Studies of Tumor-Infiltrating Lymphocytes From a Human Choroidal Melanoma

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Cell suspensions prepared enzymatically from an ocular choroidal melanoma were cultured in vitro in an effort to generate (1) melanoma tumor cell lines and (2) tumor-infiltrating lymphocytes cytotoxic for ocular melanoma cells. Even though histologic study of the tumor did not show "significant" infiltrating bone marrow-derived cells, lymphocytes were generated readily in cultures to which interleukin-2 was added. Phenotypic analysis of the cultured lymphocytes indicated that T-cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells were present. Moreover, functional studies of the cultured lymphocytes revealed NK activity, LAK activity, and most importantly, tumor antigen-specific cytotoxic T-cell activity. It was concluded that it is possible to obtain tumor cell lines and tumor-infiltrating lymphocytes from ocular tumors, both of which would be required if cellular immunotherapy of ocular tumors is contemplated. In addition, these results indicate that ocular melanomas can express unique tumor-specific antigens and that the immune system of a patient with such an ocular tumor can perceive these tumor antigens because antigen-specific precursor cytotoxic T-cells were present in the tumor-containing eye at the time of enucleation. The theoretic and therapeutic implications of these findings are discussed. Invest Ophthalmol Vis Sci 32:3198–3208, 1991

Malignant melanoma of the choroid has been treated in the past with enucleation. In recent years, controversy has arisen over this form of therapy primarily because of the high incidence of metastases that occur months to years after the surgical procedure.1–4 Opinions vary concerning the putative link between surgical extirpation of the ocular tumor and the late emergence of metastases. In an animal model, it was found that at least two factors contribute to the generation of extraocular metastases.5 First, physical manipulation of the tumor-containing eye produces showers of tumor cells that escape from the eye into the venous circulation. Acutely, tumor cells can be recovered from many distant organs. However, the disseminated cells rarely form progressively growing tumors (metastases) in normal, immunocompetent individuals. Second, if the tumor-bearing animal is immunologically incompetent, then tumor cells released during manipulation of the eye come to rest on the vascular endothelium of the lung, migrate across the endothelial surface, and form expanding metastases.6–8 These studies and others have fostered the idea that surgical manipulation itself might play a role in the seeding of tumor cells from the melanoma-containing eye. Alternative treatments that attempt to eradicate the intraocular tumor without the risk of enabling tumor cells to escape into the venous circulation are unable to prevent metastases.9–13 These results imply that the second factor, the immunologic status of the host, may also play a pathogenic role.

In recent years, there has been increasing interest in the possibility of using immunotherapy to treat both primary intraocular melanomas and their subsequent metastases. A popular version of current attempts at immunotherapy in nonocular tumors uses lymphocytes harvested from the patient’s blood14–15 or ideally from the tumor itself.16,17 These are cultured in the presence of exogenous T-cell growth factors, especially interleukin-2 (IL-2). In vitro expanded tumor-infiltrating lymphocytes (TIL) then are injected into the patient along with recombinant human IL-2. This approach to therapy has had limited success in clinical trials.18,19 However, it is labor intensive, has significant toxic side effects, and usually was attempted in cancer patients with advanced disease. Most basic and clinical investigators believe that the rationale for this therapy is sound, but that its greatest benefit might be realized if therapy were initiated early in the disease, perhaps even at the stage when only a primary tumor is known to exist.

Ocular melanomas appear to be particularly appropriate tumors in which to attempt cellular and lym-
phokine immunotherapy. The patient with a choroidal melanoma generally has a primary malignancy and no evidence of systemic metastatic disease. Moreover, the tumor tissue is spatially confined, usually slow growing, and readily obtainable. However, histologic studies of large series of choroidal melanomas indicate that only a minority (approximately 20%) of such tumors are infiltrated with microscopically obvious lymphocytes and leukocytes.20,21 Thus, it is not clear that these ocular tumors contain lymphocytes that might be used for immunotherapy. To examine these points, we evaluated tumor specimens excised from eyes enucleated from patients with a diagnosis of malignant melanoma of the choroid. We report our initial successful attempts to culture both tumor cells and TIL from a human choroidal melanoma. These cells were used to show that antigen-specific and -non-specific cytotoxic lymphocytes were present in the tumor at the time of enucleation. The implications of these results for the development of an immunotherapeutic approach to ocular melanoma are discussed.

Materials and Methods

Description of Patient and Surgery

The patient was a 24-year-old white woman referred to the Bascom Palmer Eye Institute with a 3-week history of decreased visual acuity in her right eye. She had no prior ocular problems and was in good general health with no history of previous malignancy. On examination, best-corrected visual acuity was 20/40 on the right and 20/15 on the left. Slit-lamp examination showed a dilated episcleral feeder vessel in the superonasal quadrant of the right eye. The anterior segment was otherwise unremarkable, and the lens was clear. Funduscopic examination revealed an elevated pigmented choroidal tumor in the right eye with a collar button-type configuration located in the superonasal quadrant. An associated inferior retinal detachment extending from the area of the tumor was present. Previous diagnostic evaluations showed normal liver function and normal brain, abdomen, and chest computed tomographic scans. Ultrasonographic examination revealed a large collar button-shaped, irregularly structured, medium reflective mass lesion arising from the choroid superonasally. The maximum elevation was 11.8 mm, and the largest basal diameter was 18.0 mm. A diagnosis of malignant melanoma of the choroid was made, and the patient chose enucleation as her preferred mode of therapy. An enucleation was performed, and the ocular specimen was dissected under sterile conditions immediately after removal. A portion of the tumor was submitted for immunologic studies and the remainder, for routine pathologic evaluation. The University of Miami School of Medicine human research subcommittee approved the use of human tissue in these experiments.

Preparation of a Single-Cell Suspension From the Tumor

A single-cell suspension containing melanoma cells and mononuclear cells was obtained by a modification of the collagenase treatment used to isolate TIL from metastatic melanomas.16 The tumor was minced in a Petri dish containing 7 ml of collagenase (Sigma, St. Louis, MO) at 150 units/ml and hyaluronidase type V 0.01% in RPMI-1640 with 10% pooled human AB serum. The tissue was incubated 90 min at 37°C. After this, the released cells were removed, and the debris allowed to settle. The cells in the supernatant were recovered, washed three times, and examined microscopically for the presence of mononuclear cells and tumor cells.

Culture Conditions With and Without Recombinant IL-2

The single-cell suspension was divided between three groups: (1) expansion of TIL by resuspending the cells at a concentration of $2.5 \times 10^5$ viable cells/ml in 1000 units/ml human recombinant IL-2 (provided by Dr. Eckhard Podack, Miami, FL, and Hoffmann-La Roche Inc. Nutley, NJ), (2) expansion of melanoma cells by resuspending the cells at a concentration of $2.5 \times 10^5$ viable cells/ml in the absence of exogenous IL-2, and (3) cryopreservation of the original freshly isolated cells. The culture medium consisted of RPMI-1640 supplemented with 10% heat-inactivated pooled human AB serum, 0.01 M HEPES (N-2-hydroxyethylpiperazine-N’-2 ethanesulfonic acid) buffer, 2.0 mM glutamine, and 20 μg/ml of gentamicin (all Gibco, Grand Island, NY). The cultures supplemented with human recombinant IL-2 were reestablished with fresh culture medium and IL-2 every 7 days.

Assay for Cytotoxic T-Cells

The TIL obtained by the enzymatic digestion of the tumor and cultured in vitro in the presence of IL-2 were tested for cell-mediated cytotoxicity in a conventional 4-hr $^{51}$Cr-release assay. Autologous and allogeneic melanoma cells served as target cells and were labeled with 100 μCi Na$_2$$^{51}$CrO$_4$ (New England Nuclear, Boston, MA) per $2 \times 10^5$ cells for 1 hr. Labeled target cells were washed three times in Hank’s balanced salt solution (HBSS), incubated for an additional 30 min in HBSS, washed again, and resuspended in complete RPMI-1640 medium. This pro-
tocol decreased spontaneous release. Target cells (2 \( \times 10^9 \)) were placed into wells of microtiter plates with effector cells at the following effector–target cell ratios: 40:1, 20:1, 10:1, and 5:1. Culture plates were centrifuged at 100 \( \times g \) for 1 min and incubated for 4 hr at 37°C. After incubation, the plates were centrifuged for 10 min at 200 \( \times g \), and 100 \( \mu l \) of culture supernatant was removed and counted for radioactivity. The percent specific chromium release was calculated using the standard formula. The spontaneous release of \( ^{51} \text{Cr} \) from the target cells was determined by counting supernatants from culture wells containing only tumor cells and culture medium. The total \( ^{51} \text{Cr} \) release was determined by counting supernatants with 3 N HCl.

The following experiment was done as a positive control to show that target cells from all ocular melanoma patients (patients 202, 203, 204, 205, and 209) were susceptible to cell-mediated lysis. Lymphokine-activated killer (LAK) cells were generated from the peripheral blood lymphocytes of a normal donor by the method described previously and used as effector cells in a standard 4-hr \( ^{51} \text{Cr} \)-release assay with the ocular melanoma target cells from different patients. The LAK effector cells successfully lysed ocular melanoma target cells, showing they were susceptible to cell-mediated lysis.

Assay for NK/LAK Cells

TIL obtained by the enzymatic digestion of the tumor and cultured in vitro in the presence of IL-2 were tested for the presence of natural killer (NK) and LAK cells in a conventional 4-hr \( ^{51} \text{Cr} \)-release assay. NK-sensitive K562 cells and LAK-sensitive Mel-30 cells served as target cells and were labeled with \( ^{51} \text{Cr} \) for 1 hr as described. Specific chromium release was determined as described in the assays for cytotoxic T-cells.

Phenotypic Analysis by Flow Cytometry

Flow cytometric analysis of lymphocyte subsets was performed according to a previously published method. This method allowed the simultaneous identification of cell-surface markers with the exclusion of nonnucleated acellular debris. Briefly, suspensions containing approximately \( 4 \times 10^4 \) lymphocytes were incubated with various fluorescein isothiocyanate (FITC)-labeled antibodies for 30 min, washed twice, fixed with ethanol, and resuspended in 100 \( \mu l \) of medium containing 50 mg/ml of propidium iodide (PI). Each sample was analyzed on a FACScan flow cytometric analyzer (Becton Dickinson, Mountain View, CA) for two-color analysis. We collected 10,000 PI-positive events for each sample. The percentage of FITC-positive cells for each antibody was determined after gates were set based on isotype-matched (immunglobulins G2a and G1) antibody controls. Antibodies (Becton Dickinson, Mountain View, CA) used included: CD45 (bone marrow cells), CD19 (B-cells), CD3 (T-cells), CD4 (helper T-cells), CD8 (cytotoxic T-cells), CD16 (NK cells), CD14 (monocytes), CD25 (low affinity IL-2 receptor), and histocompatibility antigen HLA-DR (polymorphic class II).

Results

Over several months, we obtained specimens of ocular melanomas from a series of patients for whom enucleation was the most appropriate form of therapy. We evaluated whether immunoreactive T-lymphocytes were present in these tumors, even though there was neither clinical nor histopathologic evidence of significant immune inflammation or destruction at the time the tumors were excised. Our overall plan was to prepare single-cell suspensions from these tumor cells and culture the cells to (1) optimize tumor cell growth and (2) optimize growth of TIL. The following experiments were performed on the first tumor specimen we obtained.

Histopathologic Characterization

The specimen consisted of the posterior portion of an eye in which a pigmented, mushroom-shaped mass, arising from the choroid, and measuring 18.0 mm in diameter and 11.8 mm in height was located (Fig. 1A). One half of the tumor was used for histopathologic examination. On routine light microscopy, the tumor was composed of abundant, tightly packed basophilic spindle-shaped cells with variable melanin pigmentation (Fig. 1B). Mitotic figures were observed infrequently (9 figures were detected after examining 40 high-power fields). Examination of many different sections of this tumor showed little evidence of TIL or other types of leukocytes. A shallow secondary retinal detachment was observed to overlie the tumor. The tumor did not extend into the optic nerve, nor was there evidence of extrascleral extension. A diagnosis of malignant melanoma of the choroid and ciliary body, spindle cell type, was made.

Characterization of Single-Cell Suspensions From Ocular Tumor Tissue

The rest of the surgically extirpated tumor mass was dissected to remove normal retina and choroid. This specimen included representative portions of basal and apical portions of the tumor mass, excluding the surrounding choroid. The tumor was minced finely with scissors and treated with collagenase as described. A single-cell suspension of \( 10.2 \times 10^6 \) total
viable cells was obtained. When examined under phase microscopy, approximately 65% of the cells contained the pigment granules characteristic of melanoma cells. In addition, a significant, but minor, proportion of the cell suspension consisted of mononuclear cells with a lymphocytic appearance. To confirm this suspicion, an aliquot of the suspended cells from the tumor was treated with fluorescein-conjugated monoclonal antibodies directed at CD3, a pan-T-cell marker, and analyzed on a FACScan flow cytometer. The results revealed that a significant number of cells (30%) stained with the CD3 marker, indicating that there were TIL in the tumor at the time it was removed. This finding encouraged us to attempt to culture and expand lymphocytes from this cell suspension. However, we first tried to culture melanoma cells from the tumor to use as a source of antigen and target cells for tumor-specific lymphocytes.

Establishment of Cultured Tumor Cell Lines

A portion of the original cell suspension, consisting of $3.4 \times 10^6$ viable, pigmented tumor cells, was suspended in RPMI-1640 and 10% pooled human AB serum and cultured at 37°C. After 10 days, when foci of cellular masses were observed visually in the culture flask, the cells were harvested and counted. The total number of viable cells in the flasks increased approximately tenfold compared with the number originally placed in culture (Fig. 2). With a phase-contrast
Fig. 2. The in vitro expansion of tumor cells from a choroidal melanoma. A single cell suspension was prepared by enzymatic digestion of the intraocular tumor, of which $3.4 \times 10^6$ viable tumor cells were resuspended in complete culture medium and incubated. Periodically (days 10, 14, and 20), the tumor cells were harvested, counted, and reestablished with fresh culture medium.

microscope, we judged that most were tumor cells; a small proportion of the cells appeared to be much smaller and resembled lymphocytes. After inspection, the cultures were reestablished with fresh medium. After another 9 days in culture, the number of tumor cells was again assessed. As shown in Figure 2, the rate of tumor cell proliferation was reduced. Only a two-fold expansion ($62 \times 10^6$ cells) was observed compared with the number of cells observed after the first 10 days in culture. At this time, the culture contained a mixed population of pigmented tumor cells with either a spindle or epithelioid configuration (Fig. 3). Few lymphoid cells were detected. The tumor cells were cultured again with fresh medium. Over the next month, proliferation of the tumor cells continued, but the rate diminished. Thus, primary explants of single-cell suspensions prepared from an ocular melanoma grew and proliferated in culture. Moreover, during this culture interval, we obtained sufficient numbers of melanoma cells to be able to use them as a source of antigen and target cells for immunologic and cytotoxic studies.

In Vitro Expansion of TIL

A second portion of the original cell suspension prepared enzymatically from the choroidal melanoma was placed in tissue culture fluid to which recombinant human IL-2, a T-lymphocyte growth factor, was added (1000 units/ml). Initially, these cultures contained $0.93 \times 10^6$ lymphocytes and $1.7 \times 10^6$ melanoma cells. The cultures were examined first microscopically 10 days later, and then 4, 6, and 10 days thereafter. Each time, the number of melanoma cells and the number of lymphocytes was determined microscopically. These results are presented in Figure 4. The pattern of melanoma cell growth in these IL-2-supplemented cultures was different from the previous cultures in which IL-2 was excluded. In the IL-2-containing cultures, the number of tumor cells barely increased during the first 10 days (compared with the rapid increase in tumor cells observed in cultures without IL-2 supplementation). By 14 days after
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Fig. 4. The in vitro expansion of tumor-infiltrating lymphocytes from a choroidal melanoma. A single cell suspension was prepared by enzymatic digestion of the intraocular tumor, of which $2.63 \times 10^6$ total viable cells ($0.93 \times 10^6$ lymphocytes and $1.7 \times 10^6$ tumor cells) were resuspended and incubated in culture medium supplemented with 1000 units/ml of human recombinant IL-2. On days 1, 14, 16, and 20 after the cultures were established, the cells were harvested, the number of lymphocytes and melanoma cells determined, and the cultures reestablished.

culture initiation, the number of melanoma cells in these cultures decreased. Their disappearance from the cultures was dramatic thereafter, and by 20 days, these cultures contained only rare tumor cells. We suspect that the difference in growth potential and survival of melanoma cells in these cultures compared with those not IL-2 supplemented was not related to any direct effect of IL-2 on melanoma cells. Rather, we believe that the progressive loss of tumor cells was related indirectly to the effects of IL-2 on the TIL also present in the cultures. In this regard, we were gratified to determine that the number of lymphocytes increased in the IL-2-supplemented cultures during the first 10 days (Fig. 4). An even more impressive expansion took place over the next 4 days. By day 16 of culture, $25.2 \times 10^6$ lymphocytes were counted, a 25-fold increase compared with the number of lymphocytes originally placed in these cultures. Of particular interest was that during the 16–20-day culture interval, the number of lymphocytes fell slightly, a reduction that paralleled in kind (if not degree) the loss of tumor cells from the same cultures.

It is pertinent that microscopic inspection of these cultures on days 10, 14, and 16 revealed multicle clusters typically composed of a central tumor cell to which one or more lymphocytes was bound (Fig. 5). The appearance of these clusters was similar to that which commonly has been observed in cultures where lymphocyte-mediated cytotoxicity of target cells occurs.23 In these assays, lymphocytes first bind to the tumor target cell, then release lytic factors that lyse the target. Because our cultures contained tumor cell–lymphocyte clusters, we suspected that these results indicated that (1) tumor cells were lost from the cultures because they were being destroyed by cytotoxic lymphocytes and (2) lymphocyte proliferation decreased after 16 days because insufficient tumor cells remained to provide the lymphocytes with antigenic stimulation (this is required for continued mitosis of antigen-specific T-cells). Subsequent experiments verified these suspicions.

Fig. 5. Phase contrast microscopy (original magnification \(\times 200\)) of tumor-infiltrating lymphocytes (arrowhead) after 18 days in cultures supplemented with 1000 units/ml of human recombinant IL-2. The few remaining tumor cells in these cultures typically formed multicle clusters (open arrowhead) consisting of a central tumor cell to which one or more lymphocytes were bound.
Cytotoxic Activity of Cultured TIL

Lymphocytes recovered from IL-2-supplemented cultures (day 18) and melanoma cells recovered from nonsupplemented cultures were used to prepare standard lymphocyte-mediated cytotoxicity assays, using $^{51}$Cr release as an indicator of tumor cell lysis. Two types of melanoma target cells were used in this assay: cells from the patient’s own (autologous) tumor and cells grown from ocular melanomas of four other patients (allogeneic). These results (Fig. 6) show that the IL-2-expanded lymphocytes from patient 203 were highly cytotoxic for the patient’s own tumor cells; a typical dilutional effect was observed as the effector-target cell ratio was reduced from 40:1 to 1:1. By contrast, the culture-expanded lymphocytes from this patient did not lyse melanoma target cells cultured from tumors of other patients, even at the highest effector-target cell ratio. The failure to lyse ocular melanoma target cells did not coincide with the percent of epithelioid or spindle-type cells present in the differing tumors. Patient 202’s target cells were all spindle type, and patient 205’s target cells were all epithelioid type. In addition, the failure to cause $^{51}$Cr release from the allogeneic tumor cells was not a result of any putative resistance on the part of these cells to lysis. The IL-2-expanded lymphocytes from one of the other patients (202) readily lysed that patient’s own tumor target cells (data not shown). Thus, the lymphocytes that were cultured from patient 203’s ocular melanoma with the aid of IL-2 had the capacity to kill the patient’s own tumor cells in an antigenically specific manner. This result indicates that the cytotoxic cells are probably T-cells and that, at the time the tumor was removed, lymphocytes with the potential to kill the tumor cells were present in the tumor-containing eye.

In other tumor culture systems, it was reported that IL-2-expanded TIL often have the capacity to lyse target cells in a nonantigen-specific fashion. Two types of nonspecific cytotoxic lymphocytes were described: NK cells, which are able to lyse K562 target cells, and LAK cells, which are lymphocytes that lyse Mel-30 target cells by a mechanism that does not use the T-cell receptor to bind tumor-specific antigens. To determine whether cells capable of mediating these types of cytotoxicity also were present among the IL-2-expanded TIL, we tested cultured lymphocytes from patient 203 for the ability to lyse K562 cells and Mel-30 cells in a standard 4-hr $^{51}$Cr-release assay. These results (Fig. 7) show that both K562 and Mel-30 target cells were lysed by the tumor-derived cells, indicating that functional NK cells and LAK cells were present among the IL-2-expanded TIL. Thus, at the time of surgical removal, this patient’s tumor contained both precursor T-cells able to kill the patient’s own tumor cells in an antigen-specific manner and lymphocytes capable of developing into nonspecific cytotoxic effector cells.

Characterization of Surface Markers on Cultured TIL

Samples of the lymphocytes expanded in these cultures and tested for cytotoxic activity also underwent flow cytometric analysis to determine expression of pertinent surface markers. By this approach, we hoped to confirm the presence of cytotoxic T- and NK cells and whether other types of leukocytes might be present. For comparison, peripheral blood leukocytes from patient 203 also were studied, as were bone marrow-derived cells present in the initial cell suspension prepared by enzymatic digestion of the choroidal melanoma. These results (Table 1) showed that most (96%) of the IL-2-expanded cells were of bone
Fig. 7. Nonantigen-specific natural killer cells and lymphokine-activated killer cells are present among tumor-infiltrating lymphocytes. TILs from patient #203 were recovered from IL-2-supplemented cultures on day 18 and tested for cytotoxic activity in a standard 4-hr $^{51}$Cr release assay using NK target cells (K562) and LAK target cells (Mel-30).

marrow origin (CD45+); however, only 41% of the cells in the original tumor cell suspension were CD45+. Approximately 60% of the IL-2-expanded cells were CD3+, and most of these cells expressed CD8, the marker of cytotoxic T-cells. A minority of T-cells expressed CD4. Note that the proportion of IL-2-expanded cells with the CD8 marker was significantly greater than that found in the original tumor-derived cell suspension, implying that this population expanded in vitro. It is relevant that a large fraction of the cultured cells had HLA-DR, a molecule that is expressed on activated T-cells. A significant number of the IL-2-expanded cells expressed CD16, a marker of NK cells, indicating that the cultured cells also were enriched for NK cells compared with the original suspension. Both B-lymphocytes and monocytes were minor populations in the original suspension and in the cultured cells. Thus, IL-2 supplementation of cultures established with single-cell suspensions from this ocular melanoma promoted the proliferation and activation of T-cells, especially cytotoxic cells. In addition, cells with NK markers also were increased.

**Discussion**

Based on our results, we drew following conclusions: (1) at the time of tumor extirpation, the eye already contained lymphocytes with the immune potential to destroy the tumor cells; (2) included among these cytotoxic lymphocytes were T-cells with antigen-specific receptors for antigens expressed uniquely on this patient’s tumor; (3) cytotoxic TIL underwent expansion in vitro after exposure of the tumor-derived cell suspension to exogenous IL-2; and (4) tumor cells were grown readily from the ocular tumor in numbers sufficient to use for immunologic studies. In immunologic terms, these results suggest that (1) choroidal melanoma cells can express unique surface an-

**Table 1. Characterization of cell surface markers on tumor-infiltrating lymphocytes**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>PBL</th>
<th>TIL at enucleation</th>
<th>TIL cultured + IL-2</th>
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<tr>
<td>CD 45</td>
<td>Bone marrow</td>
<td>99</td>
<td>41</td>
<td>96</td>
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<tr>
<td>CD 19</td>
<td>B cells</td>
<td>13</td>
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<td>7</td>
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<tr>
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<td>68</td>
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<td>60</td>
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<td>9</td>
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<td>57</td>
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<td>HLA-DR</td>
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* For comparison, three sources of lymphocytes from patient #203 were analyzed: (1) tumor-infiltrating lymphocytes (TIL) harvested from IL-2-supplemented cultures on day 18; (2) cells present in the initial cell suspension prepared by enzymatic digestion of the tumor immediately after enucleation; and (3) peripheral blood leukocytes recovered from the patient at the time of enucleation.

† The percentage of nucleated cells expressing the cell surface markers was determined on a FACScan flow cytometric analyzer. Each sample was subjected to a two-color analysis for FITC-labeled antibodies and propidium iodide, allowing for the simultaneous identification of cell surface markers with the exclusion of non-nucleated acellular debris.
tigen (tumor-specific antigens) that render the cells susceptible to immune-mediated destruction, and (2) at the time of enucleation, when the tumor is contained in the eye and is progressing slowly in size, tumor-specific T-lymphocytes already have infiltrated the eye, implying that the immune system previously perceived the antigens on the ocular tumor and responded by clonal expansion and dissemination of tumor-specific T-cells. At the technical level, our results showed that it was possible to generate both tumor cell lines and lymphocytes from ocular melanomas of individual patients. This accomplishment augurs well for the eventual use of cellular and lymphokine immunotherapy as an approach to this ocular malignancy.

It is interesting and yet perplexing that a slow-growing intraocular melanoma occurred in a patient that had evidence of tumor antigen-specific activation of the immune system. We wondered why this state of immune activation did not proceed to control and destroy the intraocular neoplasm. Although we found T-cells and other cytotoxic effector cells in the tumor with the potential to destroy the tumor cells at the time of enucleation, histologic study of this tumor did not reveal evidence of lymphocytic infiltration or a local inflammatory response. These results indicate that the failure of the immune system to destroy the tumor is not a result of (1) a failure of the immune system to perceive that a neoantigenic malignant tumor has arisen in the eye, (2) an inability of the immune system to generate precursors of tumor-specific cytotoxic cells, or (3) an anatomic barrier (the putative blood-ocular barrier) to the entry of potential effector cells into the eye with the tumor.

Recent studies from several laboratories exploring the interplay between the host immune response and ocular tumors in mice may help to explain these results. When minor histoincompatible tumor allografts are placed into the anterior chamber of the eyes of normal adult mice, progressively growing tumors emerge that (1) evoke a deviant systemic immune response characterized by impaired delayed hypersensitivity and suppressor T-cells, and (2) eventually kill their hosts by direct extraocular extension into the cranial vault. In these ocular tumor-bearing mice, precursor cytotoxic T-cells specific for antigens on the tumor cells are generated in the draining cervical lymph nodes and spleen. Moreover, these cells can be identified readily in the growing ocular tumor mass, yet no tumor rejection occurs. The analogy with our findings from this patient's tumor is striking. In the mouse model, we compared the immune response and fate of intraocular tumors with the immune responses to tumor allografts placed in the subconjunctival space. These latter tumors are rejected routinely, and (not surprisingly) tumor-specific precursor cytotoxic T-cells can be identified in the draining cervical lymph nodes and at the tumor site. However, only at subconjunctival tumor sites can we detect fully functional, terminally differentiated cytotoxic T-cells, T-helper cells capable of secreting IL-2 and IL-4 in response to tumor-associated antigens, and T-cells able to mediate tumor-specific delayed hypersensitivity (manuscript in preparation). Moreover, rejection of subconjunctival tumors begins shortly after lymphokine-secreting T-cells infiltrate the site, at which point functional cytotoxic T-cells finally appear. We interpreted these data to show that tumor rejection results from an interplay of lymphokine-secreting T-helper and precursor cytotoxic T-cells that allows the latter to differentiate into killer cells and mediate tumor regression by direct tumor target cell lysis. Because IL-4-secreting T-helper cells were sought, but not found, in murine intracameral tumors at any time (manuscript submitted) and because we found no evidence of direct cytotoxic T-cells in murine intracameral tumors or delayed hypersensitivity T-cells in the spleen, we believe that the success of the intraocular tumor depends on a failure to deliver appropriate T-cell "help" to precursor cytotoxic T-cells in the eye. We are eager to learn whether there is a similar lack of T-cell "help" among lymphocytes that infiltrate human choroidal melanomas.

Our study is not the first to identify TIL in ocular melanomas. However, the finding that some of the infiltrating cells in a primary ocular tumor are tumor antigen-specific cytotoxic T-cell precursors is new. Studies of TIL from primary tumors, even at nonocular sites, are uncommon. Most of the pertinent literature on TIL has come from studies of metastatic tumors that have arisen from primary tumors in the kidney (renal cell carcinoma), lung (small cell carcinoma), brain (glioblastoma), bladder, ovary, colon, and cutaneous melanomas. Common to all these studies of TIL has been the use of exogenous IL-2 to drive lymphocyte proliferation in culture and the subsequent identification of LAK cell activity in the effector cell populations generated. Much less frequently has it been reported that tumor-specific cytotoxic T-cells are generated under similar culture conditions. To date, the most convincing evidence that tumor-specific cytotoxic T-cells are present among TIL has come from studies of cell suspensions obtained from metastatic cutaneous melanomas. In these studies, tumor-specific lysis of melanoma cells was reported to be mediated by CD3+, CD4+, CD8+ lymphocytes. The cytotoxic activity of these cells was blocked when monoclonal antibodies that are specific for CD3 molecules (intimately associated on the cell surface with the T-cell receptor for antigen) were added to the assay or when antibodies were added that bind to major
histocompatibility complex class I molecules on the surface of the target melanoma cells. Phenotypic analysis of TIL from our patient's tumor confirmed that CD8+ T-cells and NK cells were increased during the culture interval.

Although ocular and subcutaneous skin melanomas share a common cellular origin, the pathobiology of these two malignancies is different. Whereas cutaneous melanomas can grow rapidly, are highly metastatic, and express various melanoma-associated antigens, choroidal melanomas grow slowly, are spatially confined, rarely metastasize until long after the primary tumor has been removed, and poorly express melanoma-associated antigens. Previous results for cutaneous melanoma patients show that tumor-specific activity is present among TIL recovered from large metastatic tumors at an advanced stage of disease. Our results reveal that small primary tumors are immunogenic and induce a tumor-specific immune response, resulting in the infiltration and accumulation of specific lymphocytes at the tumor site. These results indicate that the immune response to spontaneous small primary tumors is initiated early during tumorigenesis.

Other laboratories used animal models to demonstrate that TIL, generated similarly to the method we described, are highly effective in adoptive immunotherapy protocols. Moreover, similar protocols were used to treat metastatic melanomas in humans with encouraging preliminary results. In these protocols, cell suspensions prepared from surgically excised tumor tissue were expanded in culture with exogenous IL-2 and then injected intravenously back into the patient, also in conjunction with IL-2. We consider this approach to the therapy of ocular melanomas to be worth considering. Approximately 50% of patients whose tumor-containing eyes are enucleated have distant metastatic disease within 3–10 years, but usually not within the first 6 months after surgery. Thus, there would be ample time to obtain tumor cell lines and IL-2-expanded cytotoxic TIL from the enucleated specimen. These cells could be frozen and stored until such time as metastatic disease is diagnosed. Depending on the progress made in experimental animal systems, it may turn out that administration of TIL plus lymphokines, even before the emergence of metastases, may prevent the latter from occurring. We consider this a promising area for future research.

Key words: melanoma, cytotoxic T-cells, intraocular tumors, immunology, tumor-infiltrating lymphocytes

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References