Activation of immunoregulatory lymphocytes obtained from patients with malignant gliomas

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The responsiveness of T cells and their subsets (T-helper cells and T-suppressor cells) obtained from patients with malignant gliomas was evaluated in an effort to further define the mechanism of their impaired host immunocompetence. This study demonstrates that peripheral blood lymphocytes obtained from these patients have impaired responsiveness to a variety of mitogens including phytohemagglutinin, concanavalin A, pokeweed mitogen, and anti-T3 monoclonal antibody. The impaired lymphocyte responsiveness does not result from the inability of these cells to express receptors for a specific mitogen or antibody. The mitogenic responsiveness of purified T cells is markedly reduced when compared to values obtained from control subjects. Therefore, the decreased T cell reactivity of patients with malignant gliomas does not result simply from a diminution in the absolute number of potentially responding lymphocytes. The mitogen reactivity of the T cell subsets, CD4⁺ helper cells, and CD8⁺ cytotoxic/suppressor cells was also investigated. These results demonstrate that the responsiveness of the CD4⁺ T-helper cell subset obtained from these patients is consistently diminished as compared to control values. In contrast, the reactivity of the CD8⁺ T cell subset was not nearly as dramatically impaired. Thus, these results indicate that the proliferative defect observed in T cells obtained from patients is located predominantly in the T-helper cell subset. Functional deficiencies in this important subpopulation of T lymphocytes may explain, in part, the presence of depressed immune responsiveness in patients with malignant gial tumors.

KEY WORDS: T lymphocyte, malignant glioma, brain neoplasm

It is well known that immunological reactivity is impaired in patients with a variety of malignant tumors at some point during the course of the disease. Although many mechanisms have been championed, precise definitions of these etiological events remain elusive. Exact characterization of those mechanisms involved in patients harboring primary intracranial tumors is no exception. Nevertheless, it has been demonstrated that these patients express a variety of immunological deficiencies including cutaneous anergy, depressed antibody production, diminished numbers of circulating T cells, impaired lymphocyte reactivity, and serum capable of suppressing lymphocyte activation. More recent studies indicate that these impairments may result from anomalies in normal T cell function occurring as the result of intrinsic malfunctions in the biochemical and/or genetic pathways necessary for normal activation, and/or from quantitative and/or qualitative defects in the subsets of T cells. The normal cascade of events that result in immunological responsiveness include the presence of adequate numbers and function of T-helper cells. These T cell subsets are necessary for the production of important cytokines — for example, interleukin 2 (IL-2), which are required for lymphocyte differentiation and subsequent function. Defects in the function of T-helper lymphocytes would be predicted to result in decreased cell-mediated immune responsiveness, such as generation of cytotoxic T cells and antigen and mitogen T cell activation. Recently, we have demonstrated that the production of IL-2 by lymphocytes obtained from patients with malignant gliomas is markedly decreased, suggesting that T-helper cell function in these patients indeed may not be normal. In the present study, we have addressed the question: Do the previously defined defects in T cell responsiveness extend to a variety of immunological stimuli and can these deficiencies be linked to altered T-helper cell activation? The results presented here indicate that
impaired T cell function is extensive. Moreover, T-helper cell function in these patients is not normal. These data may be interpreted as demonstrating that at least one important mechanism of impaired immune responsiveness in patients with malignant gliomas stems from functional deficiencies in the circulating T-helper cell compartment of the immune system.

Clinical Material and Methods

Study Population

Patients in this study were diagnosed as having either glioblastoma multiforme or anaplastic astrocytoma. All patients had symptoms and signs of active and progressive tumor growth. No patient was receiving irradiation or chemotherapy at the time of this study. Moreover, patients were not receiving systemic steroids or phenytoin. Controls and patients had not received any anesthetic other than local anesthesia for at least 6 weeks before the study. Both men and women were included, ranging in age from 18 to 66 years (median 40 years). Healthy male and female hospital employees, ranging in age from 22 to 58 years (median 30 years), served as normal control individuals.

Lymphocyte Preparation

Heparinized venous blood was separated on a Ficoll-Hypaque gradient. After centrifugation at 400 G for 35 minutes, the lymphocyte layer was collected, washed twice in Hanks' balanced salt solution, and adjusted to 1 x 10^7 cells/ml in RPMI 1640 medium containing 1% glutamine, nonessential amino acids, vitamins, and antibiotics.

The method employed to isolate T cells has been described previously. Briefly, equal volumes of lymphocytes (1 x 10^7 ml) and 2.5% neuraminidase-treated sheep erythrocytes (sheep red blood cells or SRBC's) were mixed, and SRBC-absorbed fetal calf serum (FCS) was added to attain a final concentration of 10%. The mixture was incubated at 4°C for 1 hour and separated on a Ficoll-Hypaque gradient. The pellet containing the rosettes was treated with 0.83% NH_4Cl to lyse the SRBC's, and the lymphocytes were washed and counted. Less than 3% of these lymphocytes were B cells and less than 1% were monocytes as determined by nonspecific esterase staining.

The T-helper cells (CD4^+ and T-suppressor/cytotoxic cells (CD8^+) were obtained by a panning technique. Briefly, plastic Petri plates (100 x 15 mm) were coated with affinity-purified anti-mouse immunoglobulin (IgG) at a concentration of 10 μg/ml in 0.05 M Tris-HCl buffer (pH 9.2) by incubation with the solution for 40 minutes at room temperature. Unbound antibody was removed by washing the plates four times with Dulbecco's phosphate-buffered saline (PBS, pH 5.2) then once with Dulbecco's PBS (pH 7.2) containing 1% FCS. Negative selection was used to obtain either CD4^+ or CD8^+ T cell subsets. Purified T cells, 3 x 10^7, were resuspended in 2 ml of culture supernatant derived from hybridomas producing either OKT4 or OKT8 monoclonal antibody and were incubated at room temperature for 20 minutes. The cells were washed twice in Dulbecco's PBS (pH 7.2) containing 5% FCS, resuspended in 3 ml of this solution, and poured into antibody-coated plates. The plates were incubated for 2 hours at 4°C. The nonadherent cells (either CD4^+ or CD8^+) were collected and the plates gently washed four times with Dulbecco's PBS (pH 7.2) containing 5% FCS. The cells were washed three times, resuspended in RPMI 1640 medium supplemented with 5% FCS, counted, their viability determined, and the percentage of CD4^+ and CD8^+ cells measured by indirect immunofluorescence using anti-OKT-4 and OKT-8 monoclonal antibodies with fluorescein-labeled (Fab')_2 fragments of mouse Ig. The CD4^+ and CD8^+ cell fractions were assessed to be approximately 95% pure.

Cell Cultures

Lymphocytes were adjusted to 1 x 10^6/ml in modified RPMI 1640 medium containing 10% FCS (complete RPMI 1640). The cells were dispensed in 200-μl volumes into 96-well microtiter plates at a final concentration of 2 x 10^5 cells/well. The triplicate cell cultures were stimulated with various concentrations of phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), or OKT-3 (anti-T3) monoclonal antibody in a volume of 10 μl. The cultures were incubated at 37°C in a moist 5% CO_2-95% air atmosphere for 72 hours with 0.5 μCi of tritiated (3H)-thymidine (specific activity 6.7 Ci/mM) added 18 hours before termination of the culture. The cells were harvested with a multiple-sample harvester, and the radioactivity was determined by scintillation counting.

Flow Microfluorometric Analysis of Lectin Binding

Purified T cells at a concentration of 1 x 10^6/ml in PBS (pH 7.4) with 0.02% sodium azide were incubated for 30 minutes at 4°C in the presence of 25 μg/ml of fluorescein-labeled PHA. Cells were washed three times in PBS with sodium azide and resuspended in 200 μl of PBS, and 10^4 cells were analyzed.

* Anti-mouse immunoglobulin obtained from Cappel Laboratories, Cochranville, Pennsylvania.
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Results

Responsiveness of Peripheral Blood Lymphocytes to Various Stimuli

We have previously shown that lymphocytes obtained from patients with malignant gliomas are significantly less responsive to the nonspecific mitogen PHA than are lymphocytes from normal individuals and control patients (with head injury and stroke). Because the responsiveness of normal subjects and control patients did not significantly differ in previous reports, the results are compared among normal subjects and patients with gliomas. This series of experiments was designed to determine if this impairment of lymphocyte activation extended to other mitogens. Accordingly, lymphocytes from patients and normal individuals were stimulated with various concentrations of PHA, ConA, PWM, and anti-T3 monoclonal antibody which specifically activate the T3-Ti receptor that has been shown to be important for antigen- as well as mitogen-driven T cell immune responsiveness. The results presented in Fig. 1 demonstrate that the reactivity of lymphocytes from patients with malignant tumors is diminished when compared to similarly stimulated cells obtained from normal subjects. These results also indicate that impaired lymphocyte responsiveness does not result from the inability of these cells to express receptors for a specific mitogen or antibody, as it is unlikely that a random selection of lymphocytes would be deficient in their ability to bind such widely varying mitogenic substances. This hypothesis is confirmed in those experiments in which purified T cells were analyzed for their ability to bind the lectin PHA. In these studies, cells were stained with fluorescein-labeled PHA and subsequently examined by flow microfluorometry. The results of a representative experiment are shown in Fig. 2. There is no difference in the PHA-binding properties of T cells obtained from the patient and control groups. Furthermore, the percentage of T cells that stain positive for PHA is identical (95% to 99%). Eliminating defective binding capabilities as the etiology of impaired lymphocyte activity suggests that the mechanism either is intrinsic to the lymphocyte itself or results from diminished numbers of potentially responding cells in the T cell network.

Responsiveness of Purified T Cells

Patients with brain tumors have been shown to have a decrease in the absolute number of circulating T cells. Therefore, it was of interest to determine if this paucity of T cells is responsible for the diminished PHA responsiveness of lymphocytes obtained from these patients. In these studies, purified populations of T cells were obtained from patients with malignant gliomas and from normal subjects. The results in Table 1 show that purified T cells from patients still exhibit impaired mitogen responsiveness when compared to equal numbers of normal T cells. Therefore, it may be concluded that decreased T cell reactivity in patients with malign-

Fig. 1. Responsiveness of peripheral blood lymphocytes (PBL) obtained from seven patients with primary malignant intracranial tumors (squares) and seven normal subjects (circles) to different mitogens in various amounts. The responses of PBL obtained from patients were significantly different (Student's t-test) from responses obtained with PBL from control subjects as follows: Panel A (concanavalin A, Con A), 1 and 10 µg, p < 0.001; 5 and 20 µg, p < 0.01; Panel B (pokeweed mitogen, PWM), 1 µg, p < 0.05; 0.1, 5, 10, and 20 µg, not significant; Panel C (phytohemagglutinin, PHA), 1 and 5 µg, p < 0.001; 10 and 20 µg, p < 0.01; 0.1 µg, not significant; Panel D (OKT-3 monoclonal antibody, anti-T3), 1 and 5 ng, p < 0.001; 10 ng, p < 0.01; 20 ng, p < 0.05; 50 ng, not significant.

Fig. 2. Flow cytometric analysis of the binding of fluorescein-labeled phytohemagglutinin (FLI) to purified T cells obtained from a normal individual (left) and a patient with a glioma (right). A total of 10,000 cells were analyzed, with 98% of the cells positive in each case. These results are typical of those obtained for three other patients with brain tumors.
nant gliomas does not result simply from a diminution in the absolute number of potentially responding lymphocytes. Two possible hypotheses remain: 1) the presence of intrinsic cellular anomalies rendering T cells incapable of activation, or 2) deficiencies in the T cell repertoire leading to diminished immunological “help” or excessive “suppression.”

Responsiveness of T-Helper (CD4+) and T-Suppressor/Cytotoxic (CD8+) Lymphocyte Subsets

The responsiveness of T cells depends on a balance of immunological help and suppression. These activities predominantly result from activation of selected subsets of T cells which have the capabilities of facilitating or inhibiting immunological activity. Facilitation is afforded by T-helper cells that are phenotypically identified as CD4+; suppression is effected by T-suppressor cells identifiable by the CD8+ antigenic marker. In this set of experiments, the reactivity of each T cell subset was examined to assess the possibility that the overall host immune incompetence of glioma-bearing patients results from alterations in the responsiveness of these important regulatory T cell subsets. The results in Table 2 demonstrate that the responsiveness of the CD4+ T cell subset obtained from patients is consistently diminished as compared to control values. In contrast, the reactivity of the CD8+ population of T cells is not nearly as dramatically impaired. In two of the patients studied, the responsiveness of this subset was normal. These data indicate that the proliferative defect observed in T cells obtained from these patients is located predominantly in the T-helper cell subset. Moreover, they suggest that the impairment of immunocompetence in patients with malignant gliomas results from deficiencies in T cell help and not from excessive or easily inducible T-suppressor cell activity.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Normal Subjects (cpm)</th>
<th>Patients† (cpm)</th>
<th>p Values‡</th>
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<tr>
<td>1</td>
<td>82,109</td>
<td>39,422</td>
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<tr>
<td>2</td>
<td>57,760</td>
<td>13,919</td>
<td>0.001</td>
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<tr>
<td>3</td>
<td>183,283</td>
<td>97,784</td>
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<tr>
<td>4</td>
<td>53,180</td>
<td>28,466</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>56,492</td>
<td>23,079</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>80,980</td>
<td>52,713</td>
<td>0.05</td>
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</tbody>
</table>

* The purified T cells were stimulated with an optimal concentration of phytohemagglutinin (PHA, 10 µg).
† The data represent the results obtained with cells from six different patients with brain tumors.
‡ The data were compared by the two-tailed Student’s t-test for independent means.

Discussion

Our initial experiments in which cells from patients with primary malignant brain tumors were used demonstrated the presence of broad suppression of host immunocompetence.3-5 Recently, we have found that diminished lymphocyte reactivity in these patients is correlated with deficiencies in the initiation and subsequent proliferation of succeeding generations of responsive lymphocytes.9

The current finding that T cell reactivity is broadly suppressed indicates that the previously reported deficiencies in lymphocyte responsiveness may now be extended to a variety of stimulatory signals. The use of purified T cells further indicates that the defect is not the result of a lack of sufficient numbers of potentially responsive lymphocytes. Moreover, the decreased mitogenic reactivity is predominantly found in the T-
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helper subset which secretes those cytokines that are vital for sustained cell growth and differentiation of both T cells and B cells, thereby resulting in immune competence. Previously, we have shown that altered lymphocyte activation is linked to decreased IL-2 production, suggesting the presence of a functional defect at the level of the T-helper cell. The report that influenza antibody production in these patients is abnormal and tends to further decline with continuing tumor growth is also consistent with altered T-helper cell activity. At the in vitro level, we have demonstrated that T-helper cells obtained from patients are defective in their ability to provide adequate help for induction of Ig synthesis by normal B cells. Thus, the data obtained in the present study confirm the presence of an anomalous T-helper cell function in patients with malignant gliomas.

The precise mechanism responsible for the diminished T-helper cell activity has yet to be completely elucidated. Such a defect could result from enhanced T-suppressor cell function. However, the observation that suppressor lymphocyte activation was not diminished, coupled with the finding that suppressor cell function in these patients is normal (not increased), indicates that other mechanisms are responsible for diminished T-helper cell activity in glioma-bearing patients. Several pieces of data are available that contribute to our current understanding of this cellular deficiency and merit discussion. It does not seem likely that decreased T-helper cell activity results from dysfunction in accessory cells. These monocytes are responsible for antigen recognition and processing and subsequent elaboration of interleukin-1 (IL-1), which is necessary for the induction of T-helper lymphocyte function. Interleukin-1 production by monocytes obtained from these patients is normal. The initial steps of immune responsiveness are thus apparently intact and normal. Following these events, however, the defects in lymphocyte activation become evident. Interleukin-2 production is markedly diminished. Moreover, the production of cytokines required for the differentiation and growth of Ig-secreting cells appears to be deficient. These defects coupled with a 25% to 30% decrease in circulating T-helper cells isolate the cellular defect responsible for the impaired immunocompetence of glioma patients to qualitative and quantitative abnormalities in T-helper lymphocyte function.

How these alterations in lymphocyte number and function are initiated is conjectural; yet there are unique features of the central nervous system, particularly its relationship with the mediators of immunity, which warrant speculative and investigational comment. These include the sharing of certain antigenic determinants among normal and malignant glial cells and thymocytes and the elaboration of immunoregulatory cytokines by normal and malignant glial cells. The synthesis of antibody that may be cross-reactive with previously sequestered shared determinants on brain cells and thymocytes might result in functional elimination of the specific lymphocyte subpopulations necessary for the production of important lymphokines required for immune responsiveness. In addition, a defective blood-tumor barrier may allow egress of large quantities of immunosuppressive moieties which are capable of inhibiting lymphocyte function. Recently, we have found that cloned as well as freshly explanted glial tumor cells produce substances that profoundly suppress normal T-lymphocyte activation (in preparation). Although much is known regarding the immunobiology of patients with malignant gliomas, further work is required to completely define the mechanism responsible for impaired T-helper cell function in this group of patients. Once this is established the possibility and rationale for the use of immune modulation in the treatment of this disease may become evident.

References

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