Role of Helper T-Lymphocytes in Rejection of UV-induced Murine Skin Cancers

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ABSTRACT

The purpose of this study was to examine the role of helper T-lymphocytes (Th) in the immunological rejection of UV-induced tumors. Mice repeatedly exposed to UV radiation develop suppressor T-lymphocytes that facilitate the growth of UV-induced tumors by interfering with host immunity. These suppressor cells specifically blocked the generation of antitumor Th, suggesting that Th may be important in the immunological rejection of UV-induced tumors. The Th activity generated by a UV-induced tumor that grows progressively in normal mice was compared with that generated by a highly antigenic, regressor clone of the same tumor. The regressing tumor cell line generated a much higher amount of Th activity than the parental, regressor tumor cell line. The amount of Th activity generated by a highly antigenic, UV-induced tumor injected into normal mice, in which it regresses, was compared to the Th activity in UV-irradiated mice, in which the tumor grows progressively. Again, tumor regression was associated with a higher amount of Th activity, and this increased activity was detectable in the environment of the regressing tumor. Lyt-1* cells containing Th activity mediated the regression of a UV-induced tumor when injected with the tumor cells s.c. into immunosuppressed mice. Lyt-1* cells were cytotoxic to tumor cells in vitro but were unable to cause tumor rejection in vivo. These studies suggest that Th play a central role in the immunological rejection of UV-induced tumors.

INTRODUCTION

Most skin tumors induced in C3H mice by repeated exposure to UV radiation are highly antigenic and fail to grow when transplanted into normal syngeneic recipients (1). The UV-induced tumors grow progressively when transplanted into immunosuppressed animals and into mice chronically exposed to UV radiation (2). Treatment of mice with UV radiation generates suppressor Ts4 that specifically block immunological rejection of UV-induced tumors (3, 4). The mechanism by which these Ts prevent tumor rejection is poorly understood. Previous work by Thorn (5) demonstrated that the generation of a primary CTL response against UV-induced tumors was not prevented by UV-induced Ts, but the formation of memory CTL was decreased. Recently we found that UV-induced Ts blocked the generation of antitumor Th in vivo (6). This finding suggested that UV-induced Ts may prevent tumor rejection by blocking the production of Th; however, it is not yet clear whether Th play a role in the process of tumor rejection. The inhibition of Th production by Ts could indicate a central role for Th in the generation of antitumor effector cells. Alternatively, the Ts may inhibit many antitumor immune responses, of which only some are involved in tumor rejection.

In this study, we investigated the role of Th in the immunological rejection of UV-induced skin tumors. We found that progressively growing tumors generate much less Th activity than regressor UV-induced tumors, which are immunologically rejected. In addition, the transfer of an enriched population of Th cells from a tumor-immune animal protected UV-irradiated mice from subsequent challenge with a UV-induced tumor. Our results suggest that Th play an essential role in the immunological rejection of UV-induced skin cancers.

MATERIALS AND METHODS

Animals. Specific pathogen-free female C3H/HeNcr (Mtv-1) mice were supplied by Charles River Breeding Laboratories (Wilmington, MA) or by the animal production branch of the Frederick Cancer Research Facility (Frederick, MD). Immunosuppressed mice were produced by adult thymectomy and sublethal (450 R), whole body X-irradiation. Mice were 8 to 10 weeks old at the beginning of each experiment and were given unlimited access to NIH open-formula mouse chow and sterilized water. The animals were housed in a pathogen-free, barrier facility where ambient light was automatically controlled to produce 12-h light, 12-h dark cycles; they were maintained in an American Association for Accreditation of Laboratory Animal Care-accredited facility according to the NIH guide for Laboratory Animal Care.

UV Irradiation. Mice were exposed to UV radiation according to the procedure of Fisher and Kripke (3). The light source was a bank of six FS40 sunlamps (Westinghouse, Bloomfield, NJ). The animals' dorsal hair was removed with electric clippers once a week, and the mice were exposed to UV radiation for 1 h, three times a week, for at least 12 weeks. The incident dose rate was approximately 4 J/m²/s, and about 65% of the energy was emitted at wavelengths within the UV region (280 to 320 nm). None of the animals had developed primary tumors from the UV irradiation at the time of these experiments.

Tumor Cells. The UV-2240 and UV-2237 cell lines were established from fibrosarcomas induced in C3H/HeNcr (Mtv-1) mice by chronic UV irradiation (7). UV-2240 is a regressor tumor cell line, that grows only in UV-irradiated or immunosuppressed mice. UV-2237 grows progressively in both normal and immunosuppressed animals. UV-2237 clone 46 is a highly antigenic clone isolated from the UV-2237 cell line, which will regress when injected into normal syngeneic recipients (8). MCA-113 is a highly antigenic fibrosarcoma induced in a C3H/HeNcr (Mtv-1) mouse by chronic UV irradiation (9). This tumor regresses in both normal and UV-irradiated mice. All cell lines were routinely tested to demonstrate that they remained free of Mycoplasma. In addition, they are free of pathogenic murine viruses (Microbiological Associates, Rockville, MD). Cell lines were cultured in Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY), as previously described (6).

Spleen Cell Preparations. Spleens were removed from mice and single-cell suspensions were prepared. The cells were washed, resuspended in HBSS, and the number of viable cells was determined by trypan blue exclusion. B-Lymphocytes were purified from mice immunized with TNP-conjugated sheep RBC. Spleen cells were exposed to a concentration of 4 × 10⁶/ml in RPMI 1640 (Gibco) containing 2% fetal bovine serum and an equal volume of RPMI 1640 containing anti-Thy-1.2 (Becton Dickinson, Mountain View, CA) was added. The final dilution of Thy-1.2 monoclonal antibody was 1:166. After 30 min on ice, the cells were washed twice and resuspended in RPMI 1640 with 2% fetal bovine serum and a 1:16 dilution of rabbit H2 complement (Pel-Freez, Brown Deer, WI). The cells were incubated for 1 h at 37°C and washed twice before the number of viable cells was counted as above.

To increase the concentration of Th cells, spleen cell preparations were incubated on NW columns by using the method of Julius et al.
The NW-nonadherent cells were washed and used as an enriched source of Th. In some cases, these T-cells were then further purified into Lyt-1+ or Lyt-2+ fractions. Specific subpopulations were depleted by using monoclonal antibodies specific for the murine Lyt-1.1 and Lyt-2.1 antigens (New England Nuclear, Boston, MA). NW-nonadherent cells (4 × 10^6/ml) were mixed with an equal volume of RPMI 1640 (2% FBS) containing the Lyt-1.1 or Lyt-2.1 antibody diluted 1:1000. The cells were incubated on ice and were complement treated as described above for B-cell isolation.

Adoptive Transfer of Spleen Cells. Spleen cell populations were prepared as above and suspended at 10^6 cells/ml in HBSS. Recipients were given injections i.v. of 5 × 10^6 or 10^7 cells and challenged with tumor cells 1 day after reconstitution. For local adoptive transfer studies, purified spleen cells and 3 × 10^6 tumor cells were mixed at the indicated ratios in HBSS, and immediately injected s.c.

Peritoneal Lymphocytes. UV-induced tumors were grown as ascites in order to examine lymphocytes in the tumor environment. Three × 10^6 tumor cells were injected i.p., and at various times thereafter the animals were killed and 5 ml of HBSS was injected i.p. After massage of the abdominal area, the peritoneal fluid was withdrawn with a 22-gauge needle. The cells were spun down and then separated on NW gradient as described for T-cell populations. The NW-nonadherent cells isolated in this manner were 85 to 90% Thy 1.2-, as measured by immunofluorescence.

Helper Cell Assay. The activity of antitumor Th was measured as previously described (6). Briefly, NW-nonadherent cells (10^6) were tested for Th activity by incubating them with TNP-conjugated tumor cells (5 × 10^5) and TNP-specific B-cells (10^6). After 5 days of cocultivation, the number of B-lymphocytes stimulated to produce anti-TNP antibodies was measured in a plaque assay. Each culture was assayed in triplicate, using 8% TNP-conjugated horse RBC, guinea pig complement (Pel-Freeze), and rabbit anti-mouse IgG serum (Cappel, Cochranville, PA). The number of anti-TNP plaque-forming cells is expressed as total (direct plus indirect) plaques per culture (± SD).

Antigenic Similarity. To confirm that we had indeed transferred suppressor cells into these animals, other groups of reconstituted mice were given a s.c. injection of UV-2240 or MCA-113 cells. The UV-2240 cell line regresses in normal mice, but grows progressively in chronically UV-irradiated or immunosuppressed mice (2). As indicated in Table 1, UV-2240 grew progressively only in mice that received spleen cells from the UV-irradiated mice, implying that Ts were present. MCA-113 is a regressor tumor that does not grow in either normal or UV-irradiated mice (9). MCA-113 cells failed to grow progressively in any of the reconstituted animals, again indicating that the Ts specifically inhibit the rejection of UV-induced tumors. Thus, the specificity of the Ts in blocking the generation of Th directed against UV-induced tumors correlates with the specificity of the Ts for tumor rejection in vivo.

Th Generated against Progressor and Regressor Tumors. To investigate the importance of Th cells in the immunological rejection of UV-induced tumors, we compared the Th activity generated against progressor and regressor UV-induced tumors. UV-2237 is a UV-induced fibrosarcoma that grows progressively in syngeneic C3H mice (7). Clone 46, isolated from the UV-2237 cell line, is more antigenic than the parent tumor and regresses in normal mice, but grows progressively in immunosuppressed and UV-irradiated mice (8). The two cell lines are antigenically similar, in that immunization of mice with clone 46 protects against challenge with the parent UV-2237 tumor. C3H mice were given injections of 2 × 10^6 UV-2237 or UV-2237 clone 46 cells, and tested for Th activity at various times during the growth or regression of these tumors (Fig. 1). Five days after tumor transplantation, before palpable tumors appeared, the spleen cells from both groups of mice showed similar Th activity. At all subsequent time points, however, mice given injections of the regressor UV-2237 clone 46 tumor had a 3- to 5-fold higher amount of helper cell activity than mice bearing the progressor tumor. Mice given injections of UV-2237 cells developed palpable tumors (3 to 5 mm in diameter) by day 12, which progressed to 10 to 18 mm in diameter by day 26. No tumors were detected in mice given injections of UV-2237 clone 46 cells.

To examine regressing and progressing tumors in a different situation, we determined the Th activity generated by injecting UV-2240 cells into normal syngeneic mice, in which the tumor regresses, and into UV-irradiated mice, in which it grows progressively.
ROLE OF Th IN UV TUMOR REJECTION

Fig. 1. Antitumor Th activity in regressing and progressing tumors. On day 0, C3H mice were given injections s.c. of 2 x 10^5 cells of the progresser UV-2237 (B) or regressor UV-2237 clone 46 (4) tumor cell line. On the indicated day, 3 mice given injections of each tumor cell line were killed and their NW-nonadherent spleen cells were tested for Th activity. Cultures consisted of 10^5 TNP-primed B-cells, 5 x 10^5 TNP tumor cells, and 10^6 NW-nonadherent cells recovered from the peritoneal cavity.

Th activity was measured on day 5 of culture and is expressed as the total number of plaque-forming units (direct plus indirect) per culture; bars, SD.

| Tumor* | Days after tumor cell injection | PFC/Culture(|g|) |
|--------|--------------------------------|------------------|
| UV-2240| 4                              | 404 ± 71         |
|        | 8                              | 355 ± 31         |
|        | 11                             | 250 ± 7          |
| MCA-113| 8                              | 504 ± 40         |

* Mice were given an i.p. injection of 3 x 10^6 viable tumor cells suspended in HBSS.
| PFC, plaque-forming cells. | Anti-TNP PFC/Culture (± S.D.) of 10^5 TNP-primed B cells, 5 x 10^5 TNP-conjugated tumor cells, and 10^6 NW-nonadherent cells recovered from the peritoneal cavity.
| Five-month-old C3H mice that had been UV-irradiated for 1 h 3 times a week for 12 weeks (UV) or unirradiated controls (NR). Each time point represents the mean PFC from 3 mice.
| P ≤ 0.003 compared to PFC in NR animals using a two-sample t test.

Adoptive Transfer of Th. Our results demonstrated that Th are associated with tumor rejection, but they did not address the role Th cells may play in the rejection process. For this reason, we examined the ability of Th cells to prevent tumor growth in a local adoptive transfer assay. ATX mice, which are unable to reject UV-2240, were given injections of tumor cells mixed with various populations of lymphocytes isolated from normal or tumor-immune animals (Table 3). When 5 x 10^6 T-cells from UV-2240-immune mice were mixed with 2 x 10^6 tumor cells, the tumors failed to grow. In contrast, the UV-2240 cells grew progressively in mice given injections of the same number of tumor cells admixed with normal spleen cells. The rejection of the UV-2240 tumor was specific, because T-cells from mice immune to UV-2240 did not affect the growth of the unrelated MCA-113 tumor cell line. Some nonspecific protection against regressor tumors may be detected if a sufficiently large number of nonimmune spleen cells are transferred to the immunosuppressed recipients, as evidenced by the failure of tumors to grow in two of five mice given 10^7 normal spleen cells.

In this experiment, we transferred all T-cells that were nylon wool-nonadherent. The protective effects we observed may therefore not have been caused by the Th population alone. The phenotype of the UV tumor-specific Th cells is Thy-1.2^+ LYT-1.1^+ LYT-2^+ and L3T4a^+ (data not shown). To determine the phenotype of the T-lymphocytes that are important for tumor rejection, NW-nonadherent cells were purified from UV-2240-immune normal mice and separated into Lyt-1.1^+ and Lyt-2.1^- populations by depletion, using monoclonal antibodies and complement. The T-cell subpopulations were then injected i.v. into chronically UV-irradiated mice. The depleted subpopulations were also tested for CTL or Th activity in vitro to determine the effectiveness of the depletion procedure by means of functional assays. The reconstituted mice were challenged with 3 x 10^6 tumor cells 1 day after the lymphocyte transfers (Table 4). UV-irradiated mice that received no T-cells were susceptible to challenge with UV-2240, whereas UV-irradiated mice that received Lyt-2.1^- T-cells from tumor-immune animals were protected. UV-treated mice that received Lyt-2.1^- cells from normal mice, or Lyt-1.1^- cells from immune animals, remained susceptible to tumor challenge. The Lyt-2.1^- cells did not protect against a challenge with the unrelated MCA-113 tumor. Therefore, Lyt-1^+ Lyt-2^- spleen cells from immune mice were able to mediate immunologically specific rejection of the UV-2230 tumor, even in the presence of Ts. This population of lymphocytes exhibited Th activity, but not CTL activity, against UV-2240 cells, whereas the Lyt-1^- Lyt-2^- cells were cytotoxic, but ex-

Table 3 Growth of tumors after local adoptive transfer of T-lymphocytes

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Tumor incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2240</td>
<td>5/5</td>
</tr>
<tr>
<td>UV-2240/10^5 immune T-cells</td>
<td>0/5</td>
</tr>
<tr>
<td>UV-2240/5 x 10^5 immune T-cells</td>
<td>0/5</td>
</tr>
<tr>
<td>UV-2240/10^6 normal T-cells</td>
<td>3/5</td>
</tr>
<tr>
<td>UV-2240/5 x 10^6 normal T-cells</td>
<td>5/5</td>
</tr>
<tr>
<td>MCA-113/10^5 immune T-cells</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* ATX animals were given a s.c. injection of 2 x 10^6 tumor cells mixed with the indicated number of spleen cells in HBSS.

Number of mice with progressively growing tumor 6 weeks after tumor inoculation/number of mice given injections.

Table 4 Lyt phenotype of cells mediating rejection of tumors of UV-irradiated mice

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Splenic T-cell donors</th>
<th>Monoclonal antibody treatment</th>
<th>Tumor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>None</td>
<td>UV-2240</td>
<td>4/4</td>
</tr>
<tr>
<td>UV</td>
<td>Normal</td>
<td>Anti-Lyt-2</td>
<td>UV-2240</td>
</tr>
<tr>
<td>UV</td>
<td>Immune</td>
<td>Anti-Lyt-2</td>
<td>UV-2240</td>
</tr>
<tr>
<td>UV</td>
<td>Immune</td>
<td>Anti-Lyt-1</td>
<td>UV-2240</td>
</tr>
<tr>
<td>NR</td>
<td>None</td>
<td>Anti-Lyt-2</td>
<td>UV-2240</td>
</tr>
<tr>
<td>ATX</td>
<td>Immune</td>
<td>Anti-Lyt-2</td>
<td>MCA-113</td>
</tr>
</tbody>
</table>

* Normal C3H mice (NR), C3H mice exposed to UV irradiation (UV) for 60 min 3 times a week, or adult thymectomized, 450-R X-irradiated mice (ATX). 

Spleen cells were isolated from normal C3H or C3H mice that had been immunized 3 times with 2 x 10^6 UV-2240 cells i.p., at weekly intervals. Immune spleen cells were isolated 1 week after the third immunization, and 5 x 10^5 Lyt-1-depleted or Lyt-2-depleted T-cells were injected i.v. in 0.5 ml of HBSS.

Recipients were given injections of 3 x 10^6 UV-2240 or MCA-113 cells 1 day after spleen cell transfer. Tumor cells were suspended in HBSS and 0.3 ml was injected s.c. on the right flank.

Number of mice with progressively growing tumors 6 weeks after injection/number of mice given injections.
hibited no Th activity (Table 5). These experiments demonstrate that depletion of the appropriate subpopulations was achieved following monoclonal antibody and complement treatment.

**DISCUSSION**

The central role played by Th cells in the generation of antibodies is well established (11). However, the participation of these cells in antitumor immunity has been difficult to analyze because no appropriate assay has been available to measure their activity. The development of an indirect assay for antitumor Th cells (12) made it possible to begin to address questions of whether these cells are generated during immunization with various types of tumors (12), and what kind of antigenic specificity antitumor Th cells express (6). We are continuing this line of investigation by attempting to assess the relevance of these cells to tumor rejection in vivo. Because these Th do not have any known, unique surface markers and because they are detected indirectly, it is not possible to isolate the cells and test their antitumor activity directly. Therefore, we approached this question by asking whether tumor regression correlated in a variety of instances with the activity of Th cells.

We found that progressively growing tumors induced less Th activity in the spleen than tumors that were rejected. Furthermore, more Th activity was present locally, in the vicinity of the tumor, during tumor regression in normal mice than was observed during the growth of the same tumor in UV-irradiated mice. This finding is consistent with the results of Lill and Fortner (13), who transplanted fragments of UV-induced tumors s.c. into normal and UV-irradiated mice. They found that three times as many T-cells could be recovered from tumors regressing in the normal mice than from tumors growing progressively in UV-irradiated mice.

In a local adoptive transfer experiment we demonstrated that NW-purified T-lymphocytes from tumor-immunized mice, which contain antitumor Th cells, were able to inhibit directly the growth of the immunizing tumor in vivo. Further separation of these cells into Lyt-1- and Lyt-2- subpopulations demonstrated that the Th activity and the ability to cause tumor rejection in vivo resided in the Lyt-1-2- population, which was devoid of CTL activity. We are, of course, unable to rule out the possibility that another Lyt-1-2- cell present in the population, but lacking Th activity, is responsible for tumor regression in vivo. However, the correlations between Th activity and tumor regression and the known functions of Th in other immunological systems make Th the most reasonable candidate for being the cell that initiates tumor rejection in vivo.

The identity of the cells that actually mediate destruction of tumor cells in vivo remains a controversial question in tumor immunology. In several studies investigators have found Lyt-1-2- cells to be active in the rejection of other types of tumors (14–16). Results from other laboratories suggest, however, that Lyt-2- cytotoxic T-cells mediate the rejection of tumor cells in vivo (17, 18), and one group demonstrated that both Lyt-1- and Lyt-2- cells are necessary for the rejection of chemically induced tumors (19). In the UV-induced tumor system, it is not yet clear whether the Th actually serve as the progenitors of effector cells, as the effector cells themselves, or as cells that recruit the participation of other types of lymphoid cells. Nonetheless, these studies provide strong circumstantial evidence for an essential role of Th in the rejection of UV-induced tumors.

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**REFERENCES**

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