THE TUMOR DORMANT STATE

Comparison of L5178Y Cells Used to Establish Dormancy with Those That Emerge after Its Termination*

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During the establishment of the L5178Y cell tumor dormant state in DBA/2 mice, described in the previous communication (1), mice develop a strong cytolytic antitumor response mediated by peritoneal T cells. Despite this cell-mediated cytolytic (CMC)[1] activity, small numbers of tumor cells persist in clinically normal mice for prolonged periods. Termination of the dormant state is signaled by the appearance of overt ascitic tumors and this occurs most frequently when CMC is no longer demonstrable in the majority of clinically normal mice.

The outgrowth of tumor cells at the end of the dormant state could be due to changes in the antigenic or malignant properties of the cells, to waning host tumor suppressive activity, or to a combination of these. Phenotypic alterations within tumor cell populations during in vitro or in vivo exposure to an immune environment have been described in several experimental systems (2–5). However, in at least one tumor dormant system (6), cells emerging after long-term quiescence were found to exhibit properties identical to those cells used to produce the dormant state. In this system immunosuppressive measures released tumor cells from a dormant state and permitted their rapid outgrowth.

Tumor dormancy differs from the slowly dividing tumor cell subpopulation by the presence of some form of restraint on tumor cell growth. In the tumor dormant state, the tumors which eventually emerge must be progeny of the primary tumor, and not a newly transformed host cell. Both of these phenomena were demonstrated in Burkitt lymphoma by Fialkow et al. who found that early relapses were due to reappearance of original malignant clones whereas late relapses were due to outgrowth of new malignant cell lines (7).

The present study was undertaken to determine the origin and growth rate of tumor cells which eventually emerge from the tumor dormant state. Various properties of original and emergent L5178Y cells were compared to understand how small numbers of tumor cells avoid immune lysis, survive in an immune environment in vivo, and eventually emerge from the dormant state to form ascitic tumors.

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[1] *Abbreviations used in this paper:* C′, complement; CMC, cell-mediated cytolysis; FITC, fluorescein isothiocyanate; PC, peritoneal cell; PBS, phosphate-buffered saline; TAA, tumor-associated antigen.

Materials and Methods

Mice. DBA/2 female mice (8 wk old) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Male DBA/2 mice (8 wk old) were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts.

Tumor Cell Lines. DBA/2-derived methylcholanthrene-induced L5178Y lymphoma and P815-X2 mastocytoma cells as well as C57BL/6-derived benzanthracene-induced EL-4 lymphoma cells were obtained from Dr. Lionel Manson of the Wistar Institute, Philadelphia, Pa. Friend leukemia virus-transformed DBA/2 erythroblasts designated clone 745 were obtained from the Genetic Mutant Cell Repository, Camden, N.J. All cell lines were maintained by in vivo passage into susceptible animals and as suspension cultures in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 15% fetal calf serum.

Immunization of Tumor Recipients and Establishment of the Dormant State. L5178Y lymphoma cells (1×10^6 cells) were implanted subcutaneously on the ventral surface of DBA/2 female mice. 10 d later the resultant 1-cm diameter nodules were surgically removed. After an additional 7 d, all mice received an intraperitoneal challenge of 5×10^4 L5178Y cells. Control mice which were immunized but not challenged were free of isolatable tumor cells and lived a normal lifespan.

Definitions. Clinical normalcy, see previous communication (1). Original L5178Y tumor cells: the L5178Y cells which were maintained by weekly i.p. passage into normal DBA/2 female mice are referred to as original tumor cells. Original L5178Y cells were used in all immunization and challenge protocols. Emergent L5178Y tumor cells: the tumor cells isolated from tumors which emerged in L5178Y cell immunized and challenged mice after a prolonged clinically normal period are referred to as emergent tumor cells. The emergent tumor cells used in this report were obtained from mice with tumor burdens exceeding 5×10^8 ascitic tumor cells.

Antisera. Alloantisera against DBA/2 histocompatibility antigens was prepared as described in the previous communication (1). Xenogeneic anti-L5178Y serum was prepared in New Zealand White rabbits by a series of eight subcutaneous injections of 10^8 ascitic passaged original L5178Y cells spaced at 10-d intervals. 1 wk after the final injection, the rabbits were exsanguinated. The serum was inactivated at 56°C for 30 min and absorbed with DBA/2 thymus and P815-X2 mastocytoma cells, a DBA/2 cell line which does not cross-react with L5178Y cells in CMC assays (data not shown). The resultant absorbed rabbit anti-L5178Y (Ra-a-L5178Y) serum was shown to possess cytolytic activity against L5178Y cells but not normal DBA/2 thymocytes nor FLC-745 cells in complement-dependent assays. Anti-Thy1.2 serum was obtained from Litton Bionetics, Kensington, Md. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit serum was obtained from Cappel Laboratories Inc., Cochranville, Pa.

Karyotype Analysis. Chromosome preparations were made as described by Miller et al. (8). In this comparison 32 original tumor cells and 28 emergent tumor cells were evaluated.

CMC Assays. All CMC assays were performed as described previously (1).

Antibody-Dependent Complement-Mediated Cytolysis Assays. All assays were performed as described previously (1).

Cold Target Inhibition Assay. The ability of original and emergent L5178Y cells to competitively inhibit peritoneal cell (PC) cytolytic activity against ^{51}Cr-labeled original L5178Y cells was assayed by using a modification of the cold target inhibition assay of Ortiz de Landazuri and Herberman (9). Briefly, various numbers of unlabeled inhibitor cells were added to microtiter plate wells containing 10^6 immune PC and 10^4 ^{51}Cr-labeled original L5178Y cells. The plates were incubated for 4 h at 37°C after which supernates were collected and radioactivity determined. The percent-specific lysis values were obtained as described in the previous communication. The percent inhibition values were then calculated as follows:

$$\frac{\text{percent lysis of noninhibited group} - \text{percent lysis of inhibited group}}{\text{percent lysis of noninhibited group}} \times 100.$$

Quantitative Absorption Assay. Various numbers of original and emergent L5178Y cells were compared for their ability to absorb cytolytic antibody from Ra-a-L5178Y sera with a

modification of a previously described absorption procedure (10). Briefly, various numbers of absorbing cells, ranging from 1.25×10^5 to 3.20×10^7, were incubated in 0.5 ml of a 1:20 dilution of Ra-a-L5178Y sera at 5°C for 30 min with occasional shaking. Cells were pelleted by centrifugation at 450 *g* for 10 min. 0.1 ml of the resulting supernate was added to microtiter wells, followed by the addition of 5×10^4 (0.05 ml) ^{51}Cr-labeled original L5178Y cells. The mixture was incubated at 5°C for 15 min. 50 μl of guinea pig complement was added to the wells and the reaction mixture was shaken vigorously and incubated at 37°C for 60 min. After incubation, the plates were spun at 150 *g* for 10 min, and 0.1-ml samples were removed and assayed for radioactivity.

Indirect Immunofluorescence Assay. Original and emergent L5178Y cells were resuspended separately in phosphate-buffered saline (PBS) at a concentration of 5×10^6 cells/ml. 0.1 ml of cells was incubated with 0.1 ml of various dilutions of absorbed rabbit anti-L5178Y serum for 30 min at 5°C with constant agitation. The cells were washed three times with PBS and the pellet resuspended to 0.1 ml. 0.1 ml of a 1:10 dilution of FITC-labeled goat anti-rabbit 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 using an AO vertical illumination microscope with an HB-50 ultraviolet light source.

Neuraminidase Treatment of Original and Emergent L5178Y Cells. Original and emergent L5178Y target cells were treated with neuraminidase according to the procedure of Turianskyj and Gyenes (11). Briefly, 2×10^6 cells were first labeled by incubation in medium containing 250 μCi of $Na_2{}^{51}CrO_4$ for 45 min. After labeling, cells were washed twice in PBS and finally resuspended in 0.5 ml of PBS. 50 U of streptococcal neuraminidase (obtained from Joseph Goldschmidt, Thomas Jefferson University) was added to the cell suspension, and the mixture was incubated at 37°C for 20 min. Cells incubated in buffer served as controls. After incubation, cells were washed three times in 10 vol of PBS and resuspended in medium to yield a concentration of 1×10^5 cells/ml. Susceptibility to lysis was determined in a 4-h CMC assay as described previously (1).

Results

Cytogenetic Analysis of Emergent Tumor Cells. To determine whether the tumor cells which emerged in L5178Y cell immunized and challenged mice after a prolonged period of clinical normalcy were progeny of the tumor cells contained in the challenge inoculum rather than newly induced in the recipient host, we searched for abnormal chromosomal markers common to both original and emergent cells. In this analysis, a tumor dormant state was established in male DBA/2 mice by using the female derived original L5178Y cells, thereby permitting us to search also for a Y chromosome in the emergent tumor. An ascitic tumor emerging 218 d after L5178Y cell challenge was selected for this comparison.

The original tumor cell line had an average of 72.3 chromosomes per cell, with most cells having 73 or 74. These included copies of normal chromosomes, as well as structurally altered, or marker chromosomes. The emergent tumor cells had a slightly greater average number of chromosomes per cell, 78.7, and a somewhat wider range of chromosome numbers (62–93 compared to 61–75 for the original tumor cells). At least nine of the marker chromosomes seen in the original tumor cells were also present in the emergent tumor cells (Fig. 1). None of these marker chromosomes were seen in normal DBA/2 cells. No Y chromosome was seen. Chromosomal analysis therefore provides convincing evidence that the emergent tumor cells are progeny of the original L5178Y cell inoculum.

Analysis of Growth Rate of Emergent Tumor Cells. Because the emergent tumor cells were shown to be progeny of the original cell inoculum, the properties of these two tumor cell isolates were studied for differences which could be responsible for

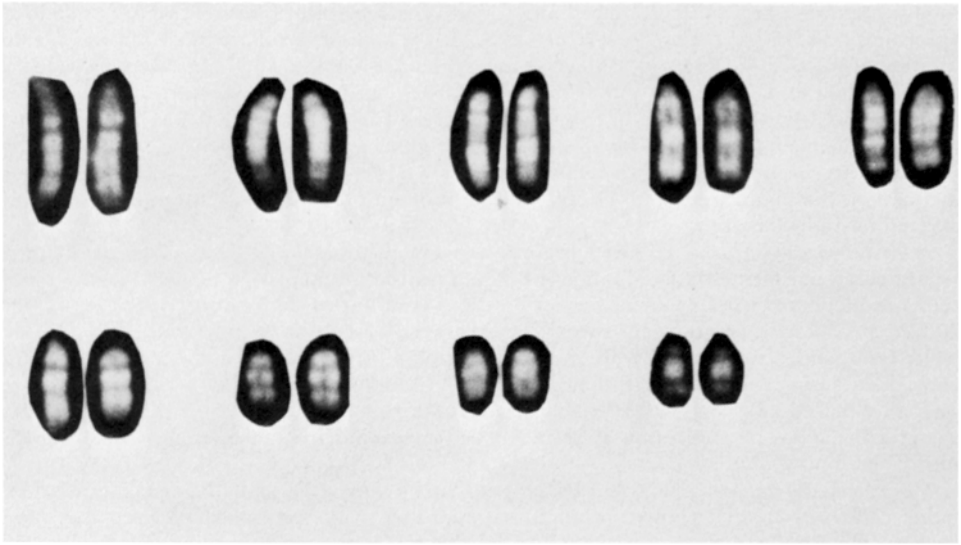

FIG. 1. Quinacrine-stained abnormal, or marker, chromosomes from the original tumor cells (left member of each pair) and the emergent tumor cells (right member of each pair). Note that for each pair the banding patterns of the two chromosomes are identical.

TABLE I

In Vivo Growth of Original and Emergent L5178Y Cells in Normal DBA/2 Mice

Inoculum	Tumor cells* recovered on day 20	Mean day of tumor associated death‡ (± SD)
5×10^4 L5178Y (Original)	3.6×10^8 (±0.3)§	24.4 (±1.4)§
5×10^4 L5178Y (Emergent)	3.2×10^8 (±0.2)	27.2 (±1.5)

* Three mice from each group were killed 20 d after i.p. injection and the number of tumor cells in the peritoneal cell population was determined by end point dilution analysis (see previous communication).

‡ Each experimental group contained six mice.

§ $P > 0.1$; not a significant difference between original and emergent cells.

emergence from the dormant state. We first compared the in vivo growth rate of emergent L5178Y cells with original L5178Y cells to determine whether the emergent cells were a slowly dividing subpopulation of the original L5178Y cells. Groups of normal age-matched DBA/2 mice were inoculated i.p. with identical numbers of cells of both types and observed for both increases in numbers of tumor cells and the times of tumor associated deaths.

As seen in Table I, the numbers of tumor cells present in the peritoneal cavity 20 d after an inoculation of 5×10^4 original or emergent L5178Y cells were not significantly different, suggesting that both original and emergent L5178Y cells have nearly equivalent population doubling times in vivo. There was also no significant difference in the times of tumor associated deaths between the two groups of mice.

Further evidence that emergent tumor cells were not merely a slowly dividing subpopulation of original L5178Y cells is provided by the rate of tumor growth seen in L5178Y immunized and challenged mice after the termination of tumor dormancy.

TABLE II

Susceptibility of Original and Emergent L5178Y Cells to CMC

Target cells	% Specific ^{51}Cr release			C57 α-DBA/2‡ Effector PC
	DBA/2 α-L5178Y Effector PC*			
	Exp. 1	Exp. 2	Exp. 3	
L5178Y (original)	83.5	79.6	85.2	93.6
L5178Y (emergent)	30.1	35.9	33.7	91.4
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P > 0.1$

* DBA/2 effector PC were obtained from L5178Y (original) immunized mice 7 d after i.p. tumor cell challenge. For exp. 1, 2, and 3, original and emergent target cells were obtained from three different, original, and emergent tumorous mice, respectively. Cells were labeled with $^{51}CrO_4$ and utilized in 18-h cytolysis assays at an effector:target ratio of 100:1.

‡ C57BL/6 effector PC were obtained from mice immunized against DBA/2 cells by two i.p. injections of 10^7 SPC spaced a week apart. PC were collected 7 d after the final injection and utilized in 18-h cytolytic assays at a 100:1 effector:target ratio. The target cells in this instance were the same as those used in exp. 1.

Emergent tumors, once detected at the end of the tumor dormant period, enlarged more rapidly, (average increase in total body weight of 2 g/d) than original L5178Y tumors growing in nonimmunized mice (0.4 g/d).

Decreased Susceptibility of Emergent Cells to CMC In Vitro. The L5178Y cells which grew out at the end of a prolonged dormant state were compared with the original L5178Y cells to detect differences which could account for their escape from dormancy. For this comparison three sets of original and emergent L5178Y cell lines were established, each line from a different tumor bearing mouse. The sets of cells were tested for susceptibility to CMC by PC from tumor dormant mice.

As seen in Table II, all three emergent L5178Y cell lines were significantly less susceptible than the original cells to lysis by peritoneal cells from tumor dormant mice. However, the original and emergent L5178Y cells were equally susceptible to lysis by alloimmune PC, indicating that the decreased lysis of emergent cells by syngeneic effectors was not due to a physiologic nonlysable state of the cells.

Examination of Emergent Cells for the Presence of an Antigen Mask. The equivalent susceptibility of original and emergent L5178Y cells to lysis by alloimmune cells suggested that antigen masking was not responsible for the decreased susceptibility of emergent cells to lysis by PC from tumor dormant mice. To investigate the possibility of antigen masking further, we measured expression of nontumor-associated antigens on original and emergent cell populations using serologic reagents. As shown inTable III, both cell lines were equally susceptible to lysis by anti-H-2^d + C′ and anti-Thy 1.2 + C′, suggesting that expression of these nontumor antigens was unaltered in the emergent cell population. We next treated original and emergent cell populations with neuraminidase. As shown in Table III, neuraminidase treatment did not alter the respective susceptibilities of these cells to CMC. These data provide strong evidence against antigen masking as a mechanism for the decreased susceptibility of emergent cells to lysis by tumor dormant PC.

Stability of Decreased Susceptibility of Emergent Cells to CMC during In Vitro Culture. The decreased susceptibility of emergent cells to CMC could be the result of in vivo antigenic modulation. To evaluate antigenic modulation, we isolated L5178Y cells

TABLE III

Comparison of Surface Cytolytic Target Antigen Expression on Original and Emergent L5178Y Cells

Treatment of target cells	Dilution or concentration	Cytolytic assay	Specific ^{51}Cr release from target cells	
			Original L5178Y	Emergent L5178Y
			%	
Anti-H-2^d	1:1,000	C′-dependent*	81.1	79.2‡
	1:2,000	"	63.5	58.6‡
	1:4,000	"	39.9	39.6‡
	1:8,000	"	19.7	20.5‡
Anti-Thy 1.2	1:80	C′-dependent*	33.7	35.2‡
	1:160	"	17.2	20.6‡
	1:320	"	8.3	10.1‡
	1:640	"	3.1	2.9‡
Neuraminidase	50 U/ml	Cell-mediated§	59.6‖	30.6‖
Buffer	–	"	58.7	31.5

* Original and emergent L5178Y cells were labeled with ^{51}Cr, washed, and incubated with various dilutions of either anti-H-2^d serum or anti-Thy1.2 serum for 90 min in the presence of guinea pig complement. Relative cell lysis was determined by the amount of ^{51}Cr released.

‡ $P > 0.15$, no significant difference in lysis of original and emergent L5178Y cells.

§ Original and emergent L5178Y cells were labeled with ^{51}Cr, washed and incubated either in buffer or in 50 U of streptococcal neuraminidase for 20 min. After incubation, labeled cells were washed three times in medium and incubated in the presence of day 7 tumor dormant PC in a 4-h CMC assay at an effector: target ratio of 100:1. CMC activity was determined by the amount of ^{51}Cr released.

‖ $P > 0.15$, no significant difference between pre-and postneuraminidase treatment susceptibility to CMC.

from a tumor emergent mouse and cultured the cells in vitro in a nonimmune environment for various periods of time and then retested their susceptibility to lysis by immune effector cells. We found that the decreased susceptibility of emergent cells to cytolysis is a stable characteristic which is maintained in a nonimmune environment for at least 35 d (more than 70 population doublings). These experiments indicate that antigenic modulation is not responsible for the decreased susceptibility of emergent cells to CMC.

Common Target Antigens on Original and Emergent L5178Y Cells. The decreased susceptibility of emergent L5178Y cells to CMC could be due either to a decreased expression of a common target antigen or to a decreased expression of a different target antigen to which the mouse had mounted an immune response during the dormant period. To differentiate between these alternatives, we attempted to inhibit the lysis of ^{51}Cr-labeled original L5178Y cells with unlabeled original and emergent L5178Y cells at several cell:cell ratios. Normal DBA/2 thymocytes and FLC-745 cells were used as potentially competing cells to control for nonspecific blocking due to cell crowding.

As seen in Fig. 2, at a 25:1 ratio both unlabeled original and emergent cells almost completely inhibited lysis of the labeled cells. At a 5:1 ratio, a discrimination could be made between the competitive activity of original and emergent L5178Y cells, with the emergent cells competing less actively than the original cells. Little or no blocking occurred with either DBA/2 thymocytes or FLC-745 cells indicating that the observed competition with L5178Y cells was not due to cell crowding. Because cold emergent cells competed with labeled original cells for PC lytic activity, we can conclude that

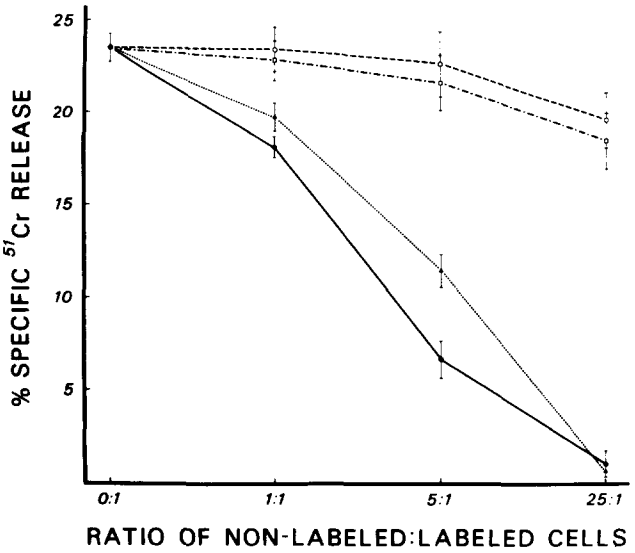

FIG. 2. Inhibition of CMC against ^{51}Cr-labeled original L5178Y cells by various numbers of original L5178Y (●), emergent L5178Y (▲), FLC-745 (□), or normal DBA/2 thymocytes (○).

the emergent L5178Y cells share a common cytolytic target antigen with the original L5178Y cells. Moreover, the lower competitive activity of emergent L5178Y cells suggested that emergent cells express less of this common antigen than the original cells.

Quantitative Expression of Tumor-Associated Antigens. To determine whether the emergent L5178Y cells express lesser amounts of a common tumor associated antigen than the original cells, we quantitated the expression of this antigen on both cell populations. These experiments utilized an immune rabbit serum prepared against original L5178Y cells. This rabbit serum was first absorbed extensively with normal DBA/2 thymocytes and a DBA/2-derived, non-L5178Y, cross-reacting tumor cell line P815-X2.

The first experiment performed with this absorbed rabbit serum tested the susceptibility of the original and emergent L5178Y cells to complement-dependent lysis. DBA/2 thymocytes were also used as a control for the completeness of absorption of antibody to H-2^d antigens. As seen in Fig. 3, this serum lysed L5178Y cells, but not normal DBA/2 thymocytes or FLC-745 cells, indicating that the serum contained antibodies to L5178Y cell tumor-associated antigen (TAA). Emergent L5178Y cells were significantly less susceptible to lysis by this serum than were original L5178Y cells. These experiments suggested that emergent L5178Y cells displayed less target antigen than the original cells. However, because no definitive assessment of quantitative differences in expression of antigen can be made from a measurement of cytolytic susceptibility, we performed an antibody absorption experiment. A 1:20 dilution of Ra-a-L5178Y serum was absorbed with varying numbers of either original or emergent cells, and the amount of cytolytic activity remaining after absorption was measured. As seen in Fig. 4, absorption of 50% of the cytolytic activity was achieved with 2.2×10^6 original L5178Y cells and with 16.4×10^6 emergent cells. Normal DBA/2 thymocytes were unable to absorb significant amounts of cytolytic activity from this serum. Original and emergent L5178Y cells have similar size ranges and

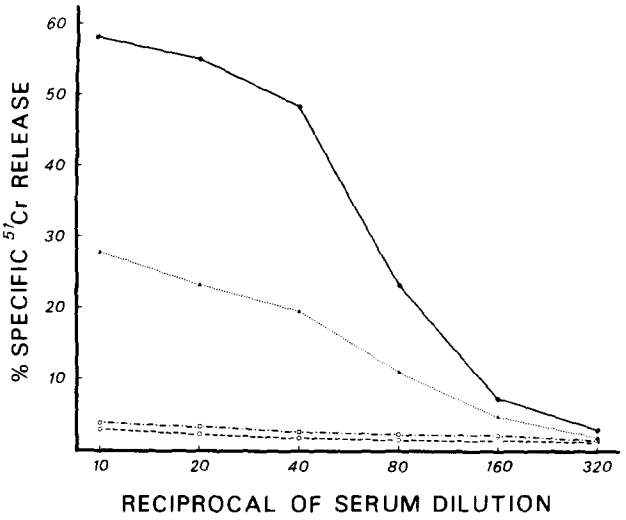

FIG. 3. Complement-dependent, antibody-mediated cytolysis of original L5178Y (●), emergent L5178Y (▲), FLC-745 (□), and normal DBA/2 thymocytes (○) by Ra-a-L5178Y serum.

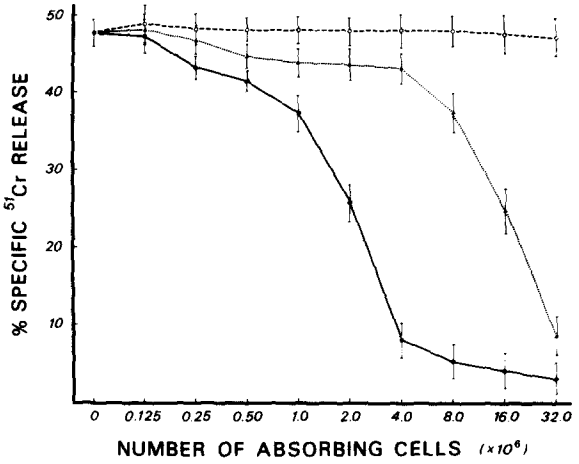

FIG. 4. Absorption of L5178Y cell cytolytic activity from rabbit anti-L5178Y serum with varying numbers of original (●) or emergent (▲) L5178Y cells, and with DBA/2 thymocytes (○). The lines represent the cytolytic activity of the rabbit serum after absorption.

surface morphologies (data not shown) and, therefore, have similar cell surface areas accessible to antibody attachment. These results indicate, therefore, that on a population basis, emergent cells express on their surface less TAA that is accessible to antibody than original L5178Y cells.

Heterogeneity of Antigen Expression on Original and Emergent L5178Y Cells. The decreased expression of TAA in the emergent L5178Y cell population may reflect either decreased antigen expression on all cells in the population or the existence of several tumor cell subpopulations, with each subpopulation expressing amounts of TAA ranging from zero to that present on the original L5178Y cells. To discriminate between these two possibilities, we performed indirect immunofluorescence assays on the original and emergent cell populations utilizing the rabbit anti-L5178Y (original

TABLE IV

Heterogeneity of Antigen Expression in Original and Emergent L5178Y Cell Populations

Cell line*	Percent of cells displaying 0-4+ immunofluorescence‡				
	4+	3+	2+	1+	0
L5178Y (Original)	—	80%	20%	—	—
L5178Y (Emergent)	—	20%	10%	65%	5%
DBA/2 Thymocytes	—	—	—	—	100%

* Tumor cells were obtained from the appropriate mice (see text) and cultured for 3 d before assay. Normal DBA/2 cells were assayed directly after removal from mice.

‡ Cells were reacted with a 1:40 dilution of rabbit anti-L5178Y (original) sera which had been extensively absorbed with normal DBA/2 tissues and with P815X-2 tumor cells. After washing, the cells were stained with fluorescein-conjugated goat anti-rabbit serum. Slides were examined by using a single-blind method of evaluation. Fluorescent staining intensity was based upon the maximum (4+) staining of L5178Y cells using a 1:40 dilution of nonabsorbed rabbit anti-L5178Y sera.

cell) sera employed in the previous section. As seen in Table IV, both original and emergent L5178Y cell populations contained cells of mixed immunofluorescent intensities with greater variation present in the emergent cell population. 20% of emergent cells stained as intensely as the majority of the original cells and ≈ 70% of the emergent cells displayed less antigen than any of the original cells observed; 5% of the emergent cells displayed no detectable tumor antigen. Thus, the decreased susceptibility of emergent cells to CMC reflects a mixed population of cells with most cells displaying less antigen than is present on the original cells. These emergent cells may have arisen from a subpopulation of the original L5178Y cells too small in number to be detected by immunofluorescence.

Discussion

In this report we describe various characteristics of tumor cells which grow out in L5178Y cell immunized and challenged mice following a prolonged period of clinical normalcy. We substantiate the presence of a true tumor dormant state in our L5178Y-DBA/2 model, and propose a mechanism by which tumor cells avoid immune cytolysis, persist in an immune environment, and eventually grow out to form overt tumors. We view tumor dormancy 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 (1). Implicit in this definition is growth restraint imposed either by the host or by cells within the tumor population. The outgrowth of tumor cells at the end of tumor dormancy must be distinguished from outgrowth due either to new transformation of host cells during remission, or to slow growth of a subpopulation of the original primary tumor. Outgrowth due to recent transformation rather than recurrence of residual tumor is known to occur in strains of mice highly susceptible to virus-induced tumors such as leukemias and mammary adenocarcinomas (12), and has been implicated in the relapse of human leukemias (13) and in Burkitt lymphomas (7). Prolonged clinical normalcy due to slow growth of residual tumor cells has been described in experimental animal systems (14) and in cases of clinical neoplasia, best illustrated with human breast cancer (15).

In the present study, tumor cells which emerged at the end of the clinically normal period were identified by karyotype analysis as progeny of the L5178Y cells contained in the challenge inoculum. In this analysis, female derived L5178Y cells were

suppressed to a dormant state in male DBA/2 mice. Tumor cells emerging from male mice were shown to lack a Y chromosome. However, because host chromosomes may be deleted after transformation (16), a more definitive analysis was required. Using chromosome banding techniques, we demonstrated that nine abnormal marker chromosomes present in original cells were also present in the emergent cells. Most abnormal chromosomes arise by unique events, and it would be extremely unlikely to have the same series of changes occur in two different tumors. In addition, both tumor lines had 70–80 chromosomes per cell, whereas diploid host cells had 40.

Investigation of the possibility that L5178Y cells grew at a slow rate during the dormant state was impeded by our inability to analyze directly the small numbers of tumor cells present in the peritoneal cavity during dormancy. However, we found that cells which emerged at the end of dormancy had in vivo growth rates when passaged in normal mice, that were equivalent to the original L5178Y cells. These results implicate a slow net growth rate of tumor cells during the dormancy state, and a dormancy-terminating event which converts the slow rate to a rapid outgrowth of tumor cells. The observation that emergent L5178Y tumors, once detected at the end of the tumor dormant period, enlarge more rapidly than original L5178Y tumors growing in nonimmunized mice may reflect a loss of tumor suppressive activity in mice bearing emergent tumors.

The L5178Y cells which grow out at the end of the dormant state were shown to be less susceptible to cell-mediated lysis by peritoneal effector cells obtained from L5178Y immunized and challenged mice. These emergent L5178Y cells may have been selected from the original L5178Y cell population by virtue of this decreased susceptibility to lysis. Immune-resistant tumor cells have been selected by exposing heterogeneous populations to cytotoxic antiserum or immune cells in vitro (3) and in vivo (2). We have demonstrated, by indirect immunofluorescence (IFA), heterogeneity in antigen expression in both original and emergent cell populations with the majority of emergent cells staining less intensely than most cells in the original population. This demonstrates that the decreased susceptibility of emergent cells is not due to the existence of a homogeneous subpopulation. There is, instead, heterogeneity with decreased expression of TAA on nearly all cells within the emergent cell population. Although no cells with such small expression of antigen were detected by IFA in the original population, the emergent cells may represent only a minute percentage of the original population with amplification of the immune selected cells during the emergent stage.

Two other explanations of the decreased susceptibility of emergent L5178Y cells to CMC were considered in our system; antigenic modulation (17); and antigen masking (18). The role of antigenic modulation in immune resistance in our tumor dormant system was ruled out by the demonstration that emergent cells did not reacquire full susceptibility to CMC during the 35 d in culture in a nonimmune environment. Antigen masking has been found to affect the expression of both normal (19) and tumor-associated surface target antigens (20), with complement-dependent lysis of susceptible cells more greatly affected than CMC (11). In our system original and emergent cells were equally susceptible both to alloimmune CMC and to complement-dependent lysis by anti-H-2^d and anti-Thy 1.2 sera indicating that masking of surface antigens on emergent cells is unlikely. Strong evidence that TAA on emergent cells were not specifically masked by neuraminic acid was provided by the demonstration

that neuraminidase treatment did not alter the susceptibility of emergent cells to CMC.

The decreased susceptibility of emergent L5178Y cells to lysis by cytolytic peritoneal effector T cells could have been responsible for their emergence from a dormant state. However, we reported earlier that emergent L5178Y cells fail to grow out rapidly when inoculated back into tumor dormant mice (21). Thus, sufficient quantities of antigen must be present on the surface of emergent cells to serve as targets for tumor suppressive activity as long as such activity exists in the mouse. We demonstrated, by cold target competition, a common target antigen on both original and emergent L5178Y cells. These experiments suggested that emergent cells may express decreased amounts of either a cytolytic target antigen or an accessory surface antigen necessary for effector cell recognition, binding, and lysis of tumor cells. Such recognition antigens may include products of the major histocompatibility complex (22), fetal antigens (23), and virion-associated antigens (24).

Quantitation of TAA expression on both original and emergent cells was performed with rabbit serum prepared against original L5178Y cells. This serum, extensively absorbed with DBA/2 tissues, possessed titrable cytolytic activity against L5178Y TAA. The observation that the susceptibilities of original and emergent cells to lysis by Ra-a-L5178Y serum paralleled their respective susceptibilities to CMC suggested that quantitative differences in expression of TAA may exist in these two populations. By measuring the ability of each population to absorb L5178Y cell lytic activity from Ra-a-L5178Y serum, we found that nearly eightfold more emergent cells than original cells were required to attain equal levels of absorption. This indicated that the original cell population displayed ≈eightfold more of serologically detectable TAA than the emergent cell population. These results were comparable to those of Fenyo et al. who found that immune resistant YAC cells displayed roughly one-tenth the TAA of fully susceptible cells (2). Biochemical analysis of the cell surface of the YAC cells has shown altered expression of Moloney virus cell surface antigen (MCSA) and reduced amounts of gp71 and H-2 antigens (25). Cell surface analysis of original and emergent L5178Y cells is underway in our laboratory. Although it is attractive to suggest a correlation between the difference in expression of serologically detected TAA on the two populations of L5178Y cells and their relative susceptibilities to CMC, such a conclusion is unwarranted until the serologic TAA is proven to be the target antigen for CMC. We have not been able, to date, to make this conclusion since Ra-a-L5178Y serum does not block lysis of L5178Y cells by effector PC (data not shown).

Our working hypothesis for the persistence of L5178Y cells in a dormant state and for their eventual outgrowth is that L5178Y cells with decreased amounts of surface antigen are selected from a heterogeneous original L5178Y cell population during the tumor dormant state. If the cells are selected early in tumor dormancy when the cytolytic immune response is at peak levels, their rapid outgrowth could be restrained by cytolytic or cytostatic effector cells. However, as tumor suppressive activity wanes, the L5178Y cells grow out to form overt tumors. The decline in cytolytic immune responses may be the result of many factors (discussed in the previous communication). The present communication provides evidence that the decline in tumor suppressive activity may be due to a decrease in the immunogenicity of emergent cells which results from decreased expression of TAA. Thus, emergence of tumor cells from the dormant state may follow immune selection of a tumor cell displaying reduced

amounts of TAA on its surface and decline in host tumor suppressive responses to a level which can no longer restrain tumor outgrowth. If this hypothesis is correct, it should be possible to prolong the tumor dormant state or eradicate all dormant tumor cells by stimulating host immune responses to them. Elucidation of the host mechanisms involved in control of the tumor dormant state in the murine model could thus serve as a basis for development of effective prolongation or eradication of tumor dormant states in man.

Summary

The tumor dormant state established in L5178Y immunized and challenged mice is characterized by a prolonged period of clinical normalcy followed by rapid tumor outgrowth. The tumor cells which emerged after termination of the tumor dormant state had abnormal marker chromosomes identical to those in the L5178Y cells used in the original challenge inoculum, indicating that the emergent tumor cells were progeny of the challenge inoculum. Original and emergent L5178Y cells had equivalent in vivo growth rates, when inoculated into normal DBA/2 mice. The emergent L5178Y cells were less susceptible than original cells to in vitro lysis by tumor dormant PC. Original and emergent L5178Y cells expressed common tumor-associated target antigens for cytolytic effector cells. Both modulation and masking of these target antigens were ruled out as mechanisms for decreased susceptibility to cell-mediated cytolysis. Immunofluorescence revealed heterogeneity in tumor-associated antigen expression within both original and emergent cell populations, with a decreased intensity of staining in the emergent population. Both populations were equally susceptible to lysis by alloimmune cells, alloantiserum, and anti-Thy 1.2 serum, but emergent cells were less susceptible to lysis by serum directed against L5178Y TAA. Quantitative absorption revealed that the emergent L5178Y cells expressed eightfold less serologically detectable TAA than the original cells. These findings indicate that the host immune response developing during establishment of the tumor dormant state selects a stable tumor cell subpopulation which expresses decreased amounts of surface tumor-associated target antigens.

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