

The effects of regional factors on the growth rate and the differentiation of mouse teratocarcinoma

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Summary Murine embryonal carcinoma cells, the pluripotent stem cells of teratocarcinoma were injected simultaneously into caudal and cranial sites on the back of syngeneic recipients in order to determine whether regional anatomical differences affect their take and growth rate and differentiation. The overall tumour take rate was higher in caudal than cranial sites, but the initial weight of tumours was higher in the cranial than caudal sites. Tumours developing in the two anatomical sites grew at the same rate with a linear increase in volume. At the end of the 4-week experimental period the differences in the size of anterior and posterior tumours were negligible and no histological differences were noted between the two groups. Our data indicate that regional factors significantly affect the take rate and the the initial growth of this murine teratocarcinoma, i.e. the establishment of solid tumours from injected stem cells. The growth rate of established tumours was not affected by regional factors.

Human teratomas are a diverse group of tumours originating in gonads and many other extragonadal locations (O'Hare, 1978). Testicular tumours are predominantly malignant (Pugh, 1976); the ovarian are most benign (Scully, 1979), whereas, the proportion of malignant and benign extragonadal tumours varies from one site to another (O'Hare, 1978). Thus, most sacrococcygeal teratomas are benign, for example, and most tumours of the anterior mediastinum are malignant. The factors determining and controlling the malignancy of human teratomas are poorly understood, although, by analogy with animal teratomas, one could speculate that various anatomical, immunological, endocrine and metabolic regulating mechanisms could be involved (Solter & Damjanov, 1979).

Since teratomas produced from mouse embryos represent an excellent replica of human neoplasia (Solter & Damjanov, 1979), we have used a transplantable, malignant murine teratoma (henceforth referred to as "teratocarcinoma") to study the possible effects of regional factors on growth rate and malignant behaviour in this tumour system.

Materials and methods

Tumours

All experiments were carried out with a new pluripotent, slightly aneuploid mouse terato-

carcinoma cell line (NF-1) that was recently established in our laboratory from a retransplantable BALB/c embryo-derived tumour (Fox *et al.*, submitted; Oosterhuis, 1983). The cells are routinely grown in Falcon tissue culture flasks in Dulbecco's modified Eagles medium (Gibco) containing 15% foetal calf serum (FCS) (Gibco) and antibiotics.

The tumour cells grown *in vitro* are adherent and have the typical features of murine embryonal carcinoma (EC) cells, the pluripotent stem cells of teratocarcinoma (Martin & Evans, 1974). One million cells injected s.c. into adult isogenic mice produce solid tumours in 85-90% of animals. The tumours consist of EC cells and various differentiated tissues. The ratio of undifferentiated and differentiated tissues is usually 2-3:1. The tumour-bearing animals die 8-12 weeks after inoculation of 10^6 cells and 6-10 weeks after inoculation of 5×10^6 cells, due to progressive local tumour growth. We never observed cessation of tumour growth due to complete terminal differentiation of tumour tissue.

Injection

Virgin, female BALB/c mice were obtained from Jackson Laboratory, Bar Harbor, Maine. All the experiments were performed on 6-8 week old animals. The animals were anaesthetized with Ketalar and the tumour cells were injected with a 26-gauge needle s.c. Tumour cell suspensions for injection were prepared by trypsinization of subconfluent cultures. They were resuspended in medium containing FCS, then washed two times in 0.9% saline and finally resuspended in 0.9% saline at concentrations of either 10×10^6 or 50×10^6

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cells ml⁻¹. The tumour cell suspensions thus prepared contained almost exclusively single cells and were virtually 100% viable. The volume of injected fluid was always 0.1 ml per inoculum. Each animal received two s.c. injections simultaneously, each containing either 10⁶ or 5 × 10⁶ cells. The injections were administered in the midline into the tissue overlying the thoracic vertebra (cranial site) or lumbar vertebra (caudal site) following the protocol described in Auerbach *et al.* (1978c). The take rate was determined at 4 time intervals and the differences between the take in the caudal and cranial location were determined by the log rank test (Peto *et al.*, 1977; Breslow, 1970).

Assessment of tumour growth

At predetermined time intervals following inoculation, all the animals were anaesthetized and palpated. Detected tumours were measured with a caliper in 3 dimensions and the volume of the tumour was determined according to the formula $V = d_1 \times d_2 \times d_3 \times \pi/6$. The formalin-fixed tumours in the first experiment were also weighed after removal of the surrounding non tumorous tissue. The results were evaluated by means of Student *t* test and the Pearson correlation coefficient test.

Histological examination

All tumours were fixed in formalin and examined histologically in the plane of their largest diameter. A semiquantitative assessment of their histological features was made by roughly estimating the ratio of fully differentiated tissues *versus* all other "undifferentiated tissues" i.e. the EC cells, the loose connective tissue stroma and all components that could not be definitively identified as a distinct somatic tissue. The components of the tumour were considered as differentiated if displaying the typical histological features of the following somatic tissues that occur predictably in most solid tumours produced by s.c. injection of the NF-1 cell line (Fox *et al.*, submitted): neural tissue, squamous epithelium, glandular and ductal epithelium, fibromuscular tissue, and cartilage (Figure 1). Furthermore, morphometry was performed on all tumours obtained by injecting 10⁶ EC cells. To this end the Merz Graticule was superimposed on each tumour slide and 50–100 intersections were counted at 25 × magnification to determine the exact volume of differentiated somatic tissues. Areas composed of necrotic tissue, which rarely exceeded 5% of the total tumour volume were excluded from the morphometric analysis. The morphometric data were evaluated by the Student *t* test.

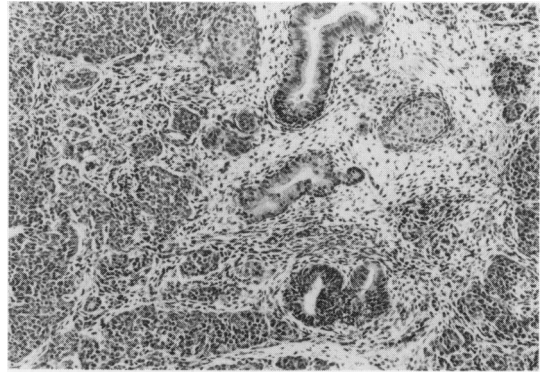


Figure 1 Area in NF-1 transplantable teratocarcinoma: well-differentiated ductal structures and foci of immature cartilage embedded in loose stroma, surrounded by solid sheets of EC cells (H and E, × 140)

Results

Take rate of tumours

Two experiments were performed: one group of animals was injected with 10⁶ cells and the second group with 5 × 10⁶ cells. The data are presented in Tables I and II, from which it may be seen that the take rate depends on the site of injection and the number of cells injected. With 10⁶ cells per inoculum the take rate was significantly higher ($P < 0.05$) in the caudal site of inoculation; moreover, the tumours emerged earlier caudally than cranially. The differences between the take rate in caudal and cranial site of inoculation were not significant with 5 × 10⁶ cells per inoculum ($P < 0.10$). However, if one combines the data from Tables I and II the differences in the take rate cranially and caudally are highly significant ($r = 7.1$ $P < 0.05$). It is of interest to note that 2/30 animals injected with 10⁶ cells did not develop tumours in either caudal or cranial sites, whereas with 5 × 10⁶ cells, all the animals developed tumours at the caudal site, but only 85% both caudally and cranially.

Both with 10⁶ and 5 × 10⁶ cells per inoculum, the caudal tumours were initially smaller than the cranial tumours ($P < 0.05$). Due to intragroup variation this difference disappeared and at the end of the experiments, there was no difference in the size of the cranial and caudal tumours (Figures 2 and 3). In both locations the tumours grew at the same rate and their volume increased linearly (r values between 0.925 and 0.999).

Histological analysis

Irrespective of their size all the tumours had essentially the same histological appearance and

Table I Take rate of tumours produced by s.c. injection of 10^6 teratocarcinoma cells

Days after inoculation	Number of animals at risk		Number of tumours			
	cranial	caudal	Observed		Expected	
			cranial	caudal	cranial	caudal
0-16	30	30	6	15	10.5	10.5
16-20	24	15	11	4	9.2	5.8
20-23	13	11	0	4	2.2	1.8
23-28	13	7	0	2	1.3	0.7
Totals			17(57%)	25(83%)	23.2	18.8

Statistical significance is determined by the log rank test for the comparison of life tables which compares observed and expected number of tumours under the hypothesis of equal life tables. For this table $\chi^2 = 3.7$, $0.05 < P < 0.10$. Using the Wilcoxon or Breslow test $z = 2.06$, $P < 0.05$.

Table II Take rate of tumours produced by s.c. injection of 5×10^6 teratocarcinoma cells

Days after inoculation	Number of animals at risk		Number of Tumours			
	cranial	caudal	Observed		Expected	
			cranial	caudal	cranial	caudal
0-13	20	20	6	10	8	8
13-16	14	10	3	4	4.1	2.9
16-20	11	6	2	3	3.2	1.8
20-24	9	3	6	3	6.7	2.3
TOTALS			17(85%)	20(100%)	22	15

Log rank test: $\chi^2 = 3.4$, $0.05 < P < 0.10$. Breslow test: $z = 1.84$, $0.05 < P < 0.10$. Combined evidence of Tables I & II, $\chi^2 = 7.1$, $P < 0.05$.

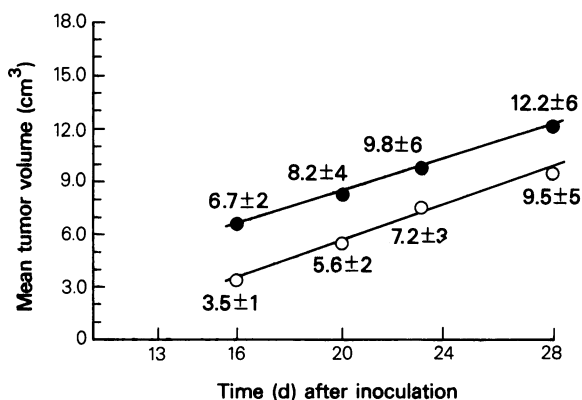


Figure 2 Growth rate of NF-1 tumours in cranial and caudal sites following simultaneous inoculation with 10^6 cells. The slope of the curve for cranial tumours was 0.46 ($r = 0.998$). The slope for the caudal tumours was 0.50 and ($r = 0.999$). Statistically there is no difference in the growth rate between the cranial and caudal tumours. The difference in the tumour size at 16 days is significant ($P < 0.05$)

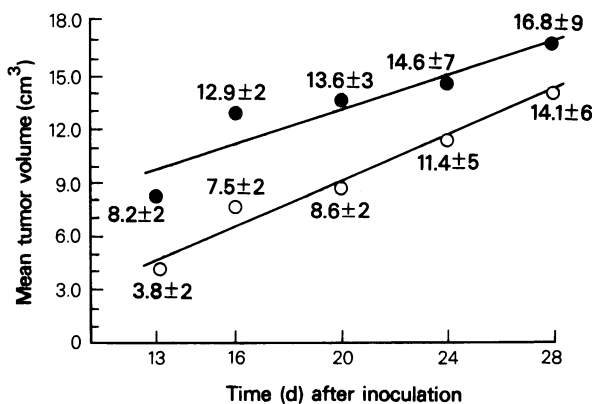


Figure 3 Growth rate of NF-1 tumours in cranial and caudal sites following simultaneous inoculation with 5×10^6 cells. The slope of the curve for cranial tumours was 0.49 ($r = 0.925$) and for the caudal tumours 0.64 ($r = 0.985$). Statistically there is no difference in the growth rate between the cranial and caudal tumours. The difference in the tumour size at 13 and 16 days is significant ($P < 0.05$).

were composed predominantly of EC cells. With few exceptions all the tumours contained at least 4 of the 5 somatic tissues that were considered to indicate differentiation of cells, but these differentiated elements rarely formed >25% of the tumour mass.

The morphometric analysis of the tumours in the first experimental group confirmed the semiquantitative estimates that there are no histological differences between the cranial and caudal tumours. The differentiated tissues formed $18 \pm 3\%$ of the volume of cranial and $17 \pm 2\%$ of the volume of the caudal tumours.

Discussion

It has been known for some time that complex regional differences regulate the evolution of carcinogen-induced skin tumours (Twort & Twort, 1936). Auerbach *et al.* (1978c) have shown that simultaneous s.c. or i.d. injections of the same number of neoplastic cells result in the formation of bigger tumours in the more cranial injection site. Regional differential growth gradients have been reported for tumour cells i.p. injected (Morrissey *et al.*, 1980) as well as for dorsally transplanted normal skin (Kubai & Auerbach, 1980). Recently, the entire problem of regional differences in tumour growth was reviewed by Auerbach & Auerbach (1982).

Prompted by the reports of Auerbach *et al.* (1978a, b, c) showing the differential growth rate of both normal and tumour cells inoculated into the cranial and caudal parts of the body we initiated the present study in an attempt to determine whether the differential growth rate affects the differentiation of the developmentally-pluripotent EC cells. However, as can be seen from our results, the growth of NF-1 teratocarcinoma did not conform with the predictions made on the basis of published data (Auerbach, 1978c) and thus this tumour could not be used for evaluating the effects of tumour growth on stem cell differentiation.

Our observations during the first 16 days post-inoculation are in good agreement with data of Auerbach *et al.* (1978c) and suggest that during the initial stages of tumour formation the anterior sites provide a distinct advantage. However after the initial period the cranial and caudal tumours apparently grow at the same rate (slope of the tumour growth line 0.46 vs. 0.50 and 0.49 vs. 0.64). The slightly steeper slope of the growth line of the caudal tumours accounts for the disappearance of the statistically-significant differences between the cranial and caudal tumours, although the anterior tumours reach an average and somewhat larger size due to the initial growth advantage.

The initial advantage for tumour growth provided by the anterior sites is contrasted with a significantly higher overall tumour take in the caudal sites. This apparent paradox cannot be explained in terms of present day knowledge.

The reasons for the differential take rate in caudal and cranial sites are generally not known, but one should obviously take into consideration various anatomical, functional, immunological determinants that have been theoretically invoked to explain some biological cranio-caudal differences (Kobayashi, 1976; Auerbach *et al.*, 1978a, b, c; Prehn & Karnik, 1979). Our data do not allow any speculations about the differences noted. However, in our future studies, we plan to explore in greater detail the immune response of the inoculated mice in order to determine whether it could account for the differential take rate and growth rate of teratocarcinoma cells. Mouse EC cells do not express antigens of the major histocompatibility system (H-2) (Stern *et al.*, 1975), and would thus not be affected by cytotoxic T cells. On the other hand, EC cells express tumour-specific antigens that could elicit a humoral immune response (Jacob, 1977) and are also attacked by natural killer cells (Stern *et al.*, 1980). These antitumour defense mechanisms could influence the take and growth rate of tumours in different anatomical locations.

The reasons for some of the discrepancies between the present data and those reported by Auerbach *et al.* (1978c) are not clear. Since these authors reported that another retransplantable murine teratocarcinoma (OTT 6050) shows distinct regional growth differences, these differences are most likely not due to the teratocarcinomatous nature of the present tumour. On the other hand, one should point out that the experiments showing a differential anterior-posterior growth rate of the OTT 6050 teratocarcinoma were terminated 8–12 days after inoculation of tumour cells, i.e. after an interval which corresponds to the earliest observation periods in the present study when we also noticed larger tumours cranially than caudally. Thus, if comparison of our data with those published by Auerbach *et al.* (1978c) were limited to this time period there would be compatibility between anterior and posterior tumour size. In order to determine whether the growth of teratocarcinomas differs in general from other mouse tumours or whether the NF-1 tumour represents a notable exception, the experiments with the OTT 6050 should be prolonged to at least one month, and other murine teratocarcinomas should be studied in parallel.

In summary, one can state that the NF-1 teratocarcinoma inoculated simultaneously into anterior and posterior sites shows a differential

initial growth rate and also a differential take rate. On the other hand, once established, the tumours grow at the same rate in both locations. Thus our data suggest that the regional factors, at least in this tumour model, affect primarily the initial establishment of the tumour.

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