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Subrenal Capsule Assay for Fresh Human Tumors in Immunocompetent Mice; an Inapproriate Technique for Non-small Cell Lung Cancer

E.A. TUENI,*† P. DUMONT,* D. JACOBOVITZ,‡ G. ATASSI,* P. ROCMANS,§ F. LEJEUNE,‖ P. DE FRANQUEN,§ P. SEMAL* and J. KLASTERSKY*

**Service de Médecine et Laboratoire d'Investigation Clinique H.J. Tagnon, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium, ‡Laboratoire de Cytologie et de Cancérologie Expérimentale, Université Libre de Bruxelles, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium, §Service de Chirurgie Thoracique, Cliniques Universitaires de Bruxelles, Hôpital Erasme, Route de Lennick, 1000 Bruxelles, Belgium and ‖Laboratoire d'Oncologie et de Chirurgie Expérimentale, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium*

Abstract—*The subrenal capsule assay (SRA) seems to present some difficulties for the evaluation of the chemosensitivity of antineoplastic agents against fresh tumor xenografts. A study was therefore carried out to verify whether two different xenografts would behave in a similar way. Tumors such as melanoma provided adequate homogeneous material for this technique, while non-small cell lung carcinoma (NSCLC) were heterogeneous since the* 1 mm^3 *specimen grafted under the renal capsule usually contained diffuse areas of necrosis and of infection. Furthermore, a large proportion of the grafted specimens,* 257 *out of* 298, *did not contain any tumor at all on microscopic examination even when they showed macroscopic growth. Added to these discrepancies, a random microscopic analysis of* 180 *adjacent fragments of NSCLC and melanomas demonstrated that the variability of heterogeneous tumors precludes any meaningful comparison between homologous tumor tissues designed to be grafted on treated and on control mice. The anti-inflammatory effect of chemotherapeutic drugs on the host's reaction to the graft is probably responsible for the differences between the macroscopic growth results observed in pieces grafted to both treated and control mice: however, it could not be simulated by hydrocortisone (HC) under our experimental conditions. This allows us to conclude that fresh tumors from NSCLC cannot be used in SRA.*

INTRODUCTION

THE DEVELOPMENT of the 6-day subrenal capsule (SRC) assay to study the chemotherapeutic sensitivity of human tumor xenografts in nude and in immunocompetent mice by Bodgen *et al.* [1–3] generated considerable enthusiasm [4–9]. Essentially, it was based on the fact that the macroscopic changes in the grafted specimens correlated well with clinical results obtained by using the same drugs in the corresponding tumors. Other investigators, however, performed histological analysis on the grafted material and demonstrated that even when human tumor cell lines were used, the macroscopic results did not always correlate with the microscopic evaluation [10–15]. These findings were confirmed by a previous study performed in our institution with *fresh* surgical explants of ovarian and non-small cell lung cancers [16]. One hypothesis that could explain the positive macroscopic results is that the grafts produce a rapid inflammatory reaction in the host mimicking the 'growth' of the tumor [13, 15, 16] which can be reduced or suppressed in the immunocompetent mice through the anti-inflammatory or immunodepressant effects of chemotherapy.

In this study, we have investigated a possible anti-inflammatory and immunosuppressive effect of the results of the SRC assay of hydrocortisone acetate, in comparison with cisplatin and etoposide [16], using fresh material from non-small cell lung tumors. Therefore a microscopic analysis of this material was performed and a comparison made with fresh melanoma samples. These 2 neoplasms were selected in order to compare a relatively homogeneous tumor (melanoma) and a heterogeneous (non-small cell lung cancer) one.

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†To whom requests for reprints should be addressed.

MATERIALS AND METHODS

SRC assay

Surgically removed non-small cell lung cancers served as fresh human tumor xenografts for implantation under the renal capsule of immunocompetent mice (male BDF_1 C57BL/6 × DBA/2 F_1 or $B_6C_3F_1$ C57BL /6 × C_3HF_1). The human tumors were fixed in Hanks media with supplemental antibiotics (penicillin and streptomycin) during transport. The longest interval from surgery to implantation was 8 h.

Initially the surgical specimen were separated, under a stereoscopic microscope fitted with an ocular micrometer, into fragments measuring approximately 1 mm^3. Following the technique described by Bogden *et al.* [2], the mouse flanks were shaved and the animals were anaesthetized with a 10% Avertine® (2,2,2-tribromoethanol) solution injected intraperitoneally. The technical details of the assay have been previously reported by our group [4].

A minor modification was introduced to allow histological evaluation of the quality of the graft prior to treatment (day 0). Instead of cutting the tumor specimen into 1 mm^3 pieces, the specimen was initially divided into fragments measuring 3 mm^3. One third of this fragment was fixed in formalin for histology; one third was implanted into a specifically designated mouse to which the drugs were administered and the last third was grafted under the renal capsule of a control mouse. The primary tumor sample, from which the implanted fragments were taken, was also fixed in buffered formalin. The mice were provided by Charles River Breeding Laboratories (Massachussets, USA).

1. *Evaluation of treatment results.* Change in tumor size (DTS), expressed as the mean tumor diameter on day 6 minus day 0, was calculated for each graft. The variation in tumor size was also expressed as a percentage relative to the initial day 0 size as shown below:

$$\frac{DTS}{TS_o} \times 100 = \frac{\text{tumor size on day 6} - \text{tumor size on day 0}}{\text{tumor size on day 0}} \times 100.$$

For an experiment to be considered assessable, control animals needed to show a DTS $\geq$ 0.5 ocular micrometer units (o.m.u.). In addition, animals with weight loss > 20% of initial body weight during the 6-day assay period were considered as non-assessable because of toxicity. For the treated groups, regression $\geq$ 20% of day 1 size was considered as a response to therapy.

2. *Drugs.* Based on studies performed by Aamdal *et al.* [5] and on our previous experiments [16], the drugs were administered on a day 2 + 3 schedule. The mice were weighed on day 2 prior to treatment and again on day 6. Cisplatin (DDP) was administered at the dose of 4 mg/kg/day, etoposide (VP-16) at the dose of 30 mg/kg/day and hydrocortisone acetate (HC) at the dose of 125 mg/kg/day.

There were 3 mice per treatment group, each having its own control.

3. *Histological evaluation of the grafts.* Serial sections (4 μm thick) were cut through the entire subrenal graft at intervals of 100–300 μm and stained with hematoxylin and eosin. All day 6 grafts of treated mice and of untreated controls were examined by the same pathologist (D.J.). An estimation was made of the areas of each graft that were constituted by human tumor or mouse cell infiltration and fibrosis by juxtaposing representative serial sections. A single mean value of these estimates was calculated for controls and for each treatment group. Serial comparison was made between the fragments grafted to the treated mice and the corresponding and adjacent fragments grafted in control mice and finally to the fragments kept in formalin at the onset of each experiment. This procedure permitted to verify the quality of the graft and to compare the day 0 to the day 6 histologic findings.

4. *Quality of sampling.* For this study, we used a fresh squamous cell lung carcinoma 10 h after the patient's death.

The fragment was taken from a presumably rapidly growing part of the tumor. After removing the necrotic and/or the infected areas, one part of the sample was separated in 20 fragments measuring 2 mm^3. Each of these fragments was then divided into 2 equal parts, as if they were destined to be grafted into experimental and control mice. All these fragments were fixed in formalin and used for histological analysis. In a similar way, 3 nodal metastases from 3 patients presenting with advanced melanoma were used. Fifteen pieces of 2 mm^3 were separated from each node and then subdivided into 2 corresponding equal parts of 1 mm^3. All these fragments were fixed in formalin and used for histological analysis.

RESULTS

SRC assay

1. *Macroscopic results.* One of the 12 experiments was not evaluable because of early deaths in control mice. The results of the 11 evaluable cases are

Table 1. Macroscopic evaluation of the grafts under the subrenal capsule

Case	Cisplatin 4 mg/kg	VP-16 30 mg/kg	Cortisone 125 mg/kg	Overall control value ($n = 9$)
1	49%	−9	−14	
Control	+19	−11 R	−3%	+5% (+0.6) ($n = 3$)
2	+17%	+8%	−15%	
T control	+28%	+12%	−1%	+11% (+1.5)
3	−40%	0	0	
T control	+10%	0	15%	+14% (1.74)
4	0	−1	0	
T control	−19 R	+32	−12 R	−1% (−0.13)
5	−10	+1	+5	
T control	+18	+13	−2	+9% (1.16)
	+1	−16	−11	
T control	−7 R	−4	−6 R	−1% (−0.15)
7	+27	+12	+10	
T control	+28	+1	+27	+18% (2.06)
8	11	10	4	
T control	4	22	7	+11% (1.23)
10	−6	−13	0	
T control	+18	+10	+11	+14% (1.87)
11	−17	−24	−4	
T control	−20 R	−8 R	−9 R	−12% (−1.44)
12	−8	+1	−2	
T control	−2	+19	+8	+8% (+1.17)

Table 2. Specimens containing tumor cells after microscopic analysis

Case	Fragments 1	Fragments 2 (chemotherapy or hydrocortisone)	Fragments 3 (control)
1	0/9	0/9	0/9
2	0/9	0/9	0/9
3	6/9	5/9	4/9
4	3/9	1/9	0/9
5	6/9	0/9	0/9
6	0/9	0/9	0/9
7	0/9	0/9	0/9
8	0/9	0/9	0/9
10	0/9	0/9	0/9
11	0/9	0/9	0/9
12	9/9	4/9	3/9
Total	24/99	10/99	7/99

reported in Table 1. By the criteria of Bogden, described above for the macroscopic evaluation of the results, case No. 11 should be excluded because of spontaneous regression of the control (> 5%). This makes 10/11 cases (90%) evaluable, if one puts together all the control mice in the same group as shown in the last column. One can see that only 2 tumors (1 and 3) responded to DDP (the response criteria being a regression of at least 20% of the tumor); no explant responded to VP-16 or to HC.

If one analyses the cases individually, i.e. each group of treated mice with respect to its own control, one can see that for DDP, cases 4, 6 and 11 had to be excluded because of spontaneous regression of the tumor in the control. Cases 1 and 3 can still be accepted for response which makes a response rate of 2/8 (25%). There were 0/9 response for VP-16, cases 6 and 11 being rejected; and 0/8 response for HC, since cases 4, 6 and 11 had also to be rejected.

2. *Microscopic results.* Microscopic analysis of almost 100 subrenal capsule grafts in the treated mice, and of 100 corresponding grafts in control mice and of 100 fragments kept in formalin from the onset of each experiment, was performed. Of these 300 histological pieces, 41 contained viable tumor. As shown in Table 2, 24/99 formalinized fragments contained tumor, 10/99 of the fragments grafted to treated mice contained tumor and 7/99 fragments grafted to control mice contained tumor.

A closer analysis revealed that among the 99 fragments of tumor of 3 mm^3 which were initially

separated into 3 equal parts, only 3 contained tumor in all the 3 parts. Three others contained tumor in fragments 1 and 3, i.e. the ungrafted part and part grafted to control mice, whereas 6 others contained tumor in fragments 1 and 2, i.e. ungrafted specimen and specimen grafted to the treated mice. Therefore, even if we accept the 3 cases where tumor was found in the control and in the untreated mice, on the assumption that treated mice may have eradicated their tumor cells, only 6% of the tumor specimens contain tumor in both treated mice and controls.

An overall estimate of fibrosis, degree of acute inflammation, necrosis, granulation phenomena and infiltration by monocytic cells was also performed. Little, if any, difference was observed for all these processes except inflammation. This was almost twice less prominent in the grafts coming from mice who were treated by DDP or VP-16 than in control grafts or in those coming from mice treated with HC. Necrosis was found in only 3/198 grafted fragments.

2. *Quality of sampling.* The microscopic study of the 20 fragments of 2 mm^3 separated into 2 equal parts coming from a fresh sample of squamous cell carcinoma of the lung, did not reveal any tumor cells in any of these 40 parts, whereas all the 2 fragments of 45 from melanomas contained tumor.

DISCUSSION

The macroscopic results obtained in the SRA described by Bogden *et al.*, and subsequently applied by many investigators, do not correlate with microscopic findings, especially when dealing with specimens from heterogeneous tumors containing areas of necrosis and fibrosis. The graft, which is evaluated on day 6, indeed contains inflammatory tissues due to the host reactions in immunocompetent animals. The difference between treated and control mice might be due to a decrease of this inflammatory reaction through chemotherapy.

In our experiment, we tried to mimic such an anti-inflammatory effect of chemotherapy by using hydrocortisone acetate. We performed our experiment on fresh specimens from non-small cell lung carcinoma. Under the criteria defined by Bogden *et al.*, the overall macroscopic results were unsatisfactory: 2/11 tumors responded to DDP but none to VP-16 or HC. The microscopic analysis which was carried out showed that only 41/300 tumor pieces of 1 mm^3 contained a tumor. Moreover, tumors were present in fragments from the same 3 mm^3 initial piece in only 12/99 cases, only half of them being adjacent one to the other.

Microscopic examination demonstrated that the inflammatory component of the subrenal tumors in the mice treated with DDP or VP-16 was almost one half compared to controls or to those receiving HC. These findings (macroscopic and microscopic) did not confirm our thesis that HC would mimic chemotherapy by diminishing the inflammatory host response, at least with the dosage used here.

In the second part of this study, we confirmed what others have already described concerning the discrepancies between macroscopic and microscopic results. The double quality control of the graft specimens proved that for heterogeneous tumors such as NSCLC, it was unlikely that one would find tumor in most of the adjacent fragments prepared for grafting. No correlation was possible between tumors grafted to treated animals and those grafted to controls since they rarely, if ever, contained homogeneous tumor material.

In order to ensure that a time delay in processing surgical specimens was not responsible for these findings, we performed a similar analysis on NSCLC samples collected at autopsy in our institution under optimal conditions. We also studied samples from 3 cases of melanoma obtained in our hospital. The microscopic findings correlated well with the first part of our experiments, since 0/40 1 mm^3 samples from the autopsy NSCLC contained malignancy, whereas 90/90 pieces obtained from the melanoma contained the tumor.

These experiments, therefore, confirm previous reports concerning discrepancies between the macroscopic and the microscopic results obtained with the SRA in immunocompetent mice. These are due to large variations in the sampling of tumor tissue especially in heterogeneous tumors such as NSCLC.

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