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Intradermal Transplantation in Mice of Small Numbers of Sarcoma Cells Followed by Tumor Growth and Regression[1]

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SUMMARY

Relatively small numbers of Sarcoma I cells were transplanted intradermally in A/WySn mice to study the course of growth when the size of the transplant is comparable to an early stage of spontaneous cancer. Transplants of 31 to 32,000 cells took one of three courses: no apparent growth; development of tumor followed by regression; or progressive growth. Transplants of 8,000 cells were the most evenly divided among these three courses and were selected for further study. Bilateral tumors took the same course to regression or to continued growth with significant frequency. There was complete correlation between regression of a tumor and immunity to a challenge made 1 to 5 weeks after the original transplant. Persistent tumors had a similar relationship, but the immunity was less complete. Limited, inapparent growth, followed by regression, was indicated in some mice, simulating immunological surveillance. Serum from such mice, when administered systemically in other mice, apparently induced enhancement of intradermal transplants.

INTRODUCTION

Numerous studies have been made on immunological responses of mice to tumor transplants of 10^6 or more cells. In order to study small transplants that might simulate early cancers, a means was sought to inject quantified suspensions of free tumor cells in circumscribed locations where the progress of resulting tumors could be readily followed. This report deals with the apparent fulfillment of these conditions by i.d.[3] transplants of 32,000 to approximately 31 tumor cells.

MATERIALS AND METHODS

Sarcoma I (6), a spindle-cell sarcoma induced by dibenz[*a,h*]anthracene in an A/J mouse, was obtained from Dr. George D. Snell in the form of ascites tumor and maintained by i.p. transfer. For i.d. transplants, dilutions of 6-day ascites tumor were made in Waymouth's chemically defined medium MAB87/3 (20) modified by the addition of 1% methylcellulose, 15 cps, (Methocel-15; Dow Chemical Co., Midland, Mich.). The osmolality (21) of this suspending fluid was 282 mOsM/kg and the pH was 7.5. The methylcellulose reduced the sedimentation rate of Sarcoma I cells to 2 mm in 15 min. A few suspensions were prepared in Hanks' balanced salt solution (10) with or without the addition of methylcellulose. When the Hanks' solution alone was used, a glass bead was placed in the barrel of the 0.25-ml syringe used for injection. Tipping the syringe so that the bead rolled from end to end maintained the cells in even suspension.

A/WySn mice (19) were used when they were 3 to 8 months old. Both sexes were used because no significant difference was found between the results obtained with male and female mice.

Transplants were made i.d. in both flanks except as later noted. Hair on the lateral aspects of the flanks was removed by clipping. Quantified cell suspensions were mixed with India ink (Special micro Indian Ink; George T. Gurr, Ltd. London, England) to a final ink dilution of 1:200. The cells and carbon suspension were drawn into a 0.25-ml siliconized glass syringe fitted with a 26-gauge, i.d.-bevel needle. The mouse was allowed to enter a woven wire cone attached horizontally to a wooden base. While the mouse was held in the restraining cone, 1 leg was extended and immobilized by placing a wire hook attached to a bead chain over the hock. The chain was placed in one of the vertical slots in a strip of metal at the end of the base so as to exert gentle tension on the leg in a selected direction. The skin was slightly tensed by pinching the inner aspect of the flank between the thumb and forefinger. The needle was inserted, bevel uppermost, into the skin for approximately 3 mm. During this procedure the syringe was about 60° to the femur and the needle was nearly parallel to the skin. Slight rotation of the flank gave controlled direction to the penetration of the needle. The orifice of the needle could be seen through the epidermis and through a portion of the dermis of the albino mouse. Tension on the skin was relaxed and 0.01 ml of suspension was injected. Mice were marked for identification with hair dye on the neck, rump, or either shoulder.

The following day the sites of all transplants were examined to determine the appearance of the tattoo marks caused by the deposit of carbon particles. If a mark was faint or absent, indicating needle penetration or rupture of the deep layer of the skin leading to s.c. leakage, the transplant was considered incomplete and was eliminated from the records.

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[3] The abbreviations used are: i.d., intradermal; N, no tumor developed; R, tumor grew followed by regression; T, tumor present on Day 42; BCG, *Bacillus Calmette-Guérin*.

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Thereafter, the mice were examined at least twice a week. Any tumors that developed were measured and gross changes were recorded. Experiments were terminated 42 days after transplantation, and autopsies were performed to determine completeness of regression, the characteristics of tumors, and to verify tattoo marks by observing the underside of the skin.

In a series of experiments, instead of concurrent transplants in both flanks, a single injection of 8000 cells was made in the left flank, followed 7 to 36 days later by a similar transplant in the right flank. In some instances, involving long intervals, a mouse died because of growth of a large tumor at the primary site before the 6-week observation period of the 2nd site was completed. Such results are not included in the tabulations.

Susceptibility of i.d. transplants to transferred serum antibodies was explored. Sera were obtained from A/WySn mice 42 days after i.d. injection of Sarcoma I. These sera were classified and pooled in accordance with the course taken by the transplants. The pooled sera were injected, 0.25 ml i.p., in a series of mice concurrently with i.d. transplants of 8000 Sarcoma I cells.

Suspensions of lyophilized solid Sarcoma I tumor (18) were injected i.p. into another group of mice. Injections of 10 mg dry weight were made 3 times a week for 2 weeks followed 1 week later by i.d. transplants of 8000 tumor cells.

RESULTS

Each transplant took 1 of 3 courses: N, R, or T. The incidence of positive transplants was related to the number of cells injected (Chart 1). When 32,000 cells were implanted, tumors (R and T) developed at 93% of the sites, but at only 6% of the sites that received approximately 31 cells. The tumors were remarkably evenly divided between those that regressed and those that continued to grow. This almost equal distribution of outcome was not influenced by the number of cells transplanted. The frequencies of dates of onset and regression of R tumors following 8000-cell implantations are shown in Chart 2. The mean day of appearance and of regression were, respectively, 10.3 ± 3.5 (S.D.) and 28.3 ± 6.4. Also represented by *bar graphs* is the duration of the tumors that appeared within 1 S.D. of the mean. Although these appeared over an 8-day range, from Day 7 to Day 14 inclusive, their mean dates of regression were nearly the same, Day 28 ± 0.6.

The maximum size to which tumors grew before regressing is an indication of the effectiveness of the destructive reactions and of the absorptive processes involved. In the series of 800-cell transplants, 37 tumors (11%) attained diameters of 9 to 12 mm before they regressed, indicating that in these instances the immune system operated effectively even in the presence of large amounts of antigen.

The results of paired, simultaneous transplants in individual mice are presented in Table 1. Bilateral tumors developed in 416 mice in the 5 groups that received injections of 125 to 32,000 cells. In each of these groups, the tumors took the same course on the 2 sides, either to regression or to continued growth in 85 ± 2% of the mice. This is in contrast ($\chi^2 = 28$; $p < 0.001$) to 50% of the mice that would be expected if growth on the 2 sides progressed independently. Sex did not influence these results. Of the total 416 mice with bilateral tumors, 85% of the males and 84% of the females developed tumors that followed the same course on both sides.

The results of 2 transplants of 8000 Sarcoma I cells each, separated by an interval of a week or more (Table 2), indicate the course of the development of immunity. None of the 77 mice with primary tumors that later regressed developed a tumor at the site of a secondary transplant made on the opposite flank 7 to 36 days after the 1st injection. Immunity developed more slowly in mice with persistent tumors at the primary site. In the experiments with time intervals of 1 or 2 weeks, 8 of the 55 secondary transplants developed into tumors. However, all of the 45 mice with persistent primary tumors tested at 3 to 5 weeks were solidly immune. Apparently, a weak immune reaction developed in at least some of the mice with primaries that did not show evidence of growth. This resistance decreased as the time interval between transplants was lengthened. Approximately 88% of the mice with N primaries did not develop tumors at the secondary site when the interval was 7 days. This fraction decreased to 50% of the mice when 3 to 5 weeks intervened between transplants.

The outcome of transplants in mice that received 0.25-ml injections of serum from isogeneic mice with bilateral N, R, or T sites is shown in Table 3. Injections of R or T serum i.p. 15 to 30 min before the i.d. transplants were made followed by a significant decrease in the incidence of R tumors (χ^2 =

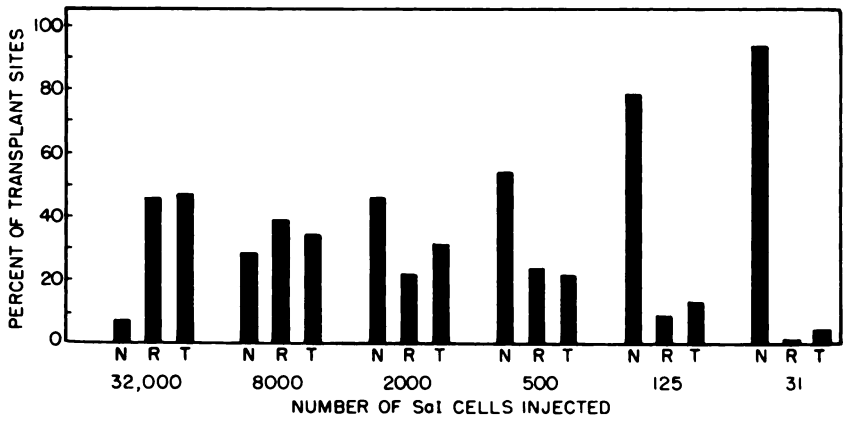

Chart 1. Effect of number of cells injected on course of transplants. *Bars*, results N, R, and T. The results are based on the following numbers of transplants: 32,000 cells, 131 sites; 8000 cells, 897 sites; 2000 cells, 277 sites; 500 cells, 262 sites; 125 cells, 121 sites; 31 cells, 148 sites. *Sal*, Sarcoma I.

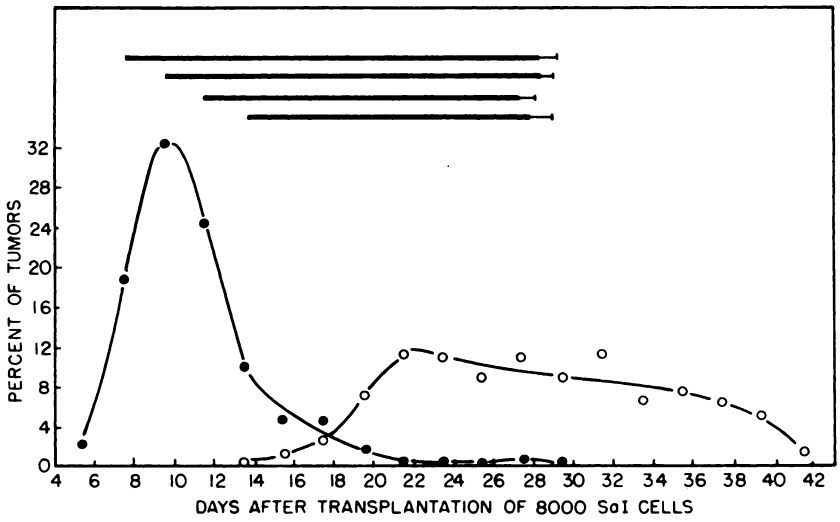

Chart 2. Percentage of 340 tumors that appeared (●) and that completely regressed (○) during 2-day periods. *Bars*, mean duration ± S. E. of tumors appearing on 2 consecutive days. *Sal*, Sarcoma I.

Table 1

Bilateral results of i.d. transplantation of Sarcoma I cells

No. of Sarcoma I cells	No tumor (N—N)	Unilateral tumors		Bilateral tumors			Total
		N—R	N—T	R—R	R—T	T—T	
32,000	2[a]	4	0	25	8	25	64
8,000	60	58	43	99	38	109	407
2,000	46	16	13	19	10	32	136
500	45	23	13	16	6	17	120
125	52	6	4	3	2	7	74
31	64	2	6	0	0	0	72
Mice with bilateral tumors							
M				90 (40)[b]	33 (15)	99 (45)	222
F				72 (37)	31 (16)	91 (47)	194
Total				162 (39)	64 (15)	190 (46)	416

[a] Numbers of mice.
[b] Numbers in parentheses, percentages.

Table 2

Delayed contralateral transplants

Results of i.d. transplantation of 8000 Sarcoma I cells made at the indicated intervals after contralateral transplants.

Primary transplant (left flank)		Interval (days)	Secondary transplant (right flank)		
Outcome	No. of sites		N	R	T
N[a]	26	7	23	2	1
R	30		30	0	0
T	31		25	2	4
N	13	14	9	2	2
R	17		17	0	0
T	24		22	0	2
N	18	21 to 36	9	5	4
R	30		30	0	0
T	45		45	0	0

[a] Not included are mice without carbon tracer in the skin and mice that were killed by primary tumor growth before the observation period for secondary transplants was completed. Controls: tumors, that either persisted or regressed, developed in 75.3 ± 9.6% of the 328 viability tests on the cell suspensions used in secondary transplants.

Table 3
Effect of serum from sarcoma-inoculated mice

i.p. injections, 0.25 ml[a]	Course of i.d. transplant sites			Total
	N	R	T	
None, controls	14 (26)[b]	15 (27)	26 (47)	55
N Serum	1 (4)	3 (14)	18 (82)	22
R Serum	16 (46)	3 (8)	16 (46)	35
T Serum	35 (47)	2 (3)	37 (50)	74

[a] Sera were obtained from bilaterally inoculated mice that yielded the same results at both sites.
[b] Percentages, given for comparison (11), are in parentheses.

Table 4
Effect of i.p. injections of lyophilized Sarcoma I tumor
Lyophilized Sarcoma I, 10 mg, was injected i.p. 3 times a week during the 3rd and 2nd week before i.d. challenge with 8000 Sarcoma I cells.

I.p. injections, 10 mg	Course of i.d. transplant sites			Total
	N	R	T	
None, controls	12 (26)[a]	8 (17)	26 (57)	46
Lyophilized Sarcoma I	35 (64)	4 (7)	16 (29)	55

[a] Percentages, given for comparison (11), are in parentheses.

4.68, $p < 0.05$, and $\chi^2 = 16.65$, $p < 0.001$, respectively) and a corresponding increase in N sites. The percentage of T results was not influenced by the injection of either of these sera, progressive tumors occurring in 47% of the controls, in 46% of the transplantation sites of mice that received R serum, and in 50% of the sites when T serum was administered. Mice that received N serum had a higher ratio of progressively growing tumors than did the controls, suggesting that pooled N serum induced passive immunological enhancement (14).

A series of 6 i.p. injections of 10 mg of lyophilized Sarcoma I tumor during the 3rd and 2nd week before challenge resulted in partial, but not complete immunity (Table 4). The incidence of tumors that followed transplantation of 8000 Sarcoma I cells was lower in the treated mice than in the controls ($\chi^2 = 14.2$; $p < 0.001$).

The carbon particles injected as a tracer with the cells did not have a significant influence on the course of tumor development. Transplants of 8000 cells each were made without carbon at 178 sites. These resulted in 52 N, 66 R, and 60 T; while controls yielded 36 N, 31 R, and 38 T ($\chi^2 = 0.79$, $p > 0.2$). Carbon tattoo marks are useful while developing an i.d. technique but would rarely be required once that is accomplished. Of a total of 3624 transplants, 3481 or 96% were proved i.d. The last 1000 were 98% successful. Presumably, ruptures in the lower layers of the skin accounted for the majority of injections that left no visible carbon at 6 weeks. In a few instances, the needle passed through the skin and the deposit was made under the muscle sheath. This led either to no growth or to a tumor that was flat with ill-defined edges during the early stages of development.

DISCUSSION

The history of Sarcoma I (6) is consonant with a minor histocompatibility difference between it and the A/WySn inbred strain of mice used in this study. The tumor was induced in an A/J mouse by dibenz[*a,h*]anthracene, an agent that has been shown (15) to give rise to tumors with immunogenic differences from their hosts. Any such minor difference, however, does not prevent uniformly successful transplants when 0.1 ml of Sarcoma I ascites tumor is injected i.p. or when small fragments of solid tumor are placed under the skin.

I.d. transplantation of small numbers of dissociated tumor cells in mice appears to present an effective means for studying early tumor growth and suppression. The i.d. site permits precise localization of the transplanted cells and minimizes their dispersion. This is especially important when very small inocula (125 or 31 cells) are used. The results of delayed contralateral transplants, and of transplants made at the time immune serum was administered systemically, indicate that the normal course of transplants can be altered by factors of both active and passive immunity.

Few studies have used mice as recipients of i.d. tumor transplants. In 1935 Besredka and Gross (4) reported regression of tumors initiated by i.d. injection of Ehrlich tumor cells in mice. Such mice were found to be immune on challenge. Similar results were later obtained with C3H mice and a methylcholanthrene-induced tumor (8). Guinea pigs have been used as recipients of i.d. transplants of diverse tumors. Leukemic tumors have grown in guinea pigs following i.d. injections of 0.1 ml of tumor brei diluted 10^{-2} to 10^{-6}. Gross *et al.* (9) observed that approximately one-half of these tumors regressed, leaving the host immune, whereas tumors resulting from s.c injections led to generalized leukemia. Holmes *et al.* (12) used the i.d. route in studies on immunity induced in guinea pigs by temporary sarcoma implants excised 30 days before challenge. Challenge with 10^7 cells i.d. led to the development of tumors in 11% of the animals. Numerous reports on the effect of BCG in suppressing tumor growth have used the i.d. route of transplantation. Zbar *et al.* (22) determined that growth of hepatoma cells is suppressed when the cells are mixed with BCG before i.d. implantation in guinea pigs. Certain mouse tumor lines also fail to grow when injected i.d. together with BCG into syngeneic mice (2). The studies with BCG were extended by Bast *et al.* (3) to include other bacteria. They observed that growth of a murine fibrosarcoma is suppressed by mixing the tumor cells with *Listeria monocytogenes* before i.d. injection of 5×10^5 tumor cells into syngeneic mice. Intratumor injection of these bacteria caused regression of early tumors, but BCG was not effective under these conditions.

The direct relation of tumor development to the number of cells transplanted, from 6% for 31 cells to 93% for 32,000 cells, suggests that proliferation and tumor development from small numbers of cells in i.d. transplants may be influenced by limiting factors analogous to those controlling the growth of tissue cultures from small-cell inocula,

where mutual interaction or local medium conditioning is important for multiplication (16).

Development of a systemic factor influencing the course of tumors in individual mice is indicated by the progress of bilateral tumors. When tumors commenced on both sides in response to simultaneous bilateral transplants, 85% regressed or grew progressively as a pair. The frequency of bilateral, identical courses was independent of the number of cells injected. One hundred twenty-five cells and 32,000 cells gave similar results, suggesting that the process linking the 2 sides developed after tumefaction had overshadowed differences in the number of cells in the original inocula.

The schedule of i.p. injections of lyophilized tumor was adopted in anticipation that the subsequent i.d. transplants would be enhanced (16). Instead, inhibition of growth resulted (Table 4). The presence of temporary enhancing antibodies is not, however, ruled out (17). Brunner (5), using another system, found that the cytotoxic antibody 19S peaked at Day 11 to 12 and then dropped off rapidly. By Day 22 to 23, when the enhancing antibody 7S was at its maximal level, 19S had diminished 25-fold. In experiments conducted by Andersson *et al.* (1), the 7S fraction attained highest concentration 12 to 15 days after immunization. The inhibition of growth following i.d. challenge in our experiments using i.p. injections of lyophilized tumor may have been facilitated by the relatively small number of cells used and by their being individual, free cells. These conditions may render cells particularly vulnerable even to low concentrations of destructive elements in the immune response (7).

The course of i.d. transplants is responsive to passively transferred seurm from immune mice. Differences in the action of the sera were related to the exposure to antigen experienced by the donors. Thus, serum from tumor-bearing mice reduced the incidence of the R course to 3%, while pooled serum from mice with bilateral N sites apparently had an enhancing effect.

Further indication of the initiation of immune reaction without palpable tumor is the fact that R tumors that appeared on day 7 to 8 regressed in 4 weeks and those that appeared a week later, on Day 13 to 14, persisted for only 3 weeks (Chart 2, *bar graphs*). Disappearance of these tumors at the same time after transplant, irrespective of their latent period, suggests that the process leading to regression started while multiplication was inapparent.

Other experiments also indicate that sites classified as no growth may in some instances have represented considerable activity. The results of delayed contralateral transplants (Table 2) suggest that some of the mice with primary transplants classified as N developed transient immunity. The antigen that brought this about originated either in the 8000 Sarcoma I cells injected or in these cells and their progeny resulting from proliferation that had been controlled by development of temporary immunity. This suggestion is in keeping with the observation of Kaliss (13) that the immune response that caused rejection of a homogeneic tumor graft decreased sharply after 1 month. Inapparent growth and regression in our experiments, simulating immunological surveillance, would be analogous on a small scale to the reactions that took place when more extensive growth occurred leading to palpable tumors, regression, and immunity.

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