Macrophages in experimental and human brain tumors

Part 2: Studies of the macrophage content of human brain tumors

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The authors have analyzed 47 tumors of the central nervous system (11 glioblastomas, nine meningiomas, three medulloblastomas, 12 assorted primary neural tumors, and 12 brain metastases) for their content of macrophages. Cell suspensions were prepared by enzymatic digestion and macrophages were quantitated by IgGEAC rosette formation. Adsorption of sensitized indicator cells (EA) to sections of tumor was used as a measure to determine the distribution of IgGFc receptor-positive cells within the tumors and to serve as a control for selective release of IgGFc receptor-positive cells by enzyme digestion. The 11 glioblastomas had a mean macrophage content of 45% (range: 8% to 78%), the nine meningiomas had a mean of 44% (range: 5% to 81%), the three medulloblastomas a mean of 6% (range: 2% to 15%), and the metastatic tumors a mean of 24% (range: 4% to 70%). Adsorption of EA demonstrated that IgGFc receptor-positive cells were distributed throughout the tumor mass, although different types of patterns were observed. There was an excellent correlation between the percent of IgGEAC positive cells in suspensions and the extent of EA adsorption to the tumor sections. Compared to systemic neoplasms, most nervous system tumors have a high macrophage content. It is possible that the high macrophage content of brain tumors is related to their immunogenicity, and may be a partial explanation for the rarity of brain-tumor metastases.

KEY WORDS • neoplasm • macrophage • tumor metastasis • brain-tumor immunology

In Part 1 of this publication we reported our studies of the macrophage content of three experimental rat brain tumors, which exhibited widely different growth characteristics and immunogenicity. When a similar type of analysis was carried out on human non-neural tumors, our results demonstrated that many of the cells comprising such tumors were in fact host-derived macrophages. Since the central nervous system (CNS) appears to differ from other organs in its relation to the immune system, we anticipated that neural tumors might differ from general neoplasms in their macrophage content. Furthermore, since it is well known that CNS tumors differ from other tumors in several important clinical respects (such as their failure to metastasize), we have attempted to correlate the macrophage content of human CNS tumors with such unique features.

Materials and Methods

Tumors

We included in the study all brain tumors removed at this center over a 1-year period. There were 11 glioblastomas, nine meningiomas, 15 other brain tumors of various histopathological types, and 12 tumors metastatic to the brain. Ten primary colon carcinomas were also included for comparison. All tumors were processed in an identical manner. After surgical removal, the fresh tissue was divided, and a portion was frozen in liquid N₂ and stored at -70°C. The remainder of the tissue was subjected to enzymatic digestion to produce single-cell suspensions as described below.
Cellular Analysis of Tumor Suspensions

Enzymatic Digestion. Necrotic tissue was removed from the tumor, the tumor tissue was minced, washed twice with Hank’s balanced salt solution (HBSS), and suspended in 0.25% trypsin diluted in Dulbecco’s phosphate buffered saline (PBS), pH 7.2, without calcium. Trypsinization was allowed to proceed for 60 minutes at 22°C to 24°C, the cell suspension was freed of remaining tissue fragments, mixed 1:1 with nutrient mixture F12 supplemented with antibiotics and 20% fetal bovine serum, sedimented at 50 G for 10 minutes, and resuspended in the same medium. The cell suspensions obtained in this manner consistently had greater than 90% viability as determined by trypan blue exclusion.

IgGEAC Rosette Assay. This assay is a simple sensitive method for detecting cells bearing IgGFc and/or C3 receptors. Sheep erythrocytes (SRBC) stored in Alsever’s solution were used within 2 weeks of receipt. The SRBC were washed three times with veronal gelatin buffer (VGB) and diluted to 5.0%. The 5.0% SRBC suspension was mixed with equal volumes of rabbit anti-SRBC (1 agglutinating unit/ml) and appropriately diluted human serum as a source of complement, incubated for 30 minutes at 37°C, washed three times with VGB, and diluted to 0.5% in HBSS.

The rosette assay was performed by mixing equal volumes of sensitized indicator cells (1.0 × 10^6/ml) with tumor cells and incubating the mixture for 5 minutes at 37°C. The mixture was sedimented at 300 G for 5 minutes, the pellet was resuspended, and the percentage of rosette-positive cells was determined. Cells with at least three attached erythrocytes were counted as positive, and at least 200 cells were counted for each determination. Further, in order to have both a functional and morphological assay of the rosetted cells, they were incubated for 60 minutes to allow erythrophagocytosis, cytocentrifuged,* stained with Wright’s agent, and examined morphologically.

Rosette Assay for Detection of IgGFc Receptor-Positive Cells. Frozen sections of tumor tissue were cut 8 to 12 μ thick, and placed on 22 × 40 mm coverslips. The sections were stored in sealed containers at −20°C and assayed within 1 week.

A 5.0% suspension of washed SRBC was incubated 1:1 with appropriately diluted rabbit anti-SRBC† for 30 minutes at 37°C, washed three times with VGB, and diluted to 1.0% in HBSS. All tumors were assayed with SRBC sensitized with a 1:1000 dilution of rabbit anti-SRBC (agglutination titer-8000, that is, 8 agglutinating units). The immunoglobulin class of the anti-SRBC antibodies in that serum was predominantly IgG. The serum was not fractionated, but passive agglutination studies with SRBC sensitized with sub-agglutinating concentrations of the antibody and goat anti-rabbit IgG established that the anti-SRBC serum contained a high concentration of IgG anti-SRBC.

The section rosette assay was performed with some minor modifications from the method that originally was described by Milgrom, et al.10 The wells of hanging drop-depression slides were filled either with the 1.0% suspension of sensitized indicator cells (EA) or with unsensitized SRBC (E). A coverslip containing the tissue section was placed over the well and the slides were inverted to allow the erythrocytes to settle onto the section and attachment to occur. The reaction mixture then was incubated for 30 minutes at 37°C, returned to the upright position, and incubated again for 30 minutes at 37°C to allow unattached erythrocytes to drop back into the well. The attachment of erythrocytes, either sensitized or unsensitized, was evaluated microscopically both quantitatively and qualitatively, and characteristic areas of the section were photographed. All analyses were performed on coded slides.

Results

Quantitation of Lymphoreticular Cells in Brain Tumor Cell Suspension

A totally unselected population of CNS tumors was assessed in the present study; each tumor that was surgically removed at this Medical Center during the prior 12 months was included, regardless of histological type. Cell suspensions were derived by trypsin digestion from all except one of the tumors. Macrophages were quantitated by IgGEAC rosette formation. The accuracy of that method for macrophage enumeration in tumors has been extensively documented previously. Although granulocytes also possess IgGFc receptors, they generally failed to form rosettes with the IgGEAC reagent.

![Table 1: IgGFc receptor-positive cells in 11 human glioblastomas](image)

*Amount of tumor surface covered by adsorbed EA given as a percentage and based on a scale of 1+ to 4+.

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*Cytocentrifuge manufactured by Shandon Southern Industries, Sewickley, Pennsylvania.
†Rabbit anti-sheep erythrocytes obtained from Baltimore Biological Laboratories, Cockeysville, Maryland.
TABLE 2

IgGFc receptor-positive cells in human meningiomas

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Histological Type</th>
<th>% EAC Rosettes</th>
<th>EA Adsorption</th>
<th>Pattern</th>
<th>Degree*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3252</td>
<td>meningotheliomatous meningioma</td>
<td>5</td>
<td>focal 10%</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>301</td>
<td>meningothelial meningioma</td>
<td>8</td>
<td>not done</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1766</td>
<td>meningioma</td>
<td>26</td>
<td>focal 5-10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>836</td>
<td>meningioma</td>
<td>30</td>
<td>diffuse 75%</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>3805</td>
<td>meningotheliomatous meningioma</td>
<td>50</td>
<td>diffuse 90%</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>7555</td>
<td>meningothelial meningioma</td>
<td>56</td>
<td>diffuse 90%</td>
<td>4+</td>
<td></td>
</tr>
<tr>
<td>4096</td>
<td>meningotheliomatous &amp; fibroblastic</td>
<td>78</td>
<td>diffuse 100%</td>
<td>4+</td>
<td></td>
</tr>
<tr>
<td>280</td>
<td>meningioma</td>
<td>81</td>
<td>diffuse 100%</td>
<td>4+</td>
<td></td>
</tr>
</tbody>
</table>

*Amount of tumor surface covered by adsorbed EA given as a percentage and based on a scale of 1+ to 4+.

The tumors were divided into groups based on their histopathological type. The cell quantitation data from 11 glioblastoma multiforme tumors are presented in Table 1. Macrophages were present in all tumors, and nine had a high macrophage content (> 30%). Adsorption of sensitized erythrocytes (EA) to sections of tumor was used to determine the distribution of IgGFc receptor-positive cells in the intact tumor and to serve as quality control for the validity of the IgGEAC results with cell suspensions. Some of the tumors exhibited a focal pattern of EA adsorption that generally results when foci of receptor-negative tumor cells are separated from areas rich in IgGFc receptor-positive cells. The remainder of the tumors exhibited diffuse EA adsorption which resulted from a mixture of IgGFc receptor-positive with negative cells. There was an excellent correlation between the macrophage percentage (IgGEAC rosettes) derived from the cell suspensions and the degree of EA adsorption to tumor sections (Table 1). Thus, tumors with low numbers of IgGEAC rosettes exhibited limited EA adsorption to tumor sections, and as the IgGEAC rosette percentage increased, the percentage of the tumor surface covered by adsorbed EA increased proportionately.

Nine meningiomas were subjected to cellular analysis and EA adsorption (Table 2). As with the glioblastomas, the meningiomas contained significant numbers of macrophages; seven contained greater than 25% IgGEAC rosette-positive cells. Again there was a good correlation between the IgGEAC data and the EA adsorption results.

The remainder of the primary brain tumors were grouped together and the data are presented in Table 3. The ependymoma, three medulloblastomas, and two of four oligodendrogliomas contained low numbers of macrophages with consistent EA adsorption patterns. The two pituitary tumors also contained low numbers of macrophages with consistent EA adsorption patterns. The remaining tumors all had high
TABLE 4

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Original Tumor</th>
<th>% EAC Rosettes</th>
<th>EA Adsorption</th>
<th>Pattern</th>
<th>Degree*</th>
</tr>
</thead>
<tbody>
<tr>
<td>11260VA</td>
<td>breast adenocarcinoma</td>
<td>23</td>
<td>focal</td>
<td>50%</td>
<td>3+</td>
</tr>
<tr>
<td>947</td>
<td>melanoma</td>
<td>4</td>
<td>diffuse</td>
<td>95%</td>
<td>3+</td>
</tr>
<tr>
<td>8820</td>
<td>bladder carcinoma</td>
<td>7</td>
<td>focal</td>
<td>&lt; 5%</td>
<td>1-</td>
</tr>
<tr>
<td>2246</td>
<td>squamous cell carcinoma, lung</td>
<td>8</td>
<td>focal</td>
<td>30-40%</td>
<td>3+</td>
</tr>
<tr>
<td>1992</td>
<td>oat-cell carcinoma, lung</td>
<td>8</td>
<td>focal</td>
<td>&lt; 5%</td>
<td>3+</td>
</tr>
<tr>
<td>2608</td>
<td>adenocarcinoma (mucin producer)</td>
<td>11</td>
<td>negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9216</td>
<td>poorly differentiated carcinoma</td>
<td>&lt; 14</td>
<td>focal</td>
<td>5%</td>
<td>3+</td>
</tr>
<tr>
<td>513</td>
<td>adenocarcinoma</td>
<td>35</td>
<td>diffuse</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>9957</td>
<td>adenocarcinoma</td>
<td>36</td>
<td>focal</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>9209</td>
<td>melanoma</td>
<td>50</td>
<td>focal</td>
<td>40-50%</td>
<td></td>
</tr>
<tr>
<td>1735</td>
<td>melanoma</td>
<td>70</td>
<td>not done</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Amount of tumor surface covered by adsorbed EA given as a percentage and based on a scale of 1+ to 4+.

A fourth group of tumors was also studied. Those were the tumors that had metastasized to the brain from other primary sites. The cell analyses of the 12 metastatic tumors are presented in Table 4. The macrophage contents of eight of the 12 tumors was relatively low (range: 4% to 23%) but the remaining four tumors all contained high relative numbers of macrophages. There were some inconsistencies between macrophage content and EA adsorption, but it appeared that these could be explained on the basis of granulocyte content. For example, one tumor had 4% macrophages but 95% of the tumor exhibited EA adsorption. Granulocytes formed 67% of the tumor-associated cells. Interestingly, all but two of the metastatic tumors exhibited a focal pattern of EA adsorption; there were distinct foci of IgGFc receptor-negative cells that were separate from the areas rich in IgGFc receptor-positive cells.

**Cellular Relationship**

In Fig. 1, the macrophage content of several groups of tumors are contrasted. Five groups of tumors were included: glioblastomas, meningiomas, medulloblastomas, tumors metastatic to the brain, and a group of non-brain (colon) tumors. The analyses of the macrophage contents of the primary colon carcinomas were performed in exactly the same manner and during the same time period as were the brain tumors. The mean macrophage content of the...
Macrophages in experimental and human brain tumors: Part 2

glioblastomas and meningiomas were the highest among these groups of tumors — 45% and 44%, respectively. The medulloblastoma group had the lowest average macrophage content (mean: 6%). The colon carcinoma group included several tumors with very low macrophage content, but the range was up to 55% and the mean was 14%. The mean macrophage content for the tumors that had metastasized to the brain was 24%.

Qualitative Analyses of Tumor-Associated Macrophages

Qualitative observations were made on the macrophage and tumor-cell populations. Tumor cells demonstrated considerable morphological heterogeneity from tumor to tumor. The tumor cells (IgGEAC rosette-negative cells) were morphologically homogeneous within the majority of tumors, but in several tumors those cells exhibited considerable size variation.

The mononuclear phagocytes from most of the tumors exhibited considerable size heterogeneity. Thus, there was variance from small cells with scant cytoplasm to extremely large cells with abundant vacuolated (foamy) cytoplasm. Most of the rosette-positive cells, however, were in the medium size range (Fig. 2). It would have been impossible to separate macrophages from tumor cells solely on the basis of size in most of the tumors, and likewise it was not possible to accurately separate macrophages from tumor cells on the basis of morphology alone. Finally, the macrophages were heterogeneous with respect to phagocytosis. That is, cells in all size ranges exhibited phagocytosis of opsonized erythrocytes during a 60-minute incubation at 37°C, but other receptor-positive cells of all sizes failed to phagocytose the Ig GEAC (Fig. 2). Thus, phagocytosis could not be used as the sole criterion for identification of tumor macrophages, since some esterase-positive and receptor-positive cells were non-phagocytic.

Discussion

The major observations of the present study of CNS tumors are as follows: 1) Most primary brain tumors have a high relative content of macrophages. As a group, the gliomas and the meningiomas contained the highest number of macrophages while the medulloblastomas contained the lowest. 2) Tumors metastatic to the brain as a group had a relatively low mean macrophage content, but several of the tumors were characterized by significant macrophage infiltration. 3) Macrophages were distributed throughout the brain-tumor tissue as determined by EA adsorption to tumor tissue sections, and there was an excellent correlation between the numbers of IgGEAC-positive cells (macrophages) in tumor-cell suspensions and the degree of EA adsorption to tissue sections. 4) The macrophage population was characterized by considerable morphological and functional heterogeneity.

One must be very cautious in interpreting the significance of these results. As a group, the primary brain tumors contained more macrophages than any other group of tumors that we have studied. The range was higher (up to 80% as compared to 55% for all other non-CNS tumors), and the mean of approximately 45% for gliomas and meningiomas was significantly higher than for other groups of non-neural tumors such as the colon carcinomas (with a mean macrophage content of 14%). These data would suggest that there may be a relationship between the high macrophage content of the CNS tumors and the fact that these tumors only rarely metastasize. This position is further strengthened by the fact that the medulloblastomas, which characteristically seed through the neuraxis,¹⁴ had a mean macrophage content of 6%. However, considerable caution must be exercised because: 1) some of the gliomas and meningiomas had low macrophage contents; 2) other

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FIG. 2. Cytocentrifuged brain-tumor preparation illustrating morphology of cell types demonstrable by rosette assay. The unlabeled small cells are antibody-coated sheep red blood cells, which are adsorbed to the surface of the macrophage on the lower left and have been phagocytized by the other two macrophages illustrated. TC = tumor cell, PM = phagocytic macrophage, and NPM = non-phagocytic macrophage. Wright, × 400.
brain tumors that do not characteristically metastasize, such as oligodendrogliomas, had a low macrophage content; and 3) some of the tumors that were metastatic to the brain were infiltrated by high numbers of macrophages. If, indeed, there is a relationship between macrophage content and metastatic potential, as has been suggested for both experimental and human non-neural tumors, it would appear that the macrophage content is not the sole factor involved.

There have been five reports which have been concerned with the lymphocytic infiltrate in brain tumors. Ridley and Cavanagh examined 93 autopsy glioma specimens and reported that a definite lymphocytic infiltrate was present in 30% of the specimens. Schiffer, et al., studied the lymphoplasmocytic infiltrate in over 200 cases of operated gliomas, and found evidence of lymphocyte infiltration in approximately 50%. Takeuchi and Barnard examined 114 surgical specimens and reported the presence of perivascular lymphocytic cuffing in 28%. More recently, Stavrou, et al., used an indirect immunofluorescence technique to study the infiltrate in five human gliomas, and found that such infiltrates are characteristically present around the periphery of the tumors. In a larger series of 200 patients with glioblastoma, Di Lorenzo, et al., reported that there was a heavy lymphocytic infiltrate in the tumors of four of eight patients who experienced long survival. To our knowledge, there has been no previous study of the macrophage content of human brain tumors.

In experimental studies on rat sarcomas, it has been proposed that the infiltration of macrophages into a tumor is a reflection of an effective immune response of the host to that tumor. In Part I of this paper, we have demonstrated that the macrophage content of several experimental neural tumors is directly related to their immunogenicity. Several studies have demonstrated that human brain tumors are antigenic and capable of eliciting a cell-mediated immune response. Thus, one possible explanation for the high macrophage content of these neural tumors is that, since they initially arise within an "immunologically privileged site," they are strongly antigenic, and consequently when there is a local breakdown of the "blood-brain barrier" due to progressive tumor growth, a specific lymphocyte-mediated immune response occurs that leads to the homing of monocytes to the area of the tumor growth.

From our experiments, it is impossible to determine whether our Fc receptor-positive cells were derived from the activation of brain microglia or from circulating monocytes that entered the tumor after leaving the blood stream. The origin of the macrophages within the brain has been an area of contention for over 50 years. Recent studies using autoradiographic techniques have concluded that most, but not all, brain macrophages are of hematogenous origin. Whatever their derivation, however, other studies have demonstrated that brain macrophages elicited by the implantation of glass coverslips into the brain are characterized by IgG and C-receptor activity. It is, therefore, possible that the tumor macrophages detected in our study had a similar dual origin (namely, from activated blood monocytes and brain microglia), and thus the growth of a primary tumor in an organ with such a high resident macrophage population may be another possible explanation for their higher macrophage content compared to systemic neoplasms.

Other investigators have demonstrated that in rats bearing tumors of varying macrophage content, there was a direct correlation between the macrophage content of the tumor and the host's ability to mount a cutaneous delayed hypersensitivity reaction. This was initially thought to be due to a sequestration of macrophages within the tumor, but more recent work has indicated that such animals in fact show an increase in peripheral monocytes, although these cells are modified so that they no longer enter sites of inflammation. Several studies of the immune response of patients with brain tumor have demonstrated that such patients have subnormal general immune competence as demonstrated by classical assays of delayed hypersensitivity such as response to skin-test antigens and lymphocyte blastogenesis. As macrophages are known to play an essential role in the genesis of such responses, it is possible that the high macrophage content of the patients' brain tumor may be related to their diminished immune response, either by a sequestration of circulating monocytes within the tumor or by some as yet unknown mechanism.

Summary

Forty-seven human CNS tumors of various types were analyzed for their content of macrophages. This analysis has indicated that both glioblastomas and meningiomas have a high content of macrophages, compared to medulloblastomas and brain metastases. It is possible that certain unique clinical and immunological features of brain neoplasms may be related to their high macrophage content.

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References


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