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Stimulation or Suppression of Metastases with Graded Doses of Tumor Cells[1]

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SUMMARY

Varying doses of syngeneic fibrosarcoma cells, injected into the left limb at the time of amputation of the right tumor-bearing limb, either augmented or inhibited development of lung metastases in mice. Two different tumors were investigated, and with each 1×10^4 live tumor cells provided complete protection from metastases. In both tumor lines, increasing the number of live tumor cells or using irradiated tumor cells at equivalent or greater dosage increased the incidence of metastases.

INTRODUCTION

Carcinogenic hydrocarbons induce tumors which have individually specific antigens (12). As isoantigens, these tumor-specific antigens can elicit an immune response which is primarily cell mediated (6, 11, 12) but which is unable to inhibit the primary tumor growth. Specific stimulation and active stimulation of host immune mechanisms for the therapy of primary tumors or their metastases have been attempted (4, 8, 13). The most encouraging results have been obtained with a nonspecific (2, 10) or nonspecific and specific (7, 9) stimulation of the lymphoid tissue.

Using a primary fibrosarcoma induced by 3,4-benzpyrene in rats, Delorme *et al.* (3) have shown failure of the lymph nodes draining the tumor to release immunoblasts specifically directed against the tumor antigens. Noninvolved lymphoid tissue, stimulated by autologous irradiated tumor cells, could increase the number of immunoblasts in the lymph (1).

This study, by measuring the appearance of spontaneous metastases, has attempted to evaluate the effect of syngeneic irradiated and live tumor inocula on an animal from which primary sarcoma has been removed. This tumor inoculum represented a graded dose of tumor cells made in the left hind limb of the experimental animal at the time that the right hind limb bearing an initial tumor inoculum was amputated.

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MATERIALS AND METHODS

Inbred male mice, 6 to 8 weeks old, of the C57BL/6J strain (Jackson Laboratory, Bar Harbor, Maine) were used. All mice were fed a standard pellet diet and water *ad libitum.*

Tumor Line Maintenance. A suspension of 1.0 mg of methylcholanthrene in 0.3 ml of trioctanoin was injected into the abdominal wall of mice about 2 months old for tumor induction. Tumors became palpable at 3 months; when the tumor was approximately 20 mm in diameter, the tumor-bearing mice were killed by cervical dislocation. Cell suspensions were prepared by enzymatic digestion of minced tumor tissue with 0.25% Pronase and 0.04% DNase for 40 min in a 37° water bath. The free tumor cells were washed twice in Hanks' solution, counted in a hemacytometer chamber, and checked for viability by trypan blue exclusion. In all tests, only suspensions containing 95% or more viable tumor cells were used in order to avoid contamination of the inoculum with dead material. Two highly immunogenic sarcomas (I and XIV) induced in our laboratory were maintained in syngeneic passages and chosen for these studies.

Radiation of Tumor Cells. Tumor cells in a plastic tube surrounded by crushed ice were irradiated with X-rays (Westinghouse Coronado apparatus) generated at 250 kV, 20 ma, and filtered by a 0.1-mm Al filter. The dose rate was 683 R/min at a tube object distance of 30 cm, and a total of 15,000 R were delivered on each occasion.

Proof of Antigenicity. Prior to their inclusion in the protocol, the tumor lines were tested for their antigenicity. Irradiated cells (1×10^6) were inoculated s.c. in the left rear limb; 19 days later, a challenge dose of 1×10^4 live tumor cells was inoculated i.m. into the opposite limb. Control groups received only the live challenge doses of the respective tumor lines. Only 6/16 mice (37%) died by 100 days when Sarcoma I was tested in this manner; 20/21 control mice (95%) died of tumor growth. When Sarcoma XIV was tested for antigenicity, only 1/20 mice (5%) died of tumor, while 6/11 (54%) of the control mice died by 100 days. Sarcoma I was used between the 18th and 28th transplant generations, and Sarcoma XIV was used in the 5th transplant generation.

Amputation Technique. Disarticulation of the right limb was performed under light ether anesthesia 20 days after the initial inoculation. The femoral vessels were ligated with 4-0 silk. The muscles around the disarticulation site were peeled, and the skin was sutured closed. Recurrence of the primary

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sarcoma occurred in 6% of the disarticulated mice. These mice were excluded from the subsequent results.

Experimental Protocol. Three methods were used to administer tumor cells to mice whose primary tumor mass had been amputated. These were: (*a*) live tumor cells i.m. in the opposite hind limb; (*b*) irradiated tumor cells i.m. in the opposite hind limb; (*c*) live tumor cells in the opposite hind limb and 6 s.c. inoculations of irradiated tumor cells in multiple sites on the trunk of the mouse. Two control groups of mice were studied for each sarcoma. One group received only a tumor inoculum and neither amputation nor any treatment; the 2nd group received the initial tumor inoculum and had amputation performed at the same time as the treated group but no other treatment was given. Colony inhibition studies with the technique of Hellström (5) were performed with Sarcoma I. At the time that the tumor-bearing limb was removed, tumor cells were placed in culture. Lymphocytes for study were obtained by sacrificing the animal 4 days later and making a suspension of inguinal and mesenteric lymph nodes. The ability of these fresh lymphocytes to inhibit colony formation in a subculture of the cells of that animal was studied.

Method of Evaluation. Two methods of evaluation were used. In the studies with Sarcoma I, both survival times of the mice and appearance of metastases at death were noted; multiple caging was used. In the studies with Sarcoma XIV, mice in each group were sacrificed at standard times, and metastases were identified simultaneously with India ink perfusion of the lung as described by Wexler (14); individual caging was used.

Survival time in the studies of Sarcoma I was measured from the day of primary inoculation of living tumor cells to the day of death. Meticulous autopsy was carried out, and the presence and distribution of metastases were recorded.

RESULTS

Sarcoma I. The studies with Sarcoma I are summarized in Tables 1 and 2. Amputation of the tumor-bearing limb at 20 days (1.5 to 2 cm, tumor size) permitted one-half of the animals in Group A to survive indefinitely without the development of metastases. Although the other 6 mice died of metastatic tumor, 4 of these had a prolonged survival. A parallel colony inhibition study of this tumor indicated increased tumor-inhibiting potential by host peripheral lymphocytes 4 days following amputation of the tumor-bearing limb as compared with normal mouse lymphocytes or with those from a mouse with a different tumor (Table 3).

Group B mice, in addition to amputation, received 1×10^4 tumor cells in the opposite limb at the time of amputation. None of these mice died of metastases and 5 of 7 lived indefinitely. The other 2 grew tumors at the implant site on the left. Increasing the dose of living tumor cells to 5×10^4 eliminated this specific effect, and all the animals died of metastases in a relatively short time. Likewise, the use of irradiated tumor cells at either of those 2 doses (Groups D and E) failed to prevent death from metastases in all animals.

All the experiments with multiple injections of irradiated tumor cells (Table 2) failed to prevent death from metastases

Table 1

Studies with Sarcoma I in C57BL/6J mice

On Day 0, 1×10^5 living cells were given to all mice in the right hind limb. Amputation of this limb was performed on Day 20, and simultaneous inoculation of varying doses of tumor cells, either living (L.C.) or irradiated (I.C.), was made in the left hind limb. The survival times were recorded, and the presence of metastases was determined at autopsy.

Group	Day 20	Individual survival time	Mean survival time[a] (days)	No. of mice with metastases
A	Amputation	47, 49, 66, 67, 71, 75, >100, >100, >100, >100, >100, >100	81+	6/12
B	Amputation + 1×10^4 L.C.	66, 69, >100, >100, >100, >100, >100	90+	0/7
C	Amputation + 5×10^4 L.C.	38, 40, 42, 43, 44, 46, 48	43	7/7
D	Amputation + 1×10^4 I.C.	38, 61, 71, 73	61	4/4
E	Amputation + 5×10^4 I.C.	41, 41, 46, 55, 58	48	5/5

Statistical data

Comparison groups	*p* value
A *vs.* B	<0.05
A *vs.* C	<0.05
A *vs.* D	Not significant
A *vs.* E	Not significant
B *vs.* C	<0.005
B *vs.* D	<0.01
B *vs.* E	<0.005

[a] In calculating mean survival time, maximum survival time has been assumed to be 100 days.

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Table 2

Studies with Sarcoma I in C57BL/6J mice

On Day 0, 1×10^5 living cells were given to all mice in the right hind limb. Amputation of this limb was performed on Day 20, and simultaneously varying doses of tumor cells, either live (L.C.) or irradiated (I.C.), were administered to the animals as shown. The I.C. were administered in 6 sites around the trunk of animal in 6 equal doses. Survival times of the mice were recorded, and the presence of metastases was determined at autopsy.

Group	Day 20	Day 24	Day 27	Individual survival (days)	Mean survival (days)	No. of mice with metastases
AA	Amputation + 5×10^4 L.C. + 6×10^6 I.C.			34, 35, 36, 42, 44, 50	40.1	6/6
BB	Amputation + 5×10^4 L.C. + 6×10^5 I.C.			37, 38, 40, 47, 65	45.4	5/5
CC	Amputation + 5×10^4 L.C. + 6×10^4 I.C.			44, 46, 46, 63, 65, 65	54.8	6/6
DD	Amputation + 5×10^4 L.C. + 6×10^3 I.C.			50, 50, 52, 54, 80	57.2	5/5
EE	Amputation + 5×10^4 L.C. + 6×10^2 I.C.			43, 52, 56, 57, 63	54.2	5/5
FF	Amputation + 5×10^4 L.C. + 6×10^6 I.C.	6×10^6 I.C.	6×10^6 I.C.	43, 44, 44, 45, 48	44.4	5/5
GG	Amputation + 5×10^4 L.C.	6×10^6 I.C.	6×10^6 I.C.	40, 50, 52, 58	50	4/4
HH	Amputation + 1×10^4 L.C. + 6×10^6 I.C.			39, 47, 47, 68, 79	56	5/5

Statistical data

Comparison groups	p value
A *vs.* all groups (AA-HH)	<0.05
B *vs.* all groups (AA-HH)	<0.003

Table 3

Colony inhibition test performed in animals bearing Sarcoma I

Lymph node cells were obtained from animals 4 days after amputation of the tumor-bearing limb. These lymph node cells were tested for their ability to inhibit colonies of syngeneic or allogeneic tumor cells. Lymph node cells from normal animals were used as controls. Reduction in colonies occurred only with lymph node cells from tumor-bearing animals when tested against syngeneic tumor cells. Cells/plate = 3000; plating efficiency = 7.3 (11.7%).

No. of cells	No. of colonies in individual replicates	Mean no. of colonies	Reduction colonies *vs.* normal lymphocytes (%)
Lymph node cells *vs.* syngeneic tumor cells			
5×10^6	175	175	45
3.5×10^6	179, 207	193	39
2.0×10^6	165, 174, 182	173	45
Lymph node cells *vs.* allogeneic tumor cells			
4×10^6	313, 317, 339	323	
2×10^6	333, 357	345	
Lymph node cells from normal mice *vs.* syngeneic tumor cells			
5×10^6	315, 316	316	

in a single mouse. The use of multiple injections of tumor on the day of amputation and 4 and 7 days later (Group FF) or a delayed injection of irradiated cells after living cell inoculum (Group GG) also failed to protect any of the animals.

The effect of varying dosages of irradiated tumor cells added to a constant number of live cells could be seen by comparing Groups AA through EE. As the total dose of irradiated cells was decreased, the mean survival time of

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animals in Group CC, DD, and EE was significantly longer than for those animals in Group AA. The antigenic potency of living cells appeared to be more critical, as already demonstrated in Table 1, since Group HH, receiving only 1 X 10^4 living cells and the same total dose of irradiated cells, had a significantly longer mean survival time than the mice in Group AA. The Group AA mice received only 5 times as many living cells as Group HH mice instead of the increase of at least 100 times in the number of cells required for the other groups where irradiated tumor cells were being compared.

Subjection of data to statistical analysis with the probability ratio and Fisher's exact chi square test indicated significant differences between the groups receiving amputation only or amputation and 1 X 10^4 live cells as compared to other groups (Tables 1 and 2).

Sarcoma XIV. Repetition of the experimental protocol with another antigenic tumor in an earlier generation and with more stringent criteria for identification of the effect of the 2nd tumor inoculum on the growth of metastases demonstrated again the same critical dose level of living tumor cells necessary for protection. This tumor was less virulent, and metastases generally appeared later than with Sarcoma I. A minimum number of mice developed metastases if only amputation was performed when the tumor reached a size of 1.5 to 2.0 cm in diameter (Table 4). Complete protection from metastases was provided by injecting 1 X 10^4 live, syngeneic tumor cells in the left leg at the time of amputation. None of the other maneuvers was as successful in preventing metastases. As with Sarcoma I, increasing the 2nd tumor inoculum to 5 X 10^4 completely altered the incidence of metastases. Irradiated tumor cells alone at either dose level appeared to stimulate the development of metastases as did the addition of irradiated cells at multiple sites to the 1 X 10^4 dose of tumor cells in the left leg. Microscopic study of the lungs in Group II mice failed to reveal any metastatic foci. Statistical analysis of these results is given in Table 4.

For both tumor lines, I and XIV, mice receiving a single tumor inoculum in the right leg and no subsequent treatment died of progressive cachexia associated with a continually growing tumor. They had a mean survival time of less than 50 days. Because death occurred before metastatic foci could be observed, these mice were not considered potential candidates for metastases and are not included in these data for comparison.

DISCUSSION

These experiments point out the insidious growth potential of tumor cells which have migrated from the primary site. The amputation in the protocol is analogous to surgical excision in the human patient, and the graded tumor inoculum in the left leg is comparable to the shed cells and those not removed by

Table 4

Studies with Sarcoma XIV in C57BL/6J mice

On Day 0, 1 X 10^5 living cells were given to all mice in the right hind limb. Amputation of the limb was performed when the tumor diameter was between 1.5 and 2.0 cm. Simultaneous inoculation of varying doses of tumor cells, either live (L.C.) or irradiated (I.C.), was made in the left hind limb. In Group VI, 6 X 10^6 I.C. were given in multiple sites as well. The animals were sacrificed at varying times after amputation, and the presence of metastases was identified by perfusion of the lungs with India ink.

Group	Treatment when tumor diameter = 1.5 to 2.0 cm	No. of mice with metastases/ total no. mice at following days after primary tumor inoculum						% of mice with metastases
		<50	55	69	77	85	>90	
I	Amputation only			0/6	2/12	0/9	0/18	4
II	Amputation + 1 X 10^4 L.C.			0/5	0/4	0/4		0
III	Amputation + 1 X 10^4 I.C.		0/1	2/8	0/4	0/4		12
IV	Amputation + 5 X 10^4 L.C.			4/7	0/3	2/4		43
V	Amputation + 5 X 10^4 I.C.			2/7	3/7			36
VI	Amputation + 1 X 10^4 L.C. + 6 X 10^5 I.C. (multiple sites)		1/4	1/5	1/6			20

Statistical data

Comparison groups	*p* value
I *vs.* IV	<0.005
I *vs.* V	<0.01
II *vs.* IV	<0.05
II *vs.* V	<0.05

All other group comparisons not significant

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the scalpel. Residual tumor cells can either grow slowly, be completely eliminated, or be stimulated to grow more rapidly. From these data, the difference may be relatively minute between a state of protection and implied heightened immunity as seen in animals receiving 1×10^4 live cells in the left leg or amputation of the tumor-bearing limb alone and the augmented tumor growth as demonstrated by most other groups receiving tumor cells in the left leg. The number of animals in the various experimental groups is relatively small, a feature that minimizes the validity of statistical analysis. Alteration of the cells by irradiation or a small increase in tumor cell inoculum was sufficient to change completely the host response to the cells already shed from the primary tumor. These cells came from the initial right leg tumor, since irradiated cells alone, which in our experience cannot grow tumors, provoked the growth of metastases.

Although the criteria for evaluation were crude, in every instance when multiple distant injections of irradiated tumor cells were made, increased growth of metastases occurred. This is most clearly demonstrated by comparing the results in Group B with those in Group HH. Identical doses of living tumor cells were injected into the left hind limb of all mice, while the mice in Group HH received an additional dose of 6×10^6 irradiated cells at 6 different sites. None of the mice in Group B developed lung metastases, and all demonstrated prolonged survival; an immunity was evoked against the tumor-specific antigens. All the mice in Group HH had metastases, a significant increase over the control group, and the tumor growth was enhanced by the additional antigenic load. Thus, the concept derived from the experiments of Alexander *et al.* (1) that other draining lymph nodes in the host can respond to the tumor antigen and release potent immunoblasts, even if the nodes draining the tumor are blocked, was not supported by our data. There is evidence from the colony inhibition studies that specifically sensitized lymphocytes are available, but their *in vivo* effectiveness is apparently altered by the cell inocula. Although no circulating antibody titer or passive transfer was sought for, the response to irradiated cells or larger doses of living cells might be considered a form of enhancement. The dangers of such a state of tumor enhancement occurring with specific tumor cell inocula with the same antigenicity as the primary tumor are clear and must be considered in any clinical program of tumor "immunotherapy."

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REFERENCES

1. Alexander, P., Bensted, J., Delorme, E. J., Hall, J. G., and Hadgett, J. The Cellular Immune Response to Primary Sarcoma in Rats. II. Abnormal Response of Nodes Draining Tumor. Proc. Roy. Soc. London Ser. B, *174:* 237–251, 1969.
2. Amiel, J.-L. Immunotherapie Active non Specifique par le BCG de la Leucemie Virale E ♂ G2 chez des Recevents Isogeniques. Rev. Franc. Etudes Clin. Biol., *12:* 912–914, 1967.
3. Delorme, E. J., Hadgett, J., Hall, J. G., and Alexander, P. The Significance of Large Basophilic Cells in the Thoracic Duct Lymph Following Antigenic Challenge. Proc. Roy. Soc. London Ser. B, *174:* 229–236, 1969.
4. Haddow, A., and Alexander, P. Immunological Method of Increasing the Sensitivity of Primary Sarcomas to Local Irradiation with X-rays. Lancet, *1:* 452–457, 1964.
5. Hellström, I. A Colony Inhibition (CI) Technique for Demonstration of Tumor Cell Destruction by Lymphoid Cells *in Vitro.* Intern. J. Cancer, *2:* 65–68, 1967.
6. Klein, G., Sjögren, H. O., Klein, E., and Hellström, K. E. Demonstration of Resistance against Methylcholanthrene. Cancer Res., *20:* 1561–1572, 1960.
7. Mathé, G., Amiel, J. L., Scharcenberg, L., Schneider, M., Cattan, A., Schlumberger, J. R., Hayat, M., and Vassal, F. Demonstration de Efficate de l'Immunotherapie Active dans la Leuceme Aigue Lymphoblastique Humaine. Rev. Franc. Etudes Clin. Biol., *13:* 454–459, 1968.
8. Matsuyama, M., Maekawa, A., Harikawa, H., Suzumori, K., Soga, K., and Iwai, K. Ineffectiveness of Autotransplantation on Growth of the Methylcholanthrene Sarcoma in Mice. Nature, *197:* 805, 1963.
9. Morton, D. L., Eilber, F. R., Joseph, W. L., Wood, W. C., Trohan, E., and Ketcham, A. S. Immunological Factors in Human Sarcomas and Melanomas: a Rational Basis for Immunotherapy. Ann. Surg., *172:* 740–749, 1970.
10. Morton, D. L., Eilber, F. R., Malmgren, R. A., and Wood, W. C. Immunological Factors Which Influence Response to Immunotherapy in Malignant Melanoma. Surgery, *68:* 158–164, 1970.
11. Old, L. J., Boyse, E. A., Clarke, D. A., and Carswell, E. A. Antigenic Properties of Chemically Induced Tumors. Ann. N. Y. Acad. Sci., *10:* 80–106, 1962.
12. Prehn, R. T., and Main, J. M. Immunity to Methylcholanthrene-induced Sarcomas in the Primary Autochthonous Host. J. Natl. Cancer Inst., *18:* 769–778, 1957.
13. Wepsic, H. T., Kronman, B. S., Zbar, B., Borson, T., and Rapp, H. J. Immunotherapy of an Intramuscular Tumor in Strain Z Guinea Pigs: Prevention of Tumor Growth of Intradermal Immunization and by Systemic Transfer of Tumor Immunity. J. Natl. Cancer Inst., *45:* 337–386, 1970.
14. Wexler, H. Accurate Identification of Experimental Pulmonary Metastases. J. Natl. Cancer Inst., *36:* 641–643, 1966.

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