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# Improvements and Limitations of the Subrenal Capsule Assay for Determining Tumour Sensitivity to Cytostatic Drugs*

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**Abstract**—*Cyclophosphamide (CY) prevented the host response from occurring in treated animals, and we therefore evaluated CY and other immunosuppressive forms of pretreatment in normal mice using the subrenal capsule assay initially for transplanted and later also for primary tumours. CY pretreatment, 4 or 4.5 Gy whole-body irradiation and cortisone were superior to silica in reducing host cell infiltration, and irradiation as pretreatment has become our routine technique. The addition of cortisone acetate to irradiation was of minor benefit in only 1/3 transplanted lines. When primary tumours were tested, the irradiation was only rarely able to completely prevent cellular infiltration. Only 7/11 ovarian tumours and 3/9 lung tumours were evaluable as tumour specimens (>50% tumour) in preirradiated mice. The degree of infiltration and fibrosis was similar in transplants in irradiated normal mice or in athymic nude mice, suggesting that these phenomena are largely due to properties of the tumours rather than of the host. The limitations of the technique to some cell lines and occasional primary tumours is obvious.*

## INTRODUCTION

THE USE of normal, immunocompetent mice in the 6-day subrenal capsule assay [1] has been questioned by us on the basis of data demonstrating that host resistance cells, observed in the graft, led to a variable assessment of efficacy of antitumour therapy for most human tumours [2]. Cyclophosphamide (CY) was alone in demonstrating good correlation between macroscopic measurement and histologic evaluation, primarily through a remarkably effective destruction of response cells. Given the known usefulness of CY as an immunosuppressive agent, we therefore initiated a program in which CY, whole-body irradiation, cortisone acetate and silica were each tested as methods of pretreatment of normal mice, after which macroscopic and microscopic evaluation of tumour response and composition were made. Combinations of these agents were also tested. Initially tumour lines, i.e. tumours in early transplant passage in nude mice, were used and later primary tumours were evaluated.

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## MATERIALS AND METHODS

### *Mice*

Only male mice were used. The normal mice used in the study were (C57BL/Rij × CBA/Rij) $F_1$ hybrids, which were 6 weeks of age when used. The nude mice were bred on a BALB/c background and used when they reached the age of 20 weeks. Nude mice were kept in laminal flow stalls, normal mice in our mouse colonies. All animals received acidified water and processed food *ad libitum*.

### *Renal capsule assay*

The technique used has been published previously [1, 2]. Briefly, after receipt of tumour material in sterile Hanks' medium, 1-mm³ specimens were prepared using a dissecting microscope and sharp dissection. A sample of the primary tumour was also placed in buffered

formalin for histological evaluation. Mice were then anaesthetized with Avertine® (2-2-2-tribromoethanol) i.p., using approximately 0.3 ml of a 25 mg/ml solution. When necessary the animals were then shaved on one flank, the skin prepared with alcohol (70%) and an incision made over the kidney. After dividing the skin and muscles, the kidney was gently externalized. With a scalpel blade (No. 10) a 5 mm long incision was made in the capsule of the kidney. Using a trochar, a single tumour specimen was then placed under the capsule. The presenting tumour surface area was then measured using a microscope grating marked in units of 0.1 mm and the abdominal incision was closed with surgical staples.

The mice were killed 6 days later and measurements of the tumour surface area were repeated. Five mice were used for each drug and 5–10 mice were used as untreated controls within each experiment. Relative tumour growth was calculated using the change in mean graft diameter $\Delta\phi$ [1]:

$$\Delta\phi = \left(\frac{l+w}{2} \text{ day } 6\right) - \left(\frac{l+w}{2} \text{ day } 0\right),$$

where $l$ = length and $w$ = width.

Statistical analysis was performed using either the Mann-Whitney $U$ test or Student's $t$ test.

*Histological procedures*

Histological examination was done for all tumour material. Sections of kidneys with the xenografted tumour were examined after the last tumour measurement. Haematoxylin/phloxin/safran or haematoxylin/eosin staining was routinely performed on these materials, which were reviewed by two of us. From the histological sections, estimates were made of the percentage of the tumour consisting of tumour cells, of infiltrating non-tumour cells and of other structures. Infiltrating cells probably originate from the host. The other non-tumour cell components included fibrosis, necrosis, haemorrhage and/or cystic areas felt to be typical of the implanted tumour. Routinely, a single mean value for percentage tumour cells is given, representing the mean of the values obtained in the group.

*Drugs and irradiation*

cis-Platinum (cisPt) was a gift from Bristol-Myers, The Netherlands. Cyclophosphamide (CY) was a gift from Asta-Werke, F.R.G. and adriamycin (ADM) was purchased from Farmitalia. All were dissolved in sterile saline. The drugs were given intravenously. Treatment was given generally 1 and 5 days after tumour implantation. Two-thirds of the $LD_{50}$ doses were used, specifically: cisPt, 8 mg/kg; CY, 200 mg/kg; ADM, 10 mg/kg; each of these doses was given twice. The procedures, drug doses and routes of administration were the same for both nude and normal mice, except that nude mice were treated only on day 1 because of increased sensitivity to drug toxicity. No health problems were identified in untreated nude mice. Pretreatment consisted of therapy, usually 24 hr prior to tumour implantation with one of the following: cortisone acetate suspension (Organon) 0.75 or 2.5 per mouse subcutaneously in a single injection; CY 200 mg/kg intravenously; 4 or 6 Gy whole-body irradiation 24 hr prior to implantation or 4.5 Gy less than 4 hr before implantation; and silica 2.5 mg/mouse of a colloidal suspension intravenously. Combinations of cortisone acetate (0.75 mg) and whole-body irradiation were also tested. During the course of the experiments reported here either a $^{137}Cs$ source (AEC, Canada) or a 5 mV Linear Accelerator (Philips) was used for irradiation.

## RESULTS

*Pretreatment of passaged tumours*

Three ovarian tumours in early (<10) passage and one colon tumour in the ±20th passage were used to test representatives of the various types of immunosuppressive agents available which, at the same time, would not be expected to have antitumour effects when given prior to tumour implantation. In Table 1 the results of no pretreatment and pretreatment with CY, $\gamma$-irradiation, cortisone, silica and the combination of cortisone and $\gamma$-irradiation are given for two ovarian tumours, XOVP and XOVS, for which all the pretreatments were evaluated. Two types of information are given in the table, the change in graft diameter, $\Delta\phi$, measured in each of the groups tested and the microscopic evaluation of the composition of the measured tumour 6 days after implantation. Relative tumour growth determination is the basis for the detection of sensitivity and tumour growth. A control for tumour growth and response to therapy is the implantation of the same tumour in nude mice, where the assay can be evaluated without immunosuppressive therapy. In comparing the data from the nude mouse to that from non-pretreated normal mice two things are apparent. First, macroscopic growth appears to be better in the normal mouse, and second, sensitivity, i.e. a significant difference between control and treated tumours at day 6, found in the nude mouse cannot be found in the normal mouse. An explanation can be found in the microscopic evaluation, where it appears that 70–90% of both XOVP and

*Table 1. Measured relative tumour growth and microscopic analysis of transplants of two human tumours of the ovary as dependent upon type of host or pretreatment and cytostatic drug treatment*

| | | | XOVP | | | | | XOVS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Microscopic | | | | | Microscopic | | |
| Mouse | Pretreatment | Treatment | Relative tumour growth ($\Delta\phi$) | No. exp. | Tumour cells (%) | Infiltration | Other | Relative tumour growth ($\Delta\phi$) | No. exp. | Tumour cells (%) | Infiltration | Other |
| Nude BALB/c | none | control | +0.33 | | 90 | - | + | +0.50 | | 90 | - | + |
| | | cyclo | +0.26 | | 90 | - | + | +0.55 | | 70 | - | + |
| | | cisPt | +0.04* | | 90 | + | + | +0.15* | | 90 | - | + |
| | | adria | +0.20 | | 90 | + | + | +0.29* | | 90 | - | + |
| Normal BCBA/F1 | none | control | +0.75 (0.45-0.95) | 4 | 10 | +++ | - | +0.97 (0.26-1.01) | 5 | - | +++ | - |
| | | cisPt | +1.02 (0.26-1.06) | 6 | 10 | +++ | - | +0.99 (0.17-1.23) | 5 | - | +++ | - |
| | | adria | +0.79 (0.37-1.11) | 6 | 20 | +++ | - | +0.75 (0.27-1.48) | 5 | 30 | ++ | - |
| | -24 hr cycle 200 mg/kg | control | +0.38 | | 90 | - | + | +0.08 | | 50 | - | ++ |
| | | cisPt | -0.14* | | 85 | - | + | +0.16 | | 80 | - | + |
| | | adria | -0.10* | | 90 | - | + | -0.03 | | 90 | - | + |
| | -24 hr silica 2.5 mg | control | +0.31 | | 10 | +++ | - | +0.44 | | 20 | +++ | - |
| | | cisPt | +0.10 | | 20 | +++ | - | +0.35 | | 30 | +++ | - |
| | | adria | +0.25 | | 10 | +++ | - | +0.29 | | 5 | +++ | - |
| | -24 hr 4 Gy $\gamma$ | control | +0.62/+1.18 | 2 | 60/65 | ++ | - | +0.43/+0.59 | 2 | 50 | ++ | + |
| | | cisPt | -0.07*/+1.05 | 2 | 85/75 | + | + | -0.25*/+0.01* | 2 | 75 | - | + |
| | | adria | +0.18/+0.70 | 2 | 90/90 | ± | ± | +0.0*/+0.14* | 2 | 75 | + | + |
| | -24 hr cortisone 2.5 mg | control | +0.28 | | 80 | + | - | +0.13 | | 90 | - | + |
| | | cisPt | +0.01 | | 90 | ± | ± | +0.12 | | 100 | - | - |
| | | adria | +0.03 | | 85 | + | ± | +0.33 | | 90 | + | - |
| | -2 hr Gy $\gamma$ | control | +0.98 (0.70-1.15) | 5 | 65 | ++ | + | +1.12 (0.98-1.20) | 3 | 25 | ++ | + |
| | | cisPt | +0.80/+0.88 | 2 | 75 | + | + | +0.54 (0.14-1.00) | 3 | 60 | + | ++ |
| | | adria | +0.53/+0.93 | 2 | 85 | + | + | +0.52 (-0.05-0.75) | 3 | 50 | + | ++ |
| | cortisone 0.75 mg | control | +0.71 (0.67-0.82) | 3 | 20 | ++ | + | | | | | |
| | | cisPt | +0.81 | | 45 | ++ | + | | | | | |
| | | adria | +0.90 | | 60 | + | + | | | | | |
| | -2 hr 4 Gy $\gamma$ +cortisone 0.75 mg | control | +0.64 (0.57-0.97) | 4 | 75 | ++ | + | +0.83/+1.03 | 2 | 70 | + | ++ |
| | | cisPt | +0.67 (0.50-0.81) | 3 | 70 | + | ++ | +0.41*/+0.23* | 2 | 45 | - | ++ |
| | | adria | +0.37* (0.34-0.67) | 3 | 70 | + | ++ | +0.84/-0.01* | 2 | 50 | - | ++ |

*Significant difference from control ($P < 0.05$).

XOVS tumours at day 6 consist of tumour cells in the nude mouse, while tumour cells never represent more than 30% of the mass (and usually 10%) measured in the normal mouse. Lymphocytes, granulocytes and macrophages form the bulk of the tumour.

These findings explain both the 'better growth' that is the larger size of the macroscopic tumours in normal mice, the extra growth representing ingrowth of host response cells, and the lack of sensitivity of the tumour cells, possibly masked by the same phenomenon [1]. For XOVS pretreatment with CY, irradiation or cortisone, or the combination of the latter two, gives histologically similar improvement in that reduction of host infiltration is induced. Much the same can be said for XOVP. In neither tumour is there an observable improvement when silica is used. For both tumours considerable variation is found in the growth of tumour and, to a lesser extent, in the distribution of cell composition in repeated experiments. Tumour growth variability also leads to differing assessments of sensitivity; correlation between experiments with the same tumour is poor.

Further, even in these passaged tumours the histological picture is always a mixed one, 100% tumour specimens being rarely found, and never in untreated animals. In theory the interpretation might be improved by multiplying for each graft the $\Delta\phi$ with the per cent tumour cells measured in the sections. However, attempts in this direction did not improve the consistency of the results.

The combination of irradiation and cortisone was tested in XOVP, XOVS and XCOLH (Table 2), an adenocarcinoma of the colon. Weight loss, and considerable morbidity was observed in the animals treated with 2.5 mg/mouse cortisone and 0.75 mg/mouse was used in the combination studies. Cortisone and irradiation were both given between 2 and 4 hr prior to tumour implantation; the shorter time was chosen since we felt that pretreatment should, in principle, be applicable to primary tumour testing, where a less than 24 hr warning is usually given.

In none of the tumour types was there a consistent difference made by the addition of cortisone to the irradiation.

Finally, in XOVL (Table 2) the irradiation dose was evaluated. Six grays offered no advantage over 4 Gy when 2-4 hr elapsed between irradiation and implantation. An increase to 4.5 Gy was made when the source changed from $\gamma$- to X-irradiation.

*Table 2. Measured relative tumour growth and microscopic analysis of two human tumours dependent upon different types of pretreatment and cytostatic drug treatment*

| Mouse | Pretreatment | Treatment | Relative tumour growth ($\Delta\phi$) | Tumour cells (%) | Microscopic Infiltration | Other |
|---|---|---|---|---|---|---|
| | | | XOVL | | | |
| Normal BCBA/$F_1$ | none | control | +0.56 | — | +++ | - - |
| | | cisPt | +0.30 | 15 | +++ | |
| | | adria | +0.62 | 5 | +++ | - - |
| | -2-4 hr 4 Gy $\gamma$ | control | +0.43 | 85 | ± | ± |
| | | cisPt | +0.32 | 80 | ± | ± |
| | | adria | +0.72 | 90 | - | + |
| | -2-4 hr 6 Gy $\gamma$ | control | +0.53 | 90 | - | + |
| | | cisPt | +0.49 | 90 | ± | ± |
| | | adria | +0.27 | 100 | - | - |
| | | | XCOLH | | | |
| Normal BCBA/$F_1$ | none | control | +0.45/+0.52 | | | |
| | | cisPt | +0.65/+0.73 | | | |
| | | adria | +0.95/+0.68 | | | |
| | -2-4 hr 4 Gy $\gamma$ | control | +0.95/+0.52 | 85/90 | + | ± |
| | | cisPt | +0.38/+0.09 | 95/90 | - | ± |
| | | adria | +0.34/+0.38 | 95/100 | - | ± |
| | -2-4 hr 4 Gy $\gamma$+ cortisone 0.75 mg | control | +0.48/+0.45 | 70/80 | + | + |
| | | cisPt | +0.35/+0.19 | 90/100 | ± | ± |
| | | adria | +0.09/+0.43 | 95/95 | - | + |

*Pretreatment of primary tumours*

In Table 3 the results for 11 primary ovarian tumours, nine lung tumours and a single testis tumour in pretreated hosts are presented. Here the relative growth of the control at day 6 and the composition of the tumour in histologic sections are given. Using the criterion of Bogden *et al.* [1] all of the ovarian tumours, 8/9 lung tumours and the single testis tumour demonstrated sufficient growth. The histologic sections demonstrated that only one of the ovarian tumours contained more than 75% tumour, six had 50–75% tumour cells, one had 25–50% and three had less than 25% tumour cells. No correlation can be found between growth and percentage tumour. For lung tumours the results are even more disappointing; tumour comprised in 1/9 more than 75%, in 2/9 50–75% and in 6/9 less than 25% of the microscopic field (5/6 no tumour cells seen). 'Growth' again occurred in the large majority of tumours. The tumour demonstrating the least tumour growth, XLO3, had histologically one of the three best scores. The remaining structures for all the primary tumours were either host response cells or other cells, or fibrotic, necrotic, haemorrhagic or cystic areas, the latter presumably of human origin. In Table 4 the results of transplantation of a number of tumours in various passages in either preirradiated normal mice or nude mice is presented. As can be seen, with the exception of the three cases where either significant host resistance cells or other structures were encountered, the macroscopic measurements are similar in both hosts. Further, with two exceptions the fraction of tumour cells in the tumour are also comparable.

Again, and even in nude mice, pure tumour is not encountered, and variability is not less than that seen previously in pretreated normal recipients. We therefore examined the individual tumours, within a single experiment, in terms of the variability of the tumour cell percentages estimated in histological sections.

In Figs 1 and 2 the variability in tumour percentage is plotted against the tumour code, for passaged and primary tumours respectively. No

*Table 3. Measured relative tumour growth and estimated percentage tumour cells upon microscopic analysis for 11 primary ovarian tumours, nine lung tumours and a single testis tumour implanted under the renal capsule of normal mice pretreated with 4.5 Gy whole-body X-irradiation 2–4 hr before implantation (five mice per group were used)*

| | Relative tumour growth ($\Delta\phi$) | Tumour cells (%) | Microscopic | |
|---|---|---|---|---|
| | | | Infiltration | Other |
| Human ovarian tumour code | | | | |
| 29 | +0.24 | 0 | +++ | - |
| 30 | +0.10 | 50 | ++ | + |
| 31 | +0.38 | 60 | ++ | + |
| 32 | +0.39 | 0 | ++ | + |
| 33 | +0.19 | 0 | +++ | - |
| 34 | +0.20 | 60 | + | + |
| 35 | +0.57 | 50 | ++ | ± |
| 36 | +0.52 | 55 | + | + |
| 37 | +0.10 | 60 | + | + |
| 38 | +0.11 | 45 | ++ | + |
| 39 | +0.46 | 85 | - | ++ |
| Human lung tumour code | | | | |
| 2 | +0.39 | 0 | +++ | - |
| 3 | -0.30 | 50 | ++ | + |
| 4 | +0.03 | 0 | + | ++ |
| 5 | +0.22 | 0 | +++ | - |
| 6 | +0.17 | 75 | ± | ++ |
| 8 | +0.15 | 0 | +++ | - |
| 9 | 0.0 | 5 | - | +++ |
| 10 | -0.11 | 0 | ± | ++ |
| 11 | -0.01 | 50 | ++ | + |
| Human testis tumour code | | | | |
| H | +0.04 | 0 | - | ++ |

*Table 4. Similar data as in Table 3 for early passage ovarian tumours and two early passage lung tumours implanted either to BALB/c nude mice or preirradiated (4.5 Gy) normal BCBA/$F_1$ mice*

| Tumour code | Passage No. | Host | Relative tumour growth ($\Delta\phi$) | Tumour cells (%) | Microscopic Infiltration | Microscopic Other |
|---|---|---|---|---|---|---|
| XLU6 | 3 | nude | +0.30 | 90 | - | + |
| | | preirr. | +0.79 | 75 | + | + |
| XLU11 | 1 | nude | -0.26 | 60 | ± | ++ |
| | | preirr. | -0.01 | 50 | + | + |
| XOVP | 11 | nude | +0.56 | 80 | ± | + |
| | | preirr. | +0.80 | 60 | + | ++ |
| XOVS | 4 | nude | +0.55 | 95 | - | + |
| | | preirr. | +1.00 | 20 | ++ | + |
| XOV30 | 3 | nude | +0.42 | 75 | - | ++ |
| | | preirr. | +0.30 | 90 | - | + |
| XOV31 | 5 | nude | +0.63 | 80 | + | - |
| | | preirr. | +0.63 | 35 | ++ | - |

tumour treatment was given, all experiments were done in pretreated hosts. In general, passaged tumours give sections in which some tumour is present in at least one of the tested grafts, although variability is great. For primary tumours many more grafts contain no tumour, overall variability is not much greater.

In order to examine the source of this variability we then examined the percentage tumour in the 1-$mm^3$ blocks of tumour prior to implantation in the mouse, or after random selection of other blocks used for implantation. In Fig. 3 the variability in the blocks can be seen for four passaged tumours and one primary tumour. Variability similar to that seen in Figs 1 and 2 is apparent, indicating that the major source of the problems lies in the original tumours rather than in the host.

## DISCUSSION

We have previously pointed out that the subrenal capsule technique, performed in normal mice, led to a discrepancy between macroscopic and histological evaluation of human tumour response due to an ingrowth of (mouse) host response cells over the 6-day period of the assay. In this paper we report our efforts to develop an immunosuppressive regimen for normal mice

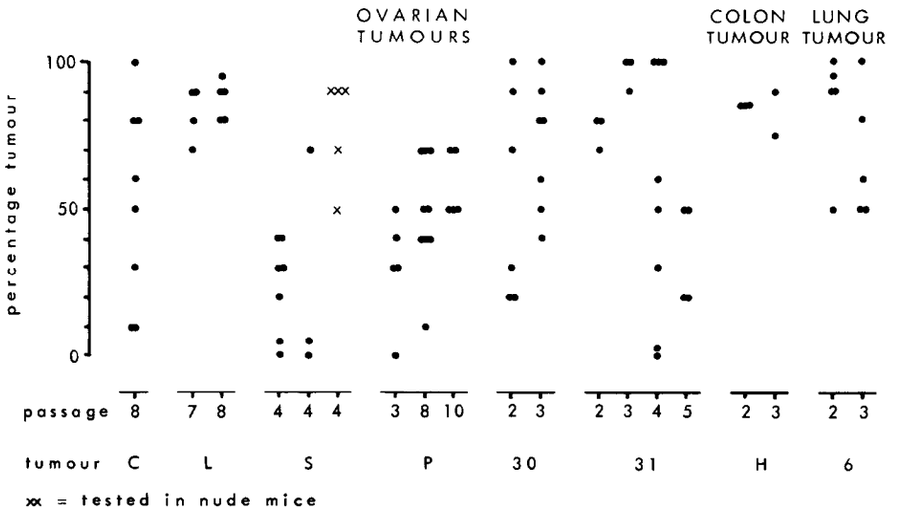


*Fig. 1. Distribution of the percentage tumour cells observed per microscopic field in individual transplants of human tumours under the kidney capsule in preirradiated normal mice after passage in nude mice.*

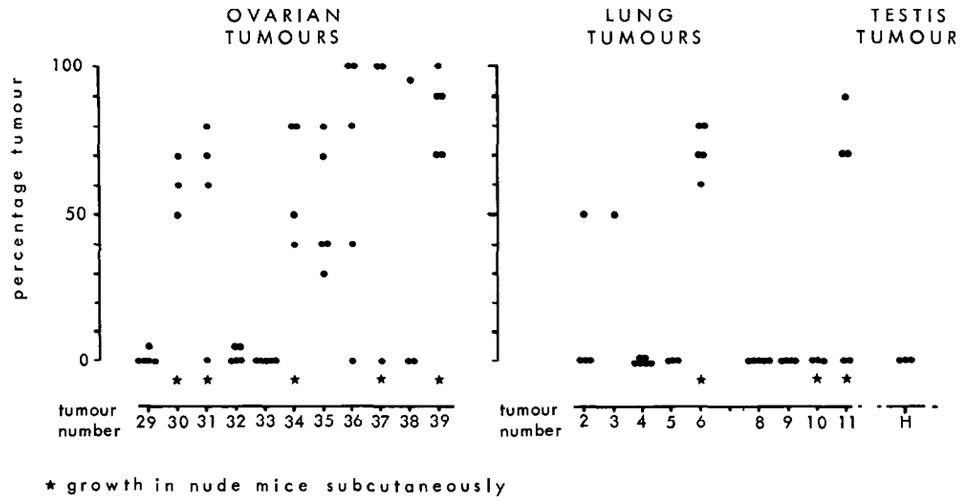


*Fig. 2. Distribution of the percentage tumour cells observed per microscopic field in individual primary transplants of human tumours under the kidney capsule of preirradiated normal mice.*

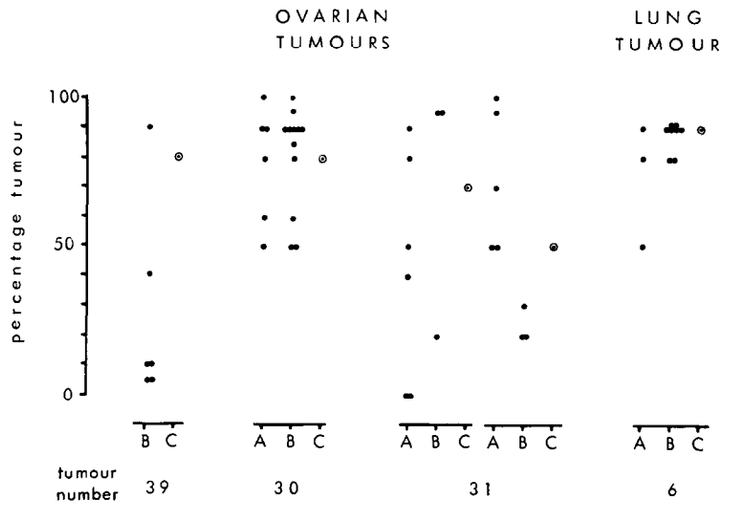


*Fig. 3. Distribution of the percentage tumour cells observed per microscopic field in individual 1-mm³ pieces of ovarian tumour selected either before (A) or after (B) implantation in preirradiated normal mice as compared to the average percentage tumour observed in the large sample (C) obtained from the nude tumour passage donor.*

which would allow better correlation between macroscopic and microscopic response and, at the same time, neither increase host toxicity nor have antitumour effects of its own. We have been partially successful; in Table 1 it can be seen that pretreatment with 4 Gy whole-body irradiation meets the two stated criteria and that cortisone in the higher concentration increased host toxicity and could be eliminated for that reason. Poor correlation between repeated experiments with the same passaged tumour suggested that the two criteria are not sufficient.

For primary tumours, one rarely has 24 hr warning before arrival, and we fortunately found that preirradiation 2-4 hr before implantation was also acceptable for reducing extrinsic host responses.

There remains the fact that even in the nude mouse, no tumour is composed only of tumour cells, and in considering this, the pretreatment results would seem more acceptable. However, there is considerable variability from experiment to experiment in the data with regard to pretreatment and treatment. When primary tumours were tested this was obvious, since, as presented in Table 3, only 6/11 ovarian tumours and 3/9 lung tumours were composed of $\geqslant 50\%$ tumour cells in histologic sections at day 6 after transplantation in control animals. This could not be measurably improved by transplantation under the renal capsule of nude mice (Table 4), implicating that the inherent heterogeneity of the human tumours was the origin of the variability. That was also demonstrated by the intraexperi-

ment variability shown in Figs 1 and 2. For most early passages it was possible to find some tumour cells in the histologic section prepared at day 6 but in some primary ovarian tumours and most primary lung tumours even this minimum requirement could not be met.

Finally, a part of the heterogeneity seen in the histologic sections at day 6 can be found prior to implantation in the 1-mm$^3$ tumour pieces. From Fig. 3 it is clear that even tumours in early passage are non-homogeneous; the single primary tumour presented is even less so. Pretreatment is thus capable of preventing host cell infiltration over the 6-day period of the assay and cannot be eliminated. However, the intrinsic heterogeneity of most but not all tumours is such that only a fraction of them, when transplanted into preirradiated hosts, will demonstrate *tumour* growth, although almost all of them will fulfill the minimal requirement for bulk growth. Results of therapy, specifically bulk reduction, may not be synonymous with tumour reduction, and thus the minimal growth requirement is not sufficient.

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