Interleukin 2 Activation of Cytotoxic T-Lymphocytes Infiltrating into Human Metastatic Melanomas

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ABSTRACT

Tumor infiltrating lymphocytes (TIL) were isolated from 22 tumors obtained from 15 patients with metastatic melanoma. In 18 of the 22 tumors, a substantial number of lymphocytes was isolated with an average lymphoid cell:tumor cell ratio of 1.26. The TIL were predominantly cytotoxic/suppressor T-lymphocytes with an average of 67% Leu4, 61% Leu2a, and 18% Leu3a cells. There were less than 2% natural killer cells, B-cells, or macrophages. An average of 3.8% (range, <0.1 to 8.6%) of freshly isolated TIL bound to autologous tumor cells. Prior to culture, none of the tumor-binding cells (TBC) was cytotoxic as judged by trypan blue exclusion. The frequency of TBC increased to 11.6% after 2 days of culture, and 10% of these TBC developed cytotoxic activity. When interleukin 2 was added to cultures, the frequency of TBC increased, and the frequency of cytotoxic TBC was 2-fold higher compared to control cultures. After 10 days of culture with interleukin 2, TIL increased in number with a concomitant disappearance of TBC, whereas there were severe decreases of lymphocytes and no decrease of tumor cells in control cultures. TIL were cultured for 8 to 10 days with recombinant interleukin 2 and tested for cytotoxicity against autologous and allogenic tumor cells and K562 targets in a 4-h ⁵¹Cr release assay. rIL2-cultured TIL from all nine patients tested exhibited the highest levels of lysis against autologous tumor cells. Of the nine TIL samples, five exhibited an apparent specificity for autologous melanoma, while four specimens killed both allogenic and autologous melanoma. The ability of TIL to kill K562 targets appeared to parallel the ability to kill allogenic targets. For comparison, recombinant interleukin 2-cultured peripheral blood mononuclear cells from the same patients were assayed for cytotoxic activity against autologous and allogenic melanomas. Unlike some TIL, none of the peripheral blood mononuclear cells exhibited specificity for autologous tumor cells. In summary, TIL isolated from metastatic melanoma patients were predominantly cytotoxic T-lymphocytes with the ability to recognize and kill autologous tumor cells after in vitro culture; interleukin 2 induced proliferation of TIL and augmented their cytotoxic activity such that they eliminated autologous tumor cells.

INTRODUCTION

Many human tumors contain an infiltration of lymphocytes. A more favorable prognosis is associated with a greater magnitude of lymphocyte infiltration in some histological tumor types, suggesting that tumor infiltration is a manifestation of immunological recognition of the tumor cells by the host (1–6). A few studies have examined the functional properties and the antigenic expression of different subpopulations of TIL.³ Results to date suggest that TIL from human tumors are primarily T-cells, although minor proportions of other lymphocytes and macrophages are sometimes present (1, 7–13). Most studies have reported that TIL have little or no cytotoxic activity against autologous tumor cells and only a low level of NK cell function (8, 9, 14–17). A few studies have demonstrated that TIL in colorectal tumors have cytotoxicity against autologous tumors but not K562, the standard NK cell target (18–20). TIL generally do not proliferate when they are cultured with autologous tumor cells (9, 21). However, Vose and coworkers reported that TIL can suppress the mitogen and tumor-induced proliferative responses of autologous blood lymphocytes, although the suppression could be reversed by the addition of interleukin 2 (8, 22).

IL2 is known to induce the differentiation of cytotoxic lymphocytes, including NK cells, LAK cells, and CTL, and to support their growth (23–28). Leu11a blood lymphocytes, a subpopulation of granular lymphocytes with high NK activity, will respond to rIL2 and then develop into LAK cells cytotoxic for fresh solid tumor cells (25). When cultured with IL2, peripheral blood lymphocytes of both cancer patients and healthy donors exhibit LAK activity against autologous and allogenic solid tumor cells (29–32).

The present study was designed to characterize certain immunological features of TIL from surgically excised metastatic melanoma and to determine the effect of IL2 on these lymphocytes. These tumor suspensions yielded a considerable number of lymphocytes which were predominantly T-lymphocytes with a cytotoxic/suppressor phenotype. Some of these TIL had the ability to bind autologous tumor cells but were not highly cytotoxic. rIL2 induced proliferation of TIL and enhanced their cytotoxic activity to the extent that they eliminated tumor cells during short-term cultures.

MATERIALS AND METHODS

Preparation of Cells. Single cell suspensions of TIL and melanoma tumor cells were prepared from 22 different surgical specimens of 15 patients with metastatic melanoma. Pathology reports confirmed the diagnosis of metastatic melanoma. None of the patients had received chemotherapy or radiation therapy prior to surgery. The surgical specimens were minced with scissors into a Petri dish (100-mm dish; Corning 25020; Corning Glassworks, Corning, NY) containing 25 ml of Dulbecco’s phosphate-buffered saline (pH 7.2) with collagenase (2 mg/ml, type V-S; Sigma), hyaluronidase (10 units/ml, type V1-S, Sigma), and DNase I (0.4 mg/ml; Sigma). After a 40-min incubation at room temperature with gentle mechanical stirring, the cells were harvested over a Ficoll-Hypaque gradient as described (25, 31). Cell viability exceeded 80%. Lymphoid cells could easily be discriminated from tumor cells based upon cell size, morphology, and the brownish cytoplasmic pigmentaion of the melanoma cells. Monoclonal antibodies (described below) specific for human lymphocytes were used to confirm the identity of lymphocytes. Tumor cells in medium supplemented with 50% fetal calf serum and 10% dimethyl sulfoxide were stored at −80°C until used. Blood and serum samples were obtained from the same patients. PBMC were isolated on Ficoll-Hypaque gradients (25). In certain experiments, TIL were separated from tumor cells by discontinuous Percoll gradient centrifugation and a cell sorter
cellsuspensions of labeledmelanoma cells were passed through acotton
(Amersham Corp., Arlington Heights, IL) for 90 mm at 37°C for K562
cr2b0 conjugated Leu15 were provided by the Becton Dickinson
previously (35). Target cells were labeled with 200 @Ci of Na231CrO4
...gamma counter. The spontaneous release did not exceed 25% of max
...supernatant were harvested, and the radioactivity was measured in a
...plates. The plates were incubated for 4 h at 37°C, and then 100 @sl of
...added at a concentration of 5 x 10' targets/well in triplicate to various
...analysis, TIL consisted of 75 to 99% Leu4* T-cells (average, 87 ± 7%), 18 ±13% Leu3a* T-helper/inducer
cells, and 61 ± 17% Leu2a* T-cytotoxic/suppressor cells (Fig. 1).
...there were few NK cells (Leu7* and Leu11a*), B-cells

RESULTS

Analysis of TIL. In 18 of 22 tumors, a substantial proportion
of lymphocytes was present in tumor cell suspensions, while
the other 4 tumors contained a small minority of lymphoid
cells (i.e., less than 5% of the cell suspension) (Table 1). The
ratio of lymphoid cells to tumor cells for all 22 tumors ranged
from <0.05 to 5.90, with an average ratio of 1.26 ± 1.72. By
fluorescence analysis, TIL consisted of 75 to 99% Leu4* T-cells
...and the frequency of TBC to autologous tumor cells were enumer

Table 1 TIL and TBC isolated from metastatic melanoma patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Ratio of lymphoid cells to tumor cells</th>
<th>% of TBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>F</td>
<td>&lt;0.05</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>M</td>
<td>3.31</td>
<td>8.4</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>M</td>
<td>0.90</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>M</td>
<td>0.51</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>F</td>
<td>0.14</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
<td>M</td>
<td>1.80</td>
<td>2.0</td>
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<td>7</td>
<td>45</td>
<td>M</td>
<td>0.20</td>
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<tr>
<td>8</td>
<td>58</td>
<td>M</td>
<td>&lt;0.05</td>
<td>&lt;0.1</td>
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<tr>
<td>9</td>
<td>57</td>
<td>M</td>
<td>2.0</td>
<td>4.5</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>M</td>
<td>0.53</td>
<td>2.5</td>
</tr>
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<td>11</td>
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<td>&lt;0.05</td>
<td>&lt;0.1</td>
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<td>12</td>
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<td>M</td>
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<td>M</td>
<td>0.67</td>
<td>5.3</td>
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<td>14</td>
<td>23</td>
<td>M</td>
<td>0.10</td>
<td>8.6</td>
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<tr>
<td>15</td>
<td>53</td>
<td>F</td>
<td>0.10</td>
<td>3.3</td>
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</table>

* Mean ± SD (n = 22).

...of lymphocytes was calculated as described (33). The proliferative response to rIL2 was measured by culturing 10

INTERLEUKIN 2 ACTIVATION OF TUMOR-INFILTRATING LYMPHOCYTES

(25, 33). Cell suspensions of TIL and tumor cells were washed twice and resuspended at a concentration of 1 to 5 x 10^6 lymphocytes/ml in RPMI 1640 medium supplemented with 10% AHS and various concentrations of rIL2 and incubated in an 8% CO_2 incubator at 37°C. PBMC at a concentration of 1 x 10^6 cells/ml were incubated under the same culture conditions. Half the volume of culture medium was exchanged every 5 days during the culture period. The rIL2 was obtained from Biogen Research Corporation (Boston, MA) and was greater than 99% pure as previously described (25, 29). The specific activity was 2.3 x 10^4 units/mg protein. The rIL2 was titrated by measuring the proliferation of IL2-dependent NK8 closed cells (23) and compared to the reference IL2 supplied by the Biological Resources Branch of the National Cancer Institute (Frederick, MD). Based on this assay, one unit of Biogen rIL2 was equivalent to approximately two standard IL2 units.

Assays of TBC and Cytotoxic TBC. The percentages of both TIL binding to autologous melanoma cells (TBC) and cytotoxic TBC of these tumors were enumerated simultaneously under a microscope. Single cell suspensions of freshly isolated TIL with tumor cells were washed twice and resuspended at a concentration of 5 x 10^6 lymphocytes/ml in RPMI 1640 medium plus 10% AHS. Tumor infiltrating lymphocytes and tumor cells cultured for up to 3 days in the presence and absence of rIL2 were washed 2 times and resuspended in medium. These cell suspensions were mixed with an equivalent volume of 0.4% trypan blue solution (GIBCO, Grand Island, NY) and pipeted gently 8 to 10 times with a Pasteur pipet to avoid nonspecific binding as described previously (33, 34). The frequency of TBC was determined by counting 300 lymphocytes and scoring for binding to tumor cells. Melanoma tumor cells were easily distinguished from lymphocytes based upon cell sizes, morphology, and brownish pigmentation. Simultaneously, TIL binding and killing of autologous tumor cells (cytotoxic TBC) were assessed using a trypan blue dye exclusion assay. The frequency of cytotoxic TBC was determined by counting 100 TBC and scoring tumor cells for viability. To test whether the binding structure was sensitive to trypsin, cells were treated for 30 min at room temperature with 0.1% trypsin, cells were treated for 30 min at room temperature with 0.1% trypsin (type V; Sigma) in phosphate-buffered saline. After washing, the frequency of TBC was determined.

Analysis of Lymphocyte Surface Markers. FITC-conjugated Leu series of monoclonal antibodies (Leu2a, 3a, 4, 10, and 11a) and phycoerythrin-conjugated Leu15 were provided by the Becton Dickinson Company, Mountain View, CA. FITC-conjugated Leu7 was produced in our laboratory (35). The OKM1 antibody was purchased from Ortho Diagnostic System, Inc., Raritan, NJ. FITC-conjugated goat anti-human IgG F(ab')2 and FITC-conjugated anti-mouse IgG were provided by Southern Biotechnology Associates, Inc., Birmingham, AL. Cells were incubated at 4°C for 30 min with 10 μl of 100 μg of FITC- or phycoerythrin-antibody per ml, or OKM1-antibody followed by FITC-conjugated anti-mouse IgG for 30 min. After washing twice, lymphocytes and TBC reacting with fluorochrome-conjugated antibodies were enumerated in wet viable cell preparations under a fluorescence microscope. Tumor cells from the 21 specimens were not stained by these antibodies with the exception of one tumor cell suspension (patient 4), which was positive for the Leu10 antigen.

^{51}Cr Release Assays and Proliferative Response to rIL2. The target cells in ^{51}Cr release assays were either noncultured melanoma cells or K562 tumor cells. Frozen aliquots of noncultured melanoma cells were thawed the morning of the assay. K562 cells were prepared as described previously (35). Target cells were labeled with 200 μCi of Na_2^{51}CrO_4 (Amersham Corp., Arlington Heights, IL) for 90 min at 37°C for K562 or room temperature for noncultured melanoma. After washing twice, cell suspensions of labeled melanoma cells were passed through a cotton column to remove cell aggregates and debris. The target cells were added at a concentration of 5 x 10^3 targets/well in triplicate to various numbers of effector lymphocytes in 96-well round-bottomed microtiter plates. The plates were incubated for 4 h at 37°C, and then 100 μl of supernatant were harvested, and the radioactivity was measured in a gamma counter. The spontaneous release did not exceed 25% of maximal release. The percentage of specific lysis was calculated as described (33). The proliferative response to rIL2 was measured by culturing 10^6 lymphocytes/well in flat-bottomed microtiter plates with and without rIL2. The cultures were pulsed 18 h before harvesting with tritiated thymidine (0.5 μCi/well; New England Nuclear). ^{3}H]Thymidine uptake was determined in triplicate samples by β-scintillation counting.

Fig. 1. Cell surface antigen expression of TIL and TBC. Single cell suspensions were prepared from 22 different metastatic tumors removed from 15 melanoma patients. The antigen expression of lymphocytes and of TBC was enumerated by fluorescence microscopy. Columns, mean of all 22 specimens; bars, SD. Stg, surface immunoglobulin.

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(surface immunoglobulin<sup>+</sup>), macrophages (OKM<sup>+</sup>), or DC antigen-positive cells (Leu10<sup>+</sup>).

In the 18 freshly isolated tumor cell suspensions with substantial numbers of lymphocytes, between 2 and 8% (average, 3.9 ± 1.9%) of the total TIL bound to autologous tumor cells (Table 1). Cell surface marker analysis of TBC showed that 82 ± 12% were Leu4<sup>+</sup> cells, 10 ± 9% were Leu3a<sup>+</sup>, and 68 ± 13% were Leu2a<sup>+</sup> T-cells (Fig. 1). There were few NK cells, B-cells, and macrophages. The tumor-cell binding could be abrogated by pretreating cells with 0.1% trypsin (data not shown).

Blood lymphocytes and lymph node cells were subjected to enzyme treatment as a control to determine whether the enzymes used for tumor disaggregation altered lymphocyte surface antigen expression. The frequency of lymphocytes positive for the Leu series of monoclonal antibodies in a fluorescence analysis was the same before and after treatment with collagenase, DNase, and hyaluronidase, indicating that these antigens were not sensitive to the enzyme treatment (data not shown).

Activation of TIL by rIL2. Single cell suspensions from 10 tumors were adjusted to 1 x 10<sup>6</sup> lymphocytes/well and cultured for 3 days in medium supplemented with 10% AHS only or with 20 units of rIL2 per ml and then tested for their proliferative response by measuring [<sup>3</sup>H]thymidine uptake (Fig. 2). In control cultures lacking rIL2, cells from only 4 of the 10 tumor cell suspensions exhibited [<sup>3</sup>H]thymidine uptake above 10<sup>3</sup> cpm. In contrast, TIL from all 10 specimens had substantial levels of proliferation when cultured with rIL2, and the [<sup>3</sup>H]thymidine uptakes were significantly higher than control cultures (Fig. 2; P = 0.0002).

The frequency of TBC and cytotoxic TBC among TIL was enumerated at serial intervals during a 3-day period in cultures containing 10% AHS alone or with 20 units of rIL2 per ml (Fig. 3). In 7 single cell suspensions from tumors, the mean frequency of TBC increased from 3.8 to 11.6 and 11.9% after 1 day of culture in the control and rIL2 cultures, respectively, and to 17.0 and 20.4% after 2 days of culture. After 3 days of incubation, the frequency increased to 25.8% in rIL2 cultures, while it remained constant at 17% in control cultures. Cytotoxic TBC, as determined by trypan blue dye exclusion, were first apparent after 2 days of culture. The mean frequency of cytotoxic TBC in rIL2 cultures was 27% after 2 days and 29% after 3 days of culture. These levels were approximately 2 times higher than those in control cultures (P = 0.02 at 2 days and P = 0.05 at 3 days).

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Elimination of Tumor Cells by rIL2-cultured TIL. Single cell suspensions of 7 surgical specimens were incubated for 3 days in medium supplemented with 10% AHS alone or with rIL2 (20 units/ml). At daily intervals, aliquots were harvested and washed once, and the percentages of TBC and cytotoxic TBC (as determined by trypan blue exclusion) were enumerated. Points and columns, mean from the 7 surgical specimens; bars, SD. The levels of cytotoxic TBC in rIL2 cultures were significantly higher than those in control cultures (P = 0.02 at 2 days and P = 0.05 at 3 days of culture, paired t test).

![Fig. 3. Time course analysis of TBC and cytotoxic cells.](image-url)
cells were completely eliminated, while the number of lymphocytes in all 6 cases actually increased from an average of 3.0 x 10^5 cells/ml to 2.73 x 10^6 cells/ml. In the cultures with the lower dose of rIL2 (20 units/ml), the number of lymphocytes increased on the average from 3.0 x 10^5 cells/ml to 1.35 x 10^6 cells/ml, and the number of tumor cells decreased from 6.0 x 10^5 cells/ml to 2.0 x 10^6 cells/ml.

Morphology of rIL2-cultured TBC. The morphology of rIL2-cultured TBC was studied at both the light and electron microscopic levels (Fig. 5). Lymphocytes which bound to the tumor cell were readily distinguishable from the melanoma because of their smaller size (Fig. 5A). These lymphocytes had a high cytoplasmic:nucler ratio with a reniform nucleus, numerous cytoplasmic organelles, and irregular nuclear contours, characteristics which are not typical of small resting lymphocytes (Fig. 5B). Some TIL binding to autologous tumor cells contained azurophilic granules (34, 36), whereas some did not.

We also analyzed the antigen expression of TIL and TBC after 6 to 8 days of culture in medium supplemented with 10% AHS and rIL2 (20 units/ml). There were no significant changes of antigen expression of either TIL or TBC from 7 different patients between freshly isolated TIL (Fig. 1) and those after culture with IL2 (data not shown).

Cytotoxicity Measured by ^51^Cr Release Assays. We analyzed rIL2-induced cytotoxicity of autologous tumor cells by TIL in ^51^Cr release assays as compared to trypan blue exclusion assays. Single cell suspensions from 2 surgical specimens (ratio of lymphoid cells to tumor cells, 0.10) were incubated with rIL2 (100 units/ml) for up to 20 days, and the lysis of ^51^Cr-labeled autologous tumor cells was assessed at 5, 10, and 20 days. In contrast to trypan blue exclusion assays, rIL2-induced cytotoxicity of autologous tumor cells as measured by ^51^Cr release assays was not detectable for up to 5 days in culture (Table 2). Significant lysis was apparent after 10 days of culture with rIL2 and increased with longer incubation periods.

Specificity of Cytotoxicity. TIL from surgical specimens and PBMC from these patients were cultured for 8 to 10 days in medium supplemented with 10% AHS and rIL2 (20 units/ml) and then assayed for cytotoxicity against autologous melanoma and a panel of allogenic melanomas (Table 3). TIL from all 9 patients exhibited the highest levels of cytotoxicity against autologous tumor cells. Six of 9 TIL samples did not demonstrate any significant cytotoxicity against allogenic tumor cells (less than 10% specific lysis). The other three TIL preparations demonstrated substantial levels of cytotoxicity.

<table>
<thead>
<tr>
<th>Table 2 rIL2-induced TIL cytotoxicity of autologous tumor cells as measured in ^51^Cr release assays</th>
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<tbody>
<tr>
<td>Patient</td>
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<td>---------</td>
</tr>
<tr>
<td>14</td>
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<table>
<thead>
<tr>
<th>Table 3 A comparison of rIL2-induced cytotoxicity of autologous and allogenic melanomas by TIL and PBMC</th>
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<tbody>
<tr>
<td>Effector cells from</td>
</tr>
<tr>
<td>Patient</td>
</tr>
<tr>
<td>TIL</td>
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</table>

| PBMC     | 4 | 13° | 11 | 19 | 14 | 16 | -° | -° | -° | -° |
|          | 5 | 40 | 13° | 10 | -° | 15 | -° | -° | -° | -° |
|          | 6 | -° | 8 | 10° | 13 | 11 | -° | -° | -° | -° |
|          | 7 | 19 | 21 | 11° | 15 | 18 | -° | -° | -° | -° |
|          | 9 | 29 | 19 | 18 | -° | 26° | -° | -° | -° | -° |
|          | 12 | 18 | 23 | 12 | -° | -° | 25° | -° | -° | -° |
|          | 13 | 16 | -° | -° | 18 | 35 | 47° | -° | -° | -° |
|          | 14 | -° | -° | -° | 32 | 40 | 26° | -° | -° | -° |

*Autologous tumor and lymphocyte combinations. The specific lysis at effector/target ratios of 10/1 and 5/1 was also evaluated and yielded similar results (data not shown). The levels of cytotoxicity of rIL2-cultured TIL against autologous targets were significantly higher than those against allogenic targets (P = 0.0001, 2-way analysis of variance).  
-°, not tested.
against some allogenic tumor cells, although the level of cytotoxicity was less than half the level when the same TIL were incubated with autologous tumor targets. For comparison, PBMC from the same 8 patients were also cultured with rIL2. All of rIL2-cultured PBMC demonstrated substantial levels of cytotoxicity against both autologous and allogenic melanoma tumors (Table 3).

We also tested the cytotoxicity of rIL2-cultured TIL and PBMC of 5 patients against the NK cell target K562 (Table 4). Cytotoxicity against autologous and allogenic tumor targets was measured simultaneously. All PBMC tested were able to kill autologous and allogenic melanoma tumor targets as well as K562 cells. In contrast, 3 of the 5 TIL did not kill K562 or autologous tumors but had significant cytotoxicity against autologous tumor targets. Two TIL samples killed all three types of targets. Thus in these 5 TIL samples, the ability to kill K562 cells appeared to parallel the ability to kill autologous targets.

To investigate whether there was a presensitization of TIL in vivo or the TIL were sensitized by coculture in vitro with tumor cells, freshly isolated TIL were separated from tumor cells by Percoll discontinuous gradient centrifugation and a cell sorter (Table 5). These purified TIL and PBMC from the same patient were incubated for 8 days in medium with 10% AHS alone or with 20 units of rIL2 per ml. In cultures without rIL2, sorted Leu2a* TIL exhibited a modest level of autologous tumor lysis, but other effectors did not exhibit significant lysis (less than 10% lysis). In rIL2 cultures, TIL purified by Percoll and sorted Leu2a* TIL exhibited comparable levels of autologous tumor lysis and proliferative responses. These levels were higher than those achieved by unseparated TIL and PBMC cultured with rIL2.

**DISCUSSION**

TIL harvested from metastatic melanoma had the following characteristics: (a) the majority of TIL were T C/S (i.e., Leu2*Leu4*); (b) some freshly isolated TIL bound to autologous tumor cells and exhibited cytotoxicity against the tumor cells after culture; (c) rIL2 induced TIL to proliferate and augmented their level of cytotoxic activity such that they eliminated autologous tumor cells during short-term cultures; and (d) rIL2-cultured TIL from some tumors appeared to have autologous tumor specificity only, while others killed allogenic tumors and K562 cells as well.

Other investigators have reported that TIL are predominantly T-cells as we observed in this study (1, 7–20). Compared to several of these reports, we found a higher frequency of T C/S lymphocytes than T H/L lymphocytes (Leu3*). Furthermore, the frequency of T C/S lymphocytes increased slightly or remained the same for up to 6 days of rIL2 culture. Within the T C/S population, those cells which express the C3 receptor (as defined by Leu15 or OKM1 antigen expression) are mostly suppressor cells, while those lacking this receptor are cytotoxic precursor and effector T-lymphocytes for Class I histocompatibility antigens (37–39). Interestingly, only a few TIL expressed the C3 receptor (OKM1); thus the vast majority of the cytotoxic/suppressor TIL had the cytotoxic cell phenotype.

Two studies on TIL from lung carcinomas and other tumor types reported that TIL did not differ significantly from blood lymphocytes in the proportion of cells with cytotoxic/suppressor, helper/inducer, or NK cell phenotypes (16, 17). Blood lymphocytes comprise an average of 8 to 10% NK cells (40), 28% cytotoxic/suppressor cells, and 43% helper/inducer cells (41). Our results differed from the previous reports in that we observed very few TIL with NK cell markers (<2%), 18% helper/inducer cells, and 61% cytotoxic/suppressor cells. The discrepancy may be due to differences in the tumor types studied (i.e., melanoma versus lung cancer and other types of neoplasms).

A small proportion of freshly isolated TIL was able to bind but not kill autologous tumor cells. The frequency of TBC increased dramatically after 1 to 2 days of culture even without any added stimulants (i.e., culture medium and autologous serum only); many of the TBC also became cytotoxic as determined by a trypan blue dye exclusion test. Addition of rIL2 to the culture induced proliferative response by TIL and increased the numbers of TBC and cytotoxic TBC. Both the proliferative response and the frequencies of TBC and cytotoxic TBC were increased and dependent upon the dose of rIL2 added (data not shown).

The frequency of TBC and cytotoxic TBC measured in trypan blue exclusion assays probably does not reflect the actual cytotoxic potential of TIL. These assays only enumerate the number of lymphocytes binding tumor cells and the number binding dead tumor cells at fixed time points. Thus, these assays do not provide a measure of the percentage of specific lysis versus.
Table 5 Lysis of autologous tumor cells by TIL separated from tumor cells

<table>
<thead>
<tr>
<th>Effector lymphocytes</th>
<th>Incubation with rIL2 (20 units/ml)</th>
<th>Proliferative response (cpm)</th>
<th>% of lysis of autologous tumor cells (effector/target)</th>
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<td>Unseparated TIL</td>
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<td>Purified TIL (by Percoll)</td>
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<td>Leu2a+ TIL</td>
<td></td>
<td></td>
<td>10</td>
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<tr>
<td>PBMC</td>
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take into account the recycling ability of cytotoxic lymphocytes nor the possibility that lymphocytes are binding tumor cells killed by other effector cells.

The rIL2-induced TIL cytotoxicity of autologous tumor cells was also measured in 51Cr release assays. While cytotoxic TIL were evident after 2 days of rIL2 culture in trypan blue exclusion assays, they were not detectable in 51Cr release assays until after 5 days of rIL2 culture. The lack of cytotoxic activity in 51Cr release assays at earlier time points may be due to cold-target inhibition of 51Cr-labeled target cells by unlabeled tumor cells remaining in the rIL2 cultures. Other experiments had shown that, at 10 days, most of the original tumor cells had been eliminated from culture (Fig. 4), while at 5 days or earlier, enough of these unlabeled tumor cells may have remained to compete with the added 51Cr-labeled tumor cells for effector cell binding.

The cytotoxic activity of rIL2-activated TIL from some patients appeared to be specific for autologous tumor cells. Other TIL showed broader target cell specificities by killing K562 and allogenic melanoma. However, in all 9 cases studied, the level of cytotoxicity against autologous targets was significantly higher than against allogenic targets (P < 0.001; Table 3). The basis for the nonspecific cytotoxicity of some TIL is unknown but may be analogous to reports that some cloned CTL kill a wide range of target cells (42–44). Alternatively, some TIL may be CTL with autologous tumor-specific activity, while others may acquire the ability to lyse a wide range of tumor targets during incubation in vitro.

While these data suggested that tumor-specific CTL were detectable in the TIL of some tumors, it was unclear whether sensitization had occurred in vivo or in the mixed lymphocyte tumor cultures in vitro. To address this issue, we analyzed the effect of rIL2 on TIL in the absence of autologous tumor cells. When TIL were purified from tumor cells by Percoll gradients prior to rIL2 culture, the proliferative response and cytotoxic activity induced by rIL2 were comparable to those of unseparated TIL (Table 5). Furthermore, sorted Leu2a+ TIL exhibited a modest level of autologous tumor lysis in culture without rIL2, and rIL2 also increased their cytotoxic activity. These data indicate that TIL have the innate ability to recognize and kill autologous tumor cells and that rIL2 will stimulate this activity.

In conclusion, these results indicated that TIL from metastatic melanoma were primarily cytotoxic T-lymphocytes which could recognize and kill autologous tumor cells after in vitro culture. Interleukin 2 induced these cells to proliferate and enhanced their cytotoxic activity such that they eliminated autologous tumor cells during short-term culture. The ability of IL2 to activate TIL in vitro is a promising finding for future immunotherapeutic trials with interleukin 2.

ACKNOWLEDGMENTS

We are grateful to Dr. Carlo Grossi for the electron and photomicrographs. We also thank Sharon Garrison and Kathy Walker for editorial assistance.

REFERENCES

INTERLEUKIN 2 ACTIVATION OF TUMOR-INfiltrATING LYMPHOCYTES


Interleukin 2 Activation of Cytotoxic T-Lymphocytes Infiltrating into Human Metastatic Melanomas

Kyogo Itoh, Arabella B. Tilden and Charles M. Balch


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