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# An *in Vivo* Clonogenic Assay for Human Tumors using Plasma Clot Diffusion Chambers Implanted in Mice*

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**Abstract**—*A modification of the* in vivo *tumor clonogenic assay using plasma clot diffusion chambers has been described which allows improved study of the cytological characteristics of the colonies cultured. Seventy-five percent of the tumors derived from malignant ascites could be cultured successfully (more than 30 small and large colonies per diffusion chamber). The cloning efficiency ranged from 0.01 to 10%. The addition of 2-mercaptoethanol, horse serum and insulin to the diffusion chambers did not affect colony formation, whereas the effect of cell-free malignant ascites added to the diffusion chambers was unpredictable. Colony growth was comparable when fresh or cryopreserved tumor cells were cultured. A linear relationship between the number of tumor cells inoculated and the number of colonies cultured was apparent in the range* $10^2$–$10^4$ *cells. Colony formation was stimulated by pre-irradiation* (8 Gy) *of the host animal and by weekly transplantation of the diffusion chambers in new mice. Intravenously administered doxorubicin penetrated the plasma clot and caused inhibition of colony formation in two experiments with melanoma cells.*

## INTRODUCTION

IN RECENT years factors influencing tumor colony growth have been exhaustively studied. Two systems—an *in vitro* agar culture system which supports the growth of clonogenic tumor cells [1–5] and *in vivo* agar diffusion chambers that isolate implanted cells from host cells while allowing exchange of nutrients, waste, drugs and presumed humoral stimulators and inhibitors [6, 7]—have received most attention. Both assays allow the study of the chemosensitivity of colony forming tumor cells. Some unexplained differences exist. Exponential cell kill after exposure of cells to cytotoxic drugs has only been shown for tumor cells cultured in diffusion chambers [8]. Lack of correlation between the results of the chemosensitivity as tested in the *in vivo* and *in vitro* cloning assays has been published recently [9]. Insight into the specific role of the tumor diffusion chamber assay is hampered by lack of knowledge of the characteristics of the colonies cultured within the diffusion chambers. The agar utilized in both culture systems hinders optimal morphology of the cultured colonies, and thus optimal investigation of colony characteristics such as morphological and immunochemical heterogeneity.

*In vivo* liquid and plasma clot diffusion chambers have been used in the study of regulation of haematopoiesis [10–13], and differences between colony-forming cells in this system and *in vitro* culture have been reported [14].

The studies reported here are designed to improve the agar diffusion chamber assay by the use of plasma clot instead of agar as semi-solid material within the chambers. This adaptation made it necessary to use a different harvesting procedure, but allowed the study of all colonies within one chamber using routine morphological techniques.

## MATERIALS AND METHODS

### *Cell suspensions*

Samples of malignant effusions were obtained by paracentesis and injected into heparinized (10 μg/ml) vacuum bottles. Patients suffered from

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ovarian carcinoma, melanoma and some other tumors both untreated and chemotherapeutically treated, but always minimally a few weeks after the last treatment. Cells were harvested by centrifugation at 150 *g* for 10 min, resuspended in Hanks' BSS + 5% heat-inactivated fetal calf serum (Gibco) and washed three times.

The resulting suspension was passed through needles of decreasing size to 23 gauge. The cell suspension was examined microscopically for aggregates. Usually only a few, small aggregates were present. In case of larger aggregates, the cell suspension was again passed through the needles until the number of cell clumps diminished. Viability (trypan blue exclusion) of all cell suspensions was recorded. Cells were used immediately and/or frozen in the presence of DMSO and stored at -196°C. Cell concentration was adjusted so as to deliver the desired number of cells to each diffusion chamber in 0.1 ml.

*Plasma clot diffusion chambers*

Diffusion chambers were made by glueing 0.20-μm pore size, 13-mm micropore filters (Amicon) to one side of plastic rings with MF-1 glue (millipore No. 70,000,000) and 0.20-μm Nucleopore filters to the other side of the rings (millipore rings No. PR 0001401). After sterilization in ethylene oxide, the diffusion chambers were allowed to ventilate for at least 12 hr. Chambers were loaded with 0.1 ml cell suspension and 0.05 ml AB citrated plasma. The loading hole was sealed by a plastic peg rendered semi-fluid by heating in a flame. Filled chambers were kept at 37°C for more than 15 min until clotting occurred within the chamber. Under general anaesthesia [subcutaneously administered fluanison (Hypnorm®, Philips Duphar) and ether], two chambers were implanted in the peritoneal cavity of female, 2-3 months old, normal or previously irradiated NMRI mice. At intervals after implantation, chambers were harvested from animals killed by ether narcosis. When a culture time of 14-21 days was required, chambers were harvested every 7 days and re-implanted in new irradiated mice.

Colonies were recovered from the chambers by a modification of the technique described by Jacobsen [15] utilizing a 60-min incubation of the chambers in a solution of 5% Ficoll (Pharmacia) and 0.5% pronase (Calbiochem, La Jolla, CA) in Hanks' BSS to dissolve any clot within the chambers. During this incubation period the chambers were kept in a diffusion chamber holder (Fig. 1).

Initial experiments showed that the pronase dissolved the clot around the colonies adhering to the nucleopore membrane perfectly, whereas the clot fixed to the microporous membrane disappeared less easily. Since staining procedures gave superior results in the presence of the microporous membrane, we routinely used diffusion chambers with a Nucleopore membrane on one side and a microporous membrane on the other side of the ring. After a 60-min incubation the diffusion chamber holder was centrifuged (150 *g*, 10 min) to sediment all colonies onto the microporous membrane. The membrane was fixed in Bouin solution for approximately 12 hr at 4°C and then stained with Giemsa or hematoxylin-eosin stains. Dehydration by successive exposures of 3 min each in 50, 70, 96 and 100% ethanol, and xylol was found preferable to air drying. Coverglasses were then mounted on slides using Entellan (Merck).

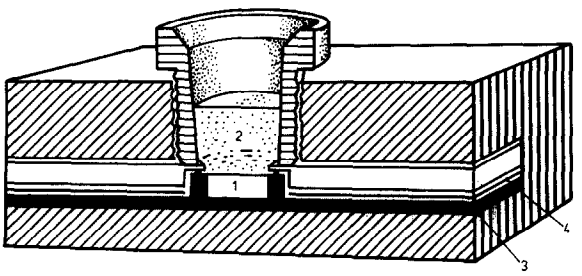


*Fig. 1. Diffusion chamber holder according to* [15]. 1, *diffusion chamber;* 2, *pronase solution;* 3, *glass slide;* 4, *filter paper.*

Colonies were then studied under a binocular microscope at magnifications up to ×400-1000. This method preserves the integrity of the majority of the colonies and enables regular histochemical (and immunochemical) staining procedures. Cells within the colonies could be counted exactly in the aggregates of less than 50 cells. In aggregates of more than 50 cells, the cells were closely adherent and the number of cells has been estimated in these colonies. Colonies were defined as 'large' aggregates (at least 50 cells) or 'small' aggregates (between 20 and 50 cells). Clusters contained between 8 and 20 cells.

*Experimental design*

Growth patterns were established for a number of ovarian tumors, melanomas, breast and lung carcinomas and some miscellaneous tumors. The effect of 2-mercaptoethanol (at a concentration of 50 μM/ml), 15% horse serum (Gibco 034-6050), 25, 50, 75 or 100% cell-free malignant ascites fluid and insulin (Iletin I, Lilly, 0.15 μg/diffusion chamber) added to the plasma clot diffusion chambers was studied. Colony growth of fresh tumor material vs cryopreserved tumor cells were compared using ovarian carcinoma cells.

Growth patterns of tumors were established in non-irradiated and in 8 Gy-irradiated host animals and in animals pretreated with cytosine

arabinoside (200 mg/kg intraperitoneally) 24 hr prior to the 8-Gy irradiation [7].

Finally, the penetration of doxorubicin given by intravenous injection in the tail vein of the mouse into the plasma clot diffusion chambers was studied. Studies were done in 2-4 mice at each time point and in each mouse two diffusion chambers containing a given sample of tumor were used. Thus each point for each experiment represented the mean value of 4-8 chambers and the value for a given day was the mean of the means for all the mice chambers harvested on that day.

Significance of difference between mean cell counts was determined by application of the non-paired, two-tailed Student's *t*-test.

## RESULTS

### *Tumor cell proliferation in diffusion chambers in 8 Gy irradiated mice*

Table 1 shows that of 59 tumors derived from malignant ascites, 45 grew more than 30 colonies when 0.5-5 × $10^4$ cells were inoculated in plasma clot diffusion chambers. The mean plating efficiency (number of colonies per 100 cells inoculated) of each tumor was fairly constant. The median plating efficiency for all tumors cultured was 0.51%, with a range of 0.01-10%.

Tables 2 and 3 show the distribution of cluster and colony numbers harvested when $10^4$ tumor cells were inoculated in each chamber.

After 3-4 days of culture a number of small clusters of tumor cells, many of them in some stage of mitosis, can be detected. Large colonies (more than 50 cells) appear only after more than 7 days of culture, depending on the types of tumor cultured. After this period small and large colonies increase not only in number but also in size. The number of clusters (less than 20 cells) remains more or less constant throughout the culture period. This growth pattern was fairly constant for all tumors that were successfully cultured.

Table 4 shows the culture results of fresh ovarian tumor cells compared with cryopreserved cells of the same original samples. No significant difference was found between those two groups at 14 days of culture.

*Table 1. Tumor cell colony growth in diffusion chambers (April 1982–October 1983)*

| | No. of tumors derived from ascites fluid | |
|---|---|---|
| | Cultured | Cultured successfully |
| Ovarian | 27 | 21 |
| Melanoma | 13 | 13 |
| Bronchus | 7 | 4 |
| Mamma | 5 | 4 |
| Others | 7 | 3 |
| Total | 59 | 45 (75%) |

*Table 2. Numbers of clusters and colonies cultured in diffusion chambers when ovarian tumor cells derived from malignant ascites were used**

| No. of cells/colony | Days of culture 7 | 14 | 21 |
|---|---|---|---|
| <20 cells | 20.1 ± 12.9 | 15.8 ± 11.3 | 19.2 ± 1.9 |
| 20-50 cells | 9.5 ± 6.2 | 13.6 ± 17 | 16.5 ± 8.7 |
| >50 cells | 0.7 ± 1.3 | 12.7 ± 2.2 | 27.5 ± 3.6 |

$10^4$ cells inoculated/diffusion chamber.
*Mean of 21 experiments.

*Table 3. Number of clusters and colonies cultured in diffusion chambers when melanoma cells derived from malignant ascites were used**

| No. of cells/colony | Day 7 of culture | Day 14 of culture |
|---|---|---|
| <20 cells | 152.8 ± 10.5 | 113.4 ± 85 |
| 20-50 cells | 20 ± 30 | 144.7 ± 14.5 |
| >50 cells | 1.9 ± 0.4 | 15.4 ± 14.3 |

$10^4$ cells inoculated/diffusion chamber.
*Mean of 11 experiments.

Tables 5 and 6 show the effects of the addition of 2-mercaptoethanol, horse serum, malignant cell-free ascites fluid and insulin to the diffusion chamber. No effect on the colony growth of ovarian carcinomas was seen when these were added to the diffusion chambers with the exception of cell-free malignant ascites fluid, the effect of which was unpredictable. In some experiments stimulation of colony growth was observed using one batch of ascites, whereas in other experiments no effect or even inhibition of

*Table 4. Comparison of fresh tumor cells or cryopreserved tumor cells on colony formation*

| | Day 7 of culture | | | Day 14 of culture | | |
|---|---|---|---|---|---|---|
| | <20* | 20-50 | >50 | <20 | 20-50 | >50 |
| Fresh material | 22 ± 7† | 7 ± 6 | 0.5 ± 6.8 | 17.2 ± 6.8 | 32.9 ± 13 | 15.2 ± 3.2 |
| Frozen material | 8 ± 4 | 2 ± 1 | 0.5 ± 0.2 | 31 ± 11 | 24 ± 15 | 15.5 ± 2.2 |

*No. of cells/colony.
†No. of clusters and colonies cultured from $10^4$ ovarian cells per diffusion chamber ± S.D.

*Table 5. Growth characteristics of tumor cells cultured in diffusion chambers, implanted in 8 Gy-pre-irradiated mice. Effect of the addition of insulin, horse serum or 2-mercaptoethanol to the diffusion chamber*

| | Day 7 of culture | | | Day 14 of culture | | |
|---|---|---|---|---|---|---|
| | <20* | 20-50 | >50 | < 20 | 20-50 | >50 |
| Control (ovarian tumors) | 50† ± 17 | 20.6 ± 16 | 2.5 ± 2.3 | 71.4 ± 11 | 114 ± 20 | 19.3 ± 2.8 |
| + insulin | 41 ± 14 | 20.2 ± 8.2 | 2.2 ± 2.0 | 53 ± 8 | 124.4 ± 23 | 22.5 ± 3.2 |
| Control (melanoma) | 113 ± 54 | 1.77 ± 1.96 | | 127 ± 59 | 71.3 ± 40 | 2.8 ± 2.6 |
| + horse serum | 92 ± 18 | 0.93 ± 0.9 | | 166 ± 38 | 85.3 ± 17 | 4.6 ± 3.1 |
| Control (ovarian tumors) | 8.85 ± 4.9 | 11.45 ± 1.1 | | 16.8 ± 3.3 | 27.7 ± 2.2 | |
| + 2-mercaptoethanol | 11.45 ± 7.1 | 11.5 ± 0.1 | | 23.4 ± 8 | 22.7 ± 7 | |

*No. of cells/colony.
†No. of clusters and colonies cultured from $10^4$ tumor cells/diffusion chamber ± S.D.

*Table 6. Effect of addition of ascites to the diffusion chambers**

| | Day 7 of culture | | | Day 14 of culture | | | Day 21 of culture | | |
|---|---|---|---|---|---|---|---|---|---|
| | <20† | 20-50 | >50 | <20 | 20-50 | >50 | <20 | 20-50 | >50 |
| Control | 12.4 ± 7.5† | 6.2 ± 4.8 | 1.8 ± 0.7 | 11.5 ± 6 | 10.7 ± 5.7 | 6.5 ± 2.8 | 40.8 ± 5 | 13.5 ± 4.5 | 41 ± 7.6 |
| Asc. 25% | 9.2 ± 1.9 | 5.0 ± 2.7 | 0.4 ± 0.3 | | | | | | |
| Asc. 50% | 19.4 ± 5.4 | 9.0 ± 2.5 | 0.1 ± 0.2 | | | | | | |
| Asc. 75% | 12.2 ± 1.2 | 6.6 ± 4.8 | 0.4 ± 0.2 | 8.6 ± 6.7 | 8 ± 7 | 4.8 ± 1.7 | 30.1 ± 7.5 | 12.0 ± 2.6 | 44 ± 10 |
| Asc. 100% | 23.4 ± 1.5 | 6.0 ± 4.5 | 0.1 ± 0.1 | | | | | | |

*Means of 6 experiments.
†No. of cells/colony.
‡No. of clusters and colonies/$10^4$ cells inoculated ± S.D.

colony growth was apparent. A linear relationship between the yield of colonies at day 14 of culture and the number of cells introduced into the chambers was demonstrated for an ovarian tumor and a melanoma over a range $10^2$–$10^4$ cells per chamber (Fig. 2). Using $10^5$ cells/chamber, colony numbers were somewhat lower than expected, possibly on the basis of crowding, cell-cell interaction or simply overlapping colonies leading to problems with quantitation.

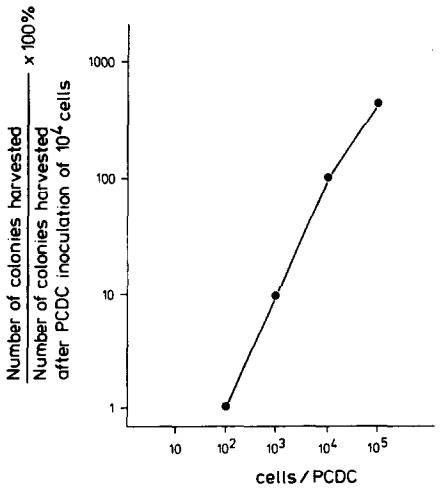


*Fig. 2. Relationship between No. of cells inoculated into the diffusion chambers and No. of colonies harvested on day 14 of culture. PCDC = plasma clot diffusion chamber.*

Morphology of the cells within the colonies was preserved rather well with the harvesting method used (Fig. 3). Cytological heterogeneity of the colonies existed within a diffusion chamber slide. Usually 2-3 kinds of colonies, containing different cell types, could be detected.

*Effect of pretreatment of the host animal on tumor-colony formation in the diffusion chamber*

Figure 4 shows the colony numbers obtained with cells cultured in diffusion chambers implanted in untreated or 8 Gy-pretreated mice. Pre-irradiation of the host animal significantly affected the number of melanoma colonies after 2 weeks of culture ($P < 0.01$). In addition to the positive effect of irradiation of the host animal, weekly harvesting, cleaning and re-implantation of the diffusion chambers also increased colony number and size.

Not presented are the data showing that the injection of cytosine arabinoside into the mice 24 hr preceding the 8-Gy irradiation increased the chance of survival after 10-14 days of culture but had no effect on the growth patterns of the implanted tumor cells.

*Effect of intravenous administered cytostatic drugs*

Figure 5 shows that doxorubicin (10 mg/kg) given as intravenous injection to the chambers

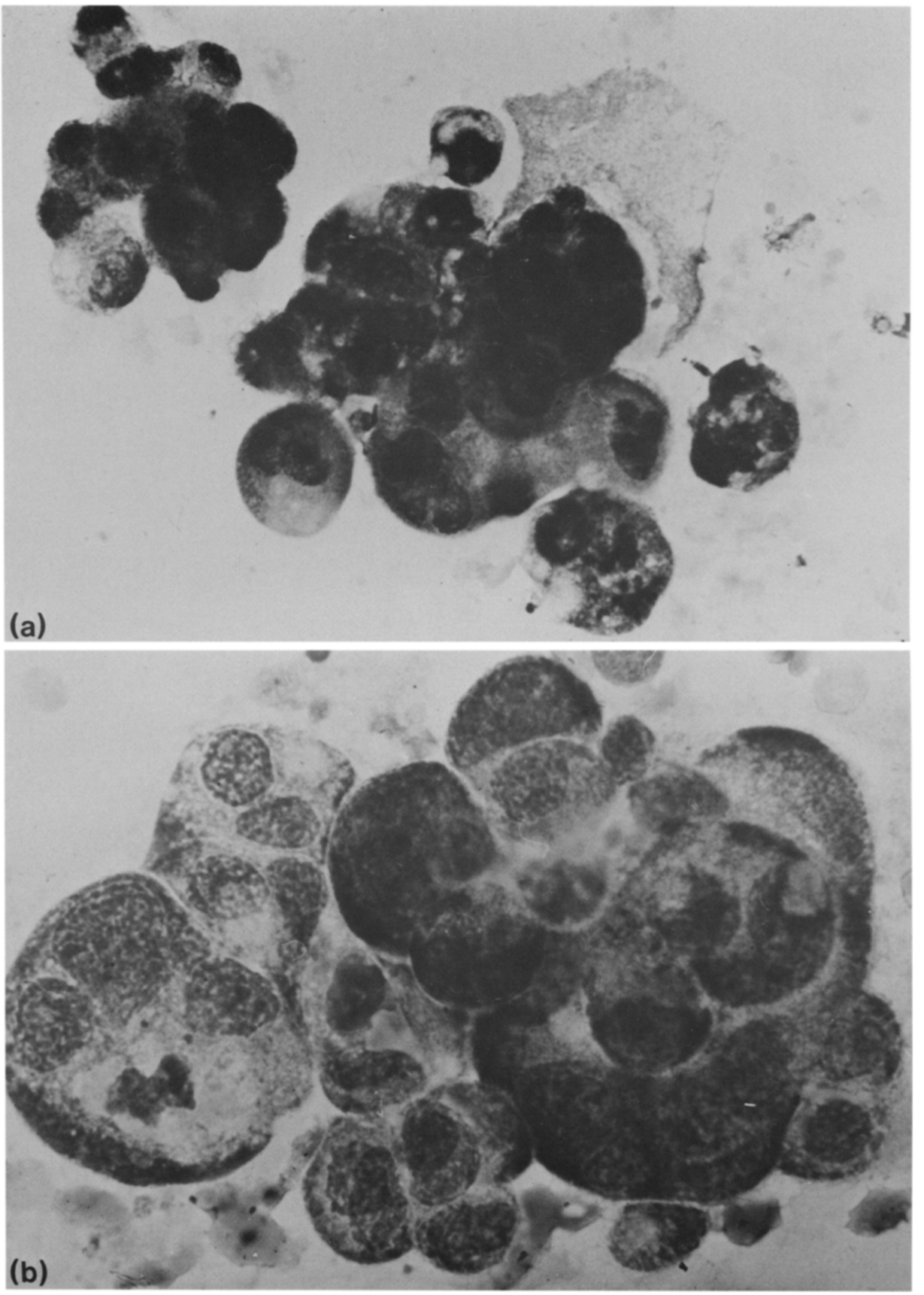


*Fig. 3. Example of oat cell (a) and ovarian tumors (b) cultured for* 7 days *in plasma clot diffusion chambers.*

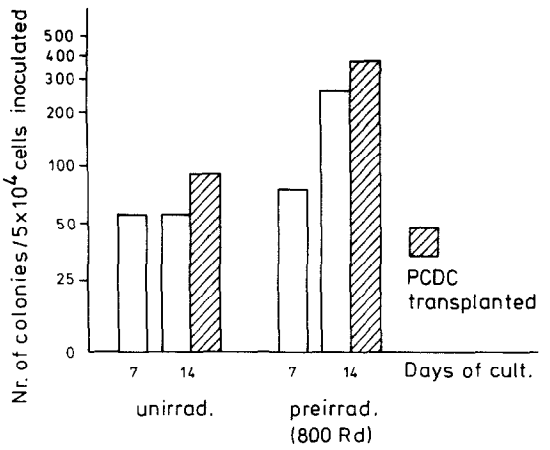


*Fig. 4. The effect of pre-irradiation (8 Gy) of the host mouse and weekly transplantation of the diffusion chambers in new mice on tumor colony growth. PCDC = Plasma clot diffusion chamber; unirrad. = animals not irradiated; preirrad. = animals irradiated (8 Gy) 24 hr before chamber implantation; PCDC transplanted = chambers transplanted on day 8 of culture in new irradiated or non-irradiated mice.*

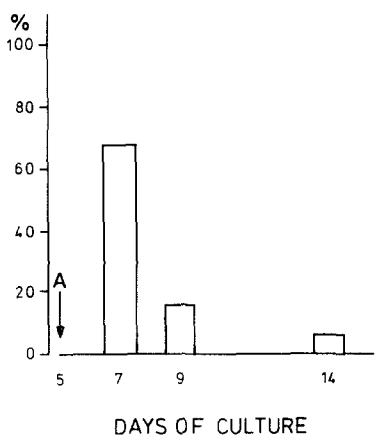


*Fig. 5. Effect of doxorubicin in intravenous administered on day 5 of culture on melanoma colony growth. No. of colonies after doxorubicin injection compared to controls. A = intravenous injection of doxorubicin.*

bearing mice on day 5 of culture penetrated the diffusion chamber, resulting in more than 90% growth inhibition on day 14 of culture in two melanoma experiments.

## DISCUSSION

Previous studies of bone marrow and tumor clonogenicity have been performed *in vitro* in agar and *in vivo* in diffusion chambers containing agar as a semi-solid medium [1, 2].

Plasma clot as a semi-solid medium inside the diffusion chamber has mainly been used in the study of hematopoiesis [10-15, 16].

The experience with plasma clot as a semi-solid medium in diffusion chambers reported here indicates that this modification of the *in vivo* diffusion chamber is suitable for the study of tumor clonogenicity and offers the advantage of improved cytology of the cultured colonies. Preliminary studies show that other techniques for characterization of the tumor cells such as immunoperoxidase, immunofluorescence, cytogenetics and electron microscopy are applicable in this assay.

Cells in diffusion chambers may die and disintegrate, or remain intact and live unchanged. Cells may also divide and/or undergo morphologic change to form colonies or clusters. Colonies in diffusion chambers can be detected after 7 days of culture, and increase in number and size during subsequent time according to a fairly constant growth pattern.

Morphology of the cultured cells is rather well preserved during the harvesting method and several tumors cultured showed clearly a heterogenous population of colonies with regard to cytological characteristics.

Cloning efficiency ranged from 0.01 to 10%, which is comparable with the *in vitro* data [5]. Seventy-five percent of all tumors were cultured successfully as defined by more than 30 small and large colonies per diffusion chamber.

It has been shown in the *in vitro* cloning assay that the addition of 2-mercaptoethanol, horse serum, insulin or ascites fluid to the culture medium leads to increased cloning efficiency in some tumors [17, 18]. In our experiments using plasma clot diffusion chambers, no effect was observed on ovarian tumor or melanoma colony growth with 2-mercaptoethanol, horse serum or insulin; malignant cell-free ascites increased the number of colonies in a dose response fashion in some tumors but had no effect or else inhibited colony formation in others. Although we got the impression that a positive effect of malignant ascites was most apparent in ovarian carcinoma, we did not study this subject further. In contrast to the experience in the *in vitro* cloning assay [P. Slee, personal communication], colony growth did not differ significantly when fresh tumor cells or cryopreserved cells from the same original sample were used, which adds to the versatility of the assay.

As reported by Smith *et al.* [7], a linear relationship exists between the number of colonies harvested and the number of cells inoculated. However, when $10^5$ cells per diffusion chamber were used the colonies were, at the time of harvesting, numerous and overlapped each other, creating problems with quantitation of colony counts. Pretreatment of the host animals by irradiation or cytostatic drugs (cyclophosphamide) has been reported to increase the rate of tumor growth in the subrenal capsule assay and the number of colonies in agar diffusion

chambers [7]. In the plasma clot diffusion chamber assay 8 Gy 1 day before chamber implantation increased colony numbers. Addition of cytosine arabinoside preceding irradiation increased the survival time of the mice but did not affect colony growth in the chambers. Weekly harvesting, cleaning and re-implantation of the chambers appeared also to be fairly important to achieve optimal colony growth. Further studies will be carried out to find out whether under each circumstance culture results are comparable in diffusion chambers carried by unirradiated or irradiated mice. Preliminary experiments show that doxorubicin given as an intravenous injection penetrates the diffusion chamber and inhibits colony formation. In the future comparisons between *in vitro* and *in vivo* colony growth of human cells will be made. In conclusion, the diffusion chamber cloning assay is laborious and expensive, but the modification reported here probably opens new ways of investigation of tumor colonies and might be worthwhile for special studies.

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