RESISTANCE OF NEOPLASMS TO IMMUNOLOGICAL DESTRUCTION: ROLE OF A MACROPHAGE CHEMOTAXIS INHIBITOR*

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A number of studies have shown that the presence of a tumor in an animal may alter that animal's capacity to respond to immunologic stimuli (1–8). Recently, a low molecular weight factor was detected in lysates of murine tumor cells but not of normal tissues. This factor interfered with macrophage accumulation in vivo and chemotactic responsiveness in vitro (3, 6, 7). Since macrophages have been implicated as major effectors of tumor immunity (9–11), it was postulated that by producing this factor, tumors might escape immunologic destruction (3, 6, 7). Indeed, it was shown that the administration of the factor to mice made them more susceptible to the development of progressive tumors when implanted with low numbers of neoplastic cells (6). In the present investigation, tumor cells of common origin but different degrees of virulence in vivo were studied to determine if the ultimate fate of the tumor in vivo corresponded to the ability of the cells to produce the macrophage chemotaxis inhibitor (MCI).[1]

Materials and Methods

Tumor. A mouse lymphoma, 6C3HED, which originated in C3H mice was purchased from The Jackson Laboratory, Bar Harbor, Maine, and maintained by serial transplantation in the calf muscles of C3HeB/FeJ mice. Single cell suspensions were prepared as described (12). These freshly prepared cells are hereafter called virulent tumor. A tumor cell line was first adapted into tissue culture in 1974 (line 1) and again in 1976 (lines 2 and 3). For adaptation to tissue culture, 2 ml of single cell suspensions were inoculated into 12 × 75-mm culture tubes (Falcon Products, Div. Becton, Dickinson & Co., Cockeysville, Md.) at a concentration of 10^6 cells/ml in RPMI-1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (Microbiological Associates, Walkersville, Md.), and incubated in humidified air

[1] *Abbreviations used in this paper:* ATXBM, lethally-irradiated adult-thymectomized mice reconstituted with thymocyte-depleted bone marrow cells; MCI, macrophage chemotaxis inhibitor; PHA, phytohemagglutinin; RIA, radioimmunoassay.

containing 5% CO_2. The cells were incubated while being monitored by periodic cell counts. Once consistent proliferation was detected, the culture was transferred to a 250-ml culture flask with sufficient medium to yield 25 ml. The cells were maintained by decanting the medium after gentle agitation to remove most cells from the bottom of the flask and by adding 25 ml of fresh medium. New flasks were propagated by seeding them with 25 ml medium containing tumor cells at 10^3–10^4/ml.

Mice. C3HeB/FeJ males of 8-12 wk of age from The Jackson Laboratory were used in all tumor experiments, and for assaying MCI.

Tumor Inoculation. Tumor cells were suspended in Hanks' balanced salt solution at the desired concentration and injected into the calf muscles of recipient mice in a 0.05-ml volume. The growth of the tumors was recorded as previously described (12).

Irradiation. Sublethal irradiation was performed as previously described (12).

Thymectomy of Mice. 4-wk-old C3H mice were thymectomized, irradiated with 850 rads, and reconstituted with bone marrow cells treated with anti-Thy 1 and complement as described (12). These are designated ATXBM mice.

Preparation of Macrophage Chemotaxis Inhibitor. Tumor cells or control tissues were suspended to contain 5×10^7 cells/ml in RPMI-1640. The lysates were prepared by sonicating the suspensions for 2 min followed by centrifugation for 10 min at $12,000\,g$. Filtrates were prepared by subjecting the lysates to ultrafiltration in Amicon CF25 cones (Amicon Corp., Lexington, Mass.). The membrane cutoff was nominally 25,000 daltons; the filtrates containing material that passed through the cones and the lysates were divided into aliquots and stored frozen at $-70°C$ until use.

Assay of Macrophage Chemotaxis Inhibitor. Inhibition of in vivo macrophage accumulation was measured as previously described (2, 3). Briefly, groups of four mice were injected subcutaneously in the thigh with 0.2 ml of the tumor or control tissue filtrates. 24 h later, the mice were injected intraperitoneally with 35 μg of phytohemagglutinin (PHA) (Burroughs Wellcome Co., Research Triangle Park, N.C.) contained in 2 ml sterile, isotonic saline. 2 days later, the mice were killed, the peritoneal cavities were lavaged, and the total and differential white cell counts were determined for the individual mice. Results are expressed as: percent inhibition = $(1 - (E/C)) \times 100$, where E equals the number of macrophages recovered from the peritoneal cavities of experimental mice treated with tumor or control tissue filtrates, and C equals the number of macrophages recovered from the peritoneal cavities of mice not treated with filtrates.

Determination of Relative Antigen Density on Tumor Cells. The antigenic density on tumor cells was estimated using an indirect radioimmunoassay (RIA). In this RIA the amount of anti-tumor antibody bound was a function of the amount of tumor antigen present on reacting cells. The details of the assay are published elsewhere (13). An antibody preparation containing mainly IgG1 (14) was incubated with 10^6 tumor cells. The cells were washed and reincubated in the presence of ^{125}I-rabbit anti-IgG1, then washed again and counted for ^{125}I. Conditions were chosen such that the amount of IgG1 bound was a linear function of the amount of tumor cells present. The relative volume of individual tumor cells was calculated from the packed volume of a known number of tumor cells. The average volume of a single cell was used to obtain the radius of the corresponding sphere. From this value, the surface area per tumor cell was calculated. Cell counts were performed in an hemocytometer. Line 1 tumor cells were >95% viable. Virulent tumor cell preparations contained <10% erythrocytes and were >95% viable.

Results

Growth Characteristics of Attenuated Cells. After introduction into culture, line 1 cells and subsequently, those of lines 2 and 3 were tested for their ability to grow in syngeneic hosts. A serial change in the growth pattern during tissue culture adaptation was observed. In the initial stages of culture, the cells behaved like the virulent tumor and were uniformly lethal to recipient animals. After a variable period in culture on the order of 4-8 wk, such cells displayed an attenuated phenotype. Fig. 1 illustrates the growth pattern of cells during this period. In this experiment, 10^5 line 1 or virulent tumor cells were inoculated into the calf muscles of syngeneic C3H mice. As expected, the virulent cells gave rise to progressively growing tumors which eventually killed their hosts.
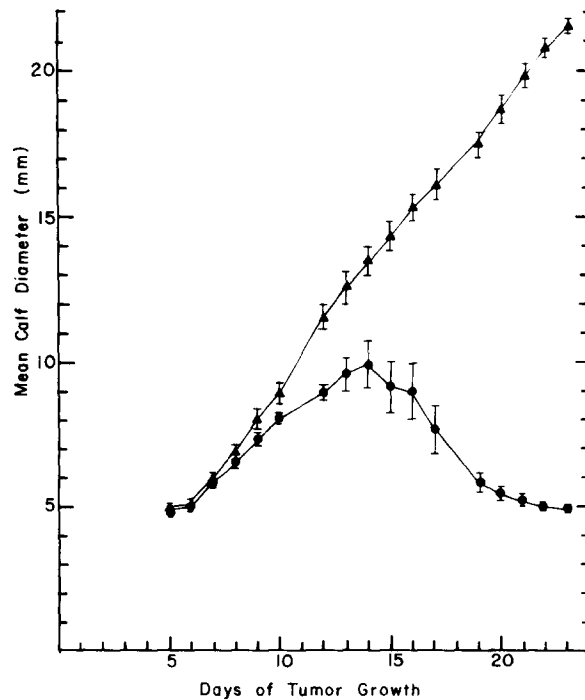
Fig. 1. Growth of virulent and line 1 cells in syngeneic hosts. Groups of four mice were inoculated with either 10^5 attenuated line 1 cells or 10^5 virulent 6C3HED cells in the left calf muscle on day 0. The mean calf diameter $\pm$ SEM is plotted versus days of tumor growth. (●), attenuated; (▲), virulent.

The cultured line 1 cells, however, produced tumors that regressed in all mice tested. After regression, no recurrence was observed over an extended period of time. Lines 2 and 3 behaved in like fashion to line 1 when similarly tested. After prolonged periods in culture exceeding 3 mo, the attenuated tumor cells failed to produce tumors in normal hosts, although they succeeded in suble-thally irradiated animals.

The regression of the line 1 tumors was dependent upon an intact host immune system. ATXBM mice were unable to cause tumors originating from 10^5 line 1 cells to regress. The data in Fig. 2 show that all ATXBM mice developed progressively growing tumors and died, whereas similarly injected normal C3H mice showed only transient tumor growth. The attenuated cells grew in ATXBM mice at a rate approximately equal to the rate of growth of virulent cells in normal syngeneic mice. The requirement for an intact immune system implies that the regression of tumor growth is an immunologic rejection phenomenon rather than an alteration of some intrinsic growth properties of the tumor. Other experiments (data not shown) demonstrated that once mice rejected tumors from cultured cells, they would not support tumor growth when subsequently challenged with either attenuated or virulent cells.

Antigen Density Is Unchanged. Since attenuated cells could grow in vivo in ATXBM mice at a rate comparable to the usual growth rate of virulent cells, factors other than a differential proliferative rate were sought to explain the
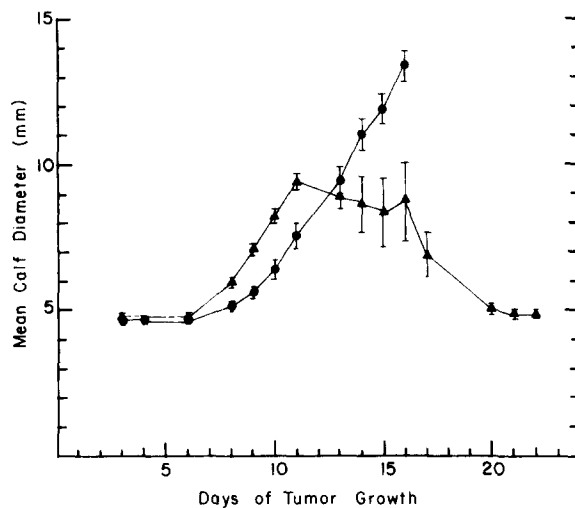
FIG. 2.   Growth of line 1 cells in normal and ATXBM syngeneic hosts. Groups of four normal and five ATXBM mice were inoculated with 10^5 attenuated line 1 cells in the left calf muscle on day 0. By day 22, all normal mice had rejected their tumors and were alive. None of the ATXBM mice showed evidence of tumor rejection, and all were dead by day 22. The mean calf diameter ± SEM is plotted versus days of tumor growth. (▲), normal; (●), ATXBM.

susceptibility of cultured cells to host defenses. The relative antigen density of the virulent and attenuated tumor cells was measured by RIA using syngeneic anti-tumor antibody. The antibody was produced by injecting syngeneic mice intraperitoneally with $\cong 10^7$ cultured cells in 0.1 ml Hanks' balanced salt solution emulsified in an equal volume of complete Freund's adjuvant at weekly intervals. The antibody was collected as ascites fluid, which was clarified by centrifugation and used directly in the RIA. The results of the RIA shown in Table I indicate that approximately the same amount of IgG1 was bound per unit of area on each tumor population. The difference in the gross amount of IgG1 anti-tumor antibody per cell could be explained simply by differences in the surface areas of the two cell populations.

Elaboration of Macrophage Chemotaxis Inhibitor. Since no gross antigenic difference was found between the virulent and attenuated cells, an attempt was made to examine other functional immunologic differences between the tumor cells. In several experiments, lysates were prepared from attenuated and from virulent cells. These were examined as unknown coded samples for their ability to inhibit macrophage accumulation in vivo. The results of these experiments (summarized in Table II) indicate that cultured cells, which displayed an attenuated phenotype with respect to tumor growth, regularly failed to produce an inhibitor of macrophage accumulation in vivo. In contrast, the virulent tumor was always found to produce this inhibitor.

Macrophage Chemotaxis Inhibitor Restores Virulence to Cultured Cells. In light of the preceding findings, we sought to determine if MCI would reconstitute the virulent tumor phenotype in the attenuated tumor. To this end, filtrates prepared from various tumors and control tissues were coded and tested for inhibitory activity as unknowns. The tests were performed by inoculating

TABLE I

Relative Antigen Density: Attenuated Versus Virulent Tumor Cells

Experi- ment	Anti-IgG$_1$/tumor cell		Surface area/tumor cell		Anti-IgG$_1$/surface cell	
	Virulent	Attenuated	Virulent	Attenuated	Virulent	Attenuated
	cpm		μ^2		*cpm/μ^2*	
I	4.2×10^{-2}	6.4×10^{-2}	3.3×10^2	3.9×10^2	1.3×10^{-4}	1.6×10^{-4}
II	1.2×10^{-2}	1.9×10^{-2}	3.1×10^2	4.4×10^2	3.9×10^{-5}	4.3×10^{-5}

Line 1 cells were used as attenuated cells and freshly harvested 6C3HED cells were used as virulent cells. The RIA and determination of surface area were performed as described in the text. The two experiments were performed on separate days.

TABLE II

An Inhibitor of Macrophage Accumulation in Virulent Neoplastic Cells

Experiment	Lysate injected in thigh	Accumulated perito- neal macrophages (10^6)	Inhibition
			%
I	Virulent	4.0 ± 0.6*	43
	Line 1	6.8 ± 1.3	3
	None	7.0 ± 0.2	—
II	Virulent	2.3 ± 0.8	65
	Line 1	6.1 ± 2.0	6
	Line 2	5.6 ± 0.7	14
	Line 2‡	5.3 ± 0.3	18
	Liver	7.3 ± 0.9	0
	Spleen	6.9 ± 0.1	0
	None	6.5 ± 0.7	—
III	Virulent	3.9 ± 0.9	38
	Line 3	6.0 ± 0.5	5
	None	6.3 ± 1.8	—

Coded tumor or control cell samples were sonicated, centrifuged, and the cell-free lysates were tested for inhibition of macrophage accumulation in vivo in response to an intraperitoneal injection of PHA. Each lysate was tested in a group of four animals.

* ± SEM.

‡ A second sample was included in this experiment under a different code letter.

10^5 tumor cells contained in a mixture consisting of 50% Hanks' solution and 50% filtrate by volume. On the 2nd and 9th days after inoculation of the tumors, the animals received an additional 0.2 ml of lysate, i.p. Two separate experiments are summarized in Table III. The results clearly show that filtrates prepared from tumors, which possess the ability to interfere with macrophage chemotaxis, were able to interfere with the rejection of the attenuated tumors. This effect was not specific, since preparations from several tumors were equally effective. The filtrates prepared from normal tissues without malignant character did not prevent regression of attenuated tumor cells.

TABLE III

Reconstitution of Virulent Phenotype in Attenuated Tumor

Experiment	Filtrate source	Tumor	Mice reject-ing
I	Liver	Line 2	4/4
	6C3HED	"	0/4
	Hepatoma*	"	4/4
II	Liver	Line 3	4/4
	Hepatoma*	"	2/4
	BP8	"	2/4
	Spleen	"	4/4
	6C3HED	"	2/4

Mice were challenged with 10^5 cultured cells and treated as described in the text. The preparation of the filtrates from 6C3HED, the BP8 fibrosarcoma, hepatoma 129, and from normal C3H tissues was previously described (3).

* The inconsistency of the results with the hepatoma has not been explained.
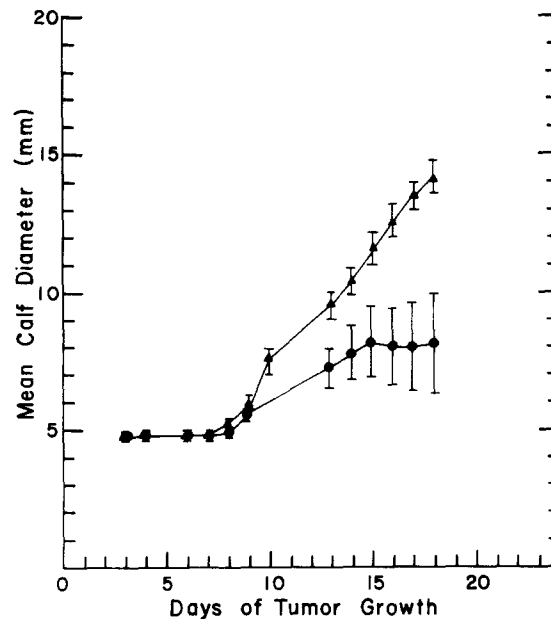

FIG. 3.   Growth of line 1 cells in normal and tumor-bearing hosts. Four mice were inoculated with 10^5 virulent 6C3HED cells in the left calf muscle on day −1. On day 0, these and four untreated mice were inoculated with 10^5 attenuated line 1 cells in the right calf muscle. By day 19, three of four untreated animals had rejected their tumors; the fourth failed to reject and died. None of the animals bearing virulent tumors showed any evidence of rejection of either tumor. The mean calf diameter ± SEM is plotted versus days of tumor growth. (●), untreated animals; (▲), animals bearing virulent tumors.

Virulent Phenotype Is Dominant.   The growth pattern of attenuated cells may be influenced by the presence of virulent cells elsewhere in the same animal. As stated, with increasing time in culture, tumor cells become less able to grow in normal mice, although they still grow in sublethally irradiated

animals. Rejection of tumor cells recently displaying the attenuated phenotype is impaired in a host bearing a virulent neoplasm. In the experiment shown in Fig. 3, three of four animals receiving only the attenuated tumor cells eventually rejected their tumors; the onset of rejection was between the 14th and 18th days. The failure of an occasional animal to reject attenuated cells was not an uncommon observation. These findings stand in marked contrast to the behavior of the attenuated cells given to hosts that had been injected 1 day previously with an equal number of virulent tumor cells in the opposite leg. In these animals, no regression of the attenuated cells was seen.

Discussion

Previous studies established a number of interesting points concerning the behavior of tumor cells in tissue culture. A number of workers showed that tumor cells maintained in tissue culture for prolonged periods of time may lose the ability to grow in the strains of mice from which they originated (15-17); these observations have been extensively confirmed. In a pertinent study, Morgan et al. (18) noted that 6C3HED behaved in this fashion, and that the nontumorigenic, or attenuated cells were immunogenic in C3H mice, conferring resistance to a challenge with virulent cells. Nontumorigenic cells become virulent only when injected in medium containing sterile ascites fluid from a virulent Ehrlich-Lettré tumor. Moore et al. (19) made similar observations in an Ehrlich ascites tumor system. In their model, attenuated cells that failed to grow in adult mice produced tumors in new born animals, suggesting an immunologic basis for attenuation.

In our work, we also observed that 6C3HED behaved in this manner; moreover, the use of an intramuscular site allowed us to detect tumor cells with tumorigenicity intermediates between virulence and complete attenuation. The failure of ATXBM mice to reject the attenuated tumor cells shows that the destruction of attenuated cells in vivo is immunologically mediated. Morgan et al. (18) were unable to detect growth of an intraperitoneal inoculum of attenuated cells in lethally irradiated mice; this finding, however, might be explained on the basis of local factors such as the resident population of peritoneal macrophages.

The reasons that mice are more resistant to tumor cells that have been serially passed in tissue culture have been previously unclear, but are of basic importance for our understanding of the mechanisms of natural resistance to neoplasms. Our studies demonstrate one clear-cut difference between attenuated and virulent neoplastic cells. Although MCI has been found in all the virulent murine neoplasms thus far examined (3, 7), neither normal murine tissues nor the attenuated neoplastic cells contain this inhibitory activity. This observation was given additional biological significance in the present experiments where it was shown that the addition of the inhibitor reconstituted the malignant phenotype of the attenuated cells in vivo. Although it remains possible that this was caused by a factor other than MCI present in the partially purified preparations, it is clear that the effect was mediated by a factor unique to malignant tissues. These experiments showed that during passage in tissue culture, the neoplastic cells lost their ability to produce an inhibitor of macrophage function, suggesting a causal relationship to the attenuated phe-

notype. A macrophage chemotaxis inhibitor has been previously demonstrated to circulate in the serum of mice with progressively growing tumors (7). This being the case, it was important to determine whether or not the attenuated cells would grow in mice already bearing virulent tumor cells. With recently attenuated tumor cells, some factor produced by the virulent tumor cells prevented rejection and the mice developed progressive tumors at both sites of cell administration; the attenuated cells had no obvious influence upon the virulent tumor residing in the opposite leg. Had concomitant immunity (20) been of significance in this experiment, the attenuated cells would have shown diminished growth. This is consistent with the observation by Eng et al. (21) that simultaneous intraperitoneal inoculation of attenuated and virulent 6C3HED cells did not prevent ascites tumor formation.

A number of workers have shown that the macrophages may play a central role in mediating immunological resistance to neoplasms (9, 10), and may be the major effectors in passive immunotherapy models (11, 12). This former contention is supported by the studies presented herein, since interference with macrophage function was shown to influence the ultimate outcome of a host-tumor relationship. In previous studies, it was suggested that the ability of neoplastic cells to subvert the host's macrophage function might be critical in determining whether or not the cells would grow successfully. It is possible that a tumor's defense against the host's immune system might be based upon the ability of neoplastic cells to prevent an effective immunological response within the tumor (1-8). Conversely, some workers have shown but failed to explain the enhancement of tumor growth by fluids from tumor-bearing animals (18, 22). The studies reported here suggest that the ability of neoplastic cells to produce an inhibitor of macrophage chemotaxis might be the central event that allows them to overcome natural resistance and thereby grow successfully. Should this prove to be a general mechanism whereby tumors evade the host's immune response, the detection, isolation, and characterization of such factors may lead to completely new approaches to immunodiagnosis and immunotherapy of malignant disease.

Summary

Several tissue culture lines of 6C3HED, a murine lymphoma, were more susceptible to immunologic destruction in vivo than the highly virulent 6C3HED line maintained by serial intramuscular transplantation. The attenuated tissue culture cells were rejected by normal syngeneic recipients, but thymectomized mice were unable to reject attenuated cells. In such mice, the growth rate of attenuated cells was equivalent to the growth rate of virulent cells in normal syngeneic mice. The increased susceptibility of attenuated cells to destruction by syngeneic hosts was shown to correlate with decreased production by the tumor cells of a macrophage chemotaxis inhibitor, and not with altered antigen density. In addition, when inhibitor isolated from virulent cells was administered to mice challenged with attenuated cells, the latter cells became virulent in vivo. When attenuated and virulent cells were administered simultaneously in the same host, the attenuated cells were able to develop into progressively growing tumors. The data suggest that the successful growth of

neoplastic cells in normal animals may require tumor cells to produce factors which subvert the ability of the host to mobilize macrophages rapidly at the tumor site.

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