Immunologic Evaluation of Spontaneous Regression of an Intraocular Murine Melanoma

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The experiments reported here describe the derivation of an immunogenic melanoma cell line from B16 melanoma by sequential in vitro mutagenization with two chemical mutagens: n-methyl n-nitro n-nitrosoguanine (MNNG) and ethane methyl sulfonate (EMS). Following in vivo screening of over 100 mutant melanoma clones, a single clone was selected for further study. When transplanted to the anterior segment of the mouse eye, the mutant melanoma (D5.1G4) underwent spontaneous resolution in 20% of the immunologically intact hosts. Tumor rejection involved extensive necrosis and culminated in phthisis of the tumor-containing eye. Histologic analysis revealed a prominent mononuclear cellular infiltrate in contrast to the parental progressor B16 melanoma. Immunologic analysis of tumor-bearing hosts showed variable cytotoxic T lymphocyte (CTL) responses but potent delayed-type hypersensitivity (DTH) responses directed against the melanoma cells. Fluorescent activated cell sorter (FACS) analysis of tumor-infiltrating cells from ocular tumors revealed a cellular response consisting mainly of CD8+ CTLs and macrophages. Cultured D5.1G4 melanoma cells demonstrated: 1) enhanced expression of class I major histocompatibility complex (MHC) antigens; 2) increased susceptibility to CTL-mediated killing; and 3) increased susceptibility to tumor necrosis factor (TNF)-mediated cytolysis. Therefore, the intraocular D5.1G4 mutant melanoma model provides important insights into the immunology and immunopathology of intraocular tumor rejection. More intensive analysis of this intraocular melanoma may yield strategies for directing the immune response toward tumor rejection while minimizing damage to normal ocular components. Invest Ophthalmol Vis Sci 31:247-257, 1990

There is ample evidence that dermal melanomas can spontaneously regress,1,2 while intraocular melanomas rarely resolve.3-5 Moreover, the presence of infiltrating mononuclear cells is indicative of a favorable response in dermal melanoma,6,7 but is not considered of prognostic significance in intraocular melanoma.8,9 One method of investigating the immune response to melanoma is to use an animal model that permits prospective studies. Murine models of intraocular melanoma have predominantly used the well characterized, metastatic B16F10 melanoma line.10-13 However, one disadvantage of using experimental murine melanomas, and B16 melanoma especially, is that they are progressor tumors: that is, when transplanted into a syngeneic host they grow rapidly and progressively, and quickly kill the host. Hence, it is difficult to evaluate the immune status of tumor-bearing hosts and to evaluate those parameters that may contribute to immune mediated tumor rejection.

One method of increasing the immunogenicity of progressive tumors is chemically to induce mutations that cause the expression of new or tumor-specific transplantation antigens (TSTA). Chemical mutagenization of tumors with alkylating agents, metabolic poisons, and DNA hypomethylating agents has been performed on a variety of tumors (reviewed in Refs. 14, 15), including murine melanomas.16-18 After treatment, progressive transplantable tumors frequently manifest a nontumorigenic phenotype when transplanted to syngeneic hosts. The host develops a strong immune response to the transplanted tumor; this response culminates in tumor rejection and a state of immune resistance to additional tumor challenges. Yet, these same tumors grow progressively in immunocompromised hosts.

Several mechanisms have been postulated to account for the increased antigenicity of mutagenized tumors. Unique (TSTA) antigens can be demonstrated on individual clones, with minimal cross-re-
activity exhibited between the clones. At the same time, chemical mutagenesis can induce increased class I major histocompatibility complex (MHC) antigen expression and thereby favor the immune interaction with the tumor. However, class I antigen expression by itself does not confer an immunogenic phenotype on the tumor cell. In order to circumvent the progressive tumor growth of B16 melanoma and to analyze how intraocular anti-melanoma immune responses contribute to tumor rejection, an immunogenic melanoma was generated from B16 melanoma by sequential treatments of in vitro cultured melanoma cells with chemical mutagens. From these experiments, one clone which demonstrated intraocular spontaneous rejection was isolated. This mutant melanoma model was evaluated histologically and immunologically in an effort to understand the immunology and pathology of intraocular tumor rejection. The current study is a first step in the long-term objective of promoting intraocular tumor rejection by stimulating a melanoma-specific immune response.

**Materials and Methods**

**Mice**

Adult female C57BL/6 (H-2b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used as experimental subjects when they were between 6 and 8 weeks of age. All animals were treated according to the ARVO Resolution on the Use of Animals in Research.

**Tumor Lines**

B16 melanoma and P815 mastocytoma were originally obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY), 1% L-glutamine, 1% vitamin solution, 1% sodium pyruvate, and 1% streptomycin/penicillin/fungizone (complete MEM). J774.1 leukemia was obtained from American Type Culture Collection; YAC-1 lymphoma (H-2a), EL-4 lymphoma (NR; H-2b), and WEHI 164.1 (H-2d) fibrosarcoma were obtained from Dr. Michael Bennett (University of Texas Southwestern Medical School). The above cells were maintained in suspension or monolayer culture in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO), 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, 0.01 M HEPES, and streptomycin/penicillin/fungizone (complete RPMI).

**Mutagenesis of B16 Melanoma**

B16 melanoma cells were treated six times with n-methyl n-nitro n-nitrosoguanine (MNNG) after a modified procedure of Marchand et al. This cell population was brought to a concentration of 6 x 10^7/ml, and 5 ml were placed in one well of a six-well flat-bottomed plate (Costar, Cambridge, MA) and incubated for 1 h at 37°C. After this incubation, 2.5 µl of ethyl methane sulfonate (EMS) was added (final concentration 0.5 µl/ml), and cells were incubated for 2 h at 37°C. The cells were washed and cultured to confluency in MEM/20% FBS. This procedure was repeated twice with 5.0 µl of EMS. Resistance to ouabain or 6-thioguanine was developed by gradually increasing the concentration of ouabain from 1 mM to 3 mM over a 1-month period or by increasing the concentration of 6-thioguanine from 8.5 µg/ml to 15 µg/ml over a 2-week period. Cells were treated with EMS and surviving cells cloned by limiting dilution.

**Monoclonal Antibodies for FACS Analysis**

Optimum dilutions of the following hybridomas were used: IgG2b rat hybridoma 30-H12, which secretes anti-Thy 1.2; IgG2a rat hybridoma 53-6.72, which secretes anti-Lyt 2.1/2.2 (anti-CD8); and IgG2b-secreting rat hybridoma GK1.5, which secretes anti-L3T4 (anti-CD4). Monoclonal antibodies (MAbs) reactive to Mac-1 (macrophages and granulocytes), B220 (B cells), and F4/80 (macrophages) were kind gifts from Dr. Pam Witte (University of Texas Southwestern Medical Center). Culture supernatants of anti-class I hybridomas (20-8-4S, 8-13-3S, and 28-14-8S) and commercially prepared mAbs (34-1-2S and 16-1-11N; Litton Bionetics, Charleston, SC) were kind gifts from Dr. Phil Tucker (University of Texas Southwestern Medical Center). Fluorescein-conjugated affinity-purified F(ab')2 fragment mouse anti-rat IgG (heavy and light chain) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Goat anti-mouse IgG and IgM were purchased from Cappell (Malvern, PA).

**Anterior Chamber Inoculations**

A modified quantitative technique for depositing a definite number of tumor cells into the anterior chamber of the mouse eye has been described. Mice were deeply anesthetized with 2.2 mg ketamine hydrochloride (Parke-Davis, Detroit, MI) given intra-
peritoneally and brief Metofane (Pittman-Moore, Washington Crossing, NJ) inhalation. In vitro grown tumor cells were washed with HBSS, and 5 μl cell suspension, containing 1 × 10^5 cells, was injected into the anterior chambers of the respective panels of mice.

**Histology**

At various intervals following intracameral tumor inoculation, mice were killed and the tumor-containing eyes fixed overnight in 10% neutral buffered formalin. Fixed eyes were dehydrated and embedded in glycol methacrylate (Sorval, Wilmington, DE). Three-micron sections were cut and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

**Isolation of Tumor-Infiltrating Cells**

Tumor-containing eyes were removed for fluorescence-activated cell sorter (FACS) analysis between days 16 and 19 after tumor inoculation and placed in complete RPMI media. The anterior segments of the eye were removed, and tumors carefully dissected free under a dissecting microscope. Pooled tumors were minced with scissors; placed in 12 ml Hanks' balanced salt solution (HBSS) containing 1.0 mg/ml collagenase (Sigma grade IV; Sigma, St. Louis, MO), 25 units hyaluronidase (Sigma), and 0.1 mg/ml DNase (Sigma); and incubated for 1.5 hr on a rocker at 37°C. The suspension was centrifuged; the tissue pellet was pressed through a wire mesh screen, washed with HBSS, and centrifuged. The cell pellet was resuspended in complete RPMI and passed through nylon mesh (Tetko, Elmsford, NY).

**FACS Analysis**

Tumor-infiltrating cell suspensions and control cell populations were incubated with primary antibodies at the appropriate dilution for 30 min on ice, washed with complete medium, and then incubated with fluorescein-labeled secondary antibody at 1:40 dilution for 30 min on ice. The cells were washed with medium and resuspended in 0.7 ml media for analysis with a Facstar Flow Cytometer (Becton Dickson, Mountain View, CA). Results reported represent percentage above background (cells stained with secondary antibody only). For determination of cell surface class I expression, in vitro maintained mutant and parental (B16) melanoma cells were exposed briefly to trypsin and washed immediately. Reaction with primary and secondary antibodies was performed as above.

**In Vitro Boosting**

A mixed lymphocyte–tumor cell culture for the expansion of antitumor-specific cytotoxic T cells was prepared as previously described. Briefly, spleen cells served as responding populations and mitomycin C-treated tumor cells (Sigma), as stimulator cells. Boosting flasks were made by adding 2.5 × 10^7 responder spleen cells and 2.5 × 10^5 stimulator tumor cells to 25 cm^2 tissue culture flasks containing 20 ml culture medium and incubating upright at 37°C in a 5% CO2 atmosphere for 6 days. Culture medium was complete RPMI supplemented with 5 × 10^-5 M 2-mercaptoethanol. In vitro-boosted alloresponsive lymph node cell effectors were obtained from BALB/c mice sensitized with mitomycin C-treated C57BL/6 spleen cells. Boosting flasks contained 3 × 10^7 responder cells mixed with 6 × 10^5 stimulator cells and incubated for 4 days.

**Treatment with Poly Inosinic:cytidylic Acid**

Poly inosinic:cytidylic acid (poly I:C; Sigma) was prepared in phosphate-buffered saline (PBS) (1.0 mg/ml), and 0.1 ml was injected intraperitoneally 24 hr before the assay.

**Cell Mediated Cytotoxicity Assays**

Cytotoxic T lymphocyte (CTL) activities were assayed by using a standard 51Cr release assay after a 6-hr incubation; natural killer (NK) activities, after a standard 4-hr incubation; and natural cytotoxic (NC) activities, after an 18-hr incubation. Tumor targets were labeled by incubation for 45 min with 200 μCi Na2 51CrO4. Labeled target cells were washed twice with HBSS and given a final wash with complete media. Effectors were serially diluted in 96-well round-bottomed microtiter plates (Costar). Effector:target ratios ranged from 100:1 to 12.5:1 and were performed in groups of 3 to 6 wells. Recombinant murine tumor necrosis factor-α (rTNF; 4 × 10^7 U/mg) (Genzyme, Boston, MA) was serially diluted in complete media. Target cells (1 × 10^5–5 × 10^5) were added, and the plates were centrifuged at 500 RPM for 3 min and then incubated in a 5% CO2 atmosphere. After the appropriate incubation, the plates were centrifuged at 1000 RPM for 5 min, and 100 μl of supernatant was collected and counted in a gamma counter. The cytotoxicity was calculated as follows:

\[
\% \text{ specific release} = \frac{\text{experimental cpm} - \text{spontaneous release cpm}}{\text{total cpm} - \text{spontaneous release cpm}}
\]
Maximum release was determined by treatment of target cells with 100 μl 1.0 M HCl, and spontaneous release was determined by incubating target cells with cytotoxicity medium.

**Delayed-Type Hypersensitivity Assay**

An assay for measuring delayed-type hypersensitivity (DTH) responses against tumor specific transplantation antigens (TSTA) has been described elsewhere. Briefly, 1 × 10⁶ mitomycin C-treated tumor cells in 25 μl of HBSS was injected into the right hind footpad; the left hind footpad served as a negative control and received 25 μl HBSS. Both footpads were measured 24 hr later, and the difference in footpad size was used as a measure of DTH. Results are expressed as: specific footpad swelling = (24-hr measurement - 0-hr measurement [experimental foot]) - (24-hr measurement - 0-hr measurement [negative control]). Statistical comparisons were made with the student t-test.

**Results**

The current study was designed to develop an immunogenic melanoma cell line that undergoes immunologic rejection after intracameral transplantation in syngeneic mice. In vitro mutagenesis with MNNG and EMS has been shown to be an effective method for producing immunogenic tumor cell lines. Accordingly, this method was applied to the well characterized B16 melanoma cell line.

In vitro cultured B16 melanoma cells were treated six times with MNNG. Surviving cells were then treated with the mutagen EMS in multiple steps. In conjunction, surviving cells were exposed to increasing concentrations of ouabain, followed by increasing concentrations of 6-thioguanine. Lastly, surviving cells were treated with EMS, and the surviving cell population was cloned by limiting dilution techniques. Samples of each clone were frozen after one in vitro passage, and other flasks were maintained in culture for in vivo experiments. After 1 month in culture, tumor lines were frozen. From this stock, cells were frozen. For subsequent experiments, vials were thawed and cultured cells used within five in vitro passages.

**Histopathology of D5.1G4 Melanoma Mutant**

Thirty-five clones derived from the final mutagenized cell population were injected intracameraly (IC), and the growth pattern was evaluated. In preliminary experiments, one clone (1D5) demonstrated intraocular spontaneous regression. This cell line was subcloned, and one clone, D5.1G4, consistently manifested spontaneous regression in 20% of mice injected IC with 1 × 10⁵ mutant melanoma cells. Spontaneous regression of this tumor after both subcutaneous and intraocular routes of inoculation occurred in a dose-response relationship to tumor inoculum (data not shown). Initial characterization of the melanoma mutant involved a histopathologic comparison with the parental B16 melanoma. It has been well documented that B16 melanoma is a highly aggressive tumor when transplanted to various tissue sites in syngeneic hosts, and likewise, grows progressively in the anterior chamber. In Figure 1A, photomicrographs of B16 melanoma demonstrate the progressive growth characteristics of this tumor in the anterior chamber. At higher magnification (Fig. 1B) several intravascular mononuclear cells are shown, yet characteristics of a host cellular response are not evident, and there is a uniform tumor cell profile of the intraocular tumor. By contrast, the histopathologic appearance of D5.1G4 melanoma is characterized by a mononuclear cellular infiltrate diffusely scattered throughout the tumor (Fig. 2A) and found within the lumen of tumor-associated blood vessels (Fig. 2B). A smaller number of other inflammatory cells (neutrophils and macrophages) were also present. By day 28, areas of tumor were undergoing focal necrosis, and at these sites vascular endothelial cell destruction was evident (Fig. 3). Other areas contained pockets of viable tumor infiltrated with host mononuclear cells (Fig. 3). Eventually, the whole tumor became necrotic, with massive destruction of normal host ocular tissue (Fig. 3 inset).

**FACS Analysis**

The first step in characterizing cellular mechanisms responsible for this pattern of tumor rejection was to identify phenotypically the tumor-infiltrating cells. To determine the background staining of the mutant tumor, in vitro cultured D5.1G4 melanoma tumor cells were stained with the panel of monoclonal antibodies indicated in Table 1. Background staining was less than 3.0% for all antibodies except anti-B220 (9.0%) (data not shown). In order to obtain a single cell suspension of the intraocular tumor, we chose to digest enzymatically the tumor dissected from the anterior chamber. This cell suspension was stained with various monoclonal antibodies recognizing cell surface antigens, and was evaluated by FACS analysis (Table 1). It appeared that a substantial number of B cells (21%) were present. However, this figure is lower (approximately 15%), considering the higher background staining with this particular antibody. Furthermore, plasma cells were identified only rarely by histology. CD8⁺ cytotoxic/suppressor T cells
Fig. 1. (A) Light micrograph, day 8, of C57BL/6 murine eye containing B16 melanoma. Note absence of host inflammatory cells in the anterior and posterior chambers (asterisks) (H&E, ×6). (B) Higher magnification of box in (A), demonstrating uniform tumor cell appearance and lack of infiltrating cells. Note normal vessel appearance (arrowhead) (H&E, ×20).

(21.4%) were more than twice as prevalent as CD4+ T helper cells (8.7%) in the T cell population. Twenty-seven percent of the cells were MAC-1+, but only 13% of these were macrophages as identified by the F4/80 antigen, and the remainder were granulocytes. Overall, these results suggest that a cell likely responsible for direct tumor cytolysis in vivo expresses Thy 1, CD8 antigens that are characteristic of cytotoxic/suppressor cells. Nevertheless, the histopathology suggests that nonspecific tumor destruction plays a role in tumor resolution, and cells responsible for this pattern (CD4+ cells and macrophages), although not dominating, are present in the tumor-infiltrating lymphocyte population.
FACS Analysis of Class I MHC Antigen Expression

The cell surface expression of class I MHC antigens is necessary for recognition of host neoplastic cells by CTLs. Indeed, the transfection of a syngeneic class I gene into B16 melanoma changed its in vivo phenotype from progressor to regressor. Therefore, the expression of class I genes by D5.1G4 mutant melanoma was evaluated. Table 2 shows the results of FACS analysis of mutant and control cells stained with various monoclonal antibodies recognizing class I antigens. Both parental B16 and D5.1G4 demonstrate low levels of K\(^b\) expression, compared to expression by EL-4 control cells. Conversely, nearly twice as many D5.1G4 melanoma cells express D\(^b\) class I antigen compared to B16. Therefore, one possible explanation for the regressor phenotype characteristic of D5.1G4 melanoma is increased expression.
Fig. 3. Light micrograph, day 28, of C57BL/6 murine eye containing D5.1G4 melanoma, demonstrating destructive resolution. Areas of focal ischemic necrosis involving vascular endothelial damage (asterisks) and pockets of viable tumor with infiltrating mononuclear cells (arrowheads) are evident (H&E, X20). Inset reveals overall pattern of destruction of the globe; a small remnant of corneal tissue is evident (arrowhead) (H&E, 6X). Box indicates area enlarged in main figure.

of class I antigens. If this were the case, then D5.1G4 melanoma would likely manifest increased sensitivity to CTL-mediated lysis. To investigate this possibility, we determined the lytic activity of in vitro-generated CTL effectors against mutant and parental B16 tumor targets.

Cytotoxic T Lymphocyte Activity

The systemic cytotoxic activity of D5.1G4 melanoma-bearing mice and of control and allo-sensitized BALB/c mice was evaluated after in vitro stimulation with mitomycin C-treated D5.1G4 cells (Figure 4). Although control cultures (naive C57BL/6) achieved slightly higher levels of cytolytic activity compared to tumor-bearing counterparts, other experimentors have shown that B16 melanoma stimulates a primary CTL response in naive syngeneic mice.28 The mean cytotoxicity for tumor-bearing hosts was not significantly different than that of controls. Nevertheless, these results demonstrate that D5.1G4 melanoma in each case was more susceptible to CTL-mediated lysis than were B16 tumor targets, and suggest that the increased expression of class I antigens was responsible. Hence, although systemic CTL responses measured were not significant, they may not reflect the antitumor activity of CD8+ cells identified within the intraocular tumor.

Natural Killer/Cytotoxic Activity

Since some of the mononuclear cells present in the tumor may be NK cells or cells mediating tumor destruction by natural cytotoxic mechanisms, these two functions were analyzed in tumor-bearing hosts. The results in Figures 5A and 5B demonstrate several important points. First, in a 4-hr assay, NK activity is

Table 1. FACS analysis of cell suspension obtained by enzymatic digestion of intraocular D5.1G4 melanoma

<table>
<thead>
<tr>
<th>Monoclonal antibody recognizing (% positive)</th>
<th>Thy 1</th>
<th>CD8</th>
<th>CD4</th>
<th>B220</th>
<th>MAC-1</th>
<th>F4/80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment no. 1</td>
<td>25.3</td>
<td>17.4</td>
<td>18.3</td>
<td>21.7</td>
<td>22.6</td>
<td>25.4</td>
</tr>
<tr>
<td>Experiment no. 2</td>
<td>28.0</td>
<td>15.1</td>
<td>15.8</td>
<td>23.7</td>
<td>29.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Average</td>
<td>29.5</td>
<td>16.2</td>
<td>16.3</td>
<td>20.5</td>
<td>27.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Data from days 16-19.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results represent the percent positive minus the percent staining with secondary antibody alone.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 2. Cell surface expression of class I antigens by different tumor lines as detected by FACS analysis

<table>
<thead>
<tr>
<th>Tumor line (% positive fluorescence)</th>
<th>KdDp</th>
<th>Kp</th>
<th>Dp</th>
<th>Ke</th>
<th>KdDp</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5.1G4</td>
<td>17.4</td>
<td>2.9</td>
<td>41.3</td>
<td>1.5</td>
<td>2.7</td>
</tr>
<tr>
<td>B16</td>
<td>15.9</td>
<td>5.5</td>
<td>19.4</td>
<td>3.7</td>
<td>2.8</td>
</tr>
<tr>
<td>BW1547</td>
<td>2.1</td>
<td>0.0</td>
<td>21.0</td>
<td>56.0</td>
<td>3.2</td>
</tr>
<tr>
<td>EL-4</td>
<td>79.2</td>
<td>48.2</td>
<td>89.5</td>
<td>0.0</td>
<td>51.2</td>
</tr>
</tbody>
</table>

Numbers represent percentage above background.
assessed usually by the ability of effectors to lyse NK-sensitive YAC-1 tumor targets. We found that normal C57BL/6 hosts have moderate levels of NK activity and that this activity could be increased by prior administration of poly I:C. By contrast, these same effectors (enhanced NK activity) were incapable of lysing B16 or D5.1G4 melanomas. Therefore, the susceptibility of the mutant tumor to NK-mediated lysis is similar to that of the parental B16. This experiment also demonstrated that tumor-bearing hosts appeared to have slightly depressed NK activity (YAC-1 lysis), although this was not significantly different than controls \((P > 0.1)\).

In 18-hr cultures, lytic activity of spleen cell effector populations mainly represents NC-mediated killing by means of secretion of tumor necrosis factor (TNF). The results in Figure 5B reveal that spleen cells from tumor-bearing hosts had impaired NC-mediated cytolytic capacities for D5.1G4 targets, yet this activity was not significantly different than the activity demonstrated by normal spleen cells \((P > 0.05)\). Paradoxically, D5.1G4 mutant melanoma cells were susceptible to cytolysis by either recombinant TNF or splenic cells from poly I:C treated hosts. Therefore, even though systemic NC activity is low, the increased susceptibility of mutant melanoma cells to TNF-mediated lysis may enhance tumor resolution intraocularly. Concurrently, TNF secretion intraocularly may contribute to nonspecific normal tissue destruction by initiation of vascular events leading to hemorrhagic necrosis.

### Delayed-Type Hypersensitivity

We have demonstrated previously that B16F10 tumor-bearing hosts exhibit a T cell dependent, cyclophosphamide-sensitive suppressor network which down-regulates the expression of DTH responses to melanoma-associated antigens (MAAs). Therefore, it was important to determine the DTH responses in mutant melanoma tumor-bearers. At various times after tumor inoculation, tumor-bearing hosts were assessed for DTH responses to D5.1G4 melanoma cells (Fig. 6). As early as day 7, significant \((P < 0.02)\) footpad swelling was detected, and by day 14, DTH responses peaked \((P < 0.01)\) and remained elevated through day 35. Consequently, one dominant anti-melanoma immune response of D5.1G4 tumor-bearers that differs from B16F10 melanoma-bearing mice is the generation of a significant and sustained DTH response.
Fig. 6. DTH responses measured in a 24-hr footpad assay in C57BL/6 mice bearing intraocular D5.1G4 melanoma compared to naive controls. Results shown are the mean value for panels of 5 mice. The segment above each bar represents ±SEM.

Discussion

Evaluating the immune response to intraocular melanoma is an important undertaking, considering the malignancy of this neoplasm. Previous murine models of intraocular B16 melanoma have been hindered due to the progressive growth of this tumor. Hence, within a short period of time (25–35 days), extraorbital extension of the tumor results in death of the host. Consequently, evaluation of the immune status of tumor-bearing hosts is limited. However, the results presented here demonstrate that chemical mutagenesis of B16 melanoma can induce an immunogenic tumor. Even so, spontaneous intraocular resolution occurs in only 20% of syngeneic hosts and parallels the rejection characteristics of a "weakly immunogenic" tumor.

In previous reports, we have categorized the rejection of murine intraocular syngeneic tumors into two general patterns. In one pattern, tumor rejection is mediated predominantly by a DTH mechanism characterized by: 1) lack of a mononuclear cellular intratumor infiltrate; 2) damage to microvascular endothelium; 3) extensive innocent-bystander destruction of normal host tissues; and 4) development of DTH, CTL, and antibody responses. A second pattern of tumor rejection appears to be mediated by CTL and demonstrates these characteristics: 1) infiltrating cell-to-tumor cell contact resulting in tumor death; and 2) localized areas of piecemeal necrosis as opposed to bulk ischemic necrosis. A similar paired histopathologic pattern has been observed by light and electron microscopic evaluation of human primary dermal melanomas.

The phenotype of the tumor infiltrating cells was evaluated by FACS analysis and provided evidence to suggest that one possible effector cell was a CTL (Thy 1+, CD8+). However, approximately equal numbers of B cells also were found, and tumor-specific systemic antibody responses were correspondingly detected (data not shown). Further investigations are underway to determine the significance of this finding. The finding that D5.1G4 melanoma was highly susceptible to TNF-mediated cytolysis implies that this mechanism may be invoked, and that it may contribute to tumor destruction. Since macrophages and CD4+ cells were identified within the tumor and tumor-bearing hosts demonstrated strong DTH responses, another likely mechanism for destructive resolution is a DTH antitumor response.

Despite the presence of CD8+ T cells in the intraocular melanomas, systemic CTL responses appeared low in tumor-bearing hosts. However, suppression of in vitro-generated CTL can be mediated by soluble melanoma antigen. Hence, the significance of the CD8+ T cells might be interpreted a number of ways. One explanation is that the CD8+ population consisted of CTL and suppressor cells; the predominance of suppressor cells (in 80% of the hosts) would eventually lead to progressive tumor growth. By contrast, emergence of a dominant CTL population (in 20% of the hosts) would lead to tumor resolution. An alternative explanation is that CTL-mediated cytolysis was insignificant and that tumor resolution occurred by means of a DTH process. However, in cases where the tumor becomes progressive, the infiltrating CD8+ cells may suppress antitumor DTH activity. Therefore, in situ immunosuppression may regulate the conversion of a regressing to a progressing tumor. To address these questions, studies are currently underway to characterize the immune status of individual mice during tumor growth and resolution.

It is established that glycoproteins encoded by the major histocompatibility complex (MHC) affect a diverse range of cellular and biochemical interactions within the immune system. Generally, class I antigens serve as restriction elements for recognition of
antigen by cytotoxic T lymphocytes. Hence, tumors that express class I antigens are potential targets for CTL-mediated killing. Furthermore, tumors may evade host CTL responses by decreasing the expression of class I molecules (reviewed in Ref. 35). The findings reported here support this hypothesis. Twice as many D5.1G4 mutant melanoma cells expressed class I MHC antigens than did parental B16 melanoma, and D5.1G4 cells were significantly more susceptible to CTL-mediated killing by both syngeneic and allogeneic effectors. In a similar manner, Nanniet al showed that nontumorigenicity correlated with tumor surface expression of class I antigens and the susceptibility to allospecific CTL effectors.36
Moreover, the histologic identification of infiltrating cells in the present studies parallels the results of Ruiter et al, who demonstrated that class I MHC antigen expression on melanocytic lesions correlated with the presence of a mononuclear cellular infiltrate.37

It is clear that melanoma cells express unique MAAs38 that serve as potent immunogens.39,40 It is possible that the immunogenicity of D5.1G4 is due to increased expression of such MAAs. If this were the case, then one would predict that D5.1G4 immune mice could reject the parental tumor. However, D5.1G4 immune hosts were unable to reject a subcutaneous challenge with the parental B16 melanoma (data not shown). Class II antigens have been detected on freshly isolated and cultured human melanoma cells37,41 and gamma interferon (IFN) treatment has been found to increase this expression.42,43 Other investigators have suggested that intratumor production of gamma IFN by host infiltrating cells provokes increased tumor MHC antigen expression.44 Nevertheless, the role of class II expression in the induction or regulation of the immune response in human melanomas is not clear. In this laboratory, preliminary in situ (intraocular) immunoperoxidase studies have demonstrated that both B16 and D5.1G4 melanomas express I-A\(^+\) antigens in vivo (unpublished data); however others have shown that in vitro cultured BL6 melanoma (C57BL/6) and 14 clones isolated from MNNG-treated BL6 were negative for class II expression.17 Consequently, since both the progressor and regressor melanoma apparently express class II antigens in vivo, it is more likely that the immunogenicity of D5.1G4 melanoma is related to the increased expression of class I antigens and tumor-specific antigens.

The current report describes the development and characterization of a murine intraocular melanoma tumor model that manifests spontaneous regression. The observation that not all hosts were capable of tumor resolution has certain advantages as a model, because the critical immune parameters responsible for tumor rejection can be evaluated and compared to hosts bearing progressive tumors. From these studies we may then better understand the significance of tumor-infiltrating cells in intraocular melanoma and the role of host immune elements in tumor resolution.

**Key Words:** melanoma, murine, spontaneous rejection, mutagenesis, immunity

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