Macrophages in experimental and human brain tumors

Part 1: Studies of the macrophage content of experimental rat brain tumors of varying immunogenicity

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Although the presence of lymphoreticular cells within tumors has been recognized for over 100 years, it is only within the last decade that the concept has arisen that standard histological examination techniques may lead to an underestimation of the true extent of tumor infiltration by lymphoreticular cells, and particularly by macrophages. The macrophage content of certain systemic tumors has been correlated with their immunogenicity and growth characteristics. Since the central nervous system is to some extent an "immunologically privileged site" and contains within it specialized reticuloendothelial cells called microglia, the authors determined the macrophage content of three rodent brain-tumor cell lines, and attempted to correlate this macrophage content with their immunogenicity and growth characteristics. Their findings indicate a direct correlation between the immunogenicity and macrophage content of these three neural tumor cell lines.

KEY WORDS • brain neoplasm • macrophage • microglia • tumor immunology

THE presence of lymphoreticular cells within tumors has been recognized for over 100 years. However, it is only within the past 5 years that the suggestion has been made that standard histological examination leads to an underestimation of the true extent of tumor infiltration by lymphoreticular cells, and particularly macrophages. Using various in vitro criteria, several investigators have demonstrated that a wide variety of experimental animal tumors contain a high relative proportion of host-derived macrophages. None of the tumors that were studied, however, were derived from the central nervous system (CNS). In the past 50 years, considerable evidence has accrued to the effect that the brain is to some extent an “immunologically privileged site,” that is, there appears to be a defect in both the afferent and efferent limbs of the immune response to an antigen placed within the CNS. Furthermore, unlike most peripheral organs, the CNS contains specialized reticuloendothelial cells called microglia, which have been considered by some to be analogous to the macrophages found in other parts of the body.

In this study, we have analyzed the macrophage content of three experimental rat brain-tumor cell lines implanted both subcutaneously and intracerebrally. We have then characterized the growth rate and immunogenicity of each of these tumors in syngeneic rats, and determined the effect on subcutaneous growth rate of treatment with a systemic antimacrophage agent.

Materials and Methods

Tumor Cells

Three tumor cell lines were studied, as follows: 1) 528 is a glioma that was originally induced in an F344 rat by the repeated injection of methylnitrosourea; 2) 

*These cells were kindly provided by Dr. Humberto Cravioto, New York University-Bellevue Medical Center.
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9L is a brain tumor that was produced in a Fisher rat and has variously been characterized as a sarcoma or a gliosarcoma; F is a glioma that was induced by a single dose of ethylnitrosourea.

Tumor Inoculation

All tumors in the present study were produced by the injection of cells maintained in tissue culture. Tumor cells were harvested from monolayers by treatment with 0.25% trypsin. The cells were washed and suspended in Hank's balanced salt solution (HBSS) at the appropriate cell concentration. In the case of subcutaneous tumors, 0.5 ml of a tumor-cell suspension was injected subcutaneously into the backs of male F344 rats weighing 175 gm.

To induce intracerebral tumors, the following technique was used. Male F344 rats were placed in a small-animal stereotaxic frame and a skin incision was made over the right frontal bone. A mark was made on the skull exactly 4.0 mm from the sagittal suture and 1.0 mm posterior to the coronal suture. With a No. 22 needle, a hole was bored through the skull at this point. A second hole was bored partially through the skull 1 mm posterior to this area, and an SC322A microscrew was then anchored to the skull. A specially machined No. 22 needle was then inserted 4.5 mm deep to the skull and then withdrawn to a depth of 3.5 mm. Through an automatic Hamilton syringe connected to this needle by means of polyethylene tubing, exactly 3 µl of tumor-cell suspension containing \(1 \times 10^4\) tumor cells was injected into the brain. After 1 minute, the needle was removed. The opening was then closed with dental cement, which was held in place by the previously placed microscrew. The area was then irrigated with antibiotic solution and the skin was closed with skin clips.

Determination of Immunogenicity

One million cells of each tumor cell line were injected subcutaneously into 24 rats. On the 12th day after inoculation, the tumors were removed by surgical ablation. The animals were left undisturbed for 14 days to check for recurrence, at which time they were inoculated with graded numbers of cells from the same tumors as were originally injected. Similar injections were carried out in rats that had previously been injected with only HBSS. It was not possible to demonstrate the immunogenicity of the 528 tumor using the above methodology, since very high numbers of cells (greater than \(10^7\)) are required to even produce tumor growth, and all such tumors are rejected by Day 9. To demonstrate the immunological nature of such rejection, three animals were given 0.5 ml of rabbit anti-rat lymphocyte serum (ALS) intraperitoneally 2 days before inoculation and twice per week thereafter. The growth rate of these tumors was then compared to the growth rate in animals who had not been immunosuppressed.

To determine if immunological specificity was present, a “crossover” experiment was carried out. In this experiment, six rats that had been previously challenged with an injection of one cell line were subsequently challenged with both the same and an opposite cell line (for instance, those immunized with F tumor cells were challenged with a suspension of either F tumor cells or 9L tumor cells and vice versa). The growth rate of the tumors was then measured and graphs constructed.

Determination of Growth Rate

Various numbers of tumor cells suspended in 0.5 ml of HBSS were injected subcutaneously in the backs of adult F344 rats. The time the tumors first appeared was noted, and at suitable intervals, the tumors were measured in two perpendicular dimensions by the use of a caliper. An approximate average area was then calculated for each tumor by multiplying these two dimensions. The mean size for the six rats used in each group was then calculated for each day and a growth curve was constructed.

Treatment with an Anti-Macrophage Agent

In order to confirm the relationship between the growth rate of the tumor and the macrophage content, a group of 20 rats was treated with a silica suspension (10 mg intravenously of silica dust, particle size less than 5 µ, on the day of tumor injection, and then the same amount intraperitoneally on the first and second days after injection). A similar group of rats received control injections of HBSS on these days. Both groups were injected subcutaneously with \(2 \times 10^4\) 9L tumor cells. The growth rates of these two groups were then compared. A further experiment was then carried out utilizing the intracerebral injection of 9L tumor cells. A group of 20 rats was treated with silica as described above. A similar group was treated with HBSS on these same days. Both groups were then injected with \(2 \times 10^4\) 9L tumor cells intracerebrally by the technique described above.

Quantitation of Macrophages in Tumor-Cell Suspensions

Necrotic tissue was carefully removed from the tumor; the tumor tissue was minced, washed twice with HBSS, and suspended in 0.25% trypsin diluted in...

†These cells were kindly provided by Drs. Marvin Barker and Charles Wilson, Brain Tumor Research Center, University of California at San Francisco.

§Small-animal stereotaxic frame manufactured by Lehigh Valley Electronics, Box 125, Fogelsville, Pennsylvania.

Silica dust obtained from Dowson & Dodson, Ltd., London, England.
Dulbecco's phosphate buffered saline (PBS), pH 7.2, without calcium. Trypsinization was allowed to proceed for 90 minutes at 22° to 24° C while the mixture was stirred in a trypsinization flask. The cell suspension was freed of undigested fragments by filtration through sterile gauze, mixed with an equal volume of F12 media supplemented with antibiotics and 20% fetal bovine serum, sedimented at 50 G for 10 minutes, and resuspended in media. The cell suspensions that were obtained in this manner consistently had greater than 80% viability as determined by trypan blue exclusion.

The percentage of cells in each tumor that could be characterized as macrophages was determined by enumeration of medium to large immunoglobulin IgGFc-receptor positive cells by an EA (sensitized indicator cell, that is, antibody-coated sheep red blood cell) rosette assay. Sheep erythrocytes (SRBC) were washed and coated with two agglutinating units (dilution 1:4000; agglutination titer of 8000) of rabbit anti-SRBC. Equal volumes of an 0.5% suspension of EA and a tumor-cell suspension (1.0 X 10^6 cells/ml) were mixed, incubated at 37° C for 5 minutes, sedimented at 300 G for 5 minutes, and resuspended. The percentage of medium to large rosetted cells was determined by counting at least 200 cells; any cell with at least three attached erythrocytes was considered as positive. Routinely, the rosetted cells were cytocentrifuged,* stained with Wright's agent, and examined to verify that the rosetted cells were macrophages. Under the conditions of the assay, nonspecific agglutination of EA did not occur.

Results

Both the 9L and F tumor cell lines that were used in the present study were fatal following intracerebral inoculations. The minimum number of cells that produced this result was 1 X 10^8 for both F and 9L. In the case of 528, however, intracerebral inoculation of as many as 5 X 10^6 cells did not lead to the death of the animal; since larger numbers of cells could not be concentrated to a suitably small volume for intracerebral injection, the remainder of experiments using this cell line were carried out only subcutaneously.

The measurement of subcutaneous tumor growth of each of these cell lines revealed that the F tumor cell line would produce a progressively growing tumor when as few as 1 X 10^3 cells were injected. These tumors increased in size quite rapidly and led to the death of the host animal in all cases (Fig. 1).

The subcutaneous injection of 1 X 10^6 or more 9L cells produced progressive tumor growth leading to death in all rats tested. Tumor growth was slower and regression occurred by Day 43 in one-third of the rats injected with 5 X 10^6 cells. If 1 X 10^5 cells were injected, then regression occurred in two-thirds of the rats by Day 43. Complete regression occurred if a smaller number of tumor cells was injected subcutaneously (Fig. 2). No growth occurred if less than 3 X 10^7 528 cells were injected subcutaneously. When either this amount or 3 X 10^8 cells were injected subcutaneously, the tumor size reached a maximum at approximately 6 days after injection, and declined progressively, so that by Day 11 no tumor was visible. In the case of recipient rats who had been immunosup-

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*Animals were immunized by a subcutaneous injection of 1 X 10^8 9L and 2.5 X 10^8 F cells, respectively. Resulting tumors were removed and 10 days later these animals, with controls, were challenged with varying doses of cells at a different subcutaneous site. This table represents the number of animals bearing tumors 25 days after challenge.

Table 1

<table>
<thead>
<tr>
<th>9L Tumor</th>
<th>F Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Cells</td>
<td>Control</td>
</tr>
<tr>
<td>1 X 10^4</td>
<td>0/6</td>
</tr>
<tr>
<td>1 X 10^5</td>
<td>1/6</td>
</tr>
<tr>
<td>1 X 10^6</td>
<td>5/6</td>
</tr>
<tr>
<td>1 X 10^7</td>
<td>6/6</td>
</tr>
</tbody>
</table>

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*Trypsinization flask manufactured by Belco Glass Co., Vineland, New Jersey.

pressed with anti-lymphocyte serum, progressive tumor growth occurred (Fig. 3).

The immunogenicity of the tumors F and 9L was evaluated by classical methods. Following surgical removal of F tumors, 92% of the animals exhibited recurrence of tumor at the operative site. The remainder of the animals were partially resistant to a challenge of $1 \times 10^4$ F cells but did in fact exhibit growth in all rats with challenges of $1 \times 10^6$ cells or greater. After surgical removal of 9L tumors, no animals exhibited tumor recurrence. The remainder of the animals were resistant to challenge with up to $1 \times 10^6$ cells (Table 1).

Tests of immunogenic specificity were carried out between tumor cell lines 9L and F. Rats injected with either HBSS or those who had removal of F tumors that had been growing subcutaneously showed a much higher rate of 9L tumor growth than did rats who had previously had a similar tumor surgically removed (Fig. 4). In the case of rats challenged with a cell suspension of F, there was no significant difference in the rate of growth between those rats that had previously had a similar tumor surgically removed, those that had been exposed previously to a 9L tumor, and those that had been previously exposed to HBSS (Fig. 5).

The growth rate of the 9L tumor in the rats treated with silica, a known antimacrophage agent, was greater than that in control rats (Fig. 6). Similarly, rats treated with silica before intracerebral inoculation of tumor cells had a decrease in mean length of survival (mean survival was 31 days in a group of 19 controls, and 22 days in 18 rats treated with silica).

Findings by investigators working with non-brain tumors have indicated that the quantity of macrophages in tumors is a determinant of tumor behavior. Because of the radically different behavior exhibited by the three different neural tumor cell lines, as demonstrated above, we wished to determine the macrophage content of each of them. The F, 9L, and 528 tumors exhibited mean macrophage contents of 9%, 24%, and 57%, respectively. We then compared the macrophage content of the F and 9L tumors when growing intracerebrally. The mean macrophage content of these two tumors growing intracerebrally was 7% and 21%, respectively (Table 2).

**Discussion**

Although many criteria such as morphology, esterase staining, and phagocytosis, have been used to enumerate a cell as a macrophage, previous work by others and in our laboratory has indicated that cells that form rosettes with antibody-coated sheep red blood cells under the conditions of our assay all express the other characteristics of macrophages, although all cells do not express all criteria at all times.

The results of these experiments demonstrate that experimental rat neural tumors, which differ widely in

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**Fig. 2.** Growth rate of tumor cell line 9L when injected subcutaneously into Fisher rats. Each point represents the average tumor size of three rats.

**Fig. 3.** Growth rate of tumor cell line 528 when injected subcutaneously into control rats and rats that were immunosuppressed with 0.5 ml of rabbit anti-rat lymphocyte serum 2 days before injection and twice per week thereafter. Each point represents the average tumor size in three rats.
their growth characteristics and immunogenicity, manifest a concomitant difference in their macrophage content: the most immunogenic tumor contained the highest number of macrophages, while the least immunogenic tumor contained the lowest. In addition, we have not noted any significant difference in the percentage of such cells in tumors that were grown either subcutaneously or intracerebrally.

These findings correlating immunogenicity and macrophage content are in agreement with those of previous workers who studied systemic carcinogen-induced neoplasms, where there also was a strong positive correlation between immunogenicity and macrophage content. It is likely that the tumor-associated macrophages represent a manifestation of the cellular immune response to tumor-associated antigens; that is, T lymphocytes are attracted to the tumors and come in contact with antigen. They then differentiate, multiply, and produce lymphokines, which attract macrophages to the tumor site and cause them to be activated.

Our experiments comparing the subcutaneous growth rate of a neural tumor cell line in control rats and animals treated with silica indicate that macrophages may have an important role in modulating the growth rate of neural tumors. Much previous work indicates that the prime effect of systemically injected silica is to markedly diminish macrophage function. The fact that such an enhanced growth rate (with a decreased rate of sur-
vival) also occurred intracerebrally is consistent with the hypothesis that there is ingress of host-derived macrophages into intracerebral as well as subcutaneous tumors. Other studies have presented convincing data that the macrophages found in experimental systemic tumors are in fact hematogenously derived; that is, if the macrophages were removed from tumor-cell suspensions that were then re-injected into the host, a comparable percentage of macrophages would shortly be found within the tumor. The data from our silica experiments support the interpretation that systemic alteration in macrophage function can lead to a concomitant alteration in macrophage function within an intracerebral neoplasm, which in turn can alter its growth rate. The fact that there was no significant difference in the macrophage contents of the intracerebral and subcutaneous tumors suggests that the tumor-associated immune response is manifested in the brain as well as in systemic sites. This interpretation must be approached cautiously, however, since the manipulation that was required for intracerebral inoculation undoubtedly led to the local breakdown of any barrier that may normally retard the free ingress of host-derived mononuclear cells into the tumor site. The question then remains as to whether systemic macrophages would have been attracted under more normal conditions. To prove this in an animal system it would be necessary to induce and analyze primary autochthonous brain tumors that have been produced by noninvasive means, such as by the transplacental administration of a carcinogen. Although we have not carried out this experiment, we have analyzed the macrophage content of a group of spontaneous human brain tumors (see Part 2 of this study) and our results do suggest that there is also a high percentage of macrophages present in patients with previously unoperated brain tumors.

If the macrophages within the brain tumors represented migration of microglial cells into the tumor from the brain itself, rather than from a hematogenous source, then one would not expect such concordance in the macrophage content between the intracerebral and subcutaneous tumors. The microglial cells of normal rats bear no markers that are characteristic of macrophages, and such cells are unreactive with specific antimacrophage serum. Thus, we believe it more likely that the macrophages we have demonstrated have a systemic derivation, as has been demonstrated for other pathologic lesions of the brain by the use of radioisotopic labeling techniques.

Several questions remain unanswered. The main one is why, despite an obvious cellular response within the tumor, such tumors continue to grow and kill the host. We believe the most likely answer is that the cellular response is “too little, too late.” By the time there are enough effector cells present within the tumor to affect its growth rate, it has already become large enough to have destroyed brain tissue and produced neurological deficits. It is also unclear whether the specific location of these tumor-associated macrophages within the tumor (that is, in the central necrotic area or at the periphery) is important in determining the growth characteristics of the tumor. Finally, it is possible that although the number of macrophages is similar in different tumors, functional activity of such cells may be heterogeneous. For example, if most of the macrophages are highly activated the tumor would be rejected, but if most have suppressed function then there may be tumor progression.

One previous study has been reported wherein macrophages were examined in experimental brain tumors. In this study utilizing morphology as the sole criterion for macrophage identification, numerous “activated” macrophages were present throughout experimental brain tumors that had been induced transplacentally with ethynitrosourea.

Summary

Three experimental rat neural tumors have been shown to differ widely in their growth characteristics and immunogenicity when injected into F344 rats. This difference appears to be correlated with the macrophage content of these tumors, which we believe is a reflection of the host’s immune response to their presence.

References

within experimental carcinomas and sarcomas. Int J Cancer 15:918–932, 1975

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