T Cell Subsets Involved in the Rejection of Metastases Arising From Intraocular Melanomas in Mice

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The role of cell-mediated immunity in the resistance to spontaneous metastasis of intraocular melanoma was studied in C57BL/6 mice harboring syngeneic B16F10 melanomas. Mice were rendered T cell-deficient by thymectomy and lethal whole-body X-irradiation. Adult thymectomized and bone marrow-restored (ATXBM) mice were selectively reconstituted with immune lymph node cell suspensions that were depleted of specific T cell subsets. The selectively reconstituted hosts were used to evaluate the role of specific T cell subsets in controlling the metastatic spread of intraocular melanomas. The results revealed that T cell-deficient ATXBM mice were highly vulnerable to the metastatic spread of intraocular melanomas. However, this susceptibility could be virtually eliminated by the infusion of either normal or specifically sensitized lymphoid cells. Negative selection experiments demonstrated that the effector cells responsible for protection against metastases resided in a population with the surface phenotype characteristic of cytotoxic T lymphocytes: Thy 1+, Lyt 1+ and Lyt 2+. Invest Ophthalmol Vis Sci 28:1397-1403, 1987

In spite of recent advances in the detection and treatment of primary neoplasms, most deaths in cancer patients are due to metastases. Although refined therapeutic strategies have resulted in a lower mortality rate for patients subjected to enucleation, metastatic melanoma remains to be the leading cause of death in individuals diagnosed with intraocular melanoma. Thus, interruption of the metastatic process should be a key consideration in planning cancer therapy.

Recently there has been a renewed interest in the immunological control of metastases. This is due in large part to promising findings in which cellular immune elements have been activated to an anti-tumor state by the use of biologic response modifiers such as interleukin 2, interferon inducers, and lymphokine activated killer (LAK) cells. The importance of cellular immunotherapeutic modalities is further emphasized by previous studies which have demonstrated that adoptively transferred immune T lymphocytes were able to eliminate disseminated tumors in mice. However, different T cell populations can confer protection against metastases depending on the tumor system under study. For example, in some tumor models, metastases are eliminated by noncytolytic T helper cells while studies with other tumor models indicate that disseminated tumor cells are controlled by Lyt 2+ cytolytic T cells. Thus, different T cell populations and different cell-mediated immune mechanisms can contribute to the control of metastases.

Results from animal studies have shown that metastases derived from intraocular mastocytomas are rapidly eliminated by cytotoxic T lymphocytes (CTL). We have recently reported that intraocular B16 melanomas have a low rate of spontaneous metastasis unless T cell immunity is compromised. The latter findings suggest that T cell-dependent immune mechanisms restrict the development of metastases that arise from intraocular melanomas. One might predict that melanoma metastases are controlled by the same T cell-dependent immune mechanisms that have recently been shown to eliminate metastases derived from intraocular mastocytomas. However, such an assumption may be misleading since these two tumors differ in several fundamental characteristics, including immunogenicity, organ preference for metastases, speed of metastatic spread from the eye, and vulnerability to cell-mediated cytolysis. Each of these characteristics can affect the immune rejection of metastases. Accordingly, it is important to identify the T cell population involved in the rejection of spontaneous metastases produced from primary intraocular melanomas. The present study addresses this problem.
Experimental Animals

Adult female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used as experimental subjects between 3 and 5 months of age. The present investigations conformed to the ARVO Resolution on the Use of Animals in Research. All surgical procedures were performed using ketamine hydrochloride anesthesia.13

Tumor Cells

B16F10 melanoma cells (C57BL/6 origin) were grown in monolayer cultures in Falcon 75 cm² tissue culture flasks (Falcon Plastics, Oxnard, CA) using Dulbecco's modified Eagle's minimum essential medium (MEM, GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), gentamicin (0.05 mg/ml; Schering Corp., Kenilworth, NJ), and vitamin solution (GIBCO). The origin and properties of the B16F10 subline of B16 melanoma have been described in detail previously.1617 B16 melanoma cells were harvested from tissue culture flasks by gentle trypsinization, washed in Hank's balanced salt solution (HBSS), and resuspended in HBSS for the various inoculations. EL-4 lymphoma cells (C57BL/6 origin) were cultured in RPMI 1640 medium (Flow Laboratories, Inc., McLean, VA) containing the same supplements described above with the exception of vitamin solution, which was omitted.

Intracameral Inoculations

A modified quantitative technique for depositing a definite number of tumor cells into the anterior chamber of the mouse eye has been described in detail elsewhere.13 Mice were anesthetized deeply with 0.66 mg of ketamine hydrochloride (Vetalar, Parke, Davis and Co., Detroit, MI) given intramuscularly. B16F10 melanoma cells, at a concentration of 10⁵ cells/5 μl, were injected intracamerally (IC) into panels of C57BL/6 mice. The eyes were examined three times per week with a dissecting microscope (×8).

Enucleation of Tumor-Containing Eyes

Tumor-containing eyes were enucleated 10–12 days after IC tumor inoculation. Mice were anesthetized deeply with ketamine hydrochloride and the tumor-containing eye subjected to traumatic enucleation as previously described.13 Briefly, the globes were squeezed ten times in rapid succession using wide blade forceps. The globes were subjected to the same degree of pressure without visibly rupturing the external surface of the eye. Mice were allowed to rest for 30–60 min and the traumatized tumor-containing eyes were enucleated with sterile curved scissors. Mild hemorrhage, when present, was arrested by gentle electrocautery or tamponade. All mice were observed three times per week and necropsied when morbidity indicated imminent death (usually day 30 post tumor inoculation).

Quantification of Spontaneous Metastases

Mice were killed when moribund and the number of lung tumor colonies was determined by inspection with a dissecting microscope.19 The lung colony assay is a simple and accurate method for evaluating spontaneous metastases of B16F10 melanomas since these tumor cells localize at the lung surface following intravenous inoculation.20 Moreover, B16F10 melanoma cells metastasize selectively to the lungs and form discrete pulmonary tumors following subcutaneous, intramuscular, intravenous, intradermal, or intracameral inoculation.13

Production of T Cell-Deficient Mice (ATXBM)

Adult C57BL/6 mice were anesthetized by intramuscular injection of sodium pentobarbital (1–2 mg/mouse) and thymectomized as previously described.22 Thymectomized mice were placed on acidified drinking water (pH 2.8) followed 2 weeks later by lethal whole body X-irradiation (850 rad, 137-Cesium source). Mice were rescued by intravenous reconstitution with 5 × 10⁶ syngeneic bone marrow cells (BM) depleted of T cells by treatment with monoclonal anti-Thy 1.2 antibody in the presence of complement (see below). Such adult thymectomized bone marrow-reconstituted mice are henceforth referred to as ATXBM mice.

Selective Reconstitution of ATXBM Mice With Specific T Cell Subsets

ATXBM mice were selectively reconstituted with various T cell subsets prior to IC challenge with B16F10 melanoma cells. Lymph node cells (LNC) were harvested from normal or intraocular tumor-bearing (day 10–14 post IC tumor inoculation) C57BL/6 mice. Panels of ATXBM mice received intravenous inocula containing 5 × 10⁶ LNC from either tumor-bearing or normal donors. Prior to adoptive transfer, the LNC suspensions were either untreated or subjected to treatment with various monoclonal antibodies. Lytic monoclonal antibodies directed against Thy 1.2, Lyt 1.2, or Lyt 2.2 were used to selectively deplete specific T cell subsets (see
below). Following in vitro treatment with antibody and complement, the remaining viable cells were injected intravenously. Although the initial LNC suspensions equaled $5 \times 10^7$ cells per recipient, the actual number of cells transferred intravenously varied depending on the specific T cell population depleted by the respective monoclonal antibody treatments.

**Monoclonal Antibodies (Mab)**

Anti-Thy 1.2 Mab (Cedarlane Laboratories, Ontario, Canada) and anti-Lyt 1.2 Mab (New England Nuclear, Boston, MA) were IgG2b isotypes. Mab anti-Lyt 2.2 (New England Nuclear) was an IgM isotype. All Mabs were supplied in ascites fluid and were shown by 5'Chromium-release assay to be cytotoxic to C57BL/6 lymphocytes in the presence of rabbit complement. The percentage of cells depleted from LNC suspensions was well within the expected range for Thy 1+, Lyt 1+,2-, and Lyt 1-,2+ T cells within this organ. All Mabs were used at a 1:1,000 dilution for depletion experiments.

**T Cell Depletions**

T cells or T cell subsets were depleted from LNC or BM suspensions by incubation with Mabs Thy 1.2, Lyt 1.2, or Lyt 2.2 plus complement. Cell suspensions were incubated ($5 \times 10^7$ cells/ml in RPMI, 0.3% bovine serum albumin) with Mabs for 45 min on ice. The cells were washed (200 X G/10 min), resuspended in RPMI, and the T cells were lysed by incubation with rabbit complement (1:10 dilution, Low-Tox-M, Cedarlane Laboratories) for 45 min at 37°C in 5% CO₂ atmosphere. Depletion of T cells or subsets was confirmed by functional assays as described elsewhere.

**Cell-Mediated Cytolysis of B16F10 Melanoma Cells**

Cell-mediated cytotoxicity (CMC) assays were performed using methodology described elsewhere. Briefly, $3 \times 10^9$ spleen cells from either normal or tumor-bearing mice were mixed with $6 \times 10^5$ X-irradiated (13,000 rads) B16F10 melanoma cells. Cells were cultured in 20 ml of RPMI 1640 medium containing 10% FCS, Hepes and $5 \times 10^{-5}$ M 2-mercaptoethanol in 25 cm² Falcon tissue culture flasks standing upright. After 4-day incubation at 37°C in 5% CO₂ atmosphere, the stimulated lymphocytes were washed in HBSS and used as effector cells for in vitro CMC assays. CMC was determined in a 20-hr ⁵¹Cr-release assay. Freshly cultured B16F10 melanoma cells and EL-4 lymphoma cells were washed in HBSS and resuspended in complete RPMI 1640. Target cells ($2 \times 10^7$) were placed into wells of Microtest II plates (Falcon Plasticware) with varying numbers of effector cells. Generally, each effector cell suspension was plated at four different effector-to-target cell ratios ranging from 100:1 to 10:1. The plates were centrifuged at 100 g for 2 min and were then incubated for 20 hr at 37°C. After incubation, the plates were centrifuged for 10 min at 200 g and 100 μl of each supernatant was removed and counted for radioactivity. The cytotoxicity was calculated as follows:

\[
\% \text{ specific release} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100
\]

The spontaneous release of ⁵¹Cr was determined by counting supernatants from culture wells containing only tumor cells and medium. The total ⁵¹Cr-release was determined by counting supernatants after three freezing/thawing cycles.

**Statistical Analysis**

Statistical significance was determined by student t-test.

**Results**

We have previously reported that an intact immune system, particularly T cell-dependent immunity, prevents the development of extensive metastases in intraocular tumor-bearing hosts. The present study was designed to identify the specific T cell subsets involved in the immune resistance to spontaneous metastases arising from intraocular melanomas. The ATXBM mouse served as the indicator system for these studies.

**Metastasis of Intraocular B16 Melanoma in T Cell-Deficient ATXBM Mice**

The first series of experiments was designed to establish the veracity of the ATXBM mouse as a model for the spontaneous metastasis of intraocular B16F10 melanomas. Four basic categories of C57BL/6 hosts were examined for the development of metastases following intracameral transplantation of B16 melanoma cells: (1) thymectomy without whole body X-irradiation and therefore without bone marrow or spleen cell infusion; (2) ATXBM but without LNC; (3) ATXBM with infusion of normal LNC; and (4) ATXBM with infusion of "immune" LNC. Immune LNC were collected from normal, immunocompetent C57BL/6 mice 10–14 days after IC inoculation.
Fig. 1. Metastasis of intraocular B16F10 melanomas in T cell-deficient ATXBM hosts. Adult thymectomized bone marrow reconstituted C57BL/6 mice were prepared as described in Materials and Methods. B16F10 melanoma cells were inoculated IC on day 0 and mice were necropsied when moribund (usually between days 28 and 32). There were 9-19 mice per experimental panel. * = P < 0.001.

of B16F10 melanoma cells. As shown in Figure 1, thymectomy alone did not jeopardize the host’s ability to reject metastases arising from intraocular melanomas. However, ATXBM mice reconstituted with bone marrow but not lymphoid cells (ie, ATXBM—no LNC) developed extensive metastases. In this group, 88% (15/17) of the hosts bore at least one pulmonary tumor focus. However, this vulnerability to metastatic tumor formation could be prevented by reconstituting the T cell-deficient ATXBM hosts with a suspension of either normal or immune LNC suspensions prior to intracameral tumor inoculation. Both panels of ATXBM mice reconstituted with lymphocyte suspensions had greatly reduced numbers of pulmonary metastases. Moreover, the number of hosts that developed metastases was sharply reduced in T cell restored hosts. Only 53% (8/15) of the mice reconstituted with normal lymphocytes had pulmonary metastases. This protective effect was even more striking in ATXBM mice reconstituted with immune lymphocytes. The incidence of metastases in this group was only 10% (2/19).

T Cell Subsets Involved in Elimination of Spontaneous Metastases Arising From Intraocular B16F10 Melanomas

The next experiments identified the T cell subsets involved in the resistance to melanoma metastases. Panels of ATXBM mice were reconstituted with immune lymphoid cell suspensions depleted of specific T cell subsets. In the first experimental panel, LNC suspensions were treated with monoclonal anti-Thy 1.2 antibody in the presence of complement as a means of removing all T cell subsets. This procedure removed approximately 50% of the nucleated cells in the lymphoid cell suspension and was within the expected range for Thy 1+ LNC, as reported by other investigators.23,24 Following antibody treatment and washing, the T cell-depleted suspensions were infused into panels of ATXBM mice. Two weeks later the hosts were challenged intracamerally with B16F10 melanoma and observed for metastases as described earlier. As shown in Figure 2, removal of T cells sharply reduced the protective effect of the lymphoid cell infusions. In this panel, 73% (8/11) of the T cell-depleted hosts developed pulmonary metastases. This was in sharp contrast to a similar experimental panel that received similar immune cell suspensions treated with complement alone. Complement treatment did not affect the immune cells since only 33% (3/9) of these hosts expressed metastatic tumor foci (Fig. 2).

Further experiments were arranged to determine the phenotype of the T cell subset responsible for resistance to melanoma metastases. Immune cell suspensions that were depleted of Lyt 1+ lymphocytes by in vitro treatment with monoclonal anti-Lyt 1.2
antibody and complement, were ineffective in protecting against metastases; 78% of these hosts (7/9) developed an average of 25 metastatic foci.

Since the Lyt 1 surface determinant can be found to varying degrees on virtually all T cells, it was important to determine if the effector cells co-expressed the Lyt 2 antigen which is characteristic of cytotoxic T lymphocytes. ATXBM hosts reconstituted with Lyt 2-depleted immune LNC were particularly vulnerable to metastatic tumor formation. Like the T cell-depleted and Lyt 1-depleted ATXBM mice, Lyt 2-depleted hosts developed extensive metastases. Removal of Lyt 2+ T cells resulted in an average of 36 metastatic tumor foci in 75% (9/12) of the mice.

Intraocular Melanoma-Bearing Mice Develop Cytotoxic T Lymphocytes

The results outlined above strongly suggest that protection against the metastatic spread of intraocular B16F10 melanoma resides in the T cell population that bears the characteristic features of cytotoxic T lymphocytes. The next series of experiments was performed to confirm the functional capacities of these cell suspensions. Lymph node and spleen cell suspensions from intraocular melanoma-bearing C57BL/6 mice were examined for the presence of tumor-specific cytotoxic T lymphocytes (CTL) since these hosts were typical lymphocyte donors for the adoptive transfer experiments reported above. As shown in Table 1, both lymph node cells and spleen cells from intraocular melanoma-bearing mice produced significant lysis of B16F10 melanoma cells. Moreover, it bears noting that the cytolysis detected in this assay was tumor-specific, since third party EL-4 lymphoma cells were not lysed by the effector cells from melanoma-bearing donors.

Discussion

The results reported here provide a number of insights into the immunobiology of metastases arising from intraocular melanomas in mice. As previously reported, immunocompetent hosts are highly resistant to the metastatic spread of intraocular B16F10 melanomas in spite of the progressive growth of the primary tumor. The T cell-dependent immune mechanisms responsible for this resistance was examined prospectively in mice that were selectively depleted of specific T cell subsets. Using this strategy for adoptive transfer of immunity, it was possible to determine that the predominant T cell subset conveying resistance to metastasis expressed the characteristic phenotype of the murine CTL: Thy 1+, Lyt 1+, and Lyt 2+.

Table 1. Anti-B16 melanoma cell-mediated cytotoxicity in lymphoid cell donors

<table>
<thead>
<tr>
<th>Experimental group*</th>
<th>Spleen</th>
<th>Lymph node</th>
</tr>
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<tbody>
<tr>
<td>B16F10 IC (day +10)</td>
<td>24.5 ± 0.6</td>
<td>18.0 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>2.1 ± 0.8</td>
<td>0.0 ± 0</td>
</tr>
<tr>
<td>B16F10 IC (day +14)</td>
<td>26.6 ± 0.7</td>
<td>16.0 ± 3.0</td>
</tr>
<tr>
<td>Control</td>
<td>10.5 ± 0.8</td>
<td>2.0 ± 2.0</td>
</tr>
</tbody>
</table>

* 1 x 10⁵ B16F10 melanoma cells injected intracameral on day 0.
† Cell-mediated cytotoxicity was determined as described in Materials and Methods. The data reported here are from one of several experiments which produced similar results. Effector-to-target cell ratio was 100:1. Lysis of third party target cells (EL-4 lymphoma cells) was less than 10%. P value for both experimental groups (lines 1 and 3) was less than 0.001.

It is not surprising that Lyt 2+ immune cells were involved in the resistance to metastases arising from intraocular melanomas since we have recently reported that metastases derived from intraocular P91 mastocytoma were rapidly eliminated by cytotoxic T lymphocytes. Previous studies with this intraocular melanoma model demonstrated that resistance to metastases was a T cell-dependent, radiosensitive process. Accordingly, one must conclude that either T cell-dependent antibodies or T cell effector systems are involved in the elimination of the metastases. The possibility that tumor-specific antibodies were involved in the rejection of melanoma metastases is unlikely since we have been unable to demonstrate a protective effect when immune serum is passively transferred to intraocular melanoma bearing mice (unpublished data). Likewise, we have been unsuccessful in demonstrating cytolytic antibody in such sera (unpublished data). The capacity of lymphoid cell suspensions to provide protection against spontaneous melanoma metastases is compelling evidence that cytotoxic T lymphocytes are involved in this process. The present findings also demonstrate that such cell suspensions contain lymphoid cells that can lyse B16F10 melanoma cells in an antigen-specific manner in vitro. The same cell suspensions might be expected to also contain T cells capable of transferring tumor-specific DTH activity which in turn might be involved in the elimination of metastasis. However, this is very unlikely since the immune spleen cell donors were sensitized by intracameral injection of B16F10 melanoma cells. Such C57BL/6 hosts are incapable of mounting delayed-type hypersensitivity (DTH) responsiveness against syngeneic B16F10 melanoma cells and contain splenic suppressor T cells that prevent the development of DTH even if recipients of such spleen cells are immunized by a conventional route. One might also raise the possibility that either natural killer (NK) or natural cytotoxic (NC) cellular immune systems were involved in
this protection. This seems unlikely for a number of reasons. First, the protection against metastases is radiosensitive and T cell-dependent while NK and NC cells are radioresistant and T cell-independent. Moreover, the ATXBM hosts reconstituted with bone marrow but not immune lymphocytes were highly vulnerable to metastases yet displayed normal NC and NK function (data not shown). However, the most compelling evidence in favor of CTL as the mediators of immunity lies in the adoptive transfer studies in which the protective effect could be dramatically reduced by the elimination of the Lyt 2+ cell population (ie, the CTL/suppressor T cell subset).

These results present an interesting paradox in which a host bears a progressively growing intraocular melanoma yet is resistant to spontaneous metastases. Why then are the CTL effectors that eliminate metastases unable to cope with the primary intraocular tumor? Our working hypothesis is that the rapid initial growth of the intraocular tumor occurs before the immune system is fully aroused. By the time CTL effector elements are generated, the primary tumor has reached a mass that is beyond the capacity of these effector cells. Thus, a sizeable tumorous mass not only survives but thrives in the presence of potentially hostile cytolytic effector cells. There is experimental precedence to support this hypothesis. For example, we have previously demonstrated a similar condition exists with intraocular P815 mastocytes in BALB/c hosts. Such hosts not only reject spontaneous metastases from the primary intraocular tumor but are fully capable of rapidly rejecting enormous numbers of P815 tumor cells injected subcutaneously. This occurs even though the host is incapable of controlling its primary intraocular tumor. Skin allograft experiments reveal a similar condition. Several investigators have shown that large tissue masses, such as skin allografts, can survive in the face of antigen-specific CTL but will be rejected if the host’s DTH immunity is restored. Therefore, it is feasible that a large foreign tissue mass—either a tumor or an organ allograft—can elicit some categories of specific immune responsiveness yet not succumb to immune rejection. By contrast, small numbers of blood-borne tumor cells or tumor emboli are vulnerable to direct cytolysis by antigen-specific CTL.

The present findings offer encouragement for the development of immunotherapeutic procedures in managing intraocular melanomas. However, caution should be exercised in the interpretation of these results since intraocular B16 melanoma differs significantly from human uveal melanoma in numerous important ways. For example, the murine tumors were transplanted into the anterior segment of the mouse eye and therefore did not originate by in situ transformation within the uveal tract, as occurs in the human counterpart. Although B16F10 melanoma is a weakly antigenic tumor and therefore resembles human melanoma, one cannot be certain that the same immune effector elements function against metastases from human intraocular melanomas. Nonetheless, there is sufficient similarity in the human and murine immune systems to suggest that such mechanisms may function in both species.

Key words: melanoma, spontaneous metastases, immune rejection, T cells

Acknowledgments

The excellent technical assistance of Ms. Elizabeth Mayhew and Ms. Jessamee Mellon is greatly appreciated. The manuscript was carefully prepared by Ms. Sara Perkins.

References


