ABSTRACT - Natural killer (NK) cells play an important role in immune surveillance against tumors. The present work aimed to study the cytotoxic activity of NK cells and T cell subsets in peripheral blood of 13 patients with primary tumors in central nervous system (CNS). As controls 29 healthy subjects with the age range equivalent to the patients were studied. The methods employed were: a) determination of cytotoxic activity of NK cells towards K562 target cells, evaluated by single cell-assay; b) enumeration of CD3+ lymphocytes and their CD4+ and CD8+ subsets defined by monoclonal antibodies; c) the identification of tumors were done by histologic and immunochemistry studies. The results indicated that adults and children with tumor in CNS display reduced percentage of total T cells, helper/inducer subset and low helper/suppressor ratio. The cytotoxic activity of NK cells was decreased in patients with CNS tumors due mainly to a decrease in the proportion of target-binding lymphocytes. These results suggest that cytotoxic activity of NK cells may be affected by the immunoregulatory disturbances observed in patients with primary tumors in CNS.

KEY WORDS: brain tumors, NK cells, T cell subsets.

Most cancers result from interaction of genetic and environment factors; however, genetic factors by themselves explains only about 5% of all cancer. The others have been attributed to environmental factors, that may interact with genetic cancer susceptibility and individual response1.

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Carcinogenesis determinants are influenced by environmental factors in about 80% of human cancers, but the variation that underlies the individual susceptibility is not well known². The prognosis in patients with malignant brain tumors, in contrast with other kinds of human systemic malignancies, has changed very little in the past 40 years. The combination of surgery, radiation and chemotherapy, only results in modest median survival³. The immunotherapy that could be an alternative approach to this problem, is of doubtful value, due to the complex relationship between the central nervous system (CNS) and the immune system⁴.

There is a close correlation between the onset, establishment and growth of a neoplasia and the ability of the immune system to inhibit it⁵. Antitumoral activity is mediated by cellular immunity through the action of natural killer (NK) cells, a subpopulation of large granular lymphocytes⁶ which acts regardless of previous sensitization⁷. These cells may be defined functionally as effector cells that mediate natural immunity having capability to spontaneously kill a variety of target cells, including tumours and virus-infected cells⁸,⁹. Although the precise mechanism of the lytic activity of NK cells is not clearly understood, granule exocytosis is generally accepted to be an important mechanism of lethal hit delivery¹⁰. Some evidence suggest that in the cytolytic event NK cells form conjugates with target cell, leading to the cross-linking of CD44 adhesion molecules and other cosignaling receptors¹¹. This initiates a sequence of events whose final step is the release of cytoplasmic perforin and granzyme B into the effector: target interface¹⁰,¹¹. Perforin polymerization and insertion into the target membrane are report to cause osmotic leakage and to provide target cells entry of granzyme B, which triggers a system leading to DNA fragmentation¹². Thus, the cooperation of these two molecules results in lysis and apoptosis of the target cell¹³.

It has been suggested that decrease in NK activity may be an important risk factor for the development of malignancy in man¹⁴. However, according to Whiteside and Herberman⁹ in a wide range of human tumors the impairment of NK activity may be a result of malignancy and not a factor that contributes to the development of cancer. Patients with advanced cancer exhibit lower NK function and global depression of T-cell responses, resultant from tumor growth and release of immunosuppressive factors¹⁵. The vast majority of evidence to support immunodysfunction in patients with brain tumors has been obtained from studies of adults with gliomas. Adults with primary CNS tumors had documented deficits in both humoral and cellular immunity¹⁶. Delayed cutaneous hypersensitivity to antigens is abnormal in patients with brain tumors. Additional evidence for abnormal cellular immune functions come from studies of mitogen blastogenic response¹⁷. However, studies of alterations of the immune system in children with brain tumors are lacking¹⁸.

In the present study we evaluated the cytotoxic activity of NK cells and T cell subsets in peripheral blood of patients with primary tumors in central nervous system.

**METHOD**

**Patients and controls.**

A group of 13 patients, 6 children and 7 adults with primary tumors in CNS seen at Hospital das Clínicas, Faculdade de Medicina de Botucatu, UNESP, São Paulo, Brazil was included in the study. The age range of children was 5-14 years and of adults was 25-74 years. Two healthy control groups were studied simultaneously, namely 14 people within age range equivalent to children group (C1 = 1-13 years) and 15 people within the age range equivalent to that adult patients (C2 = 19-65 years). The patients included in this study were not on radiotherapy or chemotherapy, neither were receiving corticosteroids. Nevertheless, we admitted patients during preoperative or postoperative phases. The histological typing and sites of CNS tumors, age and sex of the patients are expressed in Table 1. The cytotoxic activity of NK cells was performed in 10 patients. (Patients 2, 3, 4, 5, 6, 7, 8, 10, 11 and 13). Seven patients had tumors of neuroepithelial tissue, two had tumors of meningothelial cells and one had a germ cell tumor (Table 1). Informed consent was obtained from the patients and controls and the authors had approval from the Ethics Comitte of Faculdade de Medicina de Botucatu, SP, to study immunocompetence of patients and controls.
Enumeration of lymphocyte subsets.

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Hypaque density centrifugation and resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 40 mg/ml gentamicin and 10% (v:v) fetal calf serum (complete medium). Approximately 2x10^5 cells were centrifuged onto a poly-l-lysine-coated coverslip and staining reactions carried out at 4°C as described previously. Briefly, after reacting with the monoclonal antibody, the cells were treated with biotinylated horse anti-mouse IgG and fluoresceinated avidin-D and fixed in formaldehyde. At least 500 cells per sample were examined under phase and fluorescence microscopy.

Peripheral blood lymphocyte (PBL) preparations.

Peripheral blood mononuclear cells were obtained as described above. Plastic-adherent cells were depleted by incubating mononuclear cells on plastic Petri dishes (no. 3003, Falcon, Oxnard, California, USA) at 37°C for 60 min. Non-adherent cells were poured from plates after gentle shaking, washed and resuspended in complete medium. These PBL were used as effector cells for the NK cytotoxic assay.

Target cells.

The human erythroleukemic NK-sensitive cell line K562 was used throughout the experiments. The cells were grown to the stationary phase in complete medium. They were subcultured twice weekly and the day before testing. Prior to the tests, viability was assessed by trypan blue exclusion.

<table>
<thead>
<tr>
<th>I.D. Numbers</th>
<th>Tumor type</th>
<th>Classification</th>
<th>Sex</th>
<th>Age</th>
<th>Site of Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primitive neuroectodermal tumor (neuronal and glial immunoreactivity)</td>
<td>Tumors of neuroepithelial tissue</td>
<td>M</td>
<td>25</td>
<td>cerebral hemisphere, cerebellum and brain stem</td>
</tr>
<tr>
<td>2</td>
<td>Pilocytic astrocytoma</td>
<td></td>
<td>M</td>
<td>5</td>
<td>brain stem</td>
</tr>
<tr>
<td>3</td>
<td>Medulloblastoma</td>
<td></td>
<td>M</td>
<td>10</td>
<td>cerebellum</td>
</tr>
<tr>
<td>4</td>
<td>Anaplastic pilocytic astrocytoma, transitional form between tanycytic ependymoma*</td>
<td></td>
<td>M</td>
<td>11</td>
<td>spinal cord (C2-C7)</td>
</tr>
<tr>
<td>5</td>
<td>Anaplastic pilocytic astrocytoma mixed with fibrillary astrocytoma and oligodendrogioma</td>
<td></td>
<td>M</td>
<td>14</td>
<td>suprasellar and optic nerve</td>
</tr>
<tr>
<td>6</td>
<td>Low grade fibrillary astrocytoma</td>
<td></td>
<td>F</td>
<td>11</td>
<td>cerebellum and brain stem</td>
</tr>
<tr>
<td>7</td>
<td>Glioblastoma and high degree astrocytoma</td>
<td></td>
<td>F</td>
<td>74</td>
<td>cerebral hemisphere</td>
</tr>
<tr>
<td>8</td>
<td>Glioblastoma</td>
<td></td>
<td>F</td>
<td>48</td>
<td>cerebral hemisphere</td>
</tr>
<tr>
<td>9</td>
<td>Anaplastic meningioma</td>
<td>Tumors of meningothelial cells</td>
<td>M</td>
<td>60</td>
<td>cerebral hemisphere</td>
</tr>
<tr>
<td>10</td>
<td>Transitional meningioma</td>
<td></td>
<td>F</td>
<td>38</td>
<td>tentorium</td>
</tr>
<tr>
<td>11</td>
<td>Atypical meningioma</td>
<td></td>
<td>F</td>
<td>31</td>
<td>cerebral hemisphere</td>
</tr>
<tr>
<td>12</td>
<td>Transitional meningioma</td>
<td></td>
<td>F</td>
<td>50</td>
<td>cerebra hemisphere</td>
</tr>
<tr>
<td>13</td>
<td>Germinoma</td>
<td>Germ cell tumors</td>
<td>F</td>
<td>9</td>
<td>suprasellar and optic nerve</td>
</tr>
</tbody>
</table>

*electron microscopic analysis

Table 1. Distribution of patients with tumors in central nervous system according to sex, age, type and site of tumor.
Single-cell cytotoxicity assay.

PBL cells and unlabelled K562 cells were used as effector and target cells, respectively. The single-cell cytotoxicity assay on PLL-coated coverslips was performed as described by Vargas-Cortes et al.20 with minor modifications. Effector cell/target cell conjugates were formed by mixing 100ml of the effector cell suspension with an equal volume of target cells, both at a concentration of 1x10^6 cells/ml. After centrifugation at 1000 rpm for 5 min the cell pellet was incubated for 15 min at 37°C in a 5% CO₂ atmosphere. Control slides containing target cells only were prepared in the same way. The percentage of lymphocytes bound to target cells was determined by counting 500 lymphocytes (%TBC). The fraction of conjugates containing dead trypan blue-stained target cells was determined by scoring 100 conjugates. Spontaneous target cell death was determined on lymphocyte-free control coverslips by scoring the fraction of dead (trypan blue-stained) target cells in 300 cells. The fraction of target cell binding lymphocytes that were cytotoxic (A) was calculated as A = B - (B x C), where B is the fraction of conjugates containing dead target cells, and C is the fraction of spontaneously dead target cells as determined from control coverslips. The percentage of NK effector cells present in the lymphocyte sample was calculated as A x %TBC.

Statistical analysis.

The results from patients and control groups were analysed by Student’s t test with the level of significance set at p < 0.05.

RESULTS

The phenotypical distribution of peripheral blood lymphocytes was altered in patients with tumour in CNS (Table 2). In particular the percentages of CD3+ and CD4+ circulating lymphocytes and CD4:CD8 ratio were lower both in children and adults patients in relation to their respective controls of the same age range. In addition, a significant increase in proportion of CD8+ T cells was detected in the children group.

Table 2. Percentage of T cell subsets in patients with tumors in central nervous system.

<table>
<thead>
<tr>
<th>CD+ cell subsets</th>
<th>Children n = 5</th>
<th>Control 1 n = 14</th>
<th>Adults n = 7</th>
<th>Control 2 n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>49.6 ± 9.0*</td>
<td>62.2 ± 9.2</td>
<td>57.6 ± 8.6*</td>
<td>72.6 ± 3.5</td>
</tr>
<tr>
<td>CD4+</td>
<td>29.5 ± 2.7*</td>
<td>40.2 ± 4.2</td>
<td>29.7 ± 9.3*</td>
<td>48.9 ± 4.2</td>
</tr>
<tr>
<td>CD8+</td>
<td>33.0 ± 8.3*</td>
<td>22.4 ± 1.8</td>
<td>25.2 ± 5.7</td>
<td>26.7 ± 3.5</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>0.96 ± 0.2*</td>
<td>1.80 ± 0.2</td>
<td>1.12 ± 0.4*</td>
<td>1.81 ± 0.2</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± 1 standard deviation
*p < 0.05 by Student’s t test

Table 3. Frequencies of lymphocyte forming-conjugates and NK effector cells in peripheral blood lymphocytes of patients with tumors in central nervous system.

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Lymphocyte forming conjugates</th>
<th>% Lymphocyte conjugates with dead target</th>
<th>% NK effector cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n = 10)</td>
<td>6.01 ± 3.06</td>
<td>26.79 ± 14.81</td>
<td>1.38 ± 1.03</td>
</tr>
<tr>
<td>Controls (n = 19)</td>
<td>12.75 ± 1.78</td>
<td>19.36 ± 3.19</td>
<td>2.37 ± 0.46</td>
</tr>
<tr>
<td>Significance*</td>
<td>p &lt; 0.05</td>
<td>NS</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

% lymphocytes forming-conjugates were counted per 500 lymphocytes.
% lymphocyte-target conjugates with dead cells were counted per 100 conjugates.
Spontaneous dead target cells were always below 2% during the assay.
Results are expressed as mean ± 1 standard deviation.
*p Student’s t test.
The cytotoxic activity of NK cells against K562 target is presented in Table 3. The percentage of lymphocytes with the ability to recognize and bind NK-sensitive target cells forming conjugates and the proportion of NK effector cells in the total lymphocyte population were significantly lower compared to the control group. It is noteworthy that 90% (9/10) of the patients presented percentage of NK-K562 conjugates below the minimum value detected in the controls (Fig 1). The values of lytic activity of NK cells from the patients were comparable to the control group. These results suggest that the impairment of NK cell activity in patients was due probably to the diminished percentage of lymphocyte forming conjugates with targets cells.

**DISCUSSION**

This study demonstrated that patients with tumors in CNS presented a significant decrease of NK cell activity compared with control healthy individuals. The low level of NK cytotoxicity in patients reflects a defect in target cell recognition and binding, as indicated by the reduced number of conjugate formation between NK cells and K562 target cells. Although the values of lytic activity of NK cells were comparable to the control group, the defect in conjugate formation contributed to the low proportion of NK effector cells in the total NK population. These results could be observed by the single-cell assay employed that allow a direct microscopic observation of the effector-target cell interaction and an approximation of the number of active NK cells in cell preparation.

Conjugate formation between NK cells and target cells is largely mediated by the binding of NK cell surface molecules of lymphocyte function-associated antigen (LFA-1) (CD11/CD18) and CD2 to their target ligants: intercellular adhesion molecule (ICAM-1) and LFA-3 respectively. After conjugate formation, further recognition events leads to trigger NK cell lytic activity, analogous to the triggering of cytolytic T lymphocyte. Patients with severe deficiency of CD18 chain of LFA-1 adhesion molecule are deficient in NK cell activity. Antibodies directed against various epitopes of this molecule inhibited lysis at the effector cell level by preventing NK-target cell conjugate formation. Thus, the conjugate formation seems to be the initial event that induces the other events implicated in the lytic activity of NK cells and a defect in the NK binding to target cells may be of importance in this process. Probably a defect in adhesion molecules in NK surface could be involved in depression of this cell activity observed in our patients.

NK cell cytotoxic activity is usually depressed in patients with advanced cancer and this depression may be due to mechanisms that regulate NK cell in vivo. The inhibition of NK cell
activity usually occurs as a consequence of tumor invasion, which results in tumor NK cells sequestration as well as to production of cell-growth-related or other molecules produced by the tumoral cells. An immunoregulatory activity on NK cells has been attributed to human brain gangliosides being GM2 and GM3 the most potent inhibitors of human NK activity. The first stage effector-target binding was inhibited by GM2 while GM3 appears to act at a postbinding step in the lytic sequence. Ando et al. showed that GM2 blocked NK effector-target binding in the single-cell assay. Increased concentrations of GM2 and/or GM3 was observed in the circulation of patients with tumors including neuroblastoma and glioma. These gangliosides shedding from growing tumors may result in high concentration in the tumor microenvironment. Their ability to inhibit NK cytotoxicity supports the hypothesis of a role of shed tumor gangliosides in the enhancement of tumor formation and scaps from immune destruction.

Besides NK cell deficiency, patients with tumors in CNS presented a significant fall in the CD4:CD8 ratio, mainly attributed to the decrease in the proportion of helper T cells in peripheral blood. Our results are in accordance to Matsuhisa that showed significant decrease of helper T cells in peripheral blood of patients with malignant glioma. The mechanism for the reduction of CD4+ cells in patients with tumor in CNS is unclear. This could probably occurs as a consequence of these cells trapping in tumor lesions. Lymphocyte infiltration can be observed within the CNS and improved survival has been associated with a greater lymphocytic infiltration. It has already been reported that T cell infiltration is observed in brain tissue of malignant glioma patients. Imunohistochemical analysis of cell subsets of tumor infiltrating lymphocytes in surgical specimens proved that most of these lymphocytes were CD4+ (T helper/inducer) and CD8+ (T suppressor/cytotoxic) cells, but there were too few lymphocytes to kill tumor cells. Thus, although T cell infiltration is observed in brain tumor tissue, the general cellular immunity is suppressed in malignant brain tumor patients. Immunological surveillance studies of possible escape mechanisms from the host's defense immunity may clarify why tumors continue to grow.

The relationship between glial cell tumors and immune response remains unclear. Several studies reported that immunosuppression in patients with gliomas produce anergy of various degrees due to cellular immunity impairment. This impairment is associated with the histological tumor degree. Anaplastic gliomas are related with greater anergy to cutaneous tests in patients and also with lower values of blood T lymphocytes percentages. Nearly half of glioblastoma patients show poor response to mitogens, although patients with low grade tumors are able to mount positive skin test reactions.

The presence of serum bloking factors for lymphocytes activity is also described in patients with malignant tumors. Brooks et al. found that serum from glioma patients inhibited the blastogenesis of normal lymphocytes. A defective expression of cytokines such as interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α) and Interleukin-6 (IL-6) were observed in peripheral blood lymphocytes from glioma patients. It is noteworthy that in vitro cultures of gliomas cells with autologous peripheral blood mononuclear cells (PBMC) of patients produce cytokines with stimulatory (IL-6) and inhibitory (Transforming growth factor beta) activity for PBMC. On the other hand lymphokine-activated PBMC secrete IL-1 α, IL-2, IL-4, IL-6, IFN-γ and TNF-α which may modulate glioma cell proliferation. Glioma cells produce TGF-β with strong inhibition power on activation of T lymphocytes, on the generation of cytotoxic cells activated by lymphokines (LAK cells) and of NK cells. These studies indicated the existence of a glioma-lymphocyte regulatory networks that include both stimulatory and inhibitory factors from both populations, which may modulate tumor progression.

Normal and tumor astrocytes have strong relationship with immune system. Their answer to the effects of the cytokines is almost the same. Antibodies considered specific to NK cells were show to react with normal, embryonary and tumor cells of the CNS. The mechanism and the meaning of this antigenic similarity between glia and lymphoid cells are not well understood.
Meningiomas also produced similar alterations on NK and T cell functions, despite their biological behavior differences when compared with gliomas and to their meningothelial origin. The possible reasons for these findings are unknown.

These considerations would permit to conclude that our results showing the impairment of innate and cell-mediated immunity may be due to neurokines and cytokines produced by tumors and immune system. These cytokine-mediated interactions between malignant gliomas and autologous cells of the immune system may have relevance for further studies on immunotherapy in patients with CNS tumors.

REFERENCES


