Immunomagnetic separation of infiltrating T lymphocytes from brain tumors

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Tumor-infiltrating lymphocytes (TIL's) were isolated from human glioma biopsy specimens by immunomagnetic separation using T cell-specific monoclonal antibodies coupled to paramagnetic beads, and were expanded in culture with feeder cells and interleukin-2 (IL-2). The infiltrating cells from five of seven patients proliferated in culture. When tested after 2 to 3 weeks of culture, virtually all of the cells stained with antibodies against the CD2 and CD3 antigens. Most cells also expressed human leukocyte antigen class II molecules, while varying percentages of cells stained with antibodies against the IL-2 receptor and the CD4 and CD8 antigens. The cytotoxicity of the cultured TIL's against autologous and allogeneic glioma cells and the K562 and Daudi cell lines was measured and compared with that of lymphokine-activated killer (LAK) cells from the same patients. None of the TIL's showed significant cytotoxicity against these targets, whereas LAK cells lysed all of the targets.

KEY WORDS • brain neoplasm • glioma • tumor-infiltrating lymphocyte • immunomagnetic separation

The disappointing results of surgery, radiation therapy, and chemotherapy in the treatment of patients with malignant glioma have motivated the search for other treatment modalities. Immunotherapy has for many years been an attractive candidate, since it potentially has the ability of discriminating between malignant and normal tissue. The present availability of recombinant interleukin-2 (IL-2) in large quantities has prompted the experimental and clinical evaluation of adoptive immunotherapy.\textsuperscript{11,23,26,28-29} Recently, experiments from several laboratories have demonstrated the ability of IL-2-cultured peripheral blood lymphocytes to lyse both autologous and allogeneic tumor cells in vitro.\textsuperscript{2,6,12,19,32} Techniques have also been described for expanding in vitro the subpopulations of lymphocytes that infiltrate resected tumors.\textsuperscript{1,14,24} These IL-2-activated cells appear to be much more specific and cytotoxic than IL-2-activated cells derived from peripheral blood.\textsuperscript{30}

The purpose of this study was to examine the cytotoxic capability of T cells separated from resected glioblastomas after activation in vitro with IL-2. The technique of immunomagnetic separation\textsuperscript{5} was used to isolate specific subpopulations of cells, in this case cells bearing CD2 and CD3 determinants, or the T cell receptor \( \alpha \)-\( \beta \) heterodimer. Our results indicate that these T cells are not cytotoxic to transformed cell lines normally lysed by lymphokine-activated killer (LAK) cells, nor are they cytotoxic to autologous or allogeneic glioma cells.

Materials and Methods

Biopsy Specimens

Biopsy specimens were obtained from seven patients with glioma. All of the patients included in this study underwent operative debulking and had received no prior irradiation or chemotherapy. They were all receiving steroids (dexamethasone, 9 to 16 mg daily) at the time of evaluation. Clinical data are shown in Table 1.

Isolation of Peripheral Blood Mononuclear Cells and Glioma Cells

Peripheral blood mononuclear cells were isolated from defibrinated blood by Ficoll-metrizoate (Lymphoprep) flotation.\textsuperscript{*} The glioma specimens were taken during surgery, avoiding the most necrotic areas of the tumor. The tissue pieces were washed three times in a Petri dish to remove contaminating blood. Thereafter, the tissue was minced with a scalpel and suspended in

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* Lymphoprep obtained from Nyco, Oslo, Norway.
Immunomagnetic separation of glioma-infiltrating lymphocytes

TABLE 1
Summary of clinical data in seven patients with glioma

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Symptoms</th>
<th>Tumor Location</th>
<th>Histology</th>
<th>Days Dose (mg)</th>
<th>Steroid Therapy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42, F</td>
<td>seizures, headache</td>
<td>rt temporal</td>
<td>glioblastoma</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>33, F</td>
<td>seizures, headache</td>
<td>rt parieto-occipital</td>
<td>astrocytoma</td>
<td>&gt; 4</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>38, M</td>
<td>seizures</td>
<td>rt frontal</td>
<td>astrocytoma</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>62, M</td>
<td>dysphasia, right hemiparesis</td>
<td>lt temporoparietal</td>
<td>glioblastoma</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>61, M</td>
<td>seizures</td>
<td>rt parieto-occipital</td>
<td>glioblastoma</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>61, M</td>
<td>seizures, lt hemiparesis</td>
<td>lt frontal</td>
<td>glioblastoma</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>44, F</td>
<td>seizures, headache, dysphasia</td>
<td>lt temporal</td>
<td>anaplastic glioma</td>
<td>&gt; 3</td>
<td>16</td>
</tr>
</tbody>
</table>

* Duration of treatment with dexamethasone preoperatively; average daily dose during this period.

a solution of 1 mg/ml Type I A collagenase and 0.2 mg/ml Type II deoxyribonuclease (DNAse) in RPMI 1640 medium. This suspension was forced through a steel mesh, leaving the most fibrous tissue on the mesh. To further disaggregate the cells, the suspension was vigorously agitated with a Pasteur pipette for 5 minutes. Contaminating erythrocytes were removed by Percoll centrifugation as follows: two parts of tumor cell suspension were mixed with three parts of stock isotonic Percoll† and centrifuged at 2600 rpm (700 G at the top of the tube). The cells that floated to the surface were collected and washed. Infiltrating lymphocytes were isolated by immunomagnetic separation as described below. The remainder of the tumor cell suspension was frozen for use as target cells in the cytotoxicity assay.

Immunomagnetic Separation of Tumor-Infiltrating Lymphocytes

The procedure for isolating tumor-infiltrating lymphocytes (TIL’s) is summarized in Fig. 1. Infiltrating lymphocytes were isolated by immunomagnetic separation using Dynabeads M-450 coated with the T lymphocyte-specific monoclonal antibodies IOT 3, BMA0110, or BMA031.‡ These antibodies recognize the CD3 antigen, the CD2 antigen, and the T cell receptor α-β heterodimer, respectively. The beads were coated with monoclonal antibodies using a two-layer technique as described by Funderud, et al., with sheep anti-mouse immunoglobulin (IgG) antibodies in the first layer and monoclonal antibody in the second layer.

The tumor cells were suspended in 5 ml of RPMI 1640 medium supplemented with 20% human serum and were mixed with beads at a ratio of two to 10 cells per bead (see Table 2). This mixture was incubated for 30 minutes at 0°C under continuous gentle mixing. The T lymphocytes that had formed rosettes with the beads were isolated using a magnet and were washed three times to remove contaminating tumor cells. Thereafter, they were placed in culture into 24-well tissue culture plates in 2 to 6 ml of RPMI 1640 medium supplemented with 10% human serum and 50 U/ml of recombinant IL-2 (ALA-125),§ together with 0.5 to 1 x 10^6 irradiated autologous peripheral blood mononuclear cells as feeder cells. The cultures were maintained for 2 to 3 weeks in medium containing IL-2, splitting the wells and changing medium when required. On Days 8 to 12, new feeder cells (0.5 to 1 x 10^6 per well) were added.

Flow Cytometry

Flow cytometric analysis of cultured TIL’s was performed by means of indirect immunofluorescence and a Cytofluorograph‖ as described previously. The following antibodies were used in this study: OKT4A, OKT8, OKT3, and OKT11A, which recognize the antigens CD4, CD8, CD3, and CD2, respectively; the

† Percoll obtained from Pharmacia, Uppsala, Sweden.
‡ Dynabeads M-450 manufactured by Dynal A.S., Oslo, Norway; monoclonal antibody IOT 3 obtained from Immunootech, Marseille, France; and BMA0110 and BMA031 obtained from Behringwerke AG, Marburg, West Germany.
§ Recombinant interleukin-2, ALA-125, obtained from AMGen Biologicals, Amersham, United Kingdom.
‖ Cytofluorograph, Model 50-H, manufactured by Ortho Instruments, Westwood, Massachusetts.
### TABLE 2

Yield of tumor cells and tumor-infiltrating lymphocytes in specimens from seven patients with glioma

<table>
<thead>
<tr>
<th>Case No.</th>
<th>No. of Cells in Specimen/gm Tumor Tissue</th>
<th>Bead Specificity For Separation of T Cells From Tumor Cells</th>
<th>Ratio of Beads:Total Cells</th>
<th>No. of T Cells/gm Tumor Tissue</th>
<th>No. of T Cells Culture Period (days)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>$160 \times 10^6$</td>
<td>T cell receptor $\alpha$-$\beta$</td>
<td>0.1</td>
<td>54,000</td>
<td>no growth</td>
</tr>
<tr>
<td>2</td>
<td>$3.8 \times 10^6$</td>
<td>T cell receptor $\alpha$-$\beta$</td>
<td>0.2</td>
<td>6700</td>
<td>$12 \times 10^6$</td>
</tr>
<tr>
<td>3</td>
<td>$7.1 \times 10^6$</td>
<td>T cell receptor $\alpha$-$\beta$</td>
<td>0.2</td>
<td>24,000</td>
<td>$72 \times 10^6$</td>
</tr>
<tr>
<td>4</td>
<td>$8.5 \times 10^6$</td>
<td>T cell receptor $\alpha$-$\beta$</td>
<td>0.2</td>
<td>4100</td>
<td>$35 \times 10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$4.7 \times 10^6$</td>
<td>T cell receptor $\alpha$-$\beta$</td>
<td>0.2</td>
<td>1500</td>
<td>$78 \times 10^6$</td>
</tr>
<tr>
<td>6</td>
<td>$27.5 \times 10^6$</td>
<td>CD2 &amp; CD3</td>
<td>0.5 each</td>
<td>488,000</td>
<td>$22 \times 10^6$</td>
</tr>
<tr>
<td>7</td>
<td>$12.4 \times 10^6$</td>
<td>CD2 &amp; CD3</td>
<td>0.5 each</td>
<td>53,000</td>
<td>no growth</td>
</tr>
</tbody>
</table>

OKT4a antibody, which reacts with human leukocyte antigen (HLA) class II molecules; the anti-Leu-11b antibody, which recognizes the CD16 antigen (Fc receptor for IgG); and the anti-Tac antibody, which reacts with the 55-kD chain of the IL-2 receptor (CD25).* Fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG and IgM was used as the second-layer antibody.†

#### Preparation of LAK Cells

The LAK cells were generated by culturing peripheral blood mononuclear cells for 5 days at $1 \times 10^6$/ml in RPMI 1640 medium supplemented with 10% human serum and 50 U/ml of recombinant IL-2 in upright Falcon 3013 flasks. Half of the medium was replaced with fresh medium supplemented with IL-2 on Days 2 and 4.

#### Cytotoxicity Assay

The cytotoxicity of the TIL's and LAK cells from glioma patients against fresh cryopreserved autologous and allogeneic glioblastoma cells, K562 erythroleukemia cells, and Daudi (Burkitt's lymphoma) cells was measured. Whereas K562 cells are susceptible to lysis by natural killer (NK) cells, Daudi cells are not. The cell lines were kept in culture in RPMI 1640 medium supplemented with 10% fetal calf serum and were subcultured three times weekly. Eighteen hours before the assay, $4 \times 10^6$ target cells were incubated in 4 ml of medium supplemented with 20% human serum and 5 $\times 10^6$ Bq Na$_2$CrO$_4$. On the morning of the assay the target cells were washed three times at 0°C, and the effector cells were washed once. Both were resuspended in medium supplemented with 20% human serum. Two thousand labeled target cells in 100 $\mu$l medium were added to varying numbers of effector cells ($160,000, 40,000, 10,000, and 2500 \ldots all in 100 $\mu$l medium) in round-bottomed 96-well microtiter plates for a final volume of 200 $\mu$l/well. The plates were incubated for 4 hours and harvested with the Skatron supernatant harvesting system‡ as described previously. The percentage of specific chromium release was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100,$$

based on the mean counts per minute (cpm) of triplicate wells for the experimental release and the mean cpm of 12 replicates for the spontaneous and maximal release.

#### Results

**Yield of Tumor-Infiltrating Lymphocytes**

T lymphocytes were isolated from gliomas by immunomagnetic separation using beads coated with T cell-specific monoclonal antibodies. In specimens from Cases 1 to 5, beads coated with antibodies against the T cell receptor $\alpha$-$\beta$ heterodimer were used at a bead-to-cell ratio of 1:5 (1:10 in Case 1). In specimens from Cases 6 and 7, a mixture of anti-CD2- and anti-CD3-specific beads was used, each at a bead-to-cell ratio of 1:2. The yield of TIL's is shown in Table 2. The cells were cultured in medium containing IL-2 for 2 to 3 weeks, until they reached sufficient numbers to allow analysis of surface antigens and cytotoxicity. The TIL's failed to grow in culture of specimens from two of the patients (Cases 1 and 7).

**Surface Markers**

The surface antigenic profile of the cultured TIL's was studied using indirect immunofluorescence and flow cytometry. The results in specimens from Cases 2 to 6 are given in Fig. 2. Virtually all of the cells stained with antibodies against the CD2 and CD3 antigens. Most cells were also positive for HLA class II. There was considerable heterogeneity between the specimens from these five patients in the reactivity of cultured TIL's with anti-CD4 and anti-CD8 antibodies. The reactivity with antibodies against the IL-2 receptor Tac-
Immunomagnetic separation of glioma-infiltrating lymphocytes

peptide (CD25) varied also, and to some extent correlated with the reactivity with anti-CD4 antibodies. Only a minor fraction of the cells (2% to 7%) stained with anti-CD16 antibodies (Fc receptor for IgG).

Cytotoxicity

The cytotoxic capacity of LAK cells and cultured TIL's from these same five patients is compared in Fig. 3. This graph demonstrates the mean of the cytotoxicity of effector cells from all five patients against K562, Daudi, allogeneic glioma, and autologous glioma cells. In Fig. 3c, TIL cytotoxicity against allogeneic glioma cells is shown for only three of the five patients (Cases 2, 3, and 6). None of the specimens from these patients showed significant cytotoxicity against any of the targets in the TIL cultures, whereas the LAK cells lysed all of the targets.

Discussion

The purpose of this study was to determine if T lymphocytes infiltrating brain tumors could be separated, enriched, and expanded in vitro using the immunomagnetic separation technique. Additionally, the cytotoxic capability of these T cells following IL-2 activation and expansion was tested against cultured cell lines as well as autologous and allogeneic glioma cell targets obtained from fresh biopsies.

From all of the seven tumor biopsy specimens tested, it proved possible to isolate cells bearing either the T cell receptor or the CD2 and CD3 antigen complexes. However, cells from two of these biopsies failed to grow in culture. The number of T lymphocytes obtained varied greatly from 1500 to 488,000/gm of tissue, corresponding to 0.03% to 1.8% of the total number of cells. The variation in the number of T cells present was much less (12 x 10⁶ to 78 x 10⁶) after 2 to 3 weeks in culture with IL-2 and feeder cells. All of the cell cultures underwent significant proliferation. The T cells isolated using immunomagnetic separation were relatively free from tumor cells. This would allow for maximum proliferation since tumor cells have been shown to produce immunoinhibitory factors like prostaglandin E₂ and other glioma-specific suppressor factors. Additionally, the combined presence of IL-2 and particle-bound antibodies against the T cell receptor/CD3 complex forms a potent mitogenic signal.

After 2 to 3 weeks in culture, all of the cells expressed CD2 and CD3 antigens, identifying them as T cells. They also demonstrated HLA class II molecules, indicating activation of the T cells. Very few of the cells demonstrated the CD16 antigen, which is associated with cytotoxic IL-2-activated NK-like cells capable of lysing both NK-sensitive and NK-resistant hematopoietic tumor cell targets.

The results shown in Fig. 3 demonstrate that none of our T cell cultures were cytotoxic against any of the tumor cell targets used. On the other hand, in all of the patients tested in this study it was possible to generate LAK cells from the peripheral blood lymphocytes which were cytotoxic against the same target cell panel, consisting of cell lines and autologous and allogeneic glioma cells. The selection criteria in these experiments were the presence of either the T cell receptor αβ heterodimer or the CD2 or CD3 complex. This should therefore exclude the possible generation of LAK cells, since the precursors to these cells are generally considered to be CD3-negative.

The lack of cytotoxic cells in these cultures is most probably not related to the immunomagnetic separation technique since LAK cells exposed to magnetic beads coated with anti-CD8 monoclonal antibody maintain their cytotoxicity after separation.

FIG. 2. Reactivity of cultured tumor-infiltrating lymphocytes in specimens from five glioma patients (Cases 2 to 6) with antibodies against various T cell antigens and human leukocyte antigen (HLA) class II molecules. Each patient is represented by a different symbol.

FIG. 3. Cytotoxicity of lymphokine-activated killer cells (triangles) and cultured tumor-infiltrating lymphocytes (TIL's, squares) against K562 cells (a), Daudi cells (b), allogeneic glioma cells (c), and autologous glioma cells (d). Values are the mean cytotoxicity of effector cells from five patients, except in c, which demonstrates TIL cytotoxicity against allogeneic glioma cells in specimens from Cases 2, 3, and 6 only. Vertical bars represent standard error of the mean.
more, mixed lymphocyte culture-activated peripheral blood lymphocytes retain their cytotoxicity against allogeneic T cell blasts in cell-mediated lympholysis after immunomagnetic separation.18

In contrast to the results presented here, Miescher and coworkers11 have recently reported the generation of cytotoxic tumor-infiltrating T cell clones from glioma patients. However, these authors were working with cloned cell lines. It is conceivable that, in the populations of T cells that we examined, there could exist subpopulations of cytotoxic cells which could later be cloned as cytotoxic T cell lines. If this subpopulation was small, it would be below the level of detection in our cytotoxic assay. This argument is strengthened by the fact that Miescher and coworkers found a very small number of clones that were cytotoxic toward autologous glioma targets.

The effects of possible contamination from peripheral blood lymphocytes among the infiltrating populations have not been considered either in our study or in studies published previously by others. Recent publications have demonstrated that it is possible to generate cytotoxic T lymphocytes from the peripheral blood of glioma patients by first culturing them with autologous tumor cells.13,22 Therefore, cytotoxic T cells produced from putative TIL's could in fact be derived from the contaminating peripheral blood that is always present in resected biopsy specimens. Additionally, a small subset of CD3-T lymphocytes co-expressing the Leu-19 antigen has previously been reported. This subpopulation has been shown to demonstrate non-HLA-restricted cytotoxicity toward a number of target cell types, including some normally considered to be resistant to NK cells.10,27

One of the main purposes of the experiments reported here was to determine whether the T cells infiltrating into gliomas were sensitized toward the glioma cells in the tumor itself (that is, in situ). If this were the case the only signal considered necessary for further expansion of the cytotoxic cells was the addition of IL-2. Therefore, our negative results seem to indicate that few, if any, stimulated cytotoxic T cells can be found in gliomas. Since it is possible to generate cytotoxic T cells from peripheral blood in the presence of autologous tumor cells, it seems that tumor-infiltrating T cells are not a particularly good candidate when considering eventual immune therapy. It might be better to search for putative cytotoxic infiltrating cells among more NK-like cell types. The immunomagnetic separation method is well suited for this type of study since it allows the controlled separation of small subpopulations of cells from the heterogeneous cell populations found in tumor biopsy specimens. The application of this technique to other monoclonal specificities is the object for further studies.

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