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Experimental Techniques for Testing the Sensitivity of Bladder Tumours to Antineoplastic Drugs

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Summary. A number of laboratory tests can be employed to examine the sensitivity of human bladder tumour cells to various chemotherapeutic agents. - Their principles and methods, and some preliminary results, are described with special reference to certain in vitro and in vivo cytotoxicity tests and to heterotransplantation in the hamster. Satisfactory agreement has sometimes been observed between experimental results and clinical responses, but our experience is still very limited. - The employment of several such tests would probably lead to a greater degree of reliability in the laboratory assessment of the sensitivity of bladder tumours to cytotoxic drugs.

Key words: Bladder papilloma, bladder carcinoma, tomour chemotherapy, screening of anticancer compounds

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It is apparent from reports about the local or systemic chemotherapy of bladder tumours that only occasional successes are achieved. Tumours that appear identical, from either a cystoscopic or histopathological standpoint, may exhibit widely differing sensitivities to the various chemotherapeutic agents. It would be of the greatest value, therefore, if it were possible to develop a method for testing the sensitivity of every vesical tumour to the presently available drugs.

Few investigations have been made into in vitro sensitivity tests for bladder tumours (1). With respect to in vivo studies, after initial attempts to transplant bladder carcinomas into the anterior chamber of the guinea pig eye, or into the mouse brain (2, 3), we (4, 5) and others (6, 11) showed that heterotransplantation of bladder tumours was feasible in the cheek pouch of the golden hamster.

The present report is a brief survey of investigations into the feasibility and practical value of applying these and other experimental methods to determination of the susceptibility of bladder tumours to chemotherapeutic agents.

Methods and Results

A) In Vivo Experiments

1. Cytotoxicity in vivo. A very simple approach to this problem is afforded by the suggestion of Lunglmayr et al. (12), that the effect of a topical antineoplastic treatment can be evaluated by the examination of exfoliated cells. In cases of papillary bladder tumours treated by intravesical instillation of thiotepa, smears should be obtained before and after treatment. After staining by the usual cytological methods, microscopical examination will reveal cellular necrosis and degenerative changes as an indication of a successful response. Cytological examination before and after therapy has been employed in all patients treated by the intravesical infusion of the following drugs: thiotepa, peptidic complex of 1- sarcolysin ("Peptichemio"), adriamycin, daunomycin, demecolcine and the glucoside of 4' demethylepipodophyllotoxin -B-D- thenylidene (VM 26). Exfoliated cells were counted and graded from + to ++++, according to the extent of the degenerative cellular changes. Unfortunately, no consistent data were obtained probably because severe degenerative changes were usually present before treatment. The cytological method proved of value

in few cases (Figs. 1 and 2), but no consistent relationship was observed between the occurence of a clinical response and increased degenerative changes in exfoliated cells.

2. Intranuclear visualization of antineoplastic drugs in the tumour cells. Demonstration of the penetration of antineoplastic drugs into the nuclei of neoplastic cells can be considered as an index of their potential efficacy, because no drug can exert an action unless it can cross cellular and nuclear membranes to reach its target sites. Visualization of an anticancer agent following in vivo contact with a bladder tumour may be done by employing radioactive compounds and autohistoradiography, or by the use of fluorescent substances.

Some antibiotics with anticancer activity, such as daunomycin and adriamycin, are naturally fluorescent. Adriamycin solutions, when excited at 465 mμ show two characteristic peaks of emission at 540 and 584 mμ. A non fluorescent drug, Peptichemio, can be chemically bound to a fluorescent compound, such as tetracycline. In either case, the presence of these drugs in the nuclei can be investigated by examing unstained smears of exfoliated neoplastic cells by fluorescence microscopy. Fluorescence is apparent in the nuclei of bladder cancer cells following either systemic

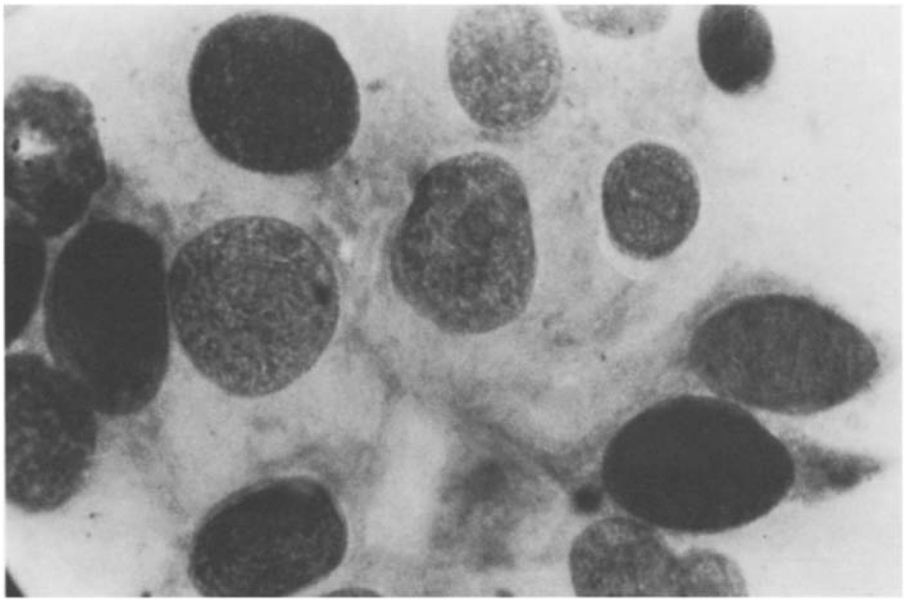

Fig. 1. Exfoliative cytology smear from a transitional cell carcinoma, grade III, before treatment

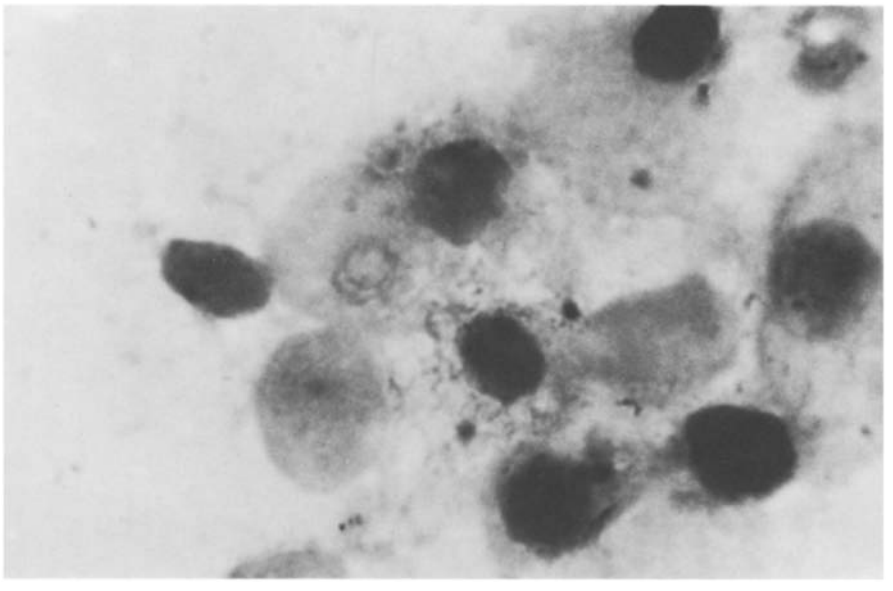

Fig. 2. Smear from the same patient after prolonged topical treatment with adriamycin. Severe degenerative changes are evident in the cells

or intravesical administration of Peptichemio linked to tetracycline. Inconstant results were obtained following intravesical treatment with adriamycin. A well pronounced orange-rosy nuclear fluorescence was seen in only two out of 9 cases. In both instances, the patients had anaplastic, deeply infiltrating bladder carcinomas. One patient received a continuous daily bladder perfusion of adriamycin, at a dose of 40 mg in 2 000 ml of sterile water, for 5 consecutive days, whilst the other received a single instillation of 10 mg of adriamycin for 40 minutes (Fig. 3). In 3 other cases there was only a faint green-yellowish nuclear fluorescence, and in the remaining two cases fluorescence was absent. There was no definite relationship between the appearance of nuclear fluorescence and the histology of the tumour, the amount of the drug administered, or the duration of contact of adriamycin solution with the neoplasm. It was also impossible to demonstrate any correlation between nuclear fluorescence and clinical response.

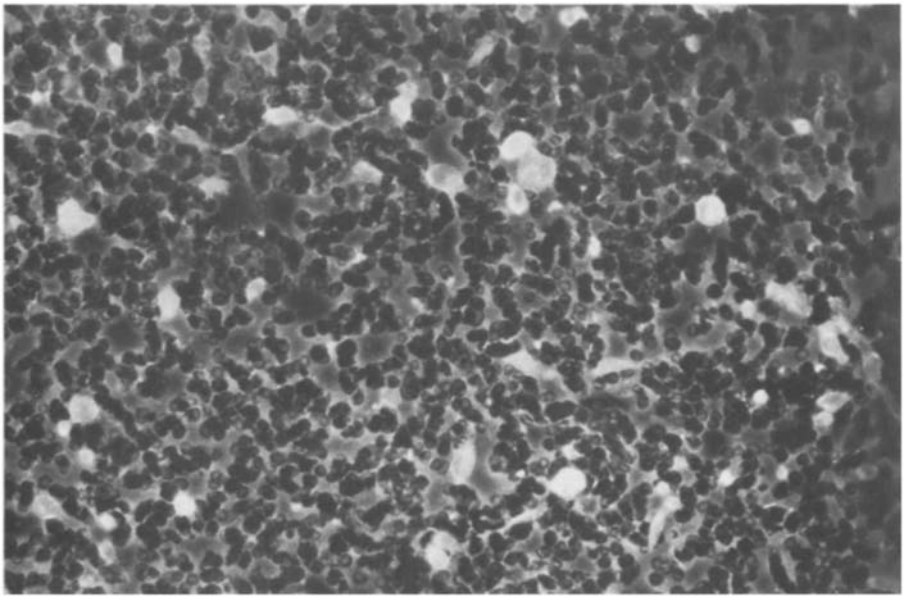

Fig. 3. Fluorescent nuclei of exfoliated cells from a transitional cell carcinoma of the bladder treated topically with adriamycin

3. Heterotransplantation in the laboratory animal. A sterile suspension of tumour cells (in hamster serum or Eagle's medium) is injected between the mucosa and the underlying stromal layer in the everted cheeks of young hamsters. Regular examination of the implant in anaesthesized animals will demonstrate the growth or rejection of the graft. If "take" occurs, the tumour becomes vascularized and starts to grow, usually 1-2 weeks after implantation. The diameter of the nodule can be measured with calipers and its volume is easy to calculate.

Growth usually follows an exponential pattern, with a doubling time of 2-3 days. If, during this phase, groups of animals are treated with different drugs, growth curves can be obtained and compared with those of untreated controls.

In the present experiments, 7 bladder tumours were transplanted by this technique. Growth was obtained in 3 cases and the other 4 neoplasms were rejected. Serial transplantation from one animal to another was successfully achieved up to 41 times for one tumour. This histological appearance and the karyotype of the growth remained unchanged throughout the different generations of transplants.

The majority of the drugs tested, namely vinblastine, methotrexate, carzinophilin, thiotepa and cyclophosphamide, had no appreciable effect on the growth of the transplanted tumours; mithramycin and vincristine produced a slight effect; and, a transient inhibition of growth was induced by intraperitoneal sarcolysin and intravenous 5 - fluorouracil (5-FU). The latter was ineffective if administered intraperitoneally, whereas actinomycin D displayed the opposite behaviour, whether given either alone or in association with X-rays.

Mitomycin C (MMC) had a very marked effect. The administration of a single dose of 2 mg/kg brought about almost complete regression of a transplanted tumour for more than a month, after which it started to grow again. Complete regression of the tumour was obtained with doses of MMC equal to or greater than 6 mg/kg (Fig. 4). Irradiation with 1 000 r was followed by temporary inhibition of growth, and 1 500 r completely stopped tumour growth and produced total regression. Similar results have been obtained by Kaufman et al. (7, 9, 10, 11) in other experiments.

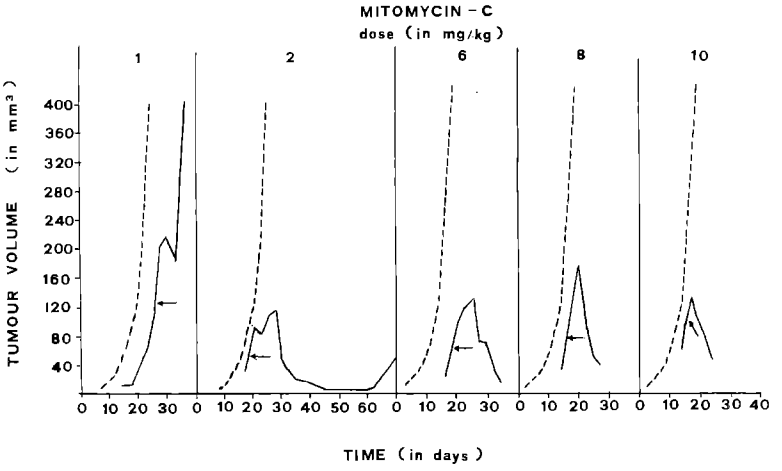

Fig. 4. Effect of mitomycin C (MMC) on growth of a transplanted bladder tumour. Broken line represents average growth curve of 25 control animals. Solid lines represent growth of a single tumour when treated with different doses of MMC. Arrows indicate time at which MMC was administered by intraperitoneal injection

B) In Vitro Experiments

1. Inhibition of protein and DNA synthesis in cell cultures. In additional experiments, the efficacy of various drugs has been assessed by measuring the uptake of radioactive precursors in vitro by cultures of bladder cancer cells, using a modification of Byfield and Stein's technique (13).

Protein synthesis was studied with tritiated leucine, and ^{3}H-thymidine was employed for measurement of the synthesis of deoxyribonucleic acid (DNA). Figures 5 and 6 show marked inhibition of protein synthesis in bladder carcinoma cells in vitro by 5-FU and, to a greater extent, by MMC. DNA synthesis in cultures of cells from another bladder tumour was inhibited by 5-FU, adriamycin and Peptichemio in increasing order of efficacy.

2. Dehydrogenase inhibition in agar plates. The test is based on the toxic effect of chemotherapeutic agents in inhibiting the dehydroenase activity of susceptible neoplastic cells. The experiments were performed according to the technique described by Di Paolo and Dowd (14); a fragment of tumour is minced and a fine cell suspension in serum is prepared. After mixing with non toxic nutrient agar, the mixture is poured into Petri dishes and allowed to solidify. Paper discs containing the drugs to be tested are placed on the surface of the agar plates. The dishes are kept for 12 h at 4° C, and at 37° for 5 h, after which they are covered with methylene blue and incubated anaerobically. When the plates are examined, decolourisation of the dye occurs only in areas where dehydrogenase

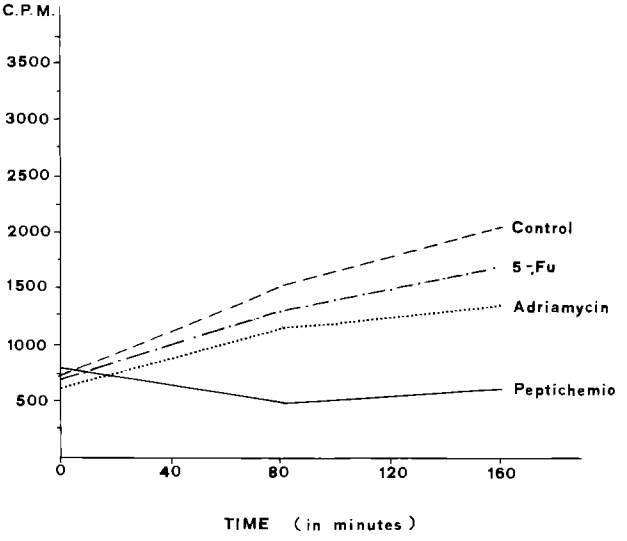

Fig. 5. Effect of 5-Fu, adriamycin and a peptidic complex of sarcolysin (Peptichemio) on incorporation of H^{3}-leucine (protein synthesis) in a cell culture from a transitional cell carcinoma of the bladder

activity of the tumour cells has not been inhibited. The presence of a blue halo around a drug-containing disc indicates that the latter is capable of exerting a cytotoxic effect, and thus inhibiting cellular dehydrogenases.

The diameter of each circular area of non-decolourisation is recorded as a quantitative measure of the effect (Fig. 7). Reliable results were obtained in 8 cases, as shown in Table 1.

Experiments 3 and 4 were carried out using fragments of tumour from the same patient at different times. It should be noted that, after the first transurethral resection (TUR), this patient received chemotherapy selected according to the results of the agar plate test. She was given systemic VM 26 for 2 days, followed by Peptichemio for 3 days. Check cystoscopy 2 weeks after completion of the treatment showed partial regression and an-

Table 1. In vitro sensitivity test for bladder tumours using the agar plate method. Each figure represents the diameter of the dehydrogenase inhibition area in mm

Drug	Experiment N							
	1	2	3	4	5	6	7	8
1 Thiotepa	-	13	14	16	-	11	16	18
2 Peptichemio	27	17	24	26	17	0	0	22
3 5-FU	22	-	0	8	-	30	17	-
4 Methotrexate	0	9	0	-	0	28	29	-
5 Daunomycin	0	24	27	22	22	13	33	19
6 Adriamycin	0	0	0	-	0	0	0	0
7 Mitomycin C	-	0	0	-	-	-	-	0
8 Bleomycin	-	0	-	13.5	21	0	0	0
9 VM-26	-	21	21	24	20	0	24	24
10 Vincristine	-	-	-	-	0	0	0	0

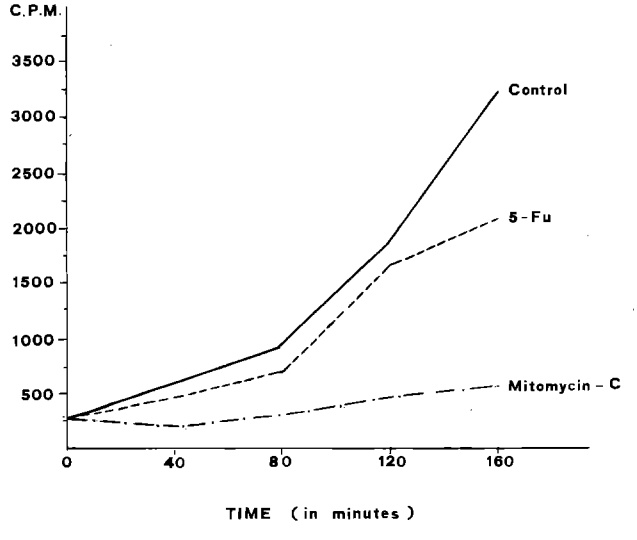

Fig. 6. Effect of 5-FU and mitomycin C on incorporation of H^3-thymidine (DNA synthesis) in cell culture of a bladder carcinoma

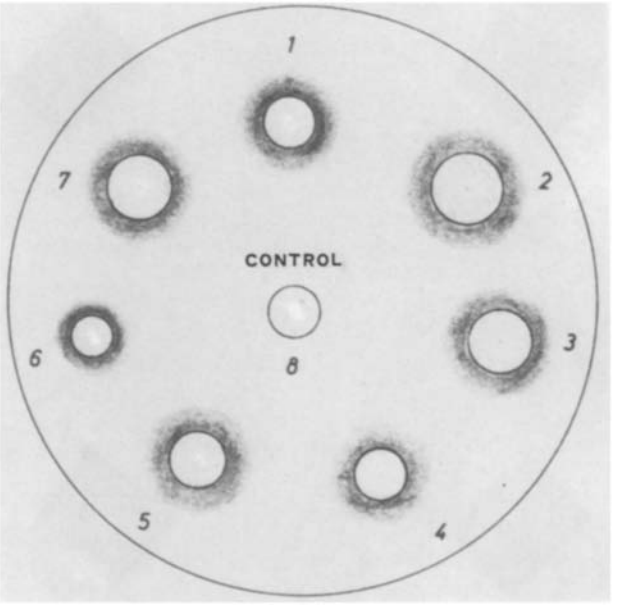

Fig. 7. Model of in vitro test on agar plates. Each paper disc corresponds to a different drug. A drug-free disc is placed in the centre as the control

other TUR resulted in complete removal of the tumour. The TUR fragments were used again in an agar plate test, but no evaluable results were obtained because of the lack of any dye decolourisation. This was interpreted as evidence of cell toxicity following the previous treatment. The patient had a further TUR for a small recurrence 3 months later. A sensitivity test was performed again and she was subsequently treated prophylactically with intravesical daunomycin, without further recurrences.

Other correlations between laboratory and clinical data were possible in some cases: No. 7 - partial regression following topical sequential treatment with VM 26 and Peptichemio; N 5-no clinical response to bleomycin despite in vitro sensitivity and No. 7-no objective response to bleomycin and a subsequent in vitro test was also negative. Clinical results are not yet available from the other cases.

Discussion

The study is still in progress and many data await confirmation, but certain points are worth making now: -

1. Cytological examination of exfoliated cells, before and after topical treatment, presents the advantages of readiness and simplicity. It can reveal signs of cytotoxicity in some cases and can be repeated as often as desired. Unfortunately, it is not reliable as the cells that exfoliate, either spontaneously or after vesical rinsing, are those that are already necrotic or degenerating, irrespective of the treatment. The present experimental findings have confirmed these theoretical drawbacks.

2. Visualization of an anticancer drug in the nucleus of a neoplastic cell is not necessarily a sign of activity. Attempts to study the penetration of fluorescent drugs into the cells have led to some puzzling observations.

If passage of adriamycin across the cellular and nuclear membranes is not just the result of passive staining of necrotic or degenerating cells, the diverse findings in different cases may represent the effect of metabolic differences between morphologically identical tumours. At any rate, it would seem illogical to use topically a drug that does not penetrate into the tumour cell even after prolonged contact. The method is simple and no expensive equipment is required. However, the present observations are only preliminary and they require further confirmation.

3. Criticism of the value of heterotransplantation should be directed first to a tumour - host relationship which is very different from that of a naturally occurring neoplasm in the human bladder. The number of cells in the reproductive cycle and the cytokinetics of the transplanted tissue are also very different from those of a slowly growing bladder carcinoma in man.

The correlation between the laboratory and the clinical results was, however, quite encouraging. A patient, from whom an epidermoid bladder cancer had been transplanted into the hamster, showed a temporary cessation of measurable tumour growth whilst receiving 5-FU, although he eventually died. Vinblastine had had no effect, but irradiation of a metatatic nodule in the skin resulted in tumour necrosis. These observations were in agreement with results obtained in the hamster. Unfortunately, MMC and thiotepa, both of which were active against the transplanted tumour, were not used in the patient. Their efficacy in the experimental model corresponds to general clinical experience.

Heterotransplantation of bladder tumours seems, therefore, to offer an interesting model for testing drug sensitivity, despite the theoretical objections mentioned above. Unfortunately, its wide application to patients with bladder cancer is impractical, due to the cost, complexity and length of time required before even preliminary results are available.

4. The same theoretical objections can also be applied to the use of culture systems in which cells proliferate very actively, unlike the low mitotic index of transitional cell tumours in man. The study of inhibition of uptake of radioactive precursors in protein and nucleic acid synthesis is, nevertheless, by no means devoid of interest. The agreement with the clinical results was quite good but, again, experience of the test is still limited. Regrettably, this technique, too, is complex, it requires expensive equipment and it cannot be employed routinely for clinical purposes.

5. The agar plate test is based on the principle of in vitro cytotoxicity and can explore only one facet of the effects of chemotherapeutic agents on tumour cells. Its advantages are that it is simple and allows simultaneous comparison of a larger number of drugs than the previous methods. Furthermore, it has given results consistent with those obtained clinically in experiments with neoplasms at different sites.

The agar plate test cannot be used to study drugs that need to be activated in vivo, such as cyclophosphamide and, probably, adriamycin. This may be the reason why the latter drug was invariably found to be inactive in vitro, while its deoxylated analogue, daunomycin, was almost always highly effective. The significance of the lack of in vitro activity of

MMC is obscure, especially if it is remembered that it was the most effective of the drugs tested in the hamster and it does not seem to need activation. Perhaps an inhibitor of MMC was present in the agar or its diffusion into the medium was hindered. The differences between the patterns of drug sensitivity shown by the tumours studied with this technique is striking. It has already been emphasized that tumours taken from the same patient at various times exhibited comparable behaviour in vitro. Thus, variation of the in-vitro sensitivities of different tumours is unlikely to be due to technical difficulties. Instead, it probably reflects genuine biochemical differences between apparently identical neoplasms.

Conclusion

In conclusion, a simple, accurate and reliable laboratory method for testing the sensitivity of tumours to drugs still remains to be discovered, and bladder tumours are no exception to this rule. The relative ease with which viable neoplastic material can be obtained by transurethral biopsy renders these lesions particularly suitable for this type of investigation.

Comparison of the results obtained from individual tumours by applying several of the methods available now is of research interest and it may also have some practical value, especially in view of the limited value of in vitro tests for drugs that require in vivo activation.

Only further experience and careful comparison of laboratory data with clinical results will permit us to define the practical value of the various cancer cell sensitivity tests.

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