Dexamethasone-induced abolition of the inflammatory response in an experimental glioma model: a flow cytometry study

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Object. Commonly used for management of cerebral edema in patients with brain tumors, steroid medications also have immunosuppressive functions. To characterize the effects of steroids on the central nervous system’s response to tumors more clearly, flow cytometry was used to quantify the extent of inflammatory cell infiltration in an immunogenic rat glioma model.

Methods. Freshly prepared 11-day-old intracranial C6 tumors that had been excised from dexamethasone-treated and untreated rats were labeled ex vivo with monoclonal antibodies against CD11b/c, CD45, and CD8a antigens. The extent of microglia (CD11b/c–highly positive, CD45–slightly positive cell), macrophage (CD11b/c–highly positive, CD45–highly positive cell), lymphocyte (CD11b/c–negative, CD45–highly positive cell), and cytotoxic T-cell (CD8a–positive cell) infiltration into each rat’s tumor, tumor periphery, and contralateral tumor-free hemisphere was analyzed using flow cytometry.

Microglia and lymphocytes constituted a significant component of infiltrating cells in this model, comprising 23 ± 3% and 33 ± 5% of viable cells, respectively. Macrophages, on the other hand, accounted for only 9 ± 1% of infiltrating cells.

Treatment of rats with a 7-day course of low-dose dexamethasone (0.1 mg/kg/day) resulted in a greater than 50% inhibition of microglia (p = 0.03) and lymphocyte (p = 0.001) infiltration into tumors. Increasing the dexamethasone dose to 1 mg/kg/day further abolished lymphocyte infiltration (89% inhibition, p = 0.001) but had no additional inhibitory effect on microglia invasion. Macrophage infiltration of tumors was not inhibited at the dexamethasone doses used in this study (p = 0.42).

Conclusions. Flow cytometry is a valuable technique for characterizing tumor-associated inflammatory cells in gliomas. Even at low doses, dexamethasone was found to inhibit significantly the infiltration of brain tumors by lymphocytes and microglia. These findings should be considered when experimental immunotherapeutic strategies are evaluated for clinical application.

Key Words • brain neoplasm • glioma • macrophage • microglia • dexamethasone • flow cytometry
Dexamethasone and immune response of glioma

helpful in designing more effective immune system–based therapies.

Perhaps one obstacle in studying leukocyte trafficking in gliomas is the difficulty associated with quantifying inflammatory cells in tumor samples. Immunohistochemical methods are commonly used to characterize tumor-associated inflammatory cells. The application of this technique, however, is limited by its inability to distinguish cells bearing similar antigens and by its inaccuracy in measuring positive-staining cells. Widely used in the field of immunology, FACS is an alternative method to separate and quantify individual cell types in a heterogeneous cell population. We recently reported the use of this technique in characterizing the extent of tumor-infiltrating inflammatory cells in experimental gliomas. The ability to stain tissue simultaneously with multiple antibodies permits identification of cells carrying similar surface antigens (such as microglia and macrophages) as well as quantification of infiltrating inflammatory cells in glioma models.

To study the role of dexamethasone in the CNS reaction to brain tumors, we used FACS to characterize the immune response in an immunogenic rat glioma model. We noted that, even at low doses, dexamethasone inhibited lymphocyte infiltration into rat C6 tumors. Although the number of tumor-associated microglia and macrophages also decreased, higher doses of dexamethasone were needed to elicit this response.

Materials and Methods

Cell Culture and Antibodies

Rat C6 cells were propagated in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and HEPES (0.01 mol/ml). All antibodies—FITC-conjugated anti–rat CD45 (clone OX-1), PE-conjugated anti–rat CD11b/c (clone OX-42), PE-conjugated anti–rat CD8a (clone OX-8), and FITC-conjugated anti–Ki67 (clone B56)—as well as isotype controls were used at a dilution of 1:100.

Tumor Implantation

All animals were housed and handled in accordance with the University of Wisconsin Research Animal Resources Center guidelines. Cells were harvested by trypsinization, counted, and resuspended in 1.2% methylcellulose. Female Wistar rats weighing 100–200 g each were anesthetized with intraperitoneal administration of ketamine (50–90 g/kg) and xylazine (10 mg/kg), then immobilized in a stereotactic head frame. Through a small burr hole, 5 μl of methylcellulose containing 10^6 tumor cells was injected into the brain at the coronal suture, 4 mm lateral to the midline and 5 mm deep into the left frontal lobe, by using a Hamilton syringe. Sham-operated animals received injections of an equal volume of methylcellulose without tumor cells.

Dexamethasone Treatment

Four days after tumor implantation, the animals were given daily intraperitoneal injections of dexamethasone (0.1 or 1 mg/kg) or PBS (control animals) for a total duration of 7 days until their brains were harvested for flow cytometry.

Tissue Preparation

Approximately 11 days after tumor implantation, the animals were anesthetized and perfused with 300 ml ice-cold PBS containing 1000 U/L heparin. Each rat brain was removed and the tumor, tumor periphery, and contralateral tumor-free hemisphere were isolated. Because of technical inaccuracies encountered during dissection of small tumors, and because of potential invasion into the contralateral hemisphere by larger tumors, only tumors localized to one hemisphere and measuring 3 to 6 mm in diameter were used for the analysis.

Approximately 5-mm^3 portions of each tissue sample were minced and triturated on ice in DMEM containing 10% FBS. Only one sample from each location was processed from each animal. The tissue suspension was forced through a sterile 70-μm filter and centrifuged at 300 G for 5 minutes.

Flow Cytometry

Flow cytometry was used to assess inflammatory cell infiltration in tumors, tumor periphery, and contralateral hemisphere. Following tissue preparation, the cells were resuspended in cold PBS, incubated with the appropriate antibodies for 1 hour at 4°C, washed once in PBS, and resuspended in 250 μl of PBS containing 1 μg/ml propidium iodide. Approximately 10^5 live gated events were assessed on a flow cytometer by using a 15-mW 488-nm-wavelength air-cooled argon ion laser and a No. 635 red diode. Propidium iodide–positive cells and debris, which accounted for 40 to 50% of total detected particles, were excluded from each analysis.

Statistical Analysis

Student’s t-test was performed using commercially available statistical computer software to compare differences in the magnitude of cell infiltration among treatment groups. Differences were considered to be significant at a probability level less than 0.05. Data are expressed as the means ± SD.

Results

Characterization of Glioma-Infiltrating Cells

The C6 glioma model was selected for these studies because this allogeneic glioma model has been shown to be highly immunogenic. Moreover, using FACS, we recently demonstrated that intracranial C6 tumors contain a significant amount of infiltrating cells composed of microglia, macrophages, and lymphocytes. This glioma model, therefore, allowed us to study not only trafficking of lymphocytes, but also of other inflammatory cells. Furthermore, because the C6 cells were negative for CD11b/c and CD45 antigens in vitro, any ex vivo staining of CNS
findings were consistent with those in our previous re-
abundant in tumors than in the tumor periphery. These
contrast to lymphocytes and T cells, which were more
tumor, as there were in the tumors themselves. This was in
there were as many microglia and macrophages in the tu-
hand, comprised only 9

cells, respectively (Fig. 2). Macrophages, on the other
try. The degree of CD45 staining was used to differentiate tumor-
infiltrating macrophages from microglia, as described by Sedg-
wick, et al. Whereas macrophages (red events) stained strongly
with both antibodies, microglia (blue events) demonstrated weaker
CD11b/c staining. The remaining stained cells, that is, CD45–positive
but CD11b/c–negative cells (green events) were used as a rough es-
timate of lymphocyte infiltration.

**Effect of Dexamethasone on Glioma Inflammatory
Reaction**

To examine the effect of steroid medications on inflam-
atory cell infiltration in tumors, the animals were inject-
ed with two different doses of dexamethasone shortly after
tumor implantation. The higher dose of dexamethasone (1
mg/kg/day) was selected because it has been shown to be
effective in reducing cerebral edema in similar animal
models. Although there was no significant change in tu-

**Effect of Dexamethasone on Proliferation of Tumor-
Associated Macrophages**

To test whether the observed decrease in microglia
frequency in dexamethasone-treated animals was due to a
decline in their migration or inhibition of their prolifera-
tion, FACS was used to measure the proliferative index
of CD11b/c–positive cells (microglia and macrophages) and
CD11b/c–negative cells (tumor cells and lymphocytes) by
using Ki67 labeling (Fig. 3). Interestingly, in untreated
animals as many as 45 ± 5% of CD11b/c–positive cells
were found to be Ki67 positive (Fig. 4). When animals
were treated with dexamethasone, microglial cell prolifera-
tion was inhibited by as much as 64% (p = 0.002), sug-
esting that the observed decrease in their accrual in

tumors was due in part to the antiproliferative effect
dexamethasone on these cells. In contrast to CD11b/
c–positive cells, high doses of dexamethasone had no
growth-inhibitory effects on tumor cells (Fig. 4; p = 0.34).

**Discussion**

In the present study, flow cytometry was used to evalu-
ate the effect of dexamethasone on the inflammatory re-

dose. Dexamethasone significantly inhibited infiltration of both
lymphocytes and microglia, and, at higher doses, com-
pletely abolished lymphocyte invasion of these tumors.
Despite the widespread use of steroid medications in neu-
rooncology, to our knowledge this is the first quantitative
report to address the effect of dexamethasone on the traf-
ficking of inflammatory cells in gliomas. Having direct
clinical relevance, these observations also highlight the in-
vestigational value of FACS to the field of neurooncology.

**Application of Flow Cytometry to Glioma Immunology
Research**

A number of studies have documented the presence
of inflammatory cells in brain tumors (for a review see
de Micco). Despite these observations, the exact role of
these cells in glioma biology remains unclear. Although
some investigators have proposed that lymphocytes act as antineoplastic cells (by showing a direct correlation between the extent of their infiltration and the prolonged survival of patients with gliomas),\textsuperscript{4,17} others have failed to substantiate this observation.\textsuperscript{6} One of the explanations for these conflicting reports may be related to the method used in measuring the degree of immune response in glioma samples. Because of the variation in the distribution pattern of inflammatory cells within tumors, and because of the heterogeneous structure of glial cells, quantification of immune cells using histochemical techniques may be imprecise and biased by the tissue site examined. By analyzing a tumor homogenate, FACS can separate specific cell types from a heterogeneous cell population and provide an overall estimation of the number of inflammatory cells in tumor samples. Although variability in tissue yield and possible downregulation of surface receptors during tissue preparation can affect the accuracy of measurements of each cell type, this technique is nevertheless valuable in comparing differences among experimental groups.

Flow cytometry also permits the detection and study of inflammatory cells not previously discernible when using histochemical methods. One such example is the study of microglia, which express surface antigens similar to those of macrophages. The differential expression of CD45, however, has been reported by Sedgwick and co-workers\textsuperscript{22} to be a useful method to distinguish microglia from macrophages in the CNS. We recently applied this technique to characterize and study tumor-associated macrophages in a rat glioma model.\textsuperscript{2} In contrast to macrophages, we noted a marked response of microglia with a wide distribution pattern involving the intracranial tumor, tumor periphery, and contralateral hemisphere. In view of the fact that microglia have been reported to secrete a variety of cytokines, we inferred from these observations that tumor-associated microglia play a role in glioma biology. Although these cells may have an antineoplastic function, secretion of IL-10 by microglia (an immunosuppressive cytokine with glial motility functions) or growth factors (such as basic fibroblast growth factor and insulin-like growth factor–1) can potentially contribute to glioma progression.\textsuperscript{11,18,26,27} Flow cytometry, we believe, is a valuable tool for better characterization of the function of microglia in brain tumors.

In the present study, FACS also allowed us to measure proliferation of both CD11b/c-positive cells (microglia and macrophages) and CD11b/c-negative cells (tumor...
cells and lymphocytes) in the same tissue samples. A proliferative index of 45% in CD11b/c-positive cells strongly indicated that a significant number of microglia and macrophages were actively dividing within the C6 tumors. Although we recently demonstrated active migration of microglial cells toward glioma cells in vitro, this observation offers another explanation for accumulation of microglia and macrophages in experimental gliomas. Perhaps in addition to secretion of chemoattractants, local release of growth factors by glioma cells may also contribute to the accrual of macrophages in these tumors. Furthermore, because most CD11b/c-positive cells expressed low levels of CD45, this finding also suggests that local CNS microglia, and not blood-borne monocytes, accounted for most of the glioma-associated macrophages. If migration of blood-borne monocytes were the only source of glioma macrophages, then one would have expected most CD11b/c-expressing cells to be quiescent and to express high levels of CD45 antigen.

Effect of Dexamethasone on Tumor-Associated Macrophages

More prevalent in C6 tumors, microglia were found to be more prone to dexamethasone inhibition than macrophages. At the doses examined, dexamethasone inhibited infiltration of microglia without influencing macrophage trafficking. To explain the observed decrease in microglia infiltration, the proliferative index of CD11b/c-positive cells (mostly microglia) was compared with that of CD11b/c-negative cells (mostly tumor cells). Although dexamethasone did not affect the propagation of neoplastic cells, it significantly inhibited microglia proliferation. This observation is consistent with other reports demonstrating the inhibition of microglia growth in vitro by dexamethasone. Although dexamethasone has been recently shown to induce apoptosis in monocytes, it is not clear whether programmed cell death also played a role in the observed reduction in tumor-associated microglia.

FIG. 3. Representative histogram of an intracranial C6 tumor stained with CD11b/c and Ki67 antibodies demonstrating active proliferation of CD11b/c-positive cells (upper-right quadrant).

FIG. 4. Bar graph showing proliferative indices of CD11b/c-negative cells (mostly tumor cells) and CD11b/c-positive cells (mostly microglia) in control and dexamethasone (Dex)-treated animals. There is a significant decrease in the proliferation of microglia (p < 0.05) but not tumor cells (p > 0.3) in response to dexamethasone treatment. Each bar represents the means ± SD in the same seven to 10 animals used for measurements of inflammatory reaction.

Effect of Dexamethasone on Lymphocyte Infiltration

The antiinflammatory functions of glucocorticoid agents have been well studied. Steroid medications can inhibit IL-2 production by T cells, suppress natural killer cell function, and induce programmed cell death in immature and activated lymphocytes. Considering these immunosuppressive functions, it was not surprising to see a decrease in lymphocyte infiltration into C6 tumors following dexamethasone administration. The observed complete obliteration of tumor-associated lymphocytes, however, was unexpected and underscores the local immunosuppressive potential of dexamethasone in patients with gliomas.

Clinical Relevance of Findings

Augmentation of the host immune reaction against tumors is a promising treatment strategy that is currently being evaluated for a number of cancers including malignant gliomas. With potential application to humans, generation of tumor-specific cytotoxic lymphocytes accomplished using vaccine strategies is being actively investigated in animal glioma models. Because these experimental strategies rely heavily on cytotoxic lymphocyte migration to tumor sites, any immunosuppressive factor can significantly influence their clinical efficacy. Although the C6 glioma model does not truly represent a primary human brain tumor, our observations may have implications in the development of novel glioma therapies. Because dexamethasone was capable of inhibiting the host immune response to C6 gliomas even at low doses, and because most glioma patients receive dexamethasone for treatment of cerebral edema, we propose that any experimental immune-based therapy should also be tested in dexamethasone-treated animals before its clinical application is considered.

Our observations also point out another possible mechanism by which steroid medications can reduce tumor-induced cerebral edema. By inhibiting the trafficking of inflammatory cells into brain tumors, dexamethasone...
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can also prevent the release of proinflammatory cytokines that can potentially exacerbate brain edema. For example, dexamethasone has been reported to inhibit the release of nitric oxide and a variety of proinflammatory cytokines such as tumor necrosis factor-α and IL-6 by microglia. By reducing microglia trafficking and proliferation and cytokine release, dexamethasone can theoretically diminish the extent of cerebral edema in these tumors. Whether inflammatory cells are a major contributor to the development of cerebral edema in gliomas, however, is not clear and should be examined further.

Conclusions

We have demonstrated that, even at low concentrations, dexamethasone can inhibit the inflammatory response in a glioma model and significantly inhibit the proliferation of tumor-associated macrophages. It would be interesting to examine whether these inhibitory effects are reversible after dexamethasone withdrawal.

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References


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