THE TUMOR DORMANT STATE

Quantitation of L5178Y Cells and Host Immune Responses during the Establishment and Course of Dormancy in Syngeneic DBA/2 Mice*

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Treatment of primary tumors in patients is often followed by a prolonged clinical remission which terminates with the occurrence of a tumor morphologically identical to the primary. Such secondary tumors could have been formed by proliferation of tumor cells transformed near the end of remission by the same oncogenic agent that induced the primary tumor or by outgrowth of residual primary tumor cells that remained dormant throughout the clinical remission (1, 2). The tumor dormant state, can be defined as "a state in which tumor cells persist in a host for prolonged periods of time with little net growth" and which is terminated by an event which permits their rapid outgrowth.

Hadfield (3) first drew attention to the concept of the dormant cancer cell in 1954 when he noted the extremely long time interval between the excision of many human tumors and the occurrence of histologically identical tumors thereafter. His observation that these late-arising tumors, once clinically evident, had growth rates comparable to the primary tumor, indicates that until the event permitting tumor emergence occurred, tumor outgrowth was under restraint. The appearance of tumors, morphologically identical to but at sites distant from the primary tumor, years after apparent successful treatment of the primary, suggests that residual metastases persisted in a dormant state at these sites throughout the clinical remission (4). In addition, the finding in unselected autopsies of an incidence of carcinoma in situ that is far greater than the incidence of overt tumors of the same kind in the population (2), suggests that a tumor dormant state may sometimes precede primary tumor outgrowth.

In spite of the probable frequent occurrence of clinical tumor dormancy, only a few animal models have been developed for studying the tumor dormant state. Experimental tumor dormant states have been achieved by implantation of tumor cells into an avascular site (5), by deprivation of hormones on which tumor growth is dependent (6), and by excision of a primary tumor after metastasis has occurred (7). Oncogenic viruses have also been suppressed to a dormant state by using immunostimulants (8). The dormant state has been broken by vascularization of a formerly avascular tumor

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site (5), administration of hormones (6), immunosuppression (7), and surgical stress (9).

We have previously reported (10) the occurrence of a tumor dormant state in DBA/2 mice after their immunization and subsequent challenge with syngeneic lymphoma cells L5178Y. These mice remained clinically normal for as long as 320 d before the onset of rapid tumor cell outgrowth. Tumor cells were isolated from the peritoneal cell (PC)[1] population of these mice during the period of clinical normalcy. In this paper, we report on the early events involved in the establishment of the tumor dormant state and relate these events to the subsequent prolonged clinical remission and eventual termination of tumor dormancy.

Materials and Methods

Mice. DBA/2 female mice (8 wk of age) were obtained from The Jackson Laboratory, Bar Harbor, Maine

Tumor Cell Lines. DBA/2-derived methylcholanthrene-induced L5178Y lymphoma cells and benzanthracene-induced EL-4 lymphoma cells derived from a C57BL/6 mouse were obtained from Dr. Lionel Manson of the Wistar Institute, Philadelphia, Pa. The L5178Y lymphoma is an immunogenic, weakly metastatic cell line in which as few as 10 cells injected i.p. into normal DBA/2 mice routinely produce ascitic tumors and death in nearly 100% of mice (11). Friend leukemia virus-transformed DBA/2 erythroblasts designated clone 745 (GM86) were obtained from the Genetic Mutant Cell Repository, Camden, N. J. Both lymphoma lines were maintained by weekly i.p. passage of 10^7 cells into normal syngeneic mice. Additionally, all lines were maintained in vitro as suspension cultures in RPMI-1640 growth medium (pH 7.0) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cultures were incubated at 37°C in a humidified chamber containing a 5% CO_2 atmosphere.

Immunization of Tumor Recipients and Establishment of the Dormant State. L5178Y lymphoma cells (1×10^6 cells per mouse) were implanted subcutaneously on the ventral surface of DBA/2 mice. 10 d later the resultant 1 cm diameter nodules were surgically excised, and 7 d later all mice were challenged intraperitoneally with 5×10^4 L5178Y cells. Control mice which were immunized but not challenged were free of isolatable tumor cells and lived a normal lifespan.

Clinical Normalcy. Immunized and challenged mice were judged to be clinically normal if: (*a*) the total body weight did not exceed 30 g, (*b*) the abdomen did not appear distended, (*c*) no macroscopic tumor foci were present in the peritoneal cavity, and (*d*) the total peritoneal cell count did not exceed 10^7 cells. Tumor dormant mice were considered to have passed into a tumor emergent phase when their total PC count exceeded 10^7 cells.

Antisera. Alloantiserum was prepared in C57BL/6 mice by a series of 6 i.p. injections of 10^7 L5178Y cells at 10-d intervals. 2 wk after the last injection all mice were exsanguinated and the serum fraction prepared. This serum contained high titers of cytolytic antibody active against normal and transformed DBA/2 cells, but not against cells of C57BL/6 origin. AKR anti-C3H thymocyte serum (anti-Thy 1.2) was purchased from Litton Bionetics (Kensington, Md.). Nonconjugated as well as fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG sera were obtained from Cappel Laboratories, Inc. (Cochranville, Pa.).

Enumeration of Tumor Cells. Single cell suspensions of various cell populations were prepared in supplemented RPMI-1640 at a concentration of 10^7 cells/ml. 150 μl of each suspension was added into the first well of each row of flat-bottomed microtiter plates. A series of threefold dilutions was then made into the remaining wells in each row. The plates were incubated for 10–14 d at 37°C in a 5% CO_2 humidified atmosphere and examined daily for tumor cell outgrowth. The highest dilution of each suspension which yielded positive in vitro tumor outgrowth after incubation for 10–14 d was identified, and the number of tumor cells in the

[1] *Abbreviations used in this paper*: AR, absorbance ratio; C′, complement; CMC, cell-mediated cytolysis; DPC, days post challenge; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PC, peritoneal cell; PBS, phosphate-buffered saline.

original suspension was calculated. Culture wells were scored positive for tumor outgrowth when microscopic observation showed increasing numbers of large lymphoblastoid cells which continued to proliferate after transfer into new culture wells. Cells from such positive wells were invariably tumorigenic when implanted into normal DBA/2 mice.

Cell Preparation

EFFECTOR CELLS. Mice were killed by cervical dislocation. Spleen cell and inguinal lymph node suspensions were prepared in supplemented RPMI-1640 by gentle flushing and aspiration through an 18-gauge needle. Debris was removed by passage through a nylon mesh screen. PC were harvested by vigorous lavage with phosphate-buffered saline (PBS). Peripheral blood leukocyte suspensions were prepared from whole heparinized blood by centrifugation through Ficoll-Hypaque according to the procedure of Bøyum (12). After washing, the test cells were resuspended to the appropriate concentration in supplemented RPMI-1640.

ADHERENT AND NONADHERENT EFFECTOR CELLS. Peritoneal cells prepared as above were diluted to a concentration of 10^7 cells/ml in supplemented RPMI-1640. 0.1 ml of this suspension was placed into replicate 6-mm flat-bottomed microtiter plate wells, and suspensions were incubated at 37°C in a humidified CO_2 atmosphere for 2 h. The nonadherent cells were then removed by gentle washing with 3 vol of supplemented RPMI-1640. These cells were washed three times in 1640, resuspended to 0.1-ml vol, and transferred to fresh microtiter plate wells. The initial wells containing adherent cells received 0.1 ml of RPMI-1640. These subpopulations served as effector cells in 18-h ^{51}Cr-release assays as described below.

ENRICHED T AND B SUBPOPULATIONS OF EFFECTOR CELLS. Peritoneal effector cells were assayed for sensitivity to treatment with either anti-Thy 1.2 + C′ or anti-Ig + C′. Briefly, 4×10^6 PC in 0.4 ml of supplemented RPMI-1640 were incubated with either mouse anti-Thy 1.2 serum (Litton Bionetics) + C′ or rabbit anti-mouse Ig (Cappel Laboratories) + C′ at 37°C for 1 h with occasional agitation. The anti-Thy 1.2 and anti-Ig sera were used at a final dilution of 1:10. Fresh frozen guinea pig serum served as the C′ source and was used at a final dilution of 1:10. Controls included sera alone and C′ alone. After treatment, cells were washed three times in media and diluted to the original volume. Treated cell suspensions were assayed for their cytolytic activity against ^{51}Cr-labeled L5178Y cells in the cell-mediated cytolysis (CMC) assay described below.

TUMOR CELL TARGETS. Tumor cells obtained from tissue culture or the peritoneal cavity of tumor-bearing mice were resuspended in 1 ml of supplemented RPMI-1640 at a concentration of 5×10^6 cells/ml and incubated at 37°C for 90 min with 150 μCi of $Na_2{}^{51}CrO_4$ (sp act 200–500 mCi/mg, New England Nuclear, Boston, Mass.). After incubation, the labeled cells were washed four times and resuspended in supplemented RPMI-1640.

CMC ASSAY. A modification of the procedure described by Thorn et al. (13) was used for CMC assays. Briefly, 0.1 ml of effector cells (10^7/ml) was added in triplicate or quadruplicate into the wells of a flat-bottomed microtiter plate (Costar, Cambridge, Mass.) followed by the addition of 0.1 ml of target cells (10^5/ml) giving an effector:target ratio of 100:1.

The plates were incubated for 18 h at 37°C in a 5% CO_2 humidified atmosphere. After incubation, a 0.1-ml aliquot was removed from each well and counted in a well-type gamma counter (model 1185, Nuclear Chicago, Des Plaines, Ill.). Spontaneous release of ^{51}Cr, ranging from 16 to 28%, was determined by incubating the target cells in medium alone for 18 h. Maximum release of ^{51}Cr was determined by hypotonic lysis of tumor targets and ranged from 78 to 93%. The percentage of cytolysis was determined according to the formula:

$$\text{Percent cytolysis} = \frac{\text{cpm released in test sample} - \text{cpm of spontaneous release}}{\text{total releasable cpm} - \text{cpm of spontaneous release}} \times 100.$$

Antibody-Dependent Complement-Mediated Cytolysis Assay. Serial twofold dilutions of heat-inactivated serum from tumor cell immunized and challenged mice were prepared in supplemented RPMI-1640. 50 μl vol of each serum dilution were plated in triplicate or quadruplicate into the wells of a round-bottomed microtiter plate (Falcon Labware, Oxnard, Calif.) followed by the addition of 0.05 ml of a 1:6 dilution of fresh-frozen guinea pig serum as a source of complement. Finally, 0.1 ml of labeled target cells (2×10^5 cells/ml) was added to each well.

The plates were sealed with adhesive plastic tape, shaken vigorously for 30 s, and incubated for 90 min at 37°C in a 5% CO_2 humidified atmosphere. After incubation, the plates were

centrifuged at 200 *g* for 10 min. A 0.1-ml aliquot was removed from each well and counted in a well-type gamma counter. Spontaneous release was determined by incubation of target cells in medium alone. Additionally, cytolysis by serum or complement was determined by incubation of target cells in the presence of either serum alone or complement alone. Spontaneous release, as well as serum or complement release, ranged from 3 to 7%. The percentage of cytolysis was determined as described above.

Indirect Radioimmunoassay. The presence of cytophilic serum antibody was determined by a modification of a radioimmunoassay procedure described by Sparks et al. (14). Briefly, 10^6 L5178Y cells in 0.1 ml were added to 0.1 ml of serially diluted heat-inactivated serum obtained from tumor cell immunized and challenged mice. The mixture was incubated at R.T. for 60 min with constant agitation. After incubation, the cells were washed eight times with PBS containing 10% FCS (PBS-FCS). To the cell pellet was added 35 μl of a 1:100 dilution of ^{125}I-labeled goat anti-mouse immunoglobulin. The mixture was incubated at 5°C for 60 min with constant shaking. Afterward, the cells were washed nine times in PBS-FCS and the cell pellet counted in a well-type counter. The amount of specific binding is expressed as an absorbance ratio (AR) which is determined by:

$$\text{AR} = \frac{\text{cpm bound with experimental serum}}{\text{cpm bound with normal mouse serum}} \times 100.$$

Indirect Immunofluorescence Assay. L5178Y cells were resuspended in PBS-FCS at a concentration of 5×10^6 cells/ml. 0.1 ml of cells was incubated with 0.1 ml of various dilutions of sera obtained from L5178Y cell immunized and challenged mice for 30 min at 5°C. The cells were washed three times with PBS and the pellet resuspended to 0.1 ml. 0.1 ml of a 1:5 dilution of FITC-labeled goat anti-mouse serum was added and the mixture reincubated at 5°C for 30 min. After further washing with PBS, cells were placed on clean glass microscope slides and sealed with glass cover slips. Slides were examined at × 930 magnification with an AO vertical illumination microscope with an HB-50 ultraviolet light source.

Results

I. *Enumeration of L5178Y Cells in the Peritoneal Cavity.* Because the events to be quantitated during the establishment of the L5178Y cell dormant state are to be placed within the framework of cumulative mortality of the tumor dormant mice, we present in Fig. 1 A the kinetics of tumor associated deaths in normal and L5178Y immunized DBA/2 mice inoculated with 50,000 L5178Y cells i.p. A similar cumulative mortality curve has been published previously (10).

In our analysis of the establishment of L5178Y cell dormancy, we first measured the number of tumor cells in the peritoneal cavity of L5178Y cell immunized and challenged mice. Immunized but nonchallenged mice lived a normal lifespan, remaining free of tumor cells in their peritoneal and spleen cell populations. Suspensions of PC were prepared from immunized mice on various days with respect to i.p. L5178Y cell challenge and serial threefold dilutions of these suspensions were cultured in wells of microtiter plates at 37°C and observed for outgrowth of tumor cells. The calculation of the number of tumor cells in the inoculum by end-point dilution analysis was based on observations in our laboratory that one to three tumor cells are capable of outgrowth in culture wells during a 14-d incubation period (our unpublished data).

Fig. 1 B illustrates that after i.p. challenge, the number of L5178Y cells increased and reached a peak in 4 d, steadily declining thereafter to lower levels ranging from 300 to 1,000 per mouse for the remainder of the tumor dormant state. Beginning at 48 d postchallenge (DPC), some mice developed ascitic tumors and these mice contained 10^7–10^8 tumor cells in the peritoneal cavity; the remaining clinically normal

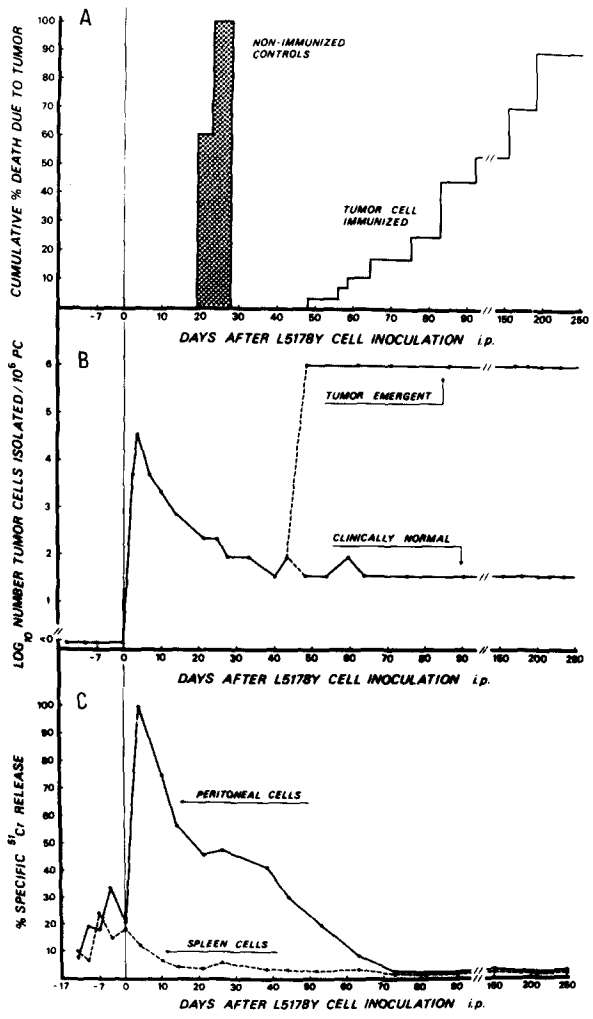

FIG. 1A. Tumor associated deaths in normal and L5178Y cell immunized mice after i.p. inoculation of 5×10^4 L5178Y lymphoma cells. The nonimmunized control group (shaded histogram) contained 10 normal DBA/2 mice. The experimental group (open histogram) contained 30 L5178Y cell immunized age-matched mice. Groups of mice which were immunized but not challenged had no incidence of tumor-associated death (not shown). FIG. 1B. Numbers of L5178Y cells isolated from the peritoneal cavity of L5178Y cell immunized mice before and after i.p. L5178Y cell challenge as measured by end point dilution analysis. Each datum point represents the mean number of tumor cells isolated from 6 to 12 individual mice. Mice were categorized as clinically normal or tumor emergent on the basis of their total PC numbers. For each datum point, the standard error was less than 12% of the mean value. FIG. 1C. Kinetics of tumor-specific cell-mediated cytotoxicity in L5178Y cell immunized and challenged mice. Each datum point represents the 18-h CMC activity of peritoneal cells (solid line) or spleen cells (dashed line) present in a pool of respective cell suspensions from three clinically normal mice/time point. The effector:target ratio in all cases was 100:1. The standard error for each point is less than 10% of the mean value. The data expressed in Fig. 1B and 1C are derived from several experiments, the values of which all fell within the indicated standard errors of the mean.

mice continued to harbor 300–1,000 tumor cells until they developed emergent tumors.

II. *Effect of Cell Density on In Vitro Isolation of L5178Y Cells.* During the course of tumor cell isolations in vitro, an observation was made which may bear on the

TABLE I

Effect of Cell Density on Outgrowth of Tumor Cells In Vitro from Cultures of Peritoneal Cells Removed from Immunized Mice on Days after L5178Y Cell Challenge

No. of peritoneal cells in each culture well	Day of peritoneal cell collection after L5178Y cell challenge					
	7		41		85	
	Tumor cell outgrowth*	No. of tumor cells present	Tumor cell outgrowth	No. of tumor cells present	Tumor cell outgrowth	No. of tumor cells present
1.0×10^6	0	6,561	0	81	+	243
3.3×10^5	0	2,187	+	27	+	81
1.1×10^5	0	729	+	9	+	27
3.7×10^4	0	243	+	3	+	9
1.2×10^4	0	81	+	1	+	3
4.1×10^3	+	27	0	0	+	1
1.4×10^3	+	9	0	0	0	0
4.6×10^2	+	3	0	0	0	0
1.5×10^2	+	1	0	0	0	0
5.0×10^1	0	0	0	0	0	0
Percent ^{51}Cr release from 10,000 L5178Y cells incubated with 10^6 peritoneal cells‡	86%		35%		3.2%	

* Determined by production of large numbers of lymphoblastoid cells in culture vessels during a 14-d incubation (see also Materials and Methods).

‡ PC cytolytic activity was determined in 18-h ^{51}Cr release assays.

suppressed state of L5178Y cells in vivo. Peritoneal cell populations collected from immunized mice on the 7th d after L5178Y cell challenge contained approximately 6,500 tumor cells per 10^6 peritoneal cells as calculated from the highest dilution well (containing 1.5×10^2 PC) in which tumor cells grew out (Table I). However, these results of tumor cell isolations were unexpected in that wells containing the highest concentrations of peritoneal cells in the same dilution series (1.2×10^4–1×10^6), failed to yield tumor cell outgrowth after 14 d of incubation. Subculture of these wells at the end of the 14-d period also failed to yield tumor cell outgrowth, whereas tumor cells could be readily subcultured from wells in which outgrowth occurred. A similar observation was made with peritoneal cells collected 41 d after L5178Y cell challenge. In this dilution series, however, a total of 81 tumor cells were calculated to be present per 1×10^6 peritoneal cells, but only the first well of the dilution series, containing 1×10^6 peritoneal cells, failed to yield tumor cell outgrowth. Finally, on day 81 after L5178Y cell challenge, 243 tumor cells were calculated to be present in 1×10^6 peritoneal cells, but no inhibition of tumor cell outgrowth was observed in any of the wells containing high concentrations of cells.

As seen in Table I, the degree of inhibition of L5178Y cell outgrowth in the high cell density wells occurred with both high (86%) and low (35%) L5178Y cytolytic activity by peritoneal cells (see section IV), but did not occur in the absence of cytolytic activity. The level of cytolytic activity correlated with the number of wells in the early part of the dilution series in which no tumor cell outgrowth occurred. Inhibition of tumor cell outgrowth in wells containing high cell densities was not due

TABLE II

Antibody to L5178Y Cells in Serum of L5178Y Cell Immunized and Challenged Mice

Days after L5178Y cell challenge	Antibody to L5178Y cells		
	Cytolytic*	Cytophilic‡	
	% ^{51}Cr Release	% Fluorescent cells	AR-binding
0	2.1	<1	1.2
10–14	3.3	<1	1.4
28–35	2.9	<1	2.6§
45–50	2.5	2	1.3
65–80	2.3	<1	ND
Normal mouse serum	1.9	2	1.0
C57BL/6 anti-DBA/2 serum	99.3	95	10.5

* Sera assayed for cytolytic activity were tested at a 1:20 final dilution and C′ was used at a 1:32 final dilution. Target cells consisted of ^{51}Cr-labeled tissue culture L5178Y targets.

‡ Sera assayed for cytophilic activity were tested at a 1:10 final dilution using tissue culture L5178Y cells.

§ A singular peak of antibody was detected in three individual experiments between days 28 and 35 with no antibody detected on other days.

to cell population effects because wells containing identical numbers of normal DBA/2 PC failed to inhibit the outgrowth of a comparable number of exogenously added L5178Y cells (data not shown). Additionally, cultures refed at 3-d intervals inhibited the outgrowth of tumor cells to the same degree as cultures which were not refed (data not shown). The inhibition of L5178Y cell outgrowth in the early culture wells could not be due to higher ratios of effector to target cells since this ratio was constant throughout the titration.

III. *Humoral Immune Responses to L5178Y Cells.* To determine whether mice immunized and challenged with L5178Y cells produce anti-tumor antibodies, we bled mice on selected days and assayed the serum fraction for: (*a*) complement-dependent cytolytic antibody, (*b*) cytophilic antibody by indirect immunofluorescence, and (*c*) cytophilic antibody by indirect radioimmunoassay. Table II shows that cytolytic antibody was not observed at any of the times tested throughout the lifespan of the mice. Cytophilic antibody was not detected by indirect immunofluorescence assay. However, indirect radioimmunoassay revealed a small peak of cytophilic antibody activity at 31 DPC. Attempts to demonstrate antibody-dependent cell-mediated cytolysis with these sera were negative (data not shown).

IV. *Kinetics of Cell-Mediated Lysis of L5178Y Cells.* The sharp decline in tumor cell numbers in the peritoneal cavity on the 5th d after i.p. L5178Y cell challenge (Fig. 1 B) in the absence of a detectable humoral response, suggested that an active cellular immune response had been elicited against the tumor. To test this we assayed peritoneal and spleen cell populations, removed from immunized mice on selected days before and after i.p. L5178Y cell challenge, for in vitro cytolytic activity against ^{51}Cr-labeled L5178Y target cells.

As shown in Fig. 1 C, cytolytic activity was detected in both the peritoneal and

TABLE III

Characterization of Cytolytic Effector Cells in L5178Y Cell Immunized and Challenged Mice

Effector peritoneal cells		Percent ^{51}Cr release from target cells‡		
Day of cell collection after L5178Y cell challenge	Cell population*	L5178Y	FLC-745	EL-4
4	Whole	99.3%	6.7%	5.5%
	Adherent	9.1	0.6	2.0
	Nonadherent	96.4	5.1	2.6
14	Whole	46.6%	4.3%	3.9%
	Adherent	5.5	0.6	0.2
	Nonadherent	46.3	2.1	4.0
35	Whole	40.7%	3.9%	5.3%
	Adherent	6.3	1.1	1.5
	Nonadherent	39.5	3.6	4.8
60	Whole	3.7%	2.5%	1.9%
	Adherent	0.6	1.0	0.3
	Nonadherent	3.3	2.8	1.3

* Whole cell populations consisted of 10^6 PC. Triplicate samples of adherent and nonadherent subpopulations of PC were prepared as described in Materials and Methods section. During the time intervals tested, nonadherent subpopulations represented 60–70% of whole PC populations.

‡ 10^4 ^{51}Cr-labeled tumor cell targets were added to all wells and CMC activity was determined in 18-h assays.

spleen cell populations within 7 d after subcutaneous implantation of L5178Y cells. Excision of the tumor nodules was followed by increased peritoneal cell cytolytic activity which peaked 4 d postsurgery and then declined to lower levels by day 7. Intraperitoneal challenge with L5178Y cells, 7 d after tumor nodule excision, stimulated cytolytic activity which reached maximum levels 4 d after challenge and then fell sharply during the next 10 d, declining gradually thereafter to undetectable levels by the 70th d postchallenge. Spleen cell cytolytic activity remained low to undetectable throughout the postchallenge period (Fig. 1C). Lymph node and peripheral blood lymphocyte populations had no measurable cytolytic activity throughout the entire time period tested (data not shown).

V. *Characterization of the Peritoneal Effector Cells.* The cytolytic activity of PC resided solely in the nonadherent cell population throughout the 60-d period after L5178Y cell challenge (Table III). This cytolytic activity was specific for L5178Y cells and did not cross-react with EL-4 and FLC-745 cells.

As shown in Table IV the cytolytic activity of PC was abolished by treatment with anti-Thy 1.2 + C′, but not by anti-Ig + C′ treatment, indicating a T-cell lineage of the cytolytic effector cells.

Discussion

In this report we have quantitated L5178Y cell growth and host anti-tumor responses in L5178Y cell immunized and challenged syngeneic DBA/2 mice, and

TABLE IV

Effect of Anti-Thy 1.2 and Anti-Ig Treatment upon PC Anti-Tumor Cell-Mediated Cytotoxicity

Treatment*	Cytolytic activity after treatment (±SEM)‡
	%
Medium	89.9 (±2.7)
Complement	56.7 (±1.4)
Anti-Thy 1.2	71.5 (±4.4)
Anti-Thy 1.2 + complement	1.7 (±0.1)
Anti-Ig	86.3 (±0.4)
Anti-Ig + complement§	89.0 (±2.3)

* Cells were treated before assay as indicated in Materials and Methods.

‡ CMC against ^{51}Cr-L5178Y cells was assayed in an 18-h assay with peritoneal cells removed from mice 5 d postchallenge at an effector:target ratio of 100:1 based on initial cell concentrations before treatment.

§ Comparable treatment of normal DBA/2 spleen cells with anti-Ig + complement reduced a subsequent lipopolysaccharide response by 94% but had no effect upon the Con A response (data not shown).

related these to the establishment and course of tumor dormancy. After i.p. challenge of immunized mice, the L5178Y tumor cell population passes through four phases: (*a*) increase in cell numbers, (*b*) reduction in cell numbers, (*c*) dormancy, and (*d*) emergence. The initial growth of tumor cells after i.p. inoculation is logarithmic for ≈4 d, during which time a secondary cell-mediated cytolytic anti-tumor response against L5178Y cells is stimulated. Tumor cell numbers and cytolytic activity both reach maximum levels at about the 4th d after challenge and both decline rapidly thereafter. The numbers of tumor cells fall to approximately 300–1,000 cells per mouse by the 30th-d after challenge and remain at this level for the duration of the dormant state.

The level of cytolytic activity of peritoneal cells is probably greater than our measurements would indicate, because the unlabeled tumor cells present in the peritoneal cell population could be expected to compete with ^{51}Cr-labeled L5178Y target cells for lysis by T cells (15). The coexistence of peak tumor cell numbers and maximum cytolytic activity 4 d after tumor cell challenge indicates that tumor cell growth proceeded while the secondary cytolytic response was being generated. However, after attainment of peak cytolytic activity, almost all of the tumor cells (>99%; Fig. 1 B) were eliminated over a 25-d period. The cells in the tumor population which survive must possess certain characteristics for escape from immune cytolysis. The data to be reported in the accompanying paper (15) suggests that this escape involves decreased expression of cell surface tumor antigen. Such decreased tumor antigen expression could result from antigenic modulation (16), immune selection (17), or antigen masking (18). Also, expression of surface antigen has been reported to vary quantitatively as a cell passes through stages in the cell cycle (19–21). Thus a tumor cell arrested in a specific stage of the cell cycle could display insufficient surface antigen for it to be lysed.

Recently, Gelfant (22) proposed that tumor cells can be subdivided into four distinct categories: (*a*) cycling cells, (*b*) noncycling G_1-blocked cells, (*c*) noncycling G_2-blocked cells, and (*d*) noncycling G_0-blocked cells, and speculated that the major pool of potentially proliferative tumor cells resides in the noncycling G_1-blocked subpop-

ulation. The finding that continuous exposure of both experimental and human tumor cells to tritiated thymidine fails to label all cells within the population supports the concept of noncycling cell pools (23–25). In tumor dormancy, tumor cells may escape by remaining in a noncycling stage until host anti-tumor activity has declined.

An essential component in tumor dormancy is control of tumor cell outgrowth which may be host imposed or intrinsic to some members of the tumor population. This restraint can be manifested as arrest of cell division, a slowed rate of cell proliferation or a normal rate of proliferation offset by a commensurate degree of cell death. A variety of host mechanisms for restraint of proliferating tumor cells has been described (26–29). In our tumor dormant model CMC persisted at low levels throughout the early stages of the tumor dormant period. A comparison of tumor-associated deaths and PC cytolytic activity (Fig. 1 A and 1 C) reveals that tumor emergence occurred most frequently after day 70, during the period when CMC was no longer demonstrable in the remaining clinically normal mice. Because CMC activity was determined with peritoneal cells pooled only from clinically normal mice, we are not able to comment on the immune status of the mice in which tumor emergence occurred before day 70. However, the prolonged period of clinical normalcy after the disappearance of detectable CMC activity suggests that other host tumor suppressive mechanisms, possibly cytostatic in nature, may be operative in later maintenance of the tumor dormant state.

The gradual decline in cell-mediated cytolytic activity described here could be the result of a number of previously described immunologic phenomena. Among these are: (*a*) an age-related decline in immune responsiveness, (*b*) absence of sufficient immunogenic stimulation needed to maintain an ongoing response (15, 30), (*c*) immune paralysis due to constant low level stimulation over prolonged periods of time (31), (*d*) the production and slow release by tumor cells of immune suppressive substances (32), and finally, (*e*) the generation of suppressor cells capable of inhibiting cell-mediated cytolysis (33). Inhibition of CMC by increasing numbers of potentially competitive cold tumor cell targets within the PC population is not a likely consideration because only clinically normal mice (i.e. total PC $< 10^7$) were assessed for CMC activity, and these mice consistently harbored $<10^3$ tumor cells in their peritoneal cell population.

The in vitro suppression of tumor cell outgrowth observed only in culture wells containing the highest number of peritoneal effector cells suggests the possible importance of localized effector cell density upon the outcome of host-tumor cell interactions in tumor dormant mice. In vitro suppression of tumor outgrowth was evident only during the period when CMC activity was demonstrable and the degree of suppressive activity, as reflected by the density of peritoneal cells required to inhibit tumor cell outgrowth, declined during the period of decreasing CMC activity. Thus the in vitro inhibition of tumor cell outgrowth is seemingly related to CMC activity yet is cell density dependent. This may reflect increased effector cell viability, more efficient cell recruitment for killing, or the production of greater concentrations of tumor suppressive factors in high cell density culture wells.

A number of reports describe the generation of tumor specific humoral immune responses (34–36). In our experiments only a late transient peak of low level cytophilic antibody was detected after L5178Y cell immunization and challenge. Benjamini et al. (37) have described a cell-mediated tumor-specific response, exclusive of any homoral immunity, after immunization of syngeneic hosts with mitomycin C-treated

EL-4 cells. Recently, Fridman et al. (32) have described an immunoglobulin binding factor, released by L5178Y cells, which suppresses the in vitro plaque response of mouse spleen cells to sheep erythrocytes. Although the activity of this factor has only been assayed in vitro, it is possible that such a factor may be responsible for suppression of humoral anti-tumor responses in vivo without affecting the early cell-mediated immune responses.

The events leading to the breakdown of tumor dormancy and subsequent emergence of tumor remain elusive. In our system tumor emergence appears to be related both to a decline in host anti-tumor CMC activity and to presence of a tumor cell subpopulation which manifests increased resistance to CMC (15). In the tumor dormant model of Fisher and Fisher (9), surgical trauma was sufficient stimulus to provoke tumor emergence. Various substances, including hormones, heavy metals, and nucleotides have been found to be capable of tumor cell stimulation (22). Treatments causing immunosuppression such as anti-lymphocyte serum (38), X-irradiation or thoracic duct drainage (7) have also been shown to stimulate growth of dormant tumor cells.

The demonstration that immunized mice can suppress a tumor cell challenge to a dormant state suggests that tumor dormancy may be a common phenomenon which often goes unrecognized and is mistaken for tumor rejection, because experiments are usually terminated before the suppressed cells can emerge to produce overt tumors. Tumor dormancy results from the establishment of a delicate balance between tumor cell and host. The identification of mechanisms by which tumor cells escape destruction by tumor suppressive responses and yet are restrained from outgrowth should lead to an understanding of persistence of tumor cells in patients during prolonged clinical remission.

Summary

Subcutaneous implantation of DBA/2-derived L5178Y cells into DBA/2 mice followed 10 d later by nodule excision protected 100% of mice from the rapid outgrowth of an intraperitoneal challenge of L5178Y cells given 7 d postexcision. Challenged mice remained clinically normal for 48–250 d before onset of an ultimately fatal tumor outgrowth. The numbers of L5178Y cells in the peritoneal cavity increased logarithmically for 4 d after challenge and then declined to low but detectable levels which persisted throughout the clinically normal period. Cells active in 18-h in vitro cytolytic assays against ^{51}Cr-labeled L5178Y target cells were found in the peritoneal cavity. The effector cells were determined to be Thy1.2 positive. Their activity was tumor specific and reached peak levels 4 d after tumor challenge and then gradually declined to undectable levels during the following 70 d. Tumor emergence occurred most frequently during the period when CMC activity was no longer demonstrable in the remaining clinically normal mice. A transient peak of low level cytophilic anti-tumor antibody was detected about 30 d after tumor cell challenge. The temporal associations between the numbers of tumor cells and the levels of cell-mediated lysis against L5178Y cells indicate the importance of the cell-mediated cytolysis response in limiting initial tumor outgrowth and suggest its role as one of the factors responsible for long-term tumor suppression during tumor dormancy.

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