Apoptotic elimination of peripheral T lymphocytes in patients with primary intracranial tumors

LORRI A. MORFORD, PH.D., AMY R. DIX, B.A., WILLIAM H. BROOKS, M.D., AND THOMAS L. ROSZMAN, PH.D.

Department of Microbiology and Immunology, University of Kentucky Medical Center, Lexington, Kentucky

Object. Patients with gliomas exhibit severe T lymphopenia during the course of the disease. This study was conducted to determine the mechanism(s) responsible for the lymphopenia.

Methods. Using two-color fluorescent staining techniques, the authors show that significant numbers of T cells undergo apoptosis in the peripheral blood of patients with gliomas. To determine whether a glioma-derived factor(s) induces this apoptosis, rosette-purified T cells obtained from healthy donors were treated with glioma cell culture supernatant (GCCS) and examined for apoptosis. It is demonstrated that treatment of normal T cells with GCCS induced apoptosis only with concurrent stimulation of the T-cell receptor/CD3 complex. The addition of neutralizing antibodies to interleukin (IL)-10, IL-4, transforming growth factor-α, or tumor necrosis factor–β (lymphotoxin) did not rescue these T cells from apoptosis. Experiments were also conducted in which the degree of monocyte involvement in the induction of T-cell apoptosis was explored. The U937 cells were pretreated for 20 hours with a 1:20 dilution of GCCS. After the removal of GCCS, the U937 cells were cultured in transwell assays with stimulated T cells. Although control U937 cells did not induce apoptosis of the activated T cells, GCCS-pretreated U937 cells induced appreciable apoptosis in normal, stimulated T-cell cultures.

Conclusions. These data indicate that one mechanism by which gliomas cause immunosuppressive effects is the induction of monocytes to release soluble factors that promote activated T-cell apoptosis. The loss of activated T cells leads to T lymphopenia and contributes to the deficiencies in cell-mediated immunity that have been observed during testing of glioma patients’ immune function.

KEY WORDS • T lymphocyte • apoptosis • glioma • monocyte • cytokine

Glioblastomas account for approximately one third of all diagnosed brain tumors. Clinically, patients with glioma are not cachectic, and the glioma tumors themselves rarely metastasize beyond the confines of the brain. Patients with gliomas exhibit a broad range of immunological defects such as cutaneous anergy, lymphopenia, depressed immunoglobulin production, and impaired T-cell responsiveness. 5,9,21-23,31,32,34,42,43,45,57 Accordingly, we have demonstrated that T cells obtained from patients with gliomas exhibit defective delayed-type hypersensitivity responses to common recall skin test antigens and neoantigens.7 Impaired in vitro proliferation of CD3+ T cells, particularly the CD4+ subset, have been observed during testing of glioma patients’ immune function.

Although the T-cell unresponsiveness observed in patients with glioma has been well established, few explanations for the dramatic loss of CD3+ cells, particularly the CD4+ subset, have been proposed. We have demonstrated that the CD4+ T-cell numbers are reduced in these patients to a greater extent than CD8+ T cells, shifting the CD4/CD8 ratio in the peripheral blood from the normal ratio of 2:1 to almost 1:1.45 Previously, the mechanism for this lymphopenia was not known. In this study, we demonstrate that there is an increase in the number of apoptotic T cells in the peripheral blood of patients with gliomas when compared with peripheral blood T cells obtained from control volunteers. Extending the hypothesis that gliomas secrete factors with broad immunosuppressive capability, we show that the glioma cell culture supernatant (GCCS) induces apoptosis in normal activated human T cells similar to that observed in the freshly isolated T cells from patients with glioma. Furthermore, GCCS-pretreated U937 monocytes release soluble mediators that induce apoptosis in normal donor T cells. Neutralizing antibodies to IL-10, IL-4, transforming growth factor–β (TGFβ), or tumor necrosis factor–β (TNFβ1), also called lymphotoxin, alone or in combination, did not protect these cells from apoptosis. Overall, these data indicate that the induction of CD3+ T-cell apoptosis is one explanation for lymphopenia observed in these patients. To our knowledge,
these results offer the first explanation for the CD3⁺ lymphopenia observed in the peripheral blood of patients with gliomas.

**Clinical Material and Methods**

**Patient Population**

Peripheral blood was obtained from six patients shortly after tumor diagnosis but prior to surgical resection. Patients were not receiving steroid or anticonvulsant medications before blood collection. Also, none of the patients included in this study received radiation therapy or chemotherapy before blood collection. Furthermore, the patients examined in this study did not exhibit additional metastases or other comorbidities at the time of blood collection. The patients examined ranged in age from 53 to 71 years (median age 63 years). Blood obtained from healthy medical center employees was used as a control.

**Cell Isolation and Media Preparation**

Peripheral blood lymphocytes were isolated from heparinized venous blood by Ficoll-Hypaque centrifugation, and T-cell populations were obtained by rosetting as described. Complete media consisted of RPMI 1640 supplemented with 1% nonessential amino acids, minimal essential medium–vitamin solution, sodium pyruvate, penicillin/streptomycin, 10 or 12.5 mM HEPES buffer, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum.

**Cell Line Maintenance**

The SNB-19 human glioma cell line was established in 1980 and is phenotypically positive for glial fibrillary acidic protein, glia-associated antigen S-100, and plasminogen activator. These cells were maintained in RPMI medium containing 5% fetal bovine serum, penicillin/streptomycin, and 10 mM HEPES at 37°C in an incubator supplied with 5% CO₂. Cells were passaged approximately once weekly by using 0.25% trypsin in phosphate-buffered saline (pH 7.35–7.4). Supernatants from this cell line served as our source of GCCS and were centrifuged before use to remove cells and debris. Cells from the non-adherent human monocyte cell line, U937, were maintained as instructed.

**In Vitro Antigen Stimulation of T Cells and Return to the Resting State**

Purified T cells (3 × 10⁶ cells at 1.5 × 10⁵/ml) in complete RPMI were stimulated for 20 hours in 24-well plates

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**TABLE 1**

Characteristics of cells and/or tumors obtained in six patients with GBM and seven controls*

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>Age (yrs), Sex</th>
<th>Tumor Size (cm³)</th>
<th>Tumor Location</th>
<th>Tumor Grade</th>
<th>PBL/cm³ (×10⁶)‡</th>
<th>T Cells/cm³ (×10⁵)$</th>
<th>Lymphocyte Proliferation (corr cpm)</th>
<th>Increased % of Apoptosis**</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>27, F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.95</td>
<td>6.30</td>
<td>208,567.25</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>44, M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.51</td>
<td>4.81</td>
<td>229,869.42</td>
<td></td>
</tr>
<tr>
<td>BT1</td>
<td>58, M</td>
<td>71</td>
<td>Lt occipital</td>
<td>GBM III-IV</td>
<td>1.90</td>
<td>5.77</td>
<td>140,073.50</td>
<td>157.6 (C1); 169.4 (C2)</td>
</tr>
<tr>
<td>C3</td>
<td>27, M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.96</td>
<td>4.27</td>
<td>257,432.19</td>
<td></td>
</tr>
<tr>
<td>BT2</td>
<td>66, F</td>
<td>15</td>
<td>Lt temporal</td>
<td>GBM III</td>
<td>1.16</td>
<td>0.91</td>
<td>201,890.00</td>
<td>131.0 (C3)</td>
</tr>
<tr>
<td>C4</td>
<td>42, F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.80</td>
<td>9.89</td>
<td>188,991.67</td>
<td></td>
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<tr>
<td>BT3</td>
<td>71, F</td>
<td>160</td>
<td>Lt frontal</td>
<td>GBM IV</td>
<td>2.14</td>
<td>2.37</td>
<td>353,800.00</td>
<td>145.7 (C4)</td>
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<td>C5</td>
<td>39, F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.95</td>
<td>5.63</td>
<td>373,501.50</td>
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<tr>
<td>C6</td>
<td>53, M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.84</td>
<td>2.08</td>
<td>222,662.90</td>
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<td>BT4</td>
<td>53, M</td>
<td>45</td>
<td>Lt temporal</td>
<td>GBM</td>
<td>2.27</td>
<td>0.81</td>
<td>150,579.50</td>
<td>214.3 (C5); 156.8 (C6)</td>
</tr>
<tr>
<td>BT5</td>
<td>70, F</td>
<td>80</td>
<td>Lt frontal</td>
<td>GBM IV</td>
<td>1.47</td>
<td>0.69</td>
<td>45,468.90</td>
<td>134.4 (C5); 0.98 (C6)</td>
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<tr>
<td>C7</td>
<td>25, F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.13</td>
<td>13.05</td>
<td>38,841.39</td>
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<tr>
<td>BT6</td>
<td>61, F</td>
<td>12</td>
<td>Recurrent</td>
<td>GBM IV</td>
<td>0.92</td>
<td>1.88</td>
<td>5,737.25</td>
<td>109.7 (C7)</td>
</tr>
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</table>

* BT = brain tumor patients; C = designation for healthy control volunteers; corr cpm = corrected counts per minute; exp = experiment; GBM = glioblastoma multiforme; NA = not applicable; PBL = peripheral blood lymphocyte; PHA = phytohemagglutinin.

† Tumor size calculated as described in Morford, et al.

‡ The PBL/cm³ was calculated after cell isolation over Ficoll-Hypaque; mean patient PBLs ± SD = 2.16 ± 0.52 cells/cm³ of blood; mean patient T-cell number ± SD = 1.64 ± 0.55 cells/cm³ of blood.

§ Purified by rosetting and counted; mean control T-cell number ± SD = 6.58 ± 3.71 cells/cm³ of blood; mean patient T-cell number ± SD = 2.07 ± 1.93 cells/cm³ of blood.

‖ Tumor size calculated as described in Morford, et al.

§§ The T cells were isolated from whole blood as described in Clinical Material and Methods. The T cells were stimulated with PHA as described in Morford, et al. Lymphocyte proliferation is expressed as the average cpm of a stimulated cell population minus the cpm of an unstimulated (background) cell population; mean control donor response ± SD = 217,125.76 ± 99,096.87 cpm; mean patient response ± SD = 149,591.53 ± 123,339.67 cpm.

** The percentage of control apoptosis level equals the percentage of apoptotic patient T cells divided by the percentage of apoptotic control donor T cells (run on the same assay day) times 100.
with 1 μg/ml of immobilized anti-CD3 MAb (OKT3 clone) in the presence or absence of a 1:20 dilution of GCCS. Immobilization of anti-CD3 MAb to plastic was performed as previously described.26 After stimulation, cells were harvested with excess media, counted, and re-cultured in 24-well plates for 1 to 5 days at concentrations indicated in each experiment in the absence of any stimulus or GCCS to allow the cells to return to a resting state. After this rest period, cells were harvested and analyzed for apoptosis. In some experiments, 5 μg/ml of anti–IL-10 neutralizing antibody, 5 μg/ml of sheep anti–human IL-4 neutralizing antibody, 25 μg/ml of panspecific TGFβ neutralizing antibody and/or 1 μg/ml of mouse anti–human TNFβ (Lymphotoxin) neutralizing MAb were added alone, or in combination, during both the stimulation and rest periods. The concentrations of anti–IL-10 antibody, anti–IL-4 antibody, and anti–TNFβ MAB used in this study were chosen based on information from previous studies.12,29 The concentration of anti–TGFβ neutralizing antibody that we chose was based on other observations in our laboratory (data not shown).

Measurement of Apoptosis by Flow Cytometry

To measure apoptosis, 10⁶ cells per treatment group were stained using the system of Reid and colleagues.18,39 Briefly, cellular DNA was stained with Hoechst 33342 (Ho342) and loosely packed or blebbing membranes, which occur during the process of apoptosis, were stained with merocyanine 540 (MC540). As seen in Fig. 1, when used concurrently these stains allow for the separation of six distinct populations of cells. These include: Region (R)1, viable resting cells (normal DNA content [2n] and MC540neg); R2, viable cycling cells (>2n and MC540neg); R3, resting cells in early stages of apoptosis (2n and MC540bright); R4, cycling cells in early stages of apoptosis (>2n and MC540bright); R5, MC540resting; and R6, MC540apoptotic, cells in later stages of apoptosis (<2n due to DNA fragmentation). For each sample examined, 10,000 events (cells) were analyzed by flow cytometry. Whenever available, three separate aliquots (10⁶ cells) of a single donor sample or a single treatment group were stained in parallel to rule out differences caused by manual staining.
error. Unless otherwise noted, the average of these three replicates has been plotted on each graph. Standard deviations (SDs) for the replicates are noted in the figure legends.

Treatment of the U937 Line With GCCS and Transwell Assays

The human monocyte cell line U937 was pretreated for 20 hours with a 1:20 dilution of GCCS. After extensive washing, $6.67 \times 10^4$ control or GCCS-pretreated U937 cells were placed in the upper chamber of a 0.4-μm transwell insert (6.5-mm diameter). Rosette-purified T cells (1.33 $\times 10^6$ cells/well) were stimulated for 4 to 6 days in the lower chambers with 1 g/ml of immobilized anti-CD3 MAb. After stimulation, the T cells were collected and the percentage of apoptotic cells was determined by Ho342/MC540 fluorescence-activated cell sorter (FACS) analysis.

Statistical Analysis

When applicable, statistical analysis of data was performed on a personal computer running a commercially available software program (procedure Proc GLM) to perform one-way analysis of variance (ANOVA) tests.

Sources of Supplies and Equipment

The components for the RPMI media were obtained from Gibco/BRL, Grand Island, NY, with the exception of the 2-mercaptoethanol and the fetal bovine serum, which were acquired from Sigma Chemical Co., St. Louis, MO, and Intergen, Purchase, NY, respectively. The trypsin was obtained from Gibco. The anti–IL-10 and panspecific TGFB neutralizing antibodies were purchased from R & D Systems, Minneapolis, MN, and the sheep anti–human IL-4 neutralizing antibody was acquired from Genzyme, Cambridge, MA. The mouse anti-human TNFβ neutralizing MAb (9B9) was obtained from Boehringer Mannheim, Mannheim, Germany. The Hoe342 and the MC540 were purchased from Molecular Probes, Inc., Eugene, OR. The transwells were obtained from Corning Costar Corp., Cambridge, MA. The statistical software was acquired from SAS Institute, Inc., Cary, NC. The SNB-19 human cell line was obtained from Dr. Paul L. Kornbluth, University of Pittsburgh, PA, and the U937 cell line was
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obtained from the American Tissue Culture Collection, Rockville, MD.

Results

Apoptosis of T Cells From Patients With Glioma

To determine whether glioma-derived factors were causing circulating T cells in patients with glioma to undergo apoptosis, a FACS assay was used. With this assay we were able to distinguish between cell cycle position and early or late stages of the apoptotic process (Fig. 1).38,39 Viable cells with either 2n or 4n DNA content are found in the R1 and R2 quadrants of the FACS plot; early apoptotic cells are found in the R3 and R4 quadrants; late apoptotic cells are found in the R5 and R6 quadrants (see Clinical Material and Methods). Rosette-purified peripheral blood T cells from patients with glioma and from control donors were examined for membrane and DNA characteristics unique to apoptotic cells. Results presented in Fig. 2 show an increased number of apoptotic T cells in the peripheral blood of patients with gliomas when compared with peripheral blood T cells isolated from control volunteers. As depicted in Table 1, the level of patient T-cell apoptosis ranged from 0.98 to 214% of control levels. This increased T-cell apoptosis in patients’ peripheral blood often correlated with reductions in T-cell numbers and in vitro T-cell responsiveness (Table 1). Based on 

Apoptosis of Normal T Cells in Vitro After Treatment With GCCS

We have demonstrated that GCCS from cloned glioma cell lines as well as from freshly explanted tumors induces intrinsic defects in T cells obtained from normal individuals that mimic those observed in patients harboring these tumors.25,44 To investigate whether GCCS could induce apoptosis in freshly isolated T cells, rosette-purified T cells obtained from healthy individuals were stimulated with immobilized anti-CD3 MAb in the presence or absence of GCCS. After 20 hours the cells were harvested with excess media and recultured in the absence of additional GCCS and were analyzed for apoptosis. Three 10^6-cell aliquots of each treatment were stained in parallel for FACS analysis. The graph shows the average staining for each region (R3–R6). The average percentage of total apoptosis (R3 + R4 + R5 + R6) ± SD for the control group was 19.48 ± 0.37% and for the GCCS-treated group was 39.04 ± 0.60%. The GCCS-treated cells were significantly more apoptotic than control cells according to ANOVA analysis (p = 0.0001). The data depicted in this figure are representative of one of 30 independent experiments.

Role for Soluble Mediators in GCCS-Mediated Apoptosis of Normal T Cells

The signaling events associated with the induction of apoptosis may be generated either via ligand–receptor interactions as the result of cell–cell interactions or by soluble mediators. Thus, experiments were performed to determine whether GCCS-induced apoptosis was dependent on cell–cell contact or could be induced by soluble factors. If cell–cell contact plays the predominant role in mediating GCCS-induced apoptosis, the level of apoptosis will be proportional to the final number of cells placed in the culture well regardless of the volume of medium. Conversely, if GCCS-mediated apoptosis is contingent in soluble mediators, one would expect that decreasing the volume of medium in cultures containing the same number of cells would dramatically increase apoptosis as a result of concentrating the soluble mediators. Accordingly, normal T cells were treated with immobilized anti-CD3 MAb in the presence or absence of a 1:20 dilution of GCCS for 20 hours. The T cells were harvested with excess media and were recultured at different cell concentrations in either 1 ml or 2 ml of media and rested for 4 days. The data presented in Fig. 5 show that cells treated with GCCS for 20 hours and rested in the absence of additional GCCS...
Resting cells for 3 days led to only small increases of apoptosis in the lower chambers (lane 6 compared with lane 7: ANOVA, p = 0.0001) and in the upper chambers (lane 1 compared with lane 5, lane 2 compared with lane 4, lane 3 compared with lane 7, lane 4 compared with lane 8; ANOVA for all comparisons, p = 0.0001). In addition, the data indicate that soluble moieties are responsible for inducing apoptosis. This is supported by the following observations. Increasing the number of cell–cell contacts during the rest period did not increase GCCS-mediated apoptosis of normal T cells (lane 5 compared with lane 6; ANOVA, p = 0.1344; or lane 7 compared with lane 8; ANOVA, p = 0.0121). In contrast, keeping the number of cell–cell contacts constant but reducing the sample volume (lane 6 compared with lane 7: ANOVA, p = 0.0001) dramatically increased GCCS-mediated T-cell apoptosis. Resting cells for 3 days led to only small increases of apoptosis in GCCS-treated compared with untreated T cells (data not shown). This correlates with the idea that soluble factor accumulation was required to induce apoptosis. Thus, soluble moieties would accumulate at a faster rate in a well with the higher cell concentration. These data indicate that the induction of soluble mediators by GCCS has an important role in glioma-induced apoptosis.

Inhibition of GCCS-Mediated Apoptosis of Normal T Cells Not Induced by Neutralizing Antibodies to Cytokines

Two cytokines secreted by gliomas, TGFβ and IL-10, have been postulated to mediate glioma-induced immunosuppression. In addition, TGFβ has been shown to induce apoptosis of murine T-cell clones. Therefore, to test whether TGFβ or IL-10 was responsible for the observed T-cell apoptosis in this system, experiments were conducted with the inclusion of neutralizing antibody to either cytokine. Normal T cells were stimulated with immobilized anti-CD3 MAb for 20 hours in the presence or absence of GCCS as described earlier. During this stimulation period, half of the samples received either a pan-specific anti-TGFβ neutralizing antibody (Fig. 6A) or an anti–IL-10 MAb (Fig. 6B). After stimulation, T cells were harvested and recultured in the presence of the appropriate neutralizing antibody. After a 4-day rest period, the cells were collected and analyzed for apoptosis by FACS. As shown in Fig. 6, the addition of neutralizing antibody to either cytokine did not blunt GCCS-mediated T-cell apoptosis.

Cytokine Dysregulation and GCCS-Induced Apoptosis of T Cells

Cytokine dysregulation can facilitate the apoptotic elimination of T cells (for example, in acquired immunodeficiency syndrome [AIDS] and cancer). To determine whether cytokine dysregulation could mediate GCCS-induced apoptosis of T cells, normal T cells were stimulated for 20 hours with anti-CD3 MAb in the presence or absence of GCCS and were rested for 4 to 5 days. Neutralizing antibody to IL-4 or TNFβ was added to half of the cultures during both the stimulation and rest periods (Fig. 7). Results of FACS analysis demonstrate that GCCS-induced apoptosis of normal T cells was not reversed by neutralizing antibody to IL-4 or TNFβ. Addition of the Th1 cytokines IL-2 (10^4 M), or IL-12 (2.5 ng/ml) during the stimulation and rest periods did not inhibit the induction of apoptosis (data not shown). Additionally, the GCCS-induced apoptosis was not inhibited even when IL-12, anti–IL-4, and anti–TNFβ antibodies were added concurrently to the cultures (data not shown). These data indicate that T-cell death activated by GCCS cannot be abrogated with the addition of Th1 cytokines or by inhibiting Th2 cytokines.

Monocytes Treated With GCCS Induce T-Cell Apoptosis Via Soluble Mediators

Monocytes are potent regulators of T-cell function. Because a small percentage of monocytes could be found in rosette-purified T-cell preparations (generally < 2%), experiments were conducted to explore monocyte involvement in the induction of T-cell apoptosis. Using the transwell system, we tested whether GCCS-treated U937 cells could induce apoptosis of normal T cells. The T cells from normal individuals were stimulated in the lower chambers of 24-well transwell plates with anti-CD3 MAb. The upper transwell chambers contained a control population of untreated U937 cells or GCCS-pretreated U937 cells (cells that had been treated for 20 hours with GCCS and washed extensively). Apoptosis of the T cells in the lower chambers...
was monitored on Days 4, 5, and 6. The results in Fig. 8 show that GCCS-treated U937 cells induced significant levels of T-cell apoptosis, whereas control U937 cells did not. Importantly, GCCS did not induce apoptosis of the U937 cells, ruling out the possibility that monocyte breakdown products were mediating T-cell apoptosis (data not shown).

Discussion

With the results from this study we can begin to address the mechanism(s) that contribute to the impaired immune response in patients harboring gliomas. Our data show that the T-cell lymphopenia observed in patients harboring primary gliomas correlates with an increase in the percentage of apoptotic peripheral T cells. These data further indicate that T-cell apoptosis can be induced in vitro by using GCCS. Taken together, these observations reinforce the notion that glioma cells secrete immunoregulatory moieties in vivo that cause the impaired T-cell immunity observed in these patients. Moreover, these findings offer an explanation for the improvement in a patient’s immune status after tumor resection. Finally, the results implicate monocytes in the observed immune impairments noted in these patients.

We have previously demonstrated that GCCS harvested from cloned glioma cell lines and freshly explanted gliomas induces impaired immune function in T cells obtained from normal individuals. Interestingly, this impaired immune function mimics that observed in T cells obtained in patients with glioma. Using a modified in vitro culture system, our study shows that GCCS promotes the apoptosis of normal T cells and that it induces apoptosis only in T cells that have received a concurrent signal through the TCR. Thus, the elimination of T cells that have become activated in the peripheral blood in the presence of glioma-derived factors provides an explanation for the dramatic decline of CD3+ T cells observed in patients. This is consistent with and may explain our previous observations that fewer mitogen-stimulated T cells obtained from these patients are capable of entering and progressing through the cell cycle as compared with normal mitogen-stimulated T cells. One notable difference between apoptosis measured in T cells freshly isolated from patients, as compared with GCCS-treated normal T cells, was that the percentage of late-stage apoptotic cells was greatly diminished in the latter. A large number of late-apoptotic (R5 + R6) T cells were noted in cultures of normal T cells treated with GCCS compared with the number of these cells in freshly isolated samples in patients and control volunteers (Fig. 2). This indicates that late-apoptotic cells are rarely detected in freshly isolated
Fig. 6. Bar graphs showing that GCCS-mediated T-cell apoptosis cannot be halted with the addition of neutralizing antibodies to TGFβ or IL-10. A: Rosette-purified T cells were stimulated with 1 μg/ml of immobilized anti-CD3 MAb for 20 hours in the presence or absence of a 1:20 dilution of GCCS. In half of the samples 25 μg/ml of a panspecific TGFβ neutralizing antibody (Ab) was added during the stimulation period. T cells were harvested with excess RPMI-1640 media and were replated in 1-ml volumes at 3 × 10^6 cells/ml for the 4-day rest. Samples originally receiving anti-TGFβ antibody during the stimulation phase were again treated with 25 μg/ml of anti-TGFβ antibody for the 4-day rest period. After this rest period, the percentage of apoptotic T cells in each culture was determined as described in Clinical Material and Methods. The average percentage of total apoptosis ± SD for the control groups was 32.94 ± 1.36% without antibody and 34.32 ± 1.35% with antibody (p = 0.7725, ANOVA). The average percentage of total apoptosis ± SD for the GCCS-treated groups was 93.83 ± 0.61% without antibody and 95.35 ± 0.31% with antibody (p ≥ 0.5833, ANOVA). The ANOVA analysis demonstrated that the GCCS-treated cells both with and without antibody were significantly more apoptotic than control cells treated with or without antibody, respectively (p = 0.0001). The data in the figure are representative of one of two independent experiments. B: The experimental protocol was similar to that described in A, with the following alterations: neutralizing antibody to IL-10 instead of anti-TGFβ, 2.5 g/ml of anti-TGFβ during the stimulation period, and IL-10 (500 ng/ml) in the control groups. The average percentage of total apoptosis ± SD for the control groups was 2.55% with antibody (p ≥ 0.5833, ANOVA). The average percentage of total apoptosis ± SD for the GCCS-treated groups was 32.85 ± 1.7% without antibody and 32.27 ± 2.55% with antibody (p = 0.5823, ANOVA). The average percentage of total apoptosis ± SD for the GCCS-treated groups was 32.85 ± 1.7% with antibody (p ≥ 0.5833, ANOVA). The ANOVA analysis demonstrated that the GCCS-treated cells both with and without antibody added were significantly more apoptotic than control cells treated with or without antibody, respectively (p = 0.0001). The data in the figure are representative of one of 12 independent experiments.

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Cell samples because macrophages rapidly clear these dead cells in vivo. Presumably the late-apoptotic cells are present in vitro because of the paucity of phagocytic cells.

It has been well documented that low concentrations of immunosuppressant factors excreted from glioma cells can inhibit peripheral immune system function. For example, serum obtained from patients with glioma is capable of suppressing autologous and homologous normal lymphocyte function in vitro. Similarly, supernatants obtained from either fresh tumor explants or long-term glioma cell lines (that is, GCCS) inhibit normal lymphocyte function in vitro. Of importance, exposure of normal T cells to GCCS induces proliferative defects similar to those observed in T cells obtained in patients harboring this neoplasm. In addition, the presence of these factors correlates well with the size of the tumor and the fact that excision of the tumor is associated with a restoration of T-cell function. Approximately 6 weeks before radiographic and/or clinical evidence of tumor recurrence, diminished T-cell responsiveness again becomes detectable. Gliomas have been shown to synthesize and secrete multiple factors that are capable of inhibiting T-cell responsiveness, including TGFβ, 1,12,19,35 prostaglandin E2, 29 and IL-10. Although TGFβ has been implicated as the major glioma-derived immunosuppressant by some researchers, our results indicate that it does not have a pivotal role in glioma-mediated immunosuppression. Support for this comes from the following observations. First, the concentration of TGFβ in culture supernatants from gliomas does not correlate with the immunosuppressive capacity of those supernatants. Second, recent studies with the TGFβ antagonist decorin have indicated that factor(s) other than TGFβ are responsible for glioma-mediated immunosuppression. Third, the mitogen unresponsiveness observed in normal T cells as the result of treatment with GCCS is not reversed by the addition of neutralizing antibody to TGFβ, whereas TGFβ-induced immunosuppression can be reversed by this antibody (unpublished observation).

Finally, we have demonstrated that the concentrations of TGFβ in glioma culture supernatants did not correlate with the biological activity to alter peripheral blood mononuclear cell (PBMC) production of IL-10, IL-12, and interferon-γ. These observations underscore the need to completely characterize the full complement of secreted glioma-derived immunoregulatory factors.

Cytokine dysregulation has been demonstrated in both normal and disease states (for example, downmodulation of immune responses, pregnancy, AIDS, cancer, lupus, and leishmaniasis infection) that may facilitate the apoptosis of T cells. For example, TNFβ (lymphotoxin) can induce the in vitro apoptosis of normal human T lymphoblasts, presumably as a means of downmodulating an immune response. The observation that TNFβ-induced apoptosis could be affected by the addition of IL-2, IL-12, and other cytokines demonstrates how overall cytokine balance influences cell death. In studies of AIDS-related diseases, the data indicate that Th2-associated cytokines such as IL-4, IL-10, and TNFβ induce apoptosis in T cells, whereas the Th1 cytokines rescue T cells from cell death. Data are emerging indicating that glial tumors are capable of inducing a Th1-to-Th2-type cytokine shift in patients. Accordingly, we have demon-
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...strated that secretion of the Th1-type cytokine IL-2 by mitogen-stimulated T cells obtained in patients with glioma is greatly diminished.21,22 Roussel, et al.,23 have shown that tumor-infiltrating lymphocytes detected at the glioma tumor site secrete predominantly Th2-type cytokines. Finally, we have demonstrated that treatment of normal PBMCs with GCCS also leads to the predominant production of Th2-type cytokines with diminished IL-12 and elevated IL-10 production. A Th2-type cytokine shift, which would support humoral immunity at the expense of cell-mediated immune responses, corroborates observations in patients with gliomas and would explain the inefficient antitumor responses generated in these individuals.

Apoptosis in this study was not altered by the addition of Th1-type cytokines or neutralizing antibodies to Th2-type cytokines. Specifically, the addition of human recombinant IL-2 or IL-12 (data not shown), or the addition of neutralizing antibodies to IL-4, IL-10, or TNFβ (alone or in combination) did not protect normal T cells from GCCS-mediated apoptosis. Thus, it is unlikely that glioma-induced cytokine polarization involving IL-2, IL-4, IL-10, IL-12, or TNFβ is responsible for T-cell apoptosis in these patients. In addition, the immunomodulator TGFβ does not appear to be responsible for the induction of T-cell apoptosis in patients with glioma.

These data together with our previous findings lend support to the continued use of this in vitro system in defining the mechanism(s) that contribute to the immunodeficient state of patients with glioma. In this study, we have used this system to further identify contributing factors that modulate apoptosis and diminished T-cell function in these patients. Because monocytes are potent regulators of T-cell function and a small percentage of monocytes (typically < 2%) can be found in rosette-purified T-cell preparations, experiments were also performed to explore the degree of monocyte involvement in the induction of T-cell apoptosis. Others have clearly demonstrated that tumor-induced immune dysfunction can be mediated via the monocyte/macrophage population.20 In fact, we have shown that treatment of PBMCs with GCCS can alter the cytokine production of monocytes.59 In addition, we have demonstrated that GCCS induces freshly isolated normal monocytes, U937 monocyte-like cells, or THP-1 monocyte-like cells to produce soluble factors capable of inhibiting T-cell proliferation in transwell assays (unpublished observation). Thus, GCCS could act directly on the monocyte/macrophage population to induce the secretion of soluble mediators that in turn drive activated T cells into apoptosis. This is supported by the fact that pretreatment of the monocyte cell line U937 with GCCS induced these cells to secrete soluble factors that traversed a membrane to induce apoptosis in anti-CD3 MAb-stimulated T cells in a transwell assay. Another possibility to explain how GCCS-treated monocytes influence T-cell apoptosis could involve the sequestration and release of GCCS by the monocyte. Although the exact mechanism of action remains unclear, our data implicate the monocyte as a mediator of the T-cell lymphopenia observed in patients with gliomas. It is intriguing to propose that monocyte-dependent activated T-cell apoptosis confined to the CD4+ Th1 subset of T lymphocytes could explain the loss of CD4+ cells and the resultant cytokine dysregulation observed in these patients. Currently inves...

**Fig. 7.** Bar graphs showing that GCCS-induced apoptosis cannot be halted with the addition of neutralizing antibodies to IL-4 or TNFβ. A: Rosette-purified T cells were stimulated with 1 μg/ml of immobilized anti-CD3 MAb for 20 hours in the presence or absence of a 1:20 dilution of GCCS. In half of the samples 5 μg/ml of anti–IL-4 neutralizing antibody (Ab) was added during the stimulation period. The T cells were harvested with excess RPMI-1640 media and replated in 1-ml volumes at 3 × 10⁶ cells/ml for the 4-day rest. Samples in which anti–IL-4 antibody was originally added during the stimulation phase were again treated with 5 μg/ml of anti–IL-4 antibody for the 4-day rest period. After this rest period, the percentage of apoptotic T cells in each culture was determined as described in Clinical Material and Methods. The average percentage of total apoptosis ± SD for the control groups was 32.41 ± 1.5% without antibody and 59.85 ± 0.46% with antibody (p = 0.0001, ANOVA). The average percentage of total apoptosis ± SD for the GCCS-treated groups was 99.56 ± 0.05% without antibody and 99.67 ± 0.09% with antibody (p = 0.8706, ANOVA). The ANOVA analysis demonstrated that the GCCS-treated cells both with and without antibody added were significantly more apoptotic than control cells treated with and without antibody, respectively (p = 0.0001). The data are representative of one of four independent experiments. B: The experimental protocol was similar to that described in panel A, with the following alterations: neutralizing antibody to TNFβ instead of the anti–IL-4 antibody was added at 1 μg/ml during both the stimulation and rest phases, and cells were rested for 5 days at 4 × 10⁶ cells/ml. The average percentage of total apoptosis ± SD for the control groups was 50.21 ± 2.63% without antibody and 60.77 ± 2.23% with antibody (p = 0.0001, ANOVA). The average percentage of total apoptosis ± SD for the GCCS-treated groups was 98.32 ± 0.18% without antibody and 98.91 ± 0.18% with antibody (p = 0.6492, ANOVA). The ANOVA analysis demonstrated that the GCCS-treated cells both with and without antibody added were significantly more apoptotic than control cells treated with and without antibody, respectively (p = 0.0001). The data are representative of one of three independent experiments.
tigations are being conducted in which this possibility is being explored.

Conclusions
The data presented in this paper provide an explanation for the observed T-cell lymphopenia in patients harboring gliomas and lend further support to our previous observations that glioma-derived soluble factors contribute to the impaired immunity in these patients. Collectively, these data indicate a more complex model than has been previously proposed to explain the interrelationship between the glioma and the immune status of the patient. For example, we infer from the data that monocytes have a prominent role in the disease process and that TGFβ may be a lesser player than originally thought. Understanding the precise mechanisms of how glioma-derived factors modify immune potential via cytokine shifts, induction of intrinsic T-cell defects, and monocyte-associated activated T-cell death will enable the development of more beneficial immunotherapeutic regimens for patients with glioma.

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