Site-specific immune response to implanted gliomas

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Object. Immunotherapy for glioblastoma has been uniformly ineffective. The immunological environment of the
brain, with its low expression of major histocompatibility complex (MHC) molecules and limited access for inflamma-
tory cells and humoral immune effectors due to the blood–brain barrier (BBB), may contribute to the failure of immu-
notherapy. The authors hypothesize that brain tumors are protected from immune surveillance by an intact BBB at early
stages of development. To investigate the immunological characteristics of early tumor growth, the authors compared
the host response to a glioma implanted into the brain and into subcutaneous tissue.

Methods. Samples of tumors growing in the brain or subcutaneously in rats were obtained for 7 consecutive days and
were examined immunohistochemically for MHC Class I & II molecules, and for CD4 and CD8 lymphocyte markers.
Additionally, B7-1 costimulatory molecule expression and lymphocyte-specific apoptosis were examined.

Conclusions. On Days 3 and 4 after implantation, brain tumors displayed significantly lower MHC Class II expres-
sion and lymphocytic infiltration (p < 0.05). After Day 5, however, no differences were detected. The MHC Class II
expressing cells within the brain tumors appeared to be infiltrating microglia. Minimal B7-1 expression combined with
lymphocyte-specific apoptosis were detected in both brain and subcutaneous tumors. Low MHC Class II expression and
low lymphocytic infiltration at early time points indicate the importance of the immunologically privileged status of the
brain during early tumor growth. These characteristics disappeared at later time points, possibly because the increas-
ing perturbation of the BBB alters the specific immunological environment of the brain. The lack of B7-1 expression
combined with lymphocyte apoptosis indicates clonal anergy of glioma-infiltrating lymphocytes regardless of implanta-
tion site.

KEY WORDS • inflammation • brain tumor • blood–brain barrier • apoptosis • rat

High-grade gliomas are the most common primary brain tumors in adults. The prognosis for patients suffering from gliomas remains dismal, with a median survival time for patients with anaplastic astrocytomas of 25 to 35 months, and for those with glioblastoma multiforme of 9 to 12 months. Recent advances in surgical approaches for the treatment of cancer, including gliomas, immunotherapy is particularly appealing. The induction of an immune response against cells expressing tumor antigens would allow specific eradication of malignant cells while leaving the normal tissue unaffected. The identification of glioma-specific antigens such as tenascin, gp240, and altered epidermal growth factor receptor isoforms, as well as numerous studies demonstrating the infiltration of human and experimental gliomas by inflammatory cells, have led us to infer that gliomas could be sensitive to a humoral or cell-mediated immune response. Unfortunately, clinical studies in which different approaches of immunotherapy were used have shown that gliomas are resistant to antibody-guided or cellular immunotherapy.

Among other factors, the specific immunological environment of the brain has been suggested as an important reason for the failure of immunotherapy. Early studies in which allogenic tissue grafts were used have indicated that the brain is an immunologically privileged site, although this proposition has been challenged recently. One important factor contributing to the modulation of an immune response in the brain is the BBB, which regulates the entry of inflammatory cells and soluble immunoregulatory molecules into the CNS. In addition, the brain has a low expression of MHC Class I and II molecules compared with other tissues. This low level of expression could compromise the presentation of tumor antigens to MHC-restricted CD4+ or CD8+ lymphocytes. A tumor cell that expresses a foreign antigen but no MHC Class I proteins is protected from direct attack by antigen-specific T cells. Tumors growing within the CNS could potentially avoid humoral and cellular immune response because of a relative lack of MHC-restricted presentation. No studies so far have characterized the specific immunological response

Abbreviations used in this paper: BBB = blood–brain barrier; BSA = bovine serum albumin; CNS = central nervous system; IgG = immunoglobulin G; MHC = major histocompatibility complex; PBS = phosphate-buffered saline; TdT = terminal deoxynu-
cleotidyl transferase; TUNEL = TdT-mediated deoxyuridine tri-
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to a growing tumor in the CNS in contrast with one in a non-CNS site.

Our hypothesis was that tumor growth in the CNS is accompanied by lower MHC Class I and II expression and consequently lower immunopresentation compared with a site outside the CNS. This may explain the organ-specific failure of adoptive immunotherapy for brain tumors. To address this hypothesis, we examined the differences in the immunological response of the host to a malignant glioma implanted into both brain and subcutaneous tissue, with a specific focus on the level of MHC Class I and II expression and the extent of lymphocytic infiltration of the tumors.

Materials and Methods

Tumor Cell Culture

We used S635 rat glioblastoma cells which were grown in Dulbecco modified Eagle medium, 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin to approximately 75% confluence. Before implantation, cells were harvested by trypsinization, washed twice in sterile PBS, and viable cells were counted using the trypan blue dye exclusion method. The cells were then resuspended in sterile PBS to the appropriate concentration. The S635 cell line was derived from a brain tumor that had been chemically induced in Fischer 344 rats (D Bigner, personal communication).

Surgical Procedures

All procedures were performed according to the National Institutes of Health Animal Care and Use Guidelines. Fischer 344 rats were anesthetized using intraperitoneally administered ketamine and xylazine and placed in a stereotactic apparatus. The S635 glioblastoma cells (10^5), which were suspended in 5 μl of sterile PBS, were implanted into the white matter of the right hemisphere (1 mm anterior and 3 mm lateral to the bregma; depth of inoculation 3.5 mm) by using a 10-μl Hamilton subcutaneous syringe with a 32-gauge needle connected to the manipulating arm of the stereotactic apparatus. For the flank tumor model a separate set of animals was anesthetized using 2% isoflurane administered through a face mask. A total of 2 × 10^5 cells suspended in 100 μl of PBS were implanted into the subcutaneous tissue by using a 1-ml syringe and a 28-gauge needle. Preliminary studies in our laboratory demonstrated that this provided a similar tumor size at comparable time intervals in the brain and the subcutaneous space, respectively. The animals were observed daily for signs of impairment, and the size of the subcutaneous tumors was assessed by palpation. From Days 2 to 7 after tumor implantation, five animals were killed each day by decapitation after induction of deep anesthesia, the brain was removed, and the subcutaneous tumors were excised. The tissue was frozen in isopentane precooled on dry ice, embedded in optimal cutting temperature compound, and stored at −70°C.

Immunohistochemical Studies

Twenty-micron cryosections were mounted on silanated slides, fixed with 1% tissue fixative containing 0.1% Triton X-100 for 12 minutes, and washed three times with PBS. Endogenous peroxidase was quenched with 0.5% hydrogen peroxidase in methanol for 20 minutes. To block nonspecific binding, sections were incubated in PBS containing 2% BSA and 2% horse serum. All incubations in the primary antibodies (monoclonal mouse anti–rat MHC Class I clone MRC OX-18, anti–rat MHC Class II clone OX-6, anti–rat macrophage clone ED1, anti–rat CD8 clone MRC OX-8, anti–rat CD4 clone MRC OX-68, and mouse anti–rat CD80 [B7-1]) were performed overnight at 4°C at a 1:500 final dilution. Control sections were incubated on the same slide with nonimmune mouse IgG. Primary antibodies were detected using a rat-absorbed biotinylated anti–mouse IgG secondary antibody followed by the avidin–biotin complex method, according to the supplier’s directions. We used rat thymus and normal brain as positive and negative tissue controls, respectively. The stained slides were reviewed in a blinded fashion by three different investigators on three separate occasions. Rating of the immunohistochemical staining was performed using a four-grade semiquantitative scale (0 = not detected, + = minimal, ++ = moderate, and +++ = intense). Statistical analysis was performed using the Kruskal–Wallis one way analysis of variance on ranks, followed by an all-pairwise multiple comparison (Dunn method).

Lymphocyte-Specific Apoptosis Detection

The detection of apoptotic cells by using the fluorescence TUNEL assay was performed according to the kit supplier’s directions. In brief, sections were fixed in 4% paraformaldehyde for 10 minutes at room temperature. After they were washed in PBS three times for 5 minutes, the sections were incubated in CytoPure solution for 20 minutes at room temperature. After a 5-minute equilibration in the TdT labeling buffer sections were incubated for 30 minutes at 37°C in the TdT reaction buffer, which contained biotinylated dioxynucleoside triphosphate, Mn^2+, and TdT at the appropriate concentrations. Sections were immersed in stop buffer for 5 minutes and washed three times in PBS, then they were incubated in streptavidin-fluorescein solution for 20 minutes at room temperature and washed three times in PBS. Following that procedure, sections were blocked in PBS containing 2% BSA and 2% horse serum for 1 hour. Primary antibody incubation against CD4 and CD8 lymphocyte marker (clones MRC OX-68 and OX-8) was performed at a concentration of 1:100 overnight at 4°C. The primary antibody was detected with a Texas Red–labeled secondary antibody (TI-2000) by using a 1:100 dilution in PBS containing 2% BSA and 2% horse serum. Sections were mounted for immunofluorescence microscopy, for which the appropriate filters were used.

Results

Histopathological Findings, Macrophage Infiltration, and MHC Class I Expression

Hematoxylin and eosin staining of the tumor sections revealed all the morphological signs of a malignant glioma regardless of the site of implantation. The tumors were highly cellular, with marked cyttoplasmic and nuclear pleomorphism, intratumoral necrosis, and mitotic figures. The tumor margins displayed mild-to-moderate infiltration of the adjacent normal tissue by tumor cells (Fig. 1A). Infiltration of the solid tumors by ED-1–positive macrophages was sparse, with only a moderate number detected throughout the tumors. In contrast, a significant number of macrophages was consistently detected in areas of intratumoral...
FIG. 1. Photomicrographs showing histopathological and macrophage infiltration findings. A: Section of S635 glioblastoma obtained 6 days after implantation into the brain. The tumor is highly cellular, with necrotic areas in the center. Note the beginning of infiltration into the adjacent normal brain tissue, indicated by arrows (H & E, original magnification × 5). B: Immunohistochemical staining with the macrophage marker ED-1 reveals a small-to-moderate number of macrophages infiltrating the tumor tissue. Larger numbers of macrophages (arrow) were detected grouped around areas of intratumoral necrosis. Original magnification × 10. C and D: The MHC Class I staining of tumors implanted in the brain (C) or flank (D) was observed to be weakly positive. Note that MHC I staining is concentrated on the wall of small and medium-sized blood vessels in the brain–tumor border area (C, arrows), and in the area of subcutaneous tumors bordering normal skin (D). Original magnification × 20.
necrosis (Fig. 1B). The MHC Class I expression was weak but detectable in association with tumors implanted into both brain and flank (Fig. 1C and D). In the brain, staining was most apparent in the walls of small and medium sized blood vessels in the area immediately bordering the tumors, rather than in the tumor itself (Fig. 1C). In the subcutaneous tumors, the MHC Class I staining was also most apparent in the area of the tumor bordering normal skin tissue (Fig. 1D). No statistically significant difference in the intensity of MHC Class I expression was observed between the two sites of implantation or among the different days of the observation period.

Expression of MHC Class II and Lymphocytic Infiltration

In contrast with MHC Class I, MHC Class II staining revealed significantly higher expression in subcutaneous tumors compared with brain tumors on Days 3 and 4 after implantation (Fig. 2A and B; Fig. 3A). At later time points, however (Days 5 and 6), no difference in the extent of MHC Class II staining was detected (Fig. 3A). Morphologically, most of the MHC Class II-positive cells in the brain tumors appeared to be microglia displaying a small cell body with short, perpendicularly branching cell processes. Corresponding to the MHC Class II expression, significantly more CD4-positive lymphocytes were detected on Days 3 and 4 in the subcutaneous tumor sections compared with the brain tumors (Fig. 2C and D; Fig. 3B). Similarly, compared with the brain lesions, the subcutaneous tumors showed a significantly higher infiltration by CD8-positive lymphocytes at Days 3 and 4 (Fig. 2E and F; Fig. 3C). At all later time points, however, tumors implanted at both sites showed an intense infiltration with CD4- and CD8-positive lymphocytes with no significant difference in the extent of infiltration between brain and subcutaneous tissue (Fig. 3B and C). Whereas in the subcutaneous tumors no significant differences were detected between sections obtained on early and late days, in the brain a significantly higher infiltration with both CD4 and CD8 lymphocytes was detected on Days 5 and 6 compared with Day 3. The lymphocytes were distributed throughout the tumor and contiguous normal tissue with occasional clustering, although no perivascular cuffing was detected.

Expression of B7-1 and Lymphocyte Specific Apoptosis

In both flank and brain tumors, CD80 staining revealed a complete absence of the B7-1 costimulatory molecule during the earlier time points, and weak-to-moderate expression was found only at sites of intratumoral necrosis at later time points (Fig. 4). Double labeling for lymphocytes and apoptosis (TUNEL assay) revealed a large number of apoptotic CD4-positive lymphocytes within the tumor at later time points (Fig. 5); the same pattern was observed for CD8 lymphocytes. The extent of lymphocyte apoptosis correlated with the extent of lymphocyte infiltration over time; hence, only a few apoptotic lymphocytes were found in the brain tumors at Days 3 and 4. At later time points no difference in the extent of lymphocyte apoptosis was detected between brain and flank tumors.

Discussion

Shirai and Murphy and Sturm were the first to observe that the brain reacts differently to transplantation of homologous tissue than most other organs of the body. Further studies by Medawar and Greene confirmed these results and established the brain as an immunologically privileged site. Scheinberg, et al., directly compared the response to a tumor homograft in the brain and in subcutaneous tissue. Whereas none of the subcutaneously implanted tumors survived, 35% of the intracerebral tumors survived and grew expansively, demonstrating a lesser extent of antitumor immune surveillance within the brain. Morantz, et al., demonstrated the lack of immune surveillance in a de novo glioma model in rats. Immunosuppressive treatment applied to rats that grew ethyl nitrosourea–induced primary brain tumors did not alter the incidence of intracerebral tumors, although there was a significant increase in the development of nonneural tumors in organs other than the CNS. The authors concluded that the pharmacological suppression of the immune function did not affect the frequency of intracerebral tumors because immune surveillance is not operative with respect to tumors within the CNS. This lack of immune surveillance within the CNS may explain why immunotherapies for malignant gliomas have failed. In fact, animal studies have shown that, although tumors growing in the lung or liver were effectively treated using adoptive immunotherapy, the same tumors growing in the brain were completely resistant to the treatment.

One reason for the diminished immune response to tumors in the brain may be the low expression of MHC molecules within the CNS. Although MHC Class II expression has been detected in human gliomas, other reports indicate a downregulated expression pattern in astrocytic tumors. Our results show that in the early days of tumor growth, the ones implanted in the brain display significantly lower expression of MHC Class II protein than the same tumors implanted in subcutaneous tissue. Correspondingly, early in its development, the infiltration of the tumor tissue with CD4 and CD8 lymphocytes is significantly lower in the intracerebral tumors compared with those growing in the flank. We propose that these findings reflect the early infiltrative growth of human gliomas in which tumor cells can escape the immune surveillance of the host. At later time points, however, both MHC Class II expression and lymphocyte infiltration (both CD4 and CD8) increase in brain tumors to the same level observed in peripheral tumors.

Another important regulator of the immune response within the CNS is the BBB. The morphological characteristics of the BBB are the paucity of endocytotic vesicles, which limits transcellular flux, and the formation of intercellular tight junctions, which severely restricts paracellular flux as well as movement of inflammatory cells into the CNS. Pollack and Lund have shown in a study in which nonsyngeneic neural grafts were used that all animals in whom the BBB was transiently disrupted with an intracarotid artery infusion of hypertonic mannitol showed increased rejection of intracerebral grafts, compared with saline-infused control animals. In a study from our own laboratory, we demonstrated that alteration of the BBB induced by the chronic application of vascular endothelial growth factor facilitated the immune response to a viral antigen. It is well known that brain tumor–associated vessels lack normal BBB function, which is thought to be the cause of peritumoral edema. Morphological studies in a rat gli-
The oman model demonstrated that, beginning 5 days after implantation the brain tumors grew beyond a diameter of approximately 1 mm and new capillary sprouts began to penetrate the tumor. At that time, the capillaries increasingly lacked the morphological features of the BBB. These findings correspond to our results, which show that starting 5 days after implantation, the differences in MHC Class II expression and lymphocytic infiltration between brain and

![Image](image.png)

**Fig. 2.** Photomicrographs showing a comparison of the early immune response between tumors implanted in the brain (A, C, and E) and in subcutaneous flank tissue (B, D, and F). The tumors shown here were examined at Days 3 and 4 postimplantation.  

A: The MHC Class II expression in the brain tumor is minimal and limited to the area of the tumor. The adjacent normal white matter does not show MHC Class II–expressing cells.  
B: The subcutaneous tumors and the adjacent normal tissue show a marked expression of MHC Class II.  
C: Almost no CD4-positive lymphocytes were detected in the brain tumors.  
D: In contrast, the subcutaneous tumors showed significant infiltration with CD4-positive lymphocytes, particularly at the tumor margins (arrows).  
E and F: The tumors in the brain (E) displayed significantly lower infiltration with CD8-positive lymphocytes compared with the tumors in subcutaneous tissue (F). Original magnification × 10.
flank tumors disappear, suggesting that alteration of the BBB may be a crucial factor for regulation of MHC molecule expression. This is consistent with the observations of Pedersen, et al., which demonstrate that the circumventricular organs in the rat brain, which lack the BBB, display higher MHC Class I and II expression. We propose that the enhanced MHC expression in growing brain tumors is due to the exposure to blood-borne positive regulators; this exposure is normally prevented by the BBB. In particular, treatment with cytokines, including interferon, has been shown to increase MHC Class I and II expression in vivo.

An additional finding in our study was that tumor growth was unaffected in the brain and in the subcutaneous tissue, despite a marked lymphocytic infiltration, a phenomenon that has been demonstrated before. This observation indicates that, although a tumor-specific immune response has been initiated, it is somehow rendered ineffective. Early studies indicate that patients harboring a glioma have a depressed cell-mediated immune response. Further studies have identified a number of potential factors released by glioma cells that may underlie the observed negative modulation of T-cell function.

Recently, the requirement for a second or “costimulatory” signal in addition to the antigen-MHC II complex for the activation of T cells has been established. The best-characterized costimulatory molecules are the family of B7 proteins. Antigen presentation by MHC Class II molecules in the absence of B7 costimulation results in an antigen-specific unresponsiveness and apoptotic T-cell death, which is called clonal anergy. In our study, B7-1 expression was undetectable in both brain and flank tumors, except around necrotic areas at later time points. Correspondingly, lymphocyte-specific apoptosis, which may be induced by the lack of B7-1-mediated costimulation, was clearly evident. It is possible that low levels of B7-1 expression contribute to the lack of an effective immune response against gliomas. This aspect could be exploited therapeutically. Indeed, the functional importance of B7-1 expression for an effective immune response against glioma cells has recently been demonstrated in vitro and in vivo. In those studies, transfection of tumor cells with the B7-1 gene led to primary tumor rejection and the establishment of a sustained immune response against the tumor.
ment of protective immunity against the tumor cells. Induction of B7-1 expression may, therefore, have clinical relevance for improvement of immunotherapy directed against malignant gliomas.

Conclusions

We found significantly lower MHC Class II expression during the early days after tumor cell implantation in tumors implanted in the brain compared with the same tumor cell line implanted subcutaneously. This, combined with correspondingly lower lymphocytic infiltration of the brain tumors, is in accordance with the concept of the brain as an immunologically privileged site. At later time points of tumor growth these site-specific differences disappeared, possibly because of increasing perturbation of the BBB. The tumors at both implantation sites grew uninhibited despite massive lymphocytic infiltration. Within the tumors we detected a tumor-specific lack of B7-1 costimulatory protein and a large number of lymphocytes undergoing cell death via apoptosis. These findings are consistent with the inability of infiltrating lymphocytes to mount an immune response due to clonal anergy of these lymphocytes against tumor cells.

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