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Comparison between Macroscopic and Microscopic Evaluation of Tumour Responsiveness using the Subrenal Capsule Assay*

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Abstract—*Using the subrenal capsule assay in normal mice, a histologic evaluation was made of 8 human primary ovarian tumours and 3 human colon, 2 lung and 5 ovarian carcinomas growing in serial passage in nude mice. The results of the evaluation indicated that there is a tumour- and drug-dependent correlation between the macroscopically and microscopically evaluated effects, with cyclophosphamide demonstrating excellent concordance but adriamycin and cisplatin both demonstrating consistently more tumour cell killing on histologic analysis than could be appreciated macroscopically. Leukocyte infiltration and fibrosis were greatly increased by the latter 2 drugs, leading to unrepresentative macroscopic measurements. Variable amounts of host cell infiltration can also be demonstrated in the untreated control when normal mice are used. The use of nude mice decreases the discrepancy between macroscopic and microscopic evaluation.*

INTRODUCTION

THE SUBRENAL location for implantation of fresh human tumour material was reported and popularized by Bogden, and has been used both for the screening of new drugs and for prospective tumour sensitivity testing [1, 2]. Its advantages include a high success rate [3, 4], a short (6-day) delay before a result can be given and a possibly more realistic pharmacokinetic profile, more in agreement with the clinical situation than *in vitro* exposure.

While the technique was initially developed for use with nude mice, later publications have stressed the usefulness of a 6-day time period and normal, immunocompetent mice [5]. The use of normal mice, even over the shorter time period, immediately raises the question as to the effect host reponse cells might have on the growth of the tumour explant. Further, the macroscopic evaluation of a tumour mass at day 6 might include not only tumour cells but also host lymphocytes and macrophages, influencing the measurement and, therefore, the evaluation of growth or antitumour effect. To date no histologic evaluation of the subrenal capsule assay has been published; the results of our investigation are reported here.

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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 F1 hybrids (Rijswijk) and CD2F1 hybrids (Zentralinstitut für Versuchstiere, Hannover), 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 laminar flow stalls, normal mice in our mouse colonies. All animals received acidified water and processed food *ad libitum*.

Renal capsule technique

After receipt of tumour material in sterile Hanks' BSS medium, 1 × 1 × 1-mm specimens were prepared using a dissecting microscope and sharp dissection. A sample of the primary tumour was also placed in formalin for histological

evaluation. Normal or nude 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 subcutaneous tissues, 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 0.1 mm and the abdominal incision closed with surgical staples.

The mice were again opened for tumour measurement 6 or 7 days later. The staples were then removed, the kidneys were again gently brought outside the body and measurements of the tumour surface area were repeated. In the first experiments where drugs were tested the final measurement was performed 7 days after implantation; later this time was changed to 6 days. Five mice were used for each drug and 5–10 mice were used as untreated controls within each experiment.

Subcutaneous inoculation in nude mice

One flank of the mice was cleaned with alcohol and, using a pincet and scissors, the skin divided. Blocks of tumour from 1 to 5 mm^3 were implanted subcutaneously. The sites were palpated weekly for 6 months or until obvious tumour growth was noted.

Histological procedures

Histological examination was done from fresh tumour material and after the first passage, when it had grown subcutaneously in the flank of nude mice. Sections of kidneys with the xenografted tumour were investigated after the last tumour measurement. Three sections were initially examined per kidney. Later, routinely, a single section was evaluated when homogeneity was demonstrated. Hematoxilin/phloxine/safran staining was routinely performed on these materials, which were reviewed by one of us. Specimens for histological analysis were not always available.

Drugs

cis-Platinum (cisPt) was a gift from Bristol-Myers, The Netherlands. Cyclophosphamide (CY) was a gift from Asta-Werke, F.R.G., 5-fluorouracil (5FU) was a gift of the NCI-NIH, Bethesda, MD, U.S.A. and adriamycin (ADM) was purchased. All were dissolved in sterile saline. The drugs were given intravenously, except CY which was given subcutaneously. Treatment was given 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, 5FU, 175 mg/kg, ADM, 10 mg/kg; each of these doses were given twice. The procedures, drug doses and route of administration were the same for nude and normal mice, except that nude mice were treated only on day 1 because of increased drug toxicity. No health problems were identified in untreated nude mice.

Tumours

The ovarian carcinomas were obtained at the time of scheduled operative procedures. Fresh tumour specimens were placed in sterile Hank's BSS and transplanted within 4 hr of operation. The human tumor xenograft lines were studied in passages as shown in Table 4. By histologic examination the 3 colon cancers were well and poorly differentiated adenocarcinomas and 2 lung tumours were squamous cell carcinomas. All ovarian tumours were adenocarcinomas of variable grade.

RESULTS

Ovarian tumours were treated with CY, cisPt, ADM and 5FU. The results of treatment are seen in Table 1, where the ratio of mean treated area to mean control area is expressed as a percentage of the control value. Two different tests of response were made: statistical analysis using the Mann–Whitney U test and the additional arbitrary requirement that treated area be less than 40% of control area. Several tumours responded to CY using both criteria, a few to 5FU but none to cisPt, and only 1 responded to ADM using the stricter ($<40\%$) criterion. Of note is that ADM in particular appeared to stimulate tumour growth, resulting in larger than control macroscopic specimens in 11/20 transplants. The range of the individual values in a typical experiment can be seen in Fig. 1 for 4 primary tumour tests demonstrating rather low variability. Figure 1 also allows comparison of our test results in Table 1 to the method chosen by Bogden *et al.* to assess tumour sensitivity [3]. When the histological sections were examined many tumours were found to have apparently responded to cisPt and ADM, but because of the heavy ingrowth of lymphocytes and fibroblasts, this shrinkage could not be appreciated in the macroscopic measurement (Table 2). For example, tumours S, T and U responded to neither cisPt nor ADM macroscopically (Table 1, Fig. 1), but microscopically, relative responses to ADM were seen in all three (Table 2). CY did not present any problems in this regard because of the striking disappearance of

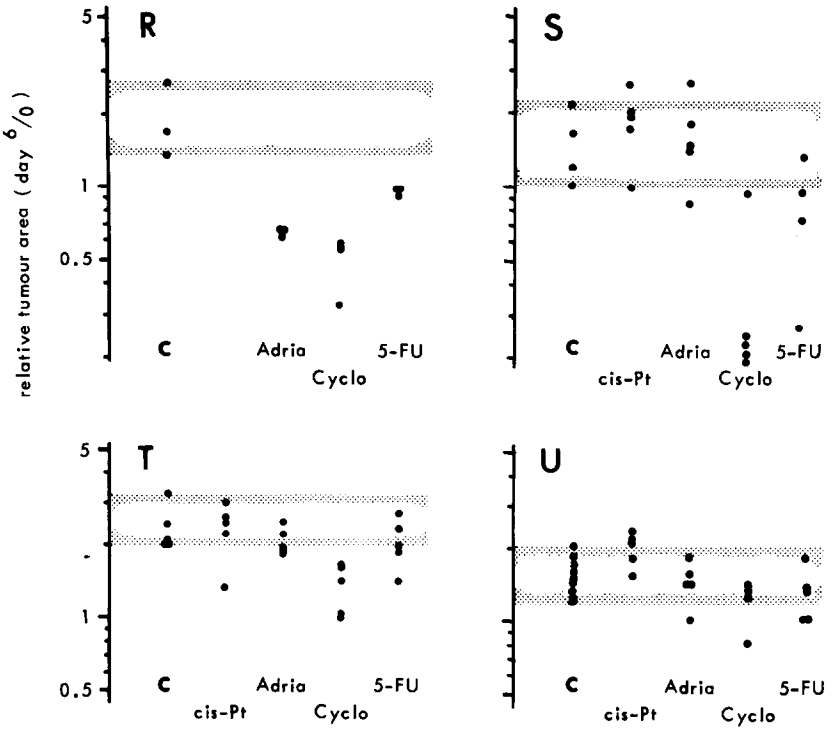


Fig. 1. Graphic representation of the results obtained using the subrenal capsule assay in 4 primary ovarian tumours. Plotted are the individual data points within the indicated control and treated groups of normal mice. Ordinate: relative tumour area at day 6 compared to day 0; abscissa: the indicated groupings. The shaded area represents the extremes of the control group data.

Table 1. Sensitivity of 19 ovarian tumours measured using the subrenal capsule assay in normal mice

	CisPt	ADM	CY	5FU
XOv.A	215	n.t.	70	116
XOv.B	124	115	n.t.	147
XOv.C	191	154	88.5	n.t.
XOv.D	110	112	75.8	n.t.
XOv.E	70–91	184–167	85*–47*	81–104
XOv.F	108	98	69*	n.t.
XOv.G	115	113	85	n.t.
XOv.H	93	85	n.t.	n.t.
XOv.L	92–124	148–92	85	75
XOv.O	92	n.t.	n.t.	n.t.
XOv.P	82	94	70	70
XOv.Q	102	104	67*	70*
XOv.R	65	33*	29*	51*
XOv.S	146	128	28*	62
XOv.T	99	87	58*	89
XOv.U	133	96	79	85
XOv.V	75*	99	70*	74*
XOv.24	119	76	38*	56
XOv.25	97	108	20*	28*

The figures in the body of the table represent:

$$\frac{\text{surface area (mm}^2\text{) of treated sample}}{\text{surface area (mm}^2\text{) of control}} \times 100.$$

Underlined ciphers represent values below the arbitrarily chosen 40% reduction level. All tests were done on primary tumours except for XOv.E and Xov.L, in which 2nd and 3rd transplant generations were tested.
*Significant ($P < 0.05$) with the Mann-Whitney U test.

Table 2. Histologic analysis of tumour and/or host tissues in the subrenal capsule technique in normal mice

	Test result	Relative contribution of:		
	% control surface area	tumour cells	host resistance cells	fibrosis
Ovarian A/3				
control	100	++	+	+
CY	61	+++	-	-
ADM	80	+	++	+
cisPt	126	+	++	+
Ovarian E/3				
control	100	++	++	-
CY	57	++++	-	-
ADM	120	+	+++	-
cisPt	101	++	+++	-
Ovarian F/1				
control	100	++	+	-
CY	69	+	+	+
ADM	98	++	++	+
cisPt	108	+	++	++
Ovarian G/1				
control	100	++	++	++
CY	85	++	-	+++
ADM	113	+	+++	+++
cisPt	115	+	++	++++
Ovarian L/2				
control	100	+	+++	-
CY	85	++++	-	-
ADM	148	+	++	-
cisPt	92	+	++	-
Ovarian P/2				
control	100	++	+	±
CY	64	+/+	-	-
ADM	140	++	++	+
cisPt	98	+/+	++	+++
Ovarian S/3				
control		++	+	-
CY	28*	++	+	-
ADM	128	+	++	+
cisPt	146	+	++	-
Ovarian T/1				
control	100	++	++	++
CY	58*	+	-	-
ADM	89	+	++	+++
cisPt	99	++	++	+++
Ovarian U/1				
control	100	+	±	++
CY	79	±	-	+++
ADM	96	+	-	+++
cisPt	133	++	-	+++
Ovarian V/1				
control	100	++	++	-
CY	70*	+	+	++
ADM	99	+	+++	++
cisPt	75*	+	+++	++
Ovarian 24/1				
control	100	++	++	++
CY	38*	+	-	+
ADM	76	+++	++	++
cisPt	119	+++	+	++
Ovarian 25/1				
control	100	+++	+++	±
CY	20*	++	-	±
ADM	108	++	+++	+
cisPt	97	+	++	+

The figure after the tumour code represents the passage of the tumour.
*See footnote to Table 1.

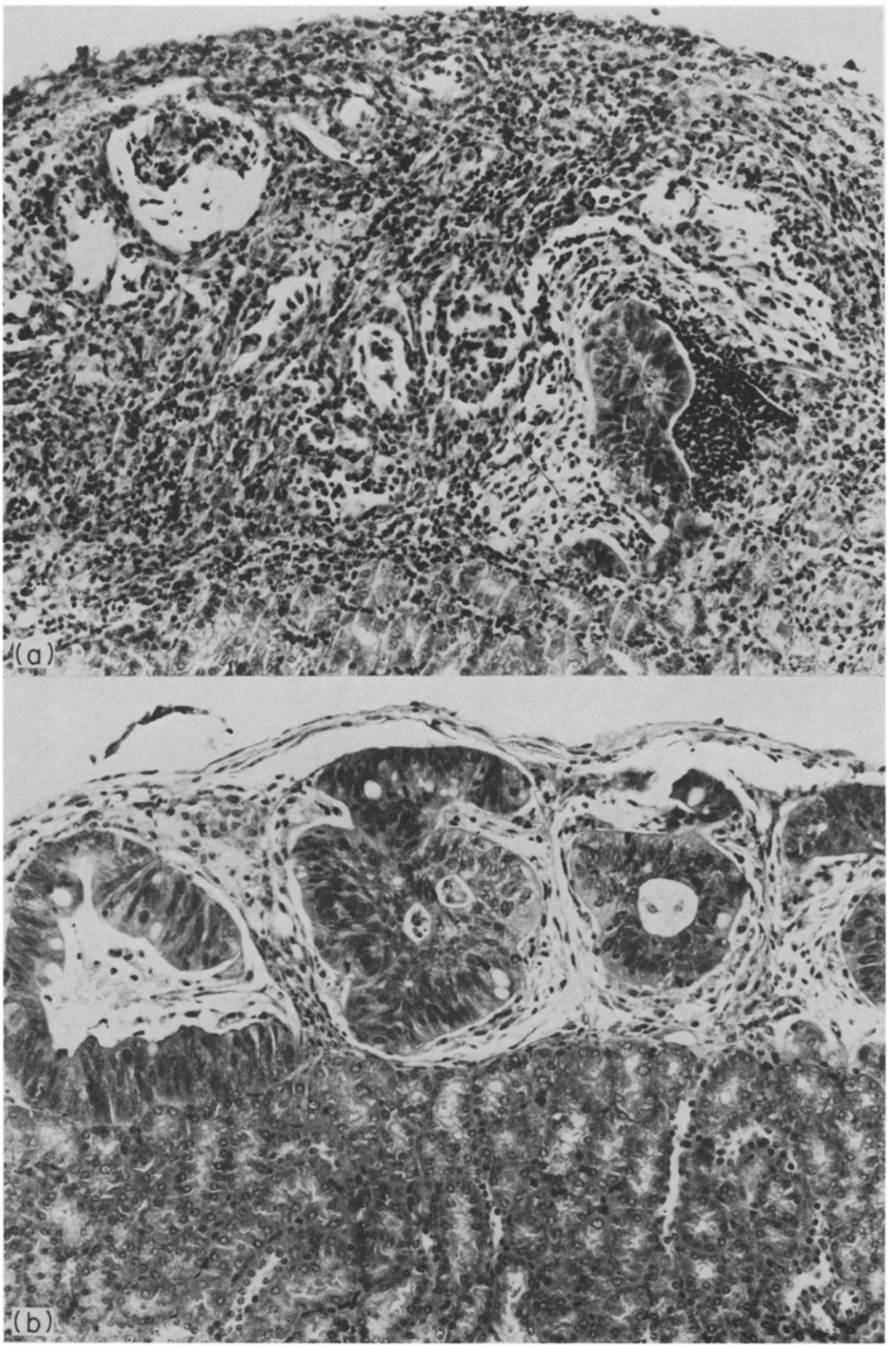


Fig. 2 (a,b)

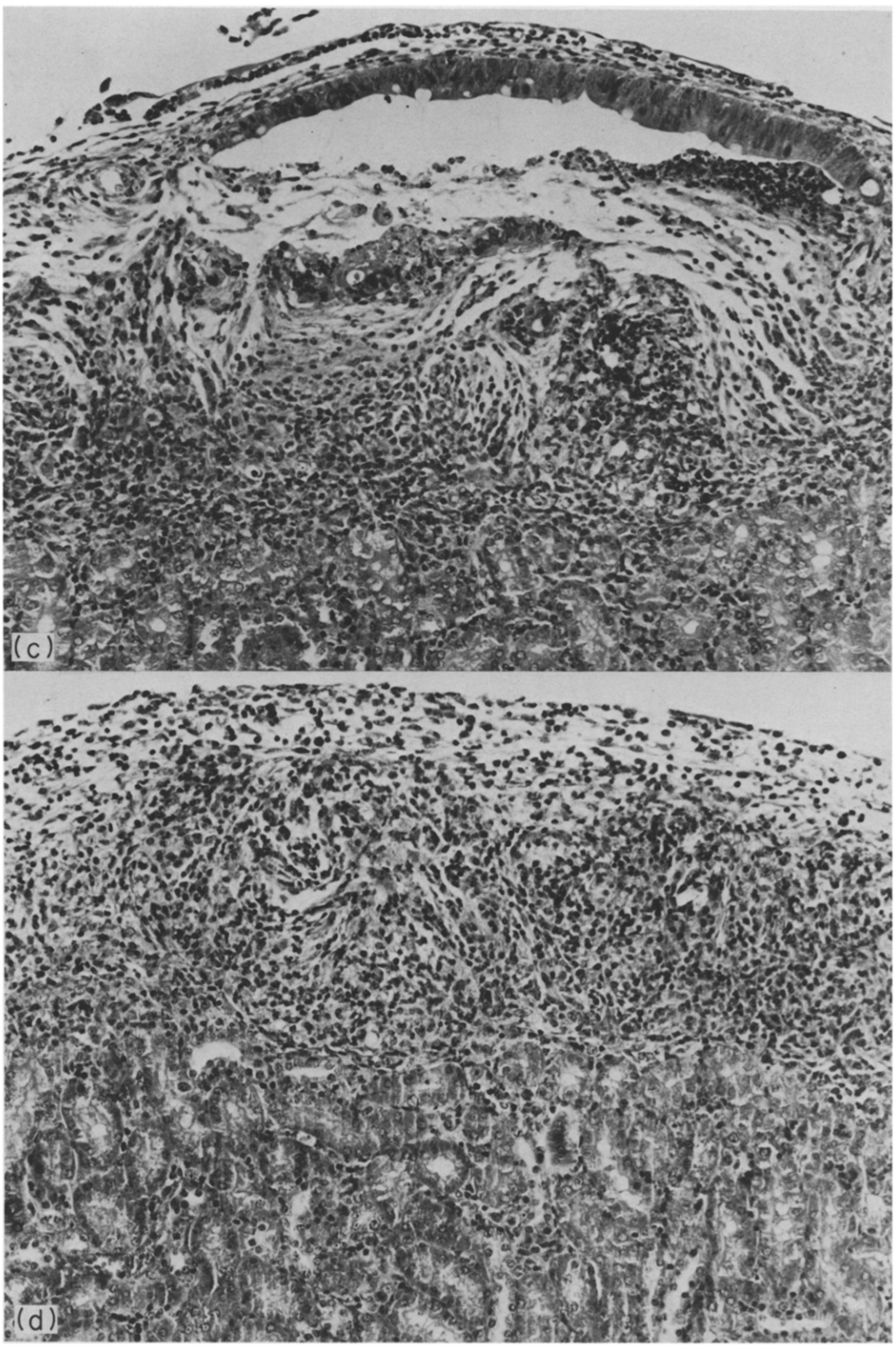


Fig. 2. Photomicrographs of the site of subrenal implantation of ovarian tumour S in normal mice 6 days *after implantation. Panel A, untreated control; panel B, CY-treated; panel C, cisPt-treated; panel D, ADM-treated. Overwhelming host cell infiltration is apparent in all but the CY-treated group.*

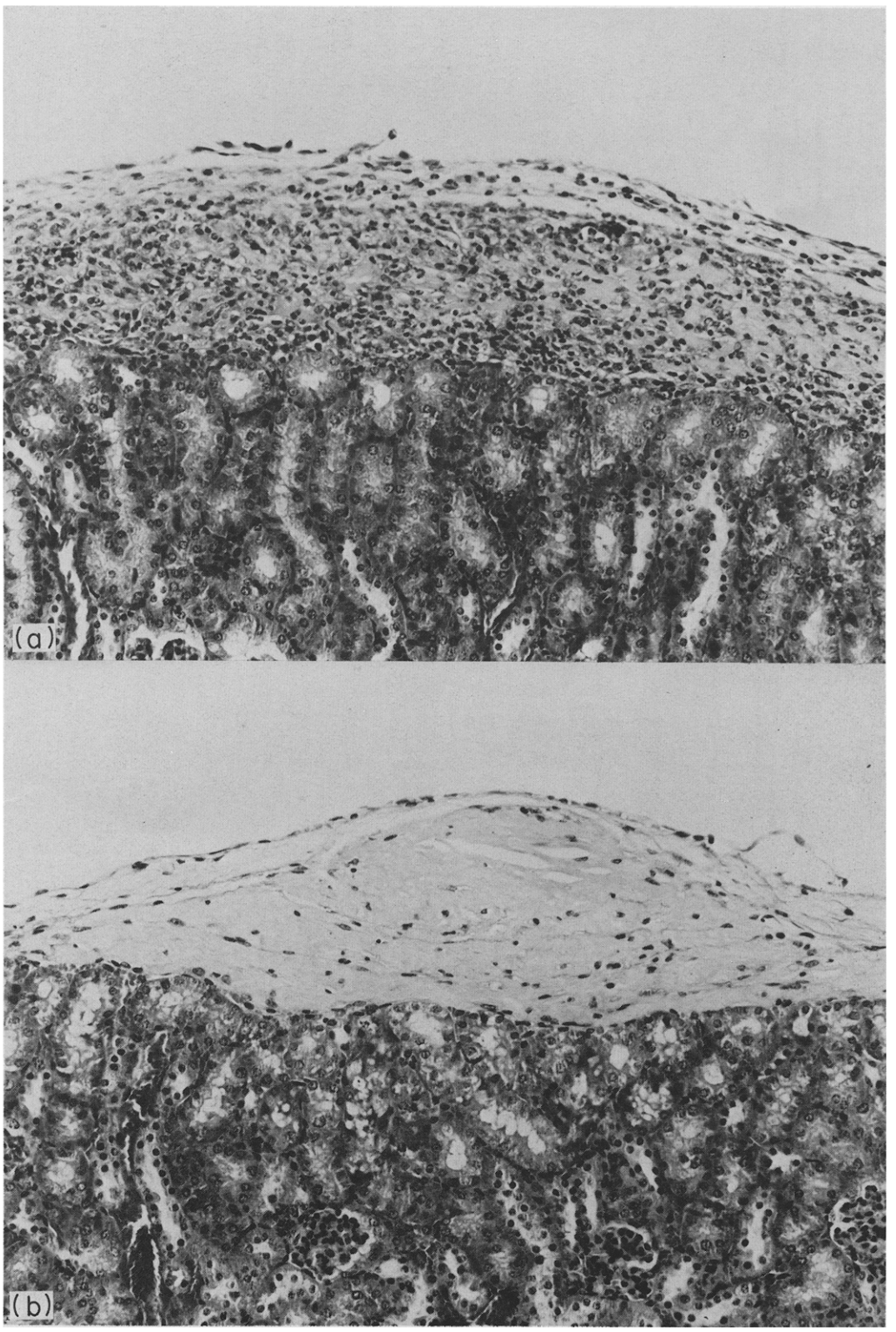


Fig. 3 (a,b)

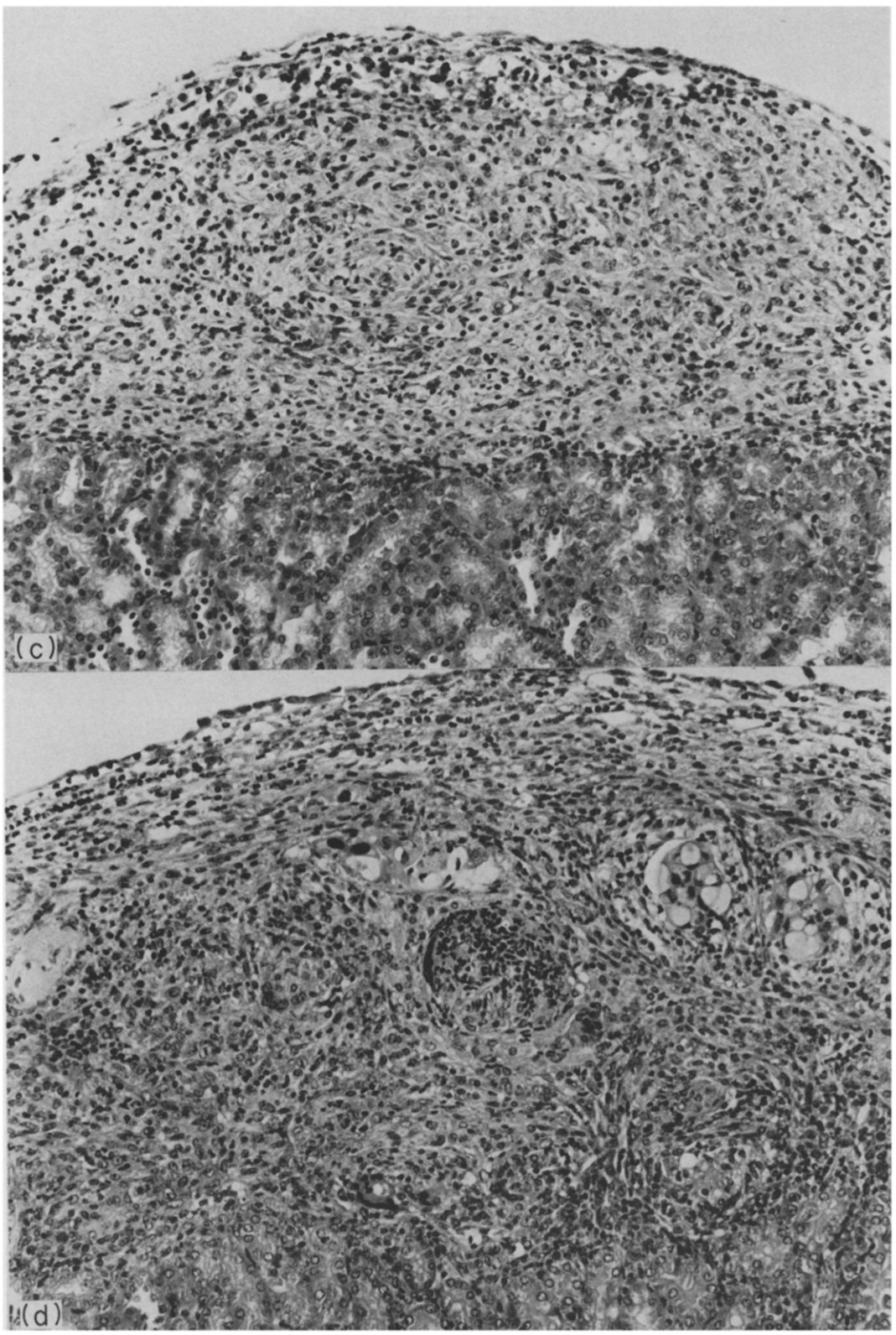


Fig. 3. Photomicrographs of the site of subrenal implantation of ovarian tumour P in normal mice 6 days *after implantation. Panel A, untreated control; panel B, CY-treated; panel C, cisPt-treated; panel D, ADM-treated.*

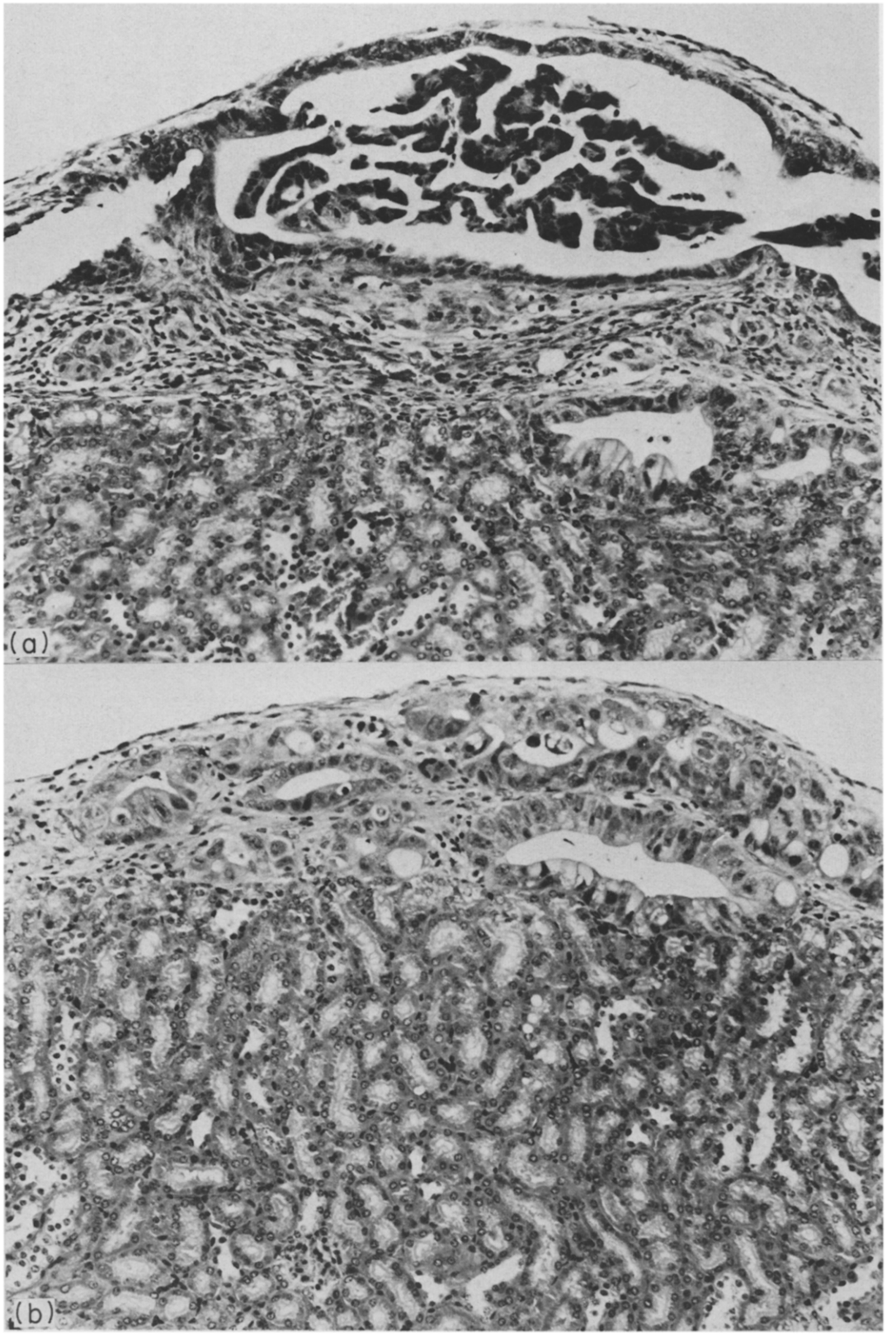


Fig. 4 (a,b)

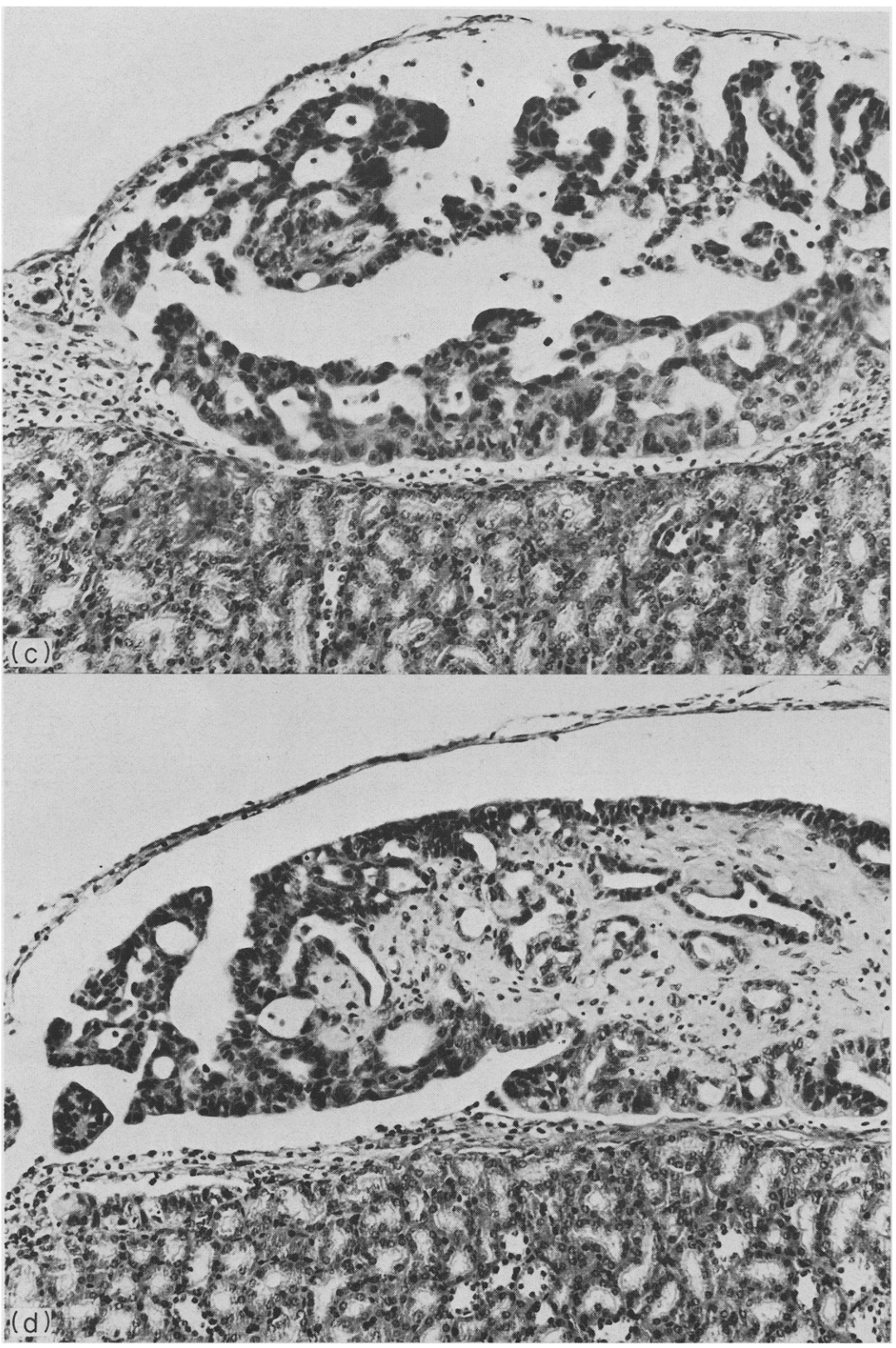


Fig. 4 (*c*,*d*)

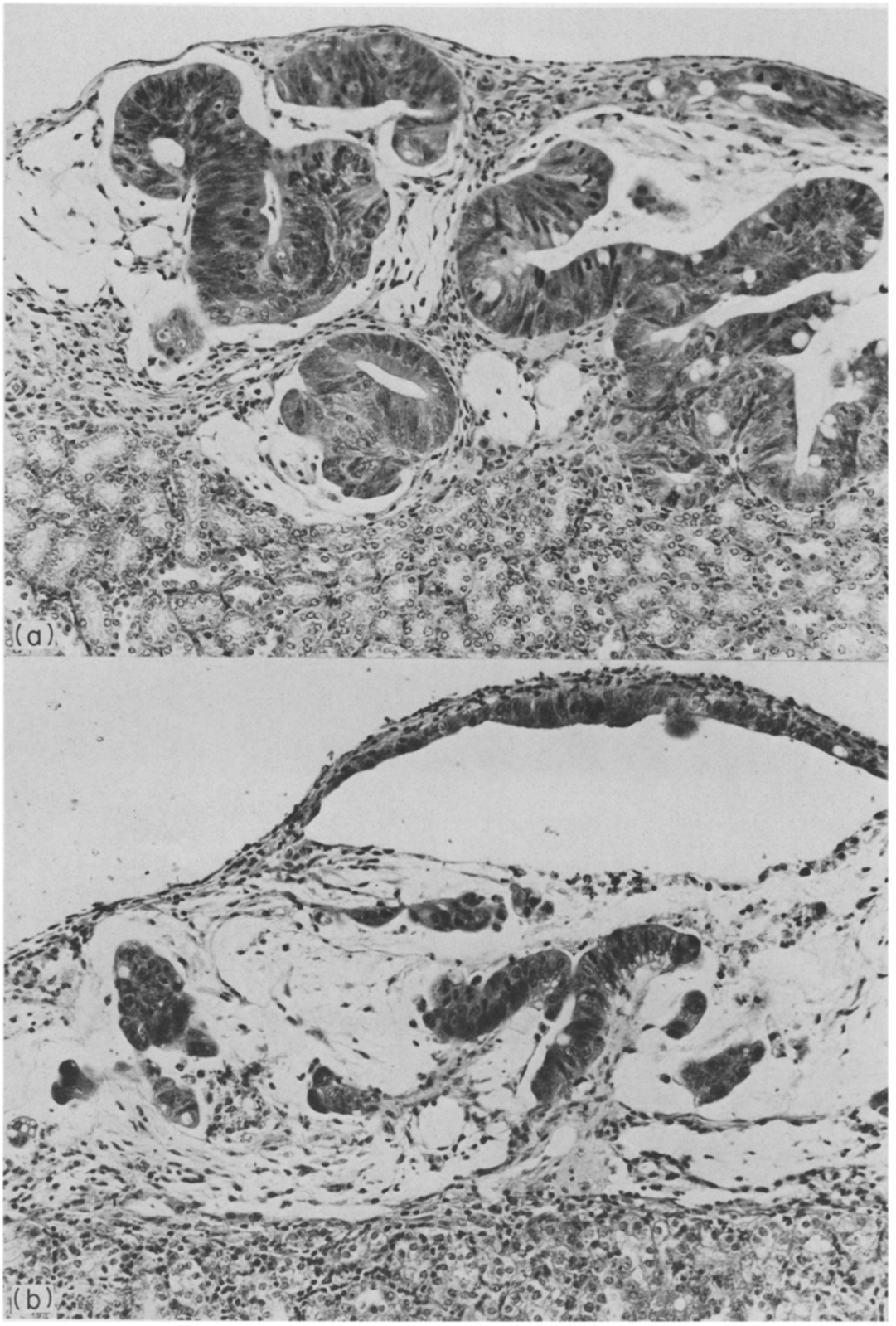
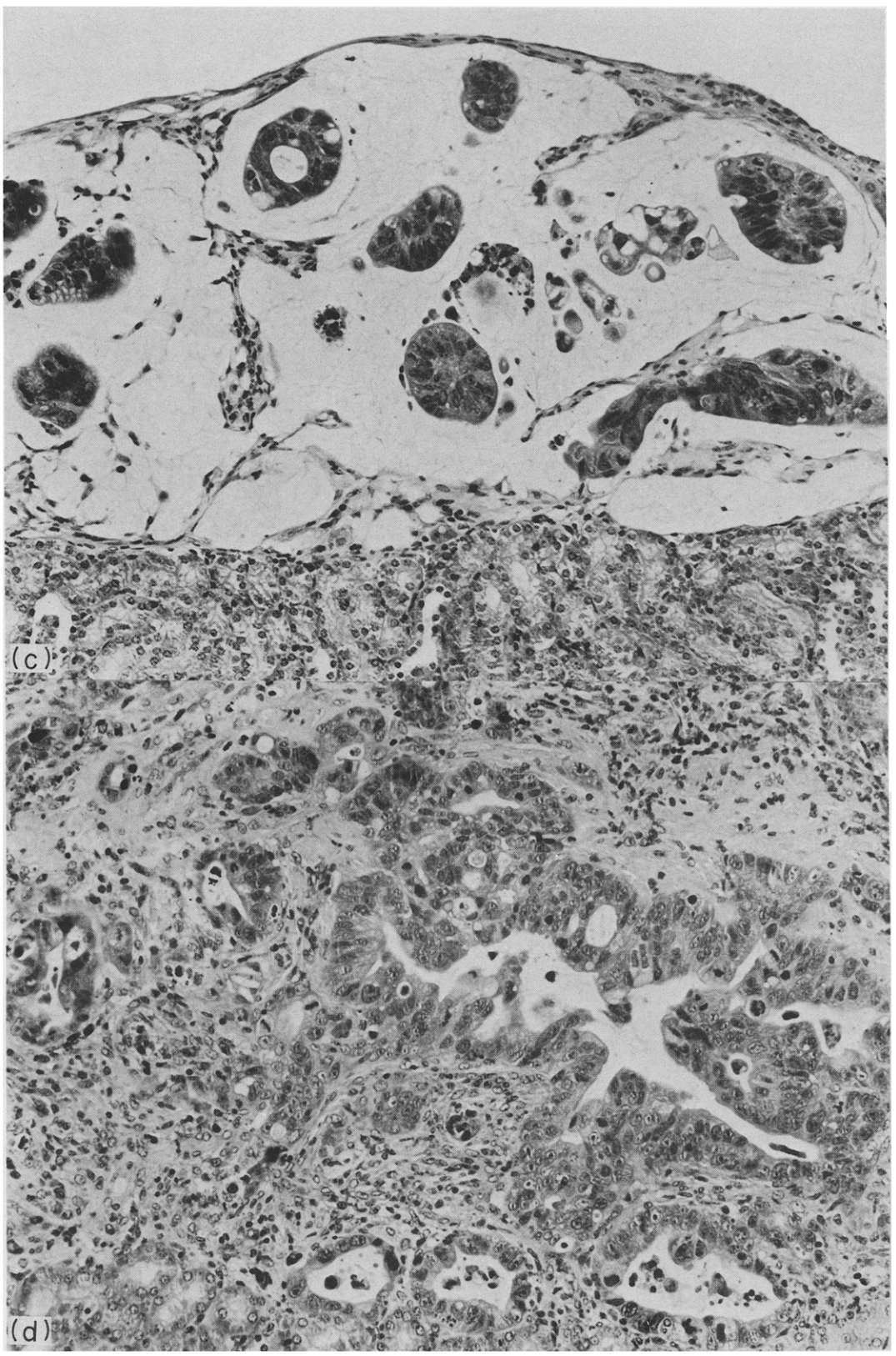


Fig. 5 (a,b)



Figs 4 and 5. Photomicrographs of the site of subrenal implantation of ovarian tumours P and S in nude mice 6 days after implantation. Panel A, untreated control; panel B, CY-treated; panel C, cisPt-treated; panel D, ADM-treated. In comparison to figs 2 and 3, markedly clear tumour histology is apparent without interference by host cells.

lymphocytes and macrophages in animals treated; the macroscopic evaluation measured 'pure' tumour or fibrotic debris.

Two examples of these remarkably consistent effects can be seen in Figs 2 and 3 for two different primary ovarian tumours, each treated with no drug (control), CY, ADM or cisPt. The 'purest' tumour (or tumour rest) sections are seen in the CY-treated group; in the untreated controls infiltration of lymphocytes and fibrosis are apparent. In the cisPt and ADM groups far more infiltration is apparent, and it is also quite clear that the bulk of the 'tumour' measured is not formed by tumour cells. When these same tumours were tested for their response to the same drugs using nude mice quite different responses were seen.

The experience obtained from multiple replicated experiments demonstrating resistance of ovarian tumours P and S to ADM and cisPt could not be duplicated for nude mice. In Table 3 tumour P appears significantly sensitive to cisPt and tumour S becomes significantly sensitive to ADM when tested in nude mice, with some variation noted between different passages. The apparent CY effects on tumour S in normal mice would appear to be largely through reduction of host cells, since the tumour in nude mice failed to respond. Unresponsiveness is unlikely to have arisen from the reduced dosage used, since sensitivity to ADM at similarly reduced dosage occurred. Histologic specimens were equally striking (Figs 4 and 5) by the absence of leukocyte infiltration in both the untreated control and the treated groups of nude mice. The 3 colon and 2 lung carcinomas studied showed all the same features. Differences in relative tumour size and the degree of host resistance and fibrosis can be seen in Table 4 for the lung and colon xenografts in nude and normal mice. In immunocompetent mice most or part of the tumours were replaced by granulation tissue due to an immune-mediated inflammation. The residual tumour cells showed signs of degeneration and necrosis, and no mitoses were observed. Small amounts of fibrous tissue containing blood vessels were also seen. In

Table 3. Responsiveness of two ovarian tumours to cytostatics in normal or nude mice

	XOv.P				XOv.S			
	Normal		Nude		Normal		Nude	
Passage:	1	2	6	8	1	4	2	4
10 mg/kg ADM	94	140	58*	84	128	104	54*	69
5 mg/kg cisPt	82	98	69	67*	146	102	97	57
200 mg/kg CY	70	64	60*	95	28*	53	80	95
175 mg/kg 5FU	70		34*		62		76*	

Figures represent $\frac{\text{surface area of treated sample}}{\text{surface area of contol}} \times 100$.

*Significantly different from control with the Mann-Whitney *U* test.

Table 4. Histological analysis of tumours growing under the renal capsule of immunocompetent and nude mice

Tumour No.	Mouse	Relative contribution of: relative tumour size on day 6	tumour cells	host resistance cells	Fibrosis
CXF 243/4	normal	3.27	±	++	+
	nude	2.16	++	-	+
CSF 164/12	normal	2.00	-	+++	+
	nude	1.49	+++	-	+
CSF 158/11	normal	2.23	±	+++	+
	nude	1.35	++	-	+
LXF 211/6	normal	0.97	-	+++	±
	nude	1.08	+++	-	+
LXF 247/2	normal	1.60	±	+++	-
	nude	0.81	++	-	-

CXF and LXF, colon and lung cancer xenograft Freiburg. The figure after the tumour No. represents the passage of the tumour. Tumour size was expressed as the product of the 2 diameters, relative values on day 6 were calculated by size on day 6/size on day 0.

contrast, the tumours growing in nude mice consisted of viable tumour cells and of small amounts of fibrous tissue. No granulation tissue was observed. We next analysed the influence of host immunocompetence on the growth of the same tumours in the control situation. The untreated control growth is, of course, a crucial parameter since not only are all treated tumour areas compared to the area of the control in order to determine response, but it may also be the best indicator of the validity of a given test, since no conclusion with regard to tumour effect can be drawn in the absence of control tumour growth. In Tables 4 and 5 the macroscopic evaluation of growth in the nude and normal mouse of ovarian tumours P and S and of colon and lung xenografts (Freiburg) can be seen. At the 6-day assay point tumours originating from these tumours are larger in normal than in nude mice and a significant difference can be seen between the tumour volumes of ovarian tumour P. This result was also obtained with a colon tumour (unpublished data, Rijswijk) and formed the basis of our decision to use normal mice. However, in view of the differences between the histology seen in Figs 2 and 3 in immunocompetent mice and in Figs 4 and 5 in nude mice and the data of Table 4, this decision may not have been justified.

DISCUSSION

The findings of variable lymphocyte infiltration with consequent obliteration of tumour in the control specimens and drug-dependent modifications of both tumour and host cell volume brings into question the usefulness of the subrenal capsule assay in normal mice for either retrospective or prospective sensitivity testing. Certainly a histological verification of the macroscopic results seems useful, since in our series there were 15/15 negative tests for cisPt and 14/15 for ADM in normal mice. These results were termed negative since no response could be scored macroscopically, while there was clear evidence of activity in the microscopic sections and, as shown, the same tumour in nude mice could be shown to respond. The responses in the nude mice occurred despite a lower drug concentration, and in the testing of tumours of uniformly low and sometimes identical passage (Table 3). The ultimate evaluation of the accuracy of the assay is dependent, of course, on the tumour response in the patient. Preliminary data would suggest that the subrenal capsule assay can perform well using this criterion [6].

Our results could be questioned on the basis that our treatment schedule and methods of evaluating drug responses differ from those found in the published literature. However, our total doses are nearly identical to the total doses suggested and the histological findings are unlikely to be influenced by the treatment schedule used. Further, we have compared the two methods of response analysis and have found only minor differences. Further, our conclusions are based on the testing of primary patient material and may be of lesser importance to screening with established tumour lines.

The striking effects of CY on host resistance and local inflammatory infiltration have encouraged us to evaluate the pretreatment effects of a single CY injection. This may be a manner in which macroscopically negative results can be avoided without otherwise affecting the results of sensitivity testing. Low-dose total-body irradiation may also prove beneficial. Preliminary results are encouraging.

Further research seems warranted but unquestioning acceptance of the macroscopical evaluation would seem misplaced when normal mice are used for the subrenal capsule assay.

Table 5. Mean growth (surface area) of tumour under the subrenal capsule after 6 days

Ovarian tumour	Immunocompetent 'normal'		Immunodeficient 'nude'
XOv. P	1.95 ± 0.107	$P < 0.01$	1.38 ± 0.1007
XOv. S	2.11 ± 0.135	n.s.	1.32 ± 0.76

REFERENCES

1. BOGDEN AE, KELTON DE, COBB WB *et al.* A rapid screening method for testing chemotherapeutic agents against human tumor xenografts. In: HOUCHENS DP, OVEJERA AA, eds. *Proceedings of the Symposium on the Use of Athymic (Nude) Mice in Cancer Research.* New York, Gustav Fisher, 1978, 231–250.
2. GRISWOLD DP, DYKES DJ, CORBETT TH *et al.* The subrenal capsule assay as a sensitivity test for determination of the response of individual tumors to specific cancer chemotherapeutic agents. *Proc Am Assoc Cancer Res* 1982, **23**, 221.

3. BOGDEN AE, COBB WB, LEPAGE DJ *et al.* Chemotherapy responsiveness of human tumors as first transplant generation xenografts in the normal mouse. *Cancer* 1981, **48,** 10–20.
4. BOGDEN AE. Chemotherapy sensitivity of human breast tumors in the 6-day subrenal capsule (src) assay. In: *Proceedings of the 4th Annual San Antonio Breast Cancer Symposium.* 1981, Abstr. 6.
5. BOGDEN AE, HASKELL PM, LEPAGE DJ *et al.* Growth of human tumor xenografts implanted under the renal capsule of normal mice. *Exp Cell Biol* 1979, **47,** 281–293.
6. STRATTON JA, BRALY PS, RETTENMAIER MA *et al.* Predicting chemotherapeutic sensitivity for human ovarian tumors: a comparison of three assays. In: *Proceedings, 13th International Cancer Congress.* 1982, Abstr. 337.