Progressive Growth of Immunogenic Tumors: Relationship between Susceptibility of Ascites P815 Tumor Cells to T-Cell-mediated Lysis and Immune Destruction in Vivo

James R. Fahey and David L. Hines
Trudeau Institute, Inc., Saranac Lake, New York 12983

ABSTRACT

Progressive growth of the P815 mastocytoma as an ascites in either normal or immunodepressed, semisynthetic B6D2F mice resulted in the outgrowth of tumor cells resistant to lysis in vitro by tumor-specific cytotoxic T-lymphocytes (CTLs). Additional testing in vitro showed that late ascites tumor cells also developed a progressive decline in susceptibility to lysis by alloreactive CTLs. The decline in susceptibility to lysis by tumor-specific CTLs was not the result of the loss of tumor-associated antigens, since late tumor cells had the capacity to inhibit the lysis of early, log-phase growth P815 cells in a cold-target inhibition assay. Further studies showed that later, CTL-resistant tumor cells regained susceptibility to CTL lysis if they were incubated for 24 h in vitro. Studies of susceptibility to in vivo immune mechanisms demonstrated that late tumor cells were as susceptible as early tumor cells to adoptive immunotherapy with spleen cells taken from mice immunized against the early tumors. Taken together, these studies suggest that the resistance of late tumor cells to in vitro lysis by CTLs is a reversible phenomenon that may have no relevance to the expression of antitumor immunity in vivo.

INTRODUCTION

One of the more controversial issues regarding the progressive growth of immunogenic, chemically induced tumors of mice is the problem of discerning the relative importance of inadequate host immunity and tumor heterogeneity in the escape of a tumor from immunological rejection.

With regard to host immunity, there is evidence which demonstrates that mice develop, in concert with the progressive growth of an immunogenic tumor, suppressor T-cells that down-regulate a concomitant antitumor immune response before the response develops sufficiently to cause tumor regression (1). Studies in which the kinetics of the generation of suppressor cells were followed by passive transfer experiments revealed that acquisition of suppressor cells in the spleens of tumor-bearing mice coincided with the progressive loss of effector cells (2). It was found that the cells which down-regulate the immune response against the Meth A fibrosarcoma (2) and the P815 mastocytoma (3) are Ly1+2+ T-cells which prevent the generation of Ly1-2+ T-cells that express antitumor immunity. These suppressor T-cells have been shown to act in a specific fashion in that they can only suppress an immune response against the tumor which induced their generation (4). Taken together, these findings suggest that the escape of immunogenic tumors from immunologically mediated regression may be the result of the acquisition of specific T-cell suppressors of antitumor immunity.

On the other hand, the escape of immunogenic tumors from immunological rejection has also been attributed to the phenotypic heterogeneity that appears to be an inherent characteristic of many tumors. As originally postulated by Nowell (5), tumor growth results in increased genetic instability of tumor cells leading to phenotypic variability for numerous biological characteristics including antigenicity. In this connection, studies with several different experimental tumors of known immunogenicity have documented the outgrowth, in normal immunocompetent mice, of tumor cells that were antigenically different from tumor cells used to initiate the original tumor (6–9). Furthermore, these studies suggest that this was the result of T-cells exerting strong selective pressure leading to the survival and outgrowth of cells that expressed less, fewer, or no TATA in common with the original tumor. The preponderance of evidence for antigen-loss variants in these studies was based on in vitro assays of tumor cell susceptibility to lysis by CTLs generated against the original tumor. However, because a definitive role for CTLs in tumor rejection has not been demonstrated, the relevance of CTL-defined antigen-loss variants in the escape of tumors from in vivo rejection remains to be established.

Studies of the progressive growth of the P815 mastocytoma in this institute have indicated that, following partial tumor rejection by the concomitant antitumor response, i.d. tumor growth resumes as a result of the generation of specific suppressor T-cells (10, 11). However, other laboratories have explained the regrowth of a P815 ascites tumor in terms of the existence of CTL-resistant, antigen-loss variants of the P815 which grew out after most of the tumor cells have been destroyed (8, 9). The present study was designed (a) to determine whether during the i.p. growth of a P815 ascites, a concomitant antitumor immune response develops, and if there is an outgrowth of P815 tumor cells that are resistant to lysis by tumor-specific CTLs generated against cells of the early tumor, (b) to determine, if CTL-resistant cells are present, whether their outgrowth results from immunological selection for antigen-loss variants, and (c) the additional purpose of determining if CTL-resistant P815 cells become dominant, whether they are also resistant to anti-P815 immunity in vivo. It will be shown that during progressive growth of the P815 as an ascites in the peritoneal cavity of either normal or immunodepressed mice, tumor cells emerge that are less susceptible to in vitro cell-mediated lysis than are the original cells used to initiate the tumor. However, it will also be shown that this resistance to lysis is a rapidly reversible phenomenon that may not be relevant to the mechanism of escape of the tumor in vivo.

MATERIALS AND METHODS

Mice. B6D2F (C57BL/6 × DBA/2) mice and AB6F (A × C57BL/6), 8–10 weeks old, were supplied by the Trudeau Institute Animal Institute.

4 The abbreviations used are: TATA, tumor-associated transplantation antigen; CTL, cytotoxic T-lymphocyte; TXB, T-cell-deficient mouse; PBS, pH 7.0 phosphate-buffered saline; FBS, fetal bovine serum; HS, horse serum; HBSS, Hank’s balanced salt solution; MOPS, morpholinopropanesulfonic acid; i.d., intradermal.
Breeding Facility. These mice were free of known pathogens, as evidenced by the result of routine testing for the presence of pathogenic bacteria, Mycoplasma, and viruses (mouse virus profile 80-211; Microbiological Associates, Bethesda, MD).

T-Cell Deficiency. Mice were rendered T-cell deficient by thymectomy at 5 weeks of age, followed in 1 week by 1000 rads of whole-body γ-irradiation from a 137Cs source. Immediately following irradiation, the mice were reconstituted with 2 × 10^7 syngeneic bone marrow cells. The thymectomized, irradiated, bone marrow reconstituted (TXB) mice were rested at least 4 weeks before use.

Tumors. The P815 mastocytoma, syngeneic in DBA/2 mice, was originally obtained from Dr. Virginia Evans, Tissue Culture Section, National Cancer Institute. The L5178Y lymphoma was obtained from Dr. Frederick Wheelock, Hahnemann Medical College (Philadelphia, PA). Both tumors are passaged weekly in B6D2F1 mice as ascites. A new passage is initiated every 3 months from tumor stocks that are cryopreserved over liquid nitrogen in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Gibco) and 10% dimethyl sulfoxide (Mallinkrodt, Inc., St. Louis, MO). For implantation, tumor cells were harvested from peritoneal ascites in sterile PBS containing 10 units/ml heparin. Cells were washed and resuspended to the appropriate concentration in PBS.

Immune T-Cells. Anti-P815 effector cells were obtained from the draining lymph nodes of B6D2F1 mice, immunized 8 or 9 days previously by i.d. implantation of 2 × 10^6 P815 cells, freshly harvested during log phase growth from the peritoneal cavity of tumor passage mice, and admixed in PBS with 50 μg of Corynebacterium parvum. Previous studies at this Institute have shown that the cytolytic cells present on days 9 and 10 in the draining lymph node of animals immunized using this protocol are Thy 1+, Lyt 2+ cytotoxic lymphocytes (12-14). C. parvum was supplied by the Trudeau Institute. Anti-H-2d effector cells were obtained from draining lymph node of AB6F1 (H-2b) mice 7 or 8 days after i.d. implantation of 2 × 10^6 L5178Y (H-2b) cells. Effector cells were prepared by mincing lymph nodes in RPMI 1640 containing 1% HS (Sterile Systems, Logan, UT) plus 10 μM MOPS (Sigma Chemical Co., St. Louis, MO), and pressing the pieces through a fine mesh, stainless steel screen. The lymph node cells were washed once and resuspended at a concentration of 10^6 viable cells/ml in RPMI-1640 containing 10% HS plus MOPS.

Cell-mediated Cytotoxicity Assay. P815 ascites tumor cells, freshly harvested from the peritoneal cavities of B6D2F1 mice, were used as target cells. Cells (10^6) were labeled for 1 h at 37°C in an atmosphere of 5% CO₂ in air for 20 min, cell suspensions were immediately used to make cytospin preparations using a Cytospin 2 centrifuge (Shandon Southern Instruments, Inc., Sewickly, PA). The cytospins were air-dried, fixed with absolute methanol, and coated with NTB2 autoradiography emulsion diluted 1:3 with distilled H₂O. The coated slides were air-dried and exposed for 1 week at −20°C (15). The autoradiograms were developed according to the manufacturers instructions (16) and stained with Giemsa to estimate the proportion of cycling cells.

Enumerating Ascites Tumor Cells. Normal and irradiated (700 rads) B6D2F1 mice were given an i.p. implant of 10^6 P815 cells on day 0, and at 3-day intervals, groups of five mice were sacrificed to enumerate the content of tumor cells. Thirty min prior to sacrifice, each mouse was given an i.p. injection of 1 ml PBS containing 10^7 heat-killed Listeria monocytogenes to physiologically label macrophages. The mice were killed by cervical dislocation, and the peritoneal cavities were injected with 10 ml PBS containing 10^6 units/ml heparin to harvest peritoneal cells. Viable cells were estimated using trypan blue exclusion. Cytosmears made from appropriately diluted ascites samples were first stained by the Graham-Knoll method (17) to label peroxidase positive cells and then with Wright-Giemsa Quick Stain (Diff-Quik; American Scientific Products, McGraw Park, IL). Total tumor burden was calculated from a knowledge of the total number of cells in the peritoneal cavity and the percentage of tumor cells on cytospins. Tumor cells were identified based on morphology, peroxidase negativity, and absence of ingested L. monocytogenes. At least 500 cells were counted on duplicate slides.

Cold Target Inhibition Assay. The ability of unlabeled tumor cells to inhibit cell-mediated lysis of log-phase growth P815 was determined by adding 10^6, 4 × 10^5, 8 × 10^5, or 1.6 × 10^6 unlabeled tumor cells to microtiter wells containing 10^5 51Cr-labeled P815 target cells and 10^6 lymph node cells containing P815-specific CTLs. Unlabeled log-phase P815 cells were used as positive controls, and unlabeled L5178Y thymoma cells were used both as a specificity control and as a control for nonspecific inhibition due to crowding.

In Vivo Antitumor Immunity. B6D2F1 mice were immunized for use as donors of anti-P815 immune spleen cells by i.d. implantation on the belly of 2–2.5 × 10^8 P815 ascites cells admixed with 100 μg of C. parvum. The P815 ascites cells used for immunization were isolated from tumors in log-phase growth in the peritoneal cavity of B6D2F1 mice 3–5 days after i.p. implantation of 3 × 10^5 P815 cells. Injection (i.d.) of a tumor cell-C. parvum admixture results in an initial 8–to 10-day period of tumor growth followed in most animals by complete tumor regression in approximately 2 weeks (12).

The ability of spleen cells from immunized mice to transfer immunity to appropriate tumor-bearing recipients was determined with spleen cells harvested 30 days after immunization. Donor spleens were aseptically harvested, minced into small pieces, and pressed through fine mesh, stainless steel screens. The resultant single cell suspensions were washed once with Hank's balanced salt solution containing 1% fetal bovine serum, and resuspended in Hank's balanced salt solution to the equivalent per ml of the number of cells obtained from 1.5 spleens. TXB mice that had received an i.d. implant of 10^6 cells on the belly 4 days previously were infu- bed via a lateral tail vein with 1.5 spleen equivalents of immune or normal spleen cells. Tumor growth was followed by measuring two perpendicular diameters of the tumor and plotting the mean diameter against time.

Radiolabeling and Autoradiography. The proportion of dividing tumor cells in freshly harvested ascites tumors and ascites tumors incubated in vitro for 24 h was determined by autoradiography of cells exposed to a 20-min pulse of [³H]thymidine. Tumor cells were washed and resuspended at 10^6 cells/ml in RPMI 1640 containing 1% HS, 10 mM MOPS, and 1 μCi [³H]thymidine/ml (TRA-120; specific activity, 5.0 Ci/mmol; Amersham). Following incubation at 37°C in 5% CO₂ in air for 20 min, cell suspensions were immediately used to make cytospins that were prepared with a Cytospin 2 centrifuge (Shandon Southern Instruments, Inc., Sewickly, PA). The cytospins were air-dried, fixed with absolute methanol, and coated with NTB2 autoradiography emulsion diluted 1:3 with distilled H₂O. The coated slides were air-dried and exposed for 1 week at −20°C (15). The autoradiograms were developed according to the manufacturers instructions (16) and stained with Giemsa to estimate the proportion of cycling cells.

RESULTS

Lack of Effect on Growth of a P815 Ascites by Immunodepression. The growth of 10^3 P815 tumor cells implanted into the peritoneal cavity of normal mice or mice immunosuppressed by exposure to 700 rads of γ-irradiation is shown in Fig. 1. It can be seen that irradiation 2 days before tumor implantation resulted in a measurable decline in the number of host cells recovered from tumor-bearing mice. However, irradiation did not affect the number of tumor cells recoverable in that the tumor grew in a similarly progressive fashion in both groups of mice. Observation of over 80 animals gave no evidence for a partial rejection of an ascites P815 tumor by normal mice that
SUSCEPTIBILITY OF TUMOR CELLS TO IN VITRO AND IN VIVO IMMUNE MECHANISMS

Fig. 1. Changes in the numbers of host cells and tumor cells during progressive growth of P815 mastocytoma cells in the peritoneal cavity of normal or 700-rad irradiated mice. Ascites tumors were initiated by implantation of 10^5 tumor cells. Log of the number of peritoneal cells in normal B6D2F1 mice without ascites tumors.

Fig. 2. Evidence that the i.p. growth of the P815 tumor generates specific concomitant antitumor immunity. B6D2F1 mice that had been given injections i.p. of 10^6 P815 tumor cells 10 days before (tumor bearer) showed a significant difference in tumor size (P < 0.02 at all time points comparing means using Student's t test) to inhibit the growth of a challenge i.d. implant of 10^6 P815 cells (left) but not of 2 x 10^6 L5178Y cells (right). Means of 5 or 10 mice/group ± 2 SEs (bars).

would have been indicated by a decrease in the number of tumor cells recovered from the peritoneal cavity, as described by others (9). The data in Fig. 2 indicate, nevertheless, that the i.p. growth of the P815 tumor is an immunologically recognized event which leads to the generation of a state of concomitant antitumor immunity that is specific for the P815 tumor. The observed decrease in tumor cell numbers in both normal and immunosuppressed mice late in tumor growth was more apparent than real, since necropsy of tumor bearing mice at 15 and 18 days revealed numerous small, solid tumors attached to the mesentery and viscera. This finding indicated that it was not possible by peritoneal washing to harvest all of the i.p. tumor cells late in tumor growth.

Susceptibility of Tumor Cells during Early and Late Tumor Growth to In Vitro Cell-mediated Lysis. Biddison and Palmer (8) previously reported that the progressive growth of P815 tumor cells as an ascites in normal mice was accompanied by a decline in their susceptibility to in vitro lysis by P815-sensitized cytolytic T-cells. It was suggested that this phenomenon may be related to the selection, during tumor growth, for cells which have reduced expression of surface TATA recognized by CTLs (8, 9). In this regard, it was reasoned that if a reduced susceptibility to lysis was a result of immunological selection for antigen-loss variants by the concomitant immune response, then tumor cells grown as ascites in immunosuppressed mice, which should have a reduced capacity to immunoselect, should be as susceptible to CTL-mediated lysis as unselected cells taken early during the growth of P815 ascites tumors in normal mice.

P815 cells harvested at 16 and 18 days of tumor growth from normal and irradiated mice were compared to P815 cells harvested at 12 days of tumor growth (log growth phase) for susceptibility to in vitro cell-mediated lysis by tumor-specific CTLs, and also for susceptibility to lysis by allogeneic CTLs (H-2^d). As an independent source of tumor cells in log phase growth (log phase control) P815 cells from mice used to passage the tumor on a weekly basis were also tested for susceptibility to lysis. Ascites cells of the L5178Y lymphoma were used as a control for the specificity of CTL lysis. As shown in Table 1, ascites tumor cells demonstrated a decline in susceptibility to lysis by both the tumor-specific anti-P815 effector cells and the allogeneic anti-H-2^d effector cells that was associated with the time of tumor harvest. Moreover, this decline in susceptibility was seen with late tumor cells harvested from both normal mice and mice immunosuppressed by 700 rads of irradiation.

The following analysis was used to determine the significance of the apparent reduction in susceptibility to lysis of tumor cells with longer period of i.p. growth. Within each experiment, a percentage of reduction in lysis was calculated by comparing various pairs of tumor cells harvested at different time points (e.g., 12 day versus 18 day) using the formula shown in Table 2. In this way, differences in the degree of lysis between early and late tumors could be meaningfully compared between different experiments in which the absolute activity of the effector CTL populations varied considerably. These normalized changes in lysis, shown in Table 2, were then statistically tested with the nonparametric Wilcoxon Matched-Pair Signed-Rank Test (18). It can be seen in Table 2 that for all comparisons between early and late ascites tumor populations, except one (log phase control versus 16-day normal, H-2^d-specific CTLs), there was a statistically significant reduction in susceptibility to CTL lysis associated with increased time of tumor growth, regardless of whether the effector CTLs were tumor- or allospecific. In contrast, comparison of the log phase control P815 from mice used to passage the tumor to the 12-day P815 from experimental mice indicated no significant difference in susceptibility to lysis.

Inhibition by Tumor Cells from Late Tumors of Lysis of Early Tumor Cells by CTLs. To determine whether reduced susceptibility to CTL-mediated lysis is caused by a loss of expression of CTL-recognized antigens, the ability of late ascites cells to inhibit the specific lysis of ^51Cr-labeled P815 by tumor-specific CTLs was examined. Target tumor cells for ^51Cr labeling were harvested during the log-phase growth of a peritoneal ascites. Tumor cells to be compared for their ability to inhibit lysis of these log phase tumor cells were harvested from days 12 and 18 ascites tumors. Fig. 3 shows the results of one of three independent experiments which demonstrated that unlabeled tumor cells from early and late tumor were equally capable of inhibiting the lysis of log-phase cells by P815-specific CTLs. In contrast, unlabeled L5178Y cells caused inhibition of lysis only when added at high concentrations. It is apparent, therefore,
### Table 1 Susceptibility of early and late ascites tumor cells to lysis by tumor-specific and H-2d-specific CTLs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Log phase control</th>
<th>12 day 700 rads</th>
<th>12-day normal</th>
<th>16 day 700 rads</th>
<th>16-day normal</th>
<th>18-day normal</th>
<th>L5178Y control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.74</td>
<td>25.75</td>
<td>34.46</td>
<td>12.61</td>
<td>12.99</td>
<td>20.23</td>
<td>Not done</td>
</tr>
<tr>
<td>2</td>
<td>12.44</td>
<td>17.25</td>
<td>15.88</td>
<td>8.06</td>
<td>8.29</td>
<td>7.08</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>21.89</td>
<td>16.52</td>
<td>21.61</td>
<td>9.69</td>
<td>16.54</td>
<td>11.06(17-day)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>16.63</td>
<td>15.61</td>
<td>17.39</td>
<td>10.43</td>
<td>7.80</td>
<td>7.00</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>51.13</td>
<td>30.68</td>
<td>34.14</td>
<td>28.13</td>
<td>35.15</td>
<td>35.74</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>16.24</td>
<td>20.31</td>
<td>25.9</td>
<td>22.73</td>
<td>14.44</td>
<td>18.03</td>
<td>3.45</td>
</tr>
<tr>
<td>7</td>
<td>15.67</td>
<td>29.26</td>
<td>24.70</td>
<td>10.28</td>
<td>16.98</td>
<td>11.62</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### Table 2 Comparison of the susceptibility of early and late ascites tumors to CTL lysis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>12-day, 700 R</th>
<th>12-day normal</th>
<th>16-day, 700 R</th>
<th>16-day normal</th>
<th>18-day normal</th>
<th>18-day normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−0.04</td>
<td>−33.9</td>
<td>51.0</td>
<td>49.5</td>
<td>21.4</td>
<td>51.0</td>
</tr>
<tr>
<td>2</td>
<td>−38.8</td>
<td>−27.7</td>
<td>35.2</td>
<td>33.4</td>
<td>43.1</td>
<td>53.3</td>
</tr>
<tr>
<td>3</td>
<td>22.7</td>
<td>1.3</td>
<td>55.7</td>
<td>24.4</td>
<td>49.5</td>
<td>42.7</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>−4.6</td>
<td>37.3</td>
<td>54.2</td>
<td>57.9</td>
<td>33.2</td>
</tr>
<tr>
<td>5</td>
<td>40.3</td>
<td>33.2</td>
<td>45.0</td>
<td>31.3</td>
<td>30.1</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>−25.1</td>
<td>−59.5</td>
<td>−40.0</td>
<td>11.1</td>
<td>−11.0</td>
<td>−11.9</td>
</tr>
<tr>
<td>7</td>
<td>−86.8</td>
<td>−8.3</td>
<td>34.4</td>
<td>−8.4</td>
<td>25.8</td>
<td>64.9</td>
</tr>
<tr>
<td>8</td>
<td>−0.7</td>
<td>41.3</td>
<td>48.8</td>
<td>59.7</td>
<td>4.7</td>
<td>30.4</td>
</tr>
<tr>
<td>9</td>
<td>−38.9</td>
<td>−4.7</td>
<td>35.7</td>
<td>52.5</td>
<td>62.0</td>
<td>44.5</td>
</tr>
</tbody>
</table>

### Notes
- The specific release of 51Cr following a 6-h incubation of labeled target cells with CTLs. All experiments with the exception of 6 and 7 with tumor-specific CTLs and 8 and 9 with H-2d-specific CTLs were done at an effectortarget ratio of 50:1; the other experiments were done at 100:1. Each data point represents the mean of quadruplicate samples. The percentage of specific release was calculated as described in “Materials and Methods.” The spontaneous 51Cr release usually ranged from 4-9% of maximum release, and rarely exceeded 10%. There was no consistent relationship between the spontaneous 51Cr release and the time of tumor cell harvest.
- The coefficient of variation (SD/mean) for the experimental data used to calculate the percentage of specific 51Cr release usually fell between 0.1 and 0.2.
- P815 mastocytoma target cells were harvested from the peritoneal cavity of normal or 700-rad irradiated female B6D2F1 mice at the days indicated. Tumors were established by implantation of 10^3 cells. Log phase P815 tumor cells were harvested from the peritoneal cavity of female B6D2F1 mice, used to passage the tumors on a weekly basis by implantation of 3 × 10^6 cells. L5178Y lymphoma cells used as specificity controls were harvested during log phase growth from the peritoneal cavity of female B6D2F1 mice given an implant of 3 × 10^6 cells.
- Tumor-specific CTLs were harvested from the draining lymph nodes of B6D2F1 mice immunized i.d. 8 or 9 days previously with 2 × 10^6 P815 cells admixed with 50 μg C. parvum. H-2d-specific CTLs were harvested from the draining lymph nodes of AB6F1 mice immunized i.d. 7 or 8 days previously with 2 × 10^6 L5178Y lymphoma cells. CTLs used in each experiment were freshly harvested on the day the experiment was performed. Therefore, the activity of the effector cells was variable between experiments, and thus data from separate experiments could not be pooled.

### Table 3 Comparison of the susceptibility of early and late ascites tumors to CTL lysis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LPC:12 day, 700 R</th>
<th>LPC:12-day normal</th>
<th>LPC:16 day, 700 R</th>
<th>LPC:16-day normal</th>
<th>LPC:18-day normal</th>
<th>12 day, 700 R</th>
<th>12-day, normal</th>
<th>12-day, normal: 18-day normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−0.04</td>
<td>−33.9</td>
<td>51.0</td>
<td>49.5</td>
<td>21.4</td>
<td>51.0</td>
<td>62.3</td>
<td>41.3</td>
</tr>
<tr>
<td>2</td>
<td>−38.8</td>
<td>−27.7</td>
<td>35.2</td>
<td>33.4</td>
<td>43.1</td>
<td>53.3</td>
<td>47.8</td>
<td>55.4</td>
</tr>
<tr>
<td>3</td>
<td>22.7</td>
<td>1.3</td>
<td>55.7</td>
<td>24.4</td>
<td>49.5</td>
<td>42.7</td>
<td>23.5</td>
<td>48.8</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>−4.6</td>
<td>37.3</td>
<td>54.2</td>
<td>57.9</td>
<td>33.2</td>
<td>55.1</td>
<td>59.7</td>
</tr>
<tr>
<td>5</td>
<td>40.3</td>
<td>33.2</td>
<td>45.0</td>
<td>31.3</td>
<td>30.1</td>
<td>8.3</td>
<td>−2.9</td>
<td>−4.7</td>
</tr>
<tr>
<td>6</td>
<td>−25.1</td>
<td>−59.5</td>
<td>−40.0</td>
<td>11.1</td>
<td>−11.0</td>
<td>−11.9</td>
<td>44.2</td>
<td>30.4</td>
</tr>
<tr>
<td>7</td>
<td>−86.8</td>
<td>−8.3</td>
<td>34.4</td>
<td>−8.4</td>
<td>25.8</td>
<td>64.9</td>
<td>31.5</td>
<td>53.1</td>
</tr>
<tr>
<td>8</td>
<td>−0.7</td>
<td>41.3</td>
<td>48.8</td>
<td>59.7</td>
<td>4.7</td>
<td>30.4</td>
<td>11.5</td>
<td>21.0</td>
</tr>
<tr>
<td>9</td>
<td>−38.9</td>
<td>−4.7</td>
<td>35.7</td>
<td>52.5</td>
<td>62.0</td>
<td>44.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

### Notes
- The percentage of reduction in lysis was calculated for each pair of tumors using the formula:

\[
\% \text{ specific } ^{51}\text{Cr release (Tumor 1)} - \% \text{ specific } ^{51}\text{Cr release (Tumor 2)}
\]

\[
100 \times \frac{\% \text{ specific } ^{51}\text{Cr release (Tumor 1)}}{\% \text{ specific } ^{51}\text{Cr release (Tumor 1)}}
\]

- LPC, log phase control.
- Tumor-specific CTLs were harvested from the draining lymph nodes of B6D2F1 mice, used to passage the tumors on a weekly basis by implantation of 3 × 10^6 cells. H-2d-specific CTLs were harvested from the draining lymph nodes of AB6F1 mice immunized i.d. 7 or 8 days previously with 2 × 10^6 L5178Y lymphoma cells. CTLs used in each experiment were freshly harvested on the day the experiment was performed. Therefore, the activity of the effector cells was variable between experiments, and thus data from separate experiments could not be pooled.

- For each pair of tumors the Wilcoxon Matched-Pair Signed-Rank Test was used to determine the significance of the percentage of reduction in lysis values. The T statistic was calculated using the data from all experiments in which the pair of tumors was compared. A P of less than 0.05 was taken to indicate that the two tumors within the pair differed significantly in their susceptibility to CTL lysis.

- NS, not significant.
that expression of CTL-recognized antigens by early and late P815 ascites cells was the same.

Reduced Susceptibility to Cell-mediated Lysis Associated with a Decreased Proportion of Tumor Cells in Cycle. In view of the above findings, it was reasoned that the decline in susceptibility to lysis of tumor cells later in tumor growth may be due to changes in cell metabolism rather than changes in antigen expression. In this regard, it has been demonstrated that the susceptibility of some cell lines to antibody-dependent, complement-mediated lysis (19-21) or cell-mediated lysis is cell cycle dependent (22, 23).

Table 3 contains the results of three independent experiments that measured both susceptibility to CTL lysis and the proportion of cells incorporating [3H]thymidine in the same tumor cell populations from early (day 12) or late (days 16 and 18) ascites tumors, immediately after harvest and then again after 24 h incubation in vitro. Examination of the data in the first column of Table 3 reveals that within each individual experiment, the percentage of cells incorporating [3H]thymidine in freshly harvested ascites tumors was much greater in early tumor cell populations (log phase control) than in late populations. However, the next column shows that incubation of fresh tumor cells for 24 h in vitro results in roughly equivalent levels of label incorporation between early and late cells.

Similarly, the percentage of specific 51Cr release of tumor cells exposed to tumor-specific CTLs was greater within each experiment for fresh log phase growth cells than for fresh late tumor cells. One exception to this finding was the 16-day tumor cells in Table 3, experiment 2, which were as susceptible to CTL-mediated lysis as were the log phase controls; however, the percentage of cells incorporating [3H]thymidine (Table 3, Column 1) was high in both populations. Nonetheless, the last column demonstrates that within each replicate experiment, susceptibility to lysis of early and late tumor cells became equivalent after the tumor cells were incubated for 24 h in vitro and then tested for susceptibility to lysis using fresh, tumor-specific CTL. It should be pointed out that for each experiment the data in Table 3, Column 3 cannot be compared directly to the data in Table 3, Column 4 because different CTL effector populations were used at each time point. Thus, the late tumor cells reversed from a state of relative insusceptibility to lysis by CTLs to a susceptible state after 24 h in culture, and this increase in susceptibility was associated with increased [3H]thymidine incorporation.

Susceptibility of Early and Late P815 Ascites Cells to In Vivo Rejection by Passively Transferred Tumor-sensitized T-Cells. Previous studies have revealed that T-cells harvested from the spleens of mice immunized with an admixture of C. parvum and P815 tumor cells can mediate, upon adoptive transfer into T-cell deficient recipient mice, the regression of an established P815 tumor in TXB recipients (12). It was reasoned, therefore, that if selection for antigen-loss cells was the cause of progressive growth of P815 ascites tumors, then passively transferred tumor-sensitized T-cells should fail to cause the rejection of a tumor initiated by implanting tumor cells from late tumors. Fig. 4 shows the results of experiments in which immune spleen cells from immunized donors were passively transferred to TXB recipients bearing a 4-day P815 tumor initiated with either early (days 6, 9, and 11) or late (days 16 and 18) P815 ascites

![Graph of COLD TARGETS: Cr-Labeled Targets](image-url)

Fig. 3. Cold-target inhibition of lysis of early, log phase growth P815 by log phase or late P815 ascites tumor cells. Unlabeled (cold) targets were added to 51Cr-labeled cells at the ratios indicated. The percentage of specific 51Cr release at a ratio of 0:1 represents the lysis of labeled log phase P815 target cells in the absence of unlabeled, cold targets.

Table 3 DNA labeling and susceptibility to cell-mediated lysis of ascites tumor cells

<table>
<thead>
<tr>
<th>Tumor targets</th>
<th>% labeled cells</th>
<th>24-h cultured tumor</th>
<th>% specific 51Cr release</th>
<th>24-h cultured tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascites tumor</td>
<td></td>
<td></td>
<td>Ascites tumor cells</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Log phase control</td>
<td>41.69 ± 3.60</td>
<td>61.32 ± 2.64</td>
<td>42.37 ± 7.05</td>
</tr>
<tr>
<td></td>
<td>16 day</td>
<td>10.59 ± 4.07</td>
<td>53.69 ± 1.54</td>
<td>31.08 ± 5.59</td>
</tr>
<tr>
<td></td>
<td>18 day</td>
<td>9.54 ± 2.39</td>
<td>51.68 ± 5.88</td>
<td>19.02 ± 4.57</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Log phase control</td>
<td>49.83 ± 2.06</td>
<td>69.52 ± 0.94</td>
<td>21.96 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>16 day</td>
<td>37.81 ± 5.78</td>
<td>67.12 ± 6.00</td>
<td>21.79 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>18 day</td>
<td>17.73 ± 4.13</td>
<td>51.09 ± 8.65</td>
<td>11.44 ± 6.64</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>Log phase control</td>
<td>33.37 ± 2.67</td>
<td>66.13 ± 1.77</td>
<td>27.05 ± 4.88</td>
</tr>
<tr>
<td></td>
<td>16 day</td>
<td>10.60 ± 1.07</td>
<td>50.90 ± 9.88</td>
<td>20.27 ± 5.32</td>
</tr>
<tr>
<td></td>
<td>18 day</td>
<td>19.67 ± 10.82</td>
<td>47.01 ± 8.13</td>
<td>13.40 ± 7.76</td>
</tr>
</tbody>
</table>

* The percentage of labeled cells in autoradiograms of tumor cells exposed to a 20-min pulse of [3H]thymidine. Data points are mean ± SD of the percentage of labeled target cells counted in at least 200 cells on duplicate slides.

* The specific release of 51Cr following a 6-h incubation of labeled target cells with lymph node cells from B6D2F1 mice immunized i.d. 8 or 9 days previously with 2 x 10^6 log phase P815 cells admixed with 50 µg C. parvum. The effector:target cell ratio was 100:1. The effector cells used in specific release assays of 24-h cultured target cells were freshly harvested on the day of assay. Data points are mean ± SD of the percentage of specific 51Cr release from target cells harvested from three separate mice.

* P815 mastocytoma target cells were harvested from the peritoneal cavity of three normal female B6D2F1 mice/group on the days of tumor growth indicated. Tumors were established by implantation of 10^3 cells.

* Target cells harvested from 24-h cultures of ascites tumor cells incubated in RPMI 1640 plus 10% HS at 37°C in 5% CO2.
tumor cells. It can be seen that immune spleen cells were equally capable of causing regression of tumors initiated by either early or late ascites tumor cells harvested from either immunocompetent or immunosuppressed (700 rads) mice. The same result was obtained in three independent replicates of these experiments.

**DISCUSSION**

It is clear from the results of the present study that the progressive growth of P815 ascites tumors in semisynecgetic B6D2F$_1$ mice is accompanied by a loss of susceptibility of tumor cells to lysis in vitro by tumor-specific CTLs. This finding is in complete agreement with that of Biddison and Palmer (8), who used the P815Y tumor subline growing as an ascites in syngeneic DBA/2 mice. However, additional experimentation in this study has shown that loss of susceptibility to lysis by CTLs in vitro does not imply that late tumor cells have the capacity to escape immunological rejection in vivo. In this regard, it is important to point out that while tumor-specific CTLs have been demonstrated in many models of tumor immunity, no formal evidence exists which conclusively links them to tumor destruction in vivo (24). Therefore, changes in the quantitative expression of tumor cell antigens as detected by in vitro CTL assays may have little relevance to the susceptibility of the same cells to destruction by immune mechanisms in vivo.

Furthermore, since tumor cells can express multiple tumor-associated transplantation antigens (25), those antigens recognized in CTL assays may be neither necessary nor sufficient for in vivo tumor rejection.

The finding that cells from late tumors, growing in either immunosuppressed or normal mice, lost susceptibility to in vitro CTL lysis, strongly suggests that concomitant antitumor immunity is not acting as a major selective force for the outgrowth of CTL-resistant tumor cells during progressive tumor growth. The additional finding that cells from late tumors from either immunosuppressed or normal mice also lost susceptibility to lysis by alloreactive CTLs suggests that the loss of susceptibility to cytolysis is metabolically determined. This is supported by the finding that the reduced susceptibility to CTL lysis by P815 cells from days 16 and 18 ascites was not associated with a loss of the capacity to inhibit the lysis of $^{51}$Cr-labeled tumor cells from early ascites tumors by CTLs in cold-target inhibition assays (Fig. 3), thus indicating that expression of the CTL-recognized tumor antigens is retained.

It is known from the results of others (26–28) that a loss of susceptibility to CTL- and antibody-mediated, complement-dependent lysis of tumor cells during progressive growth is not limited to the P815 mastocytoma. Studies in other laboratories have shown that tumor cell susceptibility to complement and CTL-mediated lysis is reversible. Thus, implantation of the LPC1 plasmacytoma into the peritoneal cavity of histocompatible, euthymic, BALB/c mice, was found to result in the progressive loss of susceptibility of cells of the tumor to lysis by CTLs specific for either major or minor histocompatibility antigens, or for trinitrophenyl-modified tumor antigens (26). This loss of susceptibility to lysis was associated with a reduction in tumor cell proliferation (26–28) and was regained when the tumor cells were transplanted into naive mice, or placed in tissue culture. Because restoration of susceptibility to lysis occurred when LPC1 cells were put into nude mice or tissue culture, it was suggested that it was not a result of immunoselection for preexisting variants (27).

Again, cells of the L5178Y thymoma, when in a nonproliferative tumor-dormant state, show reduced susceptibility to antibody-mediated lysis, compared to proliferating cells (29). Susceptibility to lysis was restored when the dormant L5178Y cells were induced to proliferate by implantation into the peritoneal cavity of normal mice, or when cultured in vitro. It was suggested that dormancy of the L5178Y tumor depends on cells of the tumor entering the G$_0$ stage of the cell cycle.

In agreement with the aforementioned studies, we also found that CTL-resistant tumor cells can revert to a more susceptible state upon in vitro cultivation, and that reversion to susceptibility to lysis was associated with the return of the cells to cycle. Therefore, it seems reasonable to propose that the reduced susceptibility of late ascites P815 tumor cells to CTL-mediated lysis in this study resulted from their arrest in cell cycle and the accompanying cellular changes in metabolic and membrane properties. Cell cycle arrest is a well-documented phenomenon in tumor cell biology (30) that has recently been used to explain the entrance of tumors into the dormant state (31). It might be argued, based on previous studies by others (32, 33) using alloreactive CTLs to test the cell cycle dependence of P815 susceptibility to lysis, that susceptibility is independent of the stage of cell cycle. However, those studies used P815 harvested from tissue culture (33) or during exponential growth in mice (32). Therefore, it is highly unlikely that many of their target cells were in a noncycling state.

The results of this study do not agree with the findings of
others (9) indicating that stable antigen-loss variants are generated during progressive growth of a P815 ascites tumor. On the contrary, our findings strongly suggest that immunoselection for CTL-resistant tumor cells is not frequently involved in the progressive growth of the P815 tumor in a normal immunocompetent host. Indeed, recent studies in this laboratory have shown that the immunogenicity of the P815 mastocytoma and of the Meth A fibrosarcoma is extremely stable in that sublines of each tumor passed separately at different locations for 10 years retained expression of the same TATA (34).

ACKNOWLEDGMENTS

The authors thank Dr. L. Johnson for help with the statistical analysis of the data, and gratefully acknowledge the technical assistance of J. Reome and the secretarial assistance of M. Durett, D. Heneka, and H. Jarvis.

REFERENCES

Progressive Growth of Immunogenic Tumors: Relationship between Susceptibility of Ascites P815 Tumor Cells to T-Cell-mediated Lysis and Immune Destruction in Vivo

James R. Fahey and David L. Hines


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/18/4759

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.