Lymphocyte subpopulations and responsiveness in rats bearing intracranial tumors induced by avian sarcoma virus

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The mitogenic responsiveness of peripheral blood lymphocytes obtained from Fischer 344 rats inoculated with avian sarcoma virus was studied. In addition, quantitative alterations in lymphocyte subpopulations were determined in these animals. Only peripheral blood lymphocytes from rats bearing astrocytomas had significantly diminished responses to concanavalin A when compared to control responses. The percentage of lymphocyte subpopulations detected in either the peripheral blood or spleen of tumor-bearing rats did not differ from values obtained with control rats. However, rats bearing astrocytomas had a marked decrease in the absolute number of the various lymphocyte subpopulations as a result of lymphopenia. Neither the sarcoma-bearing rats nor the virus-inoculated rats that did not develop tumors exhibited this lymphopenia. In addition, sera from rats bearing astrocytomas diminished the concanavalin A reactivity of spleen cells obtained from normal rats. The results of this study establish the avian sarcoma virus-induced rat astrocytoma model as a useful immunological parallel for the human disease.

KEY WORDS • brain-tumor immunology • experimental cerebral neoplasm • experimental glioma • sarcoma virus • astrocytoma

PATIENTS with primary intracranial tumors manifest a variety of immunological dysfunctions. Chief among these are the inability to respond to common recall skin-test antigens, impaired in vitro cellular reactivity to mitogens, reduced percentage and absolute number of peripheral blood thymus-derived lymphocytes (T-cells), and the presence of a serum-blocking factor or factors. The fundamental insights into the immunological consequences of this disease gained by studying patients would be greatly facilitated by establishing a representative animal model. Of the animal models available, primary intracranial tumor induced by the avian sarcoma virus (ASV) in the Fischer 344 rat appears most suited for this study, because it has the advantages of a primary tumor with morphology and clinical behavior similar to those of human anaplastic astrocytomas.

The purpose of this report is: 1) to describe the mitogenic responsiveness of peripheral blood lymphocytes obtained from rats bearing tumors elicited by intracranial inoculation of ASV, and 2) to quantitate lymphocyte subpopulations in the peripheral blood and spleen of these animals. The results demonstrate that peripheral blood lymphocytes obtained from rats bearing malignant astrocytomas have decreased mitogenic responsiveness. The absolute number of T-cells within the peripheral blood and spleen is also significantly decreased. In addition, sera from these animals can abrogate the reactivity of spleen cells obtained from normal rats. Lymphocyte blastogenic responsiveness and the number of T-cells of animals harboring nonglial intracranial tumors (sarcomas) were normal. These data, when combined with our previous results, establish the ASV-induced rat astrocytoma model as a useful immunological parallel for the human disease.

Materials and Methods

Animals

Male Fischer 344 rats, 42 days of age, were inoculated with ASV.*

* Rats obtained from Charles River Breeding Laboratory, Wilmington, Massachusetts.
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ulated intracerebrally with 2 μl of ASV as described previously. 

Histopathology

Animals were sacrificed by cardiac puncture under ether-inhalation anesthesia at various intervals from 100 to 120 days after inoculation. Their brains were removed and placed in 10% buffered formalin. After fixation, brains were sectioned in a coronal plane, and whole sections of the cerebral hemispheres were embedded in paraffin. The presence of tumors was determined by gross examination and study of sections stained with hematoxylin and eosin. Also, Masson's trichrome, phosphotungstic acid-hematoxylin (PTAH), and Wilder's reticulum methods were used for classification of tumors. 

Gemistocytic, fibrillary, poorly differentiated, and mixed astrocytomas, fibrosarcomas, and meningial sarcomas were found. Fibrillary and poorly differentiated astrocytomas had prominent PTAH-positive processes. Gemistocytic neoplasms were identified by their abundant, homogeneous, hyaline eosinophilic cytoplasm. Sarcomas were identified by the presence of bundles or sheets of pleomorphic, spindle-shaped cells and a fine network of reticulin fibers surrounding individual tumor cells. Collagen was typically present in these neoplasms. Tumors were restricted to the brain and meninges, and were not found outside the central nervous system. They were found primarily in the cerebral cortex, basal ganglia, and white matter and appeared to originate most frequently from the subependymal region. Tumors used in this study varied in size from 4 × 5 mm to 6 × 9 mm in their greatest diameter.

Lymphocyte Preparation

Heparinized (10 μl/ml) blood obtained by cardiac puncture was layered on a 14% Ficoll-38% Hypaque gradient according to a modification of the technique described by Boyum. After centrifugation at 400 G for 35 minutes, the lymphocyte layer was aspirated, washed twice in RPMI-1640, and adjusted to 2 × 10⁶ cells/ml in RPMI-1640 supplemented with vitamins, nonessential amino acids, penicillin, streptomycin, and 10% heat-inactivated normal rat serum. Contamination with macrophages, as measured by the nonspecific esterase stain, ranged from 2% to 8%. Polymorphonuclear leukocyte contamination was less than 1%. 

Spleen-cell suspensions were prepared in RPMI-1640 as described previously, washed twice, and resuspended to 4 × 10⁶ cells/ml in supplemented RPMI-1640 as described above.

Cell Cultures

Aliquots of 250 μl of the cell suspensions were pipetted into flat-bottomed microculture wells, and 10 μl of various concentrations of concanavalin (Con A) were added. Cell cultures were performed in triplicate. The culture plates were incubated at 37°C in a 5% CO₂-air atmosphere for 56 hours, at which time 0.25 μCi of (³H)-thymidine (specific activity, 6.7 Ci/mmole) was added in a volume of 10 μl. The radioactivity incorporated into DNA was determined 16 hours later by harvesting the cells with an automatic cell harvester, and counting in a liquid scintillation counter. The data were analyzed by the two-tailed Student's t-test for independent means.

Quantitation of Thymus-Derived Lymphocytes (T-Cells)

A horse anti-rat thymocyte serum was extensively absorbed with aggregated rat serum, kidney, liver, bone marrow, and rat red blood cells according to the method of Veit and Feldman. In addition, the antiserum was further absorbed with Murphy-Strum lymphosarcoma cells, which have bone marrow-derived lymphocyte (B-cell) characteristics. This absorbed antiserum, at a 1/10 dilution, was cytotoxic for 95% of rat thymocytes and less than 5% of bone-marrow cells. Moreover, the absorbed antiserum completely abrogated the responsiveness of rat spleen cells to Con A but had no effect on plaque-forming cells generated in an in vitro system to sheep red blood cells (SRBC). An indirect fluorescent antibody assay was employed to quantitate T-cells in the spleen and peripheral blood. Lymphocytes (1 × 10⁶ in 0.1 ml) were reacted with 0.1 ml of 1/10 diluted, absorbed antiserum, incubated at 4°C for 30 minutes, and washed in media containing 0.02% sodium azide. The cells were resuspended in 0.05 ml of a 1/5 dilution of fluorescein-conjugated (Fab')₂ fragments from rabbit anti-horse immunoglobulin G (IgG), incubated at 4°C for 30 minutes, washed with medium containing 0.02% sodium azide, and prepared on coverslip slides. These preparations were examined using a Leitz Orthoplan

† RPMI-1640 manufactured by Microbiological Associates, Bethesda, Maryland.

‡ Microculture wells, No. 3040, manufactured by Falcon Plastics, Oxnard, California, and Con A obtained from Sigma Chemical Co., St. Louis, Missouri.

§ (³H)-thymidine obtained from New England Nuclear, Boston, Massachusetts.

II Automatic cell harvester manufactured by Microbiological Associates, Bethesda, Maryland.

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microscope equipped with a KP 490 interference filter and a K530 barrier field with a Ploem illuminator. Cell determinations were performed in triplicate.

Quantitation of B-Cells

Lymphocytes (2 × 10⁶ in 0.1 ml) were stained with a 1/5 dilution of fluorescein-conjugated (Fab')₂ fragments of a rabbit anti-rat immunoglobulin serum.* The cells were incubated at 4°C for 30 minutes, washed with medium containing 0.02% sodium azide, and prepared on coverslip slides. These preparations were examined as described above.

Quantitation of B-Cells

Lymphocytes (2 × 10⁶ in 0.1 ml) were stained with a 1/5 dilution of fluorescein-conjugated (Fab')₂ fragments of a rabbit anti-rat immunoglobulin serum.* The cells were incubated at 4°C for 30 minutes, washed with medium containing 0.02% sodium azide, and prepared on coverslip slides. These preparations were examined as described above.

Determination of Complement Receptor-Bearing Lymphocytes

A modified method as described by Shevach, et al., 16 was used to determine the percentage of EAC lymphocytes. Briefly, 0.2 ml of lymphocytes (2 × 10⁶) were mixed with 0.2 ml of SRBC previously sensitized with rabbit anti-sheep 19S antibody and complement obtained from C57BL mice.† The mixture was centrifuged at 50 G for 2 minutes and subsequently incubated at 37°C for 30 minutes. To facilitate lymphocyte and monocyte differentiation, euchrysin 3RX$ was added to the suspension prior to coverslip slide preparation as described previously.‡ Simultaneous ultraviolet and light microscopy were used to identify lymphocytes with more than three attached SRBC, which were designated complement, receptor-bearing lymphocyte (CRL)-rosette-forming cells (RFC).

Determination of Fc-Receptor Lymphocytes

The percentage of lymphocytes able to bind SRBC sensitized with rabbit anti-sheep 7S antibody was determined by the method of Paraskevas, et al. 18 Briefly, 0.1 ml lymphocytes (2 × 10⁶) were mixed with 0.1 ml of sensitized SRBC, the mixture centrifuged at 50 G for 2 minutes, and subsequently incubated at room temperature for 30 minutes. Their percentage of RFC was determined as described above.

Results

Splenic Lymphocyte Subpopulations

The percentage of lymphocyte subpopulations was determined in the spleens of animals bearing intracranial astrocytomas and sarcomas, normal controls, and virus-inoculated rats that exhibited no evidence of tumor growth. The results presented in Table 1 demonstrate no significant difference in the percentage of T-cells, B-cells, Fc-receptor lymphocytes (FcL), and CRL among control rats and those bearing intracranial sarcomas. However, rats with astrocytomas exhibited marked lymphopenia (Table 2), which was reflected in the absolute number of lymphocyte subpopulations detectable in the spleen. Thus, these rats had a marked diminution in the absolute number of T-cells, B-cells, FcL, and CRL, when compared to the other groups of animals.

Peripheral Blood Lymphocyte Subpopulations

Experiments similar to those carried out with splenic lymphocytes were performed with peripheral blood lymphocytes. The percentage of the various lymphocyte subpopulations in rats with tumors did not differ from those values obtained from the control rats and virus-inoculated rats that did not develop tumor (Table 3). However, comparison of the absolute

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Comparison of lymphocyte subpopulation percentages in the spleen of control and tumor-bearing rats*</td>
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</table>

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>No. of Rats</th>
<th>T-Cells</th>
<th>B-Cells</th>
<th>FcL</th>
<th>CRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>18</td>
<td>59 ± 0.8</td>
<td>33 ± 1.1</td>
<td>32 ± 1.5</td>
<td>43 ± 2.8</td>
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<tr>
<td>ASV-inoculated, no tumor</td>
<td>5</td>
<td>58 ± 2.6</td>
<td>32 ± 0.9</td>
<td>32 ± 2.2</td>
<td>44 ± 1.6</td>
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<tr>
<td>sarcoma</td>
<td>5</td>
<td>53 ± 2.5</td>
<td>39 ± 3.0</td>
<td>34 ± 5.1</td>
<td>46 ± 3.0</td>
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<tr>
<td>astrocytoma</td>
<td>15</td>
<td>56 ± 1.0</td>
<td>34 ± 1.0</td>
<td>30 ± 3.1</td>
<td>46 ± 1.7</td>
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</tbody>
</table>

* Values are mean ± standard error of the mean. ASV = avian sarcoma virus; T-cells = thymus-derived lymphocytes; B-cells = bone marrow-derived lymphocytes; FcL = Fc-receptor lymphocytes; CRL = complement receptor-bearing lymphocytes.

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tr>
<td>Comparison of absolute number of lymphocyte subpopulations in spleen of normal and tumor-bearing rats*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Nucleated Cell Num- ber (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>230 ± 18</td>
</tr>
<tr>
<td>ASV-inoculated, no tumor</td>
<td>278 ± 23</td>
</tr>
<tr>
<td>sarcoma</td>
<td>252 ± 58</td>
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<tr>
<td>astrocytoma</td>
<td>140 ± 15</td>
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</table>

* Values are mean ± standard error of the mean. ASV = avian sarcoma virus; T-cells = thymus-derived lymphocytes; B-cells = bone marrow-derived lymphocytes; FcL = Fc-receptor lymphocytes; CRL = complement receptor-bearing lymphocytes.

* Rabbit anti-rat immunoglobulin serum obtained from Cappel Labs, Inc., Downington, Pennsylvania.
† C57BL mice obtained from Jackson Laboratories, Bar Harbor, Maine.
‡ Euchrysin 3RX, Lot No. 7101, from Chroma-Gesellschaft, Schmid and Co., Stuttgart-Unterturkheim, West Germany.
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number of lymphocyte subpopulations reveals that rats with astrocytomas have significantly lower values (Table 4). This finding can be attributed to the fact that rats bearing astrocytomas have approximately one-half the number of peripheral blood lymphocytes found in control animals.

**Mitogenic Responsiveness of Peripheral Blood Lymphocytes**

When Con A activation of peripheral blood lymphocytes from the various groups of rats was compared, only the rats with astrocytomas demonstrated decreased responsiveness (Fig. 1). This decreased reactivity was observed over a broad range of Con A concentrations (1 to 50 μg).

**Effect of Sera from Astrocytoma Rats on Lymphocyte Reactivity to Con A**

Experiments were conducted to determine the effect of sera from rats with intracranial astrocytomas on the mitogenic responsiveness of peripheral blood lymphocytes from normal rats. The results presented in Fig. 2 demonstrate that sera obtained from animals with gliomas suppressed Con-A-induced reactivity of normal lymphocytes, while sera obtained from rats with intracranial sarcomas had no effect on cellular responsiveness. The suppression observed cannot be attributed to cytotoxicity because greater than 70% of the cells were viable after culture with sera from rats bearing astrocytomas.

**Discussion**

The results of this study confirm and extend our previous findings that rats bearing ASV-induced astrocytomas have marked impairment of general host

**TABLE 3**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>No. of Rats</th>
<th>T-Cells</th>
<th>B-Cells</th>
<th>FcL</th>
<th>CRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>18</td>
<td>80 ± 1.4</td>
<td>15 ± 0.9</td>
<td>16 ± 1.7</td>
<td>17 ± 1.4</td>
</tr>
<tr>
<td>ASV-inoculated, no tumor</td>
<td>5</td>
<td>79 ± 2.7</td>
<td>17 ± 1.0</td>
<td>17 ± 1.3</td>
<td>16 ± 1.1</td>
</tr>
<tr>
<td>sarcoma</td>
<td>5</td>
<td>78 ± 1.5</td>
<td>18 ± 2.9</td>
<td>18 ± 0.9</td>
<td>16 ± 1.2</td>
</tr>
<tr>
<td>astrocytoma</td>
<td>15</td>
<td>75 ± 2.9</td>
<td>16 ± 2.1</td>
<td>14 ± 1.7</td>
<td>18 ± 2.5</td>
</tr>
</tbody>
</table>

* Values are mean ± standard error of the mean. ASV = avian sarcoma virus; T-cells = thymus-derived lymphocytes; B-cells = bone marrow-derived lymphocytes; FcL = Fc-receptor lymphocytes; CRL = complement receptor-bearing lymphocytes.

**TABLE 4**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Lymphocyte Count (cells/cu mm)</th>
<th>T-Cells</th>
<th>B-Cells</th>
<th>FcL</th>
<th>CRL</th>
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</thead>
<tbody>
<tr>
<td>control</td>
<td>4185 ± 326</td>
<td>3357 ± 263</td>
<td>628 ± 49</td>
<td>670 ± 52</td>
<td>711 ± 55</td>
</tr>
<tr>
<td>ASV-inoculated, no tumor</td>
<td>5070 ± 1148</td>
<td>3905 ± 839</td>
<td>893 ± 214</td>
<td>830 ± 212</td>
<td>803 ± 171</td>
</tr>
<tr>
<td>sarcoma</td>
<td>3166 ± 580</td>
<td>2469 ± 453</td>
<td>598 ± 161</td>
<td>574 ± 101</td>
<td>516 ± 110</td>
</tr>
<tr>
<td>astrocytoma†</td>
<td>2049 ± 339</td>
<td>1494 ± 258</td>
<td>345 ± 54</td>
<td>313 ± 62</td>
<td>417 ± 80</td>
</tr>
</tbody>
</table>

* Values are mean ± standard error of the mean. ASV = avian sarcoma virus; T-cells = thymus-derived lymphocytes; B-cells = bone marrow-derived lymphocytes; FcL = Fc-receptor lymphocytes; CRL = complement receptor-bearing lymphocytes.
† Values obtained from rats bearing astrocytoma were significantly different (p < 0.05) when compared to control values.
FIG. 2. Effect of sera from rats bearing astrocytomas (circles) and sarcomas (squares) on the response of normal rat spleen cells to Con A (concanavalin A). Values represent the mean ± standard error of the mean results of experiments employing sera from six different astrocytoma- and sarcoma-bearing rats each. Control responses were obtained by culturing normal rat spleen cells in 20% normal pooled rat serum (NRS). The effect of sera from tumor-bearing rats on the response of spleen cells to Con A was assessed by culturing the cells in 10% NRS + 10% tumor-bearing serum. The response of normal rat spleen cells was 38,108 ± 3510 and 24,204 ± 2875 counts/minute at 1 and 5 μg of Con A, respectively.

immunity. Accordingly, these animals exhibit: 1) lymphopenia which involves all cellular subpopulations; 2) impaired mitogenic responsiveness of peripheral blood lymphocytes; and 3) the presence of humoral factor(s) capable of abrogating lymphocyte activation. Animals harboring intracranial sarcomas or those not developing tumors after viral inoculations had no immunological abnormality.

The observed decreased cellular mitogenic reactivity cannot be ascribed to gross changes in the percentages of T- and/or B-cells because the absolute number was similar in all cultures. This compensates for any excessive lymphopenia that might result in diminished blastogenesis. Although T-cell depletion alone cannot explain the observed diminished responsiveness, shifts within the T-cell subsets could occur, which would result in altered blastogenic activation. Previously, we have presented data indicating that shifts within the spleen cell T1 and T2 subsets may occur, thereby resulting in attenuated Con A responsiveness. Thus, one explanation of the current results may be that similar shifts occur within the peripheral blood bearing ASV-induced astrocytomas, which result in impaired mitogenic activation.

Another explanation of diminished responsiveness pivots about the generation of T-suppressor cells or suppressor macrophages. Such cellular suppressor mechanisms are generally considered to be capable of broadly abrogating immune responsiveness. Utilizing a transplantable brain-tumor model, we have shown that a subpopulation of macrophages induced in association with tumor growth is capable of suppressing various in vitro correlates of cell-mediated immunity. We are currently investigating the ASV model for the presence of similar suppressor macrophages, as well as T-suppressor cells, in efforts to explain the observed decreased responsiveness.

In addition to these possible cellular modulations, this study indicates that one or more other factors are present which are also capable of markedly influencing immune responsiveness. Accordingly, sera obtained from rats bearing malignant astrocytomas suppress lymphocyte responsiveness while similar humoral suppression was not observed in rats with intracranial sarcomas. Although precise characterization of these factors remains to be elucidated, the sera are not cytotoxic and cannot explain the observed lymphopenia. It is clear, therefore, that at least two factors, cellular and humoral, are detectable in rats with ASV-induced malignant astrocytomas. We are presently investigating the individual and collective role of each in efforts to further delineate the relationship between brain tumors and the immune response.

The uniqueness of these observations results from the finding that diminished host immunocompetence is restricted to animals harboring intracranial tumors of glial origin. Animals bearing intracranial tumors of mesenchymal origin (sarcomas) did not differ immunologically from normal rats or from those inoculated with virus that did not develop neoplasms. Thus, there appears to be a relationship between brain neoplasia and immune responsiveness. Although the central nervous system is considered as an immunologically privileged site, alteration of the blood-brain barrier occurs during malignant degeneration. This allows unimpeded access of the mediators of immunity to brain tissues, and thus neural antigens no longer remain "hidden." It may be hypothesized that immunological modulation in association with glial neoplasm is important in preventing allergic encephalitis which theoretically may occur following exposure of neural antigens to the host's immune system. In sup-
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port of this is the finding that allergic encephalitis is not readily induced in rats bearing ASV malignant astrocytomas.11

Perhaps the most important observation of the present study is that the ASV brain-tumor model is an immunological parallel of humans with histologically similar tumors. Previously, we have demonstrated that the mitogenic response of spleen cells from rats bearing ASV-induced astrocytomas is markedly suppressed.10 The present report extends this observation to include impairments in other immune parameters that correlate with similar findings in patients with malignant brain tumors. Rats bearing astrocytomas thus exhibit a marked lymphopenia similar to that observed in patients.4 In addition, peripheral blood lymphocytes from these animals have significant impairment in their ability to respond to mitogen as reported in patients.14 Moreover, the sera from these animals have the ability to suppress lymphocyte mitogenic activation, which is also a hallmark in patients with malignant brain tumors.2,3 Therefore, this model provides an excellent opportunity to precisely define the immunobiology of primary intracranial tumors and investigate the interrelationship of the central nervous system and the immune system.

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References


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