivalent to about 10,000 cells) provoke tumor formation in all treated hamsters. The grafting of 1000 of these cells or that of one spheroid induce tumor formation in 3/5th of the animals. Thus, there is no significant difference between the transplantability of cells attached to the surface or that of spheroids growing in the supernatant.

The BA5–IR cells never form spheroids and they are of lower tumorigenicity. At least 1×10^5 BA5–IR cells have to be grafted to obtain tumor formation.

Table 2. *Cells of* in vitro *passages of hamster embryo culture initially exposed to 7-methyl benz(a)anthracene* (5 *μg/ml*) *during* 24 *hr were grafted subcutaneously into newborn hamsters*

Cells	Subculture	Days in culture	Number of cells grafted/animal	Average time of tumor appearance (days)	No. of tumor-bearing animals/ No. of animals receiving graft	Days of observation after grafting
BA_5–IR*	6	135	3×10^5	—	0/6	210
	14–16	225	2×10^6	76	8/8	90
	22	350	1×10^6	41	6/6	90

*Morphological transformation: starting from the 15th subculture.

Table 3. *Grafting effects of BA10–IR spheroids or of cells obtained by trypsinization of these spheroids or of the attached cell layer* (18 *months of culture*)

Origin of cells	Number of injected cells	Preparation	Average time of tumor appearance (days)	No. of tumor-bearing animals/ No. of animals receiving graft	Days of observation after grafting
Cell layer	1000	trypsinization	38	2/5	120
Spheroids		trypsinization	61	3/5	
Spheroids		—	38	4/5	
Cell layer	10,000	trypsinization	30	5/5	60
Spheroids		trypsinization	38	5/5	
Spheroids		—	37	5/5	

5. *Morphology and transplantability of control cells*

The control cell lines T14–IR after 18 months and T55–IR after 8 months of culture show normal contact inhibited growth on the surface of the plastic bottles (Fig. 5). During this relatively long period of *in vitro* growth, they never detach spontaneously from the flask to form spheroids and never produce tumors when grafted in hamsters (Table 4).

Growth curves of the BA10–IR cells attached to the surface of the flasks and those of the control cells are shown in Fig. 6. The growth rate of BA10–IR cells is at about 10 times higher than that of the early passage T55–IR, and 2·3 times more than that of the relatively late passage T14–IR control cells.

The doubling time of T55–IR is about 41 hr; its saturation density is $1{\cdot}5 \times 10^4$ cells/cm^2.

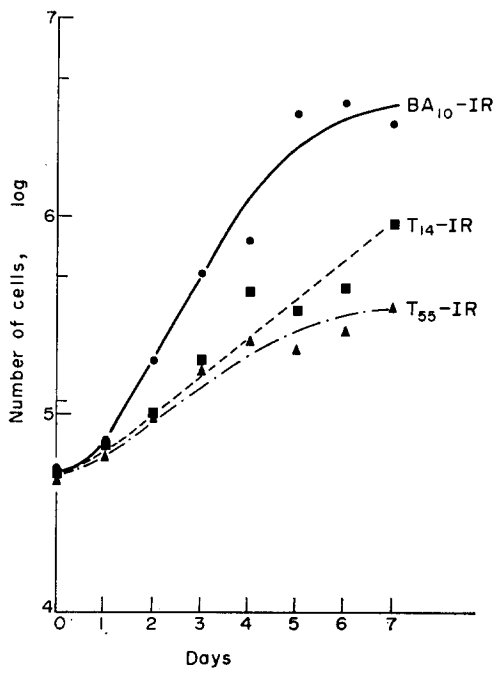


Fig. 6. In vitro *growth curves of the control cells T14–IR* (20 *months* in vitro) *and T55–IR* (8 *months* in vitro) *and of BA10–IR transformed cells* (18 *months* in vitro).

The doubling time of T14–IR is about 37 hr and its saturation density is $6{\cdot}5 \times 10^4$ cells/cm^2. The doubling time of BA10–IR is the most rapid of the three lines studied: 18 hr, and its saturation density is 15×10^4 cm^2.

6. *Karyotypes of normal and of BA10–IR cells*

After 8 and 18 months of culture respectively the karyotype of 90% of cells of the control lines show the characteristic number, e.g. 44 chromosomes (Fig. 7) [13]. After 30 months of culture, the control cells T14–IR still show a very homogenous population from the viewpoint of their karyotype (Fig. 8). At this time 16% of these control cells possess 37 to 41 chromosomes, 82% are diploid or neardiploid (41 to 46 chromosomes) and only 2% are hyperploid (59 chromosomes).

At 17 months after the treatment, BA10–IR cells possess very heterogenous chromosomes patterns (Fig. 8) 9% of cells are hypodiploid, 36% are hyperdiploid, and 55% are near triploid (61 to 73 chromosomes). Figure 9 shows a typical karyotype of BA10–IR cells, with 67 chromosomes.

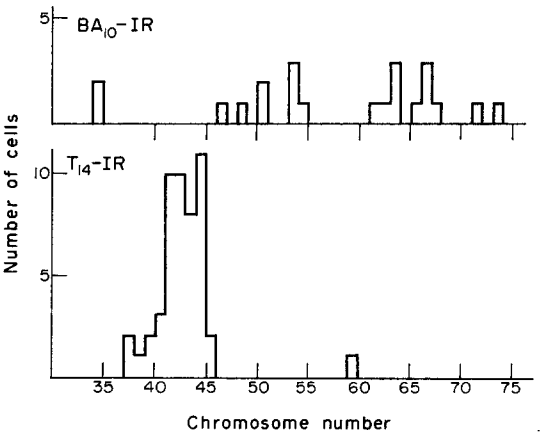


Fig. 8. *Histograms showing a homogenous karyological population of the normal cells T14–IR* (30 *months* in vitro), *and a very heterogenous one of BA10–IR transformed cells* (17 *months* in vitro).

Table 4. *Grafting effects of secondary cultures and of subcultures of control hamster embryo cells, initially exposed for* 24 *hr to a medium containing* 5% *acetone*

Cell line	Subculture	Days in culture	No. of cells grafted/animal	No. of tumor bearing animals/ No. of animals receiving graft	Days of observation after grafting
T_{14}–IR	16–19	360	1×10^6	0/12	180
	24–25	550	2×10^6	0/6	150
T_{55}–IR	8	150	$1{,}3 \times 10^6$	0/5	140

DISCUSSION

In this work and in our previous experiments we observed malignant transformation in mass cultures of hamster cmbryo cells in all cases when cultures were treated with compounds which are carcinogenic *in vivo*. The concentration of the polycyclic hydrocarbons used was determined according to the intensity of their toxic effect *in vitro* and the degree of their carcinogenicity *in vivo*. Thus, we used 5 and 10 μg/ml of 7-MBA, a rather mild carcinogenic compound *in vivo*, to transform cells in culture while in our previous studies [12] 0·05–0·1 μg/ml of benzo(a)pyrene; 7,12-dimethylbenz(a)-anthracene and 3-methylcholanthrene (highly carcinogenic compounds *in vivo*) sufficed to produce transformation. It is noteworthy that the malignant transformation appeared 7 months after the chemical treatment using 5 or 10 μg/ml of 7-methylbenz(a)anthracene, or 0·1 μg/ml of benzo(a)pyrene [12], and only a little later (8–9 months following the treatment) using 3-methylcholanthrene and 12-dimethylbenz(a)anthracene (0·1 and 0·05 μg/ml respectively) [12].

It appears therefore that to obtain *in vitro* malignant transformation, one hundred times greater quantities of the relatively mild carcinogen 7-MBA must be used of some other highly carcinogenic polycyclic hydrocarbons, as DMBA, benzo(a)pyrene, or methylcholanthrene.

The minimum number of transformed cells required to induce tumors in newborn hamsters vary according to the cell line. For cultures transformed either by 0·1 μg/ml of benzo(a)-pyrene [14] or by 10 μg/ml of 7-MBA, 500–1000, while for cultures transformed by 5 μg/ml of 7-MBA 100,000 cells were needed to obtain tumor formation.

In general, control cells originating from whole hamster embryos can not be maintained for longer periods than 3–6 months *in vitro* [15–17]. In an albumin complemented medium Matsuya and Yamane [18] cultured hamster lung cells for 3–4 months in diploid state, and for 13 months, with changes in ploidy. In an enriched Eagle medium [11], we maintain T14–IR control cells for at least 30 months *in vitro*. During the first 18 months of culture, these control cells keep their diploid state [13], and after 30 months, they are still near diploid. On the contrary, 60% of the *in vitro* transformed, malignant BA10–IR cells possess a triploid chromosome pattern (Fig. 9). We also find aneuploidy in 80% of benzo(a)pyrene transformed cells [13], but the cells of our methylcholanthrene transformed line remain near diploid [18]. According to Nachtigal *et al.* [19], recently transformed cells could remain diploid, whereas highly malignant cells would become heteroploid. The benzo(a)pyrene (0·1 μg/ml) and 7-methylbenz(a)anthracene (10 μg/ml) transformed cells are highly malignant and heteroploid, whereas the 3-methylcholanthrene (0·1 μg/ml) transformed cells are less malignant and near diploid [18]. It is necessary to study karyotypes of many other *in vitro* chemically transformed lines in order to find what proportion of lines become heteroploid.

Generally, malignant cells grow more readily in suspension than normal cells [20]. However, a particular property of BA10–IR transformed, malignant cells is that 13 months after the original chemical treatment, they grow spontaneously in the suspension and form nodules. Spheroids formation in stirred suspension of normal chinese hamster cells was reported by Sutherland *et al.* [21] and these cells were successfully used for radiobiological studies [21, 22]. Formation of spheroids by malignant cells was also observed with rat embryo cells transformed by Rous Sarcoma Virus and by malignant cells originating from mice. These studies were done with a rotative agitator in order to explain the mechanism of cell cohesiveness [23].

Acknowledgements—This work was supported by the French Ministry of Quality of Life, and by the Service d'Exploitation Industrielle des Tabacs et des Allumettes (S.E.I.T.A.).

REFERENCES

1. G. M. Badger, Relative potency of carcinogens to the skin of mice. Comparison of different systems of grading. *Brit. J. Cancer* **2,** 309 (1948).
2. E. Boyland and P. Sims, Metabolism of polycyclic compounds: The metabolism of 7,12 dimethylbenz(a)anthracene by rat liver homogenates. *Biochem. J.* **95,** (1965).
3. A. Dipple, P. D. Lawley and P. Brookes, Theory of tumour initiation by chemical carcinogens: dependence of activity on structure of ultimate carcinogen. *Europ. J. Cancer* **4,** 493 (1968).

4. H. Marquardt, T. Kuroki, E. Huberman, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, Malignant transformation of cells derived from mouse prostate by epoxides and other derivatives of polycyclic hydrocarbons. *Cancer Res.* **32,** 716 (1972).
5. P. Daudel, M. Croisy-Delcey, C. Alonso-Verduras, M. Duquesne, P. Jacquignon, P. Markovits and P. Vigny, Etudes par fluorescence d'acides nucléiques extraits de cellules en culture traitées par le méthyl-7 benzo(a)-anthracène. *C.R. Acad. Sci. Paris* **278,** 2249 (1974).
6. E. C. Miller and J. A. Miller, Low carcinogenicity of the K-region epoxides of 7-methyl benz(a)anthracene and benz(a)anthracene in the mouse and rat. *Proc. Soc. exp. Biol. (N.Y.)* **124,** 915 (1967).
7. E. Huberman, T. Kuroki, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, Transformation of hamster embryo cells by epoxides and other derivatives of polycyclic hydrocarbons. *Cancer Res.* **32,** 1391 (1972).
8. P. Daudel, D. Papadopoulo, P. Markovits, M. Hubert-Habart and L. Pichat, Effet d'un système microsomal sur le pouvoir toxique et sur l'interaction de certains hydrocarbures polycycliques cancérogènes avec des cellules en culture. *C.R. Soc. Biol. Paris* **169,** 507 (1975).
9. P. Markovits, D. Papadopoulo, S. Levy, P. Daudel and M. Hubert-Habart, Unpublished data.
10. S. Levy, D. Papadopoulo, S. Nocentini and P. Markovits, Croissance spontanée en suspension de cellules d'embryon de hamster cancérisées *in vitro* par le méthyl-7 benzo(a)anthracène, *C.R. Acad. Sci. Paris* **280,** 2905 (1975).
11. P. Markovits, J. Coppey, D. Papadopoulo, A. Mazabraud and M. Hubert-Habart, Transformation maligne de cellules d'embryon de hamster en culture par la diméthyl-7,10 benzo(c)acridine, *Int. J. Cancer* **14,** 215 (1974).
12. P. Markovits, P. Daudel, D. Papadopoulo, A. Mazabraud and M. Hubert-Habart, Sur le cancérisation chimique de cellules d'embryon de hamster en culture de masse, *Bull. Cancer* **62,** 59 (1975).
13. P. Markovits, D. Papadopoulo, M. Hubert-Habart, S. K. Mann, A. Labrecque and P. S. Sabharwal, Establishment of Normal Diploid and malignant heteroploid cell lines from non-treated and benzo(a)pyrene treated hamster Embryo cell cultures. *Experientia* **31,** 1215 (1975).
14. P. Markovits, S. Levy, D. Papadopoulo, O. Beesau, L. Chamaillard and M. Hubert-Habart, Studies on chemically treated and not treated hamster cells, maintained for 1 to 3 years in culture. In preparation.
15. Y. Berwald and L. Sachs, *In vitro* transformation of normal cells to tumor cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1965).
16. J. A. di Paolo and P. J. Donovan, Properties of syrian hamster cells transformed in the presence of carcinogenic hydrocarbons. *Exp. Cell Res.* **48,** 361 (1967).
17. G. J. Todaro and H. Green, Serum albumin supplemented medium for long term cultivation of mammalian fibroblast strains. *Proc. Soc. exp. Biol. (N.Y.)* **116,** 688 (1964).
18. Y. Matsuya and J. Yamane, Establishment of hamster fibroblast culture in albumin fortified medium, *Proc. Soc. exp. Biol. (N.Y.)* **135,** 393 (1970).
19. N. Nachtigal, N. C. Popescu and S. Nachtigal, Chromosomes of tumor induced by 3-methylcholanthrene in the golden hamster. *J. nat. Cancer Inst.* 697 (1967).
20. B. L. van Duuren, S. Witz and A. Sivak, *The Physiology of Cancer*, S. Karger, Basel, p. 2 (1974).
21. R. M. Sutherland, J. A. McCredie and W. R. Inch, Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. nat. Cancer Inst.* **46,** 113 (1971).
22. H. Dertinger and C. Lücke-Huhle, A comparative study of post-irradiation growth kinetics of spheroids and monolayers. *Int. J. Radiat. Biol.* **28,** 255 (1975).
23. B. Pejsachowicz, B. Halpern, H. Febvre and G. Barski, Différence dans les modalités d'agrégation en culture de cellules cancéreuses et de cellules témoins de même origine. *C.R. Acad. Sci. Paris,* **259,** 4891 (1964).

Europ. J. Cancer Vol. 12, pp. 877–888. Pergamon Press 1976. Printed in Great Britain

Antitumour Immunoprotection by an Immunobacterial Lectin Approach*

RENÉ VERLOES†‡, LOUIS KANAREK† and GHANEM ATASSI§

†*Laboratorium voor Chemie der Proteïnen, 65, Paardenstraat, 1640 Sint Genesius Rode, and §¶Service de Médecine et Laboratoire d'Investigation Clinique (Section de Chimie Expérimentale), Centre des Tumeurs, 1, rue Héger-Bordet, Bruxelles, Belgium*

Abstract—*By* in vivo *screening against lymphoid leukemia L*1210 *in BDF*$_1$ *mice and Ehrlich ascites carcinoma in BALB/c mice, we found that a vaccination with a synthetic antigen, built up by the cell wall of Micrococcus lysodeikticus (ATCC* 4698) *on which we coupled oligosaccharides of poly-N-acetyl-*D*-glucosamine, was able to protect to a fairly good extent against both tumours in the case of L*1210 *leukemia, hyperimmunization induced an increase in life span of* 75% *over controls and long term survivals of* 25%*; in the case of Ehrlich carcinoma, it induced an increase in life span of* 104% *and long term survivals of* 25%. *Under the present conditions, we observed that antibodies to the less immunogenic N-acetyl-*D*-glucosamine polymer were ineffective. Different antigens, antigen doses and immunization schemes were investigated. This system can be considered as an immunobacterial equivalent of both the lectins wheat germ agglutinin and concanavalin A. Although bacterial vaccines are widely used now as non-specific immunoadjuvants that stimulate the T helper cell function and non-specific macrophage immunity, we present evidence that subpopulations of antibacterial antibodies may be tumour specific.*

INTRODUCTION

FOR SOME time the cell surface has been studied as a direct mediator of cellular growth control. Many trials have been made to correlate chemical changes of the surface membrane with an alteration in cellular growth. It becomes clear that the cell surface membrane alterations may result in a loss of contact inhibition which feature is shared by all tumour cells [1–4], and in an irregular growth accompanied by a metabolic different behaviour [5]. Alterations of the cell surface of transformed cells may be detected chemically by their enhanced agglutination with wheat germ agglutinin (WGA) or Concanavalin A (ConA). Those plant proteins agglutinate tumour cells and transformed (trypsin treated) cells but not their parent normal cell lines [1–4, 6]. Tumour cell surfaces show ultrastructural changes such as microvilli, seen in mitosis in normal cells: their agglutination properties with the lectins may be explained by those changes [7]. Different intracellular structures in tumour cells showing a disrupted picture of microfilaments have been reported, whereas in normal cells a very regular pattern has been observed. [8, 9]. Those ultrastructural surface changes and intracellular distributions seen in cells derived from tumour cell cultures may be correlated and explained by the existence of a proposed [10] membrane protease that digests membrane bound substances.

Some authors provide evidence that tumour growth can be inhibited *in vivo* by ConA [11, 12], which suggests an analogous lectin behaviour on tumour cells *in vivo*. Depending on the dose, ConA suppresses [13] or stimulates immune responses [14].

Some harmful characteristics of those agglutinins are their toxic effect on normal cells as inhibitors of protein synthesis after pinocytosis by the cell, and their haemagglutinating capacity [15].

Tumours such as leukemia L1210 cannot

Accepted 21 May 1976.

*Supported in part by the contract No. N01-CM-53840 entered into with the National Cancer Institute, NIH, Bethesda, Maryland, U.S.A.

This work was partially supported by a grant from the A.S.L.K.—Kankerfonds.

‡Predoctoral fellow of the Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw.

¶This service is a member of the European Organization for Research on Treatment of Cancer (E.O.R.T.C.).

easily be agglutinated with ConA, so that one has to combine those two specificities in order to have a universal anti-tumour agent [16].

ConA binding requires the C-3, C-4, and C-6 hydroxyl groups of D-mannopyranose of D-glucopyranose [17] whereas WGA binds on a receptor containing N-acetyl-D-glucosamine (NAG).

To elicit antibodies binding on glucose determinants and other antibodies binding on NAG determinants, we made a synthetic antigen with a double immunogenic specificity.

Hypervaccination with *Micrococcus Lysodeikticus*, a gram-positive bacterium, stimulates antibodies of homogenous structure, with antibody titers up to 40 mg/ml in rabbits [18–21].

Since antimicrococcus antibodies are directed against carbohydrates such as glucose, we hoped that these antibodies could discriminate between normal and transformed cells as does ConA but without the toxicity of the lectin [15]. Anti-NAG antibodies can be considered as homologous to WGA. To take benefit of the carrier effect of the bacterium, an oligomer of NAG was coupled on the peptide moiety of the cell wall of *Micrococcus Lysodeikticus*. Since previous studies have repeatedly shown [22–24] that fractions of anti-micrococcus antibodies bind on tumour cells *in vitro* as well as on lymphocyte subpopulations, but not on erythrocytes [24], this *in vivo* approach was undertaken to investigate a possible immunoprotective effect.

MATERIAL AND METHODS

Micrococcus lysodeikticus

A suspension was made of lyophilized (ML) Micrococcus Lysodeikticus (Wortington Biochem. Corporation) after repeated washings and centrifugations at $3000 \times g$ for 15 min in Hank's balanced salt solution. The dose is indicated in each table. The vaccine was inoculated intravenously (i.v.), intraperitoneally (i.p.) or subcutaneously (s.c.).

Preparation of the oligomers of chitin

Chitin (Sigma Chem. Corp.), the naturally occurring polymer of N-acetyl-D-glucosamine, was ground in a coffee grinder for a few minutes; washed with 4 l of 0·05 M HCl and 0·05 M NaOH. Treatment by 1 % pepsin was performed in NH_4HCO_3 0·1 M, adjusted with formic acid to pH = 2·1, for 24 hr at 37°C. After repeated washings and centrifugations at $3000 \times g$, 15 min, a treatment by 1 % trypsin in 300 ml NH_4HCO_3 0·1 M, pH = 8·0 was done at 37°C for 24 hr, repeatedly washed with distilled water and dialysed against water.

Since, in strongly acid medium, the side chains of chitin are hydrolysed to yield pure polymers of N-acetyl-D-glucosamine, soft hydrolysis happened at 4°C with 11 M HCl, 48 hr under nitrogen and the insoluble fraction was removed after centrifugation ($27,100 \times g$ for 15 min).

After the addition of water, the HCl was removed by evaporation at low pressure.

We obtained 4·86 g chitin from an original 20 g weighing quantity. Amino acid analysis detected minute traces of contaminating amino acids and one single peak that eluted at the place of tyrosine. This component happened to coincide with the RF value found for pure hydrolysed N-acetyl-D-glucosamine used as a standard for calibration of the column.

Preparation of the cell walls of Micrococcus lysodeikticus

Thirty grams of ML were washed three times with water and centrifuged at $3000 \times g$ for 20 min. A suspension was made in 300 ml of water. 75 ml Duran flasks were filled with 30 ml of the ML suspension, 30 g of "glasperlen" (Braun, ϕ = 0·11–0·10 mm) and 0·2 ml of tri-*n*-butylphosphate.

In a Braun shaker the flasks were shaken under CO_2 refrigeration during 150 sec.

After elimination of the glass beads, the suspension was repeatedly washed with water and centrifuged at $3000 \times g$ for 20 min. The volume was adjusted to 1 l and 10 g sodium-dodecylsulphate were added.

The suspension was passed in a "Virtis Mixer" for 1 min and the yellow intact fraction was removed by centrifugation, $3000 \times g$ for 8 min. The supernatant was centrifuged at $27,100 \times g$ for 10 min and the white cell walls collected.

Cell walls were purified by repeated washings with 10 % formic acid and water. A final suspension was made in 200 ml NH_4HCO_3 0·1 M, pH = 2·1 with 1 % pepsin at 37°C for 24 hr, washed with water and lyophilized after extensive dialysis against water.

We obtained 4·0 ± 0·15 g insoluble cell walls. The purity of the sample was checked on a Durrum amino acid analyser and revealed the following amino acid ratios: glu: 3, gly: 3, ala: 6, tyr: 2 and lys: 2.

Coupling of the oligomers of chitin on the cell walls of Micrococcus lysodeikticus

Three grams of chitin were suspended in 40 ml of water. A suspension was made of 2 g of cell walls in 100 ml $NaHCO_3$, pH = 8·5.

The chitin was stirred at 0°C and the pH kept between 10·5 and 11·5 (5 M NaOH). Six grams of CNBr were dissolved in dimethylformamide (freshly distilled) and added to the chitin suspension. The pH was kept for 10 min between 10·5 and 11·5 after addition of the cell wall suspension and then lowered to pH = 8·6. The suspension was stirred overnight at 40°C. After centrifugation for 20 min at $3000 \times g$, the suspension was repeatedly washed with water at 4°C. After extensive dialysis against cold water and lyophilization, we obtained 4·18 g water insoluble product. Durrum acid analysis showed amino acid ratios: glu: 3, gly: 3, ala: 6, tyr: 13 and lys: 1·5.

EXPERIMENTAL ANIMALS AND TUMOURS

Mice and tumours

For all studies, 2 strains of mice and 2 tumours were used: we chose to work with leukemia L1210 because it offered an experimental model that is very well documented in literature and allows activity prediction against human leukemia [25].

As a second system we chose Ehrlich ascites carcinoma.

For each experiment 7 to 12 mice were used. Mean survival time ± the standard deviation of the treated mice and the control mice is indicated in each table.

For leukemia L1210, survivors on day 35 after tumour graft were considered as long-term survivors. A mean lifespan increase of 25 % of treated mice over controls was considered significant [26] and supported by a single tailed Student Fisher *t* test.

BDF_1 mice

Inbred (C_{57} bl/6 × DBA_2) F_1 animals were supplied by Charles River Breeding Laboratories. Those mice were observed at least for 3 weeks "in quarantaine" before we started experimenting. Male BDF_1 mice (C_{57} bl/6 female × DBA/2 male) weighing 19–23 g were used.

L1210 lymphoid leukemia was originally obtained from Mr. I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.), The L1210 leukemia is maintained in DBA/2 mice by weekly i.p. inoculation. L1210 ascites was aspirated from DBA/2 mice and a suspension was made in Hank's balanced solution; 10^5 cells in 0·1 ml solution were injected i.p. to obtain the ascitic form of leukemia, and i.v. to obtain the blood form of leukemia.

Animals were randomized into test groups and control groups. The route dose and timing of treatment is specified on each Table.

BALB/c mice

Ehrlich ascites tumour (EAT) has been transplanted for years by Cordier in our inbred strain BALB/c mice by weekly passage of 10^7 tumour cells to intact mice. Male mice weighing 19 g, or female mice weighing at least 18 g were used.

Animals were randomized into test groups and control groups.

RESULTS

The tests of activity against the two tumours are preceded by screening for immunological activity.

First, we determined an antigen dose, a route of administration and an immunization scheme. Then we screened the different antigens following different immunization schemes. In the last part of this paper we investigated if there was a direct interaction between leukemic L1210 cells and different rabbit antimicrococcus antibodies. We also determined the toxicity and mortality due to the administration of the synthetic antigen cell wall-chitin (CWC).

1. *Determination of optimal antibody response*

We vaccinated BDF_1 mice with a heat-killed (30 min at 60°C) *Micrococcus lysodeikticus* vaccine in Hank's BSS.

We administered i.v. antigen doses of 20, 40 and 80 μg. After four weeks of one injection a week, we gave three booster injections the seventh week.

Bleedings were performed in the retroorbital plexus after the second, fourth and seventh week and the serum antibody titer was determined quantitatively by agglutination and compared with rabbits antisera [19] of known titer (Table 1).

Control of body weight was used as an indication of toxicity (Table 1). The results show that the highest doses of antigen (80 μg/injection) have the same effect as the lowest

Table 1. *Determination of a good antibody response and toxicity in BDF_1 mice vaccinated with heatkilled micrococcus lysodeikticus suspension* i.v.

Antigen dose. *Micrococcus lysodeikticus* μg/injection/ 0·1 ml	Mean gain of weight in g			At 7th week	Mean antibody titer (μg/ml)		
	After 2 weeks	After 4 weeks	After 7 weeks	% control mice	After 2 weeks	After 4 weeks	After 7 weeks
Series 1 (80 μg)	1·89	3·60	4·70	72%	100	400	1600
Series 2 (40 μg)	1·80	2·73	4·79	73·5%	100	200	800
Series 3 (20 μg)	1·51	2·82	5·14	78·9%	100	200	400
Control mice 0	2·55	4·40	6·51	100%	—	—	—

Intact BDF_1 mice were injected i.v. on week, 1, 2, 3, 4 with one injection and in the 7th week, animals received 3 injections with 2 days interval.

Table 2. *Influence of the route of administration of the antigen ML on the antibodytiter in BDF_1 mice*

Antigen doses μg ml/injection	Route of administration	Antibodies values week 3 (μg/ml)	Antibodies values week 8 (μg/ml)
80	i.v.	24	750–1500
20	i.v.	24	187–400
80	i.p.	48	2250–4500
20	i.p.	48	140–280
80	s.c.	48	280–560
20	s.c.	12	140–560

Intact mice were treated by the antigen on week 1, 2, 3, 4 with one injection and on the seventh week, animals received 3 injections within 2 days interval.

ones (20 μg/injection) on the mean weight increase after 7 weeks. A slower increase in body weight of treated animals as compared to control mice was observed. However, there was no indication of toxicity since we did not observe any mortality or mean body weight loss.

2. *Influence of the route of administration of the antigen on the antibody titer*

We explored the mode of administration in BDF_1 mice with antigen doses (80 μg/injection and 20 μg/injection) using the above immunization scheme. After bleedings in the retro-orbital plexus, the serum antibody titer was determined by the agglutination technique. Values are shown in Table 2. Although the three routes of administration of the antigen yield an antibody response, the highest titers are obtained by intraperitoneal injection of 80 μg/injection (Table 2, last column).

3. *Chemotherapeutic effect of the antigens on L1210 growth*

We wanted to investigate whether there was any chemotherapeutic effect of the following antigens on L1210 growth:

Micrococcus lysodeikticus, heat-killed vaccine	(ML)
Chitin coupled with cell walls of ML	(CWC)
ML + chitin	(ML + CN)
ML + chitin coupled with cell walls of ML	(ML + CWC)
Chitin	(CN)

Intact BDF_1 mice were inoculated with 10^5 leukemic L1210 cells either i.p. or i.v. Treatment started on day 2 and was continued on days 4, 6 and 8.

Treatment doses were 50 μg/injection for the i.v. route and 400 μg/injection for the i.p. route, using the tumour inoculation route.

Body weight change and mean survival time were scored as seen in Table 3. With repeated i.v. administrations over a short period, the body weight loss is bigger with chitin than with ML or CWC.

Our results confirm a small beneficial (chemotherapeutic) anti-leukemia effect [27]: i.v. administration of insoluble chitin induces a 32% increase in lifespan of treated animals over controls ($P < 0{\cdot}03$), whereas i.p. administration is less effective.

4. *Antitumour immunoprotection by a good immune response*

Two types of immunization schemes were used. A short time vaccination, during four weeks, elicits a primary immune response.

BALB/c mice were injected with heat-killed ML. ML as well as CWC were given to BDF_1 mice. Schedules containing immunization route and dose are drawn in Tables 4a and 4b. It was seen that a small antimicrococcus response was present on the fifth week. However, this low titer immunity enhanced Ehrlich carcinoma growth in BALB/c mice as shown in Table 4a. Short times vaccination with CWC or ML did not yield any positive outcome in BDF_1 mice (Table 4b).

In a second experiment we carried out a long term (hyper)vaccination with different antigens.

As antigens we used ML (100 μg/injection), ML (80 μg) + chitin (50 μg), ML (100 μg) + CWC (50 μg), CWC (100 μg), and chitin (100 μg).

We injected the antigens i.v. during four weeks with one injection a week, and the seventh week we gave three booster injections within two days interval.

On the eighth week, mice were challenged with 10^5 L1210 cells intravenously. We used body weight as an indication of toxicity before L1210 inoculation and we recorded mean survival time as indicated in Table 5. The same immunization scheme was also used against Ehrlich ascites carcinoma. We observed that i.v. immunoprophylaxis with ML resulted in an enhanced tumour growth while vaccination with CWC yielded a good percentage (14–28%) of long-term survivors equivalent to cures. Mixed vaccination of CWC and ML was less efficient. The immunoprophylactic efficiency was confirmed on Ehrlich carcinoma: immunoprotection induced a 104% increase of survival time over controls and we obtained real cures for 25% of the tumour challenged mice as seen in Table 5. ($P < 0{\cdot}001$)

Table 3. *Chemotherapeutic effect of the antigens on survival of mice bearing* L1210 *leukemia* i.v. *and* i.p.

Antigen	Antigen doses (μg/injection)	Administration route (tumour and treatment)	Increase of weight (day 0— day of death) (g)	MST* (days)	T/C† (%)
ML	50	i.v.	0·70	10·1 ± 2·75	125 ($P < 0{\cdot}1$)
CN	50	i.v.	0·10	10·7 ± 2·86	132 ($P < 0{\cdot}08$)
ML + CN	20+20	i.v.	0·00	10·4 ± 3·77	128 ($P < 0{\cdot}05$)
ML + CWC	20+20	i.v.	0·31	9·7 ± 2·15	120
CWC	20+20	i.v.	0·00	9·9 ± 2·17	122
—	—	i.v.	0·62	8·1 ± 0·50	100
Control mice					
ML	400	i.p.	2·18	10 ± 0·53	110
CN	400	i.p.	2·28	10 ± 0·53	110
ML + CN	200+200	i.p.	2·20	9·60 ± 0·73	104
ML + CWC	200+200	i.p.	1·76	9·60 ± 0·73	104
CWC	400	i.p.	1·83	9·40 ± 0·49	102
—	—	i.p.	3·36	9·20 ± 0·57	100
Control mice					

Intact BDF_1 mice were treated on days 2, 4, 6 and 8. On day 0, mice were inoculated with 10^5 viable L1210 cells following the route indicated.

*MST = Mean survival time of the mice ± the standard deviation.

†T/C = Meah survival time of treated mice divided by that of the control expressed in percentage.

5. In vivo *study on direct interaction between rabbit antimicrococcus antibody and L1210 leukemic cells*

A previous study has indicated that rabbit antimicrococcus antibody agglutinates peripheral lymphocytes of rabbits, humans and mice [24]. No specific binding or agglutination on erythrocytes of any tested species was observed at physiological temperature (37°C) [22, 24]. This study has been confirmed by others [23] using direct immunofluorescence: rabbit antimicrococcus induces the formation of spots and caps on rabbit peripheral lymphocytes and shows a spot formation on a mouse renal adenocarcinoma culture cell line. We also observed a positive membrane fluorescence on mouse Ehrlich carcinoma with rabbit fluorescein-conjugated antimicrococcus antibody [24]. It seems that antimicrococcus antibody can distinguish between different cell lines the same way as the lectins, but with a better discriminatory capacity as far as binding is concerned. Lectins bind on all cell types including erythrocytes and fibroblasts [3].

This study was carried out to find out whether *in vitro* preincubation of antimicrococcus antibody with L1210 cells, affects the tumour growth when those incubated cells are grafted in BDF_1 mice.

Two rabbit antimicrococcus antisera—K112 and K114—and a rabbit preimmune serum P112, were precipitated repeatedly with 33% saturated $(NH_4)_2SO_2$, centrifuged at $3000 \times g$ for 15 min and dissolved in phosphate buffered saline (PBS).

After extensive dialysis of the globulin, this fraction was applied to a DEAE-cellulose ion exchange column equilibrated in the dialysis buffer (0·01 M K phosphate; pH 8·6). The purified gammaglobulin fraction was collected by elution with (0·0178 M K phosphate, pH 8·6) and lyophilized. Protein content was determined by reading the 280 mμ absorbence with a Carl Zeiss spectrophotometer and antimicrococcus antibody titers were scored by the agglutination technique. Different globulin fractions—K114 (3 mg/ml), K112 (6 mg/ml) and P112 (0 mg/ml)—were incubated vol/vol for 30 min at 37°C with a suspension of 10^4 L1210 cells in Hank's BSS.

Randomized intact BDF_1 mice were injected 0·2 ml of this suspension either i.p. or i.v. (day 0). Repeated 0·1 ml doses of the gammaglobulin fractions were injected following the tumour inoculation route on days 1, 3 and 5. Mean survival time and the percentage of long term survivors cured on day 35 are noted in Table 6.

We observed that rabbit gammaglobulin yielded a high percentage of protected animals (up to 40–80%) against L1210 growth for the i.v. route, whereas i.p. inoculation was less efficient.

6. *Toxicity of administration of CWC in BDF_1 mice*

Table 7 shows the mortality data and the body weight changes, as compared to untreated mice, after the administration i.v. to intact BDF_1 mice of various doses of CWC, once a week for four weeks. After a resting period of ten weeks, we administered CWC i.p. three times a week. Mortality and body weight changes are recorded in Table 7.

Table 4(*a*). *Effect of a primary immune response anti-micrococcus against* i.p. *inoculation of Ehrlich ascites carcinoma in Balb/C mice*

Treatment scheme	Week 0 dose (μg)	Week 2 dose (μg)	Week 4 dose (μg)	Mean serum antibody titer week 5 (μg/ml)	Week 6 dose + tumor + route	MST* (days)	T/C† (%)
Schedule I	200 i.p.	200 i.p.	100 i.p.	200	10^4 E.A.C. i.p.	28·6 ± 0·6	92 ($P < 0·001$)
Schedule II	200 i.p.	200 i.p.	0	20	10^4 E.A.C. i.p.	29·4 ± 1·4	94 ($P < 0·3$)
Schedule III	100 i.p.	100 i.p.	100 i.p. + 100 s.	100	10^4 E.A.C. i.p.	25·8 ± 1·1	82 ($P < 0·001$)
Schedule IV	100 i.p.	100 i.p.	200 s.c.	100	10^4 E.A.C. i.p.	26·4 ± 0·8	85 ($P < 0·001$)
Schedule V (control mice)	0	0	0	0	10^4 E.A.C. i.p.	31·2 ± 0·4	100

*MST = Mean survival time of the mice ± standard deviation.
†T/C = Mean survival time of the treated mice divided by that of the control, expressed in percentage.

Table 4(b) Effect of a primary immune response against i.v. *inoculation of leukemia L1210 in BDF_1 mice*

Antigen	treatment scheme	Week 0 dose (μg)	Week 2 dose (μg)	Week 4 dose (μg)	Week 6 dose + tumor + route	MST* (days)	T/C† (%)
M	Schedule I	200 s.c.	200 s.c.	200 s.c.	10^5 L1210 i.v.	9·6 ± 0·4	110
	Schedule II	200 i.p.	200 i.p.	200 i.p.	10^5 L1210 i.v.	8·2 ± 0·70	94
	Schedule III	50 s.c.	50 s.c.	50 s.c.	10^5 L1210 i.v.	9·2 ± 0·4	106
	Schedule IV	50 i.p.	50 i.p.	50 i.p.	10^5 L1210 i.v.	7·8 ± 0·3	90
	Schedule V	200 i.p. + 200 s.c.	200 i.p. + 200 s.c.	200 i.p. + 200 s.c.	10^5 L1210 i.v.	9·0 ± 0·6	103
	Schedule VI	0	0	0	10^5 L1210 i.v.	8·7 ± 0·45	100
CWC	Schedule I	200 s.c.	200 s.c.	200 s.c.	10^5 L1210 i.v.	9·0 ± 0·45	103
	Schedule II	200 i.p.	200 i.p.	200 i.p.	10^5 L1210 i.v.	9·0 ± 0·0	103
	Schedule III	50 s.c.	50 s.c.	50 s.c.	10^5 L1210 i.v.	9·0 ± 0·0	103
	Schedule IV	50 i.p.	50 i.p.	50 i.p.	10^5 L1210 i.v.	8·8 ± 0·4	101
	Schedule V	200 i.p. + 200 s.c.	200 i.p. + 200 s.c.	200 i.p. + 200 s.c.	10^5 L1210 i.v.	8·8 ± 0·4	101
	Schedule VI	0	0	0	10^5 L1210 i.v.	8·7 ± 0·45	100

*MST = Mean survival time of the mice ± standard deviation.
†T/C = Mean survival time of the treated mice divided by that of the control, expressed in percentage.

Table 5. *Hyperimmunization and immunoprophylaxis against leukemia* L1210 (i.v.) *and Ehrlich ascites carcinoma* (i.p.)

Mice + tumor		Antigen	B.W.C.* (g)	Doses μg/injection	Route	MST† (days)	T/C‡ (%)	*P* (*t* test)	% long survival animals cured on day 35
BDF_1	L1210	ML	3·64	100	i.v.	5·4 ± 1·05	65·8	($P < 0·001$)	—
BDF_1	L1210	Chitin	2·91	100	i.v.	6·9 ± 1·33	84·1	($P < 0·03$)	—
BDF_1	L1210	CWC	3·63	100	i.v.	14·33 ± 5·86	175	($P < 0.02$)	14–28%
BDF_1	L1210	ML + CWC	1·82	50 + 100	i.v.	9·0 ± 3·0	110		14–28%
BDF_1	L1210	ML + CN	1·43	80 + 50	i.v.	6·0 ± 0·75	73	($P < 0·001$)	—
BDF_1	L1210	— (control mice)	4·59	—	i.v.	8·2 ± 0·48	100		—
Balb/C	EAT	CWC	1·44	100	i.p.	45·0 ± 6·4	45/22 = 204	($P < 0.001$)	25%
Balb/C	EAT	— (control mice)	1·72	—	i.p.	22·0 ± 5·4	22/22 = 100		

Balb/C animals were inoculated intraperitoneally with 10^5 Ehrlich carcinoma cells on day 0.
BDF_1 animals were inoculated intravenously with 10^5 L1210 cells on day 0. Animals received the antigens intravenously on weeks 7, 6, 5, 4, (one injection a week) and 1, (3 injections with 2 days interval).
*Mean increase in mouse body weight (before tumor challenge).
†Mean survival time of the mice ± standard deviation.
‡Mean survival time of treated animals divided by that of the control mice expressed in percentage.

Table 6. Effect of inoculation of leukemia L1210 in male BDF_1 mice after preincubation of tumor cells with rabbit antimicrococcus globulin

Dose + tumor + route	MST (days)	% long survival animals cured on day 35	Serum
10^3 L1210 i.v.	13·2	40	K114
10^3 L1210 i.v.	18·0	80	K112
10^3 L1210 i.v.	12·0	0	P112
10^3 L1210 i.p.	13·7	20	K114
10^3 L1210 i.p.	13·6	0	K112
10^3 L1210 i.p.	13·4	0	P112

A solution of 10^4 L1210 cells were vol/vol incubated for 30 min. at 37°C respectively with rabbit globulin K114, K112 and a preimmune rabbit globulin P112.

0.2 ml of this solution was injected either i.p. or i.v. in male BDF^1 mice (18–23 g) on day 0. Repeated doses (0·1 ml) of antibody were injected following the inoculation route on days 1, 3, 5.

Table 7. Toxicity and mortality after administration of CWC in BDF_1 mice

Schedule	Dose (mg)	Freq. of injection/week	Route	Mortality % week 4	BWC* (g)
1	1·5	1	i.v.	57	1·16
2	1·25	1	i.v.	57	−0·28
3	1	1	i.v.	42	0·55
4	0	0	—	0	2·62
5	0·75	1	i.v.	29	1·52
6	0·5	1	i.v.	29	−1·03
7	0·1	1	i.v.	0	1·08
Schedule	Dose (μg)	Freq. of injection/week	Route	Mortality % week 20	BWC† (g)
1	1·5	3	i.p.	14	0·9
2	1·25	3	i.p.	14	1·95
3	1·0	3	i.p.	14	1·025
4	0	0	i.p.	—	0
5	0·75	3	i.p.	0	0·010
6	0·50	3	i.p.	0	0·766
7	0·1	3	i.p.	0	0·133

*BWC = body change weight (week 1–6)
†BWC = body weight change (week 15–20)
Male BDF_1 mice (19–25 g) were injected i.v. once a week (1–4); secondary 3 times a week injections were given i.p. on week 15 and 16.

The i.v. LD_{50} of CWC in BDF_1 mice treated for four weeks i.v. was approximately 1·1 mg/mouse or 44 mg/kg. No traces of toxicity or mortality were seen for the vaccination dose 0·1 mg/mouse or 4 mg/kg.

DISCUSSION

In this paper we provide evidence that, just like ML, the immunogenic system obtained by coupling NAG oligomers on Micrococcus lysodeikticus cell wall yields antibodies with specific antitumour activity. Vaccinations with ML show that the highest doses of antigen (80 μg/mouse/injection) have the same toxicity as the lowest ones. Serological antibody titers are the highest when the immunogen is inoculated intraperitoneally.

In our chemotherapeutic approach we obtained negative results. Our results confirm a

small beneficial chemotherapeutic anti-leukemia effect [25] for chitin.

Bacterial vaccines such as BCG and *Corynebacterium* P, [28, 29], possess a non-specific T helper cell and macrophage stimulating capacity. By lacking the required chemical peptidoglycan cross-linking peptide, ML has no adjuvant effects. [30]

Immunoprophylaxis studies show very clearly that hypervaccinations with our synthetic antigen CWC protect BDF_1 mice against L1210 challenge (with long term survivors similar to cures for 14–28% of the animals). Vaccination with *Micrococcus lysodeikticus* enhance tumour growth. The protective effect of CWC was confirmed by studying the effect of the antigen on a second mouse tumour, the Ehrlich ascites carcinoma. Here, the percentage of cures was in the same range (25%), but the mean survival time was slightly better (204%).

The tumour growth enhancing effect of antimicrococcus antibodies can be explained by the immunostimulation theory of R. Prehn [31]: a minimal immune response to tumour cells accelerates tumour growth, whereas a vigorous immune reaction is cytotoxic. Incubation of low dose (10^3) of tumour cells with antimicrococcus antibody results in the killing of tumour cells. This is shown by a bioassay that consists in grafting the incubated cells to intact mice, when a suitable inoculation route is used. Since antibodies to Gram-positive bacteria do not fix complement and function in cell mediated cytotoxicity, it is not surprising that the i.v. route is the most adequate.

Incubation of leukemic L1210 cells with rabbit antimicrococcus yielded a satisfactory outcome, when approximately 0·6 mg antibody was used for 10^3 tumour cells. Hypervaccination revealed that 2 mg/ml serum antibody titers can be obtained using the described immunization procedure. This does not correspond to a presumed tumour antibody ratio for 10^5 cells. Vaccinations with heat-killed bacteria vaccines such as pneumococci, streptococci and micrococci, yield homogenous antibodies of high titer—up to 40 mg/ml serum—in rabbits and that are anologous to myeloma proteins in humans.

It is not excluded that continued hypervaccination with the non pathogenic ML vaccine may result in homogenous antibodies of high titer in men and yield immunoprotection against considerable tumour cell doses. A simultaneous injection of SRBC and CWC results in an amplified anti-SRBC immunity expressed by serologically higher anti-SRBC haemagglutination titers [unpublished observation]. This adjuvant effect may, together with the antitumour antimicrococcus antibody obtained by vaccination with the same antigen, be responsible for the rejection of a large part of 10^5 leukemia L1210 or Ehrlich carcinoma cells. While this work was in progress, it was reported that a mixed vaccine of *Bordetella Pertussis* and *Streptococcus pyogenes* yielded specific immunoprotection against SV_{40} transformed cells in hamsters [32]. Since antibodies to M protein containing *Streptococcus* crossreact with human cardiac tissue [33], this work has no clinical interest.

No clear-cut indication for autoimmunity of antimicrococcus antibodies has been reported and vaccination for years in rabbits did not reveal any toxicity.

Acknowledgements—The authors wish to thank Mrs. F. Bournonville-Lefort, Mr. C. Schaus, Mr. M. Wynands and Mr. I. Caplier for their technical help.

REFERENCES

1. M. M. Burger, A difference in the architecture of the surface membrane of normal and virally transformed cells. *Proc. nat. Acad. Sci.* **62,** 994 (1969).
2. K. D. Noonan and M. M. Burger, Binding of (3H) ConA to normal and transformed cells. *J. biol. Chem.* **248,** 4286 (1973).
3. L. Malluci, Binding of ConA to normal and transformed cells as detected by immunofluorescence. *Nature New Biol.* **233,** 241 (1971).
4. M. M. Burger, Surface changes in transformed cells detected by lectins, *Fed. Proc.* **32,** 1, 91 (1973).
5. R. Kram and G. M. Tomkins, Pleiotypic control by cAMP: Interaction with cGMP and possible role of microtubules. *Proc. nat. Acad. Sci.* **70,** 1659 (1973).
6. M. Inbar, H. Ben-Bassat and L. Sachs, Membrane changes associated with malignancy. *Nature New Biol.* **236,** 3 (1972).
7. M. C. Willingham and I. Pastan, cAMP modulates microvillus formation and agglutinability in transformed and normal mouse fibroblasts. *Proc. nat. Acad. Sci.* **72,** 1263 (1975).

8. K. Weber and U. Groeschel-Stewart, Antibody to myosin: the specific visualization of myosin containing filaments in non-muscle cells. *Proc. nat. Acad. Sci.* **71,** 4561 (1974).
9. E. Lazarides and K. Weber, Actin antibody: the specific visualization of actin filaments in non-muscle cells. *Proc. nat. Acad. Sci.* **71,** 2268 (1974).
10. R. O. Hynes, Role of surface alterations in cell transformation: the importance of proteases and surface proteins. *Cell* **1,** 147 (1974).
11. M. M. Burger and K. D. Noonan, Restoration of normal growth by covering of agglutinin sites on tumor cell surfaces. *Nature (Lond.)* **228,** 512 (1970).
12. M. Inbar, H. Ben-Bassat and L. Sachs, Inhibition of ascites tumor development by ConA. *Int. J. Cancer* **9,** 143 (1972).
13. M. Markowitz, D. A. Person, G. L. Gitnick and R. E. Ritts, Immunosuppressive activity of ConA. *Science* **163,** 476 (1968).
14. A. Anaclerio, J. D. Waterfield and S. Möller, Induction of lymphocyte-mediated cytotoxicity against allogeneic tumor cells by ConA *in vivo*. *J. Immunol.* **113,** 870 (1974).
15. N. Sharon and H. Lis, Lectins: cell-agglutinating and sugar specific proteins. *Science* **177,** 949 (1972).
16. M. M. Burger, Surface changes in transformed cells detected by lectins. *Fed. Proc.* **32,** 1, 91 (1973).
17. I. J. Goldstein, C. E. Hollerman and E. E. Smith, Protein carbohydrate interaction. II. Inhibition studies on the interaction of Concanavalin A with Polysaccharides. *Biochemistry* **4,** 876 (1965).
18. M. van Hoegaeden, M. Wikler, R. Janssens and L. Kanarek, Antibodies to Micrococcus lysodeikticus. Restricted structural heterogeneity in hyperimmune rabbits. *Europ. J. Biochem.* **53,** 19 (1975).
19. R. Verloes, I. Immunosuppression of Micrococcus Lysodeikticus on antibody formation anti-tobacco masaic virus. II. The application of Micrococcus Lysodeikticus as a general anti-tumour vaccine. Thesis (1973). Vrije Universiteit Brussel (1973).
20. M. Wikler, Isolation and characterization of homogenous rabbit antibodies to Micrococcus lysodeikticus. *Arch. int. Physiol. biochim.* **81,** (1973).
21. M. Wikler, Isolation and characterization of homogenous rabbit antibodies to Micrococcus lysodeikticus with specificity to the peptidoglycan and to the glucose-N-acetylaminomannuronic aci polymer. *Z. Immun.-Forsch.* **149,** 193 (1975).
22. R. Verloes, G. Atassi and L. Kanarek, Anti-tumor immunoprotection by an immunobacterial lectin approach. Symp. clinical Tumor Imm., May 26–29, Brussels, 1975. *Europ. J. Cancer* (In Press).
23. P. de Baetselier, R. Hooghe, J. Grooten, C. Hamers-Casterman and R. Hamers, Interaction of antimicrococcus antibodies with the lymphoid system. Symp. clinical Tumor Imm., May 26–29, Brussels, 1975. *Europ. J. Cancer* (In Press).
24. R. Verloes and L. Kanarek, Interaction of the lectins PHA, ConA and antimicrococcus with blood cells of different species and Ehrlich carcinoma. *Arch. int. Physiol. Biochim.* (In Press).
25. E. Skipper, Kinetic behaviour versus response to chemotherapy. Prediction of response in cancer therapy. *Nat. Cancer Inst. Monograph,* **34,** 2 (1972).
26. J. Leiter, M. M. Macdonald and S. A. Schepartz, Screening data from the cancer chemotherapy national service center screening laboratories. *Cancer Res.* **24,** 665 (1964).
27. R. J. Woodmann, A. E. Sirica and J. M. Venditti, Inhibition of systematic spread of L1210 leukemia by the polycation chitosan. *Proc. Amer. Ass. Cancer Res.*, Abstracts, 335, 11 (1970).
28. D. Juy and L. Chedid, Comparison between macrophage activation and enhancement of nonspecific resistance to tumors by mycobacterial immunoadjuvants. *Proc. nat. Acad. Sci.* **72,** 4105 (1975).
29. A. Ghaffar, R. T. Cullen, N. Dunbar, M. F. A. Woodruff, Anti-tumour effect *in vitro* of lymphocytes and macrophages from mice treated with Corynebacterium Parvum. *Brit. J. Cancer* **29,** 199 (1974).
30. T. Nguyen-Dang, R. J. Turner, M. Manning and M. M. Janot, Some structural features of immunoactive compounds of bacterial and synthetic origin. *Symp. int. Past. Inst.*, Abstracts, 5, 1974.

31. R. T. Prehn, The immune reaction as a stimulator of tumor growth. *Science* **176,** 170 (1972).
32. J. L. Collins and C. L. Wust, Suppression of SV_{40} tumors after immunization with group A streptococcus pyogenes and bordetella pertussis. *Cancer Res.* **34,** 932 (1974).
33. M. H. Kaplan and M. L. Suchy, Immunology relation of streptococcal and tissue antigens. *J. exp. Med.* **119,** 643 (1964).

Europ. J. Cancer Vol. 12, pp. 889–898. Pergamon Press 1976. Printed in Great Britain

Prognostic Value of Tumour-directed, Cell-mediated Hypersensitivity Detected by Means of the Leucocyte Migration Technique in Patients with Renal Carcinoma*

MOGENS KJAER

Division of Clinical Immunology, Medical Department TA and Surgical Department C, Rigshospitalet, University Hospital of Copenhagen, Denmark

Abstract—*Tumour-directed, cell-mediated hypersensitivity (TCMH) towards soluble extracts of autologous renal carcinoma, allogeneic hypernephroma, corresponding normal renal tissue, and cell-mediated hypersensitivity towards foetal kidney tissue (FCMH) was evaluated in a prospective investigation of 49 patients with renal carcinoma and 161 control persons. TCMH and FCMH were evaluated by means of the capillary tube leucocyte migration technique (LMCT).*

Fifty-eight per cent of the patients showed reactivity towards autologous tumour, 47% towards allogeneic hypernephroma, 3% towards allogeneic normal renal tissue, and 42% towards foetal kidney tissue.

Highly significant correlations were found between detectable TCMH and FCMH and the absence of distant metastases. No correlations could be demonstrated between local macroscopic tumour invasion, the histopathology of the renal carcinomas and TCMH or FCMH.

After nephrectomy patients with evidence of TCMH and FCMH survived significantly longer than patients without. Moreover it was possible by means of TCMH to isolate a high-risk group among renal carcinoma patients without distant metastases but without detectable TCMH.

INTRODUCTION

FOR A number of years tumour-directed, cell-mediated hypersensitivity (TCMH) has been demonstrated by various *in vitro* techniques in a vast number of patients with different types of solid tumours. Several extensive reviews have been published [1–6].

There is little doubt that different types of solid tumours express antigens not present in the corresponding normal tissue, and that the tumour hosts respond with a TCMH against them [1–6].

One of the pertinent questions in human tumour immunology is whether these *in vitro* evidences of TCMH are indicators of an immunological elimination of tumour cells *in vivo* [7].

The only possible way to answer this question is to conduct prospective clinical trials in cancer patients, to evaluate TCMH with *in vitro* techniques, and to correlate the results with the survival of the patients.

It has earlier been shown by Kjaer [8–16] that human renal carcinomas express antigens not detectable in normal renal tissue leading to a TCMH in the patients; this is detectable by means of the leucocyte migration capillary tube technique (LMCT) using extracts of allogeneic hypernephroma as antigen and corresponding normal renal tissue as control.

The present paper reports on a prospective investigation of a consecutive series of patients with renal carcinoma, in which TCMH was evaluated by the LMCT, and the results correlated to the clinical stage, course, histo-

Accepted 18 May 1976.

*The present investigation was supported by The Danish Cancer Society.

pathology of the tumours, and the survival of the patients after operation.

MATERIAL AND METHODS

A material of 210 persons was investigated consisting of the following groups (Table 1):

1. A consecutive series of 49 patients with renal carcinoma demonstrated by renal arteriography, examined and followed by appointment with hospitals in the greater Copenhagen area. Histological diagnoses were the following: hypernephroma 43, transitional cell carcinoma of the renal pelvis 3, and adenopapillifery carcinoma 3 cases.

At the time of initial treatment 32 patients were without and 17 patients with clinically detectable distant metastases. The presence of distant metastases was evaluated on basis of liver function tests, X-ray examinations of lungs, vertebral column, pelvis, technetium polyphosphate, and gallium scintigraphy.

Clinical data and tumour pathology of the 49 patients are summarized in Table 2.

The first 24 consecutive patients in the series have been the subject of an earlier report [9].

2. Seventeen patients with benign renal disease: renal cyst, polycystic renal disease, hypertensive nephropathy, glomerulonephritis, pyelonephritis, hydronephrosis, renal tuberculosis, and nephrolithiasis.

3. Twenty-two patients with solid carcinoma of non-kidney origin. The diagnoses were all histologically confirmed, and comprised cases of: gastric, colonic, pancreatic, mammary, bladder, prostatic, and pulmonary carcinoma. All patients were without detectable distant metastases at the time of test.

4. Forty-five patients with various forms of non-malignant disease, examined during admission in hospital. They had no signs of malignant or benign tumours, hypertension or autoimmune diseases and had not received glucocorticoid or cytostatic treatment within the last 6 months.

This group comprised cases: duodenal ulcer, cerebral thrombosis, cholelithiasis, acute appendicitis, diverticulosis, presacral cyst, sequelae after gastric resection, haemorrhoids, stricture of the oesophagus, pilonidal cyst, oophoretic cyst, achalasia of the oesophagus, inguinal hernia, hydrocele testis, fracture, chronic pancreatitis, hemicrania, prostatic hypertrophy, and ureterolithiasis.

5. Seventy-seven members of the blood donor group of Rigshospitalet, Copenhagen, assumed healthy on basis of a questionnaire concerning earlier diseases, and of a set of blood analyses made before recognition as a blood donor. Since the upper age limit of blood donors is 65 years, the mean age of this control group is somewhat lower than that of the renal carcinoma group (Table 1).

Table 1. *Patient material*

	Men	Women	Age	Total No.
1. Renal malignant tumour	32	17	43–80, mean 59	49
2. Benign renal disease	10	7	24–78, mean 57	17
3. Carcinoma of non-kidney origin	15	7	42–83, mean 64	22
4. Various benign diseases	27	18	18–87, mean 47	45
5. Blood donors	51	26	20–65, mean 50	77
Total	135	75	—	210

PREPARATION OF TISSUE EXTRACTS FROM RENAL CARCINOMAS, CORRESPONDING NORMAL RENAL TISSUE, AND FOETAL KIDNEYS

At operation a radical nephrectomy was performed. Within 30 min after removal of the kidney specimens were obtained for preparation of soluble extracts. This was done aseptically from tumour tissue and from normal renal tissue not macroscopically invaded by tumour. The rest of the operative specimen was reserved for pathoanatomical and histopathological evaluation. The preparation of tissue extracts from renal carcinomas and corresponding renal tissues has earlier been described in detail [9, 12]. No pooling of extracts was performed.

Tissue extracts were prepared by the same procedure from pooled, human foetal kidneys originating from spontaneous or therapeutic abortions in the III trimester of gestation, and without detectable malformations. Material

from 4–6 foetuses was pooled in one batch as earlier described [9].

THE LEUCOCYTE MIGRATION CAPILLARY TUBE TECHNIQUE (LMCT)

The micromodified LMCT has earlier been described in detail [10–12].

In essence, venous blood was collected in heparin and dextran. After sedimentation of the erythrocytes, the leucocytes were washed three times in Hank's solution, and collected in 20 μl. glass capillary tubes. Each of these was placed in a 0·5 ml disposable culture chamber containing TC 199 with 10% horse serum. To the test cultures the adequate amount of tissue extract was added according to the experimental protocol. All cultures were set up at least in triplicate. After 24 hr at 37°C the migration areas of leucocytes around the capillary tube openings were studied in a projection microscope, cut, and weighed. The average migration areas of a set of tissue extract containing cultures (M_x) and a set of control cultures (M_0) containing only the culture medium are used to calculate the migration index (MI): $MI = M_x/M_0$. A MI below 1·0 accordingly indicates tissue extract induced inhibition, and a MI greater than 1·0 tissue extract induced stimulation of the leucocyte migration.

PATHOANATOMICAL AND HISTOPATHOLOGICAL EVALUATION OF RENAL CARCINOMAS

The criteria used for evaluation of renal carcinomas with regard to macroscopical invasion, microscopical invasion in renal pelvis, lymph nodes, fibrous and fatty capsule, hilus vessels, and criteria for grading of differentiation, bleeding, necrosis, and lymphocyte infiltration have earlier been discussed in detail [14].

TEST PROTOCOL

The patients were admitted consecutively to the investigation irrespective of the clinical stage at the time of initial treatment. If subsequent surgery revealed carcinoma of the kidney, the patients were included in the series. If benign disease was found, the patients served as controls. Beginning preoperatively 3–8 tests were performed in each patient. Over the total test period from the first preoperative to the last postoperative day at hospital 3–8 different tumour and normal renal tissue extracts were used for each patient. After the postoperative period the patients were followed in the out-patient clinic. Clinical stage and the course of the disease was evaluated, and death of the patients recorded.

Eighteen patients died during the observation period. Autopsy reports were available in 16/18 patients. The 2 patients in whom autopsy was not performed died in the hospital with evidence of distant metastases. They were recorded as deaths from renal carcinoma. Twelve patients were tested with autologous tumour and corresponding normal renal tissue at least twice in concentrations of 10, 50, 100, 300, 400, and 600 μg of protein per ml. With foetal kidney tissue 2 tests were performed in the preoperative period at the concentration of 100 μg per ml.

Further testing was performed with extracts of allogeneic hypernephroma and corresponding normal renal tissues, at least 3 per patient, in concentration of 10, 50, 100, 300, 400 and 600 μg of protein per ml. A total of 24 extracts from renal carcinomas and corresponding normal renal tissues were employed in the allogeneic tests of the present investigation.

The antigenic strength of each tumour extract was determined by its capacity to induce significant leucocyte migration inhibition in allogeneic combinations in hypernephroma patients without distant metastases. This capacity was expressed as a migration inhibition score (MIS) for each of the 24 different tumour extracts employed, calculated in the following way,

$$MIS = \frac{\text{number of positive tests}}{\text{total number of tests}} \times 100,$$

expressed in percent, As elsewhere discussed in detail [14] the MIS seems to be an *in vitro* correlate of tumour antigenicity *in vivo*. The MIS of the 24 tumours examined varied from 0–60%. TCMH results in the present report employing allogeneic hypernephroma extracts originate from tumours with MIS of more than 20%.

After the completion of the series of examinations with allogeneic hypernephroma and corresponding normal renal tissue a titration curve was drawn for each patient as reported in detail earlier [12–14].

Groups of control patients (groups 2, 3, and 4, Table 1) were tested as far as possible according to the same protocol, blood donors (group 5, Table 1), however, only once.

Table 2. Clinical data, tumour pathology, TCMH, and survival of 49 patients with renal carcinoma

	Clinical data						Tumour pathology										
									Microinvasion						Lymphocyte infiltration		
Tumour No.	Sex	Age	Tumour type	Distant metastases	TCMH against allogeneic hyperneph-roma Grade	FCMH	Tumour diameter (cm)	Macroscopic invasion	Pelvis	Lymph nodes	Fibrous capsule	Fatty capsule	Hillus vessels	Degree of differentiation	Stroma	Tumour	Death during the observation period
1	F	71	H	0	3	0	6	0	+	0	0	0	0	I	+	0	0
2	M	51	H	+	0	0	10	0	0	0	+	0	0	II	++	++	0
3	M	61	H	0	3	+	9	+	+	+	+	0	+	II	+++	+	+
4	F	45	H	0	2	+	3	0	0	0	0	0	0	I	+	0	0
5	M	67	H	+	0	0	5	0	0	0	0	0	0	I	+	0	+
6	M	60	H	0	1	+	6	0	0	0	+	0	0	II	++	+	0
7	M	46	H	0	0	0	3	+	+	0	+	0	+	I	+	+	+
8	F	63	H	0	0	0	5	0	+	0	0	0	0	I	+	+	0
9	M	64	H	0	1	+	10	0	0	0	+	0	0	I	+	0	0
10	M	74	H	0	1	0	6	0	+	0	+	0	+	I	+	0	0
11	M	58	H	0	1	0	6	0	0	+	+	0	0	II	+	0	0
12	M	27	AC	0	2	+	3	0	+	+	0	0	0	II	n.i.	n.i.	0
13	M	62	H	0	2	+	9	+	+	0	+	+	+	II	++	+	+
14	F	64	H	+	0	0	n.i.	0	0	0	0	0	0	II	+	0	0
15	F	51	H	0	2	+	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0
16	F	57	H	+	0	0	11	0	0	0	+	+	0	II	+++	+++	+
17	M	68	H	+	0	0	5	0	0	0	+	+	+	II	+++	+++	+
18	M	43	T	0	0	+	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	I	0	0	+
19	M	61	H	0	3	0	7	0	0	0	0	0	+	I	++	+	0
20	M	65	H	0	0	0	7	+	+	0	+	+	0	II	+++	++	+
21	M	74	H	0	2	+	3	0	0	0	+	+	0	I	+	0	0
22	M	40	H	0	3	+	3	0	0	0	+	0	0	I	+	++	0
23	F	39	H	+	0	0	5	n.i.	+	+	0	0	n.i.	II	+	+	+
24	M	61	H	+	0	0	7	+	+	0	+	+	+	II	++	0	+
25	F	53	H	0	4	+	9	0	0	0	+	0	0	I	++	+	0
26	M	64	H	+	0	0	7	+	+	0	+	+	+	II	+	0	+
27	M	67	H	+	0	n.i.	7	+	+	0	+	+	0	II	+++	+++	+
28	F	63	H	0	2	n.i.	4	+	0	0	0	0	0	II	+++	+	0
29	M	58	AC	0	0	n.i.	3	0	0	0	0	0	0	I	n.i.	n.i.	0
30	M	55	H	0	0	0	11	+	+	+	+	+	+	II	++	0	+

31	M	58	H	+	0	n.i.	3	0	0	0	0	I	n.i.	n.i.	+
32	M	72	AC	+	0	n.i	4	+	0	+	0	II	+	+	0
33	M	70	H	+	0	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	+
34	M	58	H	+	0	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0
35	F	61	H	+	0	0	9	+	0	+	0	II	+	0	0
36	F	80	H	0	2	n.i.	7	+	0	+	0	I	0	0	0
37	F	49	H	0	0	n.i.	n.i.	+	+	0	+	II	+	+	0
38	M	80	H	+	0	n.i.	11	+	0	+	0	II	+	+	0
39	F	58	H	0	2	n.i.	6	+	0	+	0	II	+	+	0
40	M	53	H	0	2	+	8	0	0	+	0	II	+	+	0
41	M	59	H	+	0	+	7	+	+	+	+	II	+	+	+
42	M	53	H	0	2	0	5	+	0	+	0	II	+	+	0
43	M	79	H	+	0	0	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0
44	F	59	H	0	2	+	3	+	0	0	0	I	+	+	0
45	F	46	H	0	0	n.i.	7	+	0	+	+	II	0	+	0
46	M	58	T	0	2	n.i.	4	n.i.	0	+	0	n.i.	+	+	+
47	M	64	H	+	2	n.i.	6	+	0	+	0	II	0	+	+
48	F	53	H	0	2	n.i.	4	0	0	+	0	I	+	+	0
49	F	63	H	0	0	n.i.	20	+	0	+	+	II	+	0	0

F = female M = male H = hypernephroma AC = adenopapillifery carcinoma T = transitional cell carcinoma of the renal pelvis n.i. = not investigated.

STATISTICAL EVALUATION

Significant leucocyte migration inhibition was defined as the mean ± 2 s.d. of the MI's in the control groups (groups 2, 3, 4, and 5) using the maximal non-toxic concentration of tumour and corresponding normal renal tissue extracts, which was 400 μg per ml in both autologous and allogeneic combinations. For foetal kidney extracts 100 μg per ml was non-toxic. The ranges have previously been discussed in detail [9, 10] and were the following: tumour and normal renal tissue extracts MI = 0·79–1·19, for foetal kidney extracts MI = 0·78–1·18. A dose-related effect of tissue extracts on the *in vitro* migration of leucocytes has earlier been the subject of several reports [10, 11]. On this basis a numerical grading of TCMH was made for each patient based on the lowest tumour extract concentration giving significant migration inhibition or stimulation in allogeneic combinations. This grading has earlier been discussed in detail [12, 14, 16] and was the following:

Grade 0: MI > 0·79, MI < 1·19 at all tissue extract concentrations employed except 600 μg/ml, which has been shown to inhibit the migration of control leucocytes [10, 12]. That is to exert a toxic = non-specific effect on the migration of leucocytes.

Grade 1: MI < 0·79, MI > 1·19 at 400 μg/ml, but not at lower concentrations.

Grade 2: MI < 0·79, MI > 1·19 at 400 and 300 μg/ml, but not at lower concentrations.

Grade 3: MI < 0·79, MI > 1·19 at 400, 300 and 100 μg/ml, but not at lower concentrations.

Grade 4: MI < 0·79, MI > 1·19 at 400, 300, 100, and 50 or 10 μg/ml.

By repeated testing only very limited variation in results was found [17]. The final TCMH grading for each patient (Table 2) was calculated on basis of the test giving most pronounced reactivity.

A computerized Fisher's exact probability test and Wilcoxon Mann–Whitney test [18] were used for comparing groups of data. Survival rates were calculated according to Biomedical Computer Programs [19], and the comparisons performed with the U-test [18]. Level of significance = 5 %.

RESULTS

Specificity of the reactivity

Thirty-three out of forty-nine patients with renal carcinoma were examined with foetal kidney tissue, 49/49 patients with allogeneic hypernephroma and corresponding normal renal tissue, and 12/49 patients with autologous renal tumour and corresponding normal renal tissue. The reactivity of the patients is shown in Table 2 and Table 3. Forty-two percent of the patients were reactive towards foetal kidney, 47 % towards allogeneic hypernephroma, and 58 % towards autologous renal tumour extracts. No postoperative depression in migration inhibition was found [17]. Migration inhibition was observed in 3 % of the tests performed with allogeneic renal tissue at the maximal non-toxic dose = 400 μg of protein/ml. Significant reactivity in renal carcinoma patients appears to be confined almost exclusively to autologous or allogeneic renal carcinoma tissue extracts.

Table 3. Reactivity of leucocytes from renal carcinoma patients towards foetal kidney tissue, allogeneic hypernephroma, and autologous renal carcinoma tissue extracts

Type of tissue extract	Grade	Patients in each group: No.		Patients in each group: %	
Foetal kidney tissue	—	14		42	
Allogeneic hypernephroma	0	26		53	
	1	4	23	8	47
	2	14		29	
	3	4		8	
	4	1		2	
Autologous renal tumour	0	5		42	
	1	3	7	25	58
	2	2		17	
	3	2		17	

Out of 63 control persons (6 patients with benign renal disease, 17 with cancer of non-kidney origin, 29 with various benign diseases, and 11 blood donors) tested with foetal kidney tissue 100 μg/ml, 2 persons = 3 % showed significant reactivity. Concerning allogeneic hypernephroma and corresponding normal renal tissue extracts, reactivity in the control groups (groups 2, 3, 4, and 5, Table 1) was as follows at the maximal non-toxic dose, 400 μg/ml [9, 10]: Group 2 + 3, allogeneic hypernephroma 5 %, allogeneic renal tissue 3 %. Group 4, allogeneic hypernephroma 1 %,

allogeneic renal tissue 3 %. Group 5, allogeneic hypernephroma 3 %, allogeneic renal tissue 6 %.

In Table 4 is shown the relation between reactivity towards allogeneic hypernephroma, autologous renal tumour, and foetal kidney tissue extracts in renal carcinoma patients examined with all three types of extracts. It appears that reactivity towards the tumour extracts and foetal kidney extracts is highly correlated suggesting the presence of identical components in the extracts.

Table 4. *Relation between reactivity towards allogeneic hypernephroma, autologous tumour, and foetal kidney tissue in patients with renal carcinoma*

	Allogeneic hypernephroma TCMH grade	
	0	1–4
Foetal kidney tissue. MI > 0·78, MI < 1·18	13	4
		$P = 0{\cdot}003$
Foetal kidney tissue. MI < 0·78, MI > 1·18	1	9
Autologous tumour. TCMH grade 0.	5	0
		$P = 0{\cdot}02$
Autologous tumour. TCMH grade 1–4	0	4

Relation between TCMH, laboratory data, and the clinical stage

For further analysis of the correlations between TCMH and clinical data responses to allogeneic hypernephroma were considered as indicative. By this criterion 23 patients had evidence of TCMH and 26 had not (Table 3).

No correlations were found between the presence of detectable TCMH and any of the following: sex, age, erythrocyte sedimentation rate, hemoglobin concentration, ABO and Rhesus blood type, percentage of granulocytes and lymphocytes in peripheral blood, side localization of tumour, tumour size, macroscopic tumour invasion in neighbouring organs, renal vein, or regional lymph nodes. However, a highly significant correlation was demonstrated between the absence of clinically detectable distant metastases and the presence of TCMH (Table 5). This correlation has been demonstrated repeatedly in previous reports [8–12, 14]. Thus it seems that TCMH reflects the dissemination of renal carcinoma, as detected by routine clinical screening for distant metastases. It was not possible to demonstrate variations in TCMH in relation to local tumour invasion or spread to the regional lymph nodes. Too few patients were present in the material to allow separate analysis of the clinical stage in relation to those patients with a high degree of TCMH (grade 3–4) as opposed to those with a low degree of TCMH (grade 1–2).

Relation between FCMH, laboratory data, and the clinical stage of the patients

The presence of detectable FCMH was evaluated against the same parameters as mentioned above. No correlations could be demonstrated except a highly significant correlation between the presence of FCMH and the absence of demonstrable distant metastases as earlier reported [9]. This correlation is shown in Table 6.

Table 5. *Relation between the presence or absence of distant metastases and TCMH*

	TCMH grade	
	0	1–4
Patients with distant metastases	17	1
Patients without distant metastases	9	22

Fisher's exact probability test: $P = 0{\cdot}0003$.

Table 6. *Relation between the presence or absence of distant metastases and FCMH*

	FCMH	
	0	+
Patients with distant metastases	10	1
Patients without without metastases	9	13

Fisher's exact probability test: $P = 0{\cdot}007$.

Relation between TCMH and FCMH and the histopathology of renal carcinomas

No correlations were found between the presence of TCMH or FCMH and any of the following: microscopic tumour invasion into the renal pelvis, fibrous capsule, fatty capsule, perirenal lymph nodes, renal vessels, degree of bleeding, necrosis, degree of tumour differentiation, degree of lymphocyte infiltration in the tumour stroma or tissue consistent with earlier reports [14].

Relation between TCMH, FCMH, clinical stage, histopathology, and the survival of the patients with renal carcinoma after operation

The postoperative observation time for the 49 patients with renal carcinoma ranged from 1–54 months, mean 16·1 months. No correlations were demonstrated between the postoperative survival time and any of the following: age, erythrocyte sedimentation rate, hemoglobin concentration, blood type, percentage of granulocytes and lymphocytes in peripheral blood, macroscopic tumour invasion in the neighbouring organs, invasion into the renal vein, microscopic invasion into the renal pelvis, perirenal lymph nodes, fibrous and fatty capsule, renal vessels, the degree of tumour differentiation, necrosis, bleeding, lymphocyte infiltration in the tumour stroma and tissue. As shown in the Figs. 1–2 highly significant correlations were found between the absence of distant metastases, evidence of TCMH and FCMH prolonged postoperative survival. It was not possible in the present material to show a difference in the survival of those patients with a high degree of TCMH (grade 3–4) as opposed to those with a low degree (grade 1–2) because of the limited number of patients with high degree responses (Table 2).

As shown in Fig. 3 women with renal carcinoma survived significantly longer after operation than men. It was not possible to demonstrate a difference between the two sexes with regard to any other parameter tested except a marginally significant difference ($P = 0{\cdot}05$) between the proportion of men and women with distant metastases (31/32 men, 4/17 women).

Immunological identification of a high-risk group in patients with renal carcinoma

In Table 7 the renal carcinoma material is classified according to the presence or absence

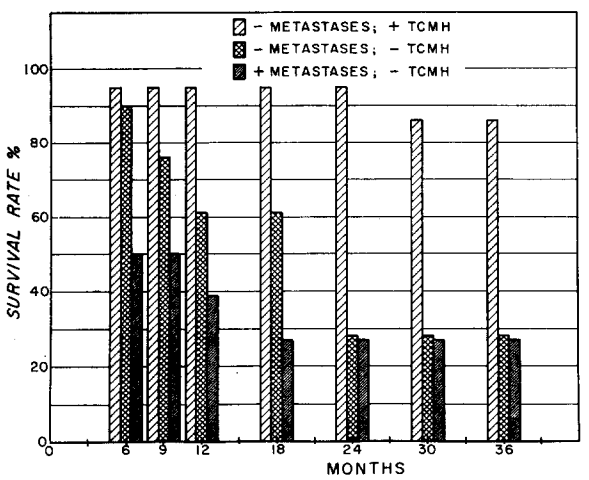


Fig. 1. Survival rates of 49 patients with renal carcinoma. Difference in survival of patients with and without clinically detectable distant metastases at the time of initial treatment U-test: at one year $P < 0{\cdot}01$, at 3 years $P < 0{\cdot}01$.

Difference in survival of patients with and without evidence of TCMH, grade 0 as opposed to grade 1–4, U-test: at one year $P < 0{\cdot}01$, at 3 years $P < 0{\cdot}01$.

Difference in survival of patients (a) with evidence of distant metastases and without evidence of TCMH, (b) patients without evidence of distant metastases and with evidence of TCMH, (c) patients without distant metastases without evidence of TCMH.

The material in group (c) was too small to allow statistical analysis.

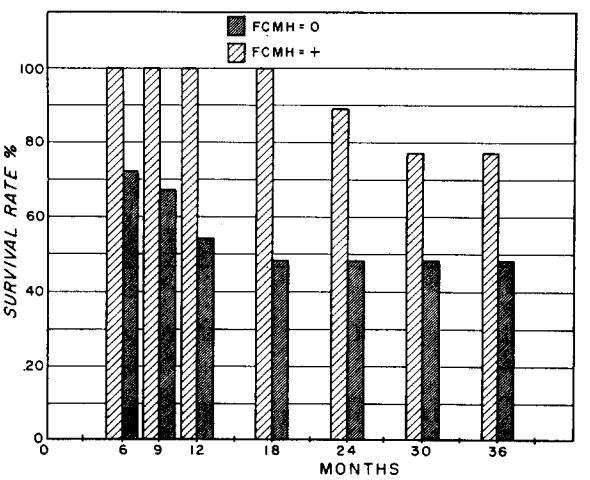


Fig. 2. Survival rates of 33 patients with renal carcinoma. Difference in survival of patients with and without evidence of FCMH, U-test: at one year $P < 0{\cdot}001$, at 3 years $P > 0{\cdot}05$.

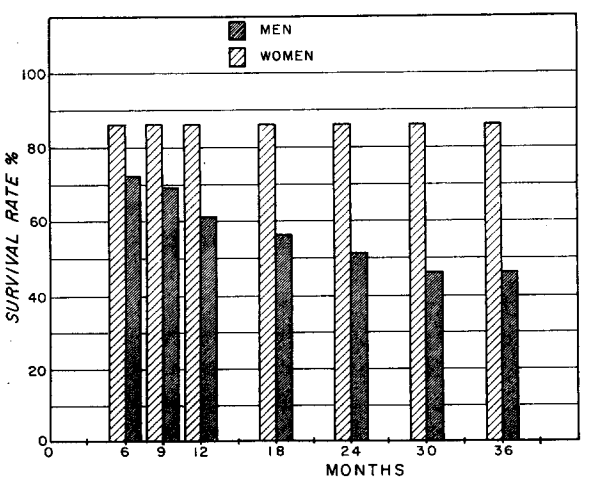


Fig. 3. Survival rates of 49 patients with renal carcinoma. Difference in survival of men and women, U-test: at one year $P < 0{\cdot}05$, at 3 years $P < 0{\cdot}01$.

Table 7. Forty-nine patients with renal carcinoma classified according to clinical stage, TCMH, and postoperative survival

			Dead	Alive	Total
Patients with distant metastases	TCMH	1–4	1	0	18
		0	11	6	
Patients without distant metastases	TCMH	1–4	3	19	31
		0	4	5	

of distant metastases, and to the presence or absence of detectable TCMH in relation to survival. Clinical stage and TCMH has been shown to be highly correlated (Table 5). Nine patients with renal carcinoma did not have detectable distant metastases, but had no evidence of TCMH, (grade 0). The hypothesis tested was that this group of patients might represent a high-risk group with regard to survival as opposed to those patients also without detectable distant metastases, but with evidence of TCMH.

As shown in Fig. 1 this group of patients had survival rates at one year between those of patients with distant metastases, and those of patients without, but with evidence of TCMH. At 3 years the patients in the high-risk group had the same survival rates as the patients with distant metastases.

This indicates that patients with renal carcinoma can be classified in a high-risk group by means of TCMH at the time of initial treatment before clinically detectable distant metastases occur.

DISCUSSION

The data on TCMH and FCMH in the present investigation indicate that antigens are found in carcinoma cells which are not present in the corresponding normal renal tissue, but possibly identical, at least in part, to antigens in foetal kidney tissue from the III trimester of gestation [9]. The antigens in renal carcinoma evoke a TCMH in the autologous host, and cross-reactivity is predominant among tumours of the same histological type. This TCMH is present in early stages of the tumour development before dissemination occurs, and can be detected by means of the LMCT. As the tumour progresses and distant metastases occur the TCMH is no longer detectable *in vitro* [9–12], but extensive leucocyte washings can restore reactivity [16], indicating that a factor (circulating-tumour antigen?) can be removed from the migrating cell population after washing. A factor re-inducing non-reactivity can be found in serum from some patients with renal carcinoma [8]. Moreover, it has been shown [14] that renal carcinomas from patients with distant metastases used in allogeneic combinations induce leucocyte migration inhibition significantly less often in renal carcinoma patients than renal carcinomas from patients with localized tumours. This indicates a progressive blocking of the antigens possibly by immunoglobulins.

The nature of the antigens in human renal carcinoma is not clear. The survival data from the present investigation indicate that the antigens may be targets in the immunological elimination of tumour cells *in vivo*. Moreover, the data on cross-reactivity between renal carcinoma and foetal kidney tissue support the hypothesis that the antigens may be of foetal origin or contain foetal components. An intriguing speculation supported by the survival data of men and women in the present material and clinical data from other materials [20], is that women may develop distant metastases more seldom than men and survive better after operation because of a possible pre-sensitization to antigens of foetal origin during pregnancy.

The identification of a high-risk group among renal carcinoma patients at the time of initial treatment by means of TCMH raises important implications regarding selection of patients for adjuvant therapy, i.e., chemotherapy, thereby may be changing the prognosis of this group of patients.

Acknowledgements—The author wants to thank Mrs. Anne Kjerulff and Mrs. Jette Hassing for competent technical assistance.

REFERENCES

1. V. Andersen, M. Kjaer and G. Bendixen, *In vitro*-demonstration of cellular hypersensitivity to tumour antigens in man. *Ser. Haemat.* **5,** 3 (1972).
2. C. O'Toole, Cell-mediated immunity to carcinoma of the urinary bladder in man. Clinical correlations of the response and the nature of the effector cells. Department of Immunology, Wenner–Gren Institute, Stockholm. Sweden (1973).
3. G. Mavligit, J. U. Gutterman, C. McBride and E. M. Hersh, Tumor-directed immune reactivity and immunotherapy in malignant melanoma. Current status. *Progr. exp. Tumor Res.* **19,** 22 (1974).
4. R. B. Herberman, Cell-mediated immunity to tumor cells. *Adv. Cancer Res.* **19,** 207 (1974).
5. G. H. Heppner, Colony inhibition and microcytotoxicity assay methods for measuring cell-mediated and associated antibody immunity *in vitro*. In *Methods in Cancer Research.* (Edited by H. Busch) Vol. VIII, 3. Academic Press (1973).
6. J. G. Sinkovics, Monitoring *in vitro* of cell-mediated immune reactions to tumors. In *Methods in Cancer Research.* (Edited by H. Busch) Vol. VIII, 107. Academic Press (1973).
7. J. P. Levy and J. C. Leclerc, Immune rejection of tumor cells: *In vivo* significance of anti-tumor *in vitro* immune reactions. *Biomedicine* **23,** 249 (1975).
8. M. Kjaer, The capillary tube leucocyte migration technique (LMT) as a method for detection of serum inhibiting activity of tumour-directed, cellular hypersensitivity in patients with renal carcinoma. *Acta path. microbiol. Scand. Sect. B.* **82,** 294 (1974).
9. M. Kjaer, *In vitro* demonstration of cellular hypersensitivity to tumour antigens by means of the leucocyte migration technique in patients with renal carcinoma. *Europ. J. Cancer* **10,** 523 (1974).
10. M. Kjaer, The dose-related effect of tumour extract on the *in vitro* migration of leucocytes from patients with renal carcinoma. *Europ. J. Cancer* **11,** 281 (1975).
11. M. Kjaer and G. Bendixen, Tumour-directed, cellular hypersensitivity detected by means of leucocyte migration technique in patients with renal carcinoma. *Ann. N.Y. Acad. Sci.*, 1976 (In press).
12. M. Kjaer and G. Bendixen, Clinical detection and monitoring of tumour-specific, cell-mediated hypersensitivity (TCMH) in man: Examinations of hypernephroma with leucocyte migration technique. In: In vitro *Methods in Cell-Mediated Immunity.* (Edited by D. Bloom and J. R. David) Academic Press, 1976, pp. 629–643.
13. M. Kjaer and T. B. Sørensen, Comparison between the direct capillary tube leucocyte migration technique (LMCT) and the direct agarose leucocyte migration technique (LMAT) as indicators of tumour-directed, cell-mediated hypersensitivity (TCMH) in patients with renal carcinoma. *Acta allerg. (Kbh.)* **31,** 141 (1976).
14. M. Kjaer and N. Christensen, Ability of renal carcinoma tissue extracts to induce leucocyte migration inhibition in patients with non-metastatic renal carcinoma: Correlation with clinical and histopathological findings. *Cancer Immunol. Immunother.* 1976 (In press).
15. M. Kjaer and M. Thomsen, General immunocompetence and tumour-directed, cell-mediated hypersensitivity *in vitro* in patients with renal carcinoma. *Acta path. microbiol. Scand. Sect. C.* 1976 (In press).
16. M. Kjaer, Effect of leucocyte washings on cellular immunity to human renal carcinoma. *Europ. J. Cancer*, 1976 (In press).
17. M. Kjaer, Unpublished data.
18. J. W. Bradley, *Distribution-free Statistical Tests.* Prentice-Hall, Englewood Cliffs. N.J., Jersey (1968).
19. *Biomedical Computer Programs.* (Edited by W. J. Dixon), University of California Publications in Automatic Computation No. 2. University of California Press, Berkeley, Ca. (1970).
20. O. Arner, C. Blanck and T. von Schreeb, Renal adenocarcinoma. Morphology—grading of malignancy-prognosis. A study of 197 cases. *Acta chir. Scand., Suppl.* **346,** (1965).

Europ. J. Cancer Vol. 12, pp. 899–905. Pergamon Press 1976. Printed in Great Britain

A Sensitive Bio-assay for Pharmacokinetic Studies of Cytosine Arabinoside in Man*

H. C. VAN PROOIJEN, G. VIERWINDEN, J. VAN EGMOND, J. M. C. WESSELS, C. HAANEN

Division of Hematology, Department of Internal Medicine, St. Radboud Hospital, University of Nijmegen, The Netherlands

Abstract—*A sensitive bio-assay is presented for the determination of plasma levels of cytosine arabinoside (Ara-C). The test is based on the inhibition of the incorporation of tritiated thymidine into DNA of rat bone marrow cells in the presence of Ara-C.*

The accuracy and the sensitivity of the test is demonstrated. Unknown plasma concentrations of Ara-C as low as 0·003 mg/l can be measured.

The plasma disappearance of Ara-C after an intravenous bolus injection in a dose of 100 mg/m², is biphasic. The half life times of these two phases vary for individuals which is illustrated in two patients with acute leukemia in which the half life time of Ara-C in the first phase was 1·8 and 1·4 min, and in the second phase 16·0 and 9·2 min respectively.

INTRODUCTION

THE PYRIMIDINE analog cytosine–arabinoside (Ara-C) has proved to be effective in the treatment of acute myeloblastic leukemia (AML) in man. The therapeutic effect of Ara-C strongly depends on the dosage and the way of administration. Complete remission is obtained in more than half of the patients treated with Ara-C in combination with other drugs like thioguanine [1] or daunorubicin [2].

To achieve optimal treatment schedules, knowledge of pharmacological parameters, such as plasma half life and effective drug concentration in relevant tissues, is required. Baguley *et al.* [3] report a significant correlation between a short half life of Ara-C in plasma and a poor response.

The drug is eliminated by glomerular filtration and by enzymatic deamination to uracil-arabinoside (Ara-U). Between 87 and 91 % of the radioactivity recovered in the urine after intravenous administration of tritiated cytosine-arabinoside (^{3}HAra-C) appeared to be Ara-U [4]. The enzyme responsible for deamination of Ara-C is deoxycytidine deaminase present in the human liver, spleen, plasma and in other tissues [5]. Individual variations in intra- and/or extracellular deaminase activity may be responsible for the observed differences in half life of Ara-C.

Several methods for the determination of Ara-C in plasma have been published [6, 7]. Baguley and Falkenhaug [3, 8] described a bio-assay based on the inhibition of tritiated thymidine (^{3}HTdR) incorporation into DNA of mouse L-cells and spleen cells or ascitic L-1210 cells in the presence of Ara-C. Concentrations as low as 0·04 mg/l plasma could be detected.

The plasma disappearance curve of Ara-C after a single i.v. injection is biphasic when plotted on a semilogarithmic scale as is shown by Ho and Frei [9] after injection of ^{3}HAra-C.

In various treatment schedules of AML a push injection of 100 mg Ara-C/m²/12 hr is given. To study interindividual differences in the second phase of the elimination curve of Ara-C after a therapeutic dose of 100 mg/m² it was found necessary to develop a more sensitive assay than those described till now. In this paper an assay is presented based on the inhibition by Ara-C of ^{3}HTdR incorporation into DNA of rat bone marrow cells. Plasma concentrations as low as 0.003 mg/l can be measured.

Accepted 9 June 1976.

*Supported by the Koningin Wilhelmina Foundation—Netherland's Organization for Cancer Research.

MATERIAL AND METHODS

Preparation of blood samples

Blood samples are taken just before and at different time intervals after a rapid injection of Ara-C in a dose of 100 mg/m^2. Blood samples of 8 ml are collected within 10 sec in precooled tubes containing 150 IU Heparin and 50 μg tetrahydrouridine and put on ice immediately. Tetrahydrouridine (THU), added to prevent enzymatic deamination of Ara-C [5], was kindly provided by Dr. Harry B. Wood, Chief Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda.

The blood samples are centrifuged at 0°C (2000 **g**, 15 min) and the plasma is ultrafiltered by centrifugation at 0°C (250 **g**, 60 min) through conical ultrafilters (Centriflo F 25, Amicon). The ultrafiltered plasma samples are stored at −70°C.

Preparation of the cell suspension

Wistar rats are sacrificed and bone marrow is obtained from both femurs by expulsion with Eagle's Minimal Essential Medium (MEM Gibco, Cat. N. F. 14). The cells are suspended and filtered through a nylon filter (pore size 100 μm to remove tissue particles and cell-clumps. The cell number is counted electronically (Coulter Counter Z.F.) and the cell suspension is diluted to a density of 2×10^6 cells/ml in MEM containing 20% foetal calf serum (Flow-lab.) buffered with 0·02 M Tris–HCL to pH 7·3 and supplemented with Spinner salts (Gibco F-15) and 2×10^{-3} M L-glutamine (Schwarz/Mann).

Determination of Ara-C

Rat bone marrow cell suspension (0·5 ml, 2×10^6 cells/ml) is added to 0·5 ml of cooled ultrafiltered plasma containing 0·6 nmol (methyl-^{3}H)-thymidine (^{3}HTdR, 1·66 Ci/mmol, Amersham). The samples are incubated at 37°C in a shaking waterbath for 150 min. After incubation the samples are placed on ice, centrifuged (1000 × **g**, 15 min) and the pellet is washed three times with 1 ml Krebs Ringer solution buffered with 35×10^{-3} M Tris–HCl to pH 7·3 and containing $0{\cdot}25 \times 10^{-3}$ M 2-deoxythymidine. To remove all acid soluble radioactivity the cells are washed twice with 1 ml 1M $HClO_4$. The residual $HClO_4$ is removed by treating the precipitate with ice cold 99% ethanol.

The sediment is dissolved in 0.2 ml Hydroxide of Hyamine (Packard) and quantitatively transferred to counting vials with 4 ml distilled water. After addition of 6 ml Instagel (Packard) the radioactivity is counted in a liquid scintillation counter (LKB 81000).

RESULTS

Aspecific effect of various human plasma samples on ^{3}H-thymidine incorporation

Experiments were performed to exclude differences in thymidine incorporation into DNA of rat bone marrow cells due to aspecific effects of human plasma. As is shown in Fig. 1 the incorporation of ^{3}HTdR into DNA varies

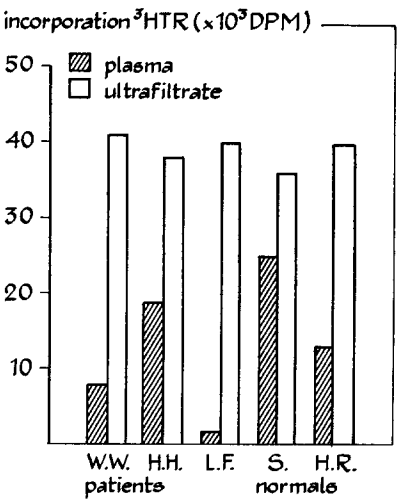


Fig. 1. Incorporation of ^{3}HTdR into DNA of 10^6 rat bone marrow cells after incubation for one hour with plasma or ultrafiltered plasma of five different individuals.

considerably after incubation with different plasma samples. This phenomenon was observed in healthy individuals as well as in leukemic patients. Removal of plasma proteins by ultrafiltration through Centriflo ultrafilters abolishes this variability in all samples almost completely and results in higher incorporation of ^{3}HTdR.

In control experiments with ^{3}HAra-C, binding to plasma proteins and adsorption to the filter was found to be negligible (< 2%).

Inhibition of ^{3}H-thymidine incorporation by cytosine arabinoside

The incorporation rate of ^{3}HTdR into DNA of rat bone marrow cells depends strongly on conditions during incubation. It is found that the incorporation per hour increases after addition of different amounts of foetal calf serum. A concentration of 20% is found optimal causing an increase in incorporation of 30% compared to the control value (Fig. 2).

In Fig. 3 it is shown that in the absence of Ara-C a linear relationship exists between the

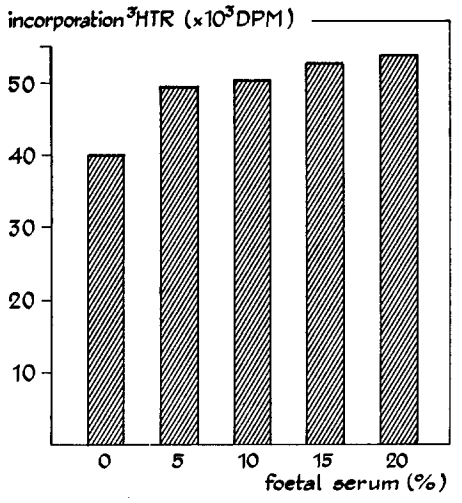


Fig. 2. Incorporation of ³HTdR into DNA 10^6 *rat bone marrow cells after incubation for one hour.*

Ultrafiltered plasma was added to the cell suspension in MEM, supplemented with different amounts of foetal serum.

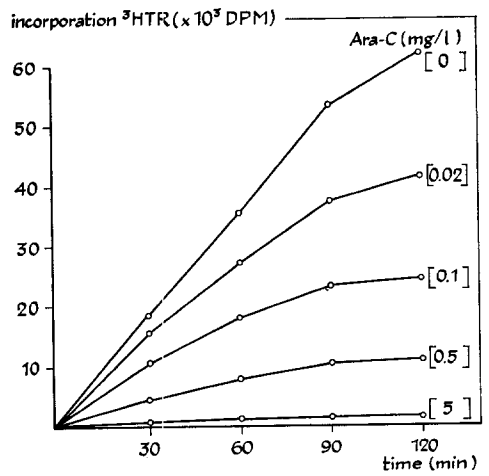


Fig. 3. Incorporation of ³HTdR into DNA of 10^6 *rat bone marrow cells after different time intervals.*

To the cell suspension in MEM an equal amount of ultrafiltered plasma was added, containing different amounts of Ara-C (concentration range 0–5 mg/l).

incorporation of ³HTdR into DNA and time. However, after 90–120 min the incorporation rate seems to decline. The same figure demonstrates that the incorporation of ³HTdR is inhibited in the presence of different amounts of Ara-C. It can be seen that the incorporation rate of ³HTdR is inhibited proportionally to the Ara-C concentration. A high concentration of Ara-C (5 mg/l) inhibits the ³HTdR incorporation almost completely within 30 min while a low concentration of Ara-C (0·02 mg/l) does not yet inhibit DNA-synthesis completely after two hours of incubation.

The determination of the calibration curve

The inhibition of ³HTdR incorporation in the presence of Ara-C can be expressed as an inhibition percentage (*IP*);

$$IP = \left[1 - \frac{A}{B}\right] \times 100\% \qquad (1)$$

In formula (1) A is the incorporation of ³HTdR in the presence of Ara-C and B the ³HTdR incorporation without Ara-C.

For the determination of unknown concentrations of Ara-C a calibration curve is made by plotting the inhibition percentages versus the known Ara-C concentrations on Log scale. The IP's are measured in triplicate for concentrations $\leq$ 0·05 mg/l and in duplicate for higher concentrations.

The known concentrations are prepared in ultrafiltered plasma samples and their *IP*'s are fitted in a least squares procedure to the equation:

$$IP = 100\left[1 - \frac{1}{a[\text{Ara-C}]^b} \times (1 - \exp(-a[\text{Ara-C}]^b))\right] \qquad (2)$$

The mathematical derivation and the meaning of parameters a and b are explained in the "appendix". A change in parameter a is related to the horizontal shift of the calibration curve and b is correlated with the slope of the steepest part of the S-curve. Figure 4 demonstrates the experimental determined inhibition percentages plotted versus the known concentrations of Ara-C. It also shows the calculated curve after determination of parameters a and b. The theoretical curve fits the experimental data rather well which gives an indication for the validity of the model from which equation (2) has been derived (see appendix).

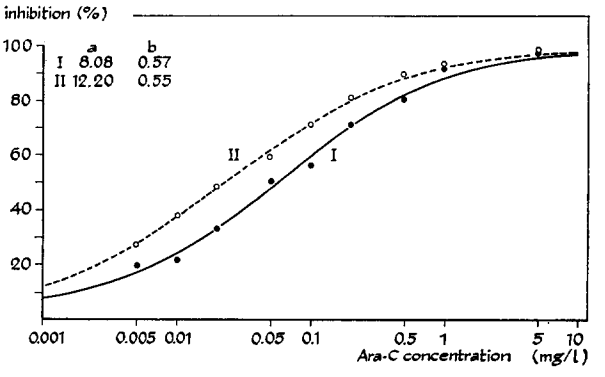


Fig. 4. The percentages of inhibition of ³HTdR incorporation into DNA of 10^6 *rat bone marrow cells is plotted versus the final Ara-C concentrations. The data are obtained with bone marrow from two different rats (○, ●) using the same Ara-C solutions. The curves are obtained by calculating parameters* a *and* b *from equation* (2) *in a least squares procedure.*

The accuracy of the determination of unknown Ara-C concentrations

The shape of the calibration curve depends on the sensitivity of rat bone marrow cells for Ara-C. To test this biological variation in sensitivity five calibration curves were processed with one set of known Ara-C concentrations and the bone marrow of five different rats. The parameters *a* and *b* and their standard deviations calculated for these curves are listed in Table 1.

Table 1. *Parameters* a *and* b (*equation* 2) *and their standard deviations calculated from five calibration curves processed with one set of known Ara-C concentrations and the bone marrow of five different rats*

Rat	*a*	S.D. *a*	*b*	S.D. *b*
1	9·5	1·2	0·58	0·04
2	8·1	0·7	0·57	0·03
3	9·8	0·4	0·56	0·01
4	12·2	0·8	0·55	0·02
5	10·0	2·1	0·67	0·07

For the analysis in the least squares procedure it was not found necessary to use a weighting scheme for the average *IP*'s of duplicate (high concentrations) and triplicate (concentrations $< 0{\cdot}05$) measurements.

The calibration curve processed with the use of bone marrow of rat 2 and 4 are plotted in Fig. 4.

The two curves are significantly different, indicating a variation in sensitivity of the bone marrow cells for Ara-C. Therefore accurate determinations of unknown concentrations will only be obtained if they are processed in the same bone marrow cell suspension as used for the calibration curve.

To get an estimate of the accuracy of the assay we calculated the standard deviation of the logarithm of an Ara-C concentration determined in the plasma of a patient. For this particular calibration curve the parameters *a* and *b* are 8·75 (s.d. 0·56) and 0·52 (s.d. 0·02 respectively. An inhibition percentage of 50% (s.d. 2%) corresponds here to a concentration of 0·076 mg/l with an error margin of 0·126 and 0·044 mg/l respectively (95% confidence level). In the experiments unknown concentrations are measured in duplicate which reduces the error margin to 0·110 and 0·052 mg/l respectively. The value 8·75 for parameter *a* is rather low (Table 1) and corresponds to a relatively insensitive rat bone marrow. Since in the outer parts of the S-curve ($IP < 80\%$ and $< 20\%$) the relative errors will increase, it is necessary to dilute the high concentrations to bring them in the linear part of the calibration curve. Higher values for parameter *a* do not change the slope of the straight part of the calibration curve. Therefore the relative error will not decrease but lower concentrations can be determined. Parameter *b* affects the slope of the S-curve. High values for *b* correspond to steeper curves which therefore reduce the relative errors in the measured concentration.

The sensitivity of the assay

Figure 3 shows that the sensitivity of the test increases with the incubation time, which can also be concluded from the mathematical approach (see appendix equation (6)).

However, Fig. 3 also demonstrates that the incorporation of ^{3}HTdR declines after 90 min of incubation which limits the increase in sensitivity and also affects the accuracy. After an incubation time of 150 min, a compromise between sensitivity and accuracy, the lower limit of the assay proves to be 0·003–0·008 mg/l depending on the sensitivity of the bone marrow. The incorporation of ^{3}HTdR in the presence of Ara-C decreases as a function of time. Therefore, preincubation with Ara-C in the absence of ^{3}HTdR will increase the sensitivity of the assay. In Fig. 6 it is shown that preincubation with Ara-C for one hour followed by incubation with ^{3}HTdR for another hour indeed increases the sensitivity of the assay considerably.

Assay of cytosine arabinoside in human plasma

After an intravenous push injection of Ara-C in a dosage of 100 mg/m^2 blood samples were taken every 2 min during the first 10 min and every 5 minutes during the next 50 min. After ultrafiltration the duplicate samples of the unknown concentrations and the samples for the calibration curve are incubated with the same rat bone marrow cell suspension. The samples collected in the first 10 min after the injection of Ara-C are diluted three times with ultrafiltered plasma containing no Ara-C to bring the concentration in the linear part of the calibration curve.

Figure 5 shows a biphasic elimination pattern of Ara-C from the blood in two AML patients before treatment. The curves are fitted with a least squares procedure* to a two-compart-

*Weighting scheme based on a constant relative error in the measured concentration.

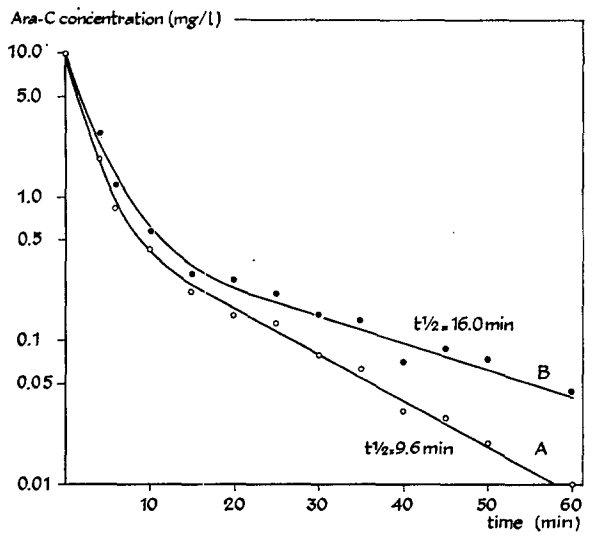


Fig. 5. Plasma disappearance of Ara-C in two AML-patients after an intravenous push injection (100 mg/m²). At different times blood was taken and Ara-C determined in the ultrafiltered plasma samples. From these data, half life times were calculated using a two compartments model.

ments model to calculate the plasma half life time of Ara-C. The half life times of the two phases are 1·4 (s.d. 0·1) and 9·4 min (s.d. 0·4) for patient A and 1·8 (s.d. 0·2) and 16·0 min (s.d. 1·8) for patient B.

DISCUSSION

The assay of Ara-C described in this paper, does not require tissue culture facilities [3] or tumor transplantation [8] and is more sensitive. The test is based on the inhibition of DNA synthesis in the presence of Ara-C.

It has been reported that the sensitivity of cells to Ara-C correlates with their metabolic activities [10]. The drug is either phosphorylated to its monophosphate by deoxycytidine kinase (K) [11, 12] and further phosphorylated to its triphosphate (Ara-CTP) or deaminated to uracil arabinoside by deoxycytidine deaminase (D) [13]. The active intracellular form of Ara-C is Ara-CTP causing inhibition of DNA synthesis most likely by inhibition of DNA polymerase [14]. The inhibition of DNA synthesis is greater and more pronounced at higher levels of Ara-CTP. Sensitive cells possess high K activities and low D activities [10, 15]. We therefore assume that the high sensitivity of rat bone marrow cells to Ara-C is due to a high K/D ratio.

The accuracy of the test is determined from the mathematical approach of the interaction between the ^{3}HTdR incorporation into DNA and the inhibition of the ^{3}HTdR incorporation by Ara-C which is elaborated in this paper.

The lower limit of the assay was 0·003 mg/l plasma.

In general the assay appears to be sufficiently sensitive to determine the plasma disappearance of Ara-C in patients. In the case of a high elimination rate, the assay can be made more sensitive at lower concentrations by a slight modification. It is shown in Fig. 6 that pre-incubation with Ara-C indeed increases the

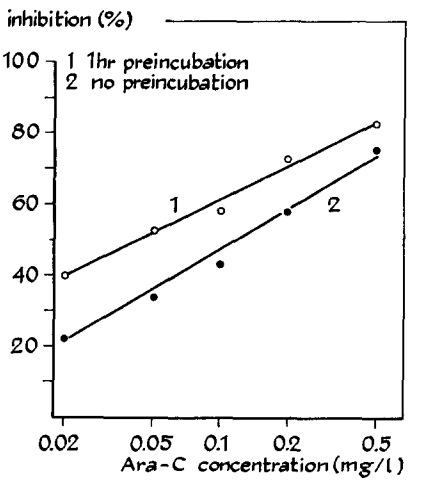


Fig. 6. The percentages of inhibition of ^{3}HTdR incorporation into DNA of 10^6 bone marrow cells is plotted versus the Ara-C concentrations. Calibration curve 2 is obtained after incubation of the cell suspension with ^{3}HTdR and Ara-C for two hours.

Calibration curve 1 is obtained after preincubation of the cell suspension with Ara-C for one hour followed by another hour of incubation with ^{3}HTdR.

sensitivity of the assay considerably. However, preincubation requires exact timing of the second incubation and therefore complicates the method considerably, since for the determination of one single disappearance curve about sixty samples have to be processed. For this reason this modification is restricted to selected cases. After an intravenous bolus injection of Ara-C the shape of the elimination curve in plasma appears to be biphasic (Fig. 5). The first phase mainly represents the rapid distribution of the drug over the total body water and the second phase reflects the elimination of the drug. Individual differences in the elimination rate correlate with the half life time of the second phase.

In this paper it is shown that a significant interindividual difference may exist in the plasma half life of Ara-C, after a bolus injection in a dose of 100 mg/m².

Dosage schedules for Ara-C related to body weight or body surface, such as used in treatment protocols in acute leukemia, do not take into consideration large variations in the elimi-

nation rate of the drug. In a number of patients treatment with Ara-C must be either ineffective or toxic. It is to be expected that the results of leukemia treatment will improve when the dosage of Ara-C can be related to optimal plasma levels during therapeutic courses.

REFERENCES

1. B. D. Clarkson, Acute myelocytic leukemia in adults. *Cancer (Philad.)* **30,** 1572 (1972).
2. D. Crowther, R. L. Powles, C. J. T. Bateman, M. E. J. Beard, C. L. Ganci, P. F. M. Wrigley, J. S. Malpas, G. Hamilton Fairly and Sir Ronald Bodley Scott, Management of adult acute myelogenous leukemia. *Brit. med. J.* **1,** 131 (1973).
3. B. C. Baguley and E. M. Falkenhaug, Plasma half life of cytosine arabinoside, in patients treated for acute myeloblastic leukemia. *Cancer Chemother. Rep.* **55** 291 (1971).
4. W. A. Creasy, R. J. Papac, M. E. Markin, P. Calabresi and A. D. Welch, Biochemical and Pharmacological studies with 1-β-D-Arabinofuranosyl cytosine in man. *Biochem. Pharmacol.* **15,** 1417 (1966).
5. G. W. Camiener, Studies of the enzymatic deamination of cytosine arabinoside. II. Properties of the deaminase of human liver. *Biochem. Pharmacol.* **16,** 1681 (1967).
6. L. J. Hanka, S. L. Kuentzel and G. L. Neil, Improved microbiological assay for cytosine arabinoside (NSC-63878). *Cancer Chemother. Rep.* **54,** 393 (1970).
7. J. Borsa, G. F. Whitmore, F. A. Valeriote, D. Collins and W. R. Bruce, Studies on the persistence of methotrexate, cytosine arabinoside and leukovorin in the serum of mice. *J. nat. Cancer Inst.* **42,** 235 (1969).
8. B. C. Baguley and E. M. Falkenhaug, Plasma half-life of cytosine arabinoside in patients with leukemia—the effect of uridine. *Europ. J. Cancer* **11,** 43 (1975).
9. D. H. W. Ho and E. Frei III, Clinical pharmacology of 1-β-D-Arabinofurasyl-cytosine. *Clin. Pharmacol. Ther.* **12,** 945 (1972).
10. D. H. W. Ho, Distribution of kinase and deaminase of 1-β-D-Arabinofurasyl-cytosine in tissues of man and mouse. *Cancer Res.* **33,** 2816 (1973).
11. M. Y. Chu and G. A. Fischer, Comparative studies of leukemic cells sensitive and resistant to cytosine arabinoside. *Biochem. Pharmacol.* **14,** 333 (1965).
12. R. L. Momparler, T. P. Brent, A. Labitan and V. Krygier, Studies on the phosphorylation of cytosine arabinoside in mammalian cells. *Molec. Pharmacol.* **7,** 413 (1971).
13. B. A. Chabner, D. G. Johns, C. N. Coleman, J. C. Drake and W. H. Evans, Purification and properties of cytidine deaminase from normal and leukemic granulocytes. *J. clin. Invest.* **53,** 992 (1974).
14. R. L. Momparler, Kinetic and template studies with cytosine arabinoside 5'-triphosphate and mammalian DNA polymerase. *Molec. Pharmacol.* **8,** 362 (1972).
15. R. L. Furner and L. B. Mellet, Kinase and deaminase activity in a variety of subcutaneous mouse tumors. *Cancer Res.* **35,** 1799 (1975).

APPENDIX

The relation between the inhibition of the ^{3}HTdR incorporation and the concentration of Ara-C expressed in equation (2) is derived from a model of two integrated processes. One process represents the incorporation of tritiated thymidine into DNA of rat bone marrow cells (DNA_c) with the incorporation rate constant k_0

$$DNA_c + {}^3HTdR \xrightarrow{k_0} DNA^* \text{ (labeled DNA)} \qquad (3)$$

Since ^{3}HTdR is available in excess (3) can be described as:

$$\frac{d\,(DNA^*)}{dt} = k_1 . DNA_c(t) \qquad (3a)$$

where $k_1 = k_0 . [{}^3HTdR]$.

The other process represents the inhibition of DNA synthesis by Ara-C with a rate constant k_2

$$DNA_c + \text{Ara-C} \xrightarrow{k_2} DNA_i \text{ (inhibited DNA)} \qquad (4)$$

It can be concluded from Fig. 3 that the inhibition of DNA synthesis in the presence of Ara-C is not linearly proportional to the Ara-C concentration. Therefore a parameter b is introduced to approach this relation:

$$\frac{\mathrm{d}\,(\mathrm{DNA}_c)}{\mathrm{d}t} = -k_2 . [\text{Ara-C}]^b . t \qquad (4a)$$

After integration it follows that the DNA-synthesis declines exponentially in time:

$$\mathrm{DNA}_c(t) = \mathrm{DNA}_0 . \exp\,(-k_2[\text{Ara-C}]^b) . t \qquad (5)$$

DNA_0 represents the initial number of rat bone marrow cells. After introduction of (5) in (3a) the rate of labeled DNA synthesis is derived.

$$\frac{\mathrm{d}\,(\mathrm{DNA}^*)}{\mathrm{d}t} = k_1 . \mathrm{DNA}_0 . \exp\,(-k_2[\text{Ara-C}]^b) . t \qquad (6)$$

Equation (6) is integrated over the incubation time and substituted into (1) to give (2).

From (6) it is obvious that the time for complete inhibition is inversely proportional to $[\text{Ara-C}]^b$ which is in good relation with the experimental findings ($b \cong 0{\cdot}5$–$0{\cdot}6$).

Europ. J. Cancer Vol. 12, pp. 907–911. Pergamon Press 1976. Printed in Great Britain

The Urinary Excretion of Pregnanetriol and Pregnenetriol in Patients with Breast Cancer. A Postulation on the Meaning of Bulbrook's Discriminant

L. GONÇALVES SOBRINHO*, L. FARO*, E. F. HENRIQUES*,
L. S. BOTELHO* and M. F. FONTES DE SOUSA†

**Department of Endocrinology and Laboratory of Endocrinology, Portuguese Cancer Institute, Lisbon and*
†Center for Applied Mathematics, Faculty of Science, University of Lisbon, Portugal

Abstract—*The ratio between 11 deoxy-17 ketosteroids and 17-hydroxycorticosteroids (Bulbrook's discriminant) and the ratio between pregnenetriol and pregnanetriol were measured in the urines of forty-one patients with advanced cancer of the breast. It was found that the two ratios were very significantly correlated. The ratio pregnenetriol/pregnanetriol reveals the efficiency of adrenal 3β hydroxysteroid dehydrogenase, an enzyme known to be blocked by oestrogens. It is therefore presumed that the Bulbrook's discriminant, of prognostic value in breast cancer patients, is an assessment of endogenous oestrogen effect.*

INTRODUCTION

IT HAS been shown that patients with advanced breast cancer who respond to endocrine ablation generally excrete more urinary androgen metabolites than patients who fail to respond [1–4]. The urinary androgen metabolites have been combined with the urinary 17-hydroxycorticosteroids (17-OHCS) in the form of a discriminant function:

80–80 (17-OHCS, mg/24 hr) + aetiocholanolone (μg/24 hr). Patients who excrete large amounts of aetiocholanolone relative to the 17-OHCS have positive discriminants and generally respond to ablation. Patients who fail to respond generally have a negative discriminant. These results have been confirmed by some workers [3, 4] but not by others [5]. It has been found that a similar degree of discrimination can be obtained by estimating the total 11-deoxy-17-oxosteroids (11-DKS) instead of a specific metabolite, aetiocholanolone [1, 2, 6, 7]. The discriminant is usually positive in normal women but decreases with age and becomes negative in old age [1]. The majority of patients with breast cancer, both in the early and advanced disease, have negative discriminants [2], and the abnormalities in steroid excretion precede by several years the clinical appearance of the disease [9].

The physiological meaning of these findings is not clear.

It has been found [10] that small quantities of oestrogen inhibit the activity of 3β-hydroxysteroid dehydrogenase in humans. The inhibition can be measured by an increase in the ratio of urinary pregn-5-ene, 3β, 17α, 20α (P_5T) to pregnane 3α, 17α, 20α triol (PT). It was possible, therefore, that the steroid abnormalities found in patients with breast cancer might result from an effect of endogenous oestrogen secretion on adrenal function. To check the validity of this assumption, the correlation between 11-DKS/17-OHCS and the P_5T/PT was studied in 41 patients with advanced breast cancer.

MATERIAL AND METHODS

Forty-one unselected patients with advanced breast cancer (local extension of the disease and/or distant metastasis) were studied. These patients had not been given chemotherapy nor had they been treated with hormones for at least a month prior to being studied.

Accepted 25 May 1976.

Twenty-four-hour urine samples were collected from each patient before and after i.m. administration of 1 mg of β 1–24 corticotrophin*.

The urines were assayed for 17-oxogenic steroids (17-OHCS) [11], 11 deoxy-17-oxosteroids (11-DKS) [12], 5β-pregnane-3α, 17α, 20αtriol (PT) and Δ5 pregnene-3β, 17α, 20αtriol (P_5T), both as glucuronides.

Method for the estimation of PT and P^5T in the urine

Reagents:

1. Glacial acetic acid
2. Acetate buffer: equal volumes of 5 M acetic acid and 5 M sodium acetate solution are mixed and the pH adjusted so that a dilution of 1 :10 with distilled water is pH 4·7.
3. Beta-glucuronidase: Sigma Chem. Co., St. Louis, Mo.
4. Benzene: B.D.H., distilled and saturated with water.
5. Sodium hydroxide-saline solution: 25% sodium chloride in N sodium hydroxide.
6. Alumina: B.D.H. 100 to 200 mesh, Brockmann, activity 2, deactivated by adding 1% water.
7. Sodium metaperiodate: 10% solution in water. Freshly prepared.
8. Dichloromethane: B.D.H. redistilled.

TECHNIQUE

An aliquot (1/40) of the 24-hr urine specimen is acidified to pH 4·6 with glacial acetic acid and 2·5 ml acetate buffer containing 25,000 units of Beta-glucuronidase added. The mixture is stirred and incubated overnight at 37°C.

The hydrolyzed urine is extracted with 50 ml of benzene. The extract is washed once with sodium hydroxide-saline solution (15 ml) and with water (3 × 10 ml).

After the final wash the extract is filtered through anhydrous sodium sulphate and a 30 ml aliquot is reduced to approximately 10 ml in a "Rotavapor" with a slight negative pressure at 37°C. A column (internal diameter 3 cm) is prepared with 3 g of standardized alumina in benzene. The extract is applied to the column and a wash of 12 ml 3% ethanol in benzene (containing the 11 DKS) is discarded.

The next 6 ml of the eluate contains P_5T. After a pre-wash with 3 ml of the same eluent, PT is collected with 15 ml of 10% ethanol in benzene.

The eluates are evaporated under an air stream at 37°C, the dry residues are dissolved in 0·2 ml of ethanol; 2 ml of 10% metaperiodate is added and the tubes are kept under darkness for 30 min.

The oxidation converts PT into aetiocholanolone and P_5T into dehydroepiandrosterone. The following morning an internal standard of 10 μg of progesterone in 10 ml water is added to each tube.

The oxidized steroids are extracted with dichloromethane. The organic phase is washed twice with 5 ml water, dehydrated with anhydrous sodium sulphate, and evaporated to dryness for solid injection in a Pye, model 14, gas–liquid chromatograph. Oven temperature 24°C; gas flow (N_2) 20 ml/min; column XE-60-2% in Gas–chrom.

This process has a 75% recovery (range of ten assays 54–83%) and its specificity has been established by combined GLC and mass spectrometry.

The lowest assessable quantity of steroid corresponds to about 0·02 mg/24 hr. For purposes of calculations when the quantity of P_5T or PT was not assessable it was arbitrarily taken as 0·01 mg/24 hr. Three patients were excluded from some of the studies because the amounts of P_5T and PT excreted could not be measured.

Spearman's method was applied for statistical investigation of the correlations and regressions. Calculations were made on a timesharing terminal of a GE 265 computer.

RESULTS

1. *Effect of synacthene on 11-DKS/17-OHCS and P_5T/PT ratios*

There is a highly significant linear correlation ($r = 0{\cdot}68$; $P < 0{\cdot}001$) between the basal and synacthene-stimulated ratios of 11-DKS/17-OHCS. High stimulated ratios are found in patients who had high basal ratios but as synacthene leads to the production of proportionately more corticosteroids than androgens, the stimulated ratios are generally lower than the basal ratios by approximately 25% (Fig. 1).

The values of the P_5T/PT ratio before and after synacthene are also highly correlated ($r = 0{\cdot}85$; $P < 0{\cdot}001$), but in this instance the stimulated values are generally some 50% above the basal ratios (Fig. 2).

*Synacthène-retard "Ciba". Kindly provided by Dr. Martinez Pinto, Ciba, Portugal.

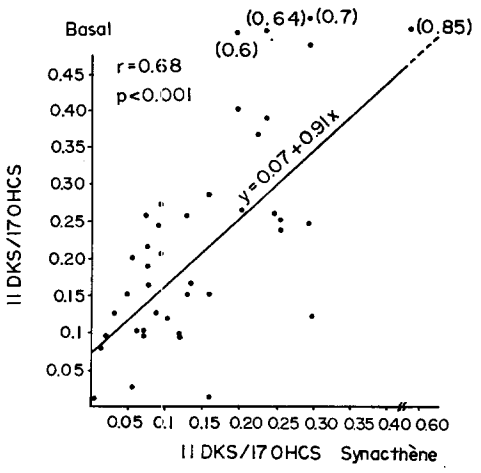


Fig. 1. *Correlation between the ratio of* 11*–DKS*/17*–OHCS before and after adrenal stimulation with synacthene.*

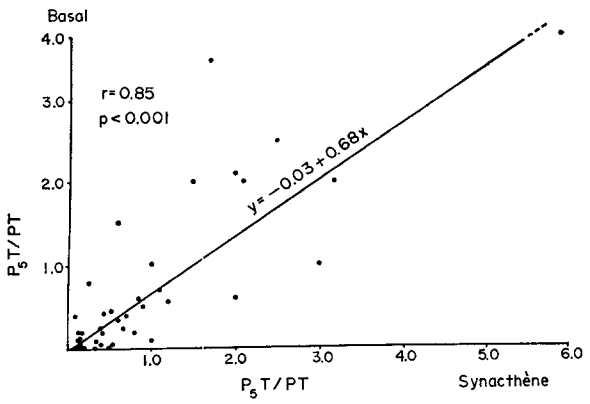


Fig. 2. *Correlation between the ratio of* P_5T/PT *before and after adrenal stimulation with synacthene.*

2. *Correlation between basal ratios of* 11*-DKS*/17*-OHCS and* P_5T/PT

There is a significant linear correlation between the basal values of the ratios 11-DKS/17-OHCS and P_5T/PT ($r=0{\cdot}5$; $P<0{\cdot}05$). In some of the urine specimens obtained from the 41 patients with breast cancer, the titres of the relevant steroids were near the limits of sensitivity of the analytical methods used. Estimates of the basal values were therefore calculated from the synacthene-stimulated values (since these are significantly related). The high titres found in the synacthene-stimulated specimens meant a reduction in measurement error.

A correlation factor (I) was calculated for each variable, defined as:

$$I = \frac{Va - Vb}{Va + Vb}$$

where *Vb* and *Va* are the basal and stimulated values, respectively. An analysis of variance was performed to estimate the "weight" of this correlation in the calculation of basal values from the stimulated values. The estimated vasal value for the 11-DKS/17-OHCS ratio could be calculated from the following function:

11-DKS/17-OHCS (basal) =
$= 1{\cdot}1 \times$ 11-DKS/17-OHCS (stimulated)
$-0{\cdot}32$ (I, 11-DKS) $+0{\cdot}36$ (I, 17-OHCS)

Similarly, for the P_5T/PT ratio, we have:

P_5T/PT (basal) =
$= 0{\cdot}68 + 0{\cdot}68 \times P_5T/PT$ (stimulated)
$-2{\cdot}81$ (I, P_5T) $+2{\cdot}28$ (I, PT)

It was then found that there is a highly significant linear correlation between the calculated basal 11-DKS/17-OHCS and P_5T/PT ratios (Fig. 3).

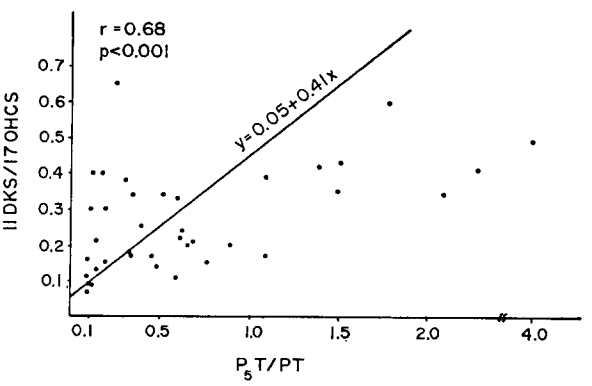


Fig. 3. *Correlation between the "estimated" basal* 11*–DKS/*17*–OHCS and* P_5T/PT *ratio.*

3. *Ratios after adrenal stimulation*

A significant correlation was found between the 11-DKS/17-OHCS and P_5T/PT ratios after adrenal stimulation with synacthene ($r = 0{\cdot}47$; $P < 0{\cdot}05$). Note, however, that stimulation lowers the former and increases the latter ratio. If the two ratios were randomly distributed, stimulation would result in a spurious negative correlation between them. This effect is responsible for some blunting in the correlation which, nevertheless, remains significant.

DISCUSSION

This study was undertaken to assess the validity of the assumption that Bulbrook's discriminant might be related to the effects of endogenous oestrogen secretion on adrenal function. The ratio of 11-DKS/17-OHCS was

compared with the P_5T/PT ratio, which is known to be affected by oestrogen inhibition of adrenal 3β-hydroxysteroid dehydrogenase. No correlation between the ratios could have been predicted on the basis of what is currently known about the biosynthetic and catabolic pathways of steroidogenesis in the adrenal glands. A significant correlation was found between the two ratios in basal conditions, when estimates of basal ratios were made from the synacthene-stimulated values, and when the stimulated values themselves were taken. Our study demonstrates that the 11-DKS/17-OHCS ratio in women with breast cancer is significantly related to the activity of adrenal 3β:hydroxysteroid dehydrogenase.

The mechanism of this dependence is not clear. It has been reported that Bulbrook's discriminant correlates linearly with the ratio of the *in vivo* production rates of dehydroepiandrosterone and cortisol [13]. As oestrogens do not seem to interfere directly with the efficiency of the adrenal 17-desmolase, it is possible that the C_{21} steroids with a $\Delta_5-3\beta$-ol configuration are more easily converted to C_{19} steroids than the corresponding Δ_4-3-one counterparts.

Adrenal stimulation with synacthene altered the proportion of 11-DKS to 17-OHCS and P_5T to PT. In the former case, the excretion of 17-OHCS increased more than that of the 11-DKS: in the latter P_5T increased more than PT. This effect accords with the findings of Bermudez *et al.* [14] who reported that after adrenal stimulation, the plasma concentrations of 17α-hydroxypregnenolone increased 2–3 times as much as those of progesterone, cortisol, and 17α hydroxyprogesterone.

It follows that the predictive value of the discriminant is likely to be affected by any stressful situation (such as hospitalization, mastectomy) in which the adrenals are overactive. In fact, these factors have been shown to affect the discriminant, although the mechanisms involved are probably multiple [14]. It is also realised that a low excretion of 17-oxo-steroids is found in debilitating disease [15] where the peripheral conversion of androgen to "classical" 17-oxosteroids is impaired and where there is an excessive conversion to uncharacterized highly polar metabolites [15].

Notwithstanding these influences, our study shows that the 11-DKS/17-OHCS ratio is related to 3β-hydroxysteroid dehydrogenase activity. This would account for the decrease in the ratio with advancing age which may be a direct consequence of the cessation of ovarian activity following the menopause. The predictive value of the ratio in selecting breast cancer patients for endocrine ablation also becomes understandable. It would seem pointless to remove the adrenals of a patient whose oestrogenic production is already negligible (assuming that regression of the tumour depends upon removal of oestrogenic stimulation).

The fact that the measurement of urinary oestrogens has no predictive value in selecting patients for ablative treatment [16, 17] does not rule out the importance of endogenous oestrogens in the course of the disease. The methods used are very crude, bear an uncertain relationship with circulating oestrogen and reckon only a fraction of total oestrogen metabolites in urine. In fact it has been reported that more direct indicators of oestrogen activity such as bioassay of urinary oestrogens [18, 19] or vaginal cytology [20] do correlate reasonably well with the response to endocrine ablative therapy in breast cancer patients.

Acknowledgements—Thanks are due to Dr. R. D. Bulbrook for his helpful criticism and revision of the manuscript.

REFERENCES

1. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, A comparison between the urinary steroid excretion of normal women and women with advanced breast cancer. *Lancet* **ii,** 1235 (1962).
2. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, Abnormal excretion of urinary steroids by women with early breast cancer. *Lancet* **ii,** 1238 (1962).
3. P. Juret, M. Hayem and A. Flaisler, A propos de 150 implantations d'yttrium radio-actif intrahypophysaire dans le traitement du cancer du sein au stade avancé. *J. Chir. (Paris)* **87,** 409 (1961).
4. S. Kumaoka, N. Sakauchi, O. Abe, M. Kusama and O. Takatani, Urinary 17-ketosteroid excretion of women with advanced breast cancer. *J. clin. Endocr.* **28,** 667 (1968).

5. A. W. Sim, R. Hobkirk, H. J. Stewart, D. W. Blair and A. P. M. Forrest, Urinary 17-ketosteroids and their fractions in women with breast cancer treated by endocrine surgery. *Brit. J. Cancer* **14,** 460 (1960).
6. M. Miller, J. A. Durant, A. G. Jacobs and J. F. Allison Alternative discriminating function for determining hormone dependency of breast cancer. *Brit. med. J.* **i,** 147 (1967).
7. B. S. Thomas, R. D. Bulbrook and J. L. Hayward, Urinary steroid values as a guide to progress in breast cancer. *Brit. med. J.* **i,** 217 (1968).
9. R. D. Bulbrook, J. L. Hayward and C. C. Spicer, Relation between urinary androgen and corticoid excretion and subsequent breast cancer. *Lancet* **ii,** 395 (1971).
10. L. G. Sobrinho, N. G. Kase and J. A. Grunt, Changes in adrenocortical function of patients with gonadal dysgenesis after treatment with estrogen. *J. clin. Endocr.* **33,** 110 (1971).
11. C. H. Gray, B. N. Baron, R. V. Brooks and V. H. T. James, A critical appraisal of a method of estimating urinary 17-oxosteroids and total 17-oxogenic steroids. *Lancet* **i,** 124 (1969).
12. B. S. Thomas, R. D. Bulbrook, J. A. Durant, H. Miller and D. M. Ross, A rapid method for the estimation of urine 11 deoxy-17 oxosteroids and its use in management of patients with breast cancer. *Clin. Biochem.* **2,** 311 (1969).
13. N. Deshpande, V. Jensen, R. D. Bulbrook and T. W. Doouss, *In vivo* steroidogenesis by the human adrenal gland. *Steroids* **9,** 393 (1967).
14. J. A. Bermudez and M. B. Lipsett, Early adrenal response to ACTH: plasma concentrations of pregnenolone, 17 hydroxy pregnenolone, progesterone and 17 hydroxiprogesterone. *J. clin. Endocr.* **34,** 241 (1972).
15. B. Zumoff, H. L. Bradlow, T. F. Gallagher and L. Hellman, Decreased conversion of androgens to normal 17-ketosteroid metabolites: a non-specific consequence of illness. *J. clin. Endocr.* **32,** 824 (1972).
16. R. A. McAllister, A. W. Sim, R. Hobkirk, H. Stewart, D. W. Blair and M. Forrest, Urinary oestrogen after endocrine ablation. *Lancet* **i,** 1102 (1960).
17. W. T. Irvine, E. H. Aitken, D. F. Rendleman and P. J. Folca, Urinary oestrogen measurements after oophorectomy and adrenalectomy for advanced breast cancer. *Lancet* **ii,** 791 (1960).
18. G. E. Block, A. B. Vial, J. D. McCarthy, C. W. Porter and F. A. Coller, Adrenalectomy in advanced breast cancer. *Sur. Gynec. Obstet.* **108,** 651 (1959).
19. C. Huggins, T. L. Y. Dao, Characteristics of adrenal dependent mammary cancers. *Ann. Surg.* **140,** 497 (1954).
20. A. A. Fracchia, H. T. Randall and J. H. Farrow, The results of adrenalectomy in advanced breast cancer in 500 consecutive cancers. *Surg. Gynec. Obstet.* **125,** 747 (1967).

Europ. J. Cancer. Vol. 12, pp. 913–923. Pergamon Press 1976. Printed in Great Britain

Heart Levels of Adriamycin in Normal and Tumor-Bearing Mice*

M. G. DONELLI, A. MARTINI, T. COLOMBO, A. BOSSI and S. GARATTINI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62–20157 Milano, Italy

Abstract—*Adriamycin accumulates in the hearts of mice bearing Lewis lung carcinoma more than in controls. The effect is more evident at longer (25 days) than earlier times (11 days) after tumor transplantation and it is more consistent at high (15 mg/kg i.v.) than at low doses (5 mg/kg i.v.). The effect is probably related to an impaired pulmonary circulation due to the presence of lung metastases but is not related to a different capacity of the heart in control versus tumor bearing animals to take up adriamycin.*

The effect is observed in Lewis lung carcinoma bearing animals is present also in mice bearing a B16 melanoma, but not in $(C_3H \times O_{20})F_1$ mice with a spontaneous mammary carcinoma or in rats transplanted with the Walker carcinosarcoma.

In most experimental conditions adriamycin accumulates less in the lungs of tumor bearing animals than in controls. Like heart and lung, other tissues show concentrations of Adriamycin much higher than in blood, but differences in AM distribution between normal and tumor bearing animals are not so evident.

Daunomycin is similar to adriamycin in this respect.

INTRODUCTION

ADRIAMYCIN (AM), an antitumoral antibiotic isolated from a mutant species of *Streptomyces peucetius*, is a 14-hydroxylated derivative of daunomycin (DM). Like its parent compound, AM is thought to intercalate with DNA and thus prevent cell division [1]. Both of these antibiotics of the anthracycline group have produced positive objective responses in acute leukemia [2, 3]. AM also has been shown to be effective against a wide variety of solid tumors [4–6]. As with other cancer chemotherapeutic agents, toxicity is a limiting factor encountered in AM therapy. Indeed alopecia, stomatitis, myelosuppression, fever, nausea and phlebitis, are some of the toxic effects of AM observed in man [4, 6, 7].

However, the major drawback in the clinical use of this antibiotic is its strong cardiac toxicity which consists of electrocardiographic alterations of a transient nature and congestive heart failure [6–9].

Various studies are currently underway to aid in understanding the mechanisms involved in AM chronic heart toxicity. In the framework of this investigation it was of interest to establish the concentrations of AM in the heart using both normal and tumor bearing animals.

Accepted 25 May 1976.

*This work was supported by NIH Contract NCI–C–72–3242. The gift of adriamycin and daunomycin from Farmitalia, Milan, is gratefully acknowledged.

MATERIAL AND METHODS

Animals and tumors

C57 Bl/6 J male mice (20 ± 2 g body weight) and CD male rats (130 ± 10 g body weight) were obtained from Charles River, Italy, $(C_3H \times O_{20})F_1$ male and female hybrid mice (30 ± 2 g body weight) were obtained from the International Center for the Provision and Study of Tumor Bearing Animals (Amsterdam, Netherlands).

The experimental tumors employed were Lewis lung carcinoma (3LL) implanted intramuscularly in the right limb of C57 Bl mice by injecting 0·1 ml of tumor homogenate diluted 1 :10, the mammary adenocarcinoma obtained in $(C_3H \times O_{20})F_1$ mice by treating castrated males with estrogens or by force-breeding females [10–12] and finally the Walker 256 carcinosarcoma transplanted subcutaneously in rats by means of a trocar (fragments of 100–200 mg tumor tissue).

The animals were kept at standard room temperature (22°C) with relative humidity (60%) and were allowed free access to food and water.

Drugs

AM was contaminated with traces of DM (demonstrable chromatographically) and both AM and DM were contaminated with their respective aglycones and other fluorescent materials.

Solutions of AM and DM were freshly prepared, protected from light, and kept cold or frozen until use. Both drugs were dissolved in water and injected intravenously at doses ranging from 5 to 15 mg/kg.

Biochemical assay of AM and DM

The levels of AM and DM in the heart and other tissues were estimated by modifying the fluorometric assay originally described for DM by Finkel *et al.* [13] which detects this compound in serum after *n*-butyl alcohol extraction without discriminating between AM or DM and its metabolites. The adaptation to tissues was performed by extracting with *n*-butyl alcohol, after deproteinizing with $AgNO_3$ according to Schwartz [14] with slight modifications.

Mice and rats were sacrificed, the tissues removed, rinsed with physiological saline and homogenized with 3 volumes of distilled water, the minimal volume being 1 ml. One millilitre of homogenate was used for each determination. To each sample a volume of 0·3 ml of a solution of 33% $AgNO_3$ in water and 0·25 ml of distilled water were added. The tubes were shaken vigorously for 20 min and centrifuged for 30 min at 1500 rev/min. A 1 ml aliquot of the supernatant was transferred into a 15 ml centrifuge tube containing 3 ml of *n*-butyl alcohol. The mixture was then shaken for 1 min and centrifuged for 3 min at 1500 rev/min.

An aliquot of the organic phase was then removed for measurement of the fluorescent intensity at 470 nm Exc and 560 nm Em (uncorrected wave-length) in an Aminco Bowman spectrofluorometer. It was noted that, if after precipitation of tissue proteins both the supernatant and the precipitate were submitted to organic solvent extraction, as originally described by Schwartz [14], the samples had a brown color and their fluorescent emission was strongly reduced. On the other hand a good drug recovery ($\sim$ 70%) can be obtained from the supernatant alone and the organic phase obtained is colorless and more suitable for fluorometric analysis.

The blank fluorescence was less than 0·1 μg for either AM or DM in 3 ml of *n*-butyl alcohol.

The method was linear in the range of drug concentrations from 1–20 μg/g. The sensitivity of the method was 1 μg/g of tissue. As *n*-butyl alcohol extracted both the injected compound and the metabolic derivatives, the drug concentrations were expressed as AM or DM equivalents. The determination of AM in serum was performed according to the method of Finkel [13]. The sensitivity was 0·5 μg/ml.

Drug levels in isolated atria

C57Bl mice bearing a 25-day-old 3LL tumor and control mice were used for the determination of drug levels in the atria.

The animals were sacrificed, the hearts removed and suspended in 15 ml of physiological salt solution (PSS) with the following composition: NaCl 118 mM, HCl 4·7 mM, $NaHCO_3$ 25 mM, glucose 11·54 mM, $CaCl_2$ 2·51 mM, $MgSO_4$ 1·19 mM, KH_2PO_4 1·19 mM in distilled water.

The bathing solution was kept at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

No attempts were made to separate the atria from the ventricles at this stage, in order to avoid damaging the atria. The whole hearts were allowed to equilibrate for 30 min before AM was added. The hearts were incubated for 30, 60, 120 min in PSS containing 2 μg/ml AM. At the end of each period the hearts were blotted on filter paper, the ventricles dissected and discarded while the atria were weighed and kept frozen (-20°C) until drug levels were determined according to the procedure described in the Methods section.

STATISTICAL ANALYSIS

The areas under the concentration versus time curve (cxt values) were measured by trapezoidal integration according to the method of Coffey [15]. Significant differences between values of normal and tumor bearing animals were assessed by means of the Student's *t* test.

RESULTS

A first series of experiments was carried out by measuring the distribution of AM in C57Bl/6 J mice bearing an intramuscular transplantation of the Lewis lung carcinoma. AM was injected at three different times: 11, 18 and 25 days, after tumor transplant.

These times have been selected because at 11 days after transplantation the primary 3LL

Table 1. *Serum levels of adriamycin (μg/ml ± S.E.) after i.v. injection (15 mg/kg) in C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma*

Time after tumor trans-plantation (days)	1 min	5 min	15 min	30 min	60 min	2 hr	3 h	cxt (120 min) (μg min/ml)
—	28·9 ± 0·7	6·9 ± 1·6	2·0 ± 0·2	1·2 ± 0·1	0·7 ± 0·02	0·5 ± 0·04	< 0·5	214 ± 18
18	21·6 ± 1·8	5·2 ± 0·3	1·5 ± 0·1	2·0 ± 0·1	1·2 ± 0·1	< 0·5	< 0·5	223 ± 16
25	32·6 ± 2·7	7·4 ± 0·9	1·9 ± 0·2	1·6 ± 0·04	0·9 ± 0·2	0·5 ± 0·04	< 0·5	218 ± 26

Table 2. *Heart levels of adriamycin (μg/g ± S.E.) after i.v. injection (15 mg/kg) of C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma*

Time after tumor trans-plantation (days)	Weight of primary tumor (g ± S.E.)	Weight of the heart (mg ± S.E.)	1 hr	3 hr	8 hr	24 hr	48 hr
—	—	94 ± 3	39 ± 4	37 ± 4	21 ± 1	9 ± 1	6 ± 0·5
11	1·7 ± 0·3	93 ± 3	39 ± 2	31 ± 1	22 ± 3	8 ± 1	7 ± 1
—	—	98 ± 4	39 ± 1	31 ± 2	14 ± 1	5 ± 1	2 ± 0·5
18	3·5 ± 0·4	99 ± 2	36 ± 1	29 ± 3	20 ± 1*	6 ± 1	3 ± 0·2
—	—	120 ± 4	39 ± 4	30 ± 2	16 ± 1	7 ± 0·5	2 ± 0·1
25	6·8 ± 0·3	96 ± 7*	33 ± 1	31 ± 0·2	24 ± 1**	10 ± 0·7**	5 ± 0·5**

Each figure is the average of 4 determinations.
*$P < 0·05$ **$P < 0·01$ vs respective controls.

is in the logarithmic phase of growth and cancer cells have already disseminated to the lung; at 18 days the first nodules are present in the lung while at 25 days the primary tumor has attained its maximal growth and the metastatic nodules in the lung are 20–25. The survival time of these mice transplanted with 3LL is 30·2 ± 1·7 days.

Table 1 shows that an intravenous injection of AM (15 mg/kg) disappears very rapidly from the serum of mice. No consistent difference has been observed in the serum concentrations of AM between normal and tumor bearing mice. Also the area described by the curve (cxt) is comparable for each group of animals. The serum level was studied only in the 18 and 25 day groups when the tumor had reached a weight of at least 3 g.

The concentration of AM in the heart of the same experimental groups are reported in Table 2. It is evident that AM accumulates in the heart like in other tissues as these concentrations are about 40 times higher than the serum levels 1 hr after AM administration. The levels of AM are persistent with time and were still measurable 48 hr after AM injections. Due to the variability of results in the determinations of heart AM, among different experiments, parallel controls were used for the three experimental 3LL groups.

While 11 days after 3LL transplant the levels and disappearance of AM from the heart are comparable to control animals, at 18 days and, more consistently at 25 days, heart concentrations of AM are higher in the 3LL group with respect to controls. The difference is, however, present only at late times beginning 8 hr after AM injection.

It was therefore of interest to observe AM distribution in other tissues. Table 3 shows a decrease of AM concentrations in the lung, particularly at 25 days after 3LL transplant, in tumor-bearing animals with respect to controls. It should be noted that at this time the lung metastases represented over 50% of the weight of the lung.

Table 4 extends and completes the significance of these findings by reporting the cxt values obtained in the heart and lung with three different doses of AM. As expected the

Table 3. Lung levels of adriamycin (μg/g ± S.E.) after i.v. injection (15 mg/kg) in C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma

Time after tumor trans-plantation (days)	Weight of primary tumor (g ± S.E.)	Weight of lung* (mg ± S.E.)	1 hr	3 hr	8 hr	24 hr	48 hr
—	—	188 ± 11	33 ± 5	45 ± 3	23 ± 3	19 ± 2	9 ± 1
11	1·7 ± 0·3	192 ± 5	40 ± 5	42 ± 3	25 ± 3	15 ± 1†	13 ± 1†
—	—	242 ± 16	33 ± 4	29 ± 2	17 ± 2	8 ± 2	5 ± 2
18	3·5 ± 0·4	277 ± 11	26 ± 3	20 ± 2†	17 ± 3	8 ± 1	7 ± 1
—	—	221 ± 15	39 ± 16	44 ± 2	32 ± 8	16 ± 2	9 ± 1
25	6·8 ± 0·3	334 ± 24‡	36 ± 1	14 ± 1‡	16 ± 7‡	7 ± 1‡	11 ± 4

*Including metastases—the weight of the visible metastases was 186 ± 15 mg at 25 days after tumor transplantation
†$P < 0·05$ ‡$P < 0·01$ vs respective controls.
Each figure is the average of 4 determinations.

Table 4. Heart and lung levels of adriamycin after different doses in C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma

Dose of AM (mg/kg, i.v.)	Exper. group	Tissue	cxt (μg min/g) 24 hr	cxt (μg min/g) 48 hr
5	Normal	Heart	4382 ± 300	—
5	3LL	Heart	5076 ± 357	—
10	Normal	Heart	10014 ± 815	13074 ± 1175
10	3LL	Heart	13792 ± 1892	17529 ± 2748
15	Normal	Heart	23652 ± 1809	30780 ± 2241
15	3LL	Heart	29631 ± 1008	40647 ± 1994*
5	Normal	Lung	11485 ± 818	18066 ± 1207
5	3LL	Lung†	7370 ± 1017*	10696 ± 1521*
10	Normal	Lung	20352 ± 2987	28862 ± 4124
10	3LL	Lung†	13692 ± 1437	22317 ± 2351
15	Normal	Lung	40296 ± 8421	57936 ± 10725
15	3LL	Lung†	19398 ± 4815*	32430 ± 7983*

Each figure is the average of 4 determinations carried out after 1, 3, 8, 24 and 48 hr after AM injection.
†Including metastases—the tumor was transplanted 25 days before the experiment.
*$P < 0·05$ vs controls.

Table 5. Tissue levels of adriamycin (μg/g ± S.E.) after intravenous injection (15 mg/kg) in C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma (25 days after transplantation)

Exper. group	Tissue	Weight (g ± S.E.)	30 min	1 hr	3 hr	8 hr	24 hr
Normal	Liver	1·311 ± 0·03	82 ± 1	82 ± 3	60 ± 4	47 ± 2	16 ± 2
3LL	Liver	1·454 ± 0·052	83 ± 1	77 ± 14	60 ± 4	43 ± 6	25 ± 2*
Normal	Kidney	0·323 ± 0·006	71 ± 3	68 ± 5	42 ± 7	32 ± 3	15 ± 1
3LL	Kidney	0·290 ± 0·011	68 ± 14	78 ± 11	43 ± 8	67 ± 9*	20 ± 2
Normal	Spleen	0·074 ± 0·003	21 ± 2	22 ± 2	28 ± 1	33 ± 2	n.d.
3LL	Spleen	0·368 ± 0·019**	22 ± 2	18 ± 3	29 ± 3	25 ± 2	n.d.
Normal	Intestine	0·360 ± 0·009	39 ± 5	21 ± 1	22 ± 1	15 ± 1	5 ± 0·2
3LL	Intestine	0·322 ± 0·021	40 ± 11	30 ± 4	24 ± 5	32 ± 6*	8 ± 1

Each figure is the average of 4 determinations.
*$P < 0·05$ **$P < 0·01$ vs respective controls.
n.d. = not determined.

cxt values increase with the dose for both tissues. In any case the values for the heart are higher for the group of animals transplanted 25 days earlier with 3LL in respect to the control group. However, only for the highest dose of AM does this difference reach statistical significance.

For the lung concentrations there is a definite decrease of the cxt values in the tumor bearing with respect to the control group at each dose of AM.

The results obtained in other experiments showing the levels of AM in liver, kidney, spleen and intestine of animals bearing a 25 day old 3LL tumor with respect to controls, are reported in Table 5.

It should be noted that the AM concentrations in the liver and kidney are considerably higher while the concentrations in the spleen and intestine are lower than the values in the heart and lung (see Tables 2 and 3) at corresponding times.

Except for occasional differences no definite trends were observed to differentiate the levels of AM in the liver, kidney, spleen and intestine in tumor bearing versus control animals.

Having observed that AM administration to 3LL bearing mice results in higher heart AM concentrations and lower lung levels with respect to controls it was decided to investigate whether these differences could also be detected in animals bearing other experimental tumors.

The B16 melanoma shows a pattern similar to the 3LL. In fact the levels of heart AM are consistently higher in tumor bearing than in control animals (Table 6). Different results were obtained with the other two experimental tumors because the heart of mice bearing a spontaneous mammary carcinoma showed, if any, lower levels of AM and the heart of rats transplanted with Walker carcinosarcoma showed no differences with respect to the relative controls (Table 6). It should be noted that the same dose of AM resulted in higher heart concentrations in CD rats with respect to mice.

As far as the lung concentrations are concerned, a decrease in tumor bearing animals with respect to controls was observed for the mammary carcinoma and also for the B16 melanoma although less marked, while no difference was detected for the Walker carcinosarcoma (Table 7). With the same dose of antibiotic the AM concentrations in the lung were highest for the $(C_3H \times O_{20})F_1$ mice followed by the CD rats and the C57Bl mice.

The last part of this study was devoted to investigating possible reasons for the increase of AM concentrations in the hearts of 3LL bearing mice. Table 8 shows that the presence of the primary tumor is not necessary for the observed effect. In fact higher levels of AM were detected in the hearts of mice where lung metastases were induced by an i.v. injection of 3LL cancer cells. Also in this experimental condition the levels of lung AM were lower in tumor bearing than in control animals (Table 8).

To establish if the heart by itself was different in its capacity to accumulate AM, because of the presence of a tumor, hearts obtained from normal or 3LL bearing mice were incubated *in vitro* in the presence of AM (2 μg/ml). Table 9 shows that no difference could be detected when the determinations of AM were performed in correspondingly isolated atria.

Finally Table 10 reports the distribution of the AM analogue daunomycin in the heart and lung. Also this drug accumulates more in the heart and less in the lung of 3LL bearing mice with respect to controls.

DISCUSSION

This study confirms and extends previous investigations showing that the distribution and/or the metabolism of several drugs is affected by the presence of a neoplastic formation [16, 17] either primary or secondary. These findings indicate that the pharmacokinetics and metabolism of antitumoral drugs studied in normal animals cannot be assumed to be the same as in tumor bearing animals.

The present results show that the accumulation of adriamycin in the heart of mice transplanted with Lewis lung carcinoma is greater than in normal animals. This effect is more evident at high (15 mg/kg, i.v.) than low (5 mg/kg i.v.) doses of AM and it is shared by the analogue daunomycin. Furthermore, it is dependent on the time of tumor transplantation, being more consistent at late (25 days) than early (11 days) times. However, this behavior of AM cannot be generalized to all experimental tumors because, while it is present in mice bearing melanoma B16, it is not evident in mice bearing a spontaneous mammary carcinoma and in rats transplanted with the Walker carcinoma.

The analyses of the factors responsible for the higher heart levels of AM in 3LL-bearing mice in respect to control mice has proven that most of the other tissues do not share a similar pattern. Particularly, in all experimental tumors investigated, a marked decrease of lung AM concentrations is present at various

Table 6. Heart levels of adriamycin (μg/g ± S.E.) after i.v. injection (15 mg/kg) in animals bearing various types of tumors

Animal strain	Tumor	Tumor weight (g ± S.E.)	Heart weight (mg ± S.E.)	30 min	1 hr	3 hr	8 hr	24 hr	48 hr	cxt (48 hr) (μg min/g)
C57Bl/6J[a]	—	—	104 ± 2	25 ± 2	24 ± 3	17 ± 1	11 ± 1	4 ± 0·4	2 ± 0·2	15811 ± 1801
C57Bl/6J	Melanoma B16	5·3 ± 0·4	89 ± 2	27 ± 4	28 ± 3	27 ± 6*	16 ± 1†	6 ± 0·7*	4 ± 0·3†	29156 ± 3315*
$(C_3H \times O_{20})F_1$[a]	—	—	109 ± 3	56 ± 6	47 ± 4	37 ± 0·1	20 ± 2	7 ± 0·4	3 ± 0·2	36495 ± 2409
$(C_3H \times O_{20})F_1$	Mammary carcinoma	10·8 ± 1·5	132 ± 3	45 ± 5	44 ± 4	32 ± 1	16 ± 2	5 ± 0·8*	9 ± 0·4	29394 ± 2934
CD[b]	—	—	679 ± 17	51 ± 2	44 ± 3	34 ± 4	29 ± 1	13 ± 1	9 ± 1	52549 ± 3495
CD	Walker carcinosarcoma	13·9 ± 0·9	658 ± 20	41 ± 2	44 ± 4	35 ± 6	27 ± 1	13 ± 3	9 ± 1	50871 ± 6681

*$P < 0.05$ †$P < 0.01$ vs respective controls [a] = mice; [b] = rats

Table 7. Lung levels of adriamycin (μg/g ± S.E.) after i.v. injection (15 mg/kg) in animals bearing various types of tumor

Animal strain	Tumor	Tumor weight (g ± S.E.)	Lung weight (mg ± S.E.)	30 min	1 hr	3 hr	8 hr	24 hr	48 hr	cxt (48 hr) (μg min/g)
C57Bl/6J[a]	—	—	195 ± 7	35 ± 2	30 ± 1	26 ± 2	24 ± 2	12 ± 1	6 ± 0·5	42401 ± 3515
C57Bl/6J	Melanoma B16	5·3 ± 0·4	160 ± 4	26 ± 3*	40 ± 5	34 ± 7	16 ± 2*	15 ± 1	9 ± 0·7*	45393 ± 5374
$(C_3H \times O_{20})F_1$[a]	—	—	162 ± 4	122 ± 6	106 ± 7	84 ± 8	69 ± 8	32 ± 1	16 ± 2	122815 ± 9879
$(C_3H \times O_{20})F_1$	Mammary carcinoma	10·8 ± 1·5	252 ± 14†	61 ± 10†	68 ± 11†	42 ± 7†	43 ± 2*	22 ± 1†	16 ± 5	80226 ± 8185*
CD[b]	—	—	1134 ± 36	88 ± 6	68 ± 6	42 ± 11	40 ± 8	28 ± 4	20 ± 2	90033 ± 14629
CD	Walker carcinosarcoma	13·9 ± 0·9	1386 ± 80	54 ± 3*	57 ± 5	38 ± 14	32 ± 5	20 ± 3	18 ± 2	71998 ± 12562

*$P < 0.05$ †$P < 0.01$ vs respective controls [a] = mice; [b] = rats.

Table 8. *Heart and lung levels of adriamycin* ($\mu g/g \pm$ *S.E.*) *after i.v. injection* (15 *mg/kg*) *in C57Bl mice normal or bearing lung metastases* 18 *days after intravenous injection of* 2×10^5 *Lewis lung carcinoma cells*

Experimental group	Tissue	Weight (mg ± S.E.)	30 min	1 hr	3 hr	8 hr	24 hr	48 hr	cxt (48 hr) (μg min/g)
Normal	Heart	100 ± 4	48 ± 5	41 ± 3	31 ± 3	20 ± 2	6 ± 0·6	3 ± 0·4	31555 ± 3228
Lung metastases	Heart	105 ± 5	48 ± 4	36 ± 5	32 ± 3	28 ± 2*	9 ± 0·3*	3 ± 1	40833 ± 3445
Normal	Lung	176 ± 12	56 ± 6	44 ± 4	35 ± 2	28 ± 2	19 ± 0·7	9 ± 1	58320 ± 3417
Lung metastases	Lung	423 ± 38†	26 ± 2†	19 ± 3†	22 ± 2†	16 ± 1†	10 ± 2†	4 ± 0·4†	32049 ± 4099†

Each figure is the average of 4 determinations.
*$P < 0{\cdot}05$ †$P < 0{\cdot}01$ vs respective controls.

Table 9. Adriamycin accumulation in isolated hearts obtained from C57Bl mice—normal or bearing a 25-day-old Lewis lung carcinoma transplanted intramuscularly

		30 min	1 hr	2 hr
Atria	Normal	9	15	18
		(7–10)	(14–15)	(12–23)
	3LL	8	15	19
		(7–8)	(15–16)	(15–23)

Adriamycin was added at the concentration of 2 μg/ml of PSS (pH 7·4). The figures are the averages of 2 pools, each formed by 2 atria.

times after the i.v. administration of different doses of AM. This difference may possibly be explained by the presence of metastatic nodules, which are evident in 3LL tumor-bearing mice [18]. As regards the other tumors. the Walker-carcinosarcoma of the rat showed only occasional metastatic nodules but almost 30% of the mice bearing mammary tumors presented large microscopic metastases to the lung; for the B16 melanoma, however, neither the weight of the lung, nor the microscopic observation in our experimental conditions could show the presence of the lung metastases described in the literature [19]. Unpublished results from this laboratory indicate that the concentration of AM in lung metastases, although higher than in the primary 3LL tumor, are lower than in the pulmonary tissue. It seems therefore possible to link the increased levels of AM in the heart with the decreased concentrations of AM in the lung. A conceivable explanation is that the widespread occlusion of the pulmonary arterioles by tumor emboli, or arteriolar constriction resulting from lymphatic permeation may bring about cardiac failure of the right heart by raising the pulmonary blood pressure [20, 21]. As a consequence, while the blood supply to the lungs is reduced, there is a relative stagnation of the blood in the heart which in turn permits a larger accumulation of AM in the myocardial tissue.

That the presence of a primary tumor is not essential for the observed effect is indicated by the fact that higher levels of AM in the heart have been observed also in mice bearing lung metastases without a primary tumor. The heart obtained from normal or tumor bearing animals does not show, per se, a difference in capacity to take up AM as shown by *in vitro* experiments.

Other factors may play a role as for instance previous studies have shown that liver microsomal enzymes of tumor bearing animals are less active than those of controls [16, 22]. An impairment of AM metabolism however should result in higher serum levels of AM in tumor bearing versus normal mice, a finding which was not detected in the present study. A firm conclusion in this respect, however, should be drawn only after the separate measurement of AM and its possible metabolites, which have been investigated in previous papers [23–25].

The significance of the increased accumulation of AM in the heart of tumor bearing animals in explaining the cardiotoxicity of AM needs further elucidation. It may be suggested, however, that certain clinical situations which are comparable to the experimental tumors utilized here, such as the existence of pulmonary metastases, may facilitate the accumulation of AM in the heart and therefore the appearance of AM induced heart failure. The knowledge of the factors which may influence the onset of AM cardiotoxicity should permit better utilization of AM.

Acknowledgements—The authors wish to thank Mr. Massimo Broggini and Miss Mariangela Mainini for the skilful technical assistance and the student Brunella Barbieri for the collaboration.

Table 10. *Heart and lung levels of daunomycin (μg/g ± S.E.) after injection (15 mg/kg i.v.) in C57Bl mice—normal or bearing intramuscular Lewis lung carcinoma*

Experimental group	Tissue	5 min	15 min	30 min	60 min	3 hr	8 hr	24 hr	cxt (24 hr) (μg min/g)
Normal	Heart	44 ± 1	41 ± 4	40 ± 2	28 ± 1	21 ± 2	8 ± 1	2 ± 0·2	14771 ± 1023
3LL	Heart	46 ± 8	48 ± 6	51 ± 10	35 ± 2*	29 ± 3*	13 ± 1*	4 ± 0·4*	20781 ± 2155*
Normal	Lung	101 ± 13	68 ± 4	80 ± 4	51 ± 3	33 ± 5	19 ± 3	5 ± 0·6	28645 ± 3752
3LL	Lung	72 ± 6	43 ± 10	51 ± 6*	23 ± 2†	25 ± 0·9	22 ± 1	9 ± 0·5*	27313 ± 1463

3LL tumors were transplanted intramuscularly 25 days before daunomycin injection.
The weight of the heart in controls was 110 ± 2 mg; in tumor bearing animals 103 ± 2 mg.
The weight of the lung in controls was 166 ± 7 mg; in tumor bearing animals 332 ± 17 mg including metastases.
The weight of the primary tumor was 7·6 ± 0·2 g.
*$P < 0.05$ †$P < 0.01$ vs respective controls.

REFERENCES

1. A. Di Marco, F. Zunino, R. Silvestrini, C. Gambarucci and R. A. Gambetta, Interaction of some daunomycin derivatives with deoxyribonucleic acid and their biological activity. *Biochem. Pharmacol.* **20,** 1323 (1971).
2. J. Bernard, Acute leukemia treatment. *Cancer Res.* **27,** 2565 (1967).
3. E. P. Cortes, R. R. Ellison and J. W. Yates, Adriamycin (NSC-123127) in the treatment of acute myelocytic leukemia. *Cancer Chemother. Rep.* **56,** 237 (1972).
4. G. Bonadonna, S. Monfardini, M. De Lena, F. Fossati-Bellani and G. Beretta, Phase I and preliminary phase II evaluation of adriamycin (NSC 123127). *Cancer Res.* **30,** 2572 (1970).
5. J. A. Gottlieb, C. S. Hill, M. L. Ibanez and R. L. Clark, Chemotherapy of thyroid cancer. *Cancer (Philad.)* **30,** 848 (1972).
6. E. Middleman, J. Luce and E. Frei III, Clinical trials with adriamycin. *Cancer (Philad.)* **28,** 844 (1971).
7. J. J. Wang, E. Cortes, L. F. Sinks and J. F. Holland, Therapeutic effect and toxicity of adriamycin in patients with neoplastic disease. *Cancer (Philad.)* **28,** 837 (1971).
8. G. Bonadonna, M. De Lena and G. Beretta, Preliminary clinical screening with adriamycin in lung cancer. *Europ. J. Cancer* **7,** 365 (1971).
9. J. A. Gottlieb, E. A. Lefrak and R. M. O'Bryan, Fatal adriamycin cardiopathy (CMY): prevention by dose limitation. *Proc. Amer. Ass. Cancer Res.* **14,** 88 (1973).
10. O. Mühlbock, Biological studies on the mammary tumor agent in different strains of mice. *Acta Un. int. Cancr.* **12,** 665 (1956).
11. J. Gelzer and P. Loustalot, 1967, Biological and chemotherapeutic studies on primary tumors of C_3H and C_3HO mice. *Int. J. Cancer* **2,** 179 (1967).
12. W. G. Hoag, Spontaneous cancer in mice. *Ann. N.Y. Acad. Sci.* **108,** 805 (1963).
13. J. M. Finkel, K. T. Knapp and L. T. Mulligan, Fluorometric determination of serum levels and urinary excretion of daunomycin (NSC-82151) in mice and rats. *Cancer Chemother. Rep.* **53,** 159 (1969).
14. H. S. Schwartz, A fluorometric assay for daunomycin and adriamycin in animal tissue. *Biochem. Med.* **7,** 396 (1973).
15. J. J. Coffey, P. E. Palm, E. P. Denine, P. E. Baronowsky and C. J. Kensler, Species differences in the physiological disposition of 3-tritylthio-L-alanine (NSC 83265). *Cancer Res.* **31,** 1908 (1971).
16. R. Rosso, M. G. Donelli, G. Franchi and S. Garattini, Impairment of drug metabolism in tumor bearing animals. *Europ. J. Cancer* **7,** 565 (1971).
17. R. Kato, A. Takanaka and T. Oshima, Drug metabolism in tumor-bearing rats. II. *In vivo* metabolisms and effects of drugs in tumor-bearing rats. *Jap. J. Pharmacol.* **18,** 245 (1968).
18. H. Wexler, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).
19. I. J. Fidler, Inhibition of pulmonary metastasis by intravenous injection of specifically activated macrophages. *Cancer Res.* **34,** 1074 (1974).
20. E. B. Greenspan, Carcinostatic endarteritis of the pulmonary vessels resulting in failure of the right ventricle. *Arch. intern. Med.* **54,** 625 (1934).
21. O. Storstein, Circulatory failure in metastatic carcinoma of the lung. *Circulation* **4,** 913 (1951).
22. R. Kato, A. Takanaka, A. Takahashi and K. Onoda, Drug metabolism in tumor-bearing rats. I. Activities of NADPH-linked electron transport and drug metabolizing enzyme systems in liver microsomes of tumor-bearing rats. *Jap. J. Pharmacol.* **18,** 224 (1968).
23. R. Rosso, C. Ravazzoni, M. Esposito, R. Sala and L. Santi, Plasma and urinary levels of adriamycin in man. *Europ. J. Cancer* **8,** 455 (1972).
24. M. A. Asbell, E. Schwartzbach, F. J. Bullock and D. W. Yesair, Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. *J. Pharmacol. exp. Ther.* **182,** 63 (1972).

25. D. W. YESAIR, E. SCHWARTZBACH, D. SHUCK, E. P. DENINE and M. A. ASBELL, Comparative pharmacokinetics of daunomycin and adriamycin in several animal species. *Cancer Res.* **32,** 1177 (1972).
26. N. R. BACHUR, R. C. HILDBRAND and J. S. JAENKE, Adriamycin and daunorubicin disposition in the rabbit. *J. Pharmacol. exp. Ther.* **191,** 331 (1974).

Europ. J. Cancer Vol. 12, pp. 925–931. Pergamon Press 1976. Printed in Great Britain

Delayed Toxicity of Epipodophyllotoxin Derivatives (VM 26 and VP 16–213), Due to a Local Effect

H. STÄHELIN

Biological and Medical Research Division, Sandoz Ltd., Basel, Switzerland

Abstract—*Our experimental results confirm previous observations that the epipodophyllotoxin derivative VM* 26 *causes delayed toxicity (with death) more than* 23 *days after three i.p. injections in mice, and that the liver seems to be involved in this late mortality. The congener of VM* 26, *VP* 16-213, *was found to have a similar effect, and late mortality was also observed in rats, both after i.p. and after intrapleural injection. One of the main characteristics of the process is the interval between drug administration and the delayed onset of wasting and consequent death. Animals dying show chronic peritonitis or pleurisy, with corresponding changes in the liver or lungs. The pathogenesis of the process is not clear, but it occurs only after i.p. or intrapleural administration. No delayed toxicity was observed in mice when the drugs were given i.v. or orally, nor in* 26*-week toxicity studies where dogs and monkeys were treated i.v. with VM* 26, *and rats and dogs received VP* 16-213 *orally. The typical feature of i.p. and intrapleural-related delayed toxicity (i.e., onset with progression after stopping treatment) was not observed during recovery periods of up to* 9 *weeks after i.v. or oral treatment. While these results support the recommendation not to use these drugs in man by the intraperitoneal or intrapleural route or to inject them into the cerebrospinal fluid, they do not support the contention that in clinical practice such a delayed toxicity may occur when the usual routes of administration (i.v. for VM* 26, *i.v. and p.o. for VP* 16-213) *are employed.*

INTRODUCTION

VM 26 (4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside) and VP 16-213 (4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside) are semisynthetic derivatives of podophyllotoxin. In their therapeutic studies, Avery, Roberts and Price [1] observed an delayed toxicity in mice cured of leukemia by intraperitoneal (i.p.) treatment with VM 26. Mice which had received i.p. injections of 6–16 mg/kg of VM 26 began to die from day 50 (after tumor inoculation) on; this occurred both in inoculated (and cured) mice as well as in mice which had received only drug, but no tumor cells. Mice died from this delayed toxicity up to 267 days after drug administration. Avery *et al.* also noted chemical peritonitis and ascites formation in the mice treated by the intraperitoneal route. No such effects and no delayed deaths were observed in mice treated subcutaneously or intramuscularly. This interesting observation raises questions of theoretical and practical importance. More extensive experiments on delayed toxicity after i.p. administration of VM 26 in mice have recently been published by Hacker and Roberts [2]. To enlarge on Avery's *et al.* findings, we did a number of experiments to find out whether such delayed lethality could also occur with VP 16-213, whether it could occur after intravenous or oral treatment (the routes used in man), and whether it could be elicited in the rat after i.p. or intrapleural treatment. We use the term delayed toxicity to express a (usually fatal) toxicity which develops late, after drug treatment, and which is apparently not due to the more acute effect of the compounds on proliferating tissues, especially the bone marrow.

Accepted 24 May 1976.

MATERIAL AND METHODS

(a) Drugs

Both drugs, VM 26 (NSC-122819) and VP 16-213 (NSC-141540), were prepared in the chemical laboratories of Sandoz Ltd., Basel. They were brought into solution or suspension in different ways (see Tables). All dosage groups, including the controls, received the same amount of solvents.

(b) Animals

The mice and rats used were raised under SPF conditions; during the experiment they were housed under conventional conditions and received Nafag pellets and water *ad libitum*. They were 6–8 weeks old at the beginning of the experiments.

The strain, number and sex of animals used in the different experiments are given in the respective table.

RESULTS

When VM 26 was given i.p. to mice thrice at 3-day intervals, there were almost no early deaths (before day 31) up to a dose of 16 mg/kg, but a large number of animals succumbed later (Table 1). At doses of 9·6 and 16 mg/kg, over 80% of the mice died from delayed toxicity. No late deaths occurred after intravenous (i.v.) application. In rats also, VM 26 produced late deaths after i.p. administration (Table 1). At a dose of 8·8 mg/kg i.p., about 60% died more than 30 days after the first of three injections.

Similar experiments were also done using the other epipodophyllotoxin derivative of clinical interest, VP 16-213. This compound, being also active by the oral route, was tested using administration by gavage as well as by i.p. and i.v. injection (Table 2). Where VP 16-213 was given on days 1, 4 and 7 to mice by the i.p. route, the highest dosage was still below the LD_{50}, and therefore only a comparatively small number of animals died, all of them later than 23 days after stopping treatment. The same schedule, but using the i.v. route, produced 25% and 92% deaths with the two higher dosages, respectively, all occurring early. The death of the one mouse which died late (day 137) after i.v. administration of 17 mg/kg was considered not to be drug-related (see below).

Table 1. *Early and delayed deaths of mice and rats after* 3 *injections of VM* 26

Animals	Treatment days	Route	Dose mg/kg/inj.	Animals dying days 1–30	Animals dying days 31–153	Survivors day 153
MD ♀	1, 4, 7,	i.p.	solvent	0	0	12/12
			3·5	0	0	12/12
			5·8	0	3	9/12
			9·6	1	10	1/12
			16	0	10	1/11
		i.v.	solvent	0	0	12/12
			3·5	0	0	12/12
			5·8	0	0	12/12
			9·6	1	0	10/11
			16	11	0	1/12
Wistar rat ♀	1, 5, 8,	i.p.	solvent	0	1	23/24
			3·0	0	0	24/24
			5·1	2	9	13/24
			8·8	7	14	3/24
			15	22	2	0/24

MD = $(MF_2 \times DBA/2)F_1$ mice.
In those groups of mice in which a total of only 11 is indicated, one mouse escaped during the experiment.
For mice, VM 26 was dissolved in DMSO and Tween 80 and further diluted with water to yield an injection volume of 20 ml/kg. In rats, for half of the animals the formulation was the same as for mice, for the other half the commercial ampule solution was used; both were further diluted with water to yield an injection volume of 10 ml/kg.

Table 2. Early and delayed deaths of mice and rats treated with VP 16-213

Animal	Treatment days	Route	Dose mg/kg/inj.	Animals dying days 1–30	Animals dying days 31–153	Survivors day 153
MD ♂	1, 4, 7	i.p.	solvent	0	0	12/12
			10	0	0	12/12
			17	0	0	12/12
			29	0	1	11/12
			50	0	5	7/12
		i.v.	solvent	0	0	12/12
			10	0	0	12/12
			17	0	1	11/12
			29	3	0	9/12
			50	11	0	1/12
BDF_1 ♀	1–6 and 8–13	i.p.	solvent	0	0	12/12
			5·0	2	0	10/12
			7·2	2	0	10/12
			10	2	7	3/12
			15	8	4	0/12
	1, 4, 8	p.o.	solvent	0	0	24/24
			100	3	0	21/24
			200	10	0	14/24
			400	14	0	10/24
Wistar rat ♀	1, 5, 8	i.p.	solvent	0	0	24/24
			6·0	0	0	24/24
			10	0	2	22/24
			17	0	13	11/24
			30	9	14	1/24
	1	i. plr.	solvent	0	0	12/12
			12	0	0	12/12
			25	0	8	4/12
			50	3	7	2/12

MD = $(MF_2 \times DBA/2)F_1$ mice. BDF_1 = $(C57/BL \times DBA/2)F_1$ mice.
For experiments with MD mice, VP 16–213 was dissolved with DMSO and Tween 80, for i.p. injection to BDF_1 mice by the use of polyethylene glycol, Tween 80 and ethanol (ampule solution), further diluted with water to yield injection volumes of 20 ml/kg. For oral administration, half of the mice received the ampule solution diluted with water to yield volumes of 10 ml/kg, for the other half of the mice VP 16–213 was given as suspension in a mixture of Miglyol 812, bee's wax and soya lecithin, diluted with vehicle to yield volumes of 10 ml/kg. I.p. administration in rats was with ampule solution or with drug dissolved in DMSO and Tween 80, each for half of the animals, diluted with water to injection volumes of 10 ml/kg. For intrapleural injection, VP 16–213 was dissolved in DMSO and Tween 80 and diluted with Hanks' solution to a volume of 5 ml/kg.

When VP 16-213 was given to mice daily (except Sundays) for two weeks by the intraperitoneal route, there was also some delayed mortality with the two higher doses (10 and 15 mg/kg) (Table 2). Oral administration to mice on days 1, 4 and 8, on the other hand, did not give rise to late deaths, neither with doses above or below the calculated LD_{50} for this experiment. The figures in Table 2 for oral administration in BDF-mice are from two separate experiments with 12 animals per dose in each; two different formulations were used in these tests, and in both of them all deaths occurred between day 3 and 18 after the first dosage; in both experiments, the mice were observed up to day 240.

In Table 2 results are also given for rats treated by the intraperitoneal and intrapleural

(i.plr.) route with VP 16-213. Intraperitoneal administration produced considerable late toxicity in this species as well, even in doses which did not elicit early mortality (as was the case in mice, both with VM 26 and VP 16-213). With the two higher doses used (17 and 30 mg/kg), over half the rats exhibited delayed mortality. A single i.plr. injection led to delayed death of more than 55 % of the animals treated with 25 or 50 mg/kg, while 12 mg/kg produced no mortality at all, and 50 mg/kg caused some early deaths (Table 2). Animals dying from a single intrapleural injection of 25 mg/kg had a median survival time of 69·5 days, the range being 47 to 131 days.

Delayed mortality occurred also in mice which were cured of leukemia L-1210 by i.p. treatment; this was observed in 13 animals inoculated with 10^5 L-1210 cells i.p. and treated with VM 26 on days 1, 4 and 7, and in 19 mice treated i.p. with VP 16-213 in the same way. In contrast to this, of 20 BDF_1 mice which had been inoculated subcutaneously (s.c.) with 10^6 L-1210 cells and were cured by i.v. treatment with VM 26, none died during the observation period of 153 days; these mice had received maximal tolerated doses, 3–4 injections within 6–12 days. Here, death was considered to be late only when it occurred more than 50 days after tumor inoculation (these results are not given in the tables).

In most experiments, there was a clear separation between early and late deaths. Thus, in the tests where the drugs were given to mice thrice by the i.p. route (Tables 1 and 2), none of the animals died between day 13 and 30. In rats treated with VM 26 i.p. (Table 1), and in rats treated with VP 16-213 i.plr. (Table 2), this separation was less clear.

As already noted by Avery *et al.* [1], there is an inverse relationship between dose and survival time in the animals dying from delayed toxicity. Thus in mice treated thrice i.p. with VM 26 (Table 1), at 5·8 mg/kg all 3 deaths were recorded between days 101 and 152; at 9·6 mg/kg, all 10 animals dying late died between days 51 and 100; and at 16 mg/kg, 8 of the 10 late deaths were registered between days 30 and 50.

Necropsy of animals dying of delayed toxicity with both drugs revealed, as the most prominent feature, severe inflammatory changes in the peritoneal cavity, including ascites formation (in the majority of cases sanguineous) and fibrinous deposits on the surfaces of the liver, spleen and elsewhere. Quite often, the abdominal organs were matted together and could hardly be separated. In those rats which died late after unilateral intrapleural injection of VP 16-213, the pleura on the injection side was thickened and adherent, and a pleural exudate, in some cases sanguineous, was present; the lungs were somewhat shrunken and reduced in weight. No marked inflammatory changes were found in the peritoneal cavity of animals dying early from i.p. administration of the compounds investigated or in solvent-treated controls.

Liver weights were always less than 1 g in mice dying late after 3 i.p. injections of both drugs, while normal or solvent-treated mice of similar body weight and age had liver weights above 1 g. The single mouse dying late after 3 i.v. administrations of VP 16-213 (Table 2) hat a liver weight of 1·92 g and was therefore considered to have succumbed to a different cause than the delayed effects seen after i.p. administration.

Not designed to detect delayed toxicity, but pertinent to the problem, are a number of prolonged toxicity tests performed with VM 26 and VP 16-213 (Griffith, unpublished results). In these experiments, a total of 80 rats, 48 dogs and 24 Rhesus monkeys were used. VM 26 was given i.v. to dogs and monkeys 3 times per week, VP 16-213 p.o. to rats and dogs daily; for both compounds, 3 dose levels were used, in each study. Treatment lasted for 26 weeks followed for part of the animals by a 6–9-week recovery period. Except for an initial, transient, statistically not significant increase in serum glutamic pyruvic transaminase (SGPT) levels in dogs receiving VP 16-213, there were no changes in the clinical chemistry values related to the liver or other organs. In the same line, there were some liver changes (macro- and microscopically) of moderate degree in some of the treated groups at the end of treatment; but they had all disappeared in the recovery animals. Leucocyte counts were reduced in all experiments with the higher doses, but normalized during weeks of recovery.

In contrast to this, pathological changes following i.p. treatment are apparently progressive despite cessation of treatment. This also became clear in a 4-week toxicity study with rats where VM 26 was given daily i.p. In this experiment, pathological findings in the peritoneal cavity (peritonitis of sero-sanguineous and lateron of fibrous character) and in the liver (thickening of the capsule, deformation of nuclei and disorganization of hepatic cords) were slight or absent at the end of treatment, but became apparent or much more severe during a 4-week recovery period (Griffith, unpublished results).

DISCUSSION

The results presented here corroborate the original finding of Avery *et al.* [1] and of Hacker and Roberts [2] that the epipodophyllotoxin derivative VM 26 produces, after i.p. injection in mice, a delayed, fatal toxicity in which the liver seems to be involved. To our knowledge, similar findings have been reported, among cytostatic drugs, only for adriamycin and cyclophosophamide. This latter compound has been found to produce delayed wasting and mortality in rats, although the route of administration was not specified in one instance [3], and the drug was given s.c. in another [4]. A similar result has been reported in mice where cyclophosphamide was given i.p. [5]. It seems unlikely, however, that in these studies the pathological process leading to death was the same as with the epipodophyllotoxins, and it was clearly different in the late deaths in rats after cyclophosphamide observed by Vahlsing *et al.* [6]. With adriamycin a delayed toxicity with wasting was found in rats after a single i.v. drug administration [7]. This observation may also explain the enhancement of delayed toxicity of VM 26 by concomitant adriamycin administration, as possibly observed by Rivera *et al.* [2].

Our studies extend the findings of Avery *et al.* and Hacker and Roberts insofar that delayed toxicity was also found with VP 16-213, the congener of VM 26, and it was found too in rats, both after i.p. and after i.plr. administration. Avery *et al.* did not observe delayed toxicity after s.c. and intramuscular (i.m.) application of VM 26 to mice. We tested both epipodophyllotoxins by those routes which are used in man, i.e., the i.v. and—for VP 16-213 only—the oral route. When VM 26 was given i.v., and VP 16-213 i.v. and orally to mice, no delayed toxic effects could be elicited, despite the use of subtoxic and toxic doses and of schedules which, with i.p. injection, produce the delayed lethal effect. In addition, there were no observations reminiscent of delayed toxicity in studies where VM 26 was given i.v. for 26 weeks to monkeys and dogs; the same holds true for toxicological experiments where VP 16-213 was given orally for 26 weeks to rats and dogs.

Fatal toxicity of VM 26 and VP 16-213 is never an immediate effect, since mice and rats do not die earlier than 3 days after a single drug injection (i.v. or i.p.), even after a 100% lethal dose. The peak of mortality occurs 5 days after injection, at a time when there is severe leucopenia and usually diarrhoea. Some animals die up to 12 days after drug administration, and this is still to be considered to be "early" mortality, due mainly to effects on proliferating tissues. Delayed toxicity kills the animals only after several weeks, in the experiments reported so far more than 2–3 weeks after i.p. drug administration has ceased. Whether it would develop in the same way under continuous drug administration has not been tested.

The mechanism by which the epipodophyllotoxins produce delayed mortality is not clear, except that it is linked to the route of drug administration: a direct contact of the compounds with the surface of serous membranes is required. These membranes then develop subacute or chronic inflammation which, besides leading to exudate formation, affects the organs they surround. Damage involving mainly the liver and perhaps the intestines in the case of i.p. injection and the lung in the case of i.plr. administration, may be due, at least in part, to compression of the organs and their blood supply by the chronic fibrosing inflammation of their envelopes. The inflammation, while being elicited by the drugs, is apparently not dependent on the continued presence of the drug for development into a more chronic state. The histology findings in the 4-week toxicity test with i.p. administration of VM 26 demonstrate that the changes in the peritoneal cavity of treated rats progressed during the "recovery" period, and the microscopic changes in the liver, while being of a very moderate degree at the end of treatment, were more pronounced four weeks after drug treatment had ceased. This is in contrast to the findings in the chronic toxicity studies with i.v. and p.o. administration, where the blood effects (leucopenia) and slight histology changes in the livers of monkeys and rats disappeared during the recovery period.

Repeated administration seems to enhance the development of delayed toxicity, since a single i.p. injection of VP 16-213 in mice up to lethal doses did not lead to any deaths between days 29 and 160, and in rats, no dose of VP 16-213, when given once i.p., produced more late than early deaths (the results of these experiments are not given in the tables). On the other hand, with i.plr. injection of VP 16-213 in rats, a single administration of 25 mg/kg of VP 16-213 killed 8 out of 12 rats later than day 30, but none earlier. Possibly the pleura is even more susceptible to direct contact with VP 16-213 than the peritoneum,

or the secondary changes in the thoracic cavity are more liable to affect vital functions than those in the peritoneal cavity.

There are strong arguments for the assumption that the epipodophyllotoxins also have a direct effect on the liver: some degree of histologic changes after i.v. administration in monkeys and after oral application in monkeys and rats; increased SGPT values found during VP 16-213 treatment in dogs (without histologic changes) in our experiments; and the clinical chemistry findings of Avery *et al.* [1] and of Hacker and Roberts [2] (elevation of plasma GPT etc. during the first 2–3 weeks). We also found that the liver weights of animals dying of early toxicity (during the first 20 days after start of treatment) were somewhat reduced The early functional changes are, however, reversible. Thus, Hacker and Roberts [2] found plasma levels of liver enzymes unaltered from week 4 to 7 after treatment; and in our experiments with oral treatment of dogs with VP 16-213, SGPT elevations disappeared during treatment.

There are reports that in man, too, VM 26 given i.p. has an irritant effect, causing severe and longlasting abdominal pain [8]. Intravenous administration of VM 26 or VP 16-213, on the other hand, does not seem to affect non-proliferative abdominal organs. No drug-induced impairments of liver function have been reported with these drugs in man, and Nissen *et al.* [8, 9] found alakaline phosphatase, SGOT, serum proteins, serum bilirubin and glucose values unchanged during prolonged treatment up to 280 days. In the same line, Rivera, Avery and Pratt could not associate variations in renal and hepatic function with drug administration in children [10].

While Avery *et al.* [1] have tentatively linked delayed toxicity after VM 26 treatment to the route of administration (i.p.) and to a consequent chemical peritonitis, Hacker and Roberts [2] were reluctant to attribute responsibility for this phenomenon to a local effect. The inability to induce delayed toxicity with i.m. and s.c. injection of VM 26 in mice [1], and our inability to observe it with i.v. and oral administration of both drugs in several species, leads to the conclusion that delayed toxicity is dependent on a local effect and therefore on the route of application (i.p. or i.plr.). The phenomenon seems to be the same with VP 16-213 and apparently of no consequence for the treatment of human patients by the recommended routes (i.v. for VM 26, i.v. or p.o. for VP 16-213). This conclusion, on the other hand, implies that administration of these compounds into any body cavities lined by a serous membrane should be avoided.

Acknowledgements—I thank Dr. R. W. Griffith for helpful suggestions and Mr. A. Trippmacher for skilful technical assistance.

REFERENCES

1. T. L. Avery, D. Roberts and R. A. Rice, Delayed toxicity of 4′-demethyl-epipodophyllotoxin 9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819) in mice. *Cancer Chemother. Rep.*, Part 1, **57,** 165 (1973).
2. M. Hacker and D. Roberts, Acute, chronic and terminal toxicity to 4′-demethylepipodophyllotoxin thenylidene glucoside (VM 26) in mice. *Cancer Res.* **35,** 1756 (1975).
3. E. E. Miller and K. C. Tsou, Comparative study of lymphoma bearing rats treated with DAAP, DMAP and cytoxan. *Proc. Amer. Ass. Cancer Res.* **7,** 50 (1966).
4. U. Stenram and H. Nordlinder, Delayed death in rats treated with cyclophosphamide. *Nature (Lond.)* **219,** 1154 (1968).
5. P. K. Kalpaktsoglou and R. A. Good, Effect of pertussis antigen and cyclophosphamide on myeloma tumor. *Cancer Res.* **30,** 2841 (1970).
6. H. L. Vahlsing, E. R. Feringa, A. G. Britten and W. K. Kinning, Dental abnormalities in rats after a single large dose of cyclophosphamide. *Cancer Res.* **35,** 2199 (1975).
7. F. S. Philips, A. Gilladoga, H. Marquardt, S. S. Sternberg and P. M. Viadl, Some observations on the toxicity of adriamycin (NSC-123127). *Cancer Chemother. Rep.*, Part 3, **6,** 177 (1975).
8. P. Dombernowsky, N. I. Nissen and V. Larsen, Clinical investigation of a new podophyllum derivative, epipodophyllotoxin, 4′-demethyl-9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819), in patients with malignant lymphoma and solid tumors. *Cancer Chemother. Rep.*, Part 1, **56,** 71 (1972).

9. N. I. NISSEN, V. LARSEN, H. PEDERSEN and K. THOMSEN, Phase I clinical trial of a new antitumor agent, 4′-demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside) (NSC-141540; VP 16-213). *Cancer Chemother. Rep.*, Part 1, **56,** 769 (1972).
10. G. RIVERA, T. AVERY and C. PRATT, 4′-demethylepipodophyllotoxin 9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819; VM 26) and 4′-demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside) (NSC-141540; VP-16-213) in childhood cancer: preliminary observations. *Cancer Chemother. Rep.*, Part 1, **59,** 743 (1975).

Europ. J. Cancer Vol. 12, pp. 933–934. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Effect of Insulin on Glucose Transport in DMBA-Induced Mammary Tumors*

JOAN THILLY HARMON and RUSSELL HILF

Department of Biochemistry and University of Rochester Cancer Center, The University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642 *(U.S.A.)*

HEUSON and his co-workers demonstrated that DMBA-induced mammary carcinomas of the rat were responsive to insulin [1–4] *in vivo*. Cohen and Hilf [5] found that 60% of DMBA-induced tumors regressed after the animals were made diabetic by streptozotocin treatment. These insulin-dependent tumors metabolized glucose to a lesser degree *in vitro* than either insulin-independent tumors (tumors that continued to grow in the diabetic host) or growing tumors in intact hosts. Since insulin can stimulate glucose transport in many different cell types [6], it seemed logical that lack of endogenous insulin may result in decreased substrate entry, which in turn could lead to tumor regression. We therefore measured glucose transport in growing, regressing and static tumors in a diabetic host and examined the effect of insulin *in vitro* on each of these tumors. The data presented here indicate that insulin appears to stimulate glucose transport in cells that were derived from an insulin-dependent tumor.

Female Sprague–Dawley rats were intubated with 7,12-dimethylbenz(a)anthracene (DMBA) (5 mg per week) for 5 weeks; each animal received a total dose of 25 mg DMBA [5]. Tumors, which arose about 5 weeks after the last carcinogen feeding, were measured weekly by calipers. Tumor size was estimated by the products of two perpendicular diameters. Diabetes was induced by a single injection of streptozotocin [5] and tumor response was classified as growing, static or regressing on the basis of change in tumor size for the 9 day period prior to sacrifice.

Glucose transport was measured using 3-O-methyl-^{14}C-D-glucose (3-OMG) (57 mCi/μmole, Amersham/Searle) *in vitro* with dissociated tumor cells as described previously [7]. Insulin at a final concentration of 10^{-10} M was incubated with cells for 15 min at 20°C; this period was found to yield maximum insulin binding under these conditions. Linear regression analysis was used to determine the best fitting straight line for glucose transport measured at 15, 30, 45 and 60 sec after addition of labeled 3-OMG. Calculation of initial velocity of 3-OMG transport was made using the slope obtained from the linear regression analysis; differences in slopes were determined by "*t*" test.

In preliminary studies, we observed an effect of insulin to increase glucose transport *in vitro* using DMBA-induced tumors that were regressing in diabetic hosts. We were presented with the opportunity to have sufficient cells to examine total glucose transport in growing, static and regressing tumors from the same diabetic host. This animal, at the time of sacrifice, had blood glucose levels of 400 mg/100 ml and $< 10^{-11}$ M serum insulin levels as determined by radioimmunoassay. The data obtained for each of these tumors are shown in Table 1. The effect of insulin (10^{-10} M) was to significantly stimulate or inhibit glucose transport in regressing or growing tumors, respectively. It is of interest that insulin under these conditions had no effect on glucose

Accepted 24 May 1976.

Reprint requests to: Dr. R. Hilf, Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 (U.S.A.)

*This research was supported by U.S. Public Health Service Grants CA-16660 and CA-11198 and a grant from the Monroe County Cancer and Leukemia Association.

Table 1. *Effect of insulin on glucose transport in DMBA-induced tumors*

Growth characteristics of tumor*	Insulin treatment *in vitro*†	Glucose transport‡ v_i^t 10^{-4} μmoles/min × 10^6 cells	% Control§
Static	−	0·312	
	+	0·258	82.7
Regressing	−	0·273	
	+	0·416	155·4¶
Growing	−	0·544	
	+	0·258	47·4¶

*Tumors were classified according to their growth over the 9 day period prior to sacrifice: static, less than 20% change; regressing, decrease greater than 20%; and growing, increase greater than 20%.

†Enzymatically dissociated tumor cells were incubated with or without 10^{-10} M porcine insulin for 15 min at 20°C.

‡Transport measurements were made in triplicate at 15, 30, 45 and 60 seconds in the presence of 1 mM (0·5 μCi/vial) 3-0-methyl(^{14}C)-D-glucose.

§Control was transport in the absence of insulin from the same tumor.

¶Significant difference ($P < 0·05$) versus control (no insulin), as determined by t test for difference in slopes.

transport in the tumor classified as having static growth.

While it is usually reported that insulin stimulates glucose transport in normal cells [6], we have observed that insulin consistently decreased glucose transport in dissociated cells of the R3230AC mammary tumor [7]. In this latter study, the response to insulin was dose-related over a physiologic range (10^{-11}–10^{-8} M). Since the R3230AC mammary tumor is insulin-independent, i.e., grows equally well or faster in the diabetic rat compared to the intact host, it was felt that a DMBA-induced tumor continuing to grow in a diabetic host might demonstrate a response of glucose transport like the R3230AC tumor to insulin. This indeed was observed. In contrast, the DMBA-induced tumor which regressed in the diabetic host demonstrated an opposite response to insulin, namely a significant stimulation of glucose transport to insulin *in vitro*.

It is possible that the lack of response in growth and glucose transport of the tumor classified as static could be attributed to a heterogenous cell population. Since all of these tumors were obtained from the same animal, it can be assumed that the divergence of the response to insulin was not due to other hormonal influences but rather to differences in the properties of the neoplasms.

Acknowledgements—Portions of this material are taken from the dissertation submitted to the University of Rochester in partial fulfillment of the requirements for the Ph.D. degree.

REFERENCES

1. J. C. Heuson and N. Legros, Effect of insulin and of alloxan diabetes on growth of the rat mammary carcinoma *in vivo*. *Europ. J. Cancer* **6,** 349 (1970).
2. J. C. Heuson and N. Legros, Effect of insulin on DNA synthesis and DNA polymerase activity in organ culture of rat mammary carcinoma and the influence of insulin pretreatment and of alloxan diabetes. *Cancer Res.* **31,** 59 (1971).
3. J. C. Heuson and N. Legros, Influence of insulin deprivation on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. *Cancer Res.* **32,** 226 (1972).
4. J. C. Heuson, N. Legros and R. Heimann, Influence of insulin administration on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in intact, oophorectomized and hypophysectomized rats. *Cancer Res.* **32,** 233 (1972).
5. N. D. Cohen and R. Hilf, Influence of insulin on growth and metabolism of 7,12-dimethylbenz(a)anthracene-induced mammary tumors. *Cancer Res.* **34,** 3245 (1974).
6. J. Elbrink and J. Bihler, Membrane transport: its relation to cellular metabolic rates. *Science* **188,** 1177 (1975).
7. J. T. Harmon and R. Hilf, Effect of insulin to decrease glucose transport in dissociated cells from the R3230AC mammary adenocarcinoma in diabetic rats. *Biochim. biophys. Acta.* In press (1976).

Europ. J. Cancer Vol. 12, pp. 935–936. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *November, 1976*

Human Cancer

B. E. Chechik, K. W. Pyke and E. W. Gelfand: Human thymus/leukemia-associated antigen in normal and leukemic cells.

H. Ito, F. Tsutsui, S. Kurihara, T. Akabayashi, T. Tobe and C. Nishimura: Serum antibodies to herpesvirus early antigens in patients with cervical carcinoma determined by anticomplement immunofluorescent technique.

P. Hersey, M. Honeyman, A. Edwards, E. Adams and W. H. McCarthy: Antigens on melanoma cells detected by leukocyte dependent antibody assays of human melanoma antisera.

A. Sell, H. Søgaard and B. Nørgaard-Pedersen: Serum alpha-fetoprotein as a marker for postoperative radiation therapy and/or chemotherapy in eight cases of ovarian endodermal sinus tumour.

A. S. Kadish, D. M. Marcus and B. R. Bloom: Inhibition of leukocyte migration by human breast cancer associated antigens.

I. Kehayov, B. Botev, V. Vulchanov and S. Kyurkchiev: Demonstration of a phase (stage) specific embryonic brain antigen in human meningioma.

H. F. Pross and M. G. Baines: Spontaneous human lymphocyte-mediated cytotoxicity against tumour target cells. I. The effect of malignant disease.

C. E. Bell, Jr. and S. Seetharam: A plasma membrane antigen highly associated with oat cell carcinoma of the lung and undetectable in normal adult tissue.

Experimental Cancer

T. Hamaoka, S. Haba, K. Takatsu and M. Kitagawa: Selective inhibition of repopulation of T lymphocytes into lymphoid organ as a mechanism of immunosuppression in tumor-bearing mice.

M. J. Embleton: Influence of cell-free tumour-associated antigen preparations on the development of immunity to chemically-induced rat tumours.

L. A. Radov, J. H. Korn and J. S. Haskill: Host immune potentiation of drug responses to a murine mammary adenocarcinoma. II. Effect of Melphalan therapy on the host immune system.

G. Klein, J. Zeutchen, P. Terasaki, R. Billing, R. Honig, M. Jondal, A. Westman and G. Clements: Inducibility of the Epstein–Barr virus (EBV) cycle and surface marker properties of EBV-negative lymphoma lines and their *in vitro* EBV-converted sublines.

L. M. Pelus and H. R. Strausser: Indomethacin enhancement of spleen cell responsiveness to mitogen stimulation in tumorous mice.

R. Levin, S. K. Ruscetti, W. P. Parks and E. M. Scolnick: Expression of feline type-C virus in normal and tumor tissues of the domestic cat.

W. T. Shier, J. T. Trotter and D. T. Astudillo: Effect of localization of L-asparaginase as its Concanavalin A conjugate on anti-tumor activity.

J.-M. Prevost, G. R. Pearson, W. C. Wallen, H. Rabin and L. F. Qualtiere: Antibody responses to membrane antigens in monkeys infected with *Herpesvirus saimiri*.

G. R. Betton: Agglutination reactions of spontaneous canine tumour cells by Concanavalin A demonstrated by an isotopic assay.

Z. Schaff, K. Lapis and P. M. Grimley: Undulating membraneous structures associated with the endoplasmic reticulum in tumour cells.

D. A. Kessler, R. S. Langer, N. A. Pless and J. Folkman: Mast cells and tumor angiogenesis.

British Journal of Cancer *November, 1976*

D. Metcalfe and S. Kolber: Stimulation of B-lymphocyte colony formation *in vitro* by sera from patients with leukaemia or lymphoma.

I. A. Smith, V. D. Courtenay and M. Y. Gordon: A colony forming assay for human tumour xenografts using agar in diffusion chambers.

R. A. Bender, W. A. Bleyer, J. C. Drake and J. L. Ziegler: *In vitro* correlates of clinical response to methotrexate therapy in acute leukaemia and Burkitt's lymphoma.

P. J. Creaven, M. H. Cohen and L. M. Allen: Methotrexate plasma decay kinetics: A possible alteration in patients undergoing gut sterilization.

H. Biran, J. L. Moake, R. C. Reed, J. U. Gutterman, E. M. Hersh, E. J. Freireich and G. M. Mavligit: Complement activation *in vivo* in cancer patients receiving C. Parvum immunotherapy.

M. A. Wainberg, V. Deutsch and D. W. Weiss: Stimulation of anti-tumour immunity in guinea pigs following immunoprophylactic treatment with the methanol extraction residue of BCG.

G. M. Macnab, J. J. Alexander, G. Lecatsas, E. M. Bay and J. M. Urbanowicz: Hepatitis B surface antigen produced by a human hepatoma cell line (PLC/PRF/5).

V. Armuth: Test for the leukaemogenic action of phorbol in intact and thymectomized mice of different strains.

I. Chouroulinkov, A. Gentil, P. L. Grover and P. Sims: Tumour initiating activities on mouse skin of dihydrodiols derived from benzo(a)pyrene.

B. H. Toh, G. C. Hard, M. N. Cauchi and H. K. Muller: Smooth muscle-associated contractile protein in renal mesenchymal tumour cells and in transformed cells derived from dimethylnitrosamine-injected rats.

R. A. Hawkins, D. Drewitt, B. Freedman, E. Killin, D. A. Jenner and E. H. D. Cameron: Plasma hormone levels and the incidence of carcinogen-induced mammary tumours in two strains of rat.

J. M. Silcock and N. J. F. Dodd: Electron spin resonance study of changes during the development of a solid Yoshida tumour. I. The ascorbyl radical.

N. J. F. Dodd and J. M. Silcock: ESR study of changes during the development of a Yoshida tumour. II. Paramagnetic metal ions.

S. C. Thompson: The effect of mouse age and sex on the colony forming efficiency of injected tumour cells.

Europ. J. Cancer Vol. 12, pp. 937–944. Pergamon Press 1976. Printed in Great Britain

Perspectives in Cancer Research

Carcinoma of the Prostate: A Focal Point for Divergent Disciplines

PHILIP H. SMITH*, MELVYN R. G. ROBINSON† and EDWARD H. COOPER‡§

**Department of Urology, St. James's Hospital, Leeds, †Department of Urology, Pontefract General Hospital, Pontefract and ‡Department of Experimental Pathology and Cancer Research, University of Leeds*

INTRODUCTION

CARCINOMA of the prostate is the commonest cancer arising in the male uro-genital tract. Clinically the disease varies from a latent carcinoma found incidentally at prostatectomy to a tumour which progresses rapidly, infiltrating and metastasising. It is largely confined to the elderly being present in occult form in at least 10% of patients at post-morten and may be diagnosed accidentally because of associated benign prostatic hypertrophy.

The age of the patients, the uncertainty of the natural history of the disease and the difficulties in determining the rate of progression make the choice of the correct line of treatment far from easy. The hope offered by the introduction of hormonal therapy in the 1940's has benefited many patients, but has also confused an already complex situation by its success, inhibiting further studies until the last ten years or so when members of the Veterans Administration made observations on the dangers of oestrogen therapy and of the similar overall mortality when large groups of patients treated with oestrogens, orchidectomy and placebo therapy were compared [1]. Clinicians are now increasingly questioning the traditional approach and are attempting to tailor the treatment to fit the individual patient.

It is necessary to assess both the prostate and the patient to define the extent and histological grade of the lesion and to evaluate the degree of dissemination; from these factors follows a prediction of the likely natural history of the condition in that individual. To establish the natural history it may also be desirable to follow any patient asymptomatic following the relief of obstructive symptoms, without treatment and also to evaluate his hormonal status in terms of the function of the testicles and anterior pituitary.

ASSESSMENT

The making of predictions of the duration, course and outcome of any chronic disease for the individual patient is essential. The patient and his relatives must be advised about the likely course of the disease so that they can manage his affairs and ensure that his remaining life span is of the best possible quality as well as duration. Clinically it is important that any therapy chosen is beneficial, and does not increase the morbidity and mortality beyond that of the natural history of the untreated disease. As accurate prognosis for the individual patient is also essential when different forms of therapy are compared in clinical trials. Usually the doctor bases prognosis on his accumulated experience aided by a distillation of knowledge from the literature.

At the present time, the prognosis is assessed by clinical staging, and by histological or cytological grading. The initial assessment is made before treatment, but it is equally important to detect as early as possible a failure of response to therapy. Newer techniques of determining prognosis and response to treatment which may be helpful in the future include enzyme estimations, the study of hormonal environment, endocrine receptors and tissue metabolism and immunology.

Accepted 1 July 1976.

§Supported by the Yorkshire Cancer Research Campaign.

The clinical stage is assessed by clinical examination, by determination of the total and prostatic acid phosphatase and by radiology of the chest and skeletal systems. Scintigraphy is increasingly useful since bone seeking isotopes such as Fluorine 18, Strontium 87 m and Technetium 99 can identify the site of skeletal metastases in carcinoma of the prostate before conventional radiography [2–4]. Also metastases in the regional lymph nodes draining the prostate may appear much earlier in the disease than skeletal or other distant metastases, and pedal lymphangiography is being increasingly used in the routine assessment of new patients [5].

The histological grade of prostatic tumours is assessed by considering the architecture of the acini, intra-cellular changes, and the presence or absence of invasion and metaplasia. The intra-cellular changes appear to be the most important in grading the tumours [6].

Esposti [6] using the trans-rectal aspiration biopsy has shown a close correlation between the cytological grading of tumours in 469 patients followed up for five years and their response to oestrogens and orchidectomy. Hendry and Williams [7] have shown that as a diagnostic technique the Franzen biopsy is reliable in 82% of cases. It causes little discomfort to the patient and therefore can be used as an Outpatient procedure and can be repeated if it unexpectedly proved to be negative.

Schubert [8] reported a series of 270 cases in which he has followed the histological progress by needle biopsy at three months after commencing therapy and by aspiration cytology at six weeks, six months and one year. He observed that needle aspirates have the great advantage that they can be repeated at regular intervals, and concluded that a positive hormone response can be judged by this method. The cytological changes observed in patients having a positive response to hormonal therapy include squamous metaplasia, hydropic degeneration, reticulation of the nuclear chromatin, and pyknosis and rupture of the nuclear membrane. Becker [9] claims that these changes become apparent in three to four weeks after orchidectomy followed by large doses of oestrogens and that if they are then absent the therapy should be changed. He also suggests that cytology should be repeated at six monthly intervals to see whether and when secondary resistance to endocrine therapy develops.

Of patients with prostatic cancer which has metastasised to the skeletal system, 75–90% have elevated serum acid phosphatase levels [10]. The extent of the increase varies considerably but tends to be moderate; Woodard [11] reported an average value of 4·0 KA units in 107 patients with bone involvement. As a monitor of the disease, serum acid phosphatase is a useful but not infallible indicator of spread to bone, or of its extent. Gursel *et al.* [12] have shown that acid phosphatase activity in the bone marrow is a more sensitive indicator of the presence of bone metastases than are serum acid phosphatases.

In many patients with bone metastases the level of serum acid phosphatase may fall in response to treatment with oestrogens or orchidectomy. Unfortunately, in long term studies it can be shown that extensive bone metastases can develop when a patient is on treatment, with little or no rise of acid phosphatase activity [10].

Lactic dehydrogenase isoenzyme, (LDH) patterns in prostatic fluid expressed by massage may prove to be useful in detecting carcinoma in glands that have no abnormality suggestive of malignancy on digital palpation. Oliver *et al.* [13] have shown that LDH V is as predominant in benign tissue associated with carcinoma of the prostate, as it is in malignant prostatic tissue. Hein, Grayhack and Goldberg [14] have demonstrated that an LDH V-I ratio exceeding 3 in prostatic fluid, in the absence of infection, should be regarded as an indicator of high risk of a malignancy in palpably normal glands.

The patient's hormonal status is also becoming increasingly recognized as an important factor since malignant prostatic tissue, like that of the normal gland, remains, at least for a time, androgen-dependent. Many studies have been made of the circulating hormones which directly or indirectly influence prostatic function. The major source of androgens is from the interstitial cells of the testis which produce testosterone. Androgens are also produced by the adrenal cortex. The interstitial cells are controlled by the interstitial cell stimulating hormone from the anterior pituitary (L.H.) which in turn is influenced by releasing hormones from the hypothalamus. The pituitary also influences the prostate by secreting prolactin which stimulates the metabolism of testosterone by the prostate.

Although testosterone production rate declines with age [15] there is no significant fall in plasma testosterone levels with age [16]. Southren [17] attributes this to a reduced metabolic clearance rate running parallel with

the fall in production rate which occurs after the second decade.

Plasma testosterone levels before treatment are not helpful in the prognosis of carcinoma of the prostate since patients with non-metastatic prostatic cancer show no significant difference in plasma testosterone levels when compared with control patients of a similar age group [16]. The mean testosterone level is, however, significantly lower in patients with metastastic disease [16, 18]. Measurement of the total prostatic hormonal environment may however be helpful. Serum levels of L.H. are significantly higher in older than younger men [19]. Also Frick [20] has shown that infusion of L.H. and of gonadotrophin releasing hormone causes a rapid rise of plasma testosterone in younger men, but not to the same extent in older men. This suggests that older men have interstitial cell atrophy and that a higher L.H. level is necessary to maintain testosterone production. This has been supported by Lunglmayr [21] who has directly stimulated the interstitial cell with human chorionic gonadotrophin. The rise in testosterone levels is less marked in older than in younger men.

In some patients with carcinoma of the prostate plasma testosterone levels may be partly maintained by increased adreno-cortical activity. Doe *et al.* [22] have shown that non-protein bound cortisol levels are elevated in such patients before treatment. Sciarra *et al.* [23] have shown that in a few orchidectomised patients plasma testosterone levels remain high. Dexamethasone therapy reduces these high levels suggesting that in these patients the adrenal cortex is making a significant contribution to plasma testosterone levels.

After the initial fall in plasma testosterone due to oestrogen therapy there is a small but significant rise [16]. This is not related to the clinical response to treatment and probably reflects increased adrenal cortical activity secondary to oestrogen therapy [22, 24] and increased plasma testosterone binding by plasma proteins [25].

We still need to know more about the direct actions of oestrogens at tissue level, and in the future tissue metabolism studies might prove helpful in determining the response to treatment.

These observations have assumed increasing importance since Bramble and Jacobs [26] put forward the hypothesis that patients with carcinoma of the prostate fall into three groups, the first having normal LH levels and normal testosterone levels in the serum, the second having an elevated LH level and a normal testosterone level and the third group having a high LH and a low testosterone level. The first group has normal interstitial cell function, the second has relative interstitial cell atrophy and the third have interstitial cell failure. It is unlikely that this third group will respond to orchidectomy, since their tumour must already be androgen independent. It is also unlikely that this group of patients will respond to oestrogen therapy unless oestrogens have a *direct* effect upon the prostate gland.

It should be noted that there is marked diurnal variation of plasma testosterone and L.H. levels which are also influenced by emotional factors and operative procedures and the measurement of LH and testosterone after the infusion of releasing hormone or chorionic gonadotrophin may prove of great value in detecting those patients with interstitial cell failure.

Bresciani [27] has shown that some carcinomas of the prostate have steroid receptors for androgens whilst others do not. However, no relationship has yet been shown between the presence or absence of androgen receptors and the response to endocrine therapy.

There is increasing clinical and experimental evidence that carcinoma of the prostate produces an immune response in the host, and in the future this may be helpful in determining prognosis for the disease. The frequent incidence of latent carcinoma suggests that many patients have a good resistance to their disease.

Tumours evoke an immune response because they lose normal tissue specific antigens and gain new tumour specific antigens. Gupta, Schuster and Christian [28] and Ablin [29] have shown the loss of normal tissue specific antigens in prostatic cancer. Weschler *et al.* [30] have demonstrated circulating tumour associated antigens, and Tannenbaum and Lattimer [31] have localized IgM in hyperplastic areas near carcinoma of the prostate.

Unfortunately, tissue culture of malignant prostatic tissue has proved difficult; therefore, specific immunological tests of host resistance to the disease cannot be performed. However, several nonspecific methods for estimating the immunological competence of patients in this disease have been reported.

Catalona, Chretien and Trahan [32] reported that patients with prostatic adenocarcinoma have a high incidence of impaired delayed hypersensitivity reaction to dinitrochlorobenzine (DNCB) there being no difference between patients with non-metastatic and metastatic disease. Williams and Castro [33] however,

found that their prostatic cancer patients gave a positive skin reaction to testing with DNCB irrespective of the stage of the disease and that their response did not differ from that in controls.

Robinson *et al.* [34] observed that lymphocyte transformation in response to stimulation with Phytohemagglutin (PHA) was depressed in patients with metastatic disease. In 24 patients close correlation was found between lymphocyte transformation and cytological grading of the tumour. During a one year follow-up only patients with a depressed lymphocyte transformation died from carcinoma of the prostate. McLaughlin *et al.* [35] found lymphocyte transformation in response to PHA was depressed in patients with carcinoma of the prostate, but in their series was not related to the clinical stage of the disease.

Gursel and Veenema [36] have detected higher levels of IgG in stage III patients with untreated carcinoma of the prostate than in stage IV patients and the level of circulating IgG and IgM is raised after cryosurgery.

Robinson *et al.* [34] and Lockwood *et al.* [37] did not find any change in lymphocyte transformation and serum immunoglobulin levels in response to oestrogen therapy. Catalona [38] has studied T-lymphocyte levels by T-rosette assay in eight patients. He found that treatment with stilbestrol 1 mg daily had no significant effect on the T-lymphocyte levels. Cyclophosphamide reduced the levels below 25% and this reduction was maintained throughout the duration of therapy but in one patient who had a good response to cytotoxic therapy with 5-fluorouracil (5 FU) there was a transient fall in levels to 50% which then rebounded to above normal levels during therapy. Clinical relapse was heralded by a further fall in T-lymphocytes.

Prediction of the outcome of therapy

In recent years it has become apparent that the application of mathematical techniques can aid the prognostic skill of the doctor and greatly increase the accuracy of forecasting. Essentially these techniques are designed to examine the inter-action of the various factors that eventually determine the force of mortality that is most likely to be operating for a given individual patient. The variations of pattern of many chronic diseases such as carcinoma of the prostate are considerable and after a life time of practice very few practitioners will have encountered all the variants or be able to structure their innate clinical prognostic skill so as to give each variable the correct weighting when attempting to give a prognosis. This is more than a purely academic exercise for these prognostic indications can determine sub-sets within a general population, for example stage III or IV of carcinoma of the prostate with the individuals in the different sub sets having considerable differences in their prognosis. Consequently in their search for new therapies, it is essential that the stratification of patients within major classes both determined by stage, should be taken into account, for bias may be introduced into any clinical trial by unwittingly including more patients with a good prognosis in one arm of the trial compared to another. This is particularly true where a single line of therapy is being pursued and survival is the end point. The general principles relating to this approach have recently been summarised by Armitage and Gehan [39]. The number of patients needed for comparison of two regimes is a function of the expected differences of the treatment [40].

As has been stated earlier in this article it is only the Veterans trial that has sufficient cases of carcinoma of the prostate for the various sub-sets in the population that make up stages III and IV to be identified. In 1974, Byar, Huse and Bailar [41] published an exponential survival model that was able to predict with remarkable accuracy the survival of patients with advanced (stages III and IV) carcinoma of the prostate based on the various combinations of presenting signs and symptoms; in their model concerned with the analysis of 1824 patients they identified eight patient characteristics some of which had more than one alternative, for example the level of phosphatase activity. They have produced formulae and tables from which it is now possible to calculate the probability of the time of death of the patient from all causes according to his status at the time of commencing treatment. Their analysis has shown that five major risk groups can be identified within the population for stages III and IV combined. The best of these groups (281 patients) had 60% survivors at seven years whilst the worst (350 patients) had less than 10% survivors at five years. An extension of this model has been made by Bayard *et al.* [42] using the step-down procedure whereby the number of variables taken into consideration are reduced progressively according to their weighting in contributing to the end point of the study. In this instance, they were concerned with the analysis of the influence of treatment upon survival, and in particular looking for the influence of treatment and the patients status at the commencement

of treatment upon their probability of dying from carcinoma of the prostate, cardio-vascular disease or other forms of death. They found that the patients with stage III die from diseases other than cancer of the prostate more frequently, but treating them with oestrogens increased the frequency of cardio-vascular deaths at the expense of death from carcinoma of the prostate. Patients in stage IV had a higher incidence of death from cancer. This trend was not influenced by orchidectomy but was increased by oestrogen, either alone or with orchidectomy. The risk from oestrogen therapy rose considerably with the patient's age and if the patient was anaemic. The detection of metastases at the onset of the therapy considerably influenced the eventual outcome and in those patients treated with either placebo or by orchidectomy alone elevated acid phosphatase levels was a highly significant risk factor. These analyses have not taken the histology of the tumour into account but Gleason *et al.* [43] also working in the Veterans group has shown that a single combination of scoring four stages with a weighting factor for histology describing both the main histology of this specimen as well as its variation in different parts of the specimen can be used as a prognostic indicator for the 4193 patients in the VACURG series.

TREATMENT

Although treatment ranges from the philosophical prescription of oestrogens to aggressive advocacy of radical prostatectomy, it is by no means certain that either course is correct. It may be more logical to reserve treatment for those patients whose symptoms demand it and it is certainly both logical and ethical to evaluate new forms of treatment as they become available.

Certain facts are, however, becoming more clear. It is now accepted that stilboestrol 1 mg per day will not suppress plasma testosterone throughout the 24-hr period [42] but that 1 mg 8-hourly is adequate for this purpose. Larger doses of stilboestrol are probably disadvantageous because of the potential cardio-vascular side-effects [44].

Orchiectomy, though superficially less attractive is probably more effective. It has none of the disadvantages of stilboestrol but imposes a minor additional surgical hazard either at the time of prostatectomy, if a biopsy result is already available, or during the convalescent period if the disease was not originally suspected.

For the patients who have failed on oestrogen therapy, orchiectomy, adrenalectomy or the use of adrenal steroids to inhibit androgen production has never become an established technique whilst hypophysectomy has achieved little in terms of patient survival despite relief of bone pain [45, 46]. The use of testosterone followed by radio-active P32 has been tried with little success [47] but the combination of P32 and Parathormone may be more hopeful [48].

Recently there has been a revival of interest in radiation therapy for carcinoma localized to the prostate gland or its immediate proximity. The Stanford group [49] have studied 405 patients treated in this way; 72% of stage II were alive at 5 years and 44% at 10 years, with only a slight decrease in survival in patients with extra capsular lesions (stage III), 51% at 5 years and 38% at 10 years. This impression has been endorsed by others [50]. Flocks *et al.* [51] have shown that interstitial radiation with radio-active gold grains can be an effective therapy.

Cryosurgery has its advocates [52], but has failed so far to become a generally adopted mode of treatment.

The Veterans Administration have shown most clearly in recent years that progress will only be made by prospective clinical trials. They have concentrated on a re-evaluation of the existing forms of therapy and have demonstrated decisively the similarity in terms of patient survival between all common forms of therapy. They have also demonstrated the statistical fact that it requires studies on large numbers of patients to determine differences between alternative forms of therapy when one is not obviously superior to another. Few other co-operative groups have the resources, or the energy, to embark upon studies of this kind and it may well be that further progress will now more quickly be made by prospective studies in smaller groups of patients designed to evaluate the potential benefits of the newer forms of treatment, either alone or in combination.

It is particularly relevant at this time to re-consider, if the information is already available, the influence of the patients hormonal status upon survival when treated by hormonal manipulation. Another area of similar priority must be to evaluate singly and in combination different cytotoxic drugs, alone or in combination with oestrogens. As yet the literature on cytocoxic therapy in cancer of the prostate is sparse [53] but limited success has been reported with Cyclophosphamide [54],

nitrogen mustard and 5-fluorouracil [55] and Mithramycin [56]. In addition, Estracyt, a combination of nitrogen mustard and oestradiol through a carbamate ester, is reported to produce remission in 50% of patients relapsing on oestrogen therapy [57, 58].

Unfortunately the data on chemotherapy is insufficient to make any real evaluation as to its place in the management. However, a word of caution is necessary to those contemplating its use as a last hope in the advanced patient, as the bone marrow reserve may be very low and consequently the risks of a fatal aplasia are very real.

At the present time the clinician treating a patient with cancer of the prostate has many problems. In the first place he must do his best to evaluate the disease in the patient and to assess the likely natural history and prognosis in the absence of treatment. In choosing treatment he should be sufficiently aware of the literature to avoid increasing the natural morbidity and mortality and in following the patient he must be sufficiently thorough to detect a failure of response to therapy or the development of dangerous side-effects at the earliest possible time.

There is a strong case to be made out for withholding treatment, other than the relief of obstruction, until the patient has symptoms which require therapy or it can be shown that the disease is progressing. In choosing treatment one must be aware that approximately 80% of patients respond to hormonal manipulation, at least for a time, suggesting that this should be the first line of attack. For the younger patient with a localised lesion however it might also be appropriate to consider high energy radiation or radical prostatectomy in the first instance.

For the patient failing on the initial treatment regime, there is an obligation not only to change the treatment but also to use the opportunity to assess newer forms of therapy including the use of cytotoxic drugs.

Acknowledgements—We would like to thank our colleagues in Leeds and members of the European Organisation for Research into the Treatment of Cancer, for various discussions which have influenced the writing of this article.

REFERENCES

1. G. T. Mellinger, J. C. Bailar, L. J. Arduino and the Veterans Administration Cooperative Urological Research Group, Treatment and survival of patients with cancer of the prostate. *Surg. Gynec. Obstet.* **124,** 1011 (1967).
2. A. C. Buck, G. D. Chisholm, M. V. Merrick and J. P. Lavender, Serial fluorine-18 bone scans in the follow-up of carcinoma of the prostate. *Brit. J. Urol.* **47,** 287 (1975).
3. R. J. Shearer, A. R. Constable, W. F. Hendry and J. D. Fergusson, Radioisotopic bone scintigraphy with the gamma camera in the investigation of prostatic cancer. *Brit. Med. J.* **2,** 362 (1974).
4. M. R. G. Robinson and A. R. Constable, Strontium 87 m and the gamma camera in the study of bone metastases from carcinoma of the prostate. *Brit. J. Urol.* **45,** 173 (1973).
5. J. C. Cerny, R. Farah, R. Rian and M. L. Weckstein, An evaluation of Lymphangiography in staging of carcinoma of the prostate. *J. Urol.* **113,** 367 (1975).
6. P. L. Esposti, Cytologic malignancy grading of prostatic carcinoma by transrectal aspiration biopsy. *Scand. J. Urol. Nephrol.* **5,** 199 (1971).
7. W. F. Hendry and J. P. Williams, Transrectal prostatic biopsy. *Brit. Med. J.* **4,** 595 (1971).
8. G. E. Schubert, Hormone dependency and histological types of prostatic cancer. *International Meeting on Hormonal Therapy of Prostatic Cancer.* Rome (1975). (In press).
9. H. Becker, Prospective study over 5 years with various hormone therapies (castration, estrogen, anti-androgen, placebo). *International Meeting on Hormonal Therapy of Prostatic Cancer.* Rome (1975). (In press).
10. O. Bodansky, *The Biochemistry of Human Cancer,* p. 67 and p. 80, Academic Press, New York.
11. H. Q. Woodard, Factors leading to elevations in serum glycerophosphatase. *Cancer (Philad.)* **5,** 236 (1952).
12. E. O. Gursel, M. Rezvan, F. A. Sy and R. J. Veenema, Comparative evaluation of bone marrow acid phosphatase and bone scanning in staging of prostatic cancer. *J. Urol.* **111,** 53 (1974).

13. J. A. Oliver, M. M. Elhilali, P. Belitsky and K. J. MacKinnon, L.D.H. Isoenzymes in benign and malignant prostatic tissue. The L.D.H. V/1 ratio as an index of malignancy. *Cancer (Philad.)* **25,** 863 (1970).
14. R. C. Hein, J. T. Grayhack and E. Goldberg, Prostatic fluid lactic dehydrogenase isoenzyme patterns of prostatic cancer and hyperplasia. *J. Urol.* **113,** 511 (1975).
15. K. Isurugi, Plasma testosterone production rates in patients with prostatic cancer and benign prostatic hyperplasia. *J. Urol.* **97,** 903 (1967).
16. M. R. G. Robinson and B. S. Thomas, The effect of hormonal therapy on plasma testosterone levels in prostatic carcinoma. *Brit. med. J.* **4,** 391 (1971).
17. A. C. Southren, G. G. Gordon and S. Tochimoto. Mean plasma concentration, metabolic clearance and basal plasma production rates of testosterone in normal men and women using a constant infusion procedure: effect of the time of day and plasma concentration on the metabolic clearance rate of testosterone. *J. clin. Endocr.* **27,** 686 (1966).
18. H. H. Young and J. R. Kent, Plasma testosterone levels in patients with prostatic cancer before and after treatment. *J. Urol.* **99,** 788 (1968).
19. Editorial British Medical Journal Hormones and the elderly testes. *Brit. med. J.* **3,** 2 (1975).
20. J. Frick, Plasma androgens and their metabolism. *International Meeting on Hormonal Therapy of Prostatic Cancer.* Rome (1975). (In press).
21. G. Lunglmayr, Plasma androgens. *International Meeting on Hormonal Therapy of Prostatic Cancer.* Rome (1975). (In press).
22. R. P. Doe, P. Dickinson, H. H. Zinneman and U. S. Seal, Elevated non protein-bound cortisol (N.P.C.) in pregnancy, during estrogen administration, and in carcinoma of the prostate. *J. clin. Endocr.* **29,** 757 (1969).
23. F. Sciarra, G. Sorcini, F. Di Silverio and V. Gagliardi, Plasma testosterone and androstenedione after orchiectomy in prostatic adenocarcinoma. *Clin. Endocr.* **2,** 101 (1973).
24. W. B. Peeling and K. Griffiths, The adrenal cortex and prostatic cancer. *Proc. roy. Soc. Med.* **67,** 1156 (1973).
25. T. Kato and R. Horton, Studies of testosterone binding globulin. *J. clin. Endocr.* **28,** 1160 (1968).
26. F. J. Bramble and H. S. Jacobs, Hormones, elderly testes and carcinoma of the prostate. *Brit. med. J.* **3,** 307 (1975).
27. F. Bresciani, Steroid receptors in prostatic cancer. *International Meeting on Hormonal Therapy of Prostatic Cancer.* Rome (1975). (In press).
28. R. K. Gupta, R. Schuster and W. D. Christian, Loss of isoantigen A.B.H. in prostate. *Amer. J. Pathol.* **70,** 439 (1973).
29. R. J. Ablin, Immunologic studies of normal, benign and malignant human prostatic tissue. *Cancer (Philad.)* **29,** 1570 (1972).
30. M. Wechsler, P. Lo Gerfo, J. Feminella and J. K. Lattimer, The cancer associated antigen test as an index to failure of complete removal of urological cancers. *Trans. Ameri. Ass. genit.-urin. Surg.* **64,** 85 (1972).
31. M. Tannenbaum and J. K. Lattimer, Immunopathology of the Prostate: Benign and Malignant. Paper read at the 16*th Congress of the International Society of Urology* Amsterdam (1973). (Not published).
32. W. J. Catalona, P. B. Chretien and E. E. Trahan, Abnormalities of cell-mediated immunocompetence in genito-urinary cancer. *J. Urol.* **3,** 229 (1974).
33. G. Williams and J. E. Castro, The diagnostic and prognostic significance of delayed hypersensitivity skin testing in patients with urological cancer. *Brit. J. Urol.* **47,** 97 (1975).
34. M. R. G. Robinson, L. S. Nakhla, R. Shearer, P. Trott and C. C. Rigby, Lymphocyte transformation in carcinoma of the prostate. *Proc.* 16*th Cong. of the International Society of Urology.* Vol. 2, p. 177. Amsterdam (1973).
35. A. P. McLaughlin, W. O. Kessler, K. Triman and R. F. Gittes, Immunologic competence in patients with urologic cancer. *J. Urol.* **111,** 233 (1974).
36. E. O. Gursel, M. R. Megalli and R. J. Veenema, Serum Immunoglobulins in patients with prostatic cancer. *Urol. Res.* **1,** 145 (1973).
37. R. Lockwood, M. Sukhia, R. N. Basu, M. C. Stewardson, J. Sheard and M. R. G. Robinson, Serum Immunoglobulins before and during estrogen treatment of carcinoma of the prostate. *Communication to British Association of Urological Surgeons* (1974). (Not published).

38. W. J. CATALONA, Effects of chemotherapy for prostatic carcinoma on T. Lymphocyte levels. *J. Urol.* **112,** 802 (1974).
39. P. ARMITAGE and E. A. GEHAN, Statistical methods for the identification and use of prognostic factors. *Int. J. Cancer* **13,** 16 (1974).
40. S. GEORGE and M. M. DESU, Planning the size and duration of a clinical trial studying the time to some critical event. *J. chron. Dis.* **27,** 15 (1974).
41. D. P. BYAR, R. HUSE, J. C. BAILAR and the VETERANS ADMINISTRATION COOPERATIVE UROLOGICAL RESEARCH GROUP, An exponential model relating censored survival data and concomitant information for prostatic cancer patients. *J. nat. Cancer Inst.* **52,** 321 (1974).
42. S. BAYARD, R. GREENBERG, D. SHOWALTER and D. BYAR, Comparison of treatments for prostatic cancer using an exponential type life model relating survival to concomitant information. *Cancer Chemother. Rep.* **58,** 845 (1974).
43. D. F. GLEASON, G. T. MELLINGER and the VETERANS ADMINISTRATION COOPERATIVE UROLOGICAL RESEARCH GROUP, Prediction and prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J. Urol.* **111,** 58 (1974).
44. J. C. BAILAR, D. P. BYAR and the VETERANS ADMINISTRATION COOPERATIVE UROLOGICAL RESEARCH GROUP, Oestrogen treatment for cancer of the prostate. *Cancer (Philad.)* **26,** 257 (1970).
45. J. D. FERGUSSON and W. F. HENDRY, Pituitary irradiation in advanced carcinoma of the prostate: analysis of 100 cases. *Brit. J. Urol.* **43,** 514 (1971).
46. G. P. MURPHY, G. REYNOSO, R. SCHOONEES, S. GAILANI, R. BOURKE, G. M. KENNY, E. A. MIRAND and D. S. SCHALCH, Hypophysectomy and adrenalectomy for disseminated prostatic cancer. *J. Urol.* **105,** 817 (1971).
47. J. G. SMART, The use of P^{32} in the treatment of severe pain from bone metastases of carcinoma of the prostate. *Brit. J. Urol.* **37,** 139 (1965).
48. E. C. K. TONG and P. FINKELSTEIN, The treatment of prostatic bone metastases with parathormone and radio-active phosphorus. *J. Urol.* **109,** 71 (1973).
49. G. R. RAY, J. R. CASSADY and M. A. BAGSHAW, Definitive radiation therapy of carcinoma of the prostate: a report on 15 years of experience. *Radiology* **106,** 407 (1973).
50. C. E. CARLTON Jr., F. DAWOOD, P. Hudgins and J. SCOTT Jr., Irradiation treatment of carcinoma of the prostate: a preliminary report based on 8 years of experience. *J. Urol.* **108,** 924 (1972).
51. R. H. FLOCKS, H. D. KERR, H. ELKINS and D. CULP. Treatment of carcinoma of the prostate by interstitial radiation with radio-active gold (Au^{198}): a preliminary report. *J. Urol.* **68,** 510 (1952).
52. R. H. FLOCKS, C. M. K. NELSON, D. L. BOATMAN, Perineal cryosurgery for carcinoma of the prostate. *J. Urol.* **108,** 933 (1972).
53. G. BRULE, S. J. ECKHARDT, T. C. HALL and A. WINKLER, Prostatic Cancer in *Drug Therapy of Cancer* p. 147. World Health Organization, Geneva (1973).
54. M. FOX, The effect of cyclophosphamide on some urinary tract tumours. *Brit. J. Urol.* **37,** 399 (1965).
55. R. H. FLOCKS, Carcinoma of the prostate, *J. Urol.* **101,** 741 (1969).
56. L. PERSKY, K. GUERRIER, R. RABIN and D. J. ALBERT, Mithramycin and metastatic carcinoma of the prostate. *J. Urol.* **104,** 884 (1970).
57. G. JONSSON and B. HOGBERG, Treatment of advanced prostatic carcinoma with Estracyt: A preliminary report. *Scand. J. Urol. Nephrol.* **5,** 103 (1971).
58. A. MITTLEMAN, S. K. SHUKLA, K. WELVAART and G. P. MURPHY, Oral estramustine phosphate (NSC-89199) in the treatment of advanced (Stage D) carcinoma of the prostate. *Cancer Chemother. Rep.* **59,** 219 (1975).

Europ. J. Cancer Vol. 12, pp. 945–950. Pergamon Press 1976. Printed in Great Britain

General Papers

Humoral Immunity in Malignant Skin Melanoma. Isolation of Melanoma Specific IgG from Melanoma Metastases*

E. KRISTENSEN†, E. LANGVAD‡ and R. REIMANN§
With the technical assistance of VIBEKE AHRENKIEL
†‡*Fibiger Laboratory*¶, *Ndr. Frihavnsgade* 70, *DK*-2100 *Copenhagen Ø, Denmark.*
§*From the Department of Plastic Surgery, The Finsen Institute, Copenhagen, Denmark.*

Abstract—*Three M potassium chloride extracts of malignant melanoma, hypernephroma and colonic carcinoma were found to consume complement in the absence of patient sera. Treatment of the tumour extracts with papain abolished the anti-complementary effect. IgG and IgA present in tumour extracts were identified in cross-over electrophoresis by means of monospecific antibodies. Purified melanoma-derived immunoglobulins were reacted in complement fixation test against the 3 M KCl extract of an established melanoma cell line with positive result.*

The presence of specific antimelanoma IgG as well as native common melanoma antigen was thus established. It is concluded that specific antimelanoma antibodies are present within the clinical tumour whereas the actual localization of these antibodies, i.e., whether present on tumour cell membranes or on infiltrating lymphocytes, remains an open question.

Sera from 93 patients suffering from stage I and II skin melanoma were assayed for circulating antibodies against antigen present in the extract of an established melanoma cell line. A positive CFT was established in 27% of the patients. The discrepancy between this finding and that of other investigators evades good explanation.

INTRODUCTION

In vitro studies have demonstrated humoral immunity against tumour-associated antigens in patients suffering from malignant skin melanoma (m.s.m.). Indirect immunofluorescence and complement-fixation tests have revealed the presence of circulating antibodies against a common cytoplasmatic antigen [1, 2], and membrane-bound antigens claimed to be unique for the individual patient [3], as well as membrane antigens shared by melanoma patients [4–7].

Accepted 9 August 1976.

*This work was supported by grants from the Danish Medical Research Council No. 512-5274 and 512-3547.

Request for reprints should be addressed to E. Kristensen.

¶Under the auspices of the Danish Cancer Society.

The results obtained are reported to be correlated to the stage of disease [7–9]. Thus, circulating antibodies were present in localized disease (stage I–II) but absent in case of dissemination. For this reason, the antibodies have been attributed the effect of preventing spread [8, 9].

MATERIAL AND METHODS

Antigen and immunoglobulin preparation

Cultured material: two mycoplasma-free established melanoma cell lines RPMI 7931 and mel-1 (kindly provided by Dr. O'Toole) served as a source of antigen. The cells were cultivated in roller bottles using a standard medium composed of 20% fetal calf serum and 80% Eagles Minimum Essential medium with a fourfold increase of the concentrations of the

vitamins and glutamine, a twofold increase of the concentrations of the essential amino acids, and a 35% increase of the glucose concentration. Penicillin 25 i.u./ml and streptomycin 25 μg/ml were added.

The melanoma cells were scraped off the roller bottles, washed 3 times in PBS and resuspended in 3 volumes of 3M KCl in phosphate buffered saline (pH 6·9). Extraction was carried out on a rotary shaker for 16 hr.

Tumour material: liver metastases from malignant melanoma were obtained at autopsy within 12 hr post mortem.

Extraction procedures: all procedures were carried out in the cold. Tumour tissue was cut into small pieces, rinsed in water and homogenized in 3 volumes of 3 M KCl in phosphate buffered saline (pH 6·9) by means of an Ultra Turrax (Janke and Kunkel KG, Staufen/Br., Germany).

The resulting homogenate was placed on a magnetic stirrer and extracted for 16 hr. The extract was centrifuged (80,000 *g*/30 min.) and dialyzed against physiological saline for 16 hr. The supernatant was concentrated by placing the dialysis bags in dry Aquacide II (Calbiochem). Protein determination was carried out according to Lowry *et al.* [10] and the antigen preparation was stored at −80°C.

Isolation of immunoglobulins (Ig) from tumour extract: Immunoglobulins were precipitated by the addition of 0·25 g ammonium sulphate per ml. Subsequent purification of immunoglobulins was performed according to Inglid and Harboe [11]. The method implies that IgM is lost.

Antisera

For immunoelectrophoresis heterologous antisera against tumour and cell extracts were raised in rabbits. Antigen extracts were emulsified in equal volumes of Freund's incomplete adjuvant. Fifty micro litre doses containing 2 mg of protein were administered at multiple intradermal sites every other week for 3 months when bleeding was commenced. The immunoglobulins were purified as above.

Patient and control sera

Ninety-three patients suffering from malignant skin melanoma in clinical stages I and II. Stage I: localized primary tumour. Stage II: primary tumour and/or regional lymph node metastases with or without cutaneous satellite metastases. For control purposes sera from 19 bladder cancer patients and 5 breast cancer patients in different clinical stages as well as sera from 16 healthy persons were investigated. Serum samples were obtained preoperatively.

Test system

Immunoelectrophoresis was performed as described by Axelsen and colleagues [12].

Complement-fixation test (CFT) was performed according to Sever [13] using the Cooke microtiter(R) system.

All tests were carried out in microplates with U-shaped wells (Cooke microtiter(R) system, No. 24A). Isotonic Veronal buffered saline was used as diluent. The antigen preparations were used in dilutions giving protein concentrations varying from 10–50 mg/ml.

The hemolytic system: a 0·6% suspension of three times washed sheep red blood cells (SRBC), sensitized by incubation with rabbit anti-SRBC hemolysin (Pasteur Institut, Paris).

The human sera (control and patient) were used either immediately or after storage at −80°C. Prior to use, sera were inactivated at 56°C for 30 min.

Each of the components involved in the CFT was added in 25 μl volumes giving a final volume of 100 μl. The undiluted sera, antigen and 1.5 units of 100% hemolytic complement were added to the plates, which were sealed and placed at 4°C for 18 hr. Following incubation the hemolytic system was added, plates were sealed again and incubated for 30 min at 37°C. After brief shaking, the results were read and scored from 0 to 4, scores 3 and 4 being considered positive.

Results

Crossed immunoelectrophoresis of the antigen preparation derived from melanoma metastases revealed the presence of a large number of proteins. Immunoglobulins IgA and IgG as well as complement component C3 were identified by monospecific antibodies (Hoechst). These proteins were present in considerable amounts.

In preliminary experiments, the 3 M KCl antigen preparation derived from fresh tumour material was found to bind C′ in the absence of serum. A similar C′ consumption could be demonstrated for 3 M KCl extracts of colonic carcinoma and hypernephroma (Table 1). Using the mercury papain extraction procedures as described by Baldwin *et al.* [14],the same tumours yielded antigen preparations which were anticomplementary only at high concentrations (Table 1) of hypernephroma and colonic tumour extracts, whereas melanoma extracts were not anticomplementary.

In contrast, 3 M KCl extracted antigen preparations derived from the established melanoma cell line (RPMI 7931) did not consume C′ in the absence of positive melanoma sera (Table 2). This antigenic material therefore was chosen as the standard antigen.

The immunoglobulins (IgG and IgA) isolated and purified from melanoma metastases showed no anticomplementary effect. CFT was performed with cell line (RPMI 7931) derived antigen and the purified immunoglobulins. Positive results were obtained in Ig dilutions up to 1/16 (Table 2). Thus, the specificity and cross-reactivity of tumour derived Ig was demonstrated.

The results obtained with patient sera are shown in Table 3. It appears from the table that the highest level of positive results (27%) was obtained at protein concentrations of 24–33 mg/ml. Using a lower protein concentration (10–15 mg/ml) either no positive results or the same number of positive results were found among melanoma patients and patients suffering from other malignancies (16–20 mg/ml). No positive results were seen with sera from healthy donors.

Discussion

Studies on humoral immunity (h.i.) in human malignant skin melanoma have yielded conflicting results. Immunofluorescence studies repeatedly have demonstrated the presence of circulating antibodies in up to 100% of mela-

Table 1. *Anticomplementary effect of tumour extracts**

Tumour type	Extract. procedure	Antigen dilutions 1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Hypernephroma	KCl	4	4	4	3	3	2	0	0
	Papain	3	0	0	0	0	0	0	0
Colonic carcinoma	KCl	4	4	4	4	3	2	0	0
	Papain	3	0	0	0	0	0	0	0
Melanoma	KCl	4	NT	3	NT	NT	NT	0	NT
	Papain	0	0	0	0	0	0	0	0

NT: not tested.
*The effect is abolished after treatment with papain.

Table 2. *Specificity of antigen and melanoma derived immunoglobulins*

	Ig Dilutions 1/2	1/4	1/8	1/16	1/32	1/64	1/128
Antigen (18 mg/ml)	0	0	0	0	0	0	0
Ig (eluted)	0	0	0	0	0	0	0
Antigen (18 ml/ml) + Ig	4	3	3	3	2	2	2

The specificity of 3M KCl extract of an established melanoma line (RPMI 7931) used as antigen source in a complement fixation test (18 mg/ml). The antibody had been eluted from melanoma metastases. It appears that neither antigen nor antibody exhibited anticomplementary effect, whereas this was the case when antigen as well as antibody was present.

Table 3. *Incidence of antibody positive sera evalu ated by complement fixation test*

Patient groups	*Antigen concentration (protein conc. mg/ml). 10–15 mg/ml no. pos./no. tested		16–20 mg/ml no. pos./no. tested		24–33 mg/ml no. pos./no. tested	
Malignant skin melanoma (SI-II)	0/40	0%	4/39	10%	8/30	27%
Other malignancies	0/8	0%	4/18	22%	1/11	10%
Persons without malignant disease	0/5	0%	0/7	0%	0/10	0%

*Antigen preparations: 3M KCl extract of established melanoma cell line (RPMI 7931).

noma patients with localized disease [7–9]. Furthermore, in the Lewis study [9] a clear-cut correlation between the disappearance of h.i. and dissemination of disease was reported. To some extent these observations are supported by the findings of Morton *et al.* [7] using CFT. In the study by Nairn *et al.* [15] as well as that by Wood and Barth [16] the presence of h.i. has been demonstrated much less consistently and the correlation between stages of disease and h.i. has not been confirmed by these authors. Furthermore, the concomitant existence of antibodies specific for a common cytoplasmatic antigen as well as antibodies with specificities directed against individual membrane antigens has not been confirmed by other workers [4–6, 15]. In these studies, the membrane bound antigen also was found to be one shared by all melanomas.

In the present study, h.i. could be demonstrated only in 27% of stage I and II patients. Similar discrepancies of findings appear in studies of h.i. in sarcoma patients. In extensive immunofluorescence studies in sarcoma, Laperevotte *et al.* [17] were unable to demonstrate h.i. in any single case. This finding contrasted with the overwhelming number of positives demonstrated by Morton and colleagues [7].

The antigen preparation used in the present CFT study was shown to react with immunoglobulins eluted from melanoma metastases, thus ensuring the presence of native melanoma antigen. Furthermore, the 3 M KCl extract of melanoma metastases was anticomplementary. This was shown to be caused by the presence of specific immunoglobulins in the clinical tumour. Treatment with papain, resulting in the removal of IgG Fc portions, abolished the anticomplementary effect of the antigen preparation. Immunoelectrophoretic analysis of 3 M KCl extracts indicated the presence of similar proteins in cell line derived as well as in tumour derived antigen preparations. It appears, therefore, that cultured cells as well as the clinical melanoma material contained the relevant native antigen recognized by circulating specific antibodies *in vivo* as well as *in vitro*. It may be concluded, therefore, that the differences in antigen extraction procedures used in this study and in the study by Eilber and Morton [18] may not be held responsible for the discrepancies observed. The CFT technique used in this study and in that by Morton *et al.* [7, 18] appears to be identical in all respects. It is not reasonable, therefore, to assume that the contrasting findings may have been caused by methodological variations resulting in lack of sensitivity.

Lewis and co-workers claim that melanoma antibodies disappear from the circulation prior to or concomitant with dissemination [9]. According to these workers, the disappearance of circulating antibodies could not be ascribed to the adsorption of the antibodies to the tumour. In contrast to other investigators [19–21], they were unable to demonstrate the presence of antibodies on the cell membranes of tumour material by means of direct immunofluorescence [22, 23]. In contrast, the present observations revealed considerable amounts of antibodies with antimelanoma specificity in melanoma metastases. This does not necessarily contradict the observations of Lewis *et al.* It may not be concluded from the present study whether the antibodies eluted from melanoma metastases are derived from the tumour cell membranes or from immunoglobulin carrying cells infiltrating the tumour [24, 25]. The fact remains, however, that apparently tumour-specific antibodies may be demonstrated in a large variety of tumours [26–28].

It appears odd, therefore, that the Lewis studies were unable to demonstrate *in vivo* membrane bound immunoglobulin by direct immunofluorescence. In the same study [22], patient sera reacted *in vitro* with autologous tumour cells giving a positive indirect immunofluorescence. No explanation is offered to elucidate why humoral antibodies should exhibit homing on the autologous cell membranes *in vitro* but not *in vivo*.

In the present study, circulating antibodies in amounts detectable by CFT were present in only 27% of patients. Therefore, the possibility exists that the tumour exerts a constant 'soaking up' effect resulting in the intratumoral accumulation of specific antibodies in the face of low or nondemonstrable levels in the circulation [7, 27].

REFERENCES

1. M. N. Muna, S. Marcus and C. Smart, Detection by immunofluorescence of antibodies specific for human malignant melanoma cells. *Cancer (Philad.)* **23,** 88 (1969).
2. F. H. Oettgen, A. Tadao, J. O. Lloyd, A. E. Boyse, E. De Harven and M. G. Mills, Suspension culture of a pigment-producing cell line derived from a human malignant melanoma. *J. nat. Cancer Inst.* **41,** 827 (1968).

3. M. G Lewis and T M. Phillips. The specificity of surface membrane immunofluorescence in human malignant melanoma. *Int. J. Cancer* **10,** 105 (1975).
4. G. Fossati, M. I. Colnaghi, G. Della Porta, N. Cascinelli and V. Veronesi. Cellular and humoral immunity against human malignant melanoma. *Int. J. Cancer* **8,** 344 (1971).
5. M. Rhomsdahl and I. S. Cox, Immunofluorescent studies of antibodies against human malignant melanoma. *Surg. Forum* **10,** 126 (1969).
6. D. L. Morton, R. A. Malmgren, E. C. Holmes and A. S. Ketcham, Demonstration of antibodies against human malignant melanoma by immunofluorescence. *Surgery* **64,** 233 (1968).
7. D. L. Morton, F. R. Eilber and R. A. Malmgren, Immune factors in human cancer: Malignant melanomas, skeletal and soft tissue sarcomas. *Progr. exp. Tumour Res.* **14,** 25 (1970).
8. G. Hamilton Fairley, Evidence for antigenicity in human tumours with reference to both melanoma and acute leukaemia. *Brit. med. J.* **4,** 483 (1970).
9. M. G. Lewis, R. L. Ikonopisov, R. C. Nairn, T. M. Phillips, G. Hamilton Fairley, D. C. Godenham and P. Alexander, Tumour specific antibodies in human malignant melanoma and their relationship to the extent of the disease. *Brit. med. J.* **3,** 547 (1969).
10. O. H. Lowry, M. J. Roseborough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 365 (1951).
11. A. Ingild and N. Harboe, Immunization, isolation of immunoglobulins, estimation of antibody titre. In *A Manual of Quantitative Immunoelectrophoresis.* (Edited by N. H. Axelsen, J. Kroell and B. Weeke) p. 184. Universitetsforlaget, Oslo (1973).
12. N. H. Axelsen, J. Kroell and B. Weeke, *A Manual of Quantitative Immunoelectrophoresis.* Universitetsforlaget, Oslo (1973).
13. J. S. Sever, Application of a microtechnique to viral serological investigations. *J. Immunol.* **88,** 320 (1962).
14. R. W. Baldwin, M. J. Embelton and M. R. Price, Inhibition of lymphocyte cytotoxicity for human colon carcinoma by treatment with solubilized tumour membrane fractions. *Int. J. Cancer* **12,** 84 (1973).
15. R. C. Nairn, A. P. P. Nind, E. P. G. Geili, D. J. Davies, J. H. Little, N. C. David and R. H. Whitehead, Antitumor immunoreactivity in patients with malignant melanoma. *Med. J. Aust.* **I,** 397 (1972).
16. W. G. Wood and R. Barth, Immunofluorescent studies of the serologic reactivity of patients with malignant melanoma against tumour-associated cytoplasmatic antigens. *J. nat. Cancer Inst.* **53,** 309 (1974).
17. I. Laprevotte, J. C. Chuat, A. M. L'Hirondel, C. Bernard, J. Pries and M. Biron, A search for antibodies against human sarcoma cells in patients' sera by indirect immunofluorescence on fixed cells. *Europ. J. Cancer* **11,** 679 (1975).
18. F. R. Eilber and D. L. Morton, Sarcoma-specific antigens: Detection by complement fixation serum from sarcoma patients. *J. nat. Cancer Inst.* **44,** 651 (1970).
19. M. Ran and I. P. Witz, Tumour-associated immunoglobulins. The elution of IG_2 from mouse tumours. *Int. J. Cancer* **6,** 361 (1970).
20. K. Irie, R. F. Irie and D. L. Morton, Evidence for *in vivo* reaction of antibody and complement to surface antigens of human cancer cells. *Science* **186,** 454 (1974).
21. R. J. Ablin, M. J. Gonder and W. Soanes, Immunohistologic studies of carcinoma of the prostate. *Oncology* **29,** 329 (1974).
22. M. G. Lewis, Immunological studies in patients with malignant melanoma—The role of circulating antibodies. *Proc. Int. Cancer Conf. Melanoma and Skin Cancer,* Sydney. V. C. N. Blight, Sydney (1972).
23. M. G. Lewis, J. W. Proctor, D. M. P. Thomson, G. Rowden and T. M. Phillips, Cellular localization of immunoglobulin within human malignant melanomata. *Brit. J. Cancer* **23,** 260 (1976).
24. B. Johansson and A. Ljunggvist, Localization of immunoglobulins in urinary bladder tumours. *Acta path. microbiol. scand.* Sect. A, **82,** 559 (1974).
25. W. A. Kopf, I. Silberberg and N. S. Cooper, Immunohistochemical study of human malignant melanoma for the presence of gamma globulin. *J. invest. Derm.* **47,** 83 (1966).

26. E. Langvad, H. Hydén, H. Wolf and N. Kroiegaard, Extracorporeal immunoadsorption of circulating specific serum factors in cancer patients. *Brit. J. Cancer* **32,** 680 (1975).
27. R. K. Gupta and D. L. Morton, Suggestive evidence for *in vivo* binding of specific antitumour antibodies of human melanomas. *Cancer Res.* **35,** 58 (1975).
28. S. Thunald, O. Tönder and O. Larsen, Immunoglobulins in eluates of malignant human tumours. *Acta path. microbiol. scand.*, Sect. A, Suppl. **236,** 97 (1973).

Europ. J. Cancer Vol. 12, pp. 951–958. Pergamon Press 1976. Printed in Great Britain

Plasma Dehydroepiandrosterone and Androsterone Sulphates, Androstenedione and Urinary Androgen Metabolites in Normal British and Japanese Women*

D. Y. WANG†, J. L. HAYWARD‡, R. D. BULBROOK†, S. KUMAOKA§, O. TAKATANI§, O. ABE¶ and J. UTSUNOMIYA‖

†*Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX,* ‡*ICRF Breast Cancer Unit, Guy's Hospital, London, SE1 9RT,* §*National Cancer Center, Tokyo,* ¶*Keio University, Tokyo,* ‖*Tokyo Medical and Dental University, Tokyo*

Abstract—*The urinary excretion of total 11-deoxy-17-ketosteroids (11-DKS), aetiocholanolone, androsterone and dehydroepiandrosterone (DHA) and the plasma concentration of dehydroepiandrosterone sulphate (DS), androsterone sulphate (AS) and androstenedione has been measured in normal Japanese and British adolescent, pre-menopausal, menopausal and post-menopausal women.*

The mean excretion of 11-DKS, androsterone and aetiocholanolone in Japanese women is significantly less than that in British women. Similarly, the concentration of plasma DS and androstenedione is significantly lower in Japanese than British women. Although not statistically significant, mean plasma AS was lower in Japanese than British women in all groups studied. The ratio of urinary androsterone (5α) to aetiocholanolone (5β) was significantly higher in all groups of Japanese women studied compared with British women, except in the adolescent group.

The amounts of all plasma and urinary steroids were significantly and negatively correlated with age in both races, except for urinary DHA in both races, aetiocholanolone in British women and 5α/5β ratio in Japanese women.

The relation between androgen status and the aetiology of breast cancer is discussed.

INTRODUCTION

WE HAVE been unable to find any convincing differences in the plasma levels of oestradiol-17β, oestrone or progesterone in adult normal British and Japanese women. The amounts of urinary metabolites in these steroid hormones were also similar in the two races [1]. It has been claimed that British women with a high risk of breast cancer excrete subnormal amounts of urinary androgen up to 10 years before diagnosis [2]. Since the purpose of the present investigations was to examine the possibility that the low incidence of breast cancer in Japan, compared with that in Western countries, was related to differences in endocrine function, the androgens were an obvious choice of investigation.

Accepted 28 June 1976.

*This study was supported in part by a grant for Cancer Research from the Ministry of Health and Welfare, Japan.

MATERIAL AND METHODS

The specimens used in this study are from the same subjects as described in a previous paper [1].

Urinary androgen metabolites were measured by the method of Bulbrook *et al.* [3] and plasma dehydroepiandrosterone and androsterone sulphates (DS and AS) by the method of Wang *et al.* [4]. Plasma androstenedione was measured by radioimmunoassay using a rabbit antiserum against androstenedione conjugated to bovine serum albumin.

The concentration of plasma DS and AS and urinary 17-oxosteroids are not related to the phase of the menstrual cycle and for this reason values at any time in the cycle have

been taken for women in the adolescent and premenopausal group for statistical analysis. Plasma androstenedione levels have been reported to change over the cycle [5–7], but in the present study there was no difference between the plasma androstenedione concentrations of women in the luteal phase of the cycle and those for women not in this phase. All values were therefore pooled for statistical analysis (Table 1). The lack of variation with time in the menstrual cycle is probably due to the fact that only single plasma specimens were obtained and the large between-person variation.

Although Table 1 shows the arithmetic means for the data on British and Japanese women, the statistical comparison has been performed after logarithmic transformation.

RESULTS

1. *Plasma DS, AS and androstenedione*

The plasma concentrations of DS, AS and androstenedione are generally higher in British than in Japanese women (Table 1). This difference is illustrated for DS and androstenedione in Figs. 1 and 2, where the individual

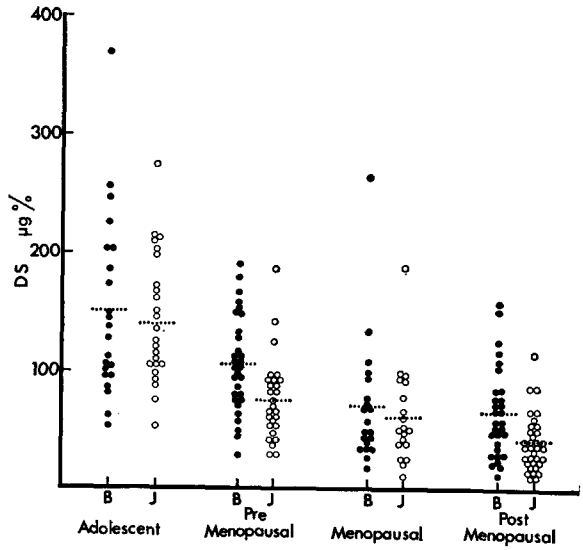


Fig. 1. Concentrations of plasma DS in Japanese and British women. B. and J. represent British and Japanese women, respectively. The dotted lines indicate the mean values of plasma DS.

Table 1. Mean levels of plasma and urinary steroids in Japanese and British women

Compound	Adolescent		Pre-Menopausal		Menopausal		Post-Menopausal	
	J	B	J	B	J	B	J	B
Plasma DS (μg%)	139 ± 53 (24)	150 ± 76 (22)	75 ± 35 (27)	105 ± 41 (30)	61 ± 40 (19)	70 ± 54 (20)	41 ± 22 (34)	65 ± 37 (30)
Plasma AS (μg%)	65 ± 38 (24)	77 ± 53 (22)	26 ± 13 (27)	34 ± 18 (30)	25 ± 15 (19)	29 ± 27 (20)	21 ± 12 (34)	28 ± 18 (30)
Plasma Androstenedione (all subjects)	155 ± 52 (25)	180 ± 84 (23)	99 ± 30 (27)	114 ± 27 (29)	64 ± 27 (18)	87 ± 27 (20)	58 ± 29 (31)	89 ± 21 (30)
Androstenedione (Luteal) (ng/100 ml)	133 ± 36 (10)	190 ± 125 (6)	108 ± 29 (17)	116 ± 27 (25)	—	—	—	—
Urinary 11-DKS (mg/day)	3·51 ± 2·24 (25)	4·25 ± 2·03 (22)	2·79 ± 1·24 (28)	4·09 ± 1·90 (30)	2·13 ± 0·83 (19)	2·44 ± 1·08 (19)	1·54 ± 0·83 (34)	2·79 ± 1·39 (29)
Androsterone	1·56 ± 1·01 (24)	2·14 ± 1·26 (21)	1·30 ± 0·59 (28)	1·75 ± 0·87 (30)	1·12 ± 0·49 (19)	1·09 ± 0·57 (18)	0·78 ± 0·44 (34)	1·16 ± 0·57 (29)
Aetiocholanolone	1·45 ± 0·80 (24)	1·81 ± 1·04 (21)	0·97 ± 0·41 (28)	1·63 ± 0·65 (30)	0·86 ± 0·36 (19)	1·09 ± 0·48 (18)	0·58 ± 0·39 (34)	1·41 ± 0·74 (29)
DHA	0·30 ± 0·31 (24)	0·31 ± 0·49 (21)	0·48 ± 0·54 (28)	0·70 ± 0·82 (30)	0·27 ± 0·28 (19)	0·20 ± 0·24 (18)	0·17 ± 0·29 (34)	0·19 ± 0·25 (29)
$5\alpha/5\beta$	1·19 ± 0·66 (24)	1·42 ± 0·80 (21)	1·40 ± 0·52 (28)	1·08 ± 0·32 (30)	1·43 ± 0·59 (19)	1·00 ± 0·27 (18)	1·46 ± 0·56 (34)	0·91 ± 0·42 (29)

All values are given as means ± S.D. The numbers in parentheses are the numbers of subjects studied. $5\alpha/5\beta$ is the ratio of androsterone to aetiocholanolone.
B and *J* represent British and Japanese women, respectively.

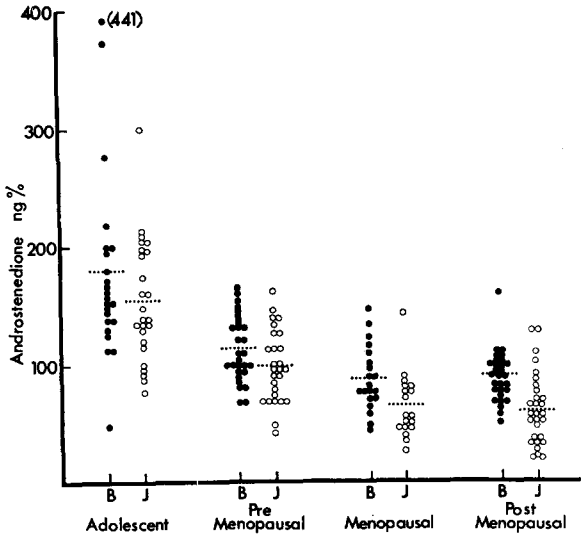


Fig. 2. Concentration of plasma androstenedione in Japanese and British women. B. and J. represent British and Japanese women, respectively. The dotted lines indicate the mean values of plasma androstenedione.

values are shown for the various menstrual status groups studied. In both races the levels of all the plasma steroids are highly significantly correlated with age (Table 2). A comparison of the mean levels of these steroids adjusted for age shows that DS ($F = 13{\cdot}0$; $P < 0{\cdot}001$) and androstenedione ($F = 24{\cdot}0$; $P < 0.001$) are significantly lower in Japanese compared with British women. There was no significant difference between the age-adjusted AS levels between the races although the mean level of AS was consistently lower in Japanese women compared with British women in all age groups studied (Table 1).

2. *Urinary androgen metabolites*

Except for urinary DHA the mean amounts of all urinary androgen metabolites excreted by Japanese women is consistently lower for the four groups examined (Table 1). As an example of these results the individual values of aetiocholanolone are shown in Fig. 3 and this is typical of the results obtained for androsterone and total 11-deoxy-17-ketosteroids (11-DKS). The amounts of urinary androgen metabolites excreted are significantly correlated with age in both races, except for DHA in both British and Japanese and aetiocholanolone in British women. Comparison of the amounts of urinary androgen metabolites, adjusted for age, showed that British women excreted significantly more total 11-DKS ($F = 21{\cdot}5$; $P < 0{\cdot}001$), aetiocholanolone ($F = 40{\cdot}6$; $P < 0{\cdot}001$) and androsterone ($F = 11{\cdot}8$; $P < 0{\cdot}001$) than

Table 2. Regression equations for age (x) on plasma or urinary steroids (y) for normal Japanese and British women

	log *y*	Intercept	Slope	*r*	*N*	*P*
Japanese	DS	2·35	−0·0128	−0·70	104	< 0·001
	AS	1·92	−0·0115	−0·60	104	< 0·001
	Δ4	2·43	−0·0119	−0·72	101	< 0·001
	11DKS	3·66	−0·0079	−0·50	106	< 0·001
	ANDRO	3·29	−0·0068	−0·43	105	< 0·001
	AETIO	3·29	−0·0090	−0·54	105	< 0·001
	DHA	2·40	−0·0068	−0·17	105	N.S.
	5α/5β*	1·16	0·0048	0·14	105	N.S.
British	DS	2·29	−0·0090	−0·52	102	< 0·001
	AS	1·90	−0·0099	−0·44	102	< 0·001
	Δ4	2·33	−0·0069	−0·65	102	< 0·001
	11DKS	3·68	−0·0049	−0·32	100	< 0·01
	ANDRO	3·40	−0·0066	−0·40	98	< 0·001
	AETIO	3·26	−0·0031	−0·19	98	N.S.
	DHA	−0·47	0·1290	0·07	98	N.S.
	5α/5β	1·55	−0·0108	−0·33	98	< 0·001

*The values for 5α/5β ratios have not been transformed to logarithms.

The abbreviations used are as follows:

Δ^4 = androstenedione
11DKS = 11-deoxy-17-ketosteroids
ANDRO = androsterone (5α)
AETIO = aetiocholanolone (5β)

Plasma DS and AS are measured as μg%.
Plasma Δ^4 measured as ng%.
Urinary ketosteroids measured as μg/day.

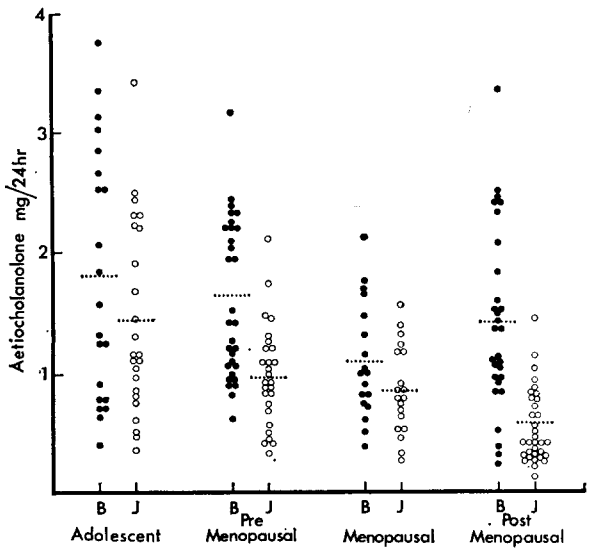


Fig. 3. Urinary execretion of aetiocholanolone in Japanese and British women. B. and J. represent British and Japanese women, respectively. The dotted lines indicate the mean values for aetiocholanolone excretion.

Japanese women. The general levels of DHA were not significantly different in the two races.

3. *Relationship between plasma and urinary androgen metabolites*

In the Japanese population studied the quantity of plasma DS, AS and androstenedione was significantly correlated with the excretion of all the urinary androgen metabolites except for the pair plasma AS and urinary DHA (Table 3). A similar situation occurs for British women except the amount of plasma androstenedione was not significantly correlated with DHA (Table 3). Furthermore, the concentration of androstenedione is significantly correlated with the plasma level of either DS or AS in both races.

However, the precise relationship between plasma steroids and urinary androgen metabolites differs in British and Japanese women.

Table 3. Regression equations for plasma and urinary steroids in normal Japanese and British women

Status	*x*	*y*	Intercept	Slope	*r*	*N*	*P*
Japanese	DS	11-DKS	807	21·6	0·71	93	< 0·001
normal	DS	Aetio	387	6·8	0·59	92	< 0·001
	DS	Andro	601	7·4	0·52	92	< 0·001
	DS	DHA	117	2·6	0·29	92	< 0·01
	AS	11-DKS	1231	35·6	0·56	93	< 0·001
	AS	Aetio	542	10·4	0·43	92	< 0·001
	AS	Andro	728	12·7	0·42	92	< 0.001
	AS	DHA	274	0·7	0·04	92	N.S.
	Δ^4	11-DKS	656	19·7	0·64	100	< 0·001
	Δ^4	Aetio	267	7·4	0·62	98	< 0·001
	Δ^4	Andro	419	8·2	0·57	98	< 0·001
	Δ^4	DHA	158	1·7	0·23	98	< 0·05
	Δ^4	DS	4·9	0·79	0·77	99	< 0·001
	Δ^4	AS	0·2	0·36	0·66	99	< 0·001
British	DS	11-DKS	1281	22·1	0·74	97	< 0·001
normal	DS	Aetio	709	8·4	0·68	93	< 0·001
	DS	Andro	501	10·6	0·70	93	< 0·001
	DS	DHA	136	2·5	0·26	93	= 0·01
	AS	11-DKS	2277	29·3	0·57	97	< 0·001
	AS	Aetio	1116	10·0	0·45	93	< 0·001
	AS	Andro	730	21·0	0·74	93	< 0·001
	AS	DHA	375	−0·2	−0·01	93	N.S.
	Δ^4	11-DKS	1767	14·3	0·46	99	< 0·001
	Δ^4	Aetio	911	5·1	0·39	97	< 0·001
	Δ^4	Andro	574	8·3	0·52	97	< 0·001
	Δ^4	DHA	260	1·0	0·11	97	N.S.
	Δ^4	DS	20·1	0·65	0·62	101	< 0·001
	Δ^4	AS	2·0	0·33	0·53	101	< 0·001

The abbreviations used are as follows:
Δ^4 = androstenedione
11-DKS = 11-deoxy-17-ketosteroids.
Andro = androsterone
Aetio = aetiocholanolone
Plasma DS and AS are measured as μg%.
Plasma Δ^4 measured as ng%.
Urinary ketosteroids measured as μg/day.

An example can be seen in Table 3 where the regressions of plasma DS on urinary 11-DKS are given for the two populations. While the regression lines are parallel, that for the British is significantly higher than that for the Japanese. In other words, for a given plasma level of DS, less 11-DKS is found in the urine of the Japanese women than in the British women.

4. *The androsterone/aetiocholanolone ratio* ($5\alpha/5\beta$ *ratio*)

The ratio of urinary androsterone and aetiocholanolone is affected by thyroid function and it has been reported that the ratio in Japanese women differs from that in British women [8]. The results obtained are shown in Table 1 and Fig. 4.

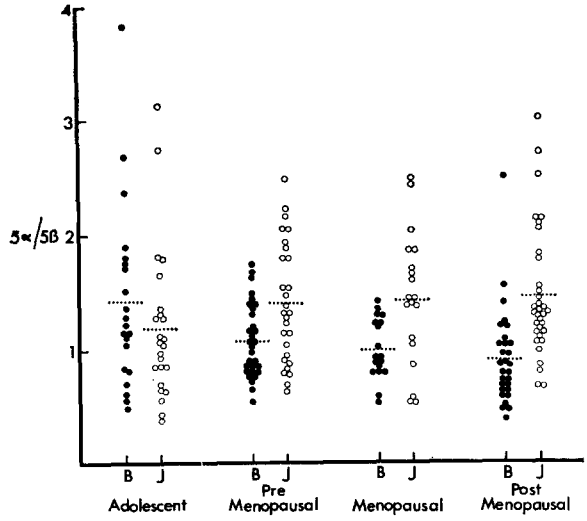


Fig. 4. *Ratio of urinary androsterone* (5α) *and aetiocholanolone* (5β) *in Japanese and British women. B. and J. represent British and Japanese women, respectively. The dotted lines indicate the mean values of the* $5\alpha/5\beta$ *ratio.*

The ratio declines with increasing age in British women but increases in Japanese. Although this change with age is significantly correlated in British women, it is not significant in Japanese (Table 2). This opposite trend in the $5\alpha/5\beta$ ratio with age for the two races is reflected by the fact that the mean ratios are not significantly different between Japanese and British adolescents but are for pre-menopausal ($P < 0{\cdot}01$), menopausal ($P < 0{\cdot}01$) and post-menopausal ($P < 0{\cdot}001$) women.

DISCUSSION

The results of a comparison of some aspects of androgenic status in normal British and Japanese women show clearly that plasma DS levels are lower in Japanese women than in British women. Furthermore, the amounts of the principal urinary androgen metabolites are also lower in the Japanese subjects, a finding which confirms earlier reports [8–10].

The levels of plasma DS and AS obtained for British women are comparable to those quoted by others for Caucasian women (see Wang *et al.* [4]). Plasma DS concentration in Japanese women has been claimed [11, 12] to fall from about 95 μg% at age 20 to about 40 μg% at 60. This compares with the mean value of 139 μg% for the youngest and 41 μg% for the oldest age groups in this study.

Since the blood and urinary ketosteroids mirror each other so effectively, and making allowances for differences in blood volume, it is most likely that Japanese women have a lower production rate of ketosteroids compared to British women.

This and other laboratories have reported that the plasma level of testosterone [10] and oestradiol [1] are similar in pre-menopausal Japanese and British women. Since androstenedione is converted to testosterone [13] and the concentrations of androstenedione are related to that of oestradiol [5, 14], it is of interest that the inter-racial differences in plasma androstenedione levels in the younger age groups are much less clearly marked than in the older age groups. Since the concentrations of plasma oestrogens are the same in both races [1] it would imply that the peripheral conversion of androstenedione to oestrogens would be different in post-menopausal British compared with Japanese women.

Whilst plasma DS is secreted mainly by the adrenal glands [15], circulating androstenedione is derived from both adrenal [16] and ovarian sources in pre-menopausal women; the latter tissue secretes 20–50% of the total blood production rate, depending on the stage of the menstrual cycle [17, 18]. It is not possible with the present data to determine what proportion of the inter-racial difference in pre-menopausal women is due to either adrenal or ovarian sources. However, in post-menopausal women it would be reasonable to assume that the difference in plasma androstenedione reflects different adrenal secretion rates for Japanese and British women.

The present results for plasma androstenedione levels in adolescent and pre-menopausal Caucasian women during the luteal phase of the menstrual cycle is in general agreement with those reported by others in the literature [5, 6, 19, 20, 21].

There is now substantial evidence showing that a subnormal androgen production and

excretion is associated with the degree of risk in Caucasian women [22–27].

In a prospective study in Guernsey, it was shown that there was an inverse correlation between the amounts of urinary aetiocholanolone and risk of breast cancer [2]. The finding that plasma androgen levels were lower in normal Japanese women than in British women and that the urinary excretion of androgen metabolites was also less, is paradoxical.

It is still possible that androgen status may be of importance in the aetiology of breast cancer in Japanese women and that Japanese women at high risk of developing breast cancer may have subnormal amounts of androgen metabolites relative to normal Japanese women. Thus, it may not be the absolute levels which are of inportance, but the relative levels. This would imply that racial differences in tissue sensitivity may have to be considered. Certainly this idea is not without precedent, since plasma testosterone concentrations are the same in Caucasian, Ainu and Japanese males, although the degree of hirsutism is different [28].

Furthermore, subnormal excretion of androgen metabolites is associated with failure to respond to endocrine ablation in Caucasian women [29]. Similar results have been obtained for Japanese patients [30]. In the clinical context, a subnormal androgen excretion is as much a "risk factor" in Japan as it is in Western countries. However, there is still a paradox in that within the Japanese population, there is a gradation in the incidence of breast cancer between rural and urban areas, the latter being higher [31]. Kodama and his colleagues have found that the excretion of urinary androgen metabolites is lower in rural than in urban Japanese women [32].

An interesting finding in this study was the change in the $5\alpha/5\beta$ ratio with age. In the Japanese population this ratio appears to remain static or to increase with age, whilst in the British population it decreased. A similar result was reported by Bulbrook *et al* in 1967 [8]. Since one factor which governs the $5\alpha/5\beta$ ratio is thyroid hormone [33], it is pertinent to consider the possible link between thyroid hormones and the aetiology of breast cancer. Various reports over a considerable period of time have implicated thyroid hormones as having a beneficial effect on the course of the disease or a protective effect against the disease [34, 35]. They have also been quoted as being a possible reason for the differences in incidence between Japanese and Caucasian women [36]. If the change in $5\alpha/5\beta$ ratio does reflect changes in thyroid activity it would imply that whilst Japanese women had fairly constant levels of circulating thyroid hormones with age, British women would experience a decline in this hormone with advancing age. It would be an attractive hypothesis to suggest that the small differences in incidence of breast cancer in the younger age groups and the divergence of the incidence curves after the menopause are related to the different patterns of thyroid activity.

The evidence that a subnormal androgenic status is a significant risk factor within Caucasian populations is persuasive. But our current results show that the correlation is not related to geographical differences in incidence. If androgenic status is a risk factor in Japanese women, then it would be necessary to postulate that other unknown factors completely override it.

REFERENCES

1. R. D. Bulbrook, M. C. Swain, D. Y. Wang, J. L. Hayward, S. Kumaoka, O. Abe and J. Utsunomiya, Plasma oestradiol-17β, oestrone and progesterone and their urinary metabolites in normal British and Japanese women. *Europ. J. Cancer* **12** (in press) (1976).
2. R. D. Bulbrook, J. L. Hayward and C. C. Spicer, Relation between urinary androgen and corticoid excretion and subsequent breast cancer. *Lancet* **ii,** 395 (1971).
3. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, Abnormal excretion of urinary steroids by women with early breast cancer. *Lancet* **ii,** 1238 (1962).
4. D. Y. Wang, R. D. Bulbrook, B. S. Thomas and M. Friedman, Determination of solvolysed sulphate esters of dehydroepiandrosterone and androsterone in human peripheral plasma by gas–liquid chromatography. *J. Endocr.* **42,** 567 (1968).
5. D. T. Baird, P. E. Burger, G. D. Heavon-Jones and R. J. Scaramuzzi, The site of secretion of androstenedione in non-pregnant women. *J. Endocr.* **63,** 201 (1974).

6. H. L. JUDD and S. E. C. YEN, Serum androstenedione and testosterone levels during the menstrual cycle. *J. clin. Endocrinol.* **36,** 475 (1963).
7. J. LOBOTSKY and C. W. LLOYD, Variations in the concentrations of androstenedione in peripheral plasma of women during the day and during the menstrual cycle. *Steroids* **22,** 133 (1973).
8. R. D. BULBROOK, B. S. THOMAS, J. UTSUNOMIYA and E. HAMAGUCHI, The urinary excretion of 11-deoxy-17-oxosteroids and 17-hydroxycorticosteroids by normal Japanese and British women. *J. Endocr.* **38,** 401 (1967).
9. H. IBAYASHI, T. FUJITA, K. MOTOHASHI. S. YOSHIDA, N. OHSAWA, S. MURAKAWA, T. YOKATA and S. OKINAKA, Determination of adrenocorticotropin in human urine by a benzoic-acid adsorption method. *J. clin. Endocrinol.* **21,** 140 (1961).
10. T. KOBOYASHI, J. LOBOTSKY and C. W. LLOYD, Plasma testosterone and urinary 17-ketosteroids in Japanese and Occidentals. *J. clin. Endocrinol* **26,** 610 (1966).
11. H. SEKIHARA, N. OHSAWA and H. IBAYASHI, A radioimmunoassay for serum dehydroepiandrosterone sulfate. *Steroids* **20,** 813 (1972).
12. H. SEKIHARA, T. YAMAJI and N. OHSAWA, Plasma dehydroepiandrosterone and dehydroepiandrosterone sulphate. *Folia endocr. japonica* **49,** 230 (1973).
13. R. L. VANDE WIELE, P. C. MACDONALD, E. GURPIDE and S. LIEBERMAN, Studies on the secretion and interconversion of the androgens. *Recent Progr. Hormone Res.* **19,** 275 (1963).
14. P. C. MACDONALD, R. P. ROMBAUT and P. K. SIITERI, Plasma precursors of estrogens. I. Extent of conversion of plasma delta-4-androstenedione to estrone in normal males and non-pregnant normal, castrate and adrenalectomised females. *J. clin. Endocrinol.* **27,** 1103 (1967).
15. E. E. BAULIEU, C. CORPECHOT, F. DRAY, R. EMILIOZZI, M. C. LEBEAU, P. MAUVAIS-JARVIS and P. ROBEL, An adrenal-secreted "androgen": Dehydroisoandrosterone sulfate. Its metabolism and a tentative generalization on the metabolism of other steroid conjugates in man. *Rec. Prog. Hormone Res.* **21,** 411 (1965).
16. R. G. WIELAND, C. DECOURCY, R. P. LEVY, A. P. ZALA and H. HIRSCHMANN, $C_{19}O_2$ steroids and some of their precursors in blood from normal human adrenals. *J. clin. Invest.* **44,** 159 (1965).
17. D. T. BAIRD, Steroids in blood reflecting ovarian function. In: *Controls of Gonadal Steroid Secretion.* (Edited by D. T. BAIRD and J. A. STRONG, p. 176. Edinburgh University Press, Edinburgh (1971).
18. R. HORTON, E. ROMANOFF and J. WALKER, Androstenedione and testosterone in ovarian venous and peripheral plasma during ovariectomy for breast cancer. *J. clin. Endocrinol.* **26,** 1267 (1966).
19. R. L. ROSENFIELD, A competitive protein binding method for the measurement of plasma androstenedione. *Steroids* **14,** 251 (1969).
20. R. HORTON, Estimation of androstenedione in human peripheral blood with ^{35}S-thiosemicarbazide. *J. clin. Endocrinol.* **25,** 1237 (1965).
21. I. H. THORNEYCROFT and S. A. TILLSON, A radioimmunoassay for androstenedione. *Steroids* **21,** 111 (1973).
22. M. J. BRENNAN, R. D. BULBROOK, N. DESHPANDE, D. Y. WANG and J. L. HAYWARD, Urinary and plasma androgens in benign breast disease. *Lancet* **i,** 1076 (1973).
23. D. Y. WANG, R. D. BULBROOK and J. L. HAYWARD, Urinary and plasma androgens and their relation to familial risk of breast cancer. *Europ. J. Cancer* **11,** 873 (1975).
24. P. PITT and G. SARFATY, Androgen metabolites in the urine of average women, nuns and women with regional and metastatic breast cancer. *Cancer Forum* **1,** 27 (1974).
25. S. WARREN, The relation of "chronic mastitis" to carcinoma of the breast. *Surg. Gynecol. Obstet.* **71,** 257 (1940).
26. D. E. ANDERSON, Genetic study of breast cancer: Identification of a high-risk group. *Cancer (Philad).* **34,** 1090 (1974).
27. J. CLEMMESON, *Statistical Studies in the Aetiology of Malignant Neoplasma. I. Review and Results,* p. 298. Copenhagen, Munksgaard (1965).

28. M. Okamoto, C. Setaishi, Y. Horiuch, K. Mashimo, K. Moriya and S. Itoh, Urinary excretion of testosterone and epitestosterone and plasma levels of LH and testosterone in the Japanese and Ainu. *J. clin. Endocrinol.* **32,** 673 (1971).
29. J. L. Hayward and R. D. Bulbrook, Urinary steroids and prognosis in breast cancer. In: *Prognostic Factors in Breast Cancer*, p. 383. (Edited by A. P. M. Forrest and P. B. Kunkler. E. & S. Livingstone, Edinburgh (1968).
30. S. Kumaoka, N. Sakauchi, O. Abe, M. Kusama and O. Takatani, Urinary 17-ketosteroid excretion of women with advanced breast cancer. *J. clin. Endocrinol.* **28,** 667 (1968).
31. E. L. Wynder, Current concepts in the aetiology of breast cancer. In: *Prognostic Factors in Breast Cancer.* (Edited by A. P. M. Forrest and P. B. Kunkler) p. 32. E. and S. Livingstone, Edinburgh (1968).
32. M. Kodama, T. Kodama, M. Yoshida, R. Totania and K. Aoki, Hormonal status of breast cancer. II. Abnormal urinary steroid excretion. *J. nat. Cancer Inst.* **54,** 1275 (1975).
33. L. Hellman, H. L. Bradlow, B. Zumoff, D. K. Fukushima and T. F. Gallagher, Thyroid-androgen inter-relations and the hypocholesteremic effects of androsterone. *J. clin. Endocrinol.* **19,** 936 (1959).
34. A. A. Loeser, A new therapy for prevention of post-operative recurrences in genital and breast cancer. A six-year study of prophylactic thyroid treatment. *Brit. med. J.* **ii,** 1380 (1954).
35. A. R. Moosa, D. A. Price Evans and E. C. Brewer, Thyroid status and breast cancer. *Ann. Roy. Coll. Surg. (England)* **53,** 178 (1973).
36. R. D. Bulbrook, B. S. Thomas and J. Utsunomiya, Urinary 11-deoxy-17-oxosteroids in British and Japanese women with reference to the incidence of breast cancer. *Nature (Lond.)* **201,** 189 (1964).

Europ. J Cancer Vol. 12, pp. 959–961. Pergamon Press 1976. Printed in Great Britain

Presence of Intramitochondrial Bodies in Avian Leukemic Myeloblasts

JAN KORB and JOSEF ŘÍMAN
Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia.

Abstract—*Electron-dense bodies of about* 50–100 *nm in diameter were detected by electron microscopy in mitochondria of chick leukemic myeloblasts cultivated by the column technique. Ninety percent of these bodies were characterized by the dimension* 50–80 *nm without any typical internal structure,* 10% *of them with diameter of* 80–100 *nm revealed the structure of intracytoplasmic A particles. These isolated virus-like particles sedimenting at the density* 1·28 *g/cm*^{3} *in sucrose gradient were analysed by electron microscopy using negative staining method. No intramitochondrial bodies were detected in mitochondria of leukemic myeloblasts cultivated in suspension culture in the same cultivation medium. The possible effect of differences in the metabolic state of the cells on the accumulation of intramitochondrial bodies were discussed.*

ELECTRON dense bodies have been observed in mitochondria of a variety of tissues and are known to be present in different amounts dependently on the composition of extra cellular fluid [1–4].

During routine electron microscopy of avian myeloblastosis virus producing chick leukemic myeloblasts cultivated in suspension cultures [5], however, only few, if any, intramitochondrial bodies have been detected.

In this paper we demonsteate that during cultivation of the cells under special conditions intramitochondrial bodies could be detected in the mitochondria of leukemic myeoblasts. About 10% of them revealed the structure and sedimentation properties of virus-like particles.

MATERIAL AND METHODS

Avian myeloblastosis virus producing cultures of chicken leukemic myeloblasts [6] growing in suspension (8.10^6 cells/ml) [5] were used both for further cultivation in the column as described elsewhere [7] and for the control morphology studies.

Leukemic myeloblasts cultivated in the column were previously used by us for the production of virus particles in the extracellular space for different short time intervals [8]. This technique allows an arbitrary and defined flow of fresh medium along the cells influencing their metabolic state. In short, leukemic myeloblasts which had been cultivated for 24 hr in a suspension culture were transferred into a glass column and immobilized on glass cullets (50–200 um). The size of the column was generally about 10 ml of bed volume/1 g of cells. The flow rate of fresh medium was then arranged to the value about 0·5–5 ml/1 min and the cells were cultivated under these conditions for different periods (30–120 min) at 38°C. The composition of cultivation medium was the same both in the suspension and the column cultivation.

The myeloblasts were prefixed with 4·5% glutaraldehyde in 0·05M cacodylate buffer (pH 7·4) either directly at cultivation temperature or at 4°C. After washing the cells were fixed with 1% osmium tetroxide in the same buffer, dehydrated in ethanol and embeded in Epon 812. Thin sections were stained either with uranyl acetate or in uranyl acetate and lead citrate [9] and observed in a Tesla BS 613 electron microscope.

The mitochondria were isolated after homogenezation of the cells by differential centrifugation and purification on sucrose gradients as described before [10]. Purified mitochondria were disrupted either by osmotic shock or in a French press at 3000 psi (both in 0·01M sodium citrate, pH 7·4 and 0·01M dithiotreitol). The suspension was placed on the top of a linear 27–67% sucrose gradient in the

Accepted 16 June 1976.

same buffer and centrifuged at 40,000 rev/min for 3 hr in SW 41 Spinco rotor. The fractions were collected and some of them prepared for electron microscopy by fixation in 2% glutaraldehyde in 0·01M sodium citrate, pH 7·4, for at least 2 hr and by negative staining with 2% phosphotungstig acid pH 7·0.

RESULTS

Leukemic myeloblasts cultivated in the glass column at a flow rate of 0·5–5 ml/1 min/1 ml bed volume revealed the presence of intramitochondrial bodies (Fig. 1 and 2). These structure were never observed in mitochondria of leukemic myeloblasts cultivated in suspension cultures. Electron dense intramitochondrial particles were preferentially stained with uranyl acetate and their dimension varied from 50 to 100 nm. Their number appears to be dependent on the conditions of the cell cultivation. Prolongation of column cultivation as well as increasing of flow rate increase the amount of intramitochondrial bodies.

The structures characterized by size of 50–70 nm in diameter lacked an inner structure, but the particles with dimensions of 80–100 nm (which represent about 10% of total number of intramitochondrial bodies) revealed an internal morphology characterized by an electronlucent center surrounded by an intensively stained ring (Fig. 3). This structure was described to be typical for intracytoplasmic A particles [11].

The appearance of the mitochondria was more or less altered by the cultivation on the column. Both large mitochondria (Fig. 1) and the reduction of cristae (Fig. 2) were observed. Since intramitochondrial bodies were observed both in intact as well as empty mitochondria, their presence was not dependent on the morphological integrity of mitochondrial structure. The dense bodies with a dimension of 50–70 nm were randomly distributed inside the mitochondria, those with a diameter of 80–100 nm were frequently, but not always, associated with the cristae.

The sedimentation profiles of the sucrose equilibrium density gradients of purified and disrupted mitochondria are given in Fig. 5. The main peak (density of 1·15 g/cm^3) consisted of morphologically non-homogeneous material without detectable presence of virions. The small peak at the position of density 1·28 g/cm^3 represented the pure fraction of particles about 100 nm in diameter. In negative staining (Fig. 4) these particles revealed the structure similar to those of intracytoplasmic A particles [12, 13].

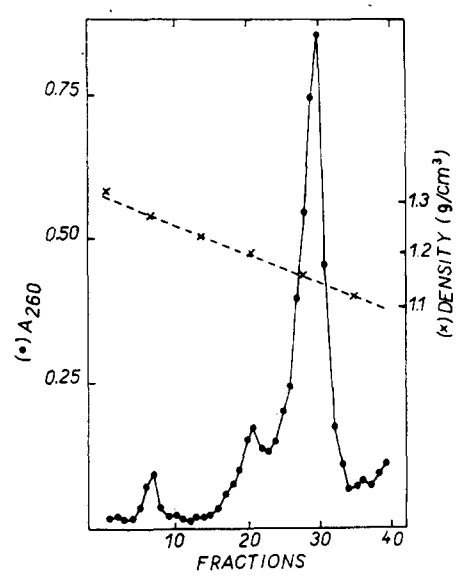


Fig. 5. Sucrose equilibrium density gradient of disrupted mitochondria isolated from avian leukemic myeloblasts.

DISCUSSION

It is clear that the differences in the cultivation conditions are of utmost importance for the appearance of intramitochondrial bodies, since we were unable to detect any of them in mitochondria of myeloblasts cultivated in suspension cultures but found them accumulated in the same organelles during column cultivation, although the composition of cultivation medium was the same.

The small bodies observed may represent either some non-specific inclusion inside of mitochondria formed during the special metabolic state of the cells, or the precursor structures of large particles. The structures of dimensions 80–100 nm revealed the resemblance of intracytoplasmic A particles in thin section electron microscopy. The possible viral character of these particles was further supported by their sedimentation at the density of 1·28 g/cm^3 and their appearance in negatively stained preparations similar to those described in other cell lines [12, 13].

Our results are consistent with the findings of Keydar *et al.* [14] who described the effect of the culture age on the presence of intramitochondrial bodies in a human cell line.

Even when negative transfections experiments with mitochondrial DNA [15, 16] appear to disprove the former suggestion that oncornaviruses are synthetized in mitochondria, the presence of virus-like particles inside of mitochondria of cells transformed by oncornaviruses seems to be established both electromicroscopically and biochemically [17–20].

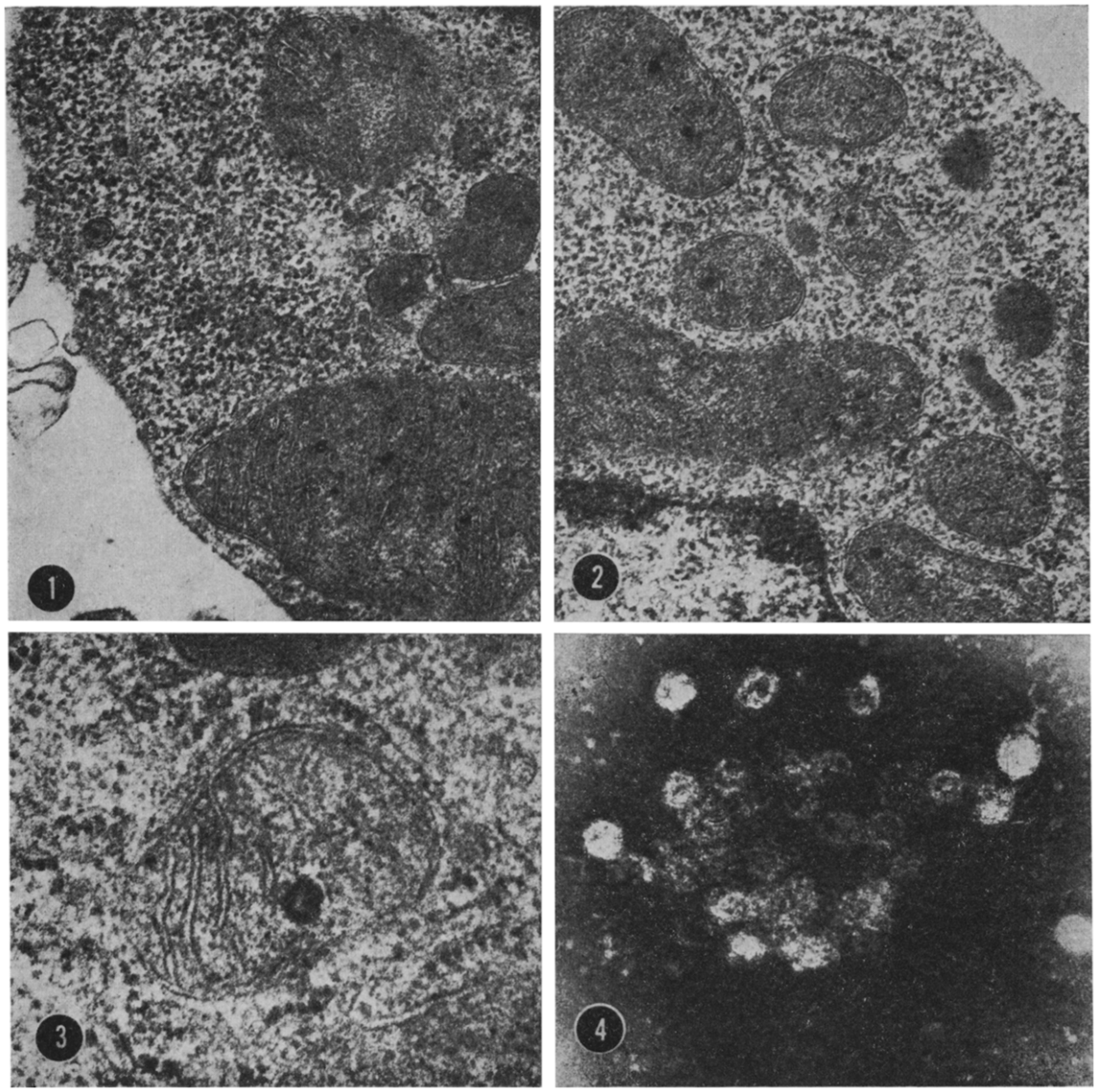


Fig. 1. *Mitochondria of avian leukemic myeloblasts with preserved inner structure (cristae) containing electron-dense bodies* 50–70 *nm in diameter.* × 30,000.

Fig. 2. *Mitochondria of avian leukemic myeloblasts devoid of inner structure with electron-dense bodies* 50–70 *nm in diameter.* × 30,000.

Fig. 3. *A mitochondria (leukemic myeloblast) containing virus-like particle* 100 *nm in diameter.* × 70,000.

Fig. 4. *Fraction of density* 1·28 *g/cm*^{3} *from disrupted mitochondria of avian leukemic myeloblasts. Negatively stained with phosphotungsticacid.* × 70,000.

(to face page 960)

The question remains as to whether the described presence of structures resembling virus-like particles inside of mitochondria of leukemic myeloblasts is an accidental process or a result of a specific metabolic state of the cells and their mitochondrial system. Since mitochondria are the structures containing all the enzymatic systems necessary for the biotransformation of energy they could represent the most convenient site for the biosythesis of some viral components or for the completion of the virus at specific metabolic state of the cells.

REFERENCES

1. L. D. Peachey, Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules. *J. Cell Biol.* **20,** 95 (1964).
2. J. Andre and V. Marinozzi, Presence dans les mitochondries de particules ressemblant aux ribosomes. *J. Microscopie* **4,** 615 (1965).
3. J. H. Martini and J. L. Matthews, Mitochondrial granules in chondrocytes, osteoblasts and osteocytes. *Clin. Orthop. Related Res.* **68,** 273 (1970).
4. E. Bonucci, M. Derenzini and V. Marinozzi, The organic–inorganic relationship in calcified mitochondria. *J. Cell Biol.* **59,** 185 (1973).
5. J. Riman and G. S. Beaudreau, Viral RNA-dependent DNA polymerase and the properties of thymidine labelled material in virions of an oncogenic RNA virus. *Nature (Lond.)* **228,** 427 (1970).
6. C. Becker, G. S. Beaudreau, W. Castle, B. W. Gibson, D. Beard and J. W. Beard, Virus of avian myeloblastosis. XXII. Influence of folic acid and other factors on growth of myeloblasts and virus synthesis in tissue culture. *J. nat. Cancer Inst.* **29,** 455 (1962).
7. J. Buzek, Cultivation of leukemic myeloblasts in the column. *Folia Biol. (Praha)* **22,** 97–100 (1976).
8. J. Korb, M. Travnicek and J. Riman, The oncornavirus maturation process: Quantitative correlation between morphological changes and conversion of genomic virion.RNA. *Intervirology* In press (1976).
9. J. H. Venable and R. Coggeshall, A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407 (1965).
10. J. Korb, Mitochondrial DNA of avian leukemic myeloblast: Isolation and electron-optical characterization. *Neoplasma* **18,** 337 (1971).
11. A. G. Bukrinskaya, G. G. Miller, E. N. Lebedeva and V. M. Zhdanov, Intracellular virus specific structure and RNAs in oncornavirus-producing human cells. *J. Virol.* **13,** 478 (1974).
12. G. H. Smith and N. A. Wivel, Isolation and partial characterisation of intracytoplasmic A particles. *Virology* **48,** 270 (1972).
13. G. H. Smith and N. A. Wivel, Intracytoplasmic A particles: Mouse mammary tumor virus nucleoprotein cores? *J. Virol.* **11,** 575 (1973).
14. J. Keydar, G. Braslawsky, T. Dajbog, S. Karby, S. T. Shinedling, F. Weiss and J. Delarea, Intramitochondrial bodies in a human cell line carrying oncornavirus-like particles. *J. nat. Cancer Inst.* **54,** 247 (1975).
15. L. Montagnier and P. Vigier, Un intermédiaire ADN infectieux et transformant du virus du sarcome de Rous dans les cellules de poule transformées par ce virus. *C. R. Acad. Sci. (Paris)* **224,** 1977 (1972).
16. J. Svoboda, I. Hlozanek, J. Korb and O. Mach, Failure to obtain transfection with XC mitochondrial DNA. *Europ. J. Cancer* **11,** 247 (1975).
17. L. Gazzolo, G. de The, P. Vigier *et al.*, Présence de particules à l'aspect de nucléocapsides associées aux mitochondries dans des cellules de hamster transformées par le virus de Rous. *C. R. Acad. Sci. (Paris)* **268,** 1668 (1969).
18. H. H. Schumacher, I. E. Szekely and S. A. Park, Virus-like particles in leukemic mitochondria. *Lancet* **i,** 7803, 601 (1973).
19. J. Kára and O. Mach, Electron microscopic evidence of the subviral oncogenic particles (virosomes) isolated from the mitochondria of Rous sarcoma cells. *Folia Biol. (Praha)* **19,** 78 (1973).
20. P. D. Lunger and H. F. Clark, Ultrastructural studies of cell-virus interaction in reptilian cell lines. I. Intramitochondrial viruses in "C" type virus producing VSV cells. *J. nat. Cancer Inst.* **50,** 111 (1973).

Europ. J. Cancer Vol. 12, pp. 963–972. Pergamon Press 1976. Printed in Great Britain

The Effect of Irradiation on the Oxygen Removal Rate of the SSB1a Rat Fibrosarcoma

T. B. CONSTABLE
Medical Research Council Cyclotron Unit, Hammersmith Hospital, London, W12 0HS, Great Britain

Abstract—*Changes in the oxygen uptake of the SSB1a rat fibrosarcoma following exposure to single doses of radiation have been measured by a technique which also enables the spatial distribution of oxygen uptake rate to be determined. The oxygen uptake rate is measured at* 91 *sampling positions within an area approximately* 5 mm *in diameter on a cut surface of recently excised tissue. A first minimum in oxygen uptake per unit volume occurs* 3 *days after irradiation, during a period of radiation-induced growth inhibition, and this may correspond to improved oxygenation of the tumour. A second decline in oxygen uptake rate coincides with the onset of regrowth and is associated with a change in the pattern of subsequent cell proliferation within the tumour. The changes in oxygen uptake rate are discussed in relation to various models for the processes of tumour reoxygenation.*

INTRODUCTION

THE RATE at which oxygen is metabolised by a tissue is likely to be altered after exposure to ionising radiation in a way which depends on the dynamic balance between the rate at which metabolism in damaged cells diminishes, or ceases, and the rate at which new cells are produced. The radiosensitivity of a tissue depends on the oxygen concentration at the time of irradiation. Changes in oxygen concentration brought about by changes in the rate of oxygen consumption after irradiation may therefore alter the pattern of radiosensitivity of a tissue so as to affect the response to fractionated schedules of radiation.

A polarographic method has been developed for measuring local oxygen uptake rates within small zones of tissue. An array of polarographic cathodes is used to obtain a distribution of the rates of oxygen uptake at 91 sampling positions in a plane hexagonal lattice of 0·5 mm spacing so that local variations in oxygen uptake rate can be studied. The basis of the method is to take a freshly excised sample of tissue and to cut through it with a sharp blade to expose a plane surface. The surface is equilibrated with a gas containing a known concentration of oxygen and oxygen diffuses into the tissue to a depth related to the oxygen uptake rate. The tissue surface is then occluded by the plane end of a glass electrode which has an array of polarographic cathodes sealed into it. The supply of oxygen to the tissue is thereby cut off and the rate at which the surface oxygen tension falls is related to the rate of consumption of oxygen in the superficial layer through which the oxygen initially penetrated. Some preliminary applications of the technique have already been reported [1] but it has now been extended to measure the effect of radiation on the oxygen uptake rate of the SSB1a rat fibrosarcoma.

The distribution of the rates of oxygen uptake within SSB1a tumours irradiated at a mean diameter of 10 mm and allowed to regrow to a diameter of 30 mm are known to differ from those of unirradiated tumours of the same size [2]. The oxygen uptake rate was uniformly low throughout the core of the pre-irradiated tumours and more normal values were only found in a thin shell at the edge of the tumour. The untreated tumours showed more inhomogeneous patterns of oxygen uptake rate with highest values towards the edge but also appreciable uptake in some central regions. These findings correlate with observed patterns of cellular proliferation within the related tumour SSB1 [3]. The present work was undertaken to investigate changes in oxygen uptake at

Accepted 14 June 1976.

short times after irradiation and also the time-scale of the development of the difference in oxygen uptake between large untreated and irradiated tumours which has just been described. It will be shown that some changes in oxygen uptake occur during the period of radiation-induced inhibition of tumour growth but that the most marked effect is closely linked to the onset of regrowth.

METHOD

The apparatus, method of calculation and experimental procedure have been described in detail in a previous publication [1]. A schematic diagram of the electrode assembly is shown in Fig. 1. The histological characteristics of the rat fibrosarcoma SSB1a and the transplantation technique have been described by Howlett *et al.* [4]. The tumour is maintained in the John's strain of Wistar rat and tumours were irradiated when they reached the standard size of 9–11 mm average diameter. The animals were immobilised with sodium amytal and breathed air during irradiations for which they were placed inside a chamber maintained at 26–28°C and irradiated by two opposed Caesium sources. At the appropriate sampling times, tumours were excised from the host, the volumes were estimated from measurements of three mutually perpendicular diameters and the tumours were cut across a principal diameter with a 4 in razor blade. The perspex measuring cuvettes will take tumour samples up to 11 mm in diameter. For tumours larger than this, a length of strip razor blade mounted around a cylinder of 11 mm diameter was used to bore down from the cut surface of the tumour to give a cylindrical section. In all cases to be described in this paper the cylindrical sections were taken from the centre of the cut surface. The tumour samples were mounted with the cut surface pressed against a 12 μm polythene membrane, care being taken to exclude air bubbles from between the two. The cuvette was placed in a double-gimbal bearing positioned below the electrode and could be brought sharply into contact with the electrode by a pneumatic mechanism (Fig. 1). Recording of the cathode currents was started shortly before the electrode and cuvette were brought into contact and continued for 20–60 sec after contact, depending on the smallest oxygen uptake rate within the tissue, and determined by monitoring the cathode currents on an oscilloscope. The samples were initially equilibrated with air and held at a temperature of 25°C. For measurements at 37°C it was necessary to pre-equilibrate each cuvette with a gas with a higher oxygen content than that of air in order for the time response of the cathode currents to be of sufficient duration to give a reasonable estimate of oxygen uptake rate. For this each cuvette had to be placed in a perspex gas chamber surrounding the electrode (Fig. 1) during the equilibration period as well as during the time of measurement. When several tumour specimens were sampled concurrently the period between successive measurements was increased above that for measurements following air equilibration. It was considered preferable to make a series of repeat measurements at 25°C on each sample within as short a period as possible after excision from the host. However, selected measurements at 37°C were made for comparison with those at 25°C.

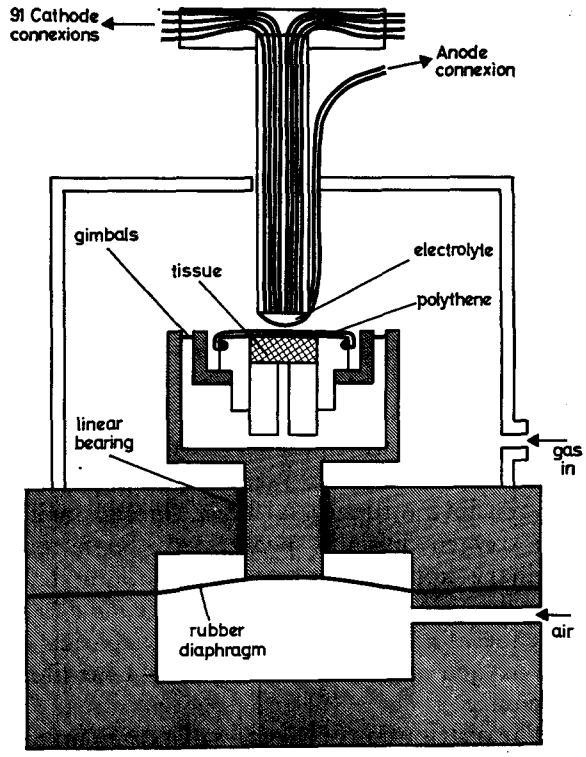


Fig. 1. *The electrode assembly. Reproduced from* (1) *by courtesy of ASP Biological and Medical Press (North-Holland division).*

The quantity estimated by this technique is the ratio of the oxygen uptake rate, to the oxygen solubility, per unit volume. In accordance with a previous publication [1] this ratio will be referred to as the oxygen removal rate, Q_R, having units of mmHg/sec. It is calculated from the equation:

$$Q_R = \frac{\pi Po}{8}\left(\frac{k}{i_0}\right)^2 \qquad [1, 5]$$

where Po is the partial pressure in mmHg of the gas with which the tissue is initially equilibrated, k is the slope of the time response curve when plotted against the square root of time and i_0 is the intercept of this plot on the ordinate axis and is equivalent to a cathode calibration factor. A computer was used to process the data.

The effect of haemoglobin

The theory behind the calculation of Q_R assumes simple diffusion properties with a linear relation between oxygen tension and oxygen solubility. There are two ways for allowing for the haemoglobin content of tissue. The tissue can be either initially equilibrated with 100% oxygen and the time course measured during a period when nearly all of the haemoglobin remains saturated, or the tissue can be exposed to carbon monoxide for a short period and then equilibrated with air. Carbon monoxide binds strongly to haemoglobin but is readily displaced from combination with respiratory enzymes by oxygen [6]. Exposure to a high concentration of carbon monoxide for five min allowed penetration to at least the depth subsequently reached by oxygen.

It has been shown previously [1] that the mean Q_R, obtained by averaging 91 cathode values measured simultaneously, for a series of SSB1a tumours, was not significantly different when measured following equilibration with 100% oxygen, equilibration with air after previous exposure to carbon monoxide, and

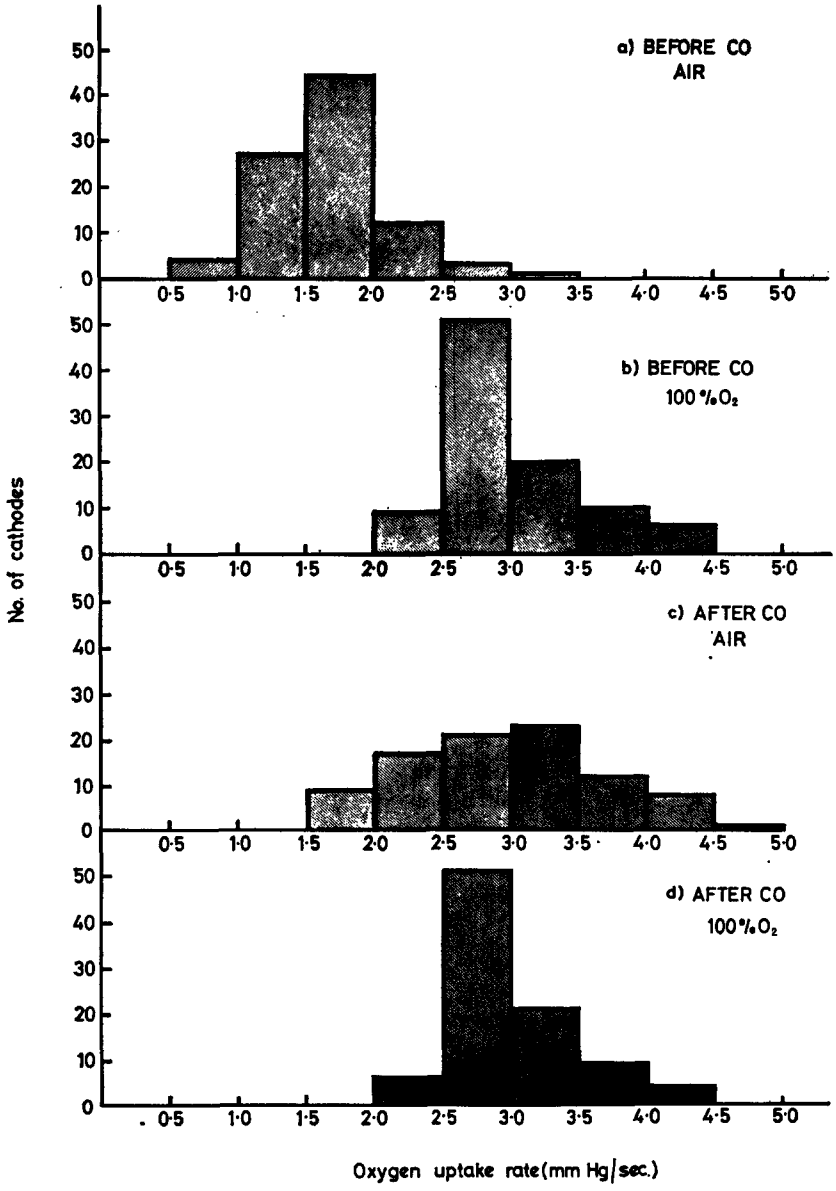


Fig. 2. *Distribution of Q_R values in the tumour SSB1a before and after exposure to carbon monoxide. Equilibration with air or* 100% O_2

equilibration with 100% oxygen after exposure to carbon monoxide. The values for equilibration with air alone were lower due to desaturation of oxyhaemoglobin during the time course. Increasing the oxygen content of the equilibration gas results in greater initial penetration of oxygen into the tissue so that the spatial resolution of the measurements is reduced. This is illustrated in Fig. 2 by histograms of the distribution of the 91 Q_R values obtained in each of the four above conditions. The mean values are identical in cases (b), (c) and (d) (Fig. 2) while that in (a) is reduced. The narrower distribution of the values for 100% oxygen equilibration (b) and (d) is associated with a reduction in spatial resolution resulting in the averaging out of some of the inhomogeneities in Q_R. The agreement between the mean values of Q_R provides a reasonable justification for the use of preliminary exposure to carbon monoxide to overcome the effect of the presence of haemoglobin in this tissue.

A plane surface produced by cutting through the tumour could leave a surface layer of damaged cells with impaired respirative capacity. This was tested by equilibrating a series of SSB1a tumours with air, 42% and 100% oxygen so as to obtain values of Q_R averaged over different depths into the tissue (Table 1). The agreement between Q_R measured under these three equilibration conditions indicated that surface damage was not significant for this tissue. It also showed that the estimate of Q_R was not distorted by storage of oxygen in the cuvette membrane or by oxygen consumption by the cathodes.

Table 1

Tumour (mean dia. in mm)	Mean Q_R ± S.D.		
	Air	42% O_2	100% O_2
1. ($\bar{d}$=9·0)	3·05 ± 0·52	3·03 ± 0·40	2·91 ± 0·44
	2·99 ± 0·39	2·92 ± 0·31	2·83 ± 0·32
	3·14 ± 0·35		
	2·84 ± 0·39		
2. ($\bar{d}$=10·1)	2·59 ± 0·31	2·91 ± 0·32	3·05 ± 0·28
	2·77 ± 0·32	2·96 ± 0·35	2·98 ± 0·36
	3·07 ± 0·40		
	3·36 ± 0·40		
3. ($\bar{d}$=10·2)	2·92 ± 0·45	2·71 ± 0·31	2·86 ± 0·32
	3·08 ± 0·46	2·92 ± 0·39	2·79 ± 0·55
	3·13 ± 0·35		
4. ($\bar{d}$=9·3)	2·84 ± 0·38	2·87 ± 0·28	2·98 ± 0·39
	2·95 ± 0·42	2·97 ± 0·29	3·03 ± 0·31
	3·26 ± 0·40		
5. ($\bar{d}$=12·8)	2·02 ± 0·31	1·95 ± 0·27	2·49 ± 0·24
	1·83 ± 0·22	2·23 ± 0·29	2·24 ± 0·25
	2·02 ± 0·28		
	2·06 ± 0·31		
6. ($\bar{d}$=9·3)	1·96 ± 0·24	2·13 ± 0·23	2·24 ± 0·25
	2·14 ± 0·26	2·44 ± 0·32	2·31 ± 0·21
	2·75 ± 0·36		

Measurements of Q_R (mmHg/sec) on SSB1a tumours following equilibration with air, 42% oxygen and 100% oxygen.

RESULTS

1. *Pattern of oxygen uptake during growth of untreated tumours*

Patterns of Q_R within irradiated tumours will be shown to be related to the size of the tumours. It is therefore appropriate to consider first the changes in Q_R which occur during growth of untreated tumours. The times at

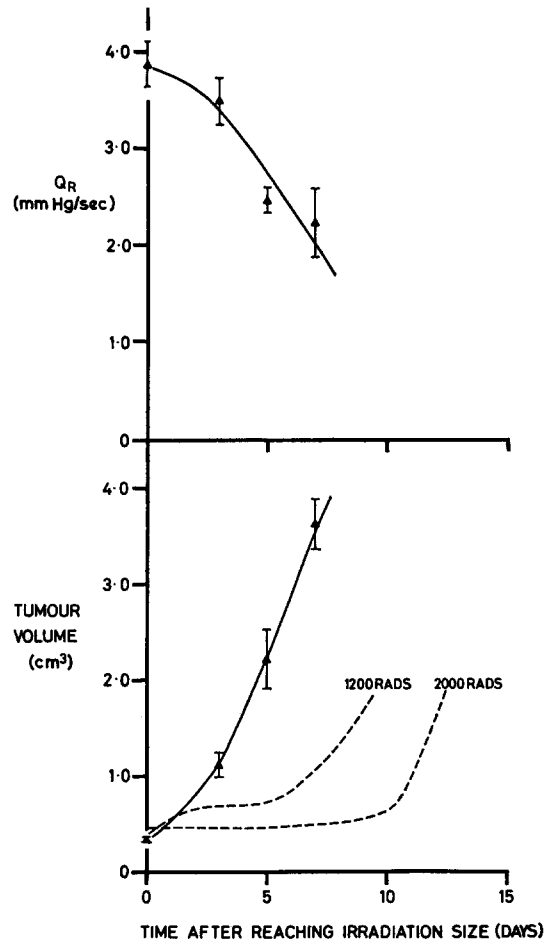


Fig. 3. *Change in Q_R with growth of untreated tumours at various times after reaching the standard irradiation size. The points represent the means and standard errors of the mean Q_R values for groups of five tumours. Volume changes of tumours exposed to* 1200 *and* 200 *rads are reproduced from Fig.* 4 *and* 8 *for comparison.*

which implanted tumours reached the standard irradiation size were noted and the tumours were allowed to continue growing for certain periods. Q_R was measured over an area of approximately 5½ mm diameter selected from the centre of the tumour (see methods). Over a period of seven days the tumour volume increased by over six times and the mean Q_R showed a progressive decrease to about one half of the control value (Fig. 3). The points on the figure, and subsequent similar figures, are derived from a triple averaging procedure. Firstly the mean value was found for 91 individual values of Q_R determined from one measurement on a tumour sample. Secondly each sample was assayed two or three times and the individual means averaged to give an overall mean for each tumour. Each point on the figure is derived from a group of usually five animals and the points represent the mean for the group together with its standard error derived from the individual tumour mean values. Therefore, these figures indicate overall changes in Q_R but not the spatial inhomogeneity in Q_R within individual tumours. Control tumours at the standard irradiation size showed a reasonably uniform pattern of Q_R across the standard electrode sampling area (an example is shown in Fig. 7) but there was some tendency for the degree of spatial inhomogeneity to be increased in very large tumours. The progressive decrease in Q_R with tumour growth is in agreement with previously published results [2] where it was also shown that Q_R was somewhat larger near the rim of large untreated tumours as compared with the centre.

2. Q_R *in tumours exposed to* 2000 *rads*

Figure 4 shows the variation of Q_R with time after exposure to 2000 rads Cs γ-rays. Each point represents the mean and standard error for a group of five animals derived as described above. The figure combines measurements from different batches of tumour implants, represented by different symbols, but is plotted in terms of relative Q_R to allow for the small variation between different control groups. The points for the controls are thus positioned at the ordinate level of 1·0 but the error bars indicate the standard error of the means of these groups. This dose of irradiation inhibited the growth of the tumours for about 10 days after which there was a sudden onset of regrowth (Fig. 4). The Q_R fell to a minimum of about 80 % of the control value 3 days after irradiation at a time when the tumour was still at its irradiation size. The Q_R recovered and then showed a second fall coincident with the start of regrowth. Changes in the initial minimum in Q_R and the association between tumour regrowth and the ultimate decline in Q_R were investigated further using two more irradiation doses.

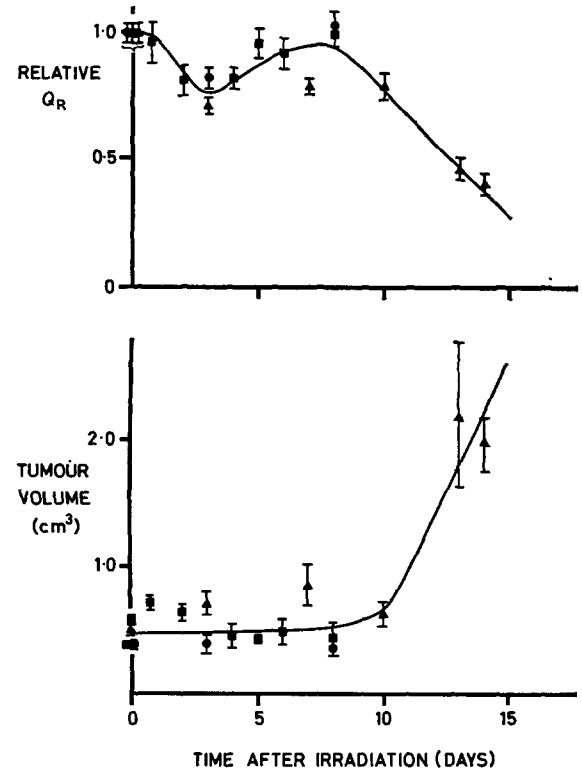


Fig. 4. Relative Q_R and tumour volume at various times after exposure to 2000 *rads. The points represent the means and standard errors of the mean Q_R values for groups of five tumours.*

3. Q_R *in tumours exposed to* 2720 *rads*

Irradiation with 2720 rads Cs γ-rays reduced Q_R at the minimum 3 days after exposure to about 70 % of the control value and the recovery time for return to the control level was perhaps increased in comparison with that for 2000 rads (Fig. 5). There was possibly a delay of one or two days in the timing of the second fall in Q_R compared to that following 2000 rads. This would be compatible with a similarly increased delay time in the regrowth of the tumour but the variation in tumour volumes at around this time did not allow a sufficiently accurate estimate of the delay time to be made. The damage to the tumour due to this higher dose of radiation may be limited by the presence of hypoxic cells and the growth delay time is certainly not much increased compared with that for 2000 rads. However, the subsequent regrowth rate after higher doses has been found to be reduced [7].

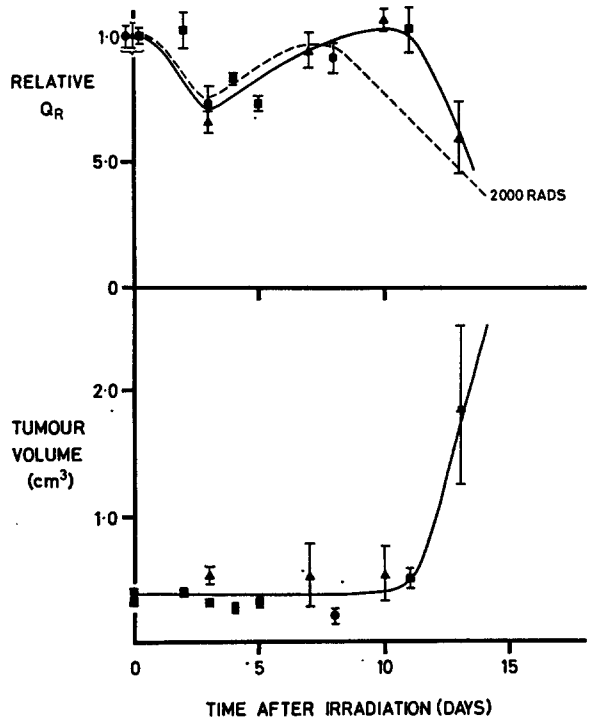


Fig. 5. Relative Q_R and tumour volume at various times after exposure to 2720 rads. The points represent the means and standard errors of the mean Q_R values or groups of five tumours. The outline of the Q_R curve for 2000 rads is reproduced from Fig. 4.

The minimum in Q_R, 3 days after irradiation, is associated with an overall decrease throughout the tumour, rather than with reductions in Q_R in certain areas only with a consequent increase in the spread of Q_R values. This is demonstrated in Fig. 6 which shows histograms of the 91 values of Q_R for four control tumours and four tumours 3 days after exposure to 2720 rads. The exact nature of the patterns of Q_R is indicated in Fig. 7 which shows examples of hexagonal arrays of Q_R values for control and 3 day-irradiated tumours. The nature of the change in Q_R after 2000 rads was similar to that described for 2720 rads.

4. *Q_R in tumours exposed to 1200 rads*

The regrowth period could be shortened by reducing the dose of irradiation. Tumours exposed to 1200 rads started to regrow 7 days after treatment. There was no minimum in Q_R corresponding to that observed 3 days after exposure to the higher doses (Fig. 8) probably due to an overlap between the first and second phases of the reduction in Q_R. The decline in Q_R again corresponded to regrowth of the tumours so that between 7 and 10 days after irradiation the mean Q_R was lower in tumours irradiated with 1200 rads than in those exposed to the higher doses of 2000 and 2720 rads.

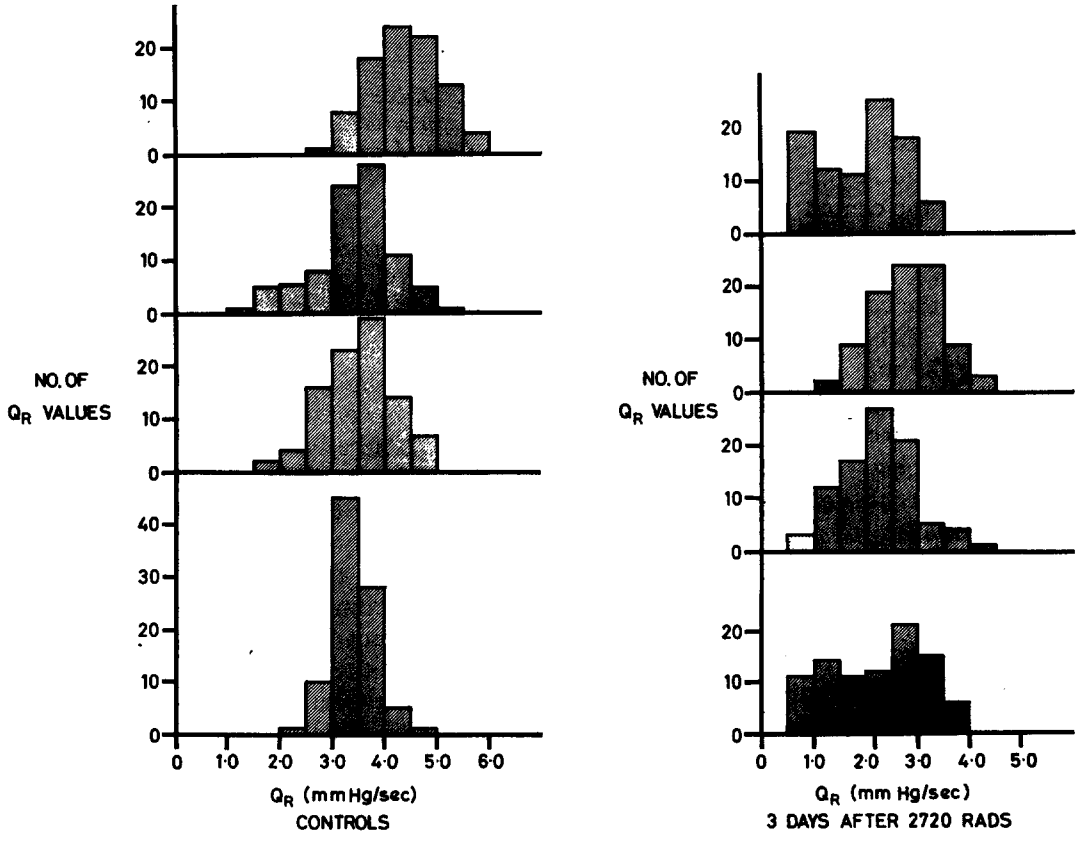


Fig. 6. Distribution of Q_R (91 values from single measurements) for four control tumours and four tumours measured 3 days after exposure to 2720 rads.

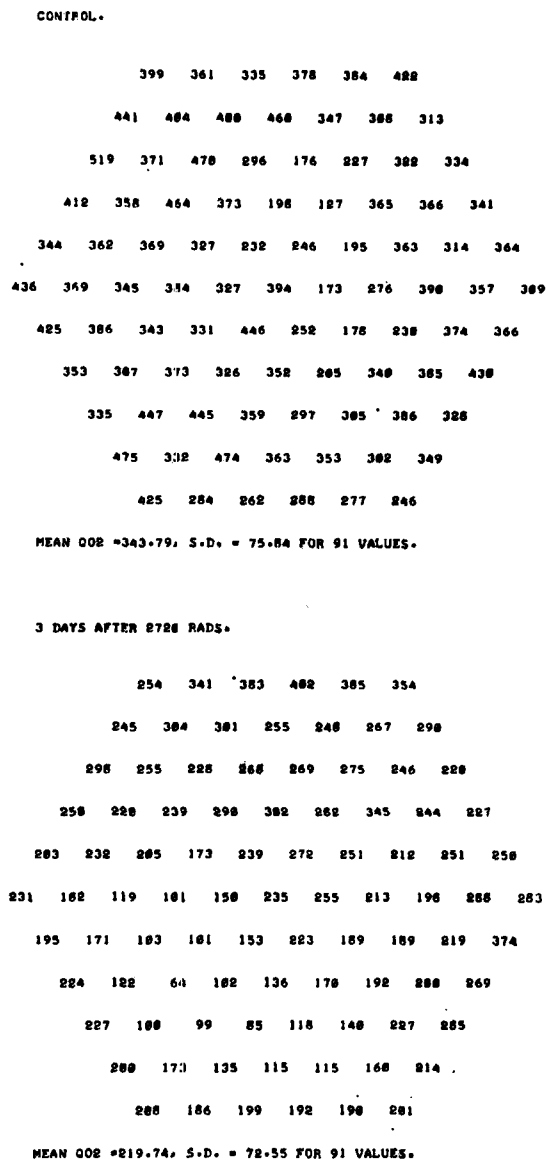


Fig. 7. Hexagonal arrays of Q_R values for a control tumour and a tumour 3 days after exposure to 2720 rads. Values are expressed as mmHg/100 sec.

5. Q_R *measured at 37 and 25°C*

In two situations corresponding to rather different values of Q_R, measurements were made at both 37 and 25°C. It was not possible to measure individual tumours at both temperatures but of groups of identically treated animals half were measured at each temperature.

For control non-irradiated tumours measured at the standard size of approximately 10 mm diameter the ratio of Q_R at 37°C to that at 25°C was just under three (Table 2). Fourteen days after exposure to 2000 rads the Q_R in the centre of SSB1a tumours was reduced to 40% of the control value (see Fig. 4) but the ratio between Q_R at 37 and 25°C was not

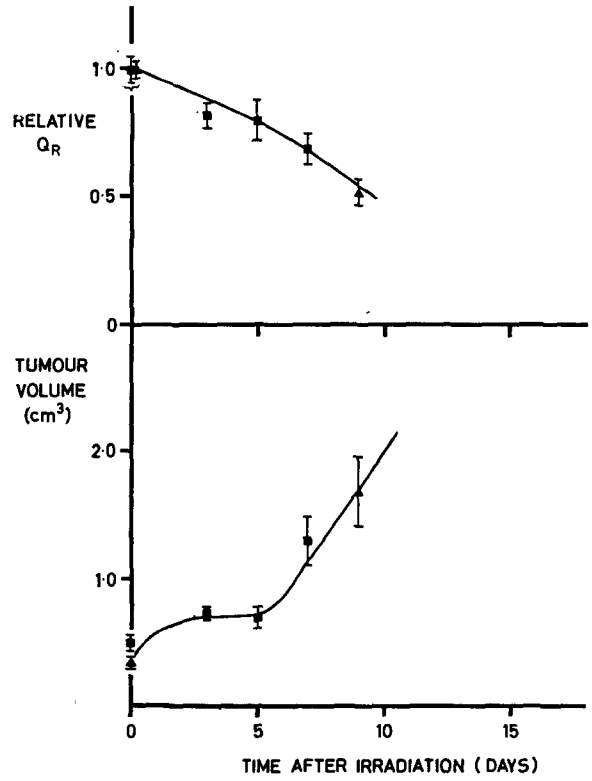


Fig. 8. Relative Q_R and tumour volume at various times after exposure to 1200 rads. The points represent the means and standard errors of the mean Q_R values for groups of 5 tumours.

Table 2

	25°C	37°C
Control tumours	4·35	13·35
	3·80	10·85
	3·80	11·45
	4·86	12·95
	3·67	10·46
	4·77	9·90
	4·36	12·36
	4·24	14·49
	3·52	14·69
	3·88	13·37
	4·29	11·56
	4·38	12·68
Mean Q_R (S.E.)	4·16(0·12)	12·34(0·44)
Ratio Q_R (37°C)/Q_R (25°C)	2·97(0·14)	
	25°C	37°C
14 *days after* 2000 rads	1·67	5·17
	1·12	5·12
	1·60	5·17
	1·52	3·17
	1·81	5·60
	2·35	
Mean Q_R (S.E.)	1·68(0·17)	4·85(0·43)
Ratio Q_R (37°C)/Q_R (25°C)	2·89(0·39)	

Mean Q_R values for control tumours at the standard irradiation size and tumours 14 days after irradiation with 2000 rads measured at 37 and 25°C.
Standard errors of overall means given in brackets.

significantly different from that for control tumours. This suggests that the patterns of response for Q_R measured at 37°C would be similar to those found at 25°C but would differ from them by a constant factor.

DISCUSSION

The capacity of a tissue for the uptake of oxygen is not a sensitive indicator of primary radiation damage (see for example the review [8]). However, most investigations into the effect of radiation on the uptake of oxygen have involved the irradiation of cell suspensions or tissue slices *in vitro* followed by incubation in buffer solutions rather than in growth media. Less study has been made of the rate of oxygen consumption in tissues irradiated *in vivo* and allowed to remain *in vivo* for various times before sampling. Ontko and Moorehead [9] have shown an increase in the endogenous respiration rate of ascites tumour cells grown in the peritoneal cavity of mice 24 hr after irradiation *in vivo* and Evans [10] has demonstrated two phases of reduction in the oxygen consumption of mouse spleen. The latter case of the mouse spleen provides an example of a secondary change in oxygen consumption due to alteration of tissue cell populations as a response to radiation damage.

In a tissue in which cell proliferation occurs, radiation injury initiates a number of processes. Damage to cells usually become apparent at the first attempt at division although some cells may pass through a small number of division cycles. The damaged cells then lyse and their components are eventually removed through the venous or lymphatic channels.

The rate of consumption of oxygen per unit volume of tissue depends principally on the cell density within the tissue but may also be affected by changes in the individual respiration per cell when cells are damaged by radiation. The density of cells within a tissue after exposure to radiation is a function of the rate at which cells die, reducing the density, and the rate at which the space once taken by the dead cells is occupied by living cells as a result of either shrinkage of the tissue or proliferation of surviving cells. The rate of oxygen uptake is influenced in addition by the rate of removal of cell debris from the tissue, since even free cellular components liberated from lysed cells may retain some capacity for the metabolism of oxygen.

The present results indicate the changes in the rate of oxygen uptake for the tumour SSB1a. The minimum in the Q_R three days after exposure to the higher doses of radiation probably corresponds to the time at which the decrease in oxygen uptake associated with the death of damaged cells and the removal of their components begins to be outweighed by the increase due to cellular repopulation.

The Q_R in the central region of untreated SSB1a tumours decreased as the tumours increased in size due to the formation of necrotic zones. In irradiated tumours, during the period when the volume remained constant, the Q_R was greater than that of untreated tumours of the same age, due to the increase in necrosis in the latter. However, following the onset of regrowth the Q_R of irradiated tumours fell rapidly. It has been suggested that either latent radiation damage to the tumour vasculature becomes expressed when proliferation of the tumour begins to increase the demand on the blood supply or that the blood supply in the centre of the tumour becomes strangulated because of oedema in necrotic tissue which has not been removed [3]. The result is the formation of an acellular non-metabolising central region in the irradiated tumours with cell proliferation, and maximum oxygen consumption [2], only occurring in a shell around the edge of the tumour. As the irradiated tumour continues to grow the width of the viable cortex remains approximately constant and the volume of the inert medulla increases ultimately becoming greater than that of the original tumour at the time of irradiation.

The tumour SSB1a does not exhibit any extensive necrosis at the standard size, of 9–11 mm diameter, used for irradiation. This may account for the lack of small-scale spatial variation in Q_R in control tumours and the acellular fibrous components of the tumour probably help to preserve its structure when damaged by irradiation. Other tumour lines have shown considerable inhomogeneity in Q_R within the standard sampling area of the electrode. In some this was true of both control and irradiated tumours whereas in others the development of inhomogeneity appeared to be a consequence of radiation damage (unpublished results).

The minimum in the Q_R of the SSB1a tumour three days after irradiation during a period when the tumour volume remains constant would coincide with an improvement in tumour oxygenation, and hence possibly radiosensitivity, provided that there is not a simultaneous reduction in blood flow. Reductions in Q_R of the order of those observed could have significant effects on tumour oxygenation particularly in situations where the hypoxic

cells form a layer only one or two cells in thickness separating viable and anoxic regions [11].

Radiobiological assays of reoxygenation have not been made for the tumour SSB1a. However, several cases have been reported where other tumours containing known fractions of hypoxic cells, usually 15–20%, have been exposed to doses of radiation sufficient to increase this fraction amongst potential surviving cells to nearly 100% immediately after irradiation, as a result of preferential killing of oxygenated cells, and have shown subsequent reoxygenation [12–15]. The rapid reoxygenation in the C3H mouse tumour of Howes [14] was associated with early shrinkage of this tumour and a diminished total demand on the blood supply. The rate of consumption of oxygen per unit volume is not likely to have shown any significant alteration in this case [2]. However, in other tumours which do not shrink so rapidly after exposure to radiation a change in the rate of oxygen consumption has been proposed as one possible factor in the early stages of reoxygenation. For example, tumour RIB_5 of Thomlinson [12] and the KHT sarcoma of Kallman [16] showed substantial reoxygenation within 24 hr of irradiation before any appreciable shrinkage of the tumour had occurred. In the case of the KHT sarcoma reoxygenation was demonstrated as early as 1 hr after the radiation exposure. These cases contrasted with that of an osteosarcoma used by van Putten [17] which also did not shrink immediately after irradiation but which, in addition, failed to reoxygenate within several days of a single dose of 1000 rads. The longer intermitotic time of the oesteosarcoma led to the suggestion that changes in the rate of oxygen uptake of this tumour were delayed as compared with other tumours. [18].

Factors which could be involved in the reoxygenation of tumours, other than a decrease in the rate of oxygen uptake, include changes in tissue water content associated with changes in tissue oxygen permeability or with changes in tumour blood flow produced by alteration of the hydrostatic pressure within the tumour or the neighbouring tissue [18]. The present results showing a relatively slow change in the rate of oxygen uptake in the tumour SSB1a, together with similar unpublished findings on other tumours, demonstrate the need for a re-examination of the processes which are involved in the early phase of radiation-induced reoxygenation of tissue.

Acknowledgements—I am most grateful to Dr. N. T. S. Evans for valuable advice on all aspects of this work. I should also like to thank Dr. R. H. Thomlinson for critical discussion of the findings.

REFERENCES

1. T. B. Constable and N. T. S. Evans, A method for measuring the spatial distribution of tissue oxygen removal rates. *Resp. Physiol.* **25,** 175 (1975).
2. T. B. Constable and N. T. S. Evans, The distribution of oxygen consumption rates in some tissues before and after X-irradiation. *Proceedings of the 2nd International Symposium on Oxygen Transport to Tissue.* Plenum Publishing Co., New York. In Press.
3. J. Denekamp and R. H. Thomlinson, The cell proliferation kinetics of four experimental tumours after acute X-irradiation. *Cancer Res.* **31,** 1279 (1971).
4. J. F. Howlett, R. H. Thomlinson and T. Alper, A marked dependence of the comparative effectiveness of neutrons on tumour line, and its implications for clinical trials. *Brit. J. Radiol.* **48,** 40 (1975).
5. N. T. S. Evans and A. R. Gourlay, The solution of a two-dimensional time-dependent diffusion problem concerned with oxygen metabolism in tissues. *J. Inst. Math. Appl.* In Press.
6. E. G. Ball, C. F. Strittmatter and O. Cooper, The reaction of cytochrome oxidase with carbon monoxide. *J. Biol. Chem.* **193,** 635 (1951).
7. J. F. Howlett, personal communication.
8. G. B. Gerber and K. I. Altman, Radiation biochemistry of tumours. In *Radiation Biochemistry* (Edited by K. I. Altman, G. B. Gerber and S. Osaka) Vol. II, p. 236. Academic Press, New York. (1970).
9. J. A. Ontko and W. R. Moorehead, Increased endogenous respiration of ascites tumour cells after radiation exposure. *Rad. Res.* **23,** 135 (1964).
10. N. T. S. Evans, Changes in the rate of oxygen consumption by mouse spleen following whole-body X-irradiation. *Rad. Res.* **35,** 465 (1968).
11. H. B. Hewitt, D. P. S. Chan and E. R. Blake, Survival curves for clonogenic cells of murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int. J. Radiat. Biol.* **12,** 535 (1967).

12. R. H. THOMLINSON, The oxygen effect and radiotherapy with fast neutrons. *Europ. J. Cancer* **7,** 139 (1971).
13. R. F. KALLMAN, Repopulation and reoxygenation as factors contributing to the effectiveness of fractionated radiotherapy. In *Frontiers of Radiation and Oncology.* (Edited by J. M. VAETH) Vol. III, p. 96. Karger, Basel (1969).
14. G. W. BARENDSEN and J. J. BROERSE, Experimental radiotherapy of a rat rhabdomysarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments, applied five times a week for several weeks. *Europ. J. Cancer* **6,** 89 (1970).
15. A. E. HOWES, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
16. R. F. KALLMAN, Discussion. In *Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy.* NCI-AEC Conference. Carmel, California. Brookhaven National Laboratory, Upton N.Y. p. 2248 (1969).
17. L. M. VAN PUTTEN, Tumour reoxygenation during fractionated radiotherapy: studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
18. R. H. THOMLINSON, Reoxygenation as a function of tumour size and histopathological type. In *Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy.* NCI-AEC Conference. Carmel, California. Brookhaven National Laboratory, Upton N.Y. p. 2242 (1969).

Europ. J. Cancer Vol. 12, pp. 973–976. Pergamon Press 1976. Printed in Great Britain

Effects of Vinblastine on Bioelectric Properties of the Frog Skin Epithelium *In Vitro*

EWA WIERASZKO and JAN KNAPOWSKI
Institute of Oncology, Warsaw, Poland; Medical Academy, Poznan, Poland

Abstract—*The influence of vinblastine on the membrane sodium transport was studied using the frog skin epithelium and short-circuit current technique. Two final concentrations of the drug in the fluid bathing the inside of the skin, namely* 1·5 *and* 3·0. $10^{-4}M$ *were used. Profound changes of the bioelectric parameters were observed: a decrease in the electric transcellular potential difference and a decrease of density of the current compensating the actual potential difference; this was accompanied by an increase in direct current (d.c.) resistance. The changes reached the greatest intensity within* 3 *hr after the addition of the drug thereafter remaining stabilized at this level. The final effect showed a statistically significant drug-concentration-dependence. A considerably worse response of the vinblastine-pretreated epithelia to vasopressin was also observed. Based upon these observations and some data obtained by other authors using different techniques, it is suggested that the drug influence the function of the cellular membrane while making it less permeable.*

INTRODUCTION

ALTHOUGH vinblastine, a cytostatic agent at the present time widely used in oncology [1] is generally believed to extend its action intracellularly (inhibition of mitosis and/or repression of DNA synthesis [2]) there are some experimental data which show that the cellular membrane may also be the target for the operation of the drug [3–5]. The cellular membrane is undoubtedly involved in the process of malignancy in pathogenic as well as in therapeutic aspects, although the relation is poorly understood as yet [6–8]. On the other hand this structure plays a leading role in the regulation of the biological transport, and this cellular activity may be rather easily assessed by electric techniques especially in epithelia [9–11]. Therefore, a series of experiments was undertaken using the frog skin epithelium in which the influence of vinblastine on sodium transcellular movement was assessed electrometrically while taking the evaluation of the membraneous mechanism of action of the agent into account.

Accepted 11 June 1976.

MATERIAL AND METHODS

Isolated sheets of the skin of the frog *R. esculenta* were mounted in plexiglass chambers with area 1·6 cm^2 exposed to the bathing Ringer solution on both sides. The solution used for all the experiments of the series contained NaCl 112·0 mM, KCl 2·0 mM, NaHCO3 2·5 mM, CaCl 1·0 mM, and its pH was adjusted to 6·8 using 0·1N HCl. The latter was necessary to realize uniform experimental conditions throughout the study as vinblastine (Vinblastin–Richter G. Budapest, Hungary) could be dissolved (in concentrations demanded i.e., 1·5 and $3{\cdot}0.10^{-4}$M) but Ringer's solution then reached pH 6·8 and could not be alkalinized. Although this degree of acidification did not seriously influence the transport activity of the epithelium, it seemed sensible to keep the same conditions for the experimental sheets of the skin before and after the addition of the drug as well as for the control sheets throughout the experiment.

Net sodium transport across the membrane was measured while using the conventional short-circuit current technique according to Ussing and Zerahn [9]; electric potential

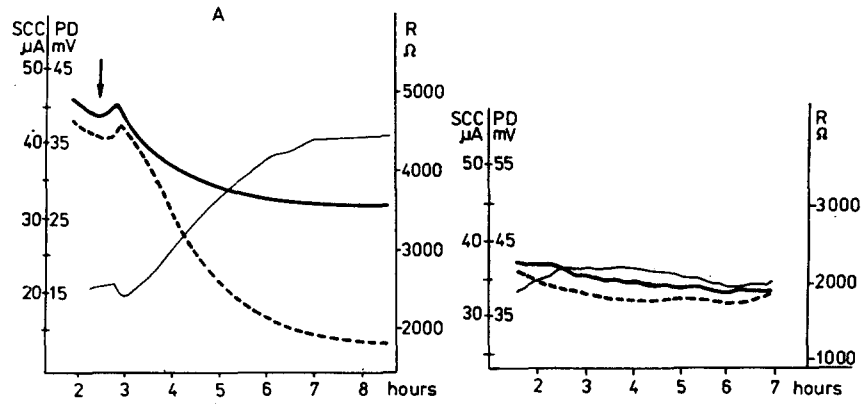


Fig. 1. Influence of vinblastine on sodium transport in vitro *measured by short-circuit current technique. Two sheets of the same skin were studied and PD, SCC and R were measured. Part A depicts typical alterations of the electrical indices observed in response to vinblastine added (↓) to the fluid bathing the inside of the skin in the concentration of* $1{\cdot}5.10^{-4}M$. *Part B depicts the electrical indices in the case when no vinblastine was added to the bathing fluid (control).*

(———)—*PD: electric potential difference across the epithelium (mV).*
(– – –)—*SCC: density of the current compensating the actual PD* ($\mu Amp.cm^{-2}$).
(———)—*R: d.c. resistance* ($\Omega.cm^2$).

difference (PD in mV), the density of the compensating current (SCC in $\mu A/cm^{-2}$) and the electrical d.c. resistance (R in $\Omega{\cdot}cm^2$) were measured as previously described [12]. Changes in the bioelectrical indices after addition of vinblastine to the fluid bathing the inner side of the skin were recorded within 4–5 consecutive hr following a control period of 1–1½ hr during which the same measurements were carried out. In order to investigate the mechanism of action of the agent vasopressin in combination with vinblastine was used in separate series of 18 experiments carried out with the same technique. After the full effect of vinblastine had been reached the antidiuretic hormone (Insipidin, A/S Benzon, Copenhagen or Vasopressin, Sandoz) at a dose of 80–100 mU/ml was added to the fluid bathing the inner side of the skin.

RESULTS

Figure 1 shows typical alterations of the electrical indices observed in one of the experiments in response to vinblastine added (↓) to the fluid bathing the inside of the skin in the concentration of $1{\cdot}5.10^{-4}$M. Profound and long-lasting variations of the lines were observed in all the experiments carried out; a decrease in PD and SCC was accompanied by an increase in R. Tenfold lower concentration of the drug had been tested in the preliminary few experiments with no effect observed in all electrical indices.

The changes which appeared when higher doses of vinblastine were added into the medium usually reached their greatest intensity within 3 hr after the addition, then remained stabilized at this level. The final effect showed statistically significant ($P < 0{\cdot}05$) concentration-dependence which is demonstrated in Table 1. In all the experiments in which vasopressin was added into the medium bathing the vinblastine-pretreated skins in order to stimulate the transport activity of the epithelium the stimulation was considerably weaker than in simultaneously studied control i.e., vinblastine non-pretreated sheets of skin. Although this stimulatory response of the experimental skins was significantly ($P < 0{\cdot}05$) smaller the effect itself was never suppressed completely (Table 2).

DISCUSSION

The results obtained seem to show quite certainly that vinblastine influences the cellular membrane of the frog skin epithelium. Significant changes of all bioelectrical indices have expressed an alteration in the transport activity of the cells which implicitly indicate a change in the function of the cellular membrane.

These results are in agreement with those obtained by Redman *et al.* who have proven

Table 1. *Effect of the addition of vinblastine to the medium bathing the inside of the isolated frog skin on bioelectric properties of the epithelium. The effect of two concentrations of the agent expressed as percent of the values recorded before the addition of vinblastine to the fluid compared with values obtained in epithelium where no vinblastine was added. The mean value of the group ∓ s.d. are given in the table. The values were read at the end of 3rd hour after addition of vinblastine to the fluid bathing*

Final concentration of vinblastine (mM)	Percent in relation to mean values before the addition of vinblastine to the medium			n
	PD	SCC	R	
0·00	111·3 ± 32·4	94·8 ± 17·8	117·0 ± 22·9	11
0·15	80·5 ± 21·5	58·1 ± 17·3	152·7 ± 40·3	11
0·30	61·2 ± 19·4	41·6 ± 11·9	147·8 ± 32·5	11

PD— electric potential difference across the epithelium.
SCC—density of the current compensating the actual PD
R— d.c. resistance
n— experiments number.

that vinblastine decreases transmembraneous potential difference in isolated apolar cells. These results can not be directly compared with ours which show the change of transcellular potential difference under the influence of the drug. However, there is an indirect identity between both results because it is known that the transporting activity of the multicellular epithelial structure "living membrane" is dependent on the transport features that take place in epithelial cells.

Our results also show the cellular mechanism of action of the drug. Presumably the drug increases the permeability of the epithelial cells while restricting the penetration of sodium ions into the cells which is followed by a decreased activity of the sodium pump. Such a mechanism of action of the drug can be directly read electrometrically in all the experiments as vinblastine always causes an increase in resistance of the epithelium. The same conclusion can be drawn indirectly from the additional series of the experiments in which vinblastine is used in combination with ADH. There is evident interaction between both these agents as far as the transport activity of the skin is concerned and the influence of the hormone on the permeability cellular membrane (increase) is unquestioned.

Since all changes in permeability of the cellular membrane are the result of an alteration of the molecular configuration of lipids and lipoproteids in the membrane, one may

Table 2. *Effect of the addition of ADH to the medium bathing vinblastine-pretreated isolated frog skin on bioelectric properties of the epithelium. ADH was added 3 hr after addition of vinblastine. The response of vinblastine-pretreated epithelia to ADH is expressed as a percentage of the values recorded before the addition of hormone to the fluid compared with the values obtained in control epithelia where hormone alone was added. The mean value of the group ± s.d. are given*

Final concentration of vinblastine (mM)	Percent in relation to mean values before the addition of ADH to the medium.			*N*
	PD	SCC	R	
0·00	159·1 ± 34·5	205·2 ± 89·4	82·2 ± 16·1	11
0·15	117·5 ± 21·0	129·1 ± 25·8	91·0 ± 7·4	9
0·30	107·5 ± 12·9	115·6 ± 18·3	94·4 ± 7·6	9

PD— electric potential difference across the epithelium
SCC—density of the current compensating the actual PD
R— d.c. resistance
N— experiments number.

suggest that vinblastine causes an impairment of the molecular arrangement of the structure which is reciprocal to that produced by the antidiuretic hormone. All the results obtained show that vinblastine make the cellular membrane less permeable. Presumably this initial effect has something to do with the final cytostatic effect of the drug.

REFERENCES

1. E. Boessen and W. Davis: *Cytotoxic Drugs in the Treatment of cancer.* E. Arnold Ltd., London (1969).
2. R. B. Livingston and S. K. Carter: *Single Agents in Cancer Chemotherapy.* Plenum Publishing Corporation, New York (1970).
3. A. W. Waddell, R. T. Robson and J. G. Edwards: Colchicine and vinblastine inhibit fibroblast aggregation. *Nature (Lond.)* **248,** 239 (1974).
4. K. Redmann, H. L. Jenssen and K. J. Kohler: Experimental and functional changes in transmembrane potential and zeta potential of single cultured cells. *Exp. Cell Res.* **87,** 281 (1974).
5. T. E. Ukena and R. D. Berlin: Effect of colchicine and vinblastine on the topographical separation of membrane function, *J. exp. Med.* **136,** 1 (1972).
6. D. F. H. Wallach, H. K. Nufermann and F. Wunderlich: Membrane aspects of neoplasia. *FEBS Lett.* **33,** 275 (1973).
7. L. Warren: The malignant cell and its membranes. *Am. J. Path.* **77,** 1, 69 (1974).
8. P. Emmelot: Biochemical properties of normal and neoplastic cell surfaces; a review. *Europ. J. Cancer* **9,** 319 (1973).
9. H. Ussing and K. Zerahn: Active transport of sodium as the source of the electric current in the short-circuited isolated frog skin. *Acta physiol. Scand.* **23,** suppl. **80,** 110 (1951).
10. J. Crabbe and P. de Weer: Relevance of sodium transport pool measurements in toad bladder tissue for the elucidation of the mechanism whereby hormones stimulate active sodium transport. *Pflugers Arch. ges. Physiol.* **313,** 197 (1969).
11. H. H. Ussing and N. A. Thorn: Effect of ADH on transport paths in toad skin. In *Transport Mechanism in Epithelia.* (Edited by H. H. Ussing and N. A. Thorn) p. 11. Munksgaard, Copenhagen (1973).
12. J. Knapowski and W. Adam. Seasonal differences in drug-and temperature-dependent changes of the sodium transport across the isolated frog skin. In *Biochemical Aspects of Renal Function.* (Edited by S. Angielski and U. C. Dubach) p. 188. Hans Huber Publ., Bern (1975).

Europ. J. Cancer Vol. 12, pp. 977–988. Pergamon Press 1976. Printed in Great Britain

Susceptibility to Lymphomas and Expression of C-Type RNA Viruses During the Graft Versus Host Reaction*

SYAMAL K. DATTA†‡ and ROBERT S. SCHWARTZ§

†*Hematology Service, New England Medical Center Hospital, and the* §*Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts* 02111, *U.S.A.*

Abstract—*The relationship between C-type RNA viruses and the development of lymphomas was examined in mice undergoing the chronic GVHR. The GVHR was induced in F_1 hybrid recipients by administration of parental spleen cells; donor strains were selected from lines that expressed abundant, few or no detectable ecotropic viruses. There was no correlation between titer of C-type RNA viruses (both ecotropic and xenotropic) and the occurrence of lymphomas. In one combination that failed to express any detectable ecotropic virus, SWR→(SWR × NZB)F_1, lymphomas occurred in relatively high incidence. The results suggested that antigen-stimulated lymphocytes, which proliferate in abundance during the GVHR, may be preferentially susceptible to malignant transformation by viruses that, in other cells, are only weakly oncogenic or even non-oncogenic.*

In the course of these experiments we also found that NZB mice possess a dominant gene (or genes) that determines high-grade expression of xenotropic virus in crosses with other strains that express little or no xenotropic virus.

INTRODUCTION

THE PATHOGENESIS of the malignant lymphomas that develop during the chronic graft versus host reaction (GVHR) is unknown [1]. Impairment of the recipient's surveillance mechanism by the GVHR is unlikely because of the lack of correlation between immunocompetence and susceptibility to lymphomas [2, 3]. Evidence that the neoplasms are due entirely to persistent immunological stimulation produced by the GVHR is only circumstantial [4, 5]. A third interpretation is that an oncogenic virus causes the neoplasms. This possibility has not been rigorously established.

The experimental system that leads to the development of lymphomas during the GVHR involves the administration of spleen cells from inbred mice to F_1 hybrid recipients. Therefore, in devising protocols it is possible to select as donors mice that spontaneously express either abundant, few or no detectable oncornaviruses. The virologic status of the donor may therefore affect the outcome without a necessary relationship to the immunological attributes of the GVHR. For example, Cornelius [6] found that lymphomas developed after injection of SJL/J spleen cells into (SJL/J × C57BL/1)F_1 recipients. But since lymphomas also developed with a similar incidence and rapidity in F_1 recipients of disrupted SJL/J spleen cells, the cause of the tumors was most likely due to transfer oncogenic viruses produced by the SJL/J cells [7]. Hays [8] carried out analogous experiments: F_1 recipients of AKR spleen cells developed thymic lymphomas and these tumors developed just as well in recipients of cell-free filtrates.

These results stand in contrast to the evidence that the induction of malignant lymphomas has an immunological basis. An analysis of 47 different parent → F_1 hybrid combinations demonstrated the importance of histo-incompatibility between donor and recipient in this process [5]. Furthermore, when the immunological reactivity of the donor cells was reduced by their enclosure in a cell-impermeable

Accepted 8 June 1976.

*Supported by USPHS Grants CA 10018 and AM 07937.

‡Scholar of the American Cancer Society (Massachusetts Division).

chamber or by rendering the prospective donor tolerant of F_1 antigens, the incidence of lymphomas in the recipients was sharply reduced. And when virus-producing AKR thymocytes—which can cause only a feeble GVHR (5)—were injected into F_1 hybrids, the incidence of lymphomas was also very low. The results of these maneuvers argue against the interpretation that the lymphomas were due solely to oncogenic viruses transferred from donors to recipients.

The recovery of ecotropic oncornaviruses from spleens of mice that do not usually express high titers of the viruses (e.g., (BALB/c × A/J)F_1 mice injected with BALB/c spleen cells) led to the conclusion that the GVHR "activated" oncogenic viruses [9]. This was supported by other experiments in which cell-free filtrates prepared from such F_1 mice provoked lymphomas when injected into newborn recipients [10]. However, later evidence challenged this view. Two independent investigations failed to confirm the finding of ecotropic viruses in the spleens of (BALB/c × A/J)F_1 mice during the early stages of the GVHR; C-type RNA viruses were detected, but they were of the xenotropic variety [11, 12]. The former studies lacked an essential control: the injection of donor parental cells into syngeneic mice (e.g., BALB/c → BALB/c). This control is essential because of age-dependent spontaneous expression of oncornaviruses, even in "low virus" strains [13, 14]. Since the donor cells take up residence in the recipients, any virus found in the F_1 may be produced by donor cells and not "activated" by an immunological process.

The present work analyzes the content of infectious ecotropic and xenotropic viruses in the spleens of F_1 mice undergoing the GVHR. Three different donor-recipient combinations were studied. The parental donor was selected from lines that spontaneously expressed abundant, few or no detectable ecotropic viruses. All three combinations were histoincompatible, thus allowing the GVHR to proceed. We failed to find any correlation between the titer of ecotropic viruses and the presence of lymphomas. Indeed, in one combination, SWR → (SWR × NZB)F_1, lymphomas developed in the absence of detectable ecotropic viruses.

MATERIAL AND METHODS

Mice

Mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, or bred in our laboratory from Jackson patental stocks. Spleen cell donors were 6–8 weeks old and both the F_1 and control parental recipients were 6–7 weeks old at the time of the first injection of spleen cells. The one exception to this was the case of SWR mice, which were 6–12 weeks old when used as donors of spleen cells for F_1 hybrid recipients. These donors were female, except where noted, whereas all other donors and receipients were males.

Induction of GVHR

F_1 recipients were given about 50×10^6 parental spleen cells intraperitoneally once weekly × 4 weeks. Donor spleens were removed aseptically, minced in chilled Ringer's solution, and pressed through tantalum gauze. Viability of the cells was determined with trypan blue and the number of living cells in the suspension was adjusted to the desired concentration.

Control mice

There were two groups of controls: uninjected F_1 hybrids and parental mice that received the same schedule of injections of parental spleen cells used for the experimental F_1 mice. The syngeneic cells used for injection of parental mice were obtained from the same pools of spleen cells that were used for injection of the F_1 hybrids.

Histopathology

Tissues were fixed in buffered formalin and stained with hematoxylin and eosin. Neoplasms were classified according to Dunn's nomenclature [15].

Titration of ecotropic virus

The UV–XC procedure used by Rowe and Pincus [16], as modified by Melief *et al.* [17], was used. Mouse fibroblasts(MEF) cultures were prepared from embryos obtained between the fourteenth and seventeenth day of gestation. The fibroblasts were trypsinized from primary lines and seeded into 60 × 15 mm plastic petri dishes (Falcon Corp., Oxnard, Calif.) at a concentration of 4×10^5 cells/4 ml media/dish. All cultures were grown and maintained in MEM 4X medium.* One day after they were planted, monolayers of fibroblasts were treated with DEAE dextran (Sigma Chemical Co.,

*Eagle's minimum essential medium with Earle's salts (1 ×), amino acids (4 ×), 2 mM L-glutamine, vitamins (4 ×), and $NaHCO_3$, supplemented with 120 U penicillin, 120 μg streptomycin/ml, and 10% unheated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.).

St. Louis, Mo.) in serum-free MEM 4X medium (25 mcg/ml), rinsed and inoculated with serial dilutions of spleen cells. Suspensions of spleen cells to be tested in this assay were prepared as previously described. Culture fluid was changed on day 4. On the seventh day after inoculation the medium was aspirated and the cultures were irradiated with 1000–1500 ergs/mm^2 of ultraviolet light. The irradiated cells were immediately overlaid with 1×10^6 XC cells/dish. Three days later the dishes were fixed and stained with methanol and giemsa. Infectious centers were recognized by the absence of growth of XC cells in areas of syncytia formation (at least three syncitia/plaque) and were distinguished from artifacts by microscopic examination. Tropism of the virus was determined by its ability to replicate preferentially on fibroblasts of either NIH Swiss or BALB/c origin. Viruses were identified as N tropic when the titer on NIH Swiss MEF was at least 30 times that on BALB/c MEF; B tropic viruses were identified when the titer on BALB/c MEF was at least 50 times that on NIH Swiss MEF. Titers were expressed as plaque forming units (PFU)/2×10^7 inoculated lymphocytes/dish.

This infectious center assay accurately reflects the *in vivo* virologic status of the animal [17]. The assay does not involve *in vitro* activation of endogenous virus during co-cultivation of spleen cells with assay cells. Previous experiments showed that virus titers obtained by this method were unaffected by mitomycin treatment of spleen cells [17] and when spleen cells from young, virus-negative mice (e.g., BALB/c, CBA) were tested, ecotropic viruses could not be activated *in vitro* either by co-cultivation or antigenic stimulation in short-term cultures [11, 12, 18]. The assay was positive only when the spleen cells to be assayed were already producing virus on the day of inoculation [18]. This is probably because most of the spleen cells die within a few days after inoculation and almost all of them are removed with the media changes during the test. Furthermore, since the MEF become confluent in growth by the fourth day of culture, they become resistant to any new infection [18].

Titration of xenotropic viruses

A modification of the method of Fischinger *et al.* [19] was employed. The assay employed the 81 cell line, which is a cloned CCC feline cell line that was transformed after single hit infection with murine sarcoma virus. This cell line grew flat and on infection with xenotropic murine C type RNA virus formed discrete foci of piled-up, rounded and loosely attached cells. The 81 cells were grown and the assay was carried out in McCoy's 5A medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with antibiotics and 15% fetal calf serum. The cells (2×10^5) were seeded into 6 cm plastic petri dishes. The next day the medium was withdrawn and the cells were treated with 2 ml of DEAE dextran in McCoy's 5A medium without serum (25 mcg/ml) for 30 min at 37°C. Serial dilutions of spleen cell suspensions in 5 ml of McCoy's 5A medium were inoculated on the 81 cells after the DEAE dextran was rinsed away. The spleen cells were from the same pool used to assay ecotropic virus. On days 3 and 10 after inoculation 3 ml of medium was added. (Similar results were obtained when on day 0 the spleen cells were inoculated in 10 ml of medium and the cultures left undisturbed until day 7). On day 7 the medium was changed completely. Foci were counted on unstained dishes on days 13–14 after infection. Titers were expressed as the number of focus forming units (FFU)/10^7 inoculated lymphocytes. In preliminary experiments we found that the number of foci that developed increased linearly and proportionally to the number of virus-producing lymphocytes inoculated. Satellite foci did not occur at lower dilutions of virus-producing lymphocytes. The infectious center technique detects both known classes of xenotropic viruses [19, 20].

Mixed lymphocyte culture (MLC)

A previously described method was employed [18]. After preparation and washing the spleen cell suspension was passed through small nylon wool plugs packed loosely in Pasteur pipettes in order to remove clumps. The stimulating cells were treated with mitomycin C (25 mcg/ml of cell suspensions) for 20 min at 37°C, washed twice, filtered and suspended in tissue culture medium. The cells were cultured in 250 ml flasks, which usually contained 10^8 mitomycin-treated and 10^8 untreated responding cells in 75 ml of final medium. The flasks were maintained at a 45° angle, which caused the cells to settle in a thin strip, for 6 days at 37°C. On the third day of incubation, the cells were resuspended and triplicate 1 ml aliquots were removed and incubated in sterile tubes with 1 μCi of ^{3}H-thymidine for 18–20 hr at 37°C. The uptake of ^{3}H-thymidine by these aliquots was then determined by a standard method. The stimulation index (SI) of the MLC was defined as the ratio of mean CPM obtained with a mixture of mitomycin treated stimulating

cells and allogeneic responding cells to the mean CPM of a mixture containing the same number of responding cells, half of which were mitomycin-treated. Following removal of aliquots of cells for determination of ^{3}H-thymidine uptake, the remaining cells were left undisturbed until the sixth day, when they were collected by centrifugation suspended in MEM 4X medium and assayed for ecotropic and xenotropic viruses.

In vitro infection of lymphocytes with xenotropic virus

A method used for the *in vitro* infection of lymphocytes by ecotropic viruses was applied to these experiments [18]. The xenotropic virus was grown by cultivating spleen cells from NZB mice on the 81 cell line and harvesting the supernatant on day 14. The supernatant was clarified by centrifugation and filtered through a 0·45 mM Millipore filter. The virus produced by this method was a pseudotype of murine sarcoma virus (rescued from the 81 cell line) with a xenotropic virus coat. The virus was used to infect 80×10^6 viable lymphocytes on the third day of the MLC in a manner described previously. Following addition of the virus, the cells were placed back in culture for an additional three days, at which time they were harvested, trypsinized, washed and assayed for the presence of xenotropic virus by the method just described. Control MLC cells were treated similarly except that tissue culture medium was used instead of virus-containing supernatant during the infection step.

RESULTS

Development of neoplasms

In preliminary experiments we ascertained that certain F_1 hybrids on an NZB background developed a high incidence of lymphomas within 6 months when injected with spleen cells from a non-NZB parent (Table 1). Some of these combinations were then selected for virological analysis. The combination BALB/c $\rightarrow$ (BALB/c $\times$ A/J)F_1 was also studied virologically because it was shown in previous work that about 30% of the hybrids developed lymphomas one year after injection of parental spleen cells [1].

Virological studies

SWR $\rightarrow$ (*SWR* $\times$ *NZB*)F_1 (*Table* 2). None of the 25 SWR mice injected with SWR spleen cells had ecotropic or xenotropic viruses in their spleens when tested at 7–11 months of age. All of the control F_1 animals had xenotropic virus in their spleens; the titers ranged from 200 to 4500 FFU/10^7 spleen cells. By contrast, ecotropic viruses were not found in these mice. The experimental F_1 mice did not differ from the control F_1 mice: none had ecotropic viruses and all were positive for xenotropic viruses. The titers of xenotropic virus in the two groups of F_1 mice did not differ significantly. There was no correlation between titer of virus and presence or absence of lymphoma. Eleven lymphoma-like lesions were found in the control SWR parental mice. These lesions were distinct from the tumors that developed in animals undergoing GVH and were identical to what Dunn has named "reticulum cell sarcoma—(Type C)" [15]. The neoplastic status of this lesion is doubtful. The incidence of true malignant lymphomas and leukemia in this strain is very low [21].

C57BL/6 $\rightarrow$ (*C57BL/6* $\times$ *NZB*)F_1 (*Table* 3). Ecotropic viruses were found in low titer in 19/27 C57BL/6 mice injected with C57BL/6 spleen cells. Because of the low titers it was not possible to determine with certainty whether the viruses were B- or N-tropic. If a titter of "0" is provisionally accepted as indicating the absence of infectious virus in the assay we employed, then 16/27 animals had B-tropic viruses in their spleens. Low titers of xenotropic viruses were found in 15/27 of the parental mice. In the control F_1 mice, by contrast, no ecotropic viruses were found and all of the animals had xenotropic viruses, often in relatively high titers (range, 50–16,000 FFU/10^7 spleen cells). Of the experimental F_1 mice, 15/26 contained ecotropic viruses, an

Table 1. *Incidence of lymphomas in various* F_1 *hybrids. In all cases the GHVR was induced by inoculation of spleen cells from the non-NZB parent. Control mice did not receive parental cells. All mice were autopsied at the age of* 8–9 *months*

F_1 Hybrid	Test	Spleen weight	Lymphoma
SWR × NZB	Control	189 ± 88	0/12
SWR × NZB	GVHR	474 ± 274	17/25
C57BL/6 × NZB	Control	91 ± 18	0/9
C57BL/6 × NZB	GVHR	436 ± 382	6/13
B10.A × NZB	Control	117 ± 17	0/5
B10.A × NZB	GVHR	257 ± 187	6/21
C57BL10 × NZB	Control	117 ± 27	0/13
C57BL10 × NZB	GVHR	449 ± 303	7/11
B10Br × NZB	Control	102 ± 14	0/13
B10Br × NZB	GVHR	177 ± 83	1/25
C3H × NZB	Control	141 ± 56	0/7
C3H × NZB	GVHR	594 ± 447	11/16

Table 2. Virus titers and lymphomas in SWR and experimental and control (SWR × NZB)F$_1$ mice. In this and succeeding tables the titers are expressed as log$_{10}$; 0 = negative, N = titer on NIH Swiss embryo fibroblasts, B = titer on BALB/c embryo fibroblasts, X = titer of xenotropic virus, L = lymphoma. Parental controls in this and all subsequent tables were injected with aliquots of the same pools of parental spleen cells used for injection of the experimental F$_1$ animals (GVHR). Both parental controls and GVH mice received about 200 × 10^6 parental spleen cells given in four weekly doses. Age is in months

F_1 Control						Parent control						GVH					
No.	Age	N	B	X	L	No.	Age	N	B	X	L	No.	Age	N	B	X	L
1.	6½	0	0	2·48	0	1.	8	0	0	0	0	1.	7½	0	0	3·20	+
2.	6½	0	0	3·00	0	2.	8	0	0	0	0	2.	7½	0	0	3·95	+
3.	6½	0	0	2·48	0	3.	8	0	0	0	0	3.	7½	0	0	2·95	0
4.	6½	0	0	3·20	0	4.	8	0	0	0	0	4.	7½	0	0	3·20	+
5.	6½	0	0	2·90	0	5.	8	0	0	0	0	5.	8½	0	0	2·65	0
6.	6½	0	0	2·48	0	6.	8	0	0	0	0	6.	8½	0	0	2·60	0
7.	5½	0	0	3·00	0	7.	8	0	0	0	0	7.	9	0	0	2·18	+
8.	5½	0	0	2·30	0	8.	8	0	0	0	0	8.	9	0	0	1·90	0
9.	6	0	0	3·20	0	9.	8	0	0	0	0	9.	9	0	0	3·70	0
10.	6	0	0	3·48	0	10.	8	0	0	0	0	10.	9	0	0	3·48	+
11.	6	0	0	3·30	0	11.	11	0	0	0	0	11.	9	0	0	3·60	+
12.	6	0	0	3·48	0	12.	11	0	0	0	0	12.	9	0	0	3·30	0
13.	6	0	0	3·30	0	13.	11	0	0	0	0	13.	9	0	0	3·18	0
14.	6	0	0	3·65	0	14.	10	0	0	0	0	14.	9	0	0	3·78	0
15.	6	0	0	3·04	0	15.	10	0	0	0	0	15.	9	0	0	3·90	0
16.	10	0	0	3·48	0	16.	7	0	0	0	0	16.	9	0	0	3·70	+
17.	10	0	0	3·18	0	17.	7	0	0	0	0	17.	9	0	0	3·20	0
18.	10	0	0	3·48	0	18.	7	0	0	0	0	18.	9	0	0	2·95	+
19.	7½	0	0	2·70	0	19.	7	0	0	0	0	19.	10	0	0	2·78	+
20.	7½	0	0	2·85	0	20.	7	0	0	0	0	20.	10	0	0	1·90	0
21.	7½	0	0	3·48	0	21.	8	0	0	0	0	21.	10	0	0	2·30	+
22.	7½	0	0	3·26	0	22.	8	0	0	0	0	22.	9	0	0	3·30	0
23.	7½	0	0	2·70	0	23.	8	0	0	0	0	23.	9	0	0	3·30	0
24.	7	0	0	3·48	0	24.	8	0	0	0	0	24.	9	0	0	3·00	0
25.	7	0	0	3·04	0	25.	8	0	0	0	0	25.	9	0	0	3·30	0
26.	7	0	0	2·90	0							26.	9	0	0	2·90	+

incidence not different from that found in the control C57BL/6 mice injected with aliquots of the same pool of cells that was used for inoculation of the F_1 animals. As in the parental line, the titers were low. And, as in the control F_1 animals, all of the experimental F_1 mice had xenotropic viruses; titers ranged from 30–2000 FFU/10^7 spleen cells. There was no correlation between the presence of ecotropic or xenotropic viruses and the presence of lymphoma in any of the mice.

B10.A → (B10.A × NZB)F$_1$ (*Table* 4). All B10.A mice injected with B10.A spleen cells had high titers of B-tropic viruses; titers on BALB/c MEF ranged from 700–45,000 PFU/2 × 10^7 spleen cells. In 18/25 cases, relatively low titers of xenotropic viruses were found (range, 0–25 FFU/10^7 spleen cells). In contrast, none of the 20 control F_1 animals had detectable ecotropic viruses and in all of them, xenotropic viruses were found in relatively higher titers than in the parental controls (range, 15–1100 FFU/10^7 spleen cells). All of the experimental F_1 mice had B-tropic viruses in their spleens; as a group the titers were lower than those found in the parental mice injected with aliquots of the same suspensions of spleen cells (range, on BALB/c MEF, 35–130,000 PFU/2 × 10^7 spleen cells). All of the experimental mice had xenotropic viruses in their spleens; the titers were intermediate between those of the parental and control F_1 mice. There was no correlation between titer of virus and lymphomas in either experimental or control animals.

BALB/c → (BALB/c × A/J)F$_1$ (*Table* 5). All parental controls contained ecotropic viruses, which appeared to be mixtures of N- and B-tropic viruses. Xenotropic viruses in moderate titers were also found in all parental animals. One control F_1 mouse had low titers of ecotropic virus, probably N-tropic, in its spleen. All of these mice had xenotropic virus in their spleens. In the experimental F_1 group, 9/10 had ecotropic viruses and in 6/9 the virus behaved like a B-tropic agent. In these six mice, the titer of virus on BALB/c MEF exceeded analogous titers in the control

Table 3. Virus titers and lymphomas in C57BL/6 and experimental and control (C57BL/6 × NZB)F$_1$ mice

F_1 Control						Parent Control						GVH					
No.	Age	N	B	X	L	No.	Age	N	B	X	L	No.	Age	N	B	X	L
1.	6	0	0	2·78	0	1.	8	0	0	0·78	0	1.	4	0	0	2·08	+
2.	6	0	0	2·00	0	2.	8	0	0	0·60	0	2.	6	0	0	3·30	+
3.	6	0	0	2·48	0	3.	8	0	1·15	0	0	3.	8	1·30	0	1·70	0
4.	6	0	0	2·48	0	4.	8	0	1·00	0	0	4.	8	1·00	0	2·60	+
5.	6	0	0	1·78	0	5.	8	0	1·18	0	0	5.	8½	0	0	2·48	+
6.	6	0	0	2·78	0	6.	8½	0	1·20	1·23	0	6.	8½	0	0·60	2·70	0
7.	9	0	0	4·20	+	7.	8½	0	1·00	1·00	+	7.	8½	0	0	3·08	0
8.	9	0	0	3·40	0	8.	8½	0	1·18	0·70	0	8.	8½	0	0	2·60	+
9.	9	0	0	3·30	0	9.	8½	0	1·26	1·30	0	9.	8½	0·60	0	3·30	+
10.	9	0	0	3·78	0	10.	9	0	0·60	0	0	10.	8½	0	0·60	2·00	0
11.	9	0	0	3·88	0	11.	9	0	0·78	0	0	11.	8½	1·00	0·78	2·30	+
12.	9	0	0	2·78	0	12.	9	0	0	0	0	12.	8½	0	0	1·48	+
13.	9	0	0	2·23	0	13.	9	0	0·48	0	0	13.	8	0	0	2·70	0
14.	9	0	0	2·15	0	14	9	0	1·20	0	0	14	8	0	0	3·11	0
15.	9	0	0	2·48	0	15.	9	0	1·08	0	0	15.	8	0	0	2·95	0
16.	9	0	0	1·78	0	16.	9	0	1·30	0	0	16.	8	0	0	2·90	0
17.	9	0	0	2·54	0	17.	9	0	0·90	0	0	17.	8	0	0	2·60	0
18.	8	0	0	3·00	0	18.	9	0	1·48	0	0	18.	8	0·70	0·48	2·00	+
19.	8	0	0	2·60	0	19.	8	0	0	0·85	0	19.	8	0	0·30	2·30	0
20.	8	0	0	2·70	0	20.	8	0	0	0·95	0	20.	8	0	0	2·81	+
21.	8	0	0	2·11	0	21.	8	1·48	0	1·30	0	21.	8	0	0	2·30	+
22.	8	0	0	2·30	0	22.	8½	1·00	0	1·43	0	22	8	0	0	2·78	+
23	8	0	0	2·48	0	23.	8½	0	0	1·00	0	23.	8	0	0	2·00	+
24.	8	0	0	1·90	0	24.	8½	0	0	1·04	0	24.	8	0	0	2·28	+
25.	8	0	0	2·78	0	25.	8½	0	0	0·48	0	25.	8	1·60	0	2·30	+
26.	8	0	0	2·18	0	26.	8½	0·60	0	0	0						
27.	8	0	0	2·30	0	27.	8½	0	1·30	0	0						
28.	8	0	0	1·70	0												

Table 4. Virus titers and lymphomas in B10.A and experimental and control (B10.A × NZB)F$_1$ mice

F_1 Control						Parent Control						GVH					
No.	Age	N	B	X	L	No.	Age	N	B	X	L	No.	Age	N	B	X	L
1.	5½	0	0	2·60	0	1.	5	2·32	4·65	0	0	1.	5	1·30	3·65	2·00	+
2.	6	0	0	2·00	0	2.	8	0·60	2·85	1·18	0	2.	5	0·60	2·90	1·18	+
3.	6	0	0	3·04	0	3.	8	0·48	2·78	1·30	0	3.	4½	0	1·90	2·60	+
4.	6	0	0	2·40	0	4.	8½	1·04	3·34	0	0	4.	6½	0	2·78	2·30	0
5.	6	0	0	1.30	0	5.	8½	1·18	3·30	0	0	5.	7	0·90	3·60	2·00	+
6.	6	0	0	3·00	0	6.	8½	2·00	3·90	1·30	0	6.	8	0	1·54	2·48	+
7.	6	0	0	2·60	0	7.	8½	1·78	3·81	1·18	0	7.	6	0	1·60	2·78	+
8.	6	0	0	2·00	0	8.	8½	1·88	3·85	1·40	0	8.	8½	0	1·81	1·85	0
9. ♀	7	0	0	2·00	0	9.	8½	2·10	4·00	1·00	0	9.	8½	0	2·20	2·00	+
10. ♀	7	0	0	1·90	0	10.	9	1·08	3·98	0	0	10.	8½	1·90	4·20	0·90	0
11. ♀	7	0	0	2·08	0	11.	9	1·30	3·90	0	0	11.	8½	0·30	2·30	1·18	+
12. ♀	7	0	0	3·00	0	12.	9	1·48	4·08	0	0	12.	8½	0	2·08	1·26	+
13. ♀	7	0	0	2·95	0	13.	9	1·18	3·90	0	0	13.	8½	0	2·48	2·26	0
14. ♀	7	0	0	1·30	0	14.	9	1·81	4·00	0	0	14.	8½	0·30	2·98	2·30	0
15. ♀	7	0	0	1·18	0	15.	9½	2·41	3·93	1·00	0	15.	8½	0	2·08	0	0
16.	8	0	0	1·78	0	16.	9½	2·11	3·85	1·30	0	16.	9	2·15	4·48	1·00	0
17.	8	0	0	2·00	0	17.	9½	2·48	4·00	1·18	0	17.	9	1·60	3·93	2·11	0
18.	8	0	0	2·30	0	18.	8	2·30	4·65	1·30	0	18.	8	2·26	3·81	1·30	0
19.	8	0	0	2·30	0	19.	8	1·78	4·48	0·60	0	19.	8	2·08	5·11	2·08	0
20.	8	0	0	1·95	0	20.	8	1·90	4·60	0	0	20.	8	1·30	3·98	1·30	0
						21.	8	1·81	4·54	0·78	0	21.	8	1·00	3·18	1·00	0
						22.	8	2·04	4·65	0·60	0						
						23.	8	2·32	4·54	0·90	0						
						24.	8	2·38	4·60	1·00	0						
						25.	8	2·18	4·40	1·18	0						

Table 5. Virus titers in BALB/c and experimental and control (BALB/c × A/J)F_1 mice

F_1 Control						Parent Control						GVH					
No.	Age	N	B	X	L	No.	Age	N	B	X	L	No.	Age	N	B	X	L
1.	8	0	0	1·70	0	1.	8	1·30	1·30	2·28	0	1.	8	1·40	2·95	2·20	0
2.	8	0	0	1·30	0	2.	8	1·18	1·20	1·78	0	2.	8	1·30	2·85	2·23	0
3.	8	0·60	0	1·04	0	3.	8	1·41	1·40	2·48	0	3.	8	0	0·48	2·08	0
4.	8	0	0	2·00	0	4.	8	2·04	1·70	2·18	0	4.	8	1·30	3·08	2·30	0
5.	8	0	0	2·18	0	5.	8	1·95	1·60	2·30	0	5.	8	2·43	2·26	2·20	0
						6.	8	2·15	1·81	1·95	0	6.	8	1·00	2·90	1·78	+
						7.	8	1·90	2·15	2·48	0	7.	8	1·18	3·04	1·48	0
						8.	8	2·00	2·20	2·00	0	8.	8	1·08	2·54	2·30	0
						9.	8	1·88	2·15	2·60	0	9.	8	0·30	0·78	1·85	0
						10.	8	1·93	2·16	2·74	0						

parental mice by a factor of at least 2 and in 5 of the 6, the factor was at least 5. All of these animals had xenotropic viruses and the titers were not different from those in control BALB/c mice injected with BALB/c spleen cells.

Attempts to infect lymphocytes with xenotropic virus.

We previously showed that lymphocytes could be infected by ecotropic viruses during *in vitro* contact with virus-producing allogeneic lymphocytes [18]. The experimental protocol employed a mixed lymphocyte culture technic in which virus produced by "stimulator" cells infected virus-negative "responding" cells. The virus-producing "stimulator" cells were treated with mitomycin, which stopped further virus production and ultimately killed the cells, so that after 6 days in culture the only cells capable of producing virus were the progeny of the originally virus-negative "responding" cells. Under these conditions infection occurred despite the Fv-1-determined tropism barrier. A similar principle was used to determine if lymphocytes stimulated by alloantigens (the immunological setting of the GVHR) could be infected by xenotropic viruses.

Table 6 shows results when NZB lymphocytes were used as "stimulator" cells and SWR or C57L lymphocytes were the "responding" cells. The alloantigens of NZB (H-2^d) lymphocytes stimulated SWR (H-2^q) or C57L (H-2^b) lymphocytes, as demonstrated by the high stimulation indices. On day 0 of the culture the NZB cells produced xenotropic virus. On day 6 of the cultures between mitomycin-treated NZB cells and either SWR or C57L cells there was no evidence of cross infection and viral replication in the responding cells. In one control mixed lymphocyte culture, where C57L cells were stimulated by SWRm, a few foci [4] were produced. This may explain the production of a few foci in the NZBm × C57L combination on day 6.

Table 6. Xenotropic virus titers in mixed lymphocyte cultures. NZB_m = NZB spleen cells treated with mitomycin. Same pool of cells were used as stimulating cells in the mixed lymphocyte cultures. On day 6 out of 200×10^6 NZB_m cells planted alone, only 10^7 cells were left that excluded trypan blue. (Results expressed as explained in Text and Ref. 18)

		Xenotropic virus F.F.U./10^7 Lymphocytes	
MLC	S.I.	Day 0	Day 6
NZB_m alone*		14,000	2,000*
NZB_m + C57L	45	7,000	6
$C57L_m$ + C57L		0	0
SWR_m + C57L	8	0	4
NZB_m + SWR	21·5	7,500	0
SWR_m + SWR		0	0
$C57L_m$ + SWR	10	0	0

*NZB_m = NZB spleen cells treated with mitomycin. Same pool of cells were used as stimulating cells in the mixed lymphocyte cultures. On day 6 out of 200×10^6 NZB_m cells planted alone, only 10^7 cells were left which excluded trypan blue.

A second attempt to infect stimulated SWR lymphocytes by cell free xenotropic virus was made by culturing the cells with virus-negative, mitomycin-treated C57L or 129 lymphocytes in the presence of a pseudotype MSV with an NZB xenotropic virus coat (Table 7). In no case was there evidence of infection, despite the allogeneic stimulation. We can therefore conclude that, despite vigorous stimulation by alloantigens, lymphocytes of SWR mice cannot be productively infected by mouse xenotropic virus.

DISCUSSION

Evidence obtained by examination of inbred mice [22], epidemiologic surveys of wild mice

Table 7. Failure of exogenous xenotropic viruses to replicate in antigen-stimulated lymphocytes. Virus from NZB lymphocytes were grown on 8C cell line to produce a pseudotype MSV with NZB xenotropic MuLV coat. Partially purified, this virus had a titer of 2000 FFU/0·2 ml. 30,000 FFU of this virus was used to infect 80×10^6 viable MLC stimulated lymphocytes

MLC	Virus*	S.I.	Xenotropic virus F.F.U./10^7 cells day 6
129_m + SWR	+		0
129_m + SWR		14	0
$C57L_m$ + SWR	+		0
$C57L_m$ + SWR		11	0

*Virus from NZB lymphocytes were grown on 8C cell line to produce a pseudotype MSV with NZB xenotropic MuLV coat. Partially purified, this virus had a titer of 2,000 F.F.U./0·2 ml. 30,000 F.F.U. of this virus was used to infect 80×10^6 viable MLC stimulated lymphocytes.

[23] and genetic analysis of the progeny of crosses between high and low leukemic strains [24] favors the view that the expression of ecotropic viruses (as detected by technics similar to ours) parallels the risk of developing lymphoreticular malignancies. However, the results of our experiments indicate that the relationship between ecotropic viruses and lymphomas may be more intricate than generally recognized. We will illustrate the complexity of the problem by analysis of our data and then suggest how the apparent discrepancies may be reconciled.

The (SWR × NZB)F_1 system was studied because we found that SWR mice failed to express any known class of C-type RNA virus, even late in life. By means of a specific radioimmunoassay Stephenson *et al.* [25] detected the p12 antigen of the xenotropic virus in embryo fibroblasts of SWR. However, these cells failed to release virus particles, even after repeated attempts to induce their production with chemical agents. In a more recent study Stephenson *et al.* [26] induced with IUDR a B-tropic virus from SWR embryo fibroblasts and spleen cells. The virus was detected only after repeated blind passage of the assay cells for six weeks. They failed, as we did, to detect ecotropic viruses in untreated spleen cells of SWR mice. The relevance of this chemically induced virus to our findings seems doubtful because, in other studies, we and others could not detect, even with blind passage, ecotropic viruses in immunologically stimulated cells from strains that are known to express these viruses spontaneously as they age [11, 12, 18].

The other parent of this F_1 hybrid, NZB, is also noteworthy because the only virus that has been isolated from this strain by us and others [27] was xenotropic. There is no evidence that the NZB xenotropic virus is oncongenic in mice. Thus, the virologic attributes of these mice and their F_1 hybrids permitted a test of the hypothesis that infectious ecotropic viruses are related to the development of malignant lymphomas during the chronic GVHR. The results were clear: malignant lymphomas developed in the absence of detectable infectious ecotropic viruses.

We admit the possibility that ecotropic viruses may have been produced by the experimental mice in quantities that we could not detect even though the assay we used was up to 1000 times more sensitive than previously described methods [17]. This assay, which reflects the *in vivo* virologic status of the animal [12, 17, 18] (see Methods section), was compared with assays of tissue extracts in three different strains of mice and was found to be a more sensitive indicator of the presence of infectious virus *in vivo* [17]. The assay does not involve *in vitro* activation of endogenous viruses [12, 17, 18] and its results parallel those of direct assay of splenic tissue for p30 by radioimmunoassay [28]. The possibility that ecotropic viruses may have been expressed only transiently and prior to performance of the test seems remote because of the previously mentioned virologic properties of the mice involved. We are aware that all C-type RNA viruses may not be detected by the methods we employed, and because the present study deals with the detection of infectious viruses, the possibility of partial expression of some viral genes is not excluded. We are continuing to study this latter question by other technics. Despite these caveats, the development of lymphomas in the absence of detectable infectious ecotropic viruses remains a contrast to previous studies [22–24].

Results of the C57BL/6 → (C57BL/6 × NZB)F_1 combination were similar to those in SWR → (SWR × NZB)F_1, but not as striking because ecotropic viruses, albeit in low titers were found in 12/26 experimental mice. Nevertheless, animals without detectable ecotropic viruses were found with lymphomas. This series and the remaining parent → F_1 combinations demonstrate the importance of a control that consists of parental mice injected with aliquots of parental spleen cells obtained from the same pool used for injection of the F_1 animals. This control was required to account for two phenomena: the occasional virus-

positive donor whose productively infected lymphocytes can contaminate the entire pool of spleen cells used in the experiment [18] (as a matter of technical convenience, spleens from 20–40 parental donors may be used in preparing suspensions of cells), and age-dependent spontaneous expression of C-type RNA viruses. An example of the first consideration is the B10.A line. About 60% of these mice express B-tropic viruses, even at an early age [28]. We found in previous experiments (unpublished) that all *pools* of spleen cells prepared from 6 week old B10.A mice contained ecotropic viruses. This would lead to infection of all experimental mice by viruses contained in the inoculum of parental spleen cells. An example of the second phenomenon is the C57BL/6 line, which is negative for ecotropic viruses early in life, but which expresses these viruses later [29]. These animals "switch on" their viral genes as they age. It can be noted in Table 3 that the incidence and titers of ecotropic viruses in control C57BL/6 mice and in experimental F_1 hybrids were virtually identical. We interpret this as an indication of age-dependent expression of ecotropic viruses by the donor cells in the hybrid and not as a phenomenon related to the GVHR.

The possibility that oncogenic C-type RNA viruses cause the lymphomas that develop during the GVHR received support when C-type RNA viruses were recovered from the spleens of (BALB/c × A/J)F_1 mice undergoing the GVHR after inoculation of BALB/c spleen cells [9]. Furthermore, cell-free filtrates prepared from the spleens of the experimental mice were oncogenic [10]. The latent period before the appearance of lymphomas was long (about 18 months) and the incidence of the tumors was relatively low [10]. In evaluating these results it is important to appreciate that B-tropic viruses isolated from normal BALB/c mice were similarly oncogenic after a long (15 months) latent period when injected into newborn hosts [13, 14]. Therefore, without the relevant control (filtrates prepared from age-matched BALB/c mice that were injected with aliquots of the same suspension of BALB/c spleen cells used to inject the F_1 hybrids), we are reluctant to accept the conclusion that transmission experiments have proved that the lymphomas of the chronic GVHR are caused by ecotropic viruses. Our data (Table 5) demonstrate that, compared to the parental control, relatively high titers of B-tropic viruses were found in (BALB/c × A/J)F_1 mice undergoing the GVHR for 6 months. These results, coupled with previous experiments in which only xenotropic viruses were found *early* in the course of the GVHR in the same F_1 mice [11, 12], suggests that in this combination B-tropic viruses are preferentially expressed relatively late in the GVHR, whereas xenotropic viruses are preferentially expressed relatively early in the reaction.

High titers of B-tropic viruses may not by themselves cause the development of lymphomas. This is demonstrated in Table 4, which shows that control B10.A mice, which were uniformly infected with very high titers of B-tropic virus, failed to develop malignancy. In previous studies of the virology of B10.A mice [28], we pointed out that these animals were infected from birth with high titers of B-tropic virus, yet the incidence of leukemia or lymphoma in one year old B10.A mice was less than 10% (unpublished).

Thus far we have seen that the development of lymphomas in the GVHR system does not correlate with the presence or titer of infectious ecotropic viruses. This finding is not without precedent. We already mentioned the low incidence of malignancy in heavily infected B10.A mice and to this we should add the example of the NZB mouse, which does not express ecotropic viruses, yet develops reticulum cell sarcomas late in life [30]. How can these results be reconciled with the generally accepted pattern of "high virus/high leukemia" –"low virus/low leukemia"? There are several possibilities. In the case of B10.A mice, the endogenous B-tropic virus seems, like its counterpart in BALB/c mice, to be only weakly oncogenic. The endogenous N-tropic virus of AKR mice is, by comparison, more oncogenic [31]. This may account for the results in AKR crosses, where the presence of the AKR virus predicts the development of leukemia [24].

We turn now to a consideration of how the host cell may contribute to the oncogenic action of these viruses. Weakly oncogenic ecotropic viruses may have an increased oncogenic potential when they infect antigen-stimulated lymphocytes. Such potential "target cells" proliferate in abundance during the chronic GVHR [32], and their interaction with weakly oncogenic viruses may be an important event in the development of lymphomas in some strains of mice. This argument is supported by the demonstration of an increased incidence of neoplasms when mice undergoing the GVHR or other types of immunologic stimulation were infected with exogenous ecotropic viruses [33–35]. Raschke *et al.* showed that when lymphocytes were

stimulated *in vitro* by either mitogen or antigen and then infected with the Abelson virus, they formed tumors when inoculated into syngeneic mice [36]. By contrast, no lymphomas developed when unstimulated lymphocytes were infected with the Abelson virus and then inoculated into syngeneic mice. Studies by Rosenberg *et al.* [37] also show that transformation of lymphocytes by the Abelson virus depends on the state of differentiation of the "target" B cell. Thus, even when titers of ecotropic viruses are very low, as in C57BL/6 → (C57BL/6 × NZB)F_1, their replication in certain antigen-driven lymphocytes may result in an outcome that differs radically from their replication in resting lymphocytes. Conventional transmission studies cannot test this hypothesis; it can receive support only if ecotropic viruses from experimental F_1 or control parental mice are shown to have preferential oncogenicity in antigen-stimulated lymphocytes. Such studies are in progress.

We stated above that xenotropic viruses have not been found to be oncogenic in the host of origin. We assume that, in order for a virus to transform a cell into a malignant state, it must be capable of penetrating and replicating within that cell. We tested this hypothesis in the case of xenotropic viruses and found that there was an absolute block to replication of exogenous xenotropic viruses by lymphocytes (Tables 6 and 7). Even when lymphocytes were stimulated by histocompatibility antigens (a situation analogous to the GVHR), they failed to permit replication of exogenous xenotropic viruses. If viral replication is an important step in viral oncogenesis, it seems unlikely that xenotropic viruses can directly transform lymphocytes. Nevertheless, the oncogenic potential of xenotropic viruses remains in contention. Similarities between the chronic GVHR and the spontaneous lymphoproliferative disorder of NZB mice are striking [30]. In each of the F_1 hybrids we studied, titers of xenotropic virus were similar to those found in NZB mice. But, as in the case of NZB mice, considerable hyperplasia of lymphoid tissue progressing to lymphoma occurred only in the experimental mice (Table 1). Therefore, further analysis of the possibility that xenotropic virus may be oncogenic under conditions of lymphoreticular proliferation seems warranted.

Finally, we call attention to the virological results in the control F_1 hybrids that were bred against an NZB background. Suppression of ecotropic viruses occurred in the (B10.A × NZB)F_1 and (C57BL/6 × NZB)F_1 mice. This was probably due to Fv-1 restriction [38] and resistance factors present in the NZB strain that restrict replication of all types of ecotropic viruses [39]. In all three hybrids, relatively high titers of xenotropic virus were found. These titers were similar to those found by the same technic in NZB mice [40]. By contrast, no xenotropic virus (SWR) or relatively low titers of xenotropic virus (C57BL/6 and B10.A) were found in the non-NZB parental line. It thus appears that NZB mice possess a dominant gene (or genes) that leads to high-grade expression of xenotropic virus. Further genetic studies relating inheritance to autoimmunity and expression of the xenotropic virus are in progress.

Acknowledgement—We are grateful to Mrs. Sylvia Johnson for her expert technical assistance, Dr. Peter Fischinger for the generous gift of the 81 cell line, and to Mrs. Pamela Senger for her expert secretarial assistance.

REFREENCES

1. M. Y. K. Armstrong, E. Gleichmann, H. Gleichmann, L. Beldotti, J. Andre-Schwartz and R. S. Schwartz, Chronic allogeneic disease. II. Development of lymphomas. *J. exp. Med.* **132,** 417 (1970).
2. C. Solnik, H. Gleichmann, M. Kavanah and R. S. Schwartz, Immunosuppression and malignant lymphomas in graft-versus-host reactions. *Cancer Res.* **33,** 2068 (1973).
3. N. H. Ruddle, M. Y. K. Armstrong and F. F. Richards, Tumor induction by immunologically activated murine leukemia virus: cellular immunity early in the graft-vs-host reaction. *J. Immunol.* **112,** 706 (1974).
4. M. Y. K. Armstrong, R. S. Schwartz and L. Beldotti, Neoplastic sequelae of allogeneic disease. III. Histological events following transplantation of allogeneic spleen cells. *Transplantation* **6,** 1380 (1967).
5. E. Gleichmann, H. Gleichmann and R. S. Schwartz, Immunologic induction of malignant lymphoma: Genetic factors in the graft-versus-host model. *J. nat. Cancer Inst.* **49,** 793 (1972).
6. E. A. Cornelius, Rapid viral induction of murine lymphomas in the graft versus host reaction. *J. exp. Med.* **136,** 1533 (1972).

7. K. S. S. Chang, L. W. Law and T. Aoki, Type-C RNA virus isolated from SJL/J mice. *J. nat. Cancer Inst.* **52,** 777 (1974).
8. E. F. Hays, Graft-versus-host reactions and the viral induction of mouse lymphoma. *Cancer Res.* **32,** 270 (1972).
9. M. S. Hirsch, P. H. Black, G. S. Tracey, S. Leibowitz and R. S. Schwartz, Leukemia virus activation in chronic allogeneic disease. *Proc. nat. Acad. Sci. (Wash.)* **67,** 1914 (1970).
10. M. Y. K. Armstrong, N. H. Ruddle, M. B. Lipman and F. F. Richard, Tumor induction by immunologically activated murine leukemia virus. *J. exp. Med.* **137,** 1163 (1973).
11. C. J. Sherr, M. M. Lieber and G. J. Todaro, Mixed splenocyte cultures and graft versus host reaction selectively induce an "S-tropic" murine type-C virus. *Cell* **1,** 55 (1974).
12. J. A. Levy, S. K. Datta and R. S. Schwartz, Xenotropic virus production during graft versus host reaction. *Clinical Immunol. Immunopathol.* January 1977 (in press).
13. R. L. Peters, G. J. Spahn, L. S. Rabstein, G. T. Kellof and R. J. Huebner, Murine C-type RNA viruses from spontaneous neoplasms: *In vitro* host range and oncogenic potential. *Science* **181,** 665 (1973).
14. R. L. Peters, G. J. Spahn, L. S. Rabstein, G. J. Kellof and R. J. Huebner, Neoplasm induction by murine type-C viruses passaged directly from spontaneous non-lymphoreticular tumors. *Nature New Biol.* **244,** 103 (1973).
15. T. B. Dunn and M. K. Deringer, Reticulum cell neoplasm, type B, or the "Hodgkin's-like lesion" of the mouse. *J. nat Cancer Inst.* **40,** 771 (1968).
16. W. P. Rowe and T. Pincus, Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. *J. exp. Med.* **135,** 429 (1972).
17. C. J. M. Melief, S. K. Datta, S. Louie, S. Johnson, M. Melief and R. S. Schwartz, Splenocyte plaque for the detection of murine leukemia virus. *Proc. Soc. exp. Biol. (N.Y.)* **149,** 1015 (1975).
18. S. K. Datta, C. J. M. Melief and R. S. Schwartz, Lymphocytes and leukemia viruses: tropism and transtropism of murine leukemia virus. *J. nat. Cancer Inst.* **55,** 425 (1975).
19. P. J. Fischinger, C. S. Blevins and S. Nomura, Simple, quantitative assay for both xenotropic murine leukemia and ecotropic feline leukemia viruses. *J. Virol.* **14,** 177 (1974).
20. R. Callahan, M. M. Lieber and G. J. Todaro, Nucleic acid homology of murine xenotropic type C virus. *J. Virol.* **15,** 1378 (1975).
21. D. D. Myers, H. Meir and R. J. Huebner, Prevalence of murine C-type RNA virus group specific antigen in inbred strains of mice. *Life Sci.* **9,** (Part II), 1071 (1970).
22. H. Meier, B. A. Taylor, M. Cherry and R. J. Huebner, Host gene control of type-C RNA tumor virus expression and tumorigenesis in inbred mice. *Proc. nat. Acad. Sci. (Wash.)* **70,** 1450 (1973).
23. M. B. Gardner, V. Klement, B. E. Henderson, H. Meier, J. D. Estes and R. J. Huebner, Genetic control of type C virus of wild mice. *Nature (Lond.)* **259,** 143 (1976).
24. F. Lilly, M. L. Duran-Reynals and W. P. Rowe, Correlation of early murine leukemia virus titer and H-2 type with spontaneous leukemia in mice of the BALB/c × AKR cross: a genetic analysis. *J. exp. Med.* **141,** 882 (1975).
25. J. R. Stephenson, R. K. Reynolds, S. R. Tronick and S. A. Aaronson, Distribution of three classes of endogenous Type-C RNA viruses among inbred strains of mice. *Virology* **67,** 404 (1975).
26. J. R. Stephenson and S. A. Aaronson, Induction of an endogenous B-tropic Type C RNA virus from SWR/J mouse embryo cells in tissue culture. *Virology* **70,** 352 (1976).
27. J. A. Levy, Xenotropic viruses: Murine leukemia viruses associated with NIH Swiss, NZB and other mouse strains. *Science* **182,** 1151 (1973).
28. C. J. M. Melief, S. Louie and R. S. Schwartz, Ecotropic leukemia viruses in congenic C57BL mice: Natural dissemination by milk-borne infection. *J. nat. Cancer Inst.* **55,** 691 (1975).
29. T. Odaka, Genetic transmission of endogenous N- and B-tropic murine leukemia viruses in low leukemia strain C57BL/6. *J. Virol.* **15,** 332 (1975).
30. J. East, Immunopathology and neoplasms in NZB and SJL/J mice. *Progr. exp. Tumor Res.* **13,** 88 (1970).

31. L. Gross, *Oncogenic Viruses*, Pergamon Press, New York (1970).
32. S. K. Datta and R. S. Schwartz, Autoimmunization and graft-versus-host reactions. *Transplant. Rev.* Vol. 31 (1976), in press. ART. 3. p. 44.
33. B. Varet, A. Cannat, N. Feingold, J. Wechsler and J. P. Levy, Enhancement of virus-induced leukemias and sarcomas in F_1 hybrid mice inoculated with parental spleen cells. *Cancer Res.* **33,** 759 (1973).
34. F. J. Rauscher, Jr, M. A. Fink and J. P. Kvedar, Enhancement of host response to subliminal doses of Rous sarcoma virus by Freund's adjuvant. *J. nat. Cancer Inst.* **30,** 645 (1963).
35. B. V. Siegel and J. I. Morton, Immunologic stimuli in relation to leukemogenesis. I. Potentiation of Rauscher viral leukemia by Freund's adjuvant. *J. nat. Cancer Inst.* **48,** 1681 (1972).
36. W. C. Raschke, P. Ralph, J. Watson, M. Sklar and H. Coon, Brief Communication: Oncogenic transformation of murine lymphoid cells by *in vitro* infection with Abelson leukemia virus. *J. nat. Cancer Inst.* **54,** 1249 (1975).
37. N. Rosenberg, D. Baltimore and C. D. Scher, *In vitro* transformation of lymphoid cells by Abelson murine leukemia virus. *Proc. nat. Acad. Sci. (Wash.)* **72,** 1932 (1975).
38. W. P. Rowe and J. W. Hartley, Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-1^b strains of mice. *J. exp. Med.* **136,** 1286 (1972).
39. J. A. Levy, Type C virus inhibitor associated with cells cultivated from New Zealand Black mice. In: *Perspectives in Virology* (Edited by Morris Pollard) Vol. IX, p. 207, Academic Press, New York (1975).
40. S. K. Datta and R. S. Schwartz, Genetics of expression of xenotropic virus and autoimmunity in NZB mice. *Nature*, in press (1977).

Europ. J. Cancer Vol. 12, pp. 989–993. Pergamon Press 1976. Printed in Great Britain

Therapeutic Effects of Cysteine Adducts of α, β-Unsaturated Aldehydes on Ehrlich Ascites Tumor of Mice*

H. M. TILLIAN, E. SCHAUENSTEIN, A. ERTL and H. ESTERBAUER
Institute of Biochemistry, University of Graz, Halbärthgasse 5, 8010 *Graz, Austria*

Abstract—*Toxicity and therapeutic effects of cysteine adducts of acroleine* (2:1), *crotonal* (2:1), *hydroxypentenal* (2:1 *and* 1:1) *on murine Ehrlich ascites tumors were investigated.*

In contrast to the free aldehydes which are highly toxic agents, the cysteine adducts proved to be practically nontoxic (up to doses of 40 *mg*/10 *g body wt.).*

Strongest therapeutic effects were observed with crotonal (2:1) *and hydroxypentenal* (1:1) *adducts. With a single dose of* 12·0 *mg*/10 *g body weight of both adducts given intraperitoneally* 24 *hours post implantation,* 30–40% *of the animals remained tumor-free through* 90 *days. Whereas the rest of the animals had significantly longer survival times* (15–50%).

After administration of 5 *single doses* (72, 96, 120, 144 *and* 168 *hours post implant.) of crotonal adduct* (2·4 *mg* / 10 *g b.w.)* 30% *of the animals remained tumor-free and the rest of the animals did not show any significant prolongation of survival time.*

With hydroxypentenal (1:1) *optimal effect was observed with* 3·5 *mg*/10 *g b.w.* 40% *of the animals remained tumor-free and the rest showed a highly significant increase of survival time* (137%). *Acroleine and hydroxypentenal* (2:1) *adducts showed only weak or no effects.*

The action of those adducts effective in inhibiting tumor growth is explained by the fact that these substances are present in aqueous solution in dissociation equilibrium releasing the free aldehydes which in the tumor cells attack especially SH-groups which are of functional importance for the DNA-synthesis.

Hence, cysteine adducts seem to be a promising depot form for α,β-unsaturated carbonyl compounds which in the free form are relatively toxic.

INTRODUCTION AND STATEMENT OF THE PROBLEM

As HAS often reported, the compound 4-hydroxypentenal (HPE) is only slightly toxic for cells of normal tissues but for tumor cells is highly toxic. The substance is a specific SH-inhibitor and blocks DNA, RNA, and protein synthesis as well as glycolysis and respiration in tumor cells [1]. Preincubation of 3 murine tumor systems *in vitro* with HPE leads after reimplantation of the tumor cells to significant increase of survival time (with concentrations of HPE = 0·16 mM) and indefinite survival (with concentrations = 0·32 mM) [1a].

When applied peritumorally, HPE causes a severe inhibition in the growth of solid tumors [2–5]. Applied intraperitoneally, however, it shows no noteworthy effect upon either ascites or solid tumors [6], because the substance is rapidly reabsorbed and inactivated in the blood, predominantly through glutathione.

As do other α,β-unsaturated aldehydes, HPE too reacts reversibly with cysteine, whereby a Michael addition product (1:1 adduct), or a thiazolidine carboxylic acid (1:2 adduct) respectively is formed [7–9]. These adducts release the aldehyde again, slowly and gradually, to the same extent as it is consumed by another HPE-affinitive compound. Consequently, they could be an appropriate storage form for the tumor inhibitory substance, hydroxypentenal.

Accepted 9 August 1976.

*This investigation was supported by the "Fonds zur Förderung der wissenschaftlichen Forschung", Vienna.

Table 1 *Cytospectrometric findings*

	N	$E_{tot,\ 560}$	moles PSH	N	$E_{tot,\ 367}$	moles DNPH
C	50	30,210		50		
T	50	25,775		50	17,606	
		4,445	$0{,}234 \cdot 10^{-14}$			$0{,}247 \cdot 10^{-14}$

N = number of cells investigated.
C = preincubated in 0·9% NaCl.
T = preincubated in HPE-Cys 1:1.

We are reporting in this paper about the therapeutic effects with cysteine adducts of hydroxypentenal and crotonal; the findings support the idea that the reversible binding with cysteine is a promising way of masking of biologically active aldehydes.

MATERIAL AND METHODS

Experimental animal: NMRI mice of own stock; both sexes; body weight at the beginning of experiment: 20–25 g.

Tumor: Ehrlich ascites tumor, strain Lettré-Heidelberg; always used 8 days after implantation.

Reagents: The reaction of cysteine with α,β-unsaturated aldehydes and the properties of the so formed adducts are described in an earlier paper [8].

For those experiments conducted in the paper at hand, the following adducts were used:

Cysteine–acroleine–2 : 1-adduct, cysteine–crotonal–2 : 1-adduct, cysteine–hydroxypentenal–2:1-adduct and cysteine–hydroxypentenal–1:1-adduct. For the first three named compounds, cysteine is added to the C_3 of the H
C=C group (Michael addition product) and
H
also the aldehyde group is converted to thiazolidine-carboxylic acid. The last named compound simply contains one molecule of cysteine
H
added to the C=C group [9]. All compounds
H
were isolated as zwitterions and, dissolved in 0·9% NaCl solution, were used in this form.

Method

For standard implantation approximately 2×10^7 cells, diluted 1:1 in 0·2 ml isotonic NaCl solution, per animal were administered intraperitoneally. Experimental and control animals received $4–5 \times 10^6$ EATC per 10 g body weight delivered in 0·25 ml isotonic NaCl (see Table 2), or $4–5 \times 10^5$ cells per 10 g body wt (see Table 3). The number of implanted cells were determined before each experiment by means of hematocrit and it was made sure, that each animal received the same quantity of cells in relation to its body weight.

RESULTS AND DISCUSSION

The reaction with cysteine leads to a drastic reduction in the toxicity of α,β-unsaturated aldehydes. With a single intraperitoneal dose, the LD_{50} for HPE is 0·8 mg/10 g body weight. For the 1:1 and 2:1 cysteine-adducts, on the other hand, even with doses of 40 mg/10 g body weight (equivalent to 22, respectively 11·6 mg HPE), still no acute toxic effect upon twenty mice was noticeable. The results were similar with the highly toxic aldehydes acroleine and crotonal, which as cysteine adducts proved practically nonpoisonous [10].

In vitro incubation (2 hr at 37°C) of EATC (2×10^6/ml) with cysteine-HPE-1 : 1-adduct (50 mM, and 100 mM final concentration), resulted in a 50%, and 100% inhibition of the incorporation of ^{3}H-thymidine respectively. These concentrations are approx. 1,000 times greater than those required for free HPE. This is to be expected from the fact that, in equilibrium, the adduct releases only small amounts of the aldehydes. Similar results were obtained with the cysteine adduct of crotonal. Compared to the control incorporation, treatment of EATC with 30 mM and 100 mM CySH-2:1 adduct inhibited the incorporation of ^{3}H-thymidine by 50% and 100% respectively.

In EATC (2×10^6 per ml) which were preincubated with cysteine-HPE-1:1-adduct (2 hours with 100 mM 37°C), cytospectrometric measurements of nuclear protein-SH groups and protein bound aldehyde groups were determined. In comparison with the control, the result was a reduction of $0{\cdot}234 \times 10^{-14}$ mole per cell in protein thiols and an increase in protein bound aldehyde groups of

Table 2. Single dose cysteine adducts after implantation with 4–5 × 10⁶ EATC/10 g body weight

Controls		Therapeutic animals							
Number	Average survival period (± s_x) in days	Total number	Died before 90th day (1)	Average survival period (± s_x) in days	Extension (%)	Survived 90th day (2)	Survived 90th day (3)	Substance (adduct)	Dose mg/10 g body weight
5 f	24·2 (8·2)	5	4	15·7 (4·3)	−35	1	0	Cys-acrolein-2:1	12·0
21 f	19·3 (8·0)	30	17	27·1 (15·4)	+40*	12	1	Cys-crotonal-2:1	12·0
5 f	32·8 (12·2)	5	5	22·5 (4·0)	−31	0	0	Cys-HPE-2:1	12·0
5 m	12·4 (0·5)	14	9	18·9 (4·5)	+52†	5	0	Cys-HPE-1:1	12·0
25 f	19·4 (10·5)	38	27	22·3 (9·2)	+15	11	0	Cys-HPE-1:1	12·0

(1) Ascites and solid tumour
(2) Ascites and solid tumor free
(3) Ascites tumor free, however with solid tumor
(± s_x) = standard deviation

m = male
f = female
* = significant
† = highly significant

Table 3. Five doses of cysteine adducts, every 72, 96, 120, 144 and 168 hr after implantation with 4–5 × 10⁵ EATC/10 g body weight

Controls		Therapeutic animals							
Number	Average survival period (± s_x) in days	Total number	Died before 90th day (1)	Average survival period (± s_x) in days	Extension (%)	Survived 90th day (2)	Survived 90th day (3)	Substance (adduct)	Dose mg/10 g body weight
10 f	22·9 (4·6)	10	7	24·1 (9·1)	+ 5	3	0	Crotonal-2:1	2·4
		10	5	22·6 (4·9)	− 1	4	1	HPE-1:1	1·75
		10	5	54·3 (21·5)	+137†	4	1		3·5
		9	6	51·5 (21·0)	+125†	2	1		7·0
20 f	14·7 (2·7)	15	4	57·2 (13·0)	+289†	0	(+)		14·0

(1) Ascites and solid tumor
(2) Ascites and solid tumor free
(3) Ascites tumor free, however with solid tumor
(± s_x) = standard deviation

m = male
f = female
* = significant
† = highly significant
(+) = Eleven animals were certainly free from ascites tumors, but died, however, between the 29th and 73rd day after implantation from exceedingly developed solid tumors.

$0{\cdot}247 \times 10^{-14}$ mole per cell [11]. SH-groups were determined by means of a specially modified staining with dihydroxydinaphtyldisulfide and Fast Blue B, protein bound aldehyde groups in form of their 2,4,-dinitrophenylhydrazone derivative. The total extinctions (E_{tot}) at 560 and 367 nm resp. were obtained by scanning each single cell and the extinction differences between control and test incubation converted into moles SH and aldehyde, resp., by the use of the corresponding extinction coefficient (see Table 1).

These cytospectrometric findings clearly show that, at least in *in vitro* experimentation, the aldehyde which is reversibly bound to cysteine is released and intracellularly bound to the protein-SH groups.

Ultimately, the finding that, through two hours long aerobic incubation of EATC (2×10^6 per ml 37°C) in a 10 mM solution of cysteine–HPE–1:1-adduct, the pretreated cells were, after reimplantation in healthy mice, prevented 100% from proliferation, is of decisive importance for therapeutic investigations [7, 12]. This is even more noteworthy, as the trypane blue staining resulted in only 21% trypane positive cells; 65% of the cells were trypane negative but showed nevertheless globular plasma extrusions (stalagmosis) [13]. The remaining 14% were morphologically intact. The control showed 14% trypane positive, 6% stalagmotic and 80% intact cells and a tumor appearance of 100%.

With this it appears that the CySH–HPE–1:1-adduct under the here named conditions, does not destroy the tumor cells, but rather inhibits, i.e., prevents their division, predominantly through inhibition of DNA synthesis, accompanied by desintegration of the cytoplasma [14].

Therapeutic effects

Table 2 compares the therapeutic effectiveness of the four investigated adducts. The cysteine–HPE–1:1-adduct which completely prevented tumor growth in 30% of the animals and clearly delayed it in the remainder of the animals, proved itself the most effective. It is noteworthy that on the 90th day, tumor-free animals also had no solid tumors. Since 100% of the controls developed solid tumors, it is certain that the substance diminishes the growth of solid tumors as well. The cysteine–crotonal–2:1-adduct showed a similarly good therapeutic effect. With cysteine–HPE–1:1-adduct, Table 3 shows the dependence of the therapeutic effect on the dose. The figures show that an optimal effect was reached with doses of 1·75, respectively 3·5 mg/10 g body weight, whereby 40% of the animals remained tumor-free. Higher doses brought, to be sure, a further increase in the survival period, but reduced the percentage of tumor-free animals.

In contrast, the two remaining adducts, namely cysteine–acroleine–2:1-, respectively cysteine–HPE–2:1-adduct were only slightly or not at all effective. Conroy *et al.* [6] reported about the latter that a comparable single dose of 2·2 mg per 10 g body weight extends significantly, around 50%, the survival time of the treated animals, all of which exhibited both solid and ascites tumors. This deviation from our results is presumably based upon differing purities of the preparations and it is to be assumed that the preparation used by Conroy *et al.* contained additional tumor effecting and toxic constituents. In fact, their preparation was considerably more toxic (LD_{50} = 12·3 mg/10 g body weight) than the compound prepared by us (see p. 990).

The various preliminary experiments, such as cytospectrometric investigations, thymidine incorporation, trypane blue test etc., give an indication as to the mode of action of the substance. From these, it is to be assumed that the aldehyde, bound to the adducts, is liberated in an enzymatic or nonenzymatic reversible reaction. The so released aldehyde then reacts, preferably, with the SH groups of functional importance for the DNA synthesis which is therewith blocked. Certainly the speed with which reversible equilibrium is set up, as well as the equilibrium state itself is of great importance for the therapeutic effectiveness of an adduct; both parameters are dependent upon the structure of the adducts, i.e., of the aldehyde. Therefore, for example, the very stable acroleine adduct [8] is more or less therapeutically ineffective, while the somewhat less stable crotonal and HPE adducts [8] are therapeutically effective. The reversible binding with cysteine appears, on the basis of our results, to be a promising way of transforming potentially tumor affecting α,β-unsaturated aldehydes into stable storage forms.

REFERENCES

1. I. J. Bickis, E. Schauenstein und M. Taufer, Wirkungen von HPE auf den Stoffwechsel von Krebs- and Normalzellen. *Monatsh. Chem.* **100,** 1077 (1969).

1a. P. J. Conroy, J. T. Nodes, T. F. Slater and G. W. White, Carcinostatic activity of 4-hydroxypentenal against transplantable murine tumour lines. *Europ. J. Cancer* **11,** 231 (1975).
2. E. Schauenstein, B. Wünschmann und H. H. Esterbauer, Über weitgehend selektive Abtötung subcutan implantierter Ehrlich-Ascites-Tumorzellen *in vivo* durch 4-Hydroxyenale, insbesondere 4-Hydroxypentenal. *Z. Krebsforsch.* **71,** 21 (1968).
3. E. Schauenstein, M. Ernet, H. Esterbauer und H. Zollner, Therapieversuche mit HPE, II. *Z. Krebsforsch.* **75,** 90 (1971).
4. E. Schauenstein, H. Zollner, M. Ernet und H. Esterbauer, Therapieversuche mit HPE, III. *Z. Krebsforsch.* **76,** 140 (1971).
5. R. Krings und H. Tritsch, Wirkung von HPE auf das Harding-Passey-Melanom der Maus. *Arch. Derm. Forsch.* **246,** 108 (1973).
6. P. J. Conroy, J. T. Nodes, T. F. Slater and G. W. White, The inhibitory effects of a 4-hydroxy-pentenal: cysteine adduct against Sarcoma 180 cells in mice. In preparation.
7. A. Ertl, Thesis, Univ. Graz (1976).
8. H. Esterbauer, A. Ertl and N. Scholz, The reaction of cysteine with α,β-unsaturated aldehydes. *Tetrahedron* **32,** 285 (1976).
9. E. Schauenstein, F. Dorner und J. Sonnenbichler, Bindung von 4-Hydroxy-2,3-enalen an Protein-SH-Gruppen. *Z. Naturforsch.* **23b,** 316 (1968).
10. H. Zollner, Unpublished communication.
11. E Schauenstein and G. Noehammer, up to now unpublished measurements.
12. H. Zollner and A. Ertl, Unpublished experiments.
13. M. Ratzenhofer, *Molekularpathologie.* Sitzungsberichte der Heidelberger Akademie der Wissenschaften; Math.-Naturwiss. Klasse, 1. Abhandlung. Springer Verlag, Berlin (1975).
14. E. Schauenstein, W. Woehl und I. Kramer, Einfluß von Hydroxyenalen auf DNS- und RNS von Tumorzellen. *Z. Naturforsch.* **23b,** 530 (1968).

Europ. J. Cancer Vol. 12, pp. 995–1001. Pergamon Press 1976. Printed in Great Britain

DNA-Binding Characteristics of Acridinylmethanesulphonanilide Drugs: Comparison with Antitumour Properties*

M. J. WARING†

Cancer Chemotherapy Laboratory, P.O. Box 1724, Auckland, and Department of Cell Biology, University of Auckland, New Zealand

Abstract—*Four drugs of the 4′-(9-acridinylamino) methanesulphonanilide (AMSA) series comprising the parent compound, a* meta-*substituted (3′) methoxy derivative and its* ortho *(2′) isomer, and an analogue having a 2-methyl substituent on the acridine nucleus have been examined for effects on the supercoiled state of closed circular duplex PM2 DNA. The parent compound and the* meta-*methoxy derivative are known to be highly active against L1210 leukaemia in mice; the other two derivatives are essentially inactive. All 4 compounds were found to remove and reverse the supercoiling of PM2 DNA consistent with the hypothesis that they bind to DNA by intercalation. Significant differences between their binding parameters and relative helix-unwinding angles were detected. The three derivatives interacted very strongly with the DNA; the parent compound less so. The helix-unwinding angle of the 2-methyl analogue was found to be smaller than that of the other three drugs. Some, but not all, of these differences could be correlated with the antitumour activity against L1210 cells* in vivo.

INTRODUCTION

AN IMPORTANT problem for the medicinal chemist concerned with the development of new antitumour drugs lies in the interpretation of structure–activity relationships. The most obvious difficulty arises from the need to distinguish changes in biological activity attributable to the specific nature and location of particular substituents introduced into a drug molecule from those which merely reflect changes in the gross hydrophilic/lipophilic character of the molecule. In their extensive studies of the antileukaemic properties of heteroaromatic compounds Cain and his collaborators [1–3] have achieved this distinction by comparing the activities of homologous compounds in which the overall hydrophilic/lipophilic balance is strictly controlled by judicious substitution with alkyl groups to produce the same chromatographic mobility under defined conditions. In this way they have succeeded in identifying portions of the molecule where substituents may be added to advantage and others where modifications are not tolerated, together with a wealth of information on subtler issues such as sites where substitutions may enhance selectivity and/or antitumour activity subject to a phenomenon of "bulk intolerance" [1–4].

As a result of this work they have developed a drug, 4′-(9-acridinylamino) methanesulphon-m-anisidide (m-AMSA, NSC 156303, structure shown in Fig. 1) which is highly effective in treating L1210 leukaemia and has shown promise in a number of other experimental tumour systems [5]. It is currently on test as a candidate drug for clinical trials.

The structure–activity relationships deduced during the research which led to the synthesis of m-AMSA were tentatively attributed to the steric and other demands of an intercalation site in double-helical DNA, assumed to constitute the probable intracellular site of action of these compounds as is widely believed to be true for acridines in general as well as other heteroaromatic drugs [6]. To date, however, no definitive study of the capacity of m-AMSA and other acridinylmethanesulphonanilides to bind to DNA by intercalation (or by other means)

Accepted 9 August 1976.

*This work was supported by the Auckland Division of the Cancer Society of New Zealand (Inc.), and in part by the Medical Research Councils of New Zealand and Great Britain.

†Permanent address: Department of Pharmacology, Medical School, Hills Road, Cambridge CB2 2QD, England.

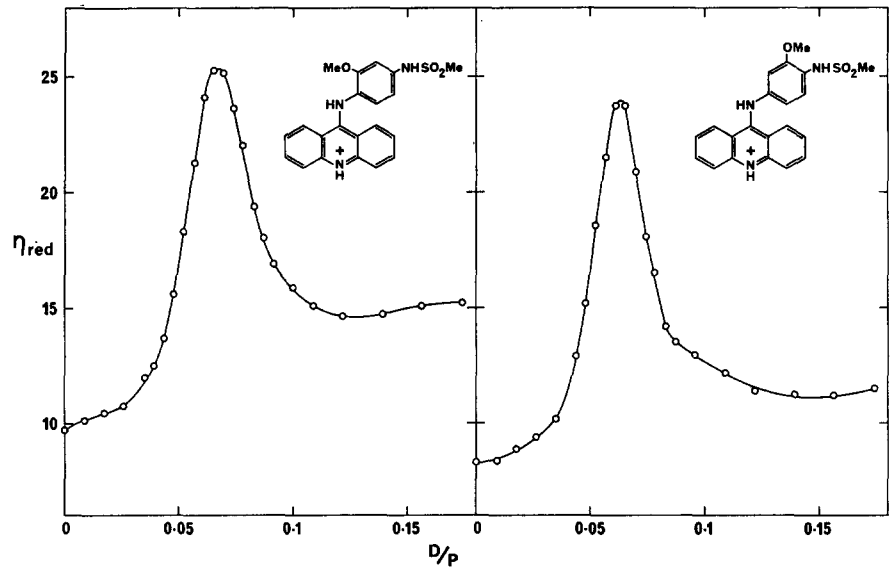


Fig. 1. *Effects of m-AMSA (left) and o-AMSA (right) on the viscosity of closed circular duplex PM2 DNA.*

The starting material was 1·0 *ml of a* 288 *μM solution of PM2 DNA in buffer; up to* 200 *μl of* 250 *μM drug solution was added in small increments. The ordinate represents the reduced viscosity in dl/g; the abscissa shows the molar ratio of added drug to DNA nucleotides.*

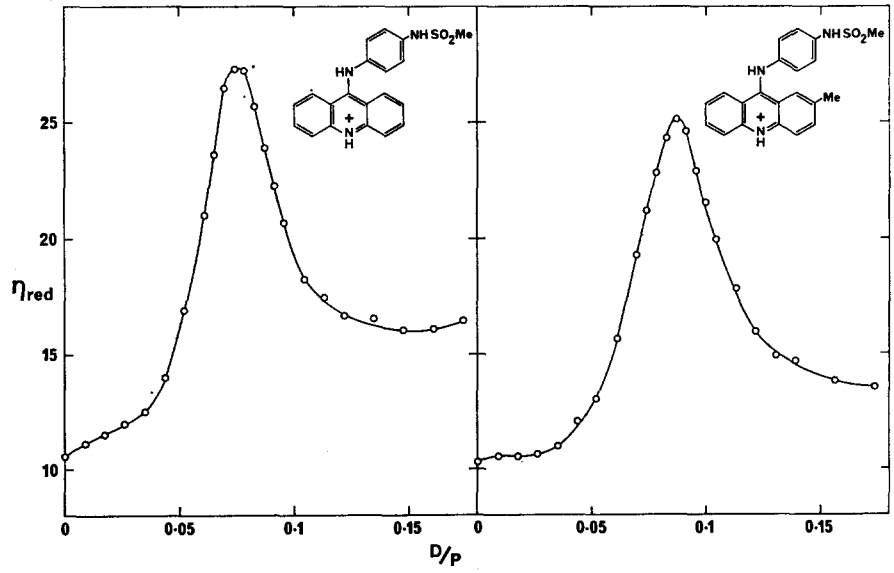


Fig. 2. *Effects of AMSA (left) and 2-methyl-AMSA (right) on the viscosity of PM 2 DNA. Conditions were as described in the legend to Fig.* 1.

has been undertaken. This paper represents a first step in that direction. Four drugs, m-AMSA, its *ortho* isomer, the parent compound and a 2-methyl analogue, were selected for study; their structural formulae are given in Figs. 1 and 2. They were chosen for their wide disparity in antileukaemic activity despite their chemical similarity. The first objective was to determine whether they were capable of intercalative binding to DNA at all. That done, an attempt was made to see whether the characteristics of their interaction with DNA might correlate with their antitumour activity as predicted by the working hypothesis of Cain *et al.* [1, 4, 7]. The experimental approach was to apply the circular DNA-binding test [8] which has previously been shown to provide information on the strength of DNA-binding as well as a geometrical characteristic, i.e., the helix-unwinding angle, of intercalative and non-intercalative DNA complexes in studies of homologous series of drugs [9–11]. Both these

factors can vary depending upon the nature and influence of substituents in DNA-binding drugs [12].

MATERIAL AND METHODS

The test drugs were synthesized by Dr. B. F. Cain at the Cancer Chemotherapy Laboratory and supplied by him in the form of anhydrous methanesulphonate salts. For convenience they are abbreviated as follows:

m-AMSA (SN 11841; NSC 156303): 4′-(9-acridinylamino) methanesulphon-m-anisidide, M_r 489·55

o-AMSA (SN 12735): 4′-(9-acridinylamino) methanesulphon-o-anisidide, M_r 489·55

AMSA (SN 11838): 4′-(9-acridinylamino) methanesulphonanilide, M_r 458·54

2-methyl-AMSA (SN 12736): 4′-(2-methyl-9-acridinylamino) methanesulphonanilide, M_r 473·55

They were checked for purity and homogeneity before and after each experiment by thin-layer chromatography. Acridone was undetectable in solutions of m-AMSA; in solutions of the other three drugs it was just detectable at the limits of sensitivity, i.e., at quite insignificant levels (I am indebted to W. R. Wilson for these analyses). Solutions were freshly prepared at 1 m*M* in glass-distilled water and diluted to yield a solution 250 μM in 10 mM NaCl immediately before use. At this salt concentration a very slow precipitation yielding crystalline nodules (presumably of a poorly soluble chloride) occurred with m-AMSA but not the other drugs. The concentration of m-AMSA in such a solution diminished by about 10% in 2 days. Because of fears that such losses of drug from solution might occur more readily in the micrometer syringe and plastic delivery catheter employed in the viscosity experiments, the concentration of drug actually delivered from the tip of the syringe system was routinely checked at the beginning and end of each experiment by spectrophotometry at the u.v. absorption maximum in the region of 260–270 nm. These measurements were also intended to verify whether drugs might be lost by adsorption to the delivery apparatus. No such losses were detected with any of the four drugs.

Ethidium bromide was a chromatographically homogeneous sample provided by May and Baker Limited, Dagenham, England. The buffer used throughout, designated 0·01 SHE, was of ionic strength 0·01 and contained 2 mM HEPES, 10 μM EDTA and 9·4 mM NaCl dissolved in glass-distilled water, adjusted to pH 7·0 at room temperature with NaOH. PM2 DNA was prepared according to the procedure of Espejo *et al.* [13] from virus and host bacteria kindly provided by Dr. R. T. Espejo. After exhaustive dialysis against 0·01 SHE buffer to remove all traces of phenol it was examined by analytical ultracentrifugation [8] to verify its intact closed circular duplex structure. Nicked circular molecules were undetectable at a sensitivity level of better than 5%. This DNA had never been exposed to ethidium bromide or any other DNA-binding drugs before use in the experiments reported here. Its concentration is expressed in terms of molarity with respect to nucleotides assuming a molar extinction coefficient at 260 nm of 6600.

Viscosity measurements were performed essentially according to the method of Revet *et al.* [14] as modified by Waring and Henley [11]. The viscometer had a 10 cm capillary of 0·4 mm bore and a bulb of volume 0·7 ml; it was filled with 1·0 ml of DNA solution and thermostatted in a large glass-sided water bath at 25·0°C±0·1°C. The flow time for water was 95·0 sec. For routine experiments the PM2 DNA concentration was 288 μM in nucleotides (OD_{260} 1·90; flow time 104·3 sec). Drugs were added in increments of 5–20 μl from an Agla precision micrometer syringe of 1 ml volume via a fine plastic (Portex) catheter inserted down the ascending limb of the viscometer; after each addition complete mixing was effected by bubbling a gentle current of air down the descending limb. Solutions were freed from contamination with traces of particulate material by brief centrifugation in glass tubes in a bench centrifuge, to avoid possible losses of drug and/or DNA by adsorption to the matrix of filter materials. Flow times were measured in triplicate to an accuracy of 0·1 sec; the average deviation of a set of measurements was 0·1–0·2 sec, i.e., approximately 0·1%. No kinetic effects attributable to the rate of complex formation were detected: there was never any consistent trend towards longer or shorter flow times during a set of replicate measurements, even at drug/nucleotide ratios where the viscosity was changing rapidly with each increment in drug concentration. Thus equilibrium must have been attained within a substantially shorter time than the 2–3 min required to make a first measurement. Reduced viscosities η_{red} were calculated by established methods and expressed in dl/g, taking into account the small dilution caused by addition of drug solutions [11, 14].

RESULTS

The characteristics of the viscometric system for measuring variation of the supercoiling of PM2 DNA have been described previously [11]. In Figs. 1 and 2 the effects of the four test drugs under standard conditions are shown. All yield the characteristic rise-and-fall response reflecting removal and reversal of the supercoiling, attributable to drug-induced unwinding of the DNA helix as required by the intercalation hypothesis [6, 8]. The responses to the various drugs exhibit characteristic similarities; in each case the reduced viscosity at the maximum of the curve (the equivalence point where the accumulated drug-induced unwinding of the helix just exactly balances the initial number of superhelical turns in the DNA circles) is about 25 dl/g, similar to the value observed for relaxed circular PM2 DNA complexes with ethidium [11]. Secondly, all the curves appear skewed as if the base-line were rising with increasing D/P; this is precisely as expected for intercalative binding of acridines, which are known to increase substantially the viscosity of linear (and presumably any other) DNA [15]. Thus the much larger changes in viscosity due to variation of the supercoiling are superimposed upon a generalized upward trend due to local extension and stiffening of the helix [15, 16].

Nevertheless, there are differences between the shapes of the curves produced by the different drugs, and the value of D/P at the maximum varies. The latter observation betokens differences in the binding parameters, helix-unwinding angles, or both. These can be investigated by performing for each drug a series of experiments at different DNA concentrations. The values of D/P at the equivalence point are determined and used to construct plots as in Fig. 3. If the interaction obeys simple

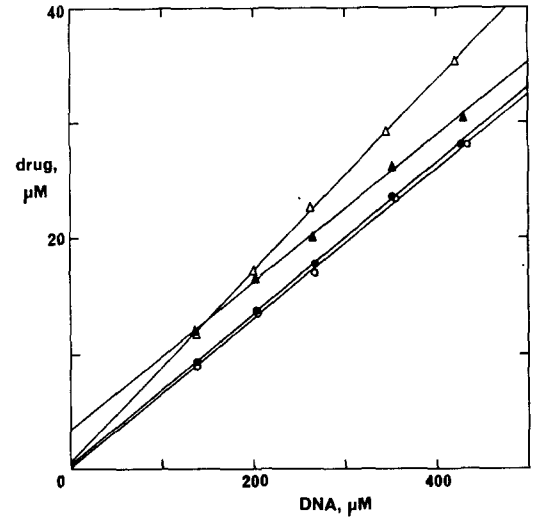


Fig. 3. Titration of the supercoiling of PM2 DNA by acridinylmethanesulphonanilide drugs.

The ordinate and abscissa show respectively the absolute concentrations of total drug and DNA nucleotides in the viscometer at the maxima of reduced viscosity plots like those presented in Figs. 1 and 2. For the different experiments the starting DNA concentration was varied between 144 and 489 μM in nucleotides. The straight lines were fitted by computer using a least-squares programme. Identification of symbols: ●, m-AMSA; ○, o-AMSA; ▲, AMSA; △, 2-methyl-AMSA.

mass-action kinetics such plots take the form of a straight line of gradient v_c, the true drug/nucleotide binding ratio at equivalence, and intercept on the drug concentration axis equal to the free drug concentration c′ in equilibrium with the relaxed circular DNA complex [11, 14]. It can be seen that the data for all four drugs satisfy the expectations of theory, leading to the parameters of interaction recorded in Table 1.

The values of v_c are directly related to the relative helix-unwinding angles of the different

Table 1. Interaction between acridinylmethanesulphonanilides and closed circular duplex PM2 DNA

Drug	v_c	c′ (μM)	ϕ_d/ϕ_e	Estimated ϕ_d	L1210 activity	Immunosuppressive activity
m-AMSA	0·0649 ± 0·0012	0·48 ± 0·44	0·786 ± 0·014	20·5° ± 0·4°	+	89%
o-AMSA	0·0645 ± 0·0019	0·10 ± 0·69	0·791 ± 0·022	20·6° ± 0·6°	−	0
AMSA	0·0635 ± 0·0020	3·40 ± 0·72	0·804 ± 0·025	20·9° ± 0·7°	+	80%
2-methyl-AMSA	0·0826 ± 0·0013	0·65 ± 0·48	0·617 ± 0·010	16·0° ± 0·3°	—	0

The equivalence binding ratio v_c, the free drug concentration c′ in equilibrium with the relaxed circular DNA complex, and the unwinding angle per bound drug molecule relative to that of ethidium ϕ_d/ϕ_e are calculated from the data presented in Fig. 3. Estimated ϕ_d values are related to an assumed value of 26° for ethidium [18-20]. Full details of the activity against L1210 leukaemia are given by Cain *et al.* [1, 2, 23]. Immunosuppressive activity is estimated in terms of plaque-forming cells produced on day 4 after injection of mice with sheep red blood cells, expressed as a percentage of a drug-free control [24].

drugs. It is accepted practice to refer these angles to that of ethidium using the relation

$$\frac{\phi_d}{\phi_e} = \frac{\nu_c \text{ (ethidium)}}{\nu_c \text{ (test drug)}}$$

where ϕ_d and ϕ_e are the helix-unwinding angles per bound test drug or ethidium molecule respectively [6, 8]. A titration with ethidium under identical conditions gave $\nu_c = 0{\cdot}051$, from which the relative helix-unwinding angles listed in Table 1 were calculated. To estimate the absolute helix-unwinding angle for any given drug it is necessary to know ϕ_e. In most previous work it has been assumed that $\phi_e \approx 12°$ as originally proposed by Fuller and Waring [17] but recent evidence suggests that the correct value should be about 26° [18–20]. Accordingly the estimated values for ϕ_d given in Table 1 are based upon the new figure. However, irrespective of whatever absolute value may ultimately be assigned to ϕ_e it should be emphasized that the relative unwinding angles ϕ_d/ϕ_e given in Table 1 are correct and provide a valid basis for comparison between the effects of different drugs.

In three cases the values of c′ are barely significantly above zero. This is not unexpected since at the low ionic strength employed here (0·01) the binding of most cationic heteroaromatic drugs to DNA is extremely tight [6]. Certainly no significant differences between the binding constants for m-AMSA, o-AMSA and 2-methyl-AMSA can be detected from these results; if differences exist they would require the application of more sensitive techniques extending to higher levels of binding for their elucidation. Such determinations are in progress [21]. However, it does appear that the interaction of the parent compound AMSA can be distinguished on this basis (Table 1). For AMSA the value of c′ is quite significantly greater than zero, in contrast to the situation with the substituted derivatives.

DISCUSSION

The first objective of the present work, to provide direct evidence that acridinylmethanesulphonanilide drugs are indeed capable of binding to DNA by intercalation, is well satisfied by the experimental results. There is no doubt that they efficiently remove and reverse the supercoiling of closed circular duplex PM2 DNA, and therefore that their interaction with DNA involves local unwinding of the helix. While this in itself does not prove that the mechanism of binding is intercalative [8, 11] that conclusion is strongly indicated if one considers the wealth of evidence that other acridines do bind in that manner (reviewed by Waring [6]). It is also supported by the magnitude of the relative helix-unwinding angles reported in Table 1 which are generally higher than those determined for proflavine (0·67–0·70 times that of ethidium [8, 22]) and 9-methylaminoacridine (0·64 times that of ethidium; unpublished observations). This is fully consistent with the view of Cain *et al.* [1] that the anilino ring attached to the 9-position of the acridine nucleus may mimic the essential 6-phenyl substituent of ethidium [10], resulting in an unwinding angle closer to that of ethidium than is typical of the simpler acridine intercalators.

The PM2 DNA-binding properties of the test drugs are, however, not simply correlated with their biological activities (Table 1). In particular the failure to detect any difference between the interaction parameters for m-AMSA and o-AMSA, the former highly active against tumour cells and the immune system while the latter is essentially inactive, shows clearly that other factors play an important role in determining activity *in vivo*. Such factors may well include the known susceptibility of these compounds to inactivation by non-enzymic thiolysis [25] as well as possible interaction with transport systems or membrane components which might affect rates of penetration into different types of cells. The overall pattern of structure-activity relationships may therefore be a composite, reflecting the differing demands of these factors as well as structural constraints imposed by the requirements of an intercalation site. Nevertheless, the comparisons which do emerge from the data in Table 1 are not without relevance to the total problem. The finding of a significantly lower helix-unwinding angle for 2-methyl-AMSA compared to the other drugs accords with the expectation that substituents applied directly to the intercalating chromophore should have particular influence on the distortion of the helix associated with its insertion. It is conceivable that this difference, albeit not large, is actually responsible for the inactivity of 2-methyl-AMSA. Conversely, it is not surprising that the presence of a methoxy substituent on the anilino ring of m-AMSA and o-AMSA does not affect the unwinding angle. What is intriguing is the apparent difference in c′ found for these derivatives (and for 2-methyl-AMSA) compared to the parent compound. The implication is that the substituted derivatives bind more tightly to PM2 DNA than does

AMSA, i.e., have a higher intrinsic binding constant. The only alternative possibility is that the frequency of binding sites in PM2 DNA is much lower for AMSA, but this is highly unlikely with such a closely related series of ligands. A higher binding constant for the methoxy derivatives could well be an outcome of the 'positioning' role for the 9-sulphonanilide ring postulated by Cain *et al.* [1]. It would also help to explain the enhanced L1210 activity of m-AMSA compared to the parent compound, but it would be necessary to invoke other factors to explain why a corresponding enhancement is not observed for o-AMSA and 2-methyl-AMSA.

In any event it is gratifying to note that the circular DNA-binding test can discriminate effectively and sensitively between the properties of such closely related drugs within a homologous series, revealing variations in both the binding parameters and the structural distortion of the DNA helix. To what extent these variations, particularly in respect of unwinding angles, can account for changes in biological activity will only be clarified when the test has been applied to a wider range of drugs, but as a potentially valuable screening procedure it offers significant advantages not least in respect of its rapidity, simplicity and economy. At present the only important limitation is the availability of pure closed circular DNA on the milligramme scale. Now that the intercalative potential of the acridinylmethanesulphonanilides has been established such studies can be employed to complement biological testing in the future development of this promising series of antitumour drugs.

Acknowledgements—I am extremely grateful to Drs. B. F. Cain, B. C. Baguley and W. R. Wilson for their advice, encouragement and provision of facilities.

REFERENCES

1. B. F. Cain, R. N. Seelye and G. J. Atwell, Potential antitumour agents. 14. Acridylmethanesulphonanilides. *J. med. Chem.* **17,** 922 (1974).
2. B. F. Cain, G. J. Atwell and W. A. Denny, Potential antitumour agents. 16. 4'(Acridin-9-ylamino) methanesulphonanilides. *J. med. Chem.* **18,** 1110 (1975).
3. B. F. Cain, G. J. Atwell and W. A. Denny, Potential antitumour agents. 17. 9-anilino-10-methylacridinium salts. *J. med. Chem.* **19,** 772 (1976).
4. B. F. Cain and G. J. Atwell, Potential antitumour agents. 19. Multiply substituted 4'-(9-acridinylamino)-methanesulphonanilides. *J. med. Chem.* **19,** 1124 (1976).
5. B. F. Cain and G. J. Atwell, The experimental antitumour properties of three congeners of the acridylmethanesulphonanilide (AMSA) series. *Europ. J. Cancer* **10,** 539 (1974).
6. M. J. Waring, Inhibitors of nucleic acid synthesis. In *The Molecular Basis of Antibiotic Action* (by E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond and M. J. Waring) p. 173. Wiley, London (1972).
7. B. F. Cain, G. J. Atwell and R. N. Seelye, Potential antitumour agents. 11. 9-anilinoacridines. *J. med. Chem.* **14,** 311 (1971).
8. M. J. Waring, Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. *J. mol. Biol.* **54,** 247 (1970).
9. M. J. Waring, Interaction of indazole analogues of lucanthone and hycanthone with closed circular duplex DNA. *J. Pharmacol. exp. Ther.* **186,** 385 (1973).
10. L. P. G. Wakelin and M. J. Waring, The unwinding of circular DNA by phenanthridinium drugs: structure-activity relations for the intercalation reaction. *Mol. Pharmacol.* **10,** 544 (1974).
11. M. J. Waring and S. M. Henley, Stereochemical aspects of the interaction between steroidal diamines and DNA. *Nucl. Acid Res.* **2,** 567 (1975).
12. M. J. Waring, Structural constraints in the binding of drugs to DNA. In *Topics in Infectious Diseases, Drug-Receptor Interactions in Antimicrobial Chemotherapy.* (Edited by J. Drews and F. E. Hahn), Vol. 1, p. 77. Springer-Verlag, Wien (1975).
13. R. T. Espejo, E. S. Canelo and R. L. Sinsheimer, DNA of bacteriophage PM2: a closed circular double-stranded molecule. *Proc. nat. Acad. Sci. (Wash.)* **63,** 1164 (1969).
14. B. M. J. Revet, M. Schmir and J. Vinograd, Direct determination of the superhelix density of closed circular DNA by viscometric titration. *Nature New Biol.* **229,** 10 (1971).

15. G. COHEN and H. EISENBERG, Viscosity and sedimentation study of sonicated DNA–proflavine complexes. *Biopolymers* **8,** 45 (1969).
16. K. E. REINERT, DNA stiffening and elongation caused by the binding of ethidium bromide. *Biochim. biophys. Acta (Amst.)* **319,** 135 (1973).
17. W. FULLER and M. J. WARING, A molecular model for the interaction of ethidium bromide with Deoxyribonucleic Acid. *Ber. Bunsenges. Physik. Chem.* **68,** 805 (1964).
18. J. C. WANG, The degree of unwinding of the DNA helix by ethidium. I. Titration of twisted PM2 DNA molecules in alkaline caesium chloride density gradients. *J. mol. Biol.* **89,** 783 (1974).
19. L. F. LIU and J. C. WANG, On the degree of unwinding of the DNA helix by ethidium. II. Studies by electron microscopy. *Biochim. biophys. Acta (Amst.)* **395,** 405 (1975).
20. D. E. PULLEYBLANK and A. R. MORGAN, The sense of naturally-occurring superhelices and the unwinding angle of intercalated ethidium. *J. mol. Biol.* **91,** 1 (1975).
21. W. R. WILSON, Personal communication.
22. W. MÜLLER, D. M. CROTHERS and M. J. WARING, A non-intercalating proflavine derivative. *Europ. J. Biochem.* **39,** 223 (1973).
23. G. J. ATWELL, B. F. CAIN and R. N. SEELYE, Potential antitumour agents. 12. 9-Anilinoacridines. *J. med. Chem.* **15,** 611 (1972).
24. B. C. BAGULEY, E.-M. FALKENHAUG, J. M. RASTRICK and J. MARBROOK, An assessment of the immunosuppressive activity of the anti-tumour compound 4′-[(9-acridinyl)amino] methanesulphon-*m*-anisidide (m-AMSA). *Europ. J. Cancer* **10,** 169 (1974).
25. W. R. WILSON, B. F. CAIN and B. C. BAGULEY, Personal communication.

Europ. J. Cancer Vol. 12, pp. 1003–1009. Pergamon Press 1976. Printed in Great Britain

A Screening for Selective Anticancer Agents Among Plant Respiratory Inhibitors*

MARIO GOSÁLVEZ, R. GARCÍA CAÑERO† and M. BLANCO
Bioquímica Experimental. Clínica Puerta de Hierro. Facultad de Medicina. Universidad Autónoma. Madrid. Spain

Abstract—*Previous work of our laboratory showed that some useful anticancer agents behave as respiratory inhibitors with some specific activity in tumor cells and that, in some cases, the effect on respiration would represent the mechanism of antitumor action. On this basis, a screening among plant compounds was undertaken to select respiratory inhibitors, selective for tumor cell respiration. Out of thirty compounds, four were found to be potent respiratory inhibitors somewhat specific for tumor cells. Two of these compounds appeared with substantial antitumor activity in mice inoculated with L1210. These results would support the interest of respiratory inhibition as a selective approach to anticancer chemotherapy.*

INTRODUCTION

WE REPORTED, previously, that the anticancer agent VM-26 inhibits NADH linked respiration at low concentrations [1] and that this respiratory inhibition seemed to be somewhat more specific for certain tumor cells than for certain normal cells, either studying the drug in *in vitro* or in *in vivo* respiration [2, 3]. The fact that the respiratory inhibition with VM-26 is obtained well within the range of chemotherapeutic doses, suggested that its mechanism of antitumor action is associated with its effect on respiration [1–3]. Respiratory inhibition and respiratory selectivity was also found by our group in other anticancer agents as ellipticene and some triazine and naphtoquinone derivatives [2, 4]. All of these studies support our view that an anticancer chemotherapy, based on specific respiratory inhibitors, must be worth investigating [5].

Accepted 14 June 1976.

*This work was supported by contract No. NCI-CM-53792 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, DHEW and by grant No. 12-199-75 from the Spanish Instituto Nacional de Previsión.

†Recipient of a fellowship from the Asociación Española Contra el Cáncer.

In this report, we present a screening for respiratory inhibitors selective for tumor cell respiration, carried out among plant compounds with chemical structure likely to interact with the mitochondrial respiratory chain. Thirty compounds were selected and studied, measuring their effects in the respiration and oxidative phosphorylation of isolated mitochondria and tumor and normal cell suspensions. Among them, four compounds were found to be potent inhibitors of cell respiration *in vitro* and *in vivo* showing some specific activity in tumor cell respiration. Two of these compounds appeared with substantial antitumor activity in mice inoculated with L1210. These results support the interest of respiratory inhibition as a selective approach to anticancer chemotherapy. Additionally, these results emphasize the interest of biochemical screenings aimed at rationally defined specific biochemical targets for anticancer chemotherapy as a means of detecting potential anticancer agents in high yield.

MATERIAL AND METHODS

Measurement of respiration and oxidative phosphorylation in rat liver mitochondria

Rat liver mitochondria were isolated by a modification of the method of Schneider [6].

Mitochondrial respiration (1 mg mitochondrial protein per ml) was measured with glutamate plus malate (5 mM), or succinate (5 mM) as substrate, in the absence (state 4 respiration) or presence (state 3 respiration) of ADP (0·3 mM). ADP:O ratios were determined as described by Estabrook [7]. The respiration was measured in a Clark-type oxygen electrode (3 ml chamber) in a medium composed of 225 mM sucrose, 20 mM KCl, 7 mM $MgCl_2$, 10 mM Tris–HCl and 5 mM Tris–Pi (pH 7·2, 22°C). The drugs were added in microliter quantities, dissolved in 50/50% dimethylformamide and dimethylsulfoxide, or in water, depending on the drug's solubility. Eight minutes of pre-incubation of the drug with the mitochondria were allowed before the determination of the respiration.

Measurements of the respiration of Ehrlich ascites tumor cells

The respiration of Ehrlich ascites tumor cells ($20–30 \times 10^6$ cells/ml), hypertriploid cells) was determined in the oxygen electrode, in a medium composed of KCl (6·16 mM), Na_2HPO_4 (9·35 mM), NaH_2PO_4 (1·65 mM) and NaCl (0·9%) (pH 7, 22°C) [8]. The respiration rate, after 15 minutes, in the presence of the drug, was compared with the control respiration of non-treated cells. As cells used in these experiments were collected from 6-day-grown Ehrlich ascites tumors (hypertriploid strain), and hemoglobin-free suspensions were prepared as described by Chance and Hess [8].

Measurement of pig leucocyte respiration

Mixed populations of leucocytes including lymphocytes were isolated by differential centrifugation, from the buffy coat of 2 liters of citrated pig blood. The respiration of the cell suspensions was measured in the Clark-type electrode, using the same medium described for the measurement of Ehrlich ascites tumor cell respiration.

Measurement of rat liver cell respiration

Anesthetized rats (nembutal) were opened and the portal vein and superior vena cava were cannulated in order to perfuse the liver with Krebs–Ringer phosphate salt solution (saturated with oxygen and CO_2). Once the liver was well-washed, the perfusion was continued with the same solution, supplemented with bovine serum albumin (2·5%), collagenase II (0·05%) and hyaluronidase (0·1%). Afterwards the liver was isolated from the rat, minced with scissors and incubated at 37°C during 20 min, with the Krebs enzyme solution, under continuous oxygen gassing. After filtering the cell syspensions with gauze, the isolated liver cells were centrifugated at 1000 rev/min and were washed twice with the Krebs–Ringer phosphate medium. The liver cell respiration was measured in a Clark-type oxygen electrode in the same assay medium that was used for the Ehrlich tumor cells.

Measurement of leukemia L1210 cell respiration

DBA/2 mice, bearing leukemia L1210 in ascites form, were received from the National Cancer Institute, and the tumors were transplanted to mice, resulting from the cross of DBA and C_3H mice, by inoculating 10^5 cells intraperitoneally. After seven days of tumor growth, cells were collected, washed, freed of blood (by a 30 sec osmotic shock) and concentrated. Cell respiration was measured in a Clark-type oxygen electrode, using the same medium that was employed for the Ehrlich ascites tumor cells.

Drugs, solvents, substrates and cofactors

All plant products were supplied in milligram quantities by Professor A. González, Instituto de Investigaciones Químicas, La Laguna, Tenerife, Spain, and almost all were new chromenes and diterpenes, extracted and purified from plants of the Canary Islands. [9–12]. The structure of these compounds is shown in Fig. 1. The chemical names are, respectively: 7H-Furo (3,2-g) (1) benzopyran-7-one, 6-(1,1-dimethyl-2-propen)- (chalepensine); 5-benzo(2-(1-methylethylen)-) furan-acrylic acid, lactone (oroselone); 4H, 6H-benzo (1,2*b*:5,4-*b*')dipyran-4-one, 8,8-dimethyl 2-methyl-5-hydroxy-10-(3,3-dimethyl-propen-2-)- (pulverochromenol); 4H, 6H-benzo(1,2*b*:5,4-*b*') dipyran-4-one, 8,8-dimethyl-2-methyl-5-hydroxy-(3,3-dimethyl-allyl-2-)-(allyl-spathelia-chromene); 6,7-furan-(2,3-dihydro)-chromen-8-ol, 2,3-dimethoxic 5-R- (pitilline)-5-benzo (2-dimethyl-hydroxymethyl-) furan-acrylic acid, lactone- (oroselol); 5,6:7,8-(2'H, 2'H-dipyran)- chromone, 2-methyl- (spathelia-bis-chromene); 3,4-furan (2,3-dihydro)-ciclodeca-5,9-diene, 6-hydroximethyl-2-(3,4-hydroximethyl-2-(3,4-hydroxi-2-en)-carboxilate-(cnicine); 7H-furo (3,2-g) (1) benzo pyran-7-one, 4-nitro-7-methoxy-(nitroxantotoxin). The compounds were used as pure products, dissolved in water or 50%/50% dimethylformamide and dimethylsylphoxide and were added to mitochondria cells in microliter quantities. Solvents were used as controls.

Substrates, salts and cofactors were purchased from Sigma Company. Mitochondrial protein was determined by the biuret reaction. Cell concentrations were calculated by cell count in a hemocytometer after vital staining.

RESULTS

Thirty purified plant compounds were selected from the stock of plant products purified by Professor González at the Instituto of Investigaciones Químicas of La Laguna (Tenerife). The compounds were selected by their chemical structure likely to interact with the mitochondrial respiratory chain and were preliminarily tested at 100 μM concentration in isolated mitochondria. Those compounds showing at least a five per cent inhibition of mitochondrial respiration with glutamate plus malate, at such concentration, were accepted in the screening. Table 1 lists the twelve compounds found with such activity and their effects in mitochondria. These twelve compounds were titrated in the isolated mitochondria at increasing concentrations and those showing a 100% inhibition of mitochondrial respiration at less than 300 μM concentrations, continued in the screening. The first six compounds of Table 1 plus the last compound of the table had such activity. These compounds were titrated in isolated mitochondria with glutamate plus malate or succinate plus or less ADP, providing thus, the evaluation of its effect on mitochondrial state 4 and state 3 respiration and on ADP:O ratio. These compounds were also assayed on the respiration of Ehrlich ascites cell suspensions to determine the concentration needed for 50% inhibition. Table 1 shows the effect of the compounds on mitochondrial respiration with glutamate plus malate or with succinate as substrate in the presence of ADP and the concentration of the compounds for 50% inhibition of the respiration of Ehrlich ascites cells. It can be observed that there is not a close relationship between the potency of the compounds in mitochondria and their potency in the cells. This would be due to differences in cell permeability for each drug. All of the seven more effective compounds except oroselona, inhibited succinate respiration, thus behaving as inhibitors of the antimycin type. None of them inhibited the mitochondrial respiration with ascorbate (not shown). Oroselona however, appeared as a pure inhibitor of the rotenone type. Trachinodiol, pulverochromenol and spathelia-bis-chromeno inhibited, partially, mitochondrial ADP:O ratio and induced a partial activation of mitochondrial respiration in state 4 (not shown), therefore behaving as mixed inhibitors-uncouplers.

Of the seven more active compounds, only trachinodiol, oroselona, chalespensine and pulverochromenol were available in sufficient quantities to be compared in the respiration of tumor and normal cells. Fig. 2 depicts the effect of increasing concentrations of these compounds on the respiration of pig leucocytes, rat liver isolated cells and mice leukemia 1210 and Ehrlich ascites cells. The cell concentration of the four types of cells was 200, 1, 50 and 50×10^6 cells/ml, respectively, as necessary to yield equivalent cell respiration (2) (50 nanoatoms O_2/min) with all the cell types. Under these conditions, the known respiratory inhibitor rotenone has exactly the same potency in the four types of cells and the cells are comparable. Differences of permeability among

Table 1. *Effects of plant compounds in mitochondrial and cell respiration*

Compound	Inhibition of mitochondrial respiration with Glutamate plus		with Succinate Malate		Inhibition of EATC Respiration	
	μM	% Inhibition	μM	% Inhibition	μM	% Inhibition
Trachinodiol (diacetate)	0·75	50	0·6	50	100	50
Chalepensine	3·5	50	50·0	50	60	50
Oroselona	7·0	50	100·0	0	90	50
Pulverochromenol	22·0	50	15·0	50	40	50
Epicandicandiol (diacetate)	25·0	50	15·0	50	30	50
Allyl spatheliachromene	38·0	50	24·0	50	600	50
Pitilline	100·0	40	—	—	—	—
Oroselol	100·0	25	—	—	—	—
Pristimerine	100·0	20	—	—	—	—
Nitroxantotoxine	100·0	10	—	—	—	—
Cnicine	100·0	5	—	—	—	—
Spathelia-bis-chromene	100·0	5	330·0	50	760	50

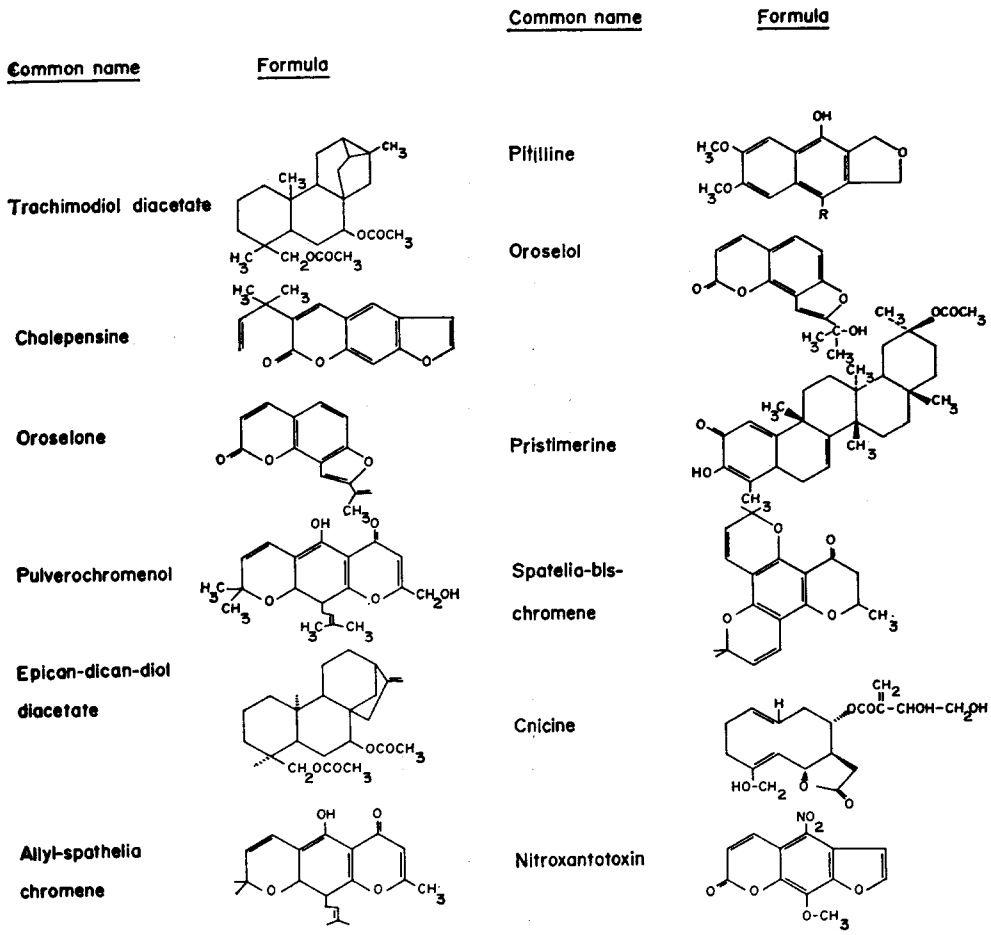


Fig. 1. *Chemical formula of principal natural compounds used in these studies.*

cell types for the different compounds, are matched by the 15 min of pre-incubation of the drug with the cells. Such conditions of equivalent respiration and pre-incubation permitted the detection of selective inhibition of Ehrlich ascites cells by VM-26, in comparison with pig leucocytes [2]. This respiratory selectivity was later confirmed *in situ* and *in vivo* between two normal and tumor tissues of the same animal [3]. Thus, in a first approximation, the experiment of Fig. 2 permits the definition of specificity of an inhibitor for tumor cell respiration. It is first apparent that oroselona is quite less active in inhibiting the respiration of the leucocytes and hepatocytes than in inhibiting the respiration of the tumor cells. The following compound with high specificity is trachinodiol. Table 2 shows the factor of specificity for each compound in each cell pair [4]. Data on spathelia-bis-chromene is also shown, which is not shown in Fig. 2. The specificity is defined by two numbers, the absolute specificity factor (ASF) and the relative specificity factor (RSF) [4]. ASF is calculated by the ratio between the maximum concentration with no effect on respiration of normal cells and the minimum concentration required for 80% inhibition of tumor cell respiration. The RSF is calculated by the ratio between the concentration necessary to inhibit 80% tumor respiration [4] and the concentration necessary to inhibit 80% normal cell respiration. Oro-

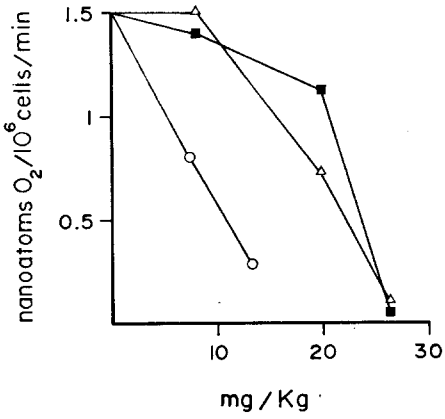


Fig. 2. *Effect of trachinodiol (black circles) oroselone (open circles), chalepensine (triangles) and pulverochromenol (crosses) on the respiration of pig leucocytes, (upper left graph), rat hepatocytes (upper right graph), leukemia* 1210 *(lower left graph) and Ehrlich ascites cells (lower right graph).*

Table 2. Specificity of plant compounds for tumor cell respiration

Compound	Leucocytes EATC		Leucocytes Leukemia 1210		Liver cells EATC		Liver cells Leukemia 1210	
	ASF	RSF	ASF	RSF	ASF	RSF	ASF	RSF
Trachinodiol (diacetate)	0·3	2·0	—	—	7·5	3·3	—	—
Chalepensine	0·12	1·1	0·15	1·5	0·12	1.1	0·15	1·5
Spathelia-bis-chromene	—	3·3	—	—	—	—	—	—
Oroselone	3·3	7·1	1·4	1·1	0·66	23·0	0·28	10·0
Pulverochromenol	0·6	2·8	0·35	1·05	0·10	0·65	0·06	0·38

selona appears to be quite specific for the two kinds of tumor cells and shows very high relative specific factors between leucocytes and Ehrlich ascites and between liver and leukemia cells. The selectivity is not so marked between leucocytes and leukemia. Trachinodiol, chalepensine, pulverochromenol and spathelia-bis-chromene also show specificity in some cell pairs. Chalepensine is the more interesting compound because it shows a good selectivity between leucocytes and leukemia cells. Selectivity in this cell pair is difficult to be demonstrated [4] due to the great similitude of both cell types.

Table 3 describes the chemotherapeutic treatment of L1210 mice with spathelia-bis-chromene, pulverochromenol and chalepensine. Trachinodiol and oroselona were not available in enough quantity for the chemotherapeutic treatments. The drugs were injected daily starting day one after inoculation of 10^5 cells per mice. Spathelia-bis-chromene induced a 62% increase in life span with no survivors after 60 days at the total dose of 7 mg/kg. Higher doses resulted in a short supervivence due to increased toxicity. Chalepensine, at the total dose of 150 mg/kg managed to yield a 60% survivors and 70% increase in life span for the non-surviving mice. These results suggest that both compounds, but above all chalepensine, have substantial chemotherapeutic activity in L1210. L1210 is the first tumor choice of chemotherapeutic screening of the National Cancer Institute in the United States and drugs inducing more than a 50% increase in life span in this tumor system are considered as potential chemotherapeutic agents [13].

To ascertain if the chemotherapeutic activity of chalepensine was due to its effects in respiration, the respiration of L1210 cells obtained from mice bearing tumors (six days grown), treated at different doses of the drug, was assayed. Fig. 3 shows that at 30 mg/kg of chalepensine, the effective chemotherapeutic dose, there is almost a 100% inhibition of cell respiration. Essentially similar results are depicted for pulverochromenol while oroselona appears to have double potency. The results with chalepensine suggest that the mechanism

Table 3. Treatment of L1210 with plant respiratory inhibitors

Compound	Daily dose (in mg/kg)	Total dose (in mg/kg)	Increase in life span	Survivors/ treated animals
Spathelia-bis-chromene	1	7	62%	0/5
	3	21	34%	0/5
	5	35	28·5%	0/5
	8	40	10%	0/5
	10	60	3%	0/5
	11	55	3·1%	0/5
Pulverochromenol	8	40	7%	0/5
	10	70	0%	0/5
Chalepensine	8	32	0%	0/5
	10	60	4%	0/5
	30	150	70%	3/5

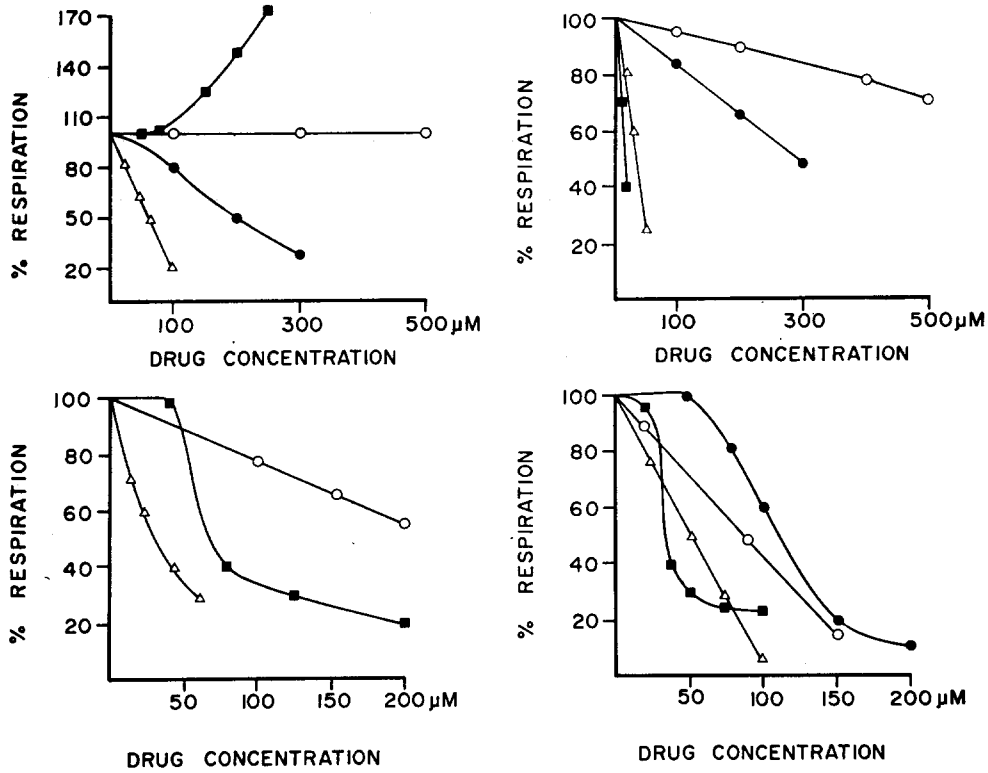


Fig. 3. *Respiration of Ehrlich ascites cells isolated from six day grown tumors, two hours after treatment with oroselone (clear circles), chalepensine (triangles) and pulverochromene (crosses) at the indicated doses.*

of inhibition of tumor growth is associated with the inhibition of cell respiration. On the other hand, pulverochromenol would have been used in chemotherapeutic treatment at too low concentrations to cause respiratory inhibition and thus, this fact would have the reason for the lack of chemotherapeutic effect with this drug.

DISCUSSION

Our results have defined chalepensine and spathelia-bis-chromene as having a substantial antitumor activity in L1210 mice and therefore these drugs behave as potential anticancer agents. Both compounds were selected out of thirty compounds as being potent respiratory inhibitors with some apparent selectivity for tumor cell respiration. Thus, it would appear that the biochemical screening employed for its selection has a high yield. The definition of the exact yield of the screening awaits the study of oroselona, trachinodiol and pulverochromene in L1210, at doses sufficient for complete inhibition of respiration.

The abdominal cavity of mice bearing an ascites tumor might behave in a similar fashion to an *in vitro* chamber, if the drug is not rapidly absorbed. Thus, it would be desirable to demonstrate that these drugs inhibit tumor growth injected parenterally. Nevertheless, it is generally accepted that the obtention of surviving animals in intraperitoneally treated tumors reflect a good therapeutic to toxic dose ratio.

The specificity of the compounds for tumor cells respiration was found by comparing normal and tumor cells of different species. The respiration of cell and mitochondria of different species is highly comparable as long as equivalent cell respiration or similar content of cytochrome is assured [2], but, however, a definite assessment of specificity should include the comparison of normal and tumor tissue of the same animal as has been done for VM-26 [3]. Nevertheless, the good therapeutic activity found with chalepensine would support its selectivity for tumor respiration. As respiratory inhibitors are highly toxic compounds, selectivity for tumor cells must be emphasized for them as a class of anticancer agent. The most specific compound found in our screening was oroselona, which has RSF factors from 7 to 23 in various cell pairs. This value means that at least 7 times more drug is necessary to affect the normal cell respiration with the same extent that tumor cell is affected. The existence of such specificity encourages the interest of

respiratory inhibition as a selective approach to anticancer chemotherapy. As activity in L1210 predicts very well potential effects in human tumors [13], the search for high values of RSF in the L1210-leucocytes pair would be desirable. Our experimental evidence and previous work [2–4] suggest strongly that there is a quantitative difference in sensitivity to some respiratory inhibitors between tumor and normal cells. The reason for such a difference would possible be based on the recently postulated differences of tumor membranes [14–17] or just be considered as a tissue difference, considering tumors in general as a characteristic tissue. The exploiting of such different sensitivity with a chemotherapeutic purpose seems appealing and emphasizes the interest of biochemical screenings aimed at rationally defined specific biochemical targets for anticancer chemotherapy.

REFERENCES

1. M. GOSÁLVEZ, J. PÉREZ-GARCÍA and M. LOPEZ, Inhibition of NADH-linked respiration with the anticancer agent VM-26. *Europ. J. Cancer* **8,** 471 (1972).
2. M. GOSÁLVEZ, M. BLANCO, J. HUNTER, M. MIKO and B. CHANCE, Effects of anticancer agents on the respiration of isolated mitochondria and tumor cells. *Europ. J. Cancer* **10,** 567 (1974).
3. M. GOSÁLVEZ, R. GARCÍA-CAÑERO and H. REINHOLD, Delayed pyridine nucleotide reoxidation induced by the anticancer agent VM-26 as measured *in vivo* and *in situ* by NADH microfluorometry. *Europ. J. Cancer* **11,** 709 (1975).
4. M. GOSÁLVEZ, R. GARCÍA-CAÑERO, M. BLANCO and C. GURUCHARRI-LLOYD, Effects and specificity on the respiration and energy metabolism of tumor cells. *Cancer Treat. Rep.* **60,** 1 (1976).
5. M. J. FIGUERAS and M. GOSÁLVEZ, Inhibition of the growth of Ehrlich ascites tumors by treatment with the respiratory inhibitor Rotenone. *Europ. J. Cancer* **9,** 529 (1973).
6. W. C. SCHNEIDER, Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. biol. Chem.* **176,** 549 (1948).
7. R. W. ESTABROOK, Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods in Enzymology* **10,** 41 (1967).
8. B. CHANCE and B. HESS, Metabolic control mechanisms. I. Electron transfer in the mammalian cell. *J. biol. Chem.* **234,** 2402 (1959).
9. A. G. GONZÁLEZ, J. P. CASTAÑEDA y B. M. FRAGA, Nuevas cromonas de la neochamelae pulverulenta, ERNDT. *An. Quim.* **68,** 447 (1972).
10. A. G. GONZÁLEZ, J. L. BRETON, B. M. FRAGA and J. G. LUIS, Constituents of labiatae—IX. Trachinodiol and trachinol, two new diterpenes from *Sideritis canariensis* Ait. *Tetrahedron Lett.* **33,** 3097 (1971).
11. A. G. GONZÁLEZ, J. D. MARTIN y C. PÉREZ, Nuevas cromonas de la neochamalae pulverulenta. *An. Quim.* **68,** 709 (1972).
12. A. G. GONZÁLEZ. Unpublished data.
13. V. BONO. (Personal communication). Drug Research and Development, Division of Cancer Treatment, Bethesda, Md.
14. J. C. ARCOS, Ultrastructural alterations of the mitochondrial electron transport chain involving electron leak: Possible basis of "respiratory impairment" in certain tumors. *J. theor. Biol.* **30,** 533 (1971).
15. J. C. ARCOS, M. J. TISORS, H. H. GOSCH and J. A. FABIAN, Sequential alterations in mitochondrial inner and outer membrane electron transport and in respiratory control during seeding of amino azo-dyes. Stability of phosphorylation. Conelation with swelling-contraction changes and tumorogenesis threshold. *Cancer Res.* **29,** 1298 (1969).
16. R. E. BARNETT, L. T. FURCHT and R. E. SCOTT, Differences in membrane fluidity and structure in contact inhibited and transferred cells. *Proc. nat. Acad. Sci.* **71,** 1992 (1974).
17. J. L. MARX, Biochemistry of cancer cells: Focus on the cell surface. *Science* **183,** 1279 (1974).

Europ. J. Cancer Vol. 12, pp. 1011–1015. Pergamon Press 1976. Printed in Great Britain

Urinary Excretion of 5-Hydroxyindoles as an indicator of *In Vivo* Growth of P815X2 Mastocytoma*

R. B. RAIKOW, L. L. SCHENKEN and S. LESHER

Cancer Research Unit, Clinical Radiation Therapy Research Center, Division of Radiation Oncology, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15212, U.S.A.

Abstract—*The extent of P815X2 growth in DBA mice is correlated with increases in urinary concentrations of 5-hydroxyindoles. Animals hosting two other mouse tumors, T1699 and S-180, did not demonstrate such increases in urinary hydroxyindoles. The concentration of 5-hydroxyindoles in the urine was not modified by immunosuppressive whole body or cytotoxic tumor-directed irradiation of hosts. Total daily urine volumes in all tumor bearing mice were variable and tended to decrease as disease advanced. This volume decrease, however, was not enough to account for the increased urinary hydroxyindole concentrations of mice bearing P815X2 tumors. Histochemical studies indicate that the P815X2 tumor cells growing* in vivo *themselves are the most probable source of the hydroxyindoles.*

INTRODUCTION

THE P815 tumor of DBA mice has some mast cell characteristics [1, 2]. Green and Day [3], who established the P815X2 subline used in the present study, showed by bioassay that it contained less than 1 μg of 5-hydroxytryptophan (5-HT) per 10^6 log phase cells. Although fluorometric assays for 5-HT are more reproducible than such bioassays, they are somewhat less sensitive. Nevertheless, histochemical fluorometric assays can serve to detect the presence of 5-HT in mast cells [4]. 5-HT is metabolized to hydroxyindoles which are subsequently excreted in the urine as hydroxyindoles. A colorimetric assay for the measurement of these metabolites of 5-HT has been described [5–7]. The concentration of hydroxyindoles in the urine of P815X2 tumor bearing mice was found to be high enough to be easily measurable by colorimetric assay. Thus we were able to monitor the *in vivo* production of 5-hydroxyindoles in mice as their P815X2 tumors developed, evaluate other tumor lines for induction of increased 5-hydroxyindole levels and also measure the effects of various irradiation treatments.

Accepted 14 June 1976.

*This work was supported by Research Training Grant CA-05224 and by D.C.T. Research Contract No. 1-CM-43730 from the National Cancer Institute.

MATERIAL AND METHODS

Twelve week old male DBA/2 mice (Texas Inbred) were used. Animals were maintained in an air-conditioned room with a 12 hr photoperiod and fed Purina lab chow and water *ad libitum*.

The cell lines used were: (1) P815X2, a subline of the P815 mastocytoma described above; (2) S-180, a transplantable mouse sarcoma; and (3) T1699, a transplantable mouse adenocarcinoma. P815X2 and S-180 were grown as solid and as ascites tumors. The T1699 tumor was grown as a solid tumor. (It will not develop as an ascites tumor in the DBA/2 mouse). In order to test the effects of tumor growth on hydroxyindole production, several specified numbers of cells were injected intraperitoneally or subcutaneously, as indicated in each experiment. Pooled daily urine samples were collected in metabolic cages. There were four mice per group.

The colorimetric assay for urinary 5-hydroxyindoles of Sjoerdsma *et al.* [6], modified by Lowenberg and Engelman [7], was used: 0·2 ml of urine sample was diluted to a final volume of 2 ml with distilled water, 1 ml of the nitrosonaphthol and 1 ml of the nitrous acid reagents were added. After 10 min (during which nitrosonaphthol-hydroxyindole coupling produces a purple pigment), the aqueous layer

was cleared by shaking with 5 ml of ethylene dichloride. The optical density of the aqueous layer was then read at 540 μm.

A histological fluorometric assay for 5-HT described by Ritzen [4] was also used. Cell smears were made from 4 day and 10 day old P815X2 ascites tumors. Peritoneal exudate was transferred directly to subbed slides. Smears were then immediately dried *in vacuo* for 30 min. After drying, slides were exposed in a desiccator for 2 hr to formaldehyde vapors formed by heating paraformaldehyde to 80°C. The paraformaldehyde was equilibrated in an atmosphere containing 50% relative humidity. Following the paraformaldehyde exposure, uncovered slides were examined under phase optics and evaluated for fluorescence following excitation at 370 μm.

Whole body or abdominal (i.e., thorax and head shielded) X-irradiation was delivered as follows: 270 kVcp; 1·5 mm Cu HVL; 20 mA; and a target to midline distance of 45 cm. The exposure rate was 100 R/min.

RESULTS

In Fig. 1 tumor size and the concentration of urinary hydroxyindoles as measured by the OD_{540} of the nitrosonaphthol-urinary hydroxyindole chromophore (hereafter referred to as OD) are plotted as a function of post-inoculum time. Various tumor lines as well as the ascites and solid modes of tumor growth were studied.

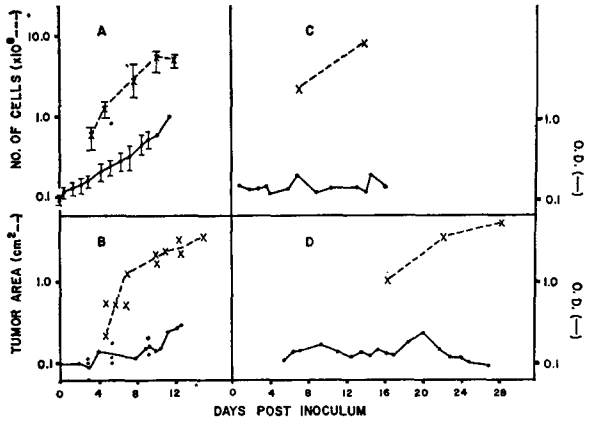


Fig. 1. OD_{540} and tumor growth vs. time; comparison of different tumour lines. Each mouse was injected with 10^6 log phase cells on day 0. The amount of urinary hydroxyindoles reflected by the optical density of the coupled nitrosonaphtol reagent (●——●) and the extent of tumor growth monitored in sample mice by total ascites cell counts or by the measurement of external tumor area (×--×) are plotted vs time. Panel A: P815X2 ascites tumor (mean value ± 2 S.E. of 7 experiments); Panel B: Subcutaneous P815X2 solid tumor; Panel C: S-180 ascites tumor; Panel D: T1699 solid tumor.

As is indicated in panel A of Fig. 1, the growth of the ascites mode of the P815X2 tumor is accompanied by a ten-fold increase in urinary hydroxyindole concentration. The data plotted in panel A represent mean values ± 2 S.E. By day 4 post inoculum, the increase in hydroxy indole concentrations is significant at the 0·05 level. The growth of solid subcutaneous P815X2 tumours also appears to be accompanied by an increase in urinary hydroxyindole concentrations (Fig. 1, panel B). However, the magnitude of the increase is less, and significant differences do not appear until later stages of tumor development. The presence of S-180 ascites tumors (panel C) or T1699 tumors (panel D) is not accompanied by such increases in urinary hydroxyindoles.

Total urine volume

The total daily volume of urine produced per mouse varied from day to day, and between individual mice to such a degree that no daily averages showed statistically significant differences. However, with all tumor bearing mice, the total daily volumes tended to decrease with advanced disease. In spite of this general tendency, the only tumor capable of inducing increased urinary hydroxyindole OD was the P815X2 mastocytoma. If corrections for decreased urine volume are made, the increase of hydroxyindoles in the urine of mice bearing P815X2 ascites tumors is reduced but not eliminated. For example, the mean total OD $[\sum (OD_{nx})\ (volume_{nx})]/n$ from urine collected on day 1 and day 8 equalled 1·45 and 6·59 respectively. This volume-corrected difference was found to be significant at the 0·05 level. In contrast, total daily hydroxyindole excretion by S-180 mice decreased in parallel to the decrease in total urinary volume.

Manipulations of P815X2

(*a*) *Inoculum size.* Varying the inoculum size resulted in corresponding shifts in the rise of hydroxindole concentrations. Following inoculum of 10^7 cells i.p., a relatively prompt increase in the urinary hydroxyindole concentration was noted (curve A of Fig. 2). The increase of urinary hydroxyindole concentration seen following a smaller inoculum of 10^4 cells (curve B of Fig. 2) produced a qualitatively similar curve, but one delayed by approximately 5 days in time of onset. This result suggested that cell populations in ascites tumors must reach a certain cell density before the detectable urinary hydroxyindole concentration begins to increase significantly above levels seen in mice without the P815X2 tumor.

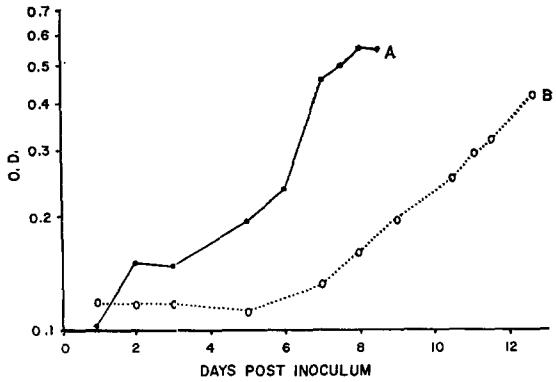


Fig. 2. OD_{540} vs time following different inocula sizes. Mice were injected I.P. with 10_4 (○ . . . ○) and 10^7 (●——●) log phase P815X2 cells and their urinary hydroxyindole concentration was monitored as above.

(*b*) *Histochemical assay of* 5-*HT.* In order to determine the relative influence of the developmental stage of P815X2 tumors on their 5-hydroxytroptophan content, cells from different aged tumors were assayed histochemically for 5-HT. The fluorometric assay for 5-HT described in Material and Methods revealed an increase in the number of significantly fluorescing cells when day 4 P815X2 ascites tumors are compared with day 10 P815X2 ascites cells (Fig. 3). Most day 4 cells were not fluorescent, but 0·16% (S.E. = 0·04%) of day 4 cells produced a bright fluorescence under u.v. (Fig. 1, panels A and B). Such brightly fluorescing cells may be 5-HT producing mastocytoma cells or normal mast cells which are routinely found in the peritoneal cavity. Their small size and density also suggest that they might be necrotic or ageing cells. In contrast, all day 10 cells were fluorescent (Fig. 3, panels C and D). Significantly, there is no change in viability between day 4 and day 10 tumors as judged by *in vitro* cloning assays [8].

(*c*) *Effect of X-irradiation.* A 1000 R/week radiotherapy schedule using abdominal exposures of 600 R on Monday and 400 R on Thursday [9] that resulted in slowed tumor growth (Fig. 4, panel A) caused little change in P815X2 induced urinary hydroxyindole OD's (Fig. 4, panel B). Furthermore, such exposure did not significantly alter daily urine volumes.

Even higher exposures to X-irradiation were used to further decrease the number of cells/mouse. Curve A of Fig. 5 shows that a single abdominal exposure of 3000 R delivered on day 3 of tumor growth did not modify the urinary hydroxyindole concentration compared to that of unirradiated mice (curve B of Fig. 5). Curve C of Fig. 5 shows that stressing healthy

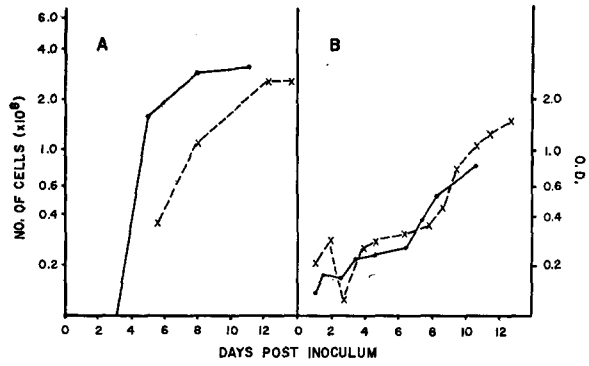


Fig. 4. Cell number and OD after several doses of abdominal X-irradiation. Mice were inoculated I.P. with 10^6 log phase P815X2 cells on day 0. Half of them were then whole-body irradiated as follows: 600 R on day 3, 400 R on day 6, 600 R on day 9 and 400 R on day 13. Panel A: Number of cells in the peritoneal cavity of sample mice killed at the time indicated; Panel B: Level of urinary hydroxyindoles vs time.

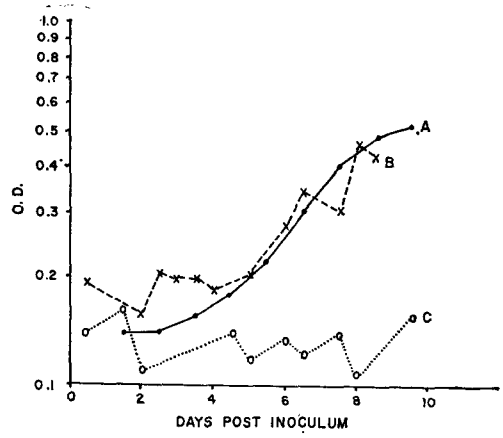


Fig. 5. Effect of 3000 R abdominal irradiation delivered on day 3 after inoculation with P815X2 cells.

Curve A (●——●): Mice were inoculated i.p. with 10^6 log phase P815X2 cells and never irradiated.

Curve B (×--×): Mice were inoculated i.p. with 10^6 log phase P815X2 cells and irradiated abdominally with 3000 R on day 3.

Curve C (○ . . . ○): Mice were never inoculated but were irradiated abdominally with 3000 R on day 3.

mice with the same irradiation dose did not affect their urinary hydroxyindole concentrations.

In an attempt to determine if a tumor-directed, acquired immune response might cause the increases in hydroxyindole excretion, mice were whole body irradiated with 500 R 2 days before they were injected with P815X2 ascites cells. Such radiation exposure suppresses the development of both humorally mediated immune responses and humorally dependent cellular immune responses [10]. However, as shown in Fig. 6, such immunosuppressive

exposures had no effect on the increase of urinary hydroxyindole concentrations seen in mice bearing P815X2 ascites cells.

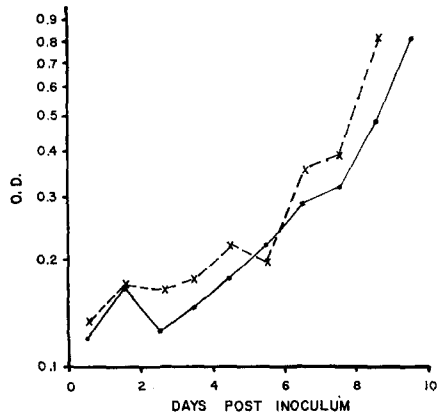


Fig. 6. Effect of immunosuppressive irradiation on OD_{540}. Mice were treated with 500 R whole-body irradiation 2 days prior to being inoculated i.p. with 10^6 log phase cells The OD_{540} reflecting urinary hydroxyindoles of the irradiated (× – – – ×) and of the unirradiated (●——●) groups are plotted with time post inoculum

DISCUSSION

The urinary concentrations of hydroxyindoles may closely reflect the production of 5-HT by P815X2 tumors *in vivo*. However before we can rely on this convenient measure of *in vivo* tumor activity, we must eliminate possible alternate sources of 5-HT in the animal. Notable among these are the normal mast cells which may be triggered to release 5-HT as a result of tumor growth. Non-specific reactions by host mice which may result in 5-HT release are eliminated by the observations that P815X2 mastocytoma was the only one of 3 tumors tested which affected the urinary hydroxyindole levels. Furthermore, the possibility that the P815X2 tumors can uniquely stimulate immune reactions, which in turn could trigger 5-HT release, was reduced by the observation that immunosuppressive whole-body irradiation delivered 2 days prior to P815X2 cell inoculation did not alter the subsequent patterns of hydroxyindole excretion. That the mastocytoma cells themselves contain 5-HT is shown by the fluorescent assays, results in Fig. 3. Since the number of fluorescent cells is greater in day 10 ascites tumors than in day 4 ascites tumors, it is probable that more cells produce 5-HT as the tumor ages. We know nothing about how and when 5-HT is released from these cells. It is possible that old tumors may contain a significantly higher portion of fragile cells which are more likely to release their 5-HT. Although the observation [8] that there is no decrease in cell clonogenicity between day 2 and day 10 argues against this. In any case, unless these cells are producing 5-HT, their breakdown will not result in urinary increases of hydroxyindoles, and the fluorometric assay shown in Fig. 3 suggests that day 4 cells are not producing 5-HT. Our evidence therefore supports the hypothesis that P815X2 cells in ascites tumors differentiate with respect to 5-HT production as the tumor ages.

The irradiation experiments show that the increase of hydroxyindoles seen with the progression of malignancy is not always correlated with total tumor cell number. It is probable that X-irradiation preferentially destroys proliferative cells. A single dose of 3000 R such as that used in Fig. 6 would destroy a substantial fraction of such cells at the time of treatment. The lack of an effect of such a dose of irradiation on the hydroxyindole production indicates that the productive cell population is radioresistant and therefore probably non-proliferative. The proliferating fraction, however, must give rise to the 5-HT producing cells whose number increases in older tumors. Therefore one would expect that reducing the proliferating fraction enough, by repeated X-ray doses, would eventually effect the increase in the rate of hydroxyindole production. Such experiments are in progress.

Mice carrying P815X2 cells as ascites have greater hydroxyindole output than those carrying P815X2 cells as solid tumors. Although the number of tumor cells in a mouse with day 10 ascites disease may indeed by higher than in a mouse with day 14 solid tumor, it is also possible that cells within an ascites tumor are especially capable of differentiation for 5-HT production.

Under what conditions differentiation, with respect to hydroxyindole production by the mastocytoma cells, takes place and to what extent these "differentiated" cells are non-proliferative are still unanswered questions that require further study.

REFERENCES

1. J. Davis and R. K. Ralph, Regulation of growth of mouse mastocytoma cells. *Cancer Res.* **35,** 495 (1975).
2. R. Schindler, M. Day and G. A. Fischer, Culture of neoplastic mast cells and

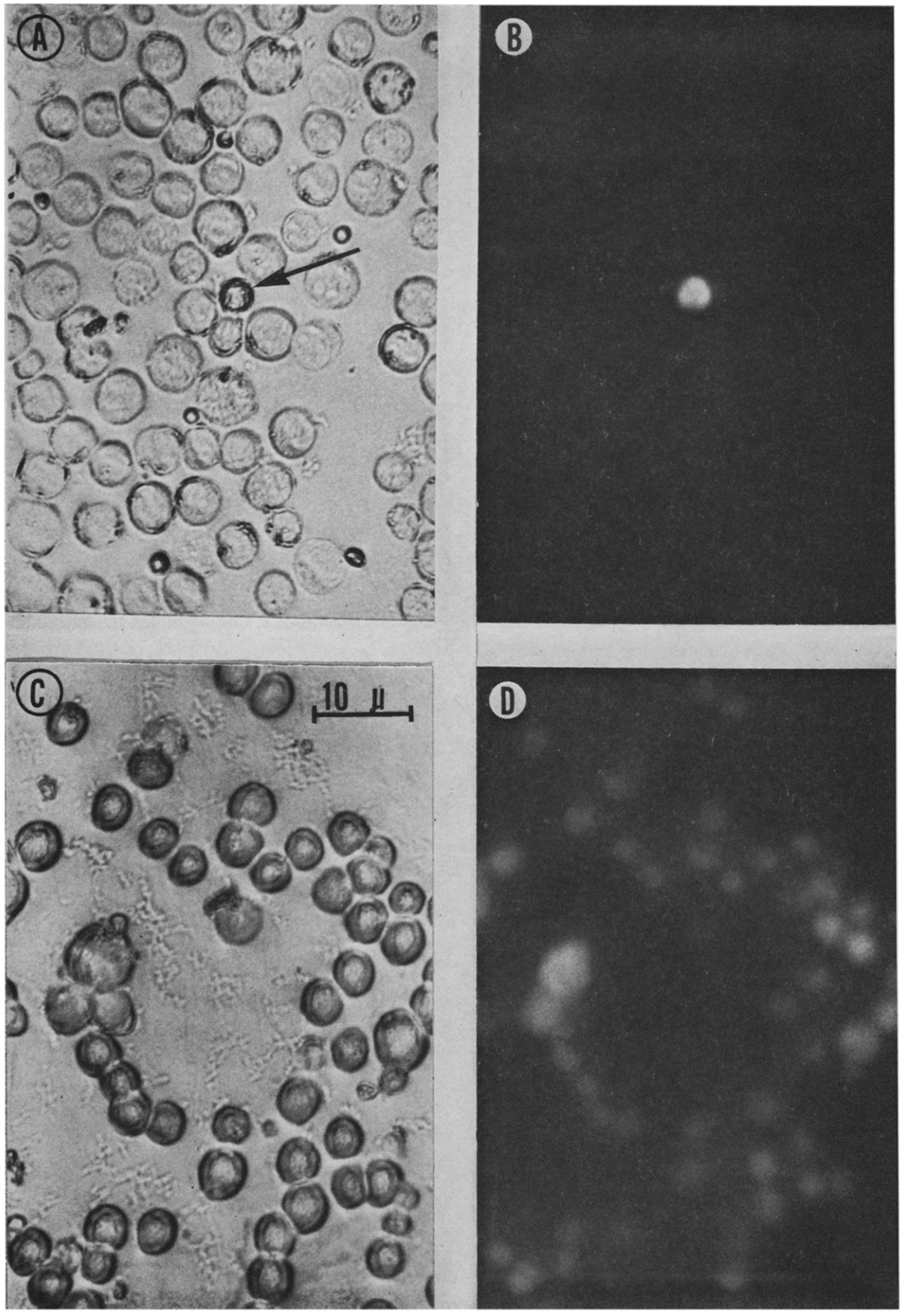


Fig. 3. Histochemical assay for 5-HT. Photomicrographs of P815X2 ascites cells treated with formaldehyde vapor to produce a fluorescent compound with 5-HT. Each panel shows the fluorescence after excitation at 370 μm next to a phase contrast photograph of the same field. Panels A & B: day 4 cells; Panels C & D: day 10 cells. All panels are at the same magnification.

(to face page 1014)

their synthesis of 5-hydroxytryptamine and histamine *in vitro*. *Cancer Res.* **19,** 47 (1959).

3. J. P. GREEN and M. DAY, Heparin, 5-hydroxytryptamine and histamine in neoplastic mast cells. *Biochem. Pharmacol.* **3,** 190 (1960).
4. M. RITZEN, Quantitative fluorescence microspectrophotometry of 5-hydroxytryptamine formaldehyde products in models and in mast cells. *Exp. Cell. Res.* **45,** 178 (1966).
5. F. LEMBECK, 5-hydroxytryptamine in the carcinoid tumor. *Nature* (*Lond.*) **172,** 910 (1953).
6. A. H. SJOERDSMA, H. WEISSBACH and S. UNDENFRIEND, Simple test for diagnosis of metastatic carcinoid (argentaffinoma). *J. Amer. med. Assoc.* **159,** 397 (1955).
7. W. LOWENBERG and K. ENGELMAN, Assay of serotonin, related metabolites and enzymes. In *Methods of Biochem. Analysis* (Edited by D. GLICK), Suppl. Vol., p. 1. Interscience Publ. John Wiley & Sons, N.Y. (1971).
8. R. F. HAGEMANN, L. L. SCHENKEN and S. LESHER, Tumor chemotherapy: efficacy dependent on mode of growth. *J. nat. Cancer Inst.* **50,** 467 (1973).
9. L. L. SCHENKEN, L. POULAKOS and R. F. HAGEMANN, Response of an experimental solid tumor to irradiation: a comparison of modes of fractionation. *Brit. J. Cancer* **31,** 228 (1975).
10. P. MORGAN, N. P. SHERWOOD, A. A. WERDEN and K. YOUNGSTROM, Studies on anaphylactic shock in the mouse. III. Single whole-body X-irradiation of 500 roentgens. *J. Immunol.* **84,** 324 (1960).

Europ. J. Cancer Vol. 12, pp. 1017–1019. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Reduction by Pituitary Grafts of Mammary Tumor Age.

Its Variability in a High Mammary Tumor Strain of Mice: Effects on Mammary DNA Synthesis*

HIROSHI NAGASAWA, REIKO YANAI and HISASHI TANIGUCHI
Pharmacology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, *Chuo-ku, Tokyo* 104, *Japan*

EVEN in high mammary tumor strains of mice maintained under the similar conditions, there may be considerable variation in mammary tumor age. Pituitary grafts at young age [1] or neonatally [2] as well as after maturity [3, 4] promote mammary tumorigenesis in mice and prolactin from the grafts has a major part in this process [3, 4]. However, in all of these experiments, the primary attention was paid to mammary tumor incidence, but neither to mammary tumor age nor to its variation. In view of the importance of mammary DNA synthesis around the time when carcinogenic agents act on the glands in mammary tumorigenesis [5–7], pituitaries grafted into very young animals should maintain high mammary DNA synthesis, which in turn might result in a less variable as well as earlier mammary tumor appearance. The present study deals with this problem.

The mammary tumor susceptible SHN strain of virgin mice [8] were used. At weaning (20–23 days of age), half of the females in each litter received 2 isologous pituitary grafts under the right kidney capsule. The other females in the litter were given no treatment and served as the control. Each mouse was checked for palpable mammary tumors every 7 days until it died or became moribund. At about 70 days of age, some mice of both groups were given an intraperitoneal injection of 50 μCi ^{3}H-thymidine (5 Ci/mmole; The Radiochemical Centre, Amersham, England) and 2 hours later ^{3}H-thymidine incorporated into mammary DNA was assayed [9] as the index of mammary DNA synthesis.

Mammary tumor incidences were 95·9% (47/49) and 88·5% (128/145) in the experimental and control groups, respectively, no significant difference being observed.

The normal distribution curves [10] of mammary tumor age are shown in Fig. 1. The mice with pituitary grafts had significantly earlier mammary tumor age than the control ($P < 0{\cdot}01$). Moreover, a marked difference in the variation of tumor age between groups was observed as shown by the standard deviation and the fiducial limit. Thirty-six per cent of the grafted mice developed mammary tumors at an age corresponding to the average age, and 70% showed tumors within one month before and after the average age. Meanwhile, only 20% of the control mice had a tumor age similar to the average, and the number of mice showing tumors one month before and after the average age was less than 35%. These indicate that the variation in mammary tumor

Accepted 11 June 1976

*Supported in part by grants-in-aid for Cancer Research from the Ministry of Education, Science and Culture (No 901039) and the Society for Promotion of Cancer Research, Japan

age is reduced by about one-half by the isografting of 2 pituitaries at weaning.

The rates of mammary DNA synthesis at 70 days of age varied between 120 and 400 dpm/μg DNA and between 13 and 710 dpm/μg DNA, mostly less than 120 dpm/μg DNA in the experimental and control groups, respectively. The averages of 230 and 151 dpm/μg DNA were not significantly different owing to the large variation in the control (Fig. 2).

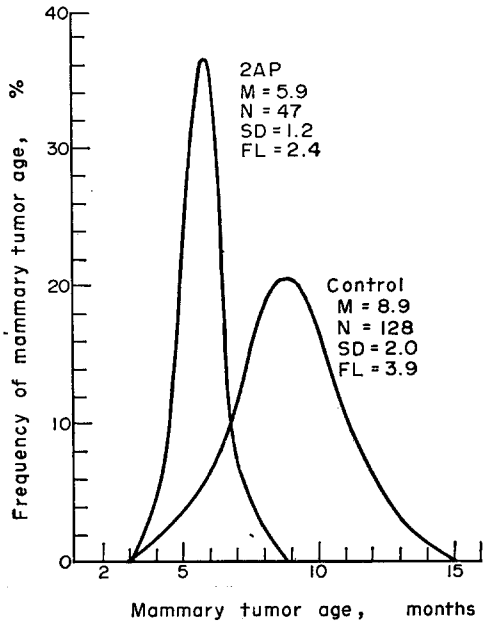


Fig. 1. Normal distribution curves of mammary tumor age. 2AP: grafted with 2 isologous pituitaries each at weaning. M: mean in months, N: number of mice, SD: standard deviation, FL: fiducial limit at P = 0·05.

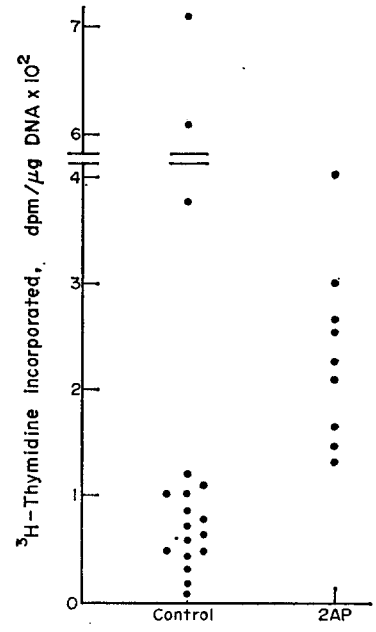


Fig. 2. ^{3}H-thymidine incorporation into mammary DNA in each group at about 70 days of age. 2AP: grafted with 2 isologous pituitaries at weaning.

The mammary gland is considered to be exposed to several carcinogenic agents throughout the lifetime of the animal. Thus, in view of the importance of mammary DNA synthesis in mammary tumor induction [5–7], the longer the period of high mammary DNA synthesis, the higher the probability of malignant transformation of mammary cells. In the present experiment, pituitary grafting at weaning markedly increased mammary DNA synthesis at 70 days of age. Mammary DNA synthesis of mammary tumor susceptible strains of mice shows peak around 70 days of age and declined thereafter, while pituitary grafting constantly maintained high mammary DNA synthesis irrespective of the host age [11]. Therefore, the marked reduction in both mammary tumor age and its variability by pituitary grafting is mostly attributable to the continuous promotion of mammary DNA synthesis by prolactin from the grafts. Bern [12] inferred that hormone stimulation of mammary gland can create conditions favorable for the action of carcinogenic agents. The data support this hypothesis and strongly suggest that one role of prolactin in mammary tumor induction is to sensitize the gland to carcinogenic agents through its constant stimulation of mammary DNA synthesis.

Acknowledgement—We thank Prof. H. A. Bern, Department of Zoology and Cancer Research Laboratory, University of California, Berkeley, California, U.S.A. and Dr. T. Mori, Zoological Institute, University of Tokyo, Tokyo, Japan for reading the manuscript and for valuable suggestions.

REFERENCES

1. D. Medina, K. B. DeOme and L. Young, Tumor-producing capabilities of hyperplastic alveolar nodules in virgin and hormone-stimulated BALB/c f. C3H and C3Hf mice. *J. nat. Cancer Inst.* **44,** 167 (1970).
2. T. Mori and H. A. Bern, Personal communication.

3. L. M. Boot, Induction by prolactin of mammary tumours in mice. *Verh. kon. Ned. Akad. Wet., Afd. Natuurk., Tweede Reeks, deel LVIII, Suppl.* 3. (1969).
4. G. Röpcke, Interaction of hypophyseal isografts and ovarian hormones in mammary tumour development in mice. *Thesis, Mondeel-Offsetdrukkerij, Amsterdam* (1975).
5. H. Nagasawa and R. Yanai, Frequency of mammary cell division in relation to age: Its significance in the induction of mammary tumors by carcinogen in rats. *J. nat. Cancer Inst.* **52,** 609 (1974).
6. H. Nagasawa, R. Yanai and H. Taniguchi, Importance of mammary gland DNA synthesis in carcinogen-induced mammary tumorigenesis in rats. *Cancer Res.* **36,** 2223 (1976).
7. R. Yanai and H. Nagasawa, Effects of pituitary graft and 2-bromo-α-ergocryptine on mammary DNA synthesis in mice in relation to mammary tumorigenesis. *J. nat. Cancer Inst.* **56,** 1055 (1976).
8. H. Nagasawa, R. Yanai, H. Taniguchi, R. Tokuzen and the late W. Nakahara, Two-way selection of a stock of Swiss albino mice for mammary tumorigenesis: Establishment of two new strains (SHN and SLN). *J. nat. Cancer Inst.* **57,** In press (1976).
9. H. Nagasawa and R. Yanai, Effects of estrogen and/or pituitary graft on nucleic acid synthesis of carcinogen-induced mammary tumors in rats. *J. nat. Cancer Inst.* **52,** 1219 (1974).
10. G. W. Snedecor, *Statistical Methods.* 5th ed., p. 194. Iowa State Univ. Press., Ames.
11. H. Nagasawa and R. Yanai, Unpublished.
12. H. A. Bern, Nature of the hormonal influence in mouse mammary cancer. *Science* **131,** 1039 (1960).

Europ. J. Cancer Vol. 12, pp. 1021–1024. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Tumour Development after 3-Methylcholanthrene in Irradiated, Thymectomized Mice Reconstituted with Syngeneic Bone Marrow*

N. ALLEGRETTI AND M. MARUŠIĆ

Department of Physiology, University of Zagreb Faculty of Medicine, Šalata 3, 41001 *Zagreb, p.o.b.* 978, *Croatia, Yugoslavia*

SPONTANEOUS malignancies were described to occur in immunologically deficient neonatally thymectomized [1] and athymic-nude mice [2] in the same proportion as in normal ones. Furthermore, athymic-nude mice were shown to undergo tumour development after 3-methylcholanthrene in the same proportion as the normal controls [3]. Results on chemical carcinogenesis obtained in immunodepressed mice are not unanimous regarding their susceptibility to tumour development [3–18]. These results bring evidence to bear on the question of the thymus role in the "immunological surveillance" of some spontaneous and chemically induced tumours in mice. In view of this, immunologically deficient mice after thymectomy, irradiation and syngeneic bone marrow reconstitution were considered a suitable model for testing the above hypothesis. This experimental model appeared advantageous in dealing with long-lived animals in comparison with athymic-nude or neonathally thymectomized mice.

CBA mice were thymectomized when 3–4 weeks old. Thymectomy was proved at the autopsy after experiment. About 4 weeks after operation mice were lethally irradiated. Irradiation constants were: 200 kV, 15 mA; 0·5 mm Cu, 1·0 mm Al filters; distance to target 40 cm; dose rate 100 R/56 sec, irradiation dose 900 R. Within 4 hr after irradiation thymectomized mice were reconstituted with 10×10^6 normal syngeneic bone marrow cells. Similarly treated CBA mice were proved to take Balb/c and LC57 skin allografts for more than 50 and 80 days, respectively. Mice were given 1 mg 3-methylcholanthrene (MCA, Fluka) in 0·1 ml seed oil subcutaneously 50 days after irradiation and reconstitution. Simultaneously, normal mice were equally treated. The animals were observed twice weekly and tumour and death appearance were scored. After administration of the drug solved in seed oil the injection sites become organized resembling the incipient tumour knot. Some of the knots regress after one to two weeks. This makes impossible the determination of the exact day of the true tumour appearance. It appeared suitable, therefore, to determine the week (not the day) of the tumour appearance after the drug in oil administration.

Figure 1 presents the tumour and death appearance in T-cell deficient and normal mice. According to the χ^2-test the tumour incidence did not differ significantly between the groups. In addition the probit analysis revealed that there was no significant difference neither in regression coefficients nor in medians (Fig. 2). This allowed tumour appearance in both groups to be considered as forming parts of one single group of data which was proved also statistically correct. It is obvious that the score of the tumour development would be rendered more in favour of the immune deficient mice

Accepted 19 March 1976.

*This work has been supported by the Research Fund of Croatia(Zagreb).

Table 1. *Tumour appearance and incidence* after carcinogenic drug in thymus deprived mice†*

Strain of mice	Method of thymus deprivation	Drug	Tumour appearance and incidence*	References
Outbred albino	Tex‡ at 3 days	BP§	accelerated, increased	4
C3H/Bi	Tex at 7 days + 300 R	MCA¶	accelerated, increased	5
C3H/Bi	Tex at 7 days + 300 R	DMBA‖	normal, normal	5
C57Bl	Tex at 3 days	MCA	accelerated, normal	6
C3H/HeN	Tex at 3 days	MCA	normal, normal	7
Swiss	Tex within 24 hr	BP	accelerated, increased	8
C57Bl	Tex at 24 hr	MCA	normal, normal	9
(C57Bl × CBA)F_1	Tex at 24 hr	MCA	normal, normal	9
C57Bl	Tex at 3 days	MCA	accelerated, normal	10
AKR	Tex at 2 days	MCA	accelerated, increased	11
(AKR × C3H)F_1	Tex at 2 days	MCA	accelerated, normal	11
C3H	Tex at 2 days	MCA	normal, normal	11
CBA	ALS**	MCA	accelerated, increased	12
C57Bl	ALS	MCA	accelerated, increased	13
C57Bl	ALS	MCA	normal, normal	14
SWR	ALS	DMBA	normal, normal	15
Swiss	Tex at 3 days	DMBA	accelerated, normal	16
Swiss	Tex at 3 days	Urethane	normal, increased	16
SWR	Tex at 3 days	DMBA	normal, increased	16
SWR	Tex at 3 days	Urethane	normal, increased	16
C3H/f	ALG††	MCA	normal, normal	17
I	ALG	MCA	normal, increased	17
C3H/f	Tex at 3 days	MCA	normal, normal	18
I	Tex at 3 days	MCA	normal, normal	18
In CBA/H background	Athymic-nude	MCA	normal, normal	3
CBA	Tex + lethal irradiation + bone marrow	MCA	normal, normal	This paper

*Tumour appearance after carcinogenic drug and incidence in comparison to normal mice.

†Thymus deprived considered mice: thymectomized within 3 days after birth, adult thymectomized and nonlethally irradiated, antilymphocyte serum treated, athymic-nude and adult thymectomized, lethally irradiated, syngeneic bone marrow reconstituted.

‡Thymectomized.

§3,4-Benzopyrene.

¶3-Methylcholanthrene.

‖3,4–9,10 Dimethylbenzanthracene.

**Antilymphocyte serum.

††Antilymphocyte gammaglubulin.

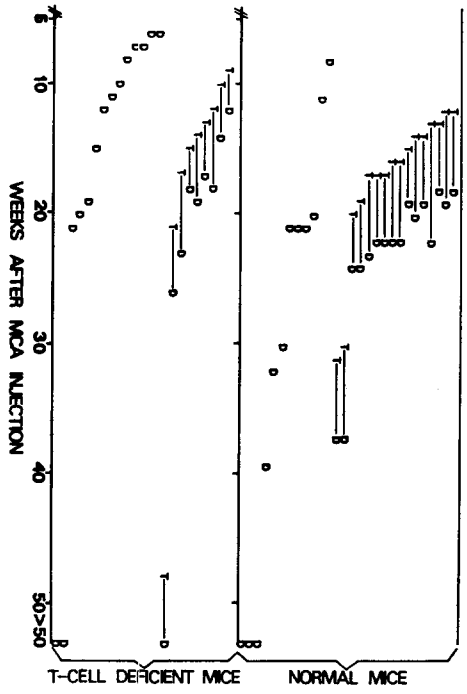


Fig. 1. Tumour and death appearance in normal and T-cell deficient CBA mice after 3-methylcholanthrene injection. T: tumour appearance; D: death appearance

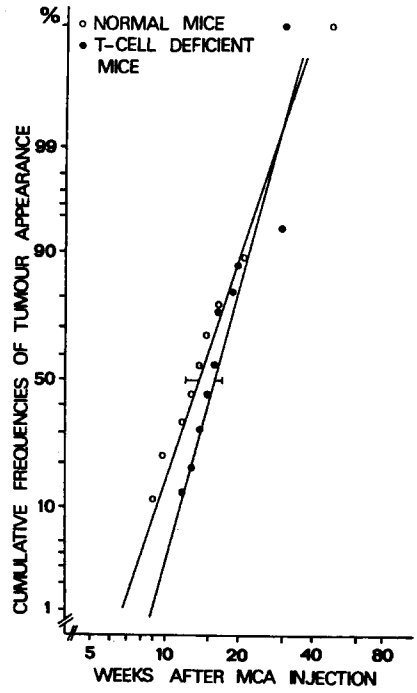


Fig. 2. Percent cumulative frequencies of tumour appearances plotted against the logarithm of weeks in normal and T-cell deficient mice. Horizontal lines depict fiducial limits of medians.

if some of the "spontaneously" dying mice in this group were not included into calculation. The spontaneous mortality, however, was not so high as to permit the exclusion of more than 4–5 mice dying without tumour. Accordingly, the incidence of tumour after MCA in immune deficient mice was, at least, of the same order as that in normal controls.

These results give good evidence that athymic, immunologically deficient mice have no greater susceptibility to MCA tumour induction and development than the normal mice. Earlier results on chemical carcinogenesis in T-lymphocyte deprived mice are summarized in Table 1. As T-lymphocyte deprived are considered mice thymectomized within 3 days after birth, adult mice thymectomized and non-lethally irradiated, antilymphocyte serum treated mice, athymic-nude mice and adult thymectomized, lethally irradiated, syngeneic bone marrow reconstituted mice. As shown many previous results were also calling in question the immunological surveillance mechanisms, particularly the role of the thymus. Accordingly, there appears good reason to conclude that those mechanisms are not generally potent and not likely to act in each malignancy. The existence of a "non-immunological surveillance" independent of the immune status of the organism may also be assumed.

Acknowledgement—The excellent technical assistance of Mrs. Gjurja Grgurić is acknowledged.

REFERENCES

1. B. H. Sandford, H. I. Kohn, J. L. Daly and S. F. Soo, Long-term spontaneous tumor incidence in neonatally thymectomized mice. *J. Immunol.* **110,** 1437 (1973).
2. J. Rygaard and C. O. Povlsen, The mouse mutant *nude* does not develop spontaneous tumours. *Acta path. microbiol. scand. Section* B, **82,** 99 (1974).
3. O. Stutman, Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice, *Science* **183,** 534 (1974).
4. J. F. A. P. Miller, G. A. Grant and F. J. C. Roe, Effect of thymectomy on the induction of skin tumours by 3,4-benzpyrene, *Nature (Lond.)* **199,** 920 (1963).
5. V. Defendi and R. A. Roosa, The role of the thymus in carcinogenesis. In *The Thymus* (Edited by: V. Defendi and D. Metcalf), Wistar Institute Press, Philadelphia, p. 121 (1964).

6. G. A. Grant and J. F. A. P. Miller, Effect of neonatal thymectomy on the induction of sarcomata in C57Bl mice. *Nature (Lond.)* **205,** 1124 (1965).
7. L. W. Law, Neoplasms in thymectomized mice following room infection with Polyoma virus. *Nature (Lond.)* **205,** 672 (1965).
8. G. A. Grant, F. J. C. Roe and M. C. Pike, Effect of neonatal thymectomy on the induction of papillomata and carcinomata by 3,4-benzpyrene in mice. *Nature (Lond.)* **210,** 603 (1966).
9. H. Balner and H. Dersjant, Neonatal thymectomy and tumor induction with methylcholanthrene in mice. *J. nat. Cancer Inst.* **36,** 513 (1966).
10. S. Johnson, The effect of thymectomy and of the dose of 3-methylcholanthrene on the induction and antigenic properties of sarcomas in C57Bl mice. *Brit. J. Cancer* **22,** 93 (1968).
11. K. Nomoto and K. Takeya, Immunologic properties in methylcholanthrene-induced sarcomas of neonatally thymectomized mice. *J. nat. Cancer Inst.* **42,** 445 (1969).
12. G. J. Cerilli and R. C. Treat, The effect of antilymphocyte serum on the induction and growth of tumour in the adult mouse. *Transplantation* **8,** 774 (1969).
13. A. G. Rabbat and H. F. Jeejeebhoy, Heterologous antilymphocyte serum (ALS) hastens the appearance of methylcholanthrene-induced tumours in mice. *Transplantation* **9,** 164 (1970).
14. J. L. Wagner and G. Haughton, Immunosuppression by antilymphocyte serum and its effect on tumors induced by 3-methylcholanthrene in mice. *J. nat. Cancer Inst.* **46,** 1 (1971).
15. N. Haran-Ghera and M. Lurie, Effect of heterologous antilymphocyte serum on mouse skin tumorigenesis. *J. nat. Cancer Inst.* **46,** 103 (1971).
16. N. Trainin and M. Linker-Israeli, Increased incidence of spontaneous lung adenomas in mice following neonatal thymectomy. *Israel J. med. Sci.* **7,** 36 (1971).
17. O. Stutman, Immunologic studies on resistance to oncogenic agents in mice. *Nat. Cancer Inst. Monogr.* **35,** 107 (1972).
18. O. Stutman, Immunological aspects of resistance to the oncogenic effect of 3-methylcholanthrene in mice. *Israel. J. med. Sci.* **9,** 217 (1973).

Europ. J. Cancer Vol. 12, pp. 1025–1026. Pergamon Press 1976. Printed in Great Britain

Letter to the Editor

Survival of VX2 Carcinoma Cells *In Vitro*

C. S. B. GALASKO* and D. W. HAYNES,
Nuffield Orthopaedic Centre, Oxford, U.K.

THE VX2 carcinoma is an animal tumour which is being used extensively to study different aspects of tumour behaviour. It is derived from a virus induced papilloma of rabbits [1], which subsequently progressed to a metastazing carcinoma [2]. In 1940 Kidd and Rous [3] showed that a squamous cell carcinoma, derived from the original papilloma, could be propagated easily in skeletal muscle. The loss of viral dependency of the tumour was reported in 1952 [4]. The VX2 carcinoma has since been free of viral characteristics and remains readily transmissible. It is maintained by serial passage in the skeletal muscle of New Zealand white rabbits. If the tumour is lost, an inoculated rabbit has to be obtained from another laboratory. This may mean international transport of the animal with the problems of quarantine.

We therefore decided to investigate whether there was any method of maintaining the VX2 carcinoma *in vitro*.

In the first experiment, tumour cell suspensions of the VX2 carcinoma were obtained by finely dividing a cubic centimeter of tumour in 5 cm^3 of sterile saline. The fluid was filtered through gauze and 1 cm^3 of the tumour cell suspension dispensed into 3 glass ampoules. There were approximately $2{\cdot}25 \times 10^6$ cells per cubic centimeter. Each ampoule was placed in ice and at intervals of 24, 48 and 72 hr the tumour cell suspension was injected into the thigh muscles of New Zealand white rabbits.

In the second experiment six ampoules, each containing 1 cc tumour cell suspension, which was obtained in the identical manner, were frozen in liquid nitrogen. At intervals varying from immediately following freezing to four weeks following freezing, the ampoules were removed from the liquid nitrogen, the tumour cell suspension allowed to thaw and then injected into the thigh muscles of New Zealand white rabbits.

In the final experiment the piece of tumour was placed in a petri dish containing tissue culture medium (TC 199) with 10% DMSO added. A tumour cell suspension was again obtained by finely dividing the tumour in the medium, filtering it through gauze and dispensing it into six glass ampoules. The ampoules were sealed and placed in a $-20°C$ refrigerator which allowed the cell suspension to freeze at approximately 1°C/min. After 1 hr they were placed in a $-70°C$ refrigerator until required.

When the tumour was reimplanted, at intervals varying from 4 hr to 13 months, the ampoules were allowed to thaw at room temperature. A fibrin clot had formed and it was impossible to resuspend the cells. The fibrin clot, with the VX2 cells enmeshed in it, was then implanted into the thigh muscle of the New Zealand white rabbit through a surgical incision. The supernatant fluid was injected into the thigh muscles of a different rabbit.

Cooling in ice

In all instances, the tumour grew normally. There was no histological difference between the ice-cooled VX2 carcinoma and the normally passaged tumour (Fig. 1a and 1b). All the rabbits developed pulmonary metastases and died from these lesions at the same time as the rabbits in whom the tumour was being passaged normally.

Freezing in liquid nitrogen

All specimens failed to grow.

Accepted 20 February 1976.

*Current address: Orthopaedic Unit, Department of Surgery, Royal Postgraduate Medical School, London W12 0HS, U.K.

Tumour frozen with DMSO

Tumours which were suspended in DMSO and stored at −70°C for periods varying between 4 hr and 6 months grew when the fibrin clot was subsequently implanted into the muscle of New Zealand white rabbits. There was no histological difference between these tumours and the normally passaged tumours (Fig. 1(c)) and these tumours were normally transmissible.

The animals also developed pulmonary metastases but these took longer to develop and the animals survived longer than the passaged animals. In our laboratory, animals in whom the VX2 has been implanted usually die within six weeks. In this experiment some of the animals survived for eight to eleven weeks but all had pulmonary metastases at autopsy. Tumour which had been stored for 13 months showed no evidence of growth several months after implantation.

The results indicate that the VX2 carcinoma can be stored *in vitro* and does not necessarily have to be passaged from one animal to another. For periods of up to 72 hr the tumour cell suspension can be kept in ice. An attempt to store it for longer periods was made by freezing the tumour cell suspension in liquid nitrogen, but the tumour was destroyed. This may have been due to too rapid cooling or using saline as the suspension medium.

Suspending the cells in TC 199 with 10% DMSO allowed the cells to remain viable when stored at −70°C for up to 6 months. A fibrin clot was formed and this had to be implanted for successful take of the tumour. The supernatant fluid did not contain sufficient viable cells to propagate the lesions.

REFERENCES

1. R. E. Shope and E. W. Hurst, Infectious papillomatosis of rabbits with note on histopathology. *J. exp. Med.* **58,** 607 (1933).
2. P. Rous and J. W. Beard, Progression to carcinoma of virus-induced rabbit papillomas (Shope). *J. exp. Med.* **62,** 523 (1935).
3. J. G. Kidd and P. Rous, Transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the virus in masked or altered form. *J. exp. Med.* **71,** 813 (1940).
4. P. Rous, J. G. Kidd and W. E. Smith, Experiments on cause of rabbit carcinomas derived from virus-induced papillomas. Loss by VX2 carcinoma of power to immunize hosts against papilloma virus. *J. exp. Med,* **96,** 159 (1952).

Europ. J. Cancer Vol. 12, pp. 1027–1028. Pergamon Press 1976. Printed in Great Britain

Recent Journal Contents (1976)

International Journal of Cancer — *December, 1976*

Human Cancer

A. S. Morrison: Histologic specificity of the effect of age at first birth on breast cancer risk.

A. Aromaa, M. Hakama, T. Hakulinen, E. Saxén, L. Teppo and J. Idänpään-Heikkilä: Breast cancer and use of rauwolfia and other antihypertensive agents in the hypertensive patients: A nationwide case-control study in Finland.

R. K. Oldham, J. L. Weese, R. B. Herberman, E. Perlin, M. Mills, W. Heim, J. Blom, D. Green, J. Reid, S. Bellinger, I. Law, J. L. McCoy, J. H. Dean, G. B. Cannon and J. Djeu: Immunological monitoring and immunotherapy in carcinoma of the lung.

G. Leclercq, M.-C. Deboel and J.-C. Heuson: Affinity of estradiol mustard for estrogen receptors and its enzymatic degradation in uterine and breast cancer cytosols.

P. G. Parsons, E. Klucis, P. D. Goss, J. H. Pope, J. H. Little and N. C. Davis: Oncornavirus-like particles in malignant melanoma and control biopsies.

Experimental Cancer

W. M. Brown and A. A. Axelrad: Effect of Friend leukemia virus on megakaryocytes and platelets in mice.

L. Weiss and D. Glaves: The immunospecificity of altered initial arrest patterns of circulating cancer cells in tumor-bearing mice.

C. G. Brooks, R. C. Rees and R. W. Baldwin: Studies on the microcytotoxicity test. I. Evidence that the effects of normal lymphoid cells on tumour cell growth in microtest plates may be caused by non-immunological modifications of the culture medium.

M. Aupoix and P. Vigier: Expression of viral proteins in mammalian cells transformed by avian sarcoma viruses.

L. Falk, F. Deinhardt, M. Nonoyama, L. G. Wolfe, C. Bergholz, B. Lapin, L. Yakovleva, V. Agrba, G. Henle and W. Henle: Properties of a baboon lymphotropic herpesvirus related to Epstein–Barr virus.

D. M. Lopez and M. M. Sigel: Tumor antigen extracts stimulate cytotoxicity against unrelated target cells.

A. Gabizon, M. Small and N. Trainin: Kinetics of the response of spleen cells from tumor-bearing animals in an *in vivo* tumor neutralization assay.

S. O. Prather and R. N. Lausch: Membrane-associated antigen from the SV40-induced hamster fibrosarcoma, PARA-7. I. Role in immune complex formation and effector cell blockade.

J. Szkudlarek, C. Radzikowski, A. Czarnomska, K. Dux and W. Bartoszewicz: Leukaemias in BN/a and BN/b mice after prolonged treatment with antilymphocyte globulin.

D. L. Buchhagen, T. Pincus, O. Stutman and E. Fleissner: Leukemogenic activity of murine type C viruses after long-term passage *in vitro*.

K. Friend, I. Hellström and K. E. Hellström: *In vitro* sensitization to embryonic antigens.

N. K. Mishra, K. J. Pant, F. O. Thomas and P. J. Price: Chemical–viral co-carcinogenesis: Requirement for leukemia virus expression for accelerated transformation.

British Journal of Cancer *December, 1976*

R. Peto: The design and analysis of randomized clinical trials which require prolonged observations of each patient. Part I: Introduction and design.

G. A. Rawlins, J. M. F. Wood and K. D. Bagshawe: Macrophage electrophoretic mobility (MEM test) with myelin basic protein.

M. E. Ludgate and J. Gough: Stimulation of autologous blood lymphocytes by malignant lymphoma cells and homogenates.

J. Ferluga, H. U. Schorlemmer, L. C. Baptista and A. C. Allison: Cytolytic effects of the complement cleavage product, C3a.

N. Matthews and R. W. Whitehead: Inhibition of K cell function by human breast cancer sera.

C. R. Franks and Y. Williams: Peripheral lymphocyte count: A prognostic index of response to hormone therapy in patients with advanced breast cancer.

P. J. Chalmers, N. Matthews and R. C. Nairn: Characterization of serum factors which modulate splenic cytotoxicity in a syngeneic rat tumour system.

J. W. Proctor: Support for the "soil seed" hypothesis and the "mechanical" theory of metastatic spread from a rat sarcoma system.

J. H. C. Ho, M. H. Ng and H. C. Kwan: Epstein–Barr virus-specific serum antibodies in nasopharyngeal carcinoma patients and controls.

R. A. Bhisey and S. M. Sirsat: Selective promoting activity of phorbol myristate acetate in experimental skin carcinogenesis.

J. W. Hopewell, D. N. Edwards and G. Wiernik: The sex dependence of human intracranial gliomata.

Brief Communication

J. R. Kirwan and E. M. Narebor: The absorption of large oral doses of 5-formytetra-hydrofolate in man.

Report

Organized by Professor David Harnden: Report on workshop on short term carcinogenicity testing. Held at the University of Birmingham, 23rd June, 1976.

Book Reviews

S. Muldal: Handbuch der Mikroskopischen Anatomie des Menschen 1/3. H. G. Schwarzacker.

D. Pearson: Paediatric Oncology Case Studies, by Rashid A. Al-Rashid, M.D. 1975, London.

D. Pearson: Wilms' Tumour: Eds. Carl Pochedly and Denis Miller.

D. P. Burkitt: Monograph on Cancer Research No. 18. Cancer in Asia: Opportunities for Prevention, Detection and Treatment. Ed: Takeshi Hirayama. Japanese Cancer Association.

A. H. W. Nias, Cell and Tissue Culture by John Paul (1975) 5th Edition. Churchill Livingstone, Edinburgh.

EUROPEAN JOURNAL OF CANCER

VOLUME 12

Page

Number 1, *January* 1976

Perspectives in Cancer Research

J. S. Tobias and M. H. N. Tattersall: Autologous marrow support and intensive chemotherapy in cancer patients 1

General Papers

George P. Tryfiates and H. P. Morris: Growth of Morris hepatoma No. 7794A with and without vitamin B6. Effect of inoculation time 9

Jacques Jami, Nazario Rubio and Evelyne Ritz: Cellular and humoral response to immunization with semi-allogeneic hybrid cells in the mouse 13

K. H. Tragl, P. Angelberger, P. Pils, R. Hruby and E. Deutsch: Distribution of ^{64}Cu-bleomycin in normal and tumour-bearing rats 19

E. Scherer, W. F. M. van Dijk and P. Emmelot: The effect of antilymphocytic and normal horse serum on growth of precancerous foci and development of tumours induced by diethylnitrosamine in rat liver 25

M. Satya Murthy, Jean Belehradek, Jr. and Georges Barski: Interspecies mouse X Akodon Urichi somatic cell hybrids: comparative immunogenicity of parental and hybrid cells 33

EORTC Brain Tumour Group: Effect of CCNU on survival, rate of objective remission and duration of free interval in patients with malignant brain glioma—first evaluation 41

Ralph R. Weichselbaum, J. Epstein, John B. Little and Paul L. Kornblith: *In vitro* cellular radiosensitivity of human malignant tumors 47

K. R. Harrap and E. W. Gascoigne: The interaction of bifunctional alkylating agents with the DNA of tumour cells 53

Clive R. Taylor: An immunohistological study of follicular lymphoma, reticulum cell sarcoma and Hodgkin's disease 61

Recent Journal Contents 77

Number 2, *February* 1976

Perspectives in Cancer Research

L. M. Van Putten, H. J. Keizer and J. H. Mulder: Synchronization in tumour chemotherapy 79

General Papers

S. Pyrhönen, T. Timonen, A. Heikkinen, K. Penttinen, O. Alftan, E. Saksela and O. Wager: Rheumatoid factor as an indicator of serum blocking activity and tumour recurrences in bladder tumours 87

A. Guclu, T. Ghose, J. Tai and Molly Mammen: Binding of chlorambucil with antitumor globulins and its effect on drug and antibody activities 95

L. Den Engelse, W. Misdorp and P. Emmelot: Hepatomas in GRS/A male mice treated with horse serum or horse anti-mouse antilymphocyte serum 101

L. Morasca, E. G. Fogar-Ottaviano and S. Garattini: Time dependent cytotoxicity of adriamycin and daunomycin in primary cultures of normal and neoplastic mammary glands 107

Alberto Mantovani, Aldo Tagliabue, Annunciata Vecchi and Federico Spreafico: Effect of *Corynebacterium parvum* on cellular and humoral antitumoral immune effector mechanisms 113

D. Vaillier, J. Vaillier and M. Donner: Modification of interstitial fluid close to chemically-induced tumours and correlation with a growth stimulation of target cells 125

S. Ohl, A. L. Carsten, A. D. Chanana, G. Chikkapa and E. P. Cronkite: Increased erythrocytic and neutrophillic progenitors in myelofibrosis with myeloid metaplasia 131

Melvin D. Reuber: Effect of age and testosterone on the induction of hyperplastic nodules, carcinomas, and cirrhosis of the liver in rats ingesting N-2-fluorenyldiacetamide 137

Toru Masoka, Yoshihisa Hasegawa, Takaaki Ueda, Takayuki Takubo, Hirotoshi, Shibata, Hiroyuki Nakamura, Noriyuki Tatsumi, Junsuke Yoshitake and Nobuyuki Senda: On the mode of decrease of leukemic cells during treatment of acute leukemia 143

R. Revoltella, M. Pediconi, M. Amini, C. Bosman and L. Bertolini: Detection of epidermal growth factor (EGF)-binding sites on HeLa and corneal epithelial cells 151

Letter to the Editor

R. A. Flickinger and S. R. Trost: Cytotoxicity of antibody-phospholipase C conjugates on cultured Friend leukemia cells 159

Erratum 161

Recent Journal Contents 163

Number 3, *March* 1976

Perspectives in Cancer Research

F. Martin, M. S. Martin and C. Bourgeaux: Fetal antigens in human digestive tumors 165

General Papers

H. H. Cotino and A. Zwaveling: Treatment of experimental liver tumors by continuous intra-arterial chemotherapy 177

Per Dombernowsky and Nis I. Nissen: Combination chemotherapy with 4′demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside) VP 16-213 (NSC 141540) in L1210 leukemia 181

H. Horn, I. Erlichman, A. Geier and I. S. Levij: Changes in morphology and hormone dependency following transplantation of rat 9,10-dimethyl-1,2-benzanthracene-induced mammary adenocarcinoma 189

R. L. Nelson, P. J. Creaven, M. H. Cohen and B. E. Fossieck, Jr.: Phase I clinical trial of a 3-day divided dose schedule of ifosfamide (NSC 109724) 195

G. Cohnen, K. Fischer, H. Ludwig and G. Brittinger: Surface morphology of normal and chronic lymphocytic leukaemia lymphocytes 199

J. W. Proctor, B. G. Auclair and M. G. Lewis: The effect of BCG on B_{16} mouse melanoma: a comparison of routes of administration on tumour growth at different anatomical sites 203

B. Vinet and W. G. Verly: Purification of a protein inhibitor of DNA synthesis in cells of hepatic origin 211

J. R. W. Masters: The effect of carcinogens on mitotic frequency in mouse lung primordia *in vitro* 219

Preston H. Grantham, Nguyen-Ba-Giao, Letitia C. Mohan, Timothy Benjamin, Peter P. Roller and Elizabeth K. Weisburger: The metabolism of 6-aminochrysene in the rat 227

Letter to the Editor

P. J. Gray and D. R. Phillips: The interaction of daunomycin with normal and leukaemic leukocyte DNA 237

E.O.R.T.C. News

H. J. Tagnon: Definition and function of E.O.R.T.C. 239
M. Staquet: The practice of cooperative clinical trials 241
Stephen L. George: The data center of the European Organization for Research on Treatment of Cancer 245
Announcement

Number 4, *April* 1976

General Papers

David M. Taylor, Kenneth D. Tew and Julie D. Jones: Effects of *cis*-dichlorodiammine platinum (II) on DNA synthesis in kidney and other tissues of normal and tumour-bearing rats 249
M. Bertschmann, K. J. Clemetson and E. F. Lüscher: Preparation and properties of antisera directed against antigens of the P-815 mastocytoma cell not shared by its syngeneic host, the DBA/2 mouse 255
K. J. Clemetson, A. Gerber, M. Bertschmann and E. F. Lüscher: Solubilization of histocompatibility and tumour-associated antigens of the P-815 murine mastocytoma cell 263
N. Deshpande, Pamela Carson, L. Di Martino and A. Tarquini: Biogenesis of steroid hormones by human mammary gland *in vivo* and *in vitro* 271
P. A. M. Walden, C. R. Pentycross, R. Leyton, P. Browne, J. Dent and P. Vernon: Leucocyte migration in response to P.P.D. in patients with trophoblastic tumours 277
R. D. Barnes: Tumour immunity in tetraparental mouse chimaeras—a review 283
Gunhild Lange Wantzin, Hans Karle, Preben Philip and Sven-Aage Killmann: The effect of daunomycin on cell proliferation and protein synthesis of human leukemic blast cells 291
John R. Sabine: Metabolic controls in precancerous liver—VII. Time course of loss of dietary feedback control of cholesterol synthesis during carcinogen treatment 299
D. Y. Wang, P. R. Goodwin, R. D. Bulbrook and J. L. Hayward: Plasma FSH and LH in post-menopausal women with breast cancer 305
A. Sellakumar, F. Stenback and J. Rowland: Effects of different dusts on respiratory carcinogenesis in hamsters induced by benzo(a)pyrene and diethylnitrosamine 313
Frida G. Van Pelt, Ada Houweling, A. M. Aarssen and P. Bentvelzen: Lack of interference in antigen production *in vitro* between B-type and C-type oncornaviruses as determined by double stain immunofluorescence 321
Recent Journal Contents 325

Number 5, *May* 1976

Perspectives in Cancer Research

J. M. Prevost: Oncogenic viruses: implications for human diseases (part 1) 327
J. Gybels, H. Adriaensen and P. Cosyns: Treatment of pain in patients with advanced cancer 341

General Papers

Costantino Mangioni, Giorgio Bolis, Nicola Natale and Luciano Morasca: Continuous low-dose cyclophosphamide (NSC-26271) therapy in advanced ovarian cancer 353
Ronald W. Pero, Tomas Bryngelsson, Adam Deutsch and Ake Nordén: Changes in the deoxyadenylate regions in 5 cases of chronic lymphocytic leukemic DNA 357
Sara Thompson and Thomas J. Slaga: The effects of dexamethasone on mouse skin initiation and aryl hydrocarbon hydroxylase 363
A. Mantovani, A. Vecchi, A. Tagliabue and F. Spreafico: The effects of adriamycin and daunomycin on antitumoral immune effector mechanisms in an allogeneic system 371
A. Mantovani, A. Tagliabue, A. Vecchi and F. Spreafico: Effects of adriamycin and daunomycin on spleen cell populations in normal and tumor allografted mice 381
Luise Pasternak, H. L. Jenssen, H. Köhler and G. Pasternak: Cross-reactions among mouse tumors of different etiology as detected by macrophage electrophoretic mobility (MEM) test 389
Stevan Adler, Katsutaro Shimaoka, Tin Han and Yoshiaki Tsukada: Multiple agent chemotherapy for advanced histiocytic lymphoma 395
B. S. Thomas, Pamela Kirby, Elizabeth K. Symes and D. Y. Wang: Plasma dehydroepiandrosterone concentration in normal women and in patients with benign and malignant breast disease 405
Recent Journal Contents (1976) 411

Number 6, *June* 1976

Perspectives in Cancer Research

Jan G. Waldenstrom: Specific activities of immunoglobulins produced in monoclonal gammapathy—maladies of derepression 413

General Papers

V. C. Jordan: Effect of tamoxifen (ICI 46,474) oninitiation and growth of DMBA-induced rat mammary carcinomata 419
James I. Durodola: Burkitt's lymphoma in Ibadan: response to various doses of cyclophosphamide and long-term survivors 425
M. Mammerickx, D. Portetelle, R. Kettmann, J. Ghysdael, A. Burny and D. Dekegel: Diagnostic tests of bovine leukemia. Comparison between an hematological test and the serological diagnosis 433
Ch. Aubert, C. Lagrange, H. Rorsman and E. Rosengren: Catechols in primary and metastatic human malignant melanoma cells in monolayer culture 441
G. Sand, A. Jortay, R. Pochet and J. E. Dumont: Adenylate cyclase and protein phosphokinase activities in human thyroid. Comparison of normal glands, hyperfunctional nodules and carcinomas 447

A. Yerushalmi: Influence on metastatic spread of whole-body or local tumor hyperthermia 455
Kent Lundholm, Ann-Christin Bylund, Jan Holm and Tore Scherstén: Skeletal muscle metabolism in patients with malignant tumor 465
A. Coudert, J. F. Dore, R. Huchet, C. Guibout and C. Santana: Increase in cell surface antigen expression by leukemic cells following *in vitro* treatment with papain 475
Kenneth R. Kase and George M. Hahn: Comparison of some response to hyperthermia by normal human diploid cells and neoplastic cells from the same origin 481
Souei Sekiya, Naohide Takayama and Hiroyoshi Takamizawa: Effects of progesterone on rat uterine adenocarcinoma cells *in vitro*: an electron microscopic study 493
Recent Journal Contents (1976) 497

Number 7, *July* 1976

Perspectives in Cancer Research

J. M. Prevost: Oncogenic viruses: implications for human diseases (Part II) 499
Willy F. Piessens and William C. Moloney: Immunological aspects of acute leukemia in man 511

General Papers

A. Tulp and J. J. M. N. Welagen: Fractionation of ascites tumour cells at 1*g*: separation of cells in specific stages of the life cycle 519
S. Karasaki, A. Simard and G. de Lamirande: Ultrastructural analysis of malignant conversion *in vitro* of epithelial cells derived from normal adult rat liver 527
K. Atkinson, P. R. M. Thomas, M. J. Peckham and T. J. McElwain: Radiosensitivity of the acute leukaemic infiltrate 535
Bridget T. Hill, L. A. Price and J. H. Goldie: The value of adriamycin in overcoming resistance to methotrexate in tissue culture 541
M. Y. Gordon and I. D. C. Douglas: Changes in proliferation rate of human bone marrow colony-forming cells measured by a cytosine arabinoside-diffusion chamber method 551
Bruno Azzarone and Delfina Pedulla: Spontaneous transformation of human fibroblast cultures derived from bronchial carcinomata 557
R. D. Rubens, R. K. Knight and J. L. Hayward: Norethisterone acetate in the treatment of advanced breast cancer 563
J. F. Smyth, A. B. Robins and C. L. Leese: The metabolism of cytosine arabinoside as a predictive test for clinical response to the drug in acute myeloid leukaemia 567
M. J. Fulker, S. J. Adamthwaite and C. K. Anderson: Stereological measurements of bladder tumour morphology 575

Letter to the Editor

N. P. Bishun, D. C. Williams and J. Reed: Cellular "modulation" in breast tumour cultures 581
Announcement 583
Recent Journal Contents (1976) 584

Number 8, *August* 1976

Perspectives in Cancer Research

Alistair J. Cochran: The biology and treatment of malignant melanoma 585

General Papers

F. Fey, W. Arnold and A. Graffi: Demonstration of the stimulation of the reticulohistiocytic system (RHS) of mice by treatment with BCG by means of biometric and histochemical techniques 595
G. Schwarze and A. Pappas: Tumor-enhancing and tumor-inhibiting *in vivo* effects of phytohaemagglutinin. Study of proliferation of transplantable mouse melanoma 599
L. A. Smets and Ch. Homburg: Immunogenicity and concanavalin A agglutinability in transplantable mouse lymphosarcoma and human leukemia 605
Maria Grazia Bernengo and Luigi Pegoraro: Deoxycytidylate deaminase activity and its template stability in human acute lymphoblastic and myeloblastic leukaemic cells 611
Michael L. Greenberg and Samuel Waxman: Sequential use of methotrexate, folinic acid, and cytosine arabinoside in the treatment of acute leukemia 617
J. P. Gmür, G. Deluigi, Chr. Sauter and P. W. Straub: Correlation of skin test reactivity and *in vitro* lymphocyte stimulation by soluble antigens in normal subjects and leukemic patients 625
Kees Nooter, Kor Van Den Berg, Jan de Vries and Peter Bentvelzen: Transfection of mouse bone marrow cells with Rauscher virus proviral DNA 633
Brent M. Vose and Michael Moore: Reactivity of peripheral blood leucocytes against human fetal cells 639
Egon Durban and Elisa M. Durban: Size distribution patterns of nuclei from normal rat liver and from two hepatomas 651

Letter to the Editor

Branka Herzog, J. C. Hendrick and P. Franchimont: Heterogeneity of carcinoembryonic antigen (CEA) in human serum 657
Recent Journal Contents (1976) 659

Number 9, *September* 1976

Perspectives in Cancer Research

Gianni Bonadonna, Angelo Lattuada and Alberto Banfi: Recent trends in the treatment of non-Hodgkin's lymphomas 661
A. J. Van Der Kogel, D. W. Van Bekkum and G. W. Barendsen: Tolerance of CNS to total body irradiation combined with chemotherapy applied for the treatment of leukemia 675

General Papers

William R. Miller: *In vitro* effects of prolactin upon testosterone metabolism by rat mammary adenocarcinomata 679
D. J. Th. Wagener, P. J. J. Van Munster and C. Haanen: The immunoglobulins in Hodgkin's disease 683
D. E. Schumm, D. F. Billmire and H. P. Morris: Inhibition of mitogen-induced lymphocyte blastogenesis by serum from tumor-bearing animals: changes with tumor growth 689
Orjan Wrange, Bo Nordenskjöld, Claes Silfverswärd, Per Ola Granberg and Jan-Åke Gustafsson: Isoelectric focusing of estradiol receptor protein from human mammary carcinoma—a comparison to sucrose gradient analysis 695
P. Juret, J. E. Couette, A. M. Mandard, A. Carré, T. Delozier, D. Brune and J. C. Vernhes: Age at menarche as a prognostic factor in human breast cancer 701
R. D. Barr, P. Sarin, G. Sarna and S. Perry: The relationship of DNA polymerase activity to cell cycle stage 705
R. I. Nicholson, M. P. Golder, P. Davies and K. Griffiths: Effects of oestradiol-17β and tamoxifen on total and accessible cytoplasmic oestradiol-17β receptors in DMBA-induced rat mammary tumours 711
Meriel P. Golder, M. Elizabeth A. Phillips, D. R. Fahmy, P. E. Preece, V. Jones, J. M. Henk and K. Griffiths: Plasma hormones in patients with advanced breast cancer treated with tamoxifen 719
R. D. Bulbrook, Margaret C. Swain, D. Y. Wang, J. L. Hayward, S. Kumaoka, O. Takatani, O. Abe and J. Utsunomiya: Breast cancer in Britain and Japan: plasma oestradiol-17β, oestrone and progesterone, and their urinary metabolites in normal British and Japanese women 725
Caroline B. Wigley and A. W. Carbonell: The target cell in the chemical induction of carcinomas in mouse submandibular gland 737

Letters to the Editor

C. B. Wigley, M. H. Thompson and P. Brookes: The nature of benzo(*a*)pyrene binding to DNA in an epithelial cell culture system 743
G. H. Zielmaker and C. M. P. M. Verhamme: Cell proliferation in the mammary glands of the mouse during prolonged unilateral lactation 747
G. J. O'Neill: Control of an EL4 lymphoma in nude mice by passively administered antibody 749
Recent Journal Contents (1976) 753

Number 10, *October* 1976

Perspectives in Cancer Research

P. Hilgard and R. D. Thornes: Anticoagulants in the treatment of cancer 755

General Papers

Sven-Aage Killmann, P. Philip and M. Baccarani: Rapid blastic transformation and early ectopic proliferation of hyperdiploid myeloblasts in chronic myeloid leukemia 763
S. Kumaoka, O. Takatani, O. Abe, J. Utsunomiya, D. Y. Wang, R. D. Bulbrook, J. L. Hayward and F. C. Greenwood: Plasma prolactin, thyroid-stimulating hormone, follicle-stimulating hormone and luteinizing hormone in normal British and Japanese women 767
T. Bates, R. D. Rubens, R. D. Bulbrook, P. R. Goodwin, D. Y. Wang, R. K. Knight and J. L. Hayward: Comparison of pituitary function and clinical response after transphenoidal and transfrontal hypophysectomy for advanced breast cancer 775
Mogens Kjaer: Effect of leucocyte washings on cellular immunity to human renal carcinoma 783
J. Wells, R. J. Berry and A. H. Laing: Reproductive survival *in vitro* of recently explanted human tumour cells following exposure to vinblastine. A suggestion for improved clinical dose schedules 793
A. Cattan, C. Pourny, Y. Carpentier, E. Cattan and M. Barouh: Antimetastasis effect of two derivatives of epipodophyllotoxin in mice 797
Aharon Yerushalmi: Treatment of a solid tumor by local simultaneous hyperthermia and ionizing radiation: dependence on temperature and dose 807
B. P. Gothoskar, H. D'Silva, H. Gharpure and S. H. Advani: Leukocyte migration studies in chronic myeloid leukemia (CML). Specificity of reactions 815
E. Dolfini, B. Azzarone, D. Pedulla, E. Ottaviano, G. de Gaetano, M. B. Donati and L. Morasca: Characterization of human fibroblasts from cancer patients: loss of fibrin clot retractile activity after *in vitro* spontaneous transformation 823
Helmut Schlag and Christine Lücke-Huhle: Cytokinetic studies on the effect of hyperthermia on Chinese hamster lung cells 827
George P. Tryfiates and Frank L. Saus: Hepatic and tumor proteins reactive with tyrosine transaminase—specific rabbit antiserum 833
Lambert Skoog, Bo Nordenskjöld, Solveig Humla and Torsten Hägerström: Effects of methotrexate on deoxyribonucleotide pools and nucleic acid synthesis in human osteosarcoma cells 839
Recent Journal Contents (1976) 847
Papers to be Published 850

Number 11, *November* 1976

Perspectives in Cancer Research

Lise Thiry: Herpes simplex virus and carcinoma of the cervix 851

General Papers

Allan Fenselau and Cedric Long: *In vitro* inhibition of cell growth by aldose phosphates 859
Hiroshi Maeda, Naoki Yamamoto and Akira Yamashita: Fate and distribution of [^{14}C] succinyl neocarzinostatin in rats 865
S. Levy, D. Papadopoulo, S. Nocentini, L. Chamaillard, O. Beesau, M. Hubert-Habart and P. Markovits: Transformation of hamster embryo cells by 7-methyl benz (a)anthracene *in vitro*: tumorigenicity, chromosome pattern and spheroid formation 871
Rene Verloes, Louis Kanarek and Ghanem Atassi: Antitumour immunoprotection by an immunobacterial lectin approach 877

Mogens Kjaer: Prognostic value of tumour-directed, cell-mediated hypersensitivity detected by means of the leucocyte migration technique in patients with renal carcinoma 889
A. C. van Prooijen, G. Vierwinden, J. van Egmond, J. M. C. Wessels and C. Haanen: A sensitive bio-assay for pharmacokinetic studies of cytosine arabinoside in man 899
L. Gonçalves Sobrinho, L. Faro, E. F. Henriques, L. S. Botelho and M. F. Fontes De Sousa: The urinary excretion of pregnanetriol and pregnenetriol in patients with breast cancer. A postulation on the meaning of Bulbrook's discriminant 907
M. G. Donelli, A. Martini, T. Colombo, A. Bossi and S. Garattini: Heart levels of adriamycin in normal and tumor-bearing mice 913
H. Stähelin: Delayed toxicity of epipodophyllotoxin derivatives (VM 26 and VP 16-213), due to a local effect 925

Letter to the Editor

Joan Thilly Harmon and Russell Hilf: Effect of insulin on glucose transport in DMBA-induced mammary tumors 933
Recent Journal Contents (1976) 935

Number 12, *December* 1976

Perspectives in Cancer Research

Philip H. Smith, Melvyn R. C. Robinson and Edward H. Cooper: Carcinoma of the prostate: a focal point for divergent disciplines 937

General Papers

E. Kristensen, E. Langvad and R. Reimann: Humoral immunity in malignant skin melanoma. Isolation of melanoma IgG from melanoma metastases 945
D. Y. Wang, J. L. Hayward, R. D. Bulbrook, S. Kumoaka, O. Takatani, O. Abe and J. Utsunomiya: Plasma dehydroepiandrosterone and androsterone sulphates, androstenedione and urinary androgen metabolites in normal British and Japanese Women 951
Jan Korb and Josef Říman: Presence of intramitochondrial bodies in avian leukemic myeloblasts 959
T. B. Constable: The effect of irradiation on the oxygen removal rate of the SSBIa rat fibrosarcoma 963
Ewa Wieraszko and Jan Knapowski: Effects of vinblastine on bioelectric properties of the frog skin epithelium *In vitro* 973
Syamal K. Datta and Robert S. Schwartz: Susceptibility to lymphomas and expression of C-type RNA viruses during the graft versus host reaction 977
H. M. Tillian, E. Schauenstein, A. Ertl and H. Esterbauer: Therapeutic effects of cysteine adducts of α,β-unsaturated aldehydes on Ehrlich ascites tumor of mice 989
M. J. Waring: DNA-binding characteristics of acridinylmethanesulphonanilide drugs: comparison with antitumour properties 995
Mario Cosálvez, R. Garcia Cañero and M. Blanco: A screening for selective anticancer agents among plant respiratory inhibitors 1003
R. B. Raikow, L. L. Schenken and S. Lesher: Urinary excretion of 5-hydroxyindoles as an indicator of *in vivo* growth of P815 × 2 mastocytoma 1011

Letters to the Editor

Hiroshi Nagasawa, Reiko Yanai and Hisashi Taniguchi: Reduction by pituitary grafts of mammary tumor age. Its variability in a high mammary tumor strain of mice: effects on mammary DNA synthesis 1017
N. Allegretti and M. Marušić: Tumour development after 3-methylcholanthrene in irradiated, thymectomized mice reconstituted with syngeneic bone marrow 1021
C. S. B. Calasko and D. W. Haynes: Survival of VX2 carcinoma cells *in vitro* 1025
Recent Journal Contents (1976) 1027

AUTHOR INDEX

Aarssen, A. M. 321
Abe, O. 725, 767, 951
Adamthwaite, S. J. 575
Advani, S. H. 815
Alftan, O. 87
Allegretti, N. 1021
Amini, M. 151
Anderson, C. K. 575
Angelberger, P. 19
Arnold, W. 595
Atassi, G. 877
Atkinson, K. 535
Aubert, Ch. 441
Auclair, B. G. 203
Azzarone, B. 557, 823

Baccarani, M. 763
Ba-Giao, N. 227
Barendsen, G. W. 675
Barnes, R. D. 283
Barouh, M. 797
Barr, R. D. 705
Barski, G. 33
Bates, T. 775
Beesau, O. 871
Belehradek Jr. J. 33
Benjamin, T. 227
Bentvelzen, P. 321, 633
Bernengo, M. G. 611
Berry, R. J. 793
Bertolini, L. 151
Bertschmann, M. 255, 263
Billmire, D. F. 689
Bishun, N. P. 581
Blanco, M. 1003
Bonadonna, G. 661
Bosman, C. 151
Bossi, A. 913
Botelho, L. S. 907
Bourgeaux, C. 165
Brittinger, G. 199
Brookes, P. 743
Browne, P. 277
Brune, D. 701
Bulbrook, R. D. 305, 725, 767, 775, 951
Burny, A. 433
Bylund, A-C. 465

Carbonell, A. W. 737
Carpentier, Y. 797
Carré, A. 701
Carson, P. 271
Carsten, A. L. 131
Cattan, A. 797
Chamaillard, L. 871
Chanana, A. D. 131
Chikkapa, G. 131
Cochran, A. J. 585
Cohen, M. H. 195
Cohnen, G. 199
Colombo, T. 913
Constable, T. B. 963
Cooper, E. H. 937
Cotino, H. H. 177
Coudert, A. 475
Couette, J. E. 701
Clemetson, K. J. 255, 263
Creaven, P. J. 195
Cronkite, E. P. 131

Datta, S. K. 977
Davies, P. 711
De Gaetano, G. 823
Dekegel, D. 433
De Lamirande, G. 527
Delozier, T. 701
Deluigi, G. 625
Den Engelse, L. 101
Dent, J. 277
Deshpande, N. 271
Deutsch, E. 19
De Vries, J. 633
Di Martino, L. 271
Dolfini, E. 823
Dombernowsky, P. 181
Donati, M. B. 823
Donelli, M. G. 913
Donner, M. 125
Dore, J. F. 475
Douglass, I. D. C. 551
D'Silva, H. 815
Dumont, J. E. 447
Durban, E. 651
Durban, E. M. 651
Durodola, J. I. 425

Emmelot, P. 25, 101
EORTC Brain Tumor Group 41
Epstein, J. 47
Erlichman, I. 189
Ertl, A. 989
Esterbauer, H. 989

Fahmy, D. R. 719
Faro, L. 907
Fenselau, A. 859
Fey, F. 595
Fischer, K. 199
Flickinger, R. A. 159
Fogar-Ottaviano, E. G. 107
Fontes De Sousa, M. F. 907
Fossieck Jr., B. E. 195
Franchmont, P. 657
Fulker, M. J. 575

Galasko, C. S. B. 1025
Garattini, S. 107, 913
Garcia Cañero, R. 1003
Gascoigne, E. W. 53
Geier, A. 189
George, S. L. 245
Gerber, A. 263
Gharpure, H. 815
Ghose, T. 95
Ghysdael, J. 433
Gmür, J. P. 625
Golder, M. P. 711, 719
Goldie, J. H. 541
Gonçalves, Sobrinho L. 907
Goodwin, P. R. 305, 775
Gordon, M. Y. 551
Gosalves, M. 1003
Gothoskar, B. P. 815
Graffi, A. 595
Granberg, P. O. 695
Grantham, P. H. 227
Gray, P. J. 237
Greenberg, M. L. 617
Greenwood, F. C. 767
Griffiths, K. 711, 719
Guclu, A. 95
Guibout, C. 475
Gustafsson, J-A. 695

Haanen, C. 683, 899
Hägerström, T. 839
Hahn, G. M. 481
Harmon, J. T. 933
Harrap, K. 53
Hasegawa, Y. 143
Haynes, D. W. 1025
Hayward, J. L. 305, 563, 725, 767, 775, 951
Heikkinen, A. 87
Hendrick, J. C. 657
Henk, J. M. 719
Henriques, E. F. 907
Herzog, B. 657
Hilf, R. 933
Hilgard, P. 755
Hill, B. T. 541
Holm, J. 465
Homburg, Ch. 605
Horn, H. 189
Houweling, A. 321
Hruby, R. 19
Hubert-Habart, M. 871
Humla, S. 839

Jami, J. 13
Jones, J. D. 249
Jones, V. 719
Jordan, V. C. 419
Jortay, A. 447
Juret, P. 701

Kanarek, L. 877
Karasaki, S. 527
Karle, H. 291
Kase, K. R. 481
Keizer, H. J. 79
Kettman, R. 433
Killmann, S.-A. 291, 763
Kjaer, M. 783, 889
Knapowski, J. 973
Knight, R. K. 563, 775
Korb, J. 959
Kornblith, P. L. 47
Kristensen, E. 945
Kumaoka, S. 725, 767, 951

Lagrange, C. 441
Laing, A. H. 793
Langvad, E. 945
Lattuada, A. 661
Leese, C. L. 567
Lesher, S. 1011
Levij, I. S. 189
Levy, S. 871
Lewis, M. G. 203
Leyton, R. 277
Little, J. B. 47
Long, C. 859
Lücke-Huhle, C. 827
Ludwig, H. 199
Lundholm, K. 465
Luscher, E. F. 255, 263

Maeda, H. 865
Mammen, M. 95
Mammerickx, M. 433
Mandard, A. M. 701
Mantovani, A. 113
Markovits, P. 871
Martin, F. 165
Martin, M. S. 165
Martini, A. 913
Marušić, M. 1021
Masoka, T. 143
Masters, J. R. W. 219
McElwain, T. J. 535
Miller, W. R. 679
Misdorp, W. 101
Mohan, L. C. 227
Moloney, W. C. 511
Moore, M. 639
Morasca, L. 107, 823
Morris, H. P. 9, 689
Mulder, J. H. 79

Nagasawa, H. 1017
Nakamura, H. 143
Nelson, R. L. 195
Nicholson, R. I. 711
Nissen, N. I. 181
Nocentini, S. 871
Nooter, K. 633
Nordenskjöld, B. 695, 839

Ohl, S. 131
O'Neill, G. J. 749
Ottaviano, E. 823

Papadopoulo, D. 871
Pappas, A. 599
Peckham, M. J. 535
Pediconi, M. 151
Pedulla, D. 557, 823
Pegoraro, L. 611
Penttinen, K. 87
Pentycross, C. R. 277
Perry, S. 705
Philip, P. 291, 763
Phillips, D. R. 237
Phillips, M. E. A. 719
Piessens, W. F. 511
Pils, P. 19
Pochet, R. 447
Portetelle, D. 433
Pourny, C. 797
Preece, P. E. 719
Prevost, J. M. 499
Price, L. A. 541
Proctor, J. W. 203
Pyrhönen, S. 87

Raikow, R. B. 1011
Reed, J. 581
Reimann, R. 945
Reuber, M. D. 137
Revoltella, R. 151
Riman, J. 959
Ritz, E. 13
Robins, A. B. 567
Robinson, M. R. G. 937
Roller, P. P. 227
Rorsman, H. 441
Rosengren, E. 441
Rowland, J. 313
Rubens, R. D. 563, 775
Rubio, N. 13

Sabine, J. R. 299
Saksela, E. 87
Sand, G. 447
Santana, C. 475
Sarin, P. 705
Sarna, G. 705
Satya Murthy, M. 33
Saus, F. L. 833
Sauter, Chr. 625
Schauenstein, E. 989
Schenken, L. L. 1011
Scherer, E. 25
Scherstén, T. 465
Schlag, H. 827
Schumm, D. E. 689
Schwartz, R. S. 977
Schwarze, G. 599
Sellakumar, A. 313
Sekiya, S. 493
Senda, N. 143
Shibata, H. 143
Silfverswärd, C. 695
Simard, A. 527
Skoog, L. 839
Smets, L. A. 605
Smith, P. H. 937
Smyth, J. F. 567

AUTHOR INDEX

Spreafico, F. 113
Stähelin, H. 925
Staquet, M. 241
Stenback, F. 313
Straub, P. W. 625
Swain, M. C. 725

Tagliabue, A. 113
Tagnon, H. J. 236
Tai, J. 95
Takamizawa, H. 493
Takatani, O. 725, 767, 951
Takayama, N. 493
Takubo, T. 143
Taniguchi, H. 1017
Tarquini, A. 271
Tatsumi, N. 143
Tattersall, M. H. N. 1
Taylor, C. R. 61
Taylor, D. M. 249
Tew, K. D. 249
Thiry, L. 851
Thomas, P. R. M. 535
Thompson, M. H. 743
Thornes, R. D. 755
Tillian, H. M. 989
Timonen, T. 87
Tobias, J. S. 1
Tragl, K. H. 19
Trost, S. R. 159
Tryfiates, G. P. 9, 833
Tulp, A. 519

Ueda, T. 143
Utsunomiya, J. 725, 767, 951

Vallier, D. 125
Van Bekkum, D. W. 675
Van Egmond, J. 899
Van Den Berg, K. 633
Van Der Kogel, A. J. 675
Van Dijk, W. F. M. 25
Van Munster, P. J. J. 683
Van Pelt, F. G. 321
Van Prooijen, A. C. 899
Van Putten, L. M. 79
Vecchi, A. 113
Verhamme, C. M. P. M. 747
Verloes, R. 877
Verly, W. G. 211
Vernhes, J. C. 701
Vernon, P. 277
Vierwinden, G. 899
Vinet, B. 211
Vose, B. M. 639

Wagener, D. J. Th. 683
Wagner, O. 87
Walden, P. A. M. 277
Waldenstrom, J. G. 413
Wang, D. Y. 305, 725, 767, 775, 951
Wantzin, G. L. 291
Waring, M. J. 995
Waxman, S. 617
Weichselbaum, R. R. 47
Weisburger, E. K. 227
Welagen, J. J. M. N. 519
Wells, J. 793
Wessels, J. M. C. 899
Wieraszko, E. 973
Wigley, C. B. 737, 743
Williams, C. C. 581
Wrange, O. 695

Yamamoto, N. 865
Yamashita, A. 865
Yanai, R. 1017
Yerushalmi, A. 455, 807
Yoshitake, J. 143

Zielmaker, G. H. 747
Zwaveling, A. 177