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Potentiation of Hamster Tumors by Normal Cells or Charcoal[1]

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SUMMARY

Admixed spleen cells from normal animals or from animals given injections of Syrian hamster type C virus significantly potentiated the growth of the transplanted D9 lymphoma of random-bred hamsters. Potentiation was measured by an increase in incidence of tumors, a shortened latent period, and a decreased 50% tumor-producing dose of tumor cells. Intermediate doses of spleen cells (10 to 100 spleen cells per tumor cell) produced the greatest potentiation. Preincubation of admixed spleen and tumor cell suspensions *in vitro* was unnecessary. Immunization to isoantigens was not responsible for potentiation, since growth of a transplantable carcinoma of inbred hamsters was also facilitated by normal spleen cells. In addition, normal kidney or liver cells increased the incidence of tumors transplanted by a small number of tumor cells. Potentiation did not occur when spleen cells were injected at a site remote from the tumor cells. Since the potentiating cells might act either as a physical barrier to host response, or by blocking normal macrophage function, we injected charcoal with tumor cells. Simultaneous treatment with charcoal facilitated the growth of the lymphoma but not that of the carcinoma. Treatment with some doses of charcoal was also effective at distant sites. Although potentiation of tumor growth by cells or charcoal may operate through different mechanisms, these phenomena should be explored in regard to outgrowth of primary tumors, tumor immunity, or enhancement of tumor growth.

INTRODUCTION

The D9 lymphoma is transplantable in random-bred hamsters and releases a hamster-specific type C virus into the blood of tumor-bearing animals (18). Since the virions are nononcogenic *in vivo* (6, 18) and noninfectious *in vitro* (16), but bud from the tumor cell membrane, attempts were made to use the apparently innocuous virus to immunize hamsters against the transplantable D9 tumor. While studying cellular immunity in this system, we noted that normal tissue cells added to the tumor cell inoculum potentiated the growth of the lymphoma. Facilitation of transplanted tumor growth has been described in murine systems after mixing tumor cells with tumor cells damaged by X-ray (14), with normal spleen cells (2), or with specifically immunized spleen cells (12). Our initial observations on potentiation or facilitation of growth of hamster tumors are described in this report.

MATERIALS AND METHODS

Tumors. The D9 lymphoma (17, 18) was transplanted s.c. in random-bred hamsters. A transplantable anaplastic carcinoma (CILT/2) derived from an adenovirus-induced hepatocellular carcinoma of inbred hamsters (K. J. McCormick, N. K. McCormick, and J. J. Trentin submitted for publication) was also used. The TPD_{50}[4] was calculated by the method of Reed and Muench (13).

Animals. Random-bred hamsters (20) were used in experiments with the D9 lymphoma; cesarian-derived inbred LSH/Lak hamsters (1) were used in experiments with the CILT/2 carcinoma. Weanling animals (8 to 20/group) were used.

Immunization. Concentrated suspensions of Syrian hamster type C virus were prepared from plasma of D9 tumor-bearing animals. Normal or infected plasma was clarified at 9000 × *g* for 5 min. Plasma pellets were prepared by sedimentation at 78,000 × *g* for 1 hr. The pellets were resuspended in phosphate-buffered saline, pH 7.2, at one-sixteenth the original volume. Pellets from viremic plasma had an antigen titer of approximately 1:120/0.025 ml, as determined by inhibition of the passive hemagglutination test for Syrian hamster type C antigens (7). Hamsters, 3 to 4 weeks old, were given s.c. injections, for 3 consecutive weeks, of 0.2 ml of virus suspension mixed (1:1) with Freund's complete adjuvant (Difco Laboratory, Detroit, Mich.). Spleens were harvested 1 week following the last injection.

Preparation of Cell Suspensions. Suspensions of tumor cells, liver, or kidney cells were prepared by trypsinization (0.25% trypsin) at room temperature. Suspensions of spleen cells were prepared by mechanical dispersion through a wire mesh. Viable cell counts were performed by the trypan blue-exclusion technique.

Charcoal. The charcoal used was 50 to 200 mesh acti-

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[4] The abbreviation used is: TPD_{50}, 50% tumor-producing dose of tumor cells.

vated coconut charcoal (Fisher Scientific Co., Fair Lawn, N. J.).

Irradiation. Irradiation (400 rads) was delivered from a Theratron 80 ^{60}Co source at a distance of 90 cm, delivering 85 rads/min.

Statistics. The Fisher exact probability (1-tailed) test was used to determine whether tumor incidence was significantly potentiated in treated groups, compared with control groups.

RESULTS

Potentiation of Tumor Production by Spleen Cells. Spleen cell suspensions were prepared from 7- to 8-week-old hamsters given injections of emulsions of Freund's complete adjuvant combined with resuspended plasma pellets from either normal or viremic hamsters. The spleen cells were mixed with D9 tumor cells, incubated at 37° for 30 min, and injected s.c. into 3- to 4-week-old weanling hamsters (20/group). Each animal received 4000 tumor cells. The ratio of spleen cells to tumor cells varied from 10:1 to 1000:1. Approximately 35% of hamsters receiving only tumor cells developed tumors (Chart 1). However, spleen cells from hamsters treated with adjuvant and virus significantly ($p < 0.003$) potentiated tumor incidence, compared with hamsters that received no spleen cells. Although increased tumor incidences also occurred in groups receiving spleen cells from animals treated with adjuvant and normal plasma concentrates, a significant difference from the control was obtained only at the highest ratio of spleen to tumor cells, *i.e.*, 1000:1 ($p = 0.03$).

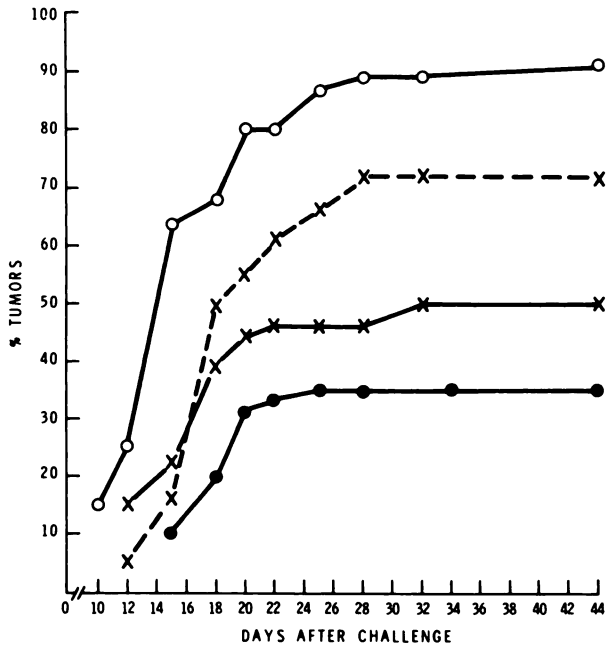

Chart 1. Potentiation of transplantable D9 lymphoma by spleen cells from hamsters given injections of Freund's complete adjuvant and concentrates of normal plasma (×) or viremic plasma (O). O, ratio of tumor cells to spleen cells from animals given injections of viremic plasma, 1:10, 1:10^2, and 1:10^3; ×- - -×, ratio of tumor cells to spleen cells from animals given injections of normal plasma, 1:10^3; ×——×, ratio of tumor cells to spleen cells from animals given injections of normal plasma, 1:10, 1:10^2; ●, tumor cells alone. Data of lines O, and ×——× were pooled from 3 and 2 groups containing a total of 56 and 32 animals, respectively.

However, further experiments showed no difference in tumor incidence between groups receiving spleen cells from hamsters treated with adjuvant and concentrates of either normal or viremic plasma (Table 1). The tumor incidence was significantly higher in all groups, compared with that of hamsters receiving tumor cells alone.

Similar effects could be obtained with spleen cells from hamsters treated with adjuvant alone or even with spleen cells from untreated animals. Thus, increased tumor incidence after spleen cells were mixed with tumor cells was nonspecific in regard to treatment of the donor animal prior to preparation of the spleen cell suspension. Experiments also showed that incubation *in vitro* was not required for the effect.

Biphasic Nature of Potentiation. In most experiments, the potentiation of tumor incidence by spleen cells was biphasic. Spleen cells from hamsters treated with adjuvant alone or adjuvant emulsified with normal or viremic plasma pellets were mixed in various ratios with D9 tumor cells (Chart 2). As the ratio of spleen cells to tumor cells increased, the tumor incidence increased significantly and then declined. A biphasic response was also noted when spleen cells from normal hamsters were tested. Optimal potentiation was usually obtained when a ratio of 10 to 100 spleen cells per tumor cell was used.

Degree of Potentiation. The effect of normal spleen cells on the TPD_{50} of the D9 tumor was determined. At the optimal dose of cells, the TPD_{50} was decreased by 10-fold. For example, the TPD_{50}'s of D9 tumor cells alone or in ratios of 1:10, 1:10^2, or 1:10^3 with normal spleen cells were $10^{2.5}$, $10^{1.5}$, $10^{1.7}$, and $10^{1.8}$, respectively.

Potentiation of Tumor in Inbred Hamsters. To exclude the possibility that an immune response of either host or donor spleen cells to isoantigens present on the random-bred D9 tumor was responsible for potentiation, the CILT/2 carcinoma of inbred LSH hamsters was also used (Table 2).

Table 1

Potentiation of D9 tumors by spleen cells from hamsters given injections of concentrates of normal or viremic hamster plasma

Spleen cells from hamsters injected with	Spleen cells: tumor cell	No. of tumors/ no. of hamsters given injections	Mean latent period (days)
Nothing		1/9	18
Normal plasma concentrate	10^2	7/10[a]	14.3[b]
	10^3	10/10[a]	13.3[b]
Viremic plasma concentrate	10^2	6/8[a]	16.3
	10^3	9/9[a]	14.6
	10^4	8/10[a]	16.8

[a] $p \leq 0.02$.
[b] $p < 0.01$, t test.

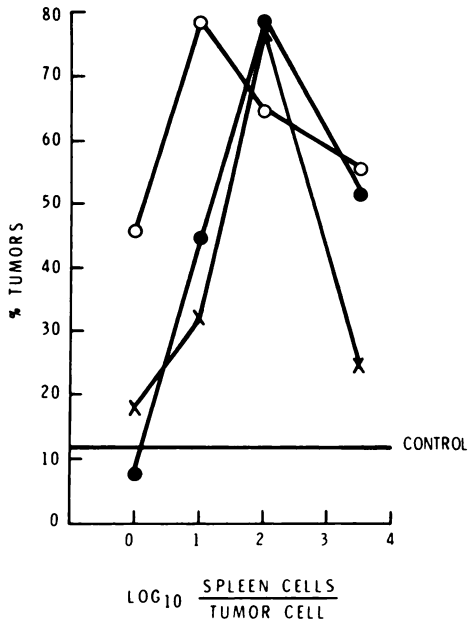

Chart 2. Biphasic response in potentiation of D9 tumor by various spleen cell-to-tumor cell ratios: O, spleen cells from hamsters given injections of Freund's complete adjuvant and a concentrate of normal plasma; ●, spleen cells from hamsters given injections of Freund's complete adjuvant and a concentrate of viremic plasma; ×, spleen cells from hamsters given injections of Freund's complete adjuvant.

Table 2
Potentiation of the CILT/2 carcinoma of inbred hamsters by normal spleen cells

Inoculum	Spleen cells: tumor cell	No. of tumors/no. of hamsters given injections in experiment	
		1	2
CILT/2		4/10	1/10
CILT/2 + spleen cells	10^1	7/8	5/10
	10^2	9/9[a]	10/10[a]

[a] $p < 0.01$.

When mixed with spleen cells from normal inbred hamsters, tumor incidence was significantly increased.

Potentiation by Normal Nonlymphoid Cells. To determine whether the potentiating effect was limited to spleen cells, the effect of suspensions of liver or kidney cells on the incidence of D9 tumors was investigated. The incidence of tumors could be increased significantly by coinoculation of tumors with normal cells from spleen, liver, or kidney (Table 3), suggesting that the ability to potentiate was not limited to immune effector cells. In a similar experiment, kidney cells mixed with CILT/2 tumor cells potentiated the appearance of these tumors (Chart 3).

Effect of Separation of Normal and Tumor Cells on Potentiation. When, instead of coinjection, normal tissue cells were injected in the opposite flank from the tumor cells, slight but statistically insignificant potentiation was observed (Chart 3).

Potentiation of Tumor Growth in Irradiated Hosts. Potentiation might represent either a local interference with a radiosensitive cell-mediated immune response of the host to tumor antigens (2), or the normal cells in the inoculum might provide metabolites for growth of the transplanted tumor cells. The D9 lymphoma cells were diluted to a point at which no tumors developed in the intact host. This cell suspension was injected into normal 4-week-old hamsters and hamsters given 400 rads of ^{60}Co irradiation, 16 to 20 hr previously. The same cell suspension was mixed with varying doses of normal cells and injected into both normal and irradiated hamsters. Tumors appeared with equal frequency in both irradiated and normal animals receiving a high dose of coinjected normal cells (Table 4).

Potentiation of Tumors by Charcoal. To determine whether coinoculated nonviable particulate matter could potentiate tumor incidence, activated charcoal was mixed and injected with D9 lymphoma cells. Doses of charcoal from 10 to 100 μg significantly increased tumor incidence (Table 5). However, the 100-μg dose appeared to be the most reliable. Higher doses tended to decrease the incidence

Table 3
Potentiation of D9 tumors by normal tissue cells

Tissue	Tissue cells: Tumor cell	No. of tumors/no. of animals receiving injections
None		3/11
Spleen	10^1	7/11
	10^2	8/10[a]
	10^3	5/9
Kidney	10^1	10/11[b]
	10^2	9/11[a]
	10^3	10/11[b]
Liver	10^1	10/11[b]
	10^2	6/9
	10^3	5/9

[a] $p < 0.05$.
[b] $p < 0.01$.

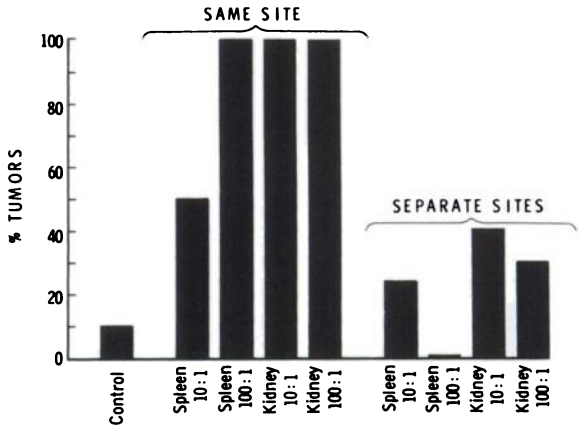

Chart 3. Effect of separation of tissue cells and tumor cells on potentiation of CILT/2 tumors. *Abscissa*, kind and ratio of tissue cells to tumor cells.

of tumors in this and other experiments. Charcoal was ineffective when used with CILT/2 carcinoma cells.

Effect of Separation of Charcoal and D9 Lymphoma Cells. Tumor incidence was increased whether charcoal was inoculated with the tumor cells, or at a separate site. With charcoal doses of 10 to 100 μg s.c. in the opposite flank, tumor incidence was increased slightly, but significantly, in some experiments at a given tumor cell dose (Table 5). However, in quantitative assays using several doses of tumor cells per charcoal dose level, charcoal was unable to consistently lower the TPD_{50} of tumor cells to a significant degree, *i.e.*, by 10-fold or more (Table 5). In some experiments, the TPD_{50} was decreased by 1 to 1.3 $\log_{10}$ by charcoal injected either at the same site or separately i.p., but not when injected s.c.

Table 4

Lack of effect of whole-body irradiation on potentiation of D9 tumor by normal spleen cells

No. of tumor cells	Spleen cells: tumor cell	No. of tumors/no. of animals receiving injections after 400 rads	None
10^1		0/8	0/8
10^2		0/8	0/8
10^1	10^1	0/8	0/8
	10^2	0/8	0/8
	10^3	0/8	0/8
10^2	10^1	0/8	0/8
	10^2	1/8	0/8
	10^3	4/8[a]	4/8[a]

[a] $p = 0.04$.

DISCUSSION

Results of these experiments indicate that potentiation of growth of transplantable tumors by admixed cells: (*a*) can be induced by spleen cells from normal donors or donors injected with normal or viremic plasma concentrates, (*b*) is sometimes biphasic with reference to spleen cell dose, (*c*) can be induced by cells from nonlymphoid tissues such as kidney or liver, (*d*) is apparently a local phenomenon, and (*e*) does not result from sensitization of host or donor spleen cells to isoantigens present on tumor cells, but could conceivably result from interference with reaction to tumor-specific antigens by a radioresistant host cell population such as macrophages.

Potentiation was detected by a significant increase in the incidence of tumors, which was accompanied by a decrease in the latent period of tumor growth. Although initial experiments utilized only one dose of tumor cells, the potentiating effect in quantitative transplantation assays at several doses of tumor cells was shown to produce a 10-fold decrease in TPD_{50} values at an optimal ratio of spleen to tumor cells.

This optimal potentiation was obtained using 10 to 100 spleen cells per tumor cell. If too few or too many spleen cells were inoculated, there was no significant difference in tumor incidence from that in control groups. Similar biphasic effects using specifically immunized spleen cells

Table 5

Potentiation of D9 tumors by charcoal

Inoculum	Tumor incidence[a] in Experiment: 1	2	3	4	5	6	Log_{10} TPD_{50} in Experiment: 7	8	9	Decrease TPD_{50} for Experiments 7, 8, and 9[b]
Tumor s.c.										
D9 cells	3/10	0/10	0/8	0/8	6/10	<1/10	2.7	2.8	2.5	
Tumor and charcoal at same site s.c.										
D9 cells + 10 μg charcoal	9/11[c]	7/10[d]		0/8			2.6			0.1
D9 cells + 100 μg charcoal			5/6[d]	5/6[d]			1.7			1.0
D9 cells + 1000 μg charcoal				2/9			2.9			−0.2
D9 cells + 1000 μg charcoal								1.5		1.3
Tumor and charcoal at separate sites										
Tumor s.c. + charcoal s.c. on opposite flank										
D9 cells + 10 μg charcoal					10/10[c]	5/10				
D9 cells + 100 μg charcoal					10/10[c]	5/10				
D9 cells + 1000 μg charcoal								2.5		0.3
Tumor s.c. + charcoal i.p.										
D9 cells + 10 μg charcoal									2.4	0.1
D9 cells + 100 μg charcoal									2.0	0.5
D9 cells + 1000 μg charcoal									1.5	1.0
D9 cells + 10000 μg charcoal									2.1	0.4

[a] No. of tumors/no. of animals treated.
[b] Log_{10} TPD_{50} of control group minus log_{10} TPD_{50} of treated group.
[c] $p < 0.05$.
[d] $p < 0.01$.

have been noted by Prehn (12), who suggested that a slight degree of tumor immunity might actually stimulate tumor growth. Using higher ratios of specifically sensitized spleen cells, he obtained specific suppression of tumor. In our experiments, suppression of tumor growth at high ratios of normal spleen cells to tumor cells might conceivably have resulted from tumor-specific sensitization of the admixed spleen cell population. In accord with this hypothesis, potentiation of tumor did not consistently decline at high input ratios of nonlymphoid tissue cells (Table 3).

In vitro stimulation of tumor cell growth by lymphoid cells has been described by several laboratories (4, 11). In addition, Fidler (5) has demonstrated that *in vitro* incubation of tumor cells and normal or immune lymphocytes results in clumping of the 2 types of cells. We have not yet attempted *in vitro* studies with our system.

Although the decline in potentiating ability at high doses of spleen cells may be directly related to immunologically competent cells in the inoculum, the phenomenon of potentiation itself probably is not. However, in the initial experiment (Chart 1), it appeared that spleen cells from D9 virus-treated hamsters specifically potentiated tumor growth, compared with the effect of low doses of spleen cells (10:1; 10^2:1), but not with higher doses (10^3:1), from hamsters treated with normal plasma concentrates. Further work did not confirm this "specific" potentiation. The subsequent negative data may have resulted from insufficient sensitization of the donors or from the lack of relevant antigens on the virion. Nevertheless, preliminary tests *in vitro* have indicated that a cell-mediated immune response to D9 virus and to D9 tumor antigen is obtained with the immunization regimen used here (S. K. Datta, K. J. McCormick, and J. J. Trentin, unpublished observations). In general, potentiation can result from both specific and nonspecific effects (12) and these effects, still under investigation for the D9 tumor, must be analyzed for each system.

Development of a tumor from a low number of cells may be potentiated by the addition of kidney or liver cells, as well as spleen cells, to the inoculum. This property and the others summarized above render immunological enhancement (9) unlikely as a mechanism of potentiation. Other possible mechanisms include: (*a*) a greater nonspecific inflammatory response (21), (*b*) a local nutrient effect by metabolizing cells, (*c*) a physical barrier to the normal immune response of the host to tumor antigens (2), or (*d*) interference with macrophage activity.

Comparable potentiation of D9-transplanted lymphoma growth was obtained in irradiated and nonirradiated hosts by 10^3 spleen cells per tumor cell. This would appear to exclude interference with a radiosensitive host-resistance phenomenon as the mechanism of potentiation. However, genetic resistance to bone marrow transplantation applies also to transplantation of both normal (19) and tumorous lymphoid tissue (15), but not to other tumors. Genetic resistance is remarkably radioresistant, and can be interfered with by a variety of substances including macrophage-blocking agents (10).

In experiments to determine whether increased particulate matter could be a factor in potentiation of tumor cells (via macrophages), we found that charcoal (100 μg) significantly increased the incidence of D9, but not of CILT/2, tumors when administered simultaneously with the tumor cells. Although slight increases in tumor incidence were noted when charcoal (10 to 100 μg) was injected s.c. in the flank opposite the tumor cells, 1000 μg of charcoal administered i.p. significantly decreased the TPD_{50} of the tumor. Slight increases in tumor incidence were also noted when normal cells were injected at sites separate from the tumor implant (Chart 3). Charcoal given s.c. at a separate site apparently is localized and does not reach its site of action as efficiently as when administered i.p.

The inability of charcoal to potentiate the CILT/2 carcinoma may be related to several possibilities. The CILT/2 tumor has a much longer latent period than the D9 lymphoma, allowing greater clearance of the charcoal. Moreover, charcoal can effectively block genetic resistance to marrow transplantation in mice (M. T. Gallagher and J. J. Trentin, unpublished observation). Genetic resistance applies to lymphomas but not to nonlymphoid or nonhemopoietic tissues. It is possible, as mentioned above, that a radioresistant mechanism akin to genetic resistance is involved in some forms of potentiation, but not others. If so, charcoal might potentiate only those tumors normally held in check by a genetic resistance-like phenomenon which would operate against lymphomas but not carcinomas.

Since macrophages are important in cytotoxic reactions to tumor cells *in vitro* (3, 8) and *in vivo* (22), macrophages may be involved in potentiation by either normal cells or charcoal. The direct action of macrophages on transplantable tumors has been demonstrated using the specific macrophage-blocking substance, carrageenan (E. Lotzova, E. Richie, and J. J. Trentin, unpublished observation). However, the relationship, if any, between the mechanisms of action of cells or charcoal in potentiating tumor growth and the relationship of these substances to the macrophage must be explored in greater detail.

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