

JNI 00502

## $(\text{Na}^+ + \text{K}^+)$ -ATPase in $\text{C}_6$ glioma and rat cerebrum

Harold J. Sheedlo, Simon Starosta-Rubinstein,  
Timothy J. Desmond and George J. Siegel

*Department of Neurology, Neuroscience Laboratory Building, University of Michigan,  
Ann Arbor, MI 48104, U.S.A.*

(Received 11 September 1986)

(Revised, received 30 December 1986)

(Accepted 30 December 1986)

*Key words:*  $(\text{Na}^+ + \text{K}^+)$ -ATPase;  $\text{C}_6$  glioma; Immunocytochemistry; (Rat cerebrum); Immunoblot; Glial fibrillary acidic protein; Myelin basic protein

### Summary

The content and distribution of the membrane-bound enzyme  $(\text{Na}^+ + \text{K}^+)$ -ATPase in a rat cerebral  $\text{C}_6$  glioma was determined by immunocytochemistry, immunoblots and enzyme assay. In the  $\text{C}_6$  glioma cell culture  $(\text{Na}^+ + \text{K}^+)$ -ATPase activity was about 20% of  $(\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+)$ -ATPase activity. However,  $(\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+)$ -ATPase activity in the cerebral  $\text{C}_6$  gliomas was very close to  $\text{Mg}^{2+}$  baseline and not significantly increased by  $\text{Na}^+$  and  $\text{K}^+$ . As shown by immunoblotting,  $(\text{Na}^+ + \text{K}^+)$ -ATPase catalytic subunit was detected in excised samples of control cerebrum and as a trace in the intracerebral portions of  $\text{C}_6$  glioma but not at all in the extracranial portions of  $\text{C}_6$  glioma or in  $\text{C}_6$  glioma cell culture.  $(\text{Na}^+ + \text{K}^+)$ -ATPase was not detected immunocytochemically in paraffin sections of the extracranial or intracerebral portions of rat cerebral  $\text{C}_6$  glioma. The absence of staining for  $(\text{Na}^+ + \text{K}^+)$ -ATPase clearly demarcated projections of glioma within normal brain. These results suggest that  $\text{C}_6$  glioma has little if any expression of  $(\text{Na}^+ + \text{K}^+)$ -ATPase in vitro or in vivo. The small amount of enzyme epitope in the intracerebral portions represents contamination by normal cerebrum in the extracts.

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Address for correspondence: Dr. Harold J. Sheedlo, Department of Neurology, Neuroscience Laboratory Building, University of Michigan, 1103 East Huron, Ann Arbor, MI 48104, U.S.A.

## Introduction

The C<sub>6</sub> glioma cell line was chemically induced by *N*-nitrosomethylurea in the rat CNS (Benda et al. 1968). Although the C<sub>6</sub> glioma has been characterized as an astrocytoma (Benda et al. 1971; Embree et al. 1971), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), believed to be a myelin and oligodendrocyte enzyme, is enriched in these cells (Zanetta et al. 1972; McMorris et al. 1985) as is the expected glial fibrillary acid protein (GFAP) (Bissell et al. 1974). Myelin basic protein (MBP) and proteolipid protein (PLP) were also thought to be characteristic of C<sub>6</sub> glioma cells as shown by electrophoresis studies (Volpe et al. 1975); however, a radioimmunoassay study failed to detect MBP in C<sub>6</sub> cultured cells (Sims et al. 1979). The C<sub>6</sub> cell line appears to have the potential to express features of both astrocytes and oligodendrocytes and the phenotype may depend on growth stage and environmental factors (Parker et al. 1980).

(Na<sup>+</sup> + K<sup>+</sup>)-ATPase, a membrane-bound enzyme, functions in the transport of sodium and potassium ions across membranes (Siegel et al. 1981). The enzyme is most heavily concentrated in gray matter in the mammalian CNS (Guth and Albers 1974; Stahl and Broderson 1976).

(Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity in cultured rat astrocytes (0.017–0.037 μmol/min/mg protein) is several-fold lower than in separated populations of rat astrocytes including their processes (0.085–0.25 μmol/min/mg protein) (Kimelberg et al. 1978; Atterwill et al. 1984). In vivo, this enzyme has been demonstrated immunocytochemically at some astrocytes and their processes, especially at the end feet (Wood et al. 1977; Ariyasu et al. 1985) and nodes of Ranvier (Wood et al. 1977; Schwartz et al. 1981; Ariyasu et al. 1985). Oligodendroglia and myelin, on the other hand, have little (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity (Zimmerman and Cammer 1982).

Data on (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity in the C<sub>6</sub> glioma are sparse. C<sub>6</sub> glioma cells have (Na<sup>+</sup> + K<sup>+</sup>)-ATPase specific activity of 0.01–0.02 μmol/min/mg protein (Kimelberg 1974; Volpe et al. 1975; Maltese and Volpe 1979; Elkouby et al. 1982), which is lower than in cultured or separated astrocytes and is only about 5% of activity found in total brain homogenates (Krivanek 1986). In a subcutaneously grown C<sub>6</sub> glioma (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity was determined to be 0.01 μmol/min/mg protein, similar to measurements in culture studies (Embree et al. 1971). Surprisingly, there are no immunocytochemical analyses of the intracerebral C<sub>6</sub> glioma. There is one reported histochemical study of rat spinal malignant astrocytoma showing absent K<sup>+</sup> *p*-nitrophenylphosphatase activity and reduced (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity (Stahl et al. 1978).

Although cells of the C<sub>6</sub> glioma seem more like astrocytes than like oligodendroglia, the relatively low (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity resembles the latter. It is possible that the enzyme in the C<sub>6</sub> glioma cells is present in reduced amounts or is expressed in a form of lower activity. Another possibility is that activity may be altered by in vivo factors. Immunochemical methods might detect enzyme epitopes and help to distinguish among these possibilities.

This study attempted to determine the effect of the rat CNS on the (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity in C<sub>6</sub> glioma homogenates and on the distribution and content of

immunoreactive enzyme in an implanted C<sub>6</sub> glioma compared to in vitro C<sub>6</sub> cultures.

## Materials and methods

### *C<sub>6</sub> glioma culture and transplantation*

The rat C<sub>6</sub> glioma cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in Ham's medium supplemented with 10% fetal calf serum according to published methods (Benda et al. 1968; Starosta-Rubinstein et al. 1987). C<sub>6</sub> cultured cells of 37 passes were used for the cerebral implantation and enzyme studies.

Adult Wistar rats were injected intracerebrally with 2.5  $\mu$ l of the C<sub>6</sub> glioma culture ( $0.5 \times 10^6$  cells). The rats were sacrificed 3 weeks after the C<sub>6</sub> glioma cell implantation (Barker et al. 1973; Starosta-Rubinstein et al. 1987).

### *Tissue preparation*

Rats were sacrificed by guillotine. Control cerebrum (contralateral hemisphere) and separate portions of extracranial (extending from surface of cerebrum through calvarium) and intracerebral C<sub>6</sub> glioma and meninges were removed and each homogenized immediately in 10 volumes of ice-cold 0.32 M sucrose, 1 mM EDTA, pH 7.4. C<sub>6</sub> glioma cell cultures were homogenized similarly in 1 volume of the same buffered sucrose. Adult mice (CBA strain) were decapitated and brains were treated as rat cerebrum. All homogenate samples were stored at  $-70^\circ\text{C}$ . The protein content of the homogenized samples was determined by the method of Lowry et al. (1951).

### *(Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity*

The cerebral, C<sub>6</sub> culture and C<sub>6</sub> glioma samples were assayed for (Na<sup>+</sup> + K<sup>+</sup>)-ATPase specific activity according to the procedure described by Bertoni and Siegel (1978). (Na<sup>+</sup> + K<sup>+</sup>)-ATPase specific activity was determined by subtracting Mg<sup>2+</sup>-ATPase from (Mg<sup>2+</sup> + Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity. Each assay was performed in triplicate with incubation for 10 min at 37°C with 4  $\mu$ g of homogenate protein in media containing 3 mM Tris-ATP, 65 mM Tris-HCl, pH 7.4, 3 mM MgCl with or without 80 mM NaCl and 10 mM KCl (Siegel et al. 1984).

### *Antigen and antisera preparation*

The preparation and characterization of mouse brain (Na<sup>+</sup> + K<sup>+</sup>)-ATPase catalytic subunit antigen and the specificity of the antisera have been described. The antisera are specific for the catalytic subunit on Western blots of whole homogenates and fractions of brain and of purified kidney enzyme (Siegel et al. 1984). The human glial fibrillary acidic protein (GFAP) and bovine myelin basic protein (MBP) antisera, kind gifts of Dr. T.J. Sprinkle (Augusta, GA), have been characterized and described previously (Uyeda et al. 1972) as well as in this laboratory (unpublished).

### *Electrophoresis and immunoblot*

Tissue homogenate samples containing 100  $\mu\text{g}$  protein were added to SDS-gel sample buffer (2.3% SDS) and electrophoresed in an SDS-polyacrylamide slab gel (6% acrylamide) system as described (Laemmli 1970). Companion gels were electrophoresed together and one gel was stained with Coomassie blue while the second gel was electroblotted to nitrocellulose (Towbin et al. 1979; Siegel et al. 1984; Sheedlo and Siegel, 1986). The nitrocellulose sheets were incubated as follows: blocking solution consisting of 5% dry milk solids in 10 mM Tris-HCl, pH 7.4 and 0.9% NaCl (TBS) (Johnson et al. 1984) for 2 h at 37°C, rabbit anti-mouse ( $\text{Na}^+ + \text{K}^+$ )-ATPase antiserum (1:400) for 2 h or overnight (18 h) at 37°C, goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP) (1:2000) for 1 h at 37°C and, lastly, in a solution containing 0.05% 4-chloro-1-naphthol, 0.03% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), 1% methanol and TBS for 3 min. The antisera were diluted with 10% normal goat serum, 5% dry milk solids in TBS. The nitrocellulose sheets were rinsed for 30 min with TBS after each incubation with the primary and secondary antisera.

### *Immunocytochemistry*

Four  $\text{C}_6$  glioma cell-injected rats were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. The cerebrum was sectioned coronally (1–2 mm thick), fixed for an additional 6 h at room temperature and embedded in paraffin.

Paraffin sections (5  $\mu\text{m}$ ) were mounted on gelatin-coated slides, incubated with 1:200 anti-mouse ( $\text{Na}^+ + \text{K}^+$ )-ATPase, 1:250 anti-GFAP, 1:250 anti-MBP antisera or 1:200 rabbit preimmune serum as described for other antisera (Siegel et al. 1984; Sheedlo and Siegel 1986). The secondary antibodies (goat anti-rabbit IgG horseradish peroxidase) were diluted 1:250. All antisera were diluted in 0.1 M phosphate-buffered saline (PBS), pH 7.4 + 1% normal goat serum. The immunostaining was visualized by treating the sections with 0.025% diaminobenzidine (DAB), 0.01%  $\text{H}_2\text{O}_2$  in 0.05 M Tris-HCl, pH 7.6 for 5 min.

## **Results**

### *Enzyme activity*

( $\text{Na}^+ + \text{K}^+$ )-ATPase activity in the  $\text{C}_6$  glioma cells was about 20% of ( $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ )-ATPase activity. However, the extracranial and intracerebral gliomas did not exhibit specific ( $\text{Na}^+ + \text{K}^+$ )-stimulated activity significantly above  $\text{Mg}^{2+}$ -baseline activity. The total ( $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ )-ATPase and  $\text{Mg}^{2+}$ -baseline activities in the extracranial and intracerebral gliomas were about double those of the  $\text{C}_6$  glioma cell culture (Table 1).

### *Immunoblot and electrophoresis*

As revealed by immunoblotting with antiserum against ( $\text{Na}^+ + \text{K}^+$ )-ATPase catalytic polypeptide, homogenates of mouse brain and rat cerebrum showed an immunoreactive band at about 100 kDa corresponding to the catalytic subunit protein (Fig. 1A, lanes 2 and 3). This band is specific for the enzyme in various

TABLE 1

( $\text{Na}^+ + \text{K}^+$ )-ATPase,  $\text{Mg}^{2+}$ -ATPase AND ( $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ )-ATPase ACTIVITY OF  $\text{C}_6$  CULTURE,  $\text{C}_6$  GLIOMA AND RAT CEREBRUM

The units of activity are  $\mu\text{mol Pi released}/\text{min}/\text{mg protein} \pm$  standard error. The number of experiments is listed in parentheses. Rat cerebrum consisted of gray and white matter.

Sample	( $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ )-ATPase	$\text{Mg}^{2+}$ -ATPase	( $\text{Na}^+ + \text{K}^+$ )-ATPase
Rat cerebrum (5)	$0.75 \pm 0.06$	$0.48 \pm 0.06$	$0.28 \pm 0.03$
$\text{C}_6$ glioma cell culture (5)	$0.18 \pm 0.02$	$0.13 \pm 0.00$	$0.04 \pm 0.01$
Extracranial $\text{C}_6$ glioma (3)	$0.36 \pm 0.03$	$0.35 \pm 0.03$	N.S. <sup>a</sup>
Intracerebral $\text{C}_6$ glioma (3)	$0.34 \pm 0.04$	$0.31 \pm 0.04$	N.S. <sup>a</sup>

<sup>a</sup> N.S.: ( $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ )-ATPase activities in the extracranial and intracerebral  $\text{C}_6$  gliomas were not significantly different from their respective  $\text{Mg}^{2+}$ -baseline activities.

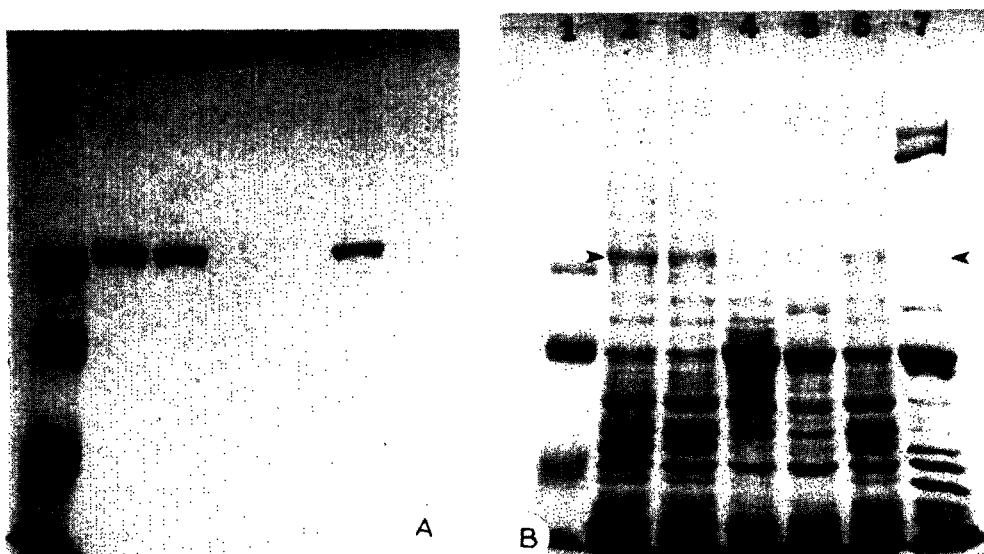


Fig. 1. *A*: Immunoblot analysis of brain homogenates and  $\text{C}_6$  glioma. Each sample consisted of  $100 \mu\text{g}$  protein. Lane 1: protein molecular weight standards — myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa); lane 2: mouse brain homogenate; lane 3: rat cerebral homogenate; lane 4:  $\text{C}_6$  glioma cell culture; lane 5: extracranial  $\text{C}_6$  glioma; lane 6: intracerebral  $\text{C}_6$  glioma; lane 7: meninges. The ( $\text{Na}^+ + \text{K}^+$ )-ATPase catalytic subunit was detected at about the 100 kDa region in the mouse brain and rat cerebral homogenates and intracerebral  $\text{C}_6$  glioma homogenate (lanes 2, 3 and 6, respectively). *B*: Coomassie blue-stained gel of brain homogenates and  $\text{C}_6$  glioma. The lanes are as indicated in Fig. 1*A* and each sample consisted of  $100 \mu\text{g}$  protein. The arrowheads indicate the ( $\text{Na}^+ + \text{K}^+$ )-ATPase catalytic subunit at about 100 kDa in the mouse brain and rat cerebral homogenates and intracerebral  $\text{C}_6$  glioma (lanes 2, 3 and 6, respectively). Very faint protein staining was detected in the 100 kDa region in the  $\text{C}_6$  glioma cell culture and extracranial  $\text{C}_6$  glioma, with no staining in the meninges homogenate.

tissues as shown earlier (Siegel et al. 1984). No other bands were detected by the antisera in whole homogenates of mouse or rat brain on immunoblots even with overnight incubation (Fig. 1A). The intracerebral  $C_6$  glioma showed a faint band at 100 kDa (Fig. 1A, lane 6), while the  $C_6$  glioma culture cells, extracranial  $C_6$  glioma and meninges exhibited no immunostaining for  $(Na^+ + K^+)$ -ATPase (Fig. 1A, lanes 4, 5 and 7). Even when immunoblots were incubated in primary antiserum for an additional 18 h, no immunostained bands were observed in samples of the  $C_6$  culture, extracranial glioma or meninges. The faint immunostaining for  $(Na^+ + K^+)$ -ATPase in the intracerebral glioma homogenate probably resulted from contamination of cerebral tissue in the excised tumor. Because of the interdigitation of the  $C_6$  glioma with the cerebrum, it was impossible to avoid extracting some cerebral tissue together with the  $C_6$  glioma. Hematoxylin-eosin (H&E)-stained paraffin sections of the excised glioma confirmed the  $C_6$  glioma and juxtaposed cerebral tissue (not shown).

The Coomassie blue-stained gel revealed a band at about the 100 kDa region only in homogenates of mouse brain, rat cerebrum and intracerebral  $C_6$  glioma (Fig. 1B, lanes 2, 3 and 6).

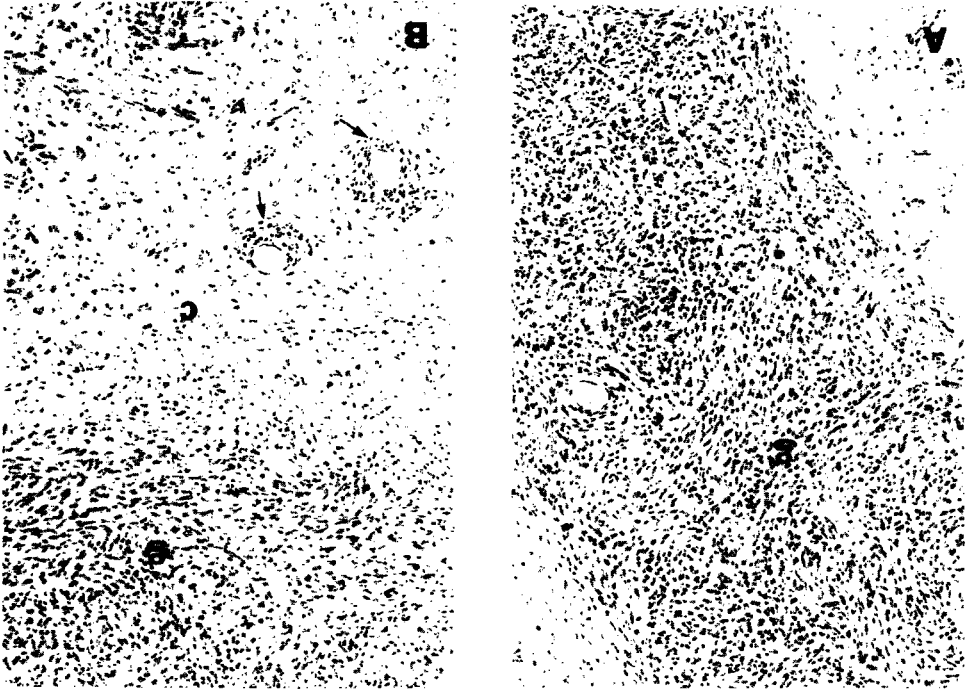


Fig. 2. Hematoxylin-eosin-stained section of  $C_6$  glioma in rat cerebrum. The dense cell mass typifies the  $C_6$  glioma (A, B). The circular cell masses (arrows) in the cerebrum are projections of the  $C_6$  glioma (A). c = rat cerebrum; g =  $C_6$  glioma. Original magnification: A, B,  $\times 120$ .

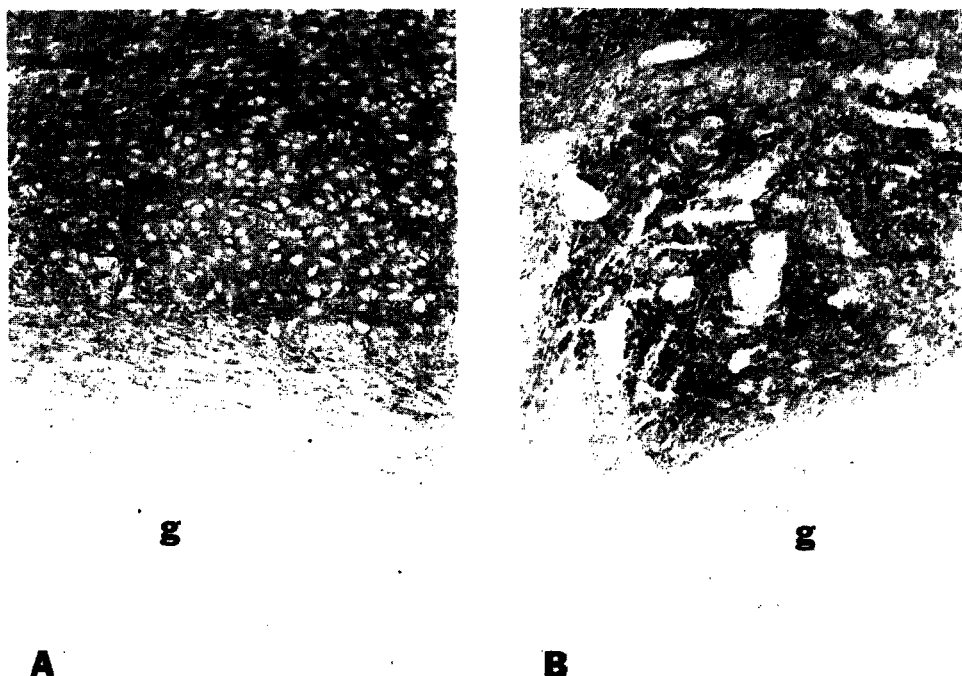


Fig. 3. Immunocytochemical localization of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in  $\text{C}_6$  glioma in rat cerebrum. No immunostaining was detected in the extracranial (A) or intracerebral  $\text{C}_6$  glioma (B). Areas within the rat caudate nucleus (cn) not immunostaining for  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  (arrows) are projections of the  $\text{C}_6$  glioma (B). g =  $\text{C}_6$  glioma; cc = cerebral cortex. Original magnification: A,  $\times 100$ ; B  $\times 80$ .

#### *Immunocytochemistry*

H&E-stained sections revealed a dense distribution of cells in the  $\text{C}_6$  glioma (Fig. 2A and B) and islands of similar cells were embedded within the rat caudate nucleus (Fig. 2B).

$(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  immunostaining in the rat cerebral  $\text{C}_6$  glioma was extremely faint when compared to the staining in the adjacent cerebral region in both the extracranial (Fig. 3A) or the intracerebral  $\text{C}_6$  glioma (Fig. 3B). No cells or cell processes within the  $\text{C}_6$  glioma could be distinguished by  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  immunostaining. Within the caudate nucleus, islands of tissue devoid of immunostain for  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  were observed. These were finger-like projections of the  $\text{C}_6$  glioma into normal parenchyma (Fig. 3B).

The normal appearance of astrocytes stained for GFAP can be seen in the rat caudate nucleus. Typically, the astrocyte cell bodies were large and the processes elongated (Fig. 4A and B). In the  $\text{C}_6$  glioma, in contrast, only short processes and small cell bodies were immunostained for GFAP and the density of stain was fainter than in the surrounding rat cerebrum (Fig. 4A and B).

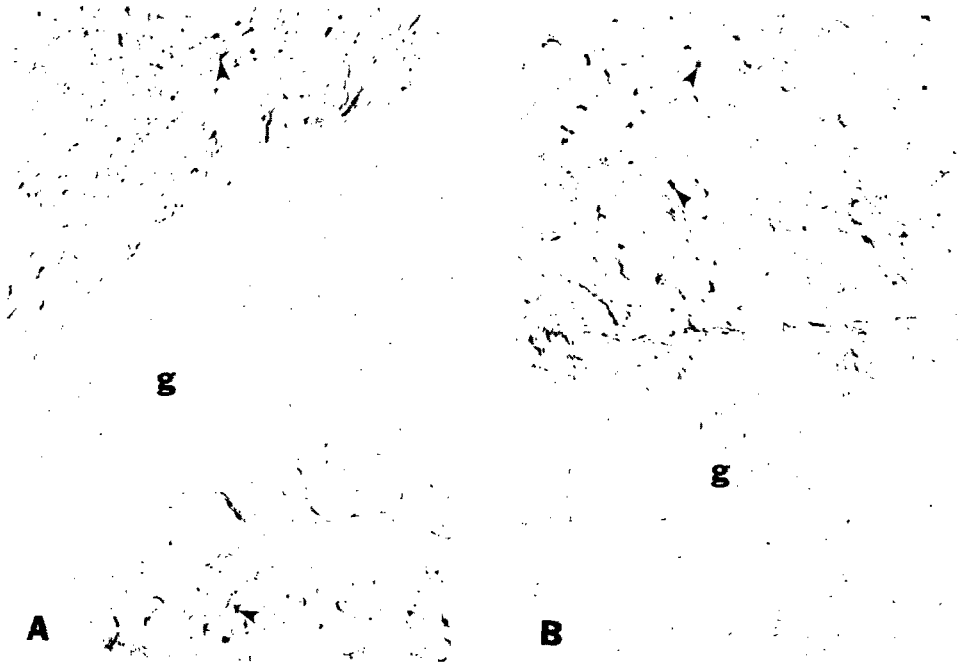


Fig. 4. Immunocytochemical localization of GFAP in  $C_6$  glioma in rat cerebrum. GFAP immunostaining is associated with astrocytes (arrowheads) and their processes in the rat caudate nucleus. Immunostaining for GFAP in the  $C_6$  glioma is very faint and appears to be localized to short processes and small cell bodies (A, B). g =  $C_6$  glioma. Original magnification: A, B,  $\times 120$ .

No MBP-immunostained cellular structures were detected in the  $C_6$  glioma, while, as expected, MBP-immunostained nerve fibers were evident in the rat caudate nucleus (Fig. 5).

Sections of rat cerebrum with implanted  $C_6$  glioma incubated with rabbit preimmune serum showed no specific immunostaining (Fig. 6).

## Discussion

( $Na^+ + K^+$ )-ATPase activity in rat cerebral homogenates, as determined in this study (Table 1), was similar to the activity measured in various whole brain homogenates in other studies (Krivanek 1986). The lack of significant ( $Na^+ + K^+$ )-ATPase activity in the  $C_6$  glioma might be due to altered kinetic characteristics or to decreased concentrations of the enzyme. The near-absence of ( $Na^+ + K^+$ )-ATPase immunostaining on paraffin sections of the cerebrally implanted  $C_6$  glioma (Fig. 3) or on immunoblots (Fig. 1A) is consistent with assays of ( $Na^+ + K^+$ )-ATPase activity and indicates that the concentration of this enzyme in the  $C_6$  glioma is much reduced or absent compared to normal cerebral cortex or separated astrocytes.





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Fig. 5. Immunocytochemical localization of MBP in  $C_6$  glioma in rat cerebrum. No MBP immunostaining is evident in the  $C_6$  glioma, although nerve fibers in the cerebral region surrounding the  $C_6$  glioma are densely immunostained. Regions of the cerebrum not immunostained for MBP are  $C_6$  glioma projections (arrow). g =  $C_6$  glioma. Original magnification:  $\times 120$ .

Absence of MBP immunostaining in the  $C_6$  glioma (Fig. 5) confirms the work of Sims et al. (1979) who detected no MBP in  $C_6$  glioma cells by radioimmunoassay. Myelin-associated glycoprotein (MAG) also could not be detected immunocytochemically in the  $C_6$  glioma in rat cerebrum (unpublished results). Therefore, the  $C_6$  cells do not appear to express features of oligodendrocytes under these conditions.

Immunostaining for the astrocytic marker GFAP in the  $C_6$  glioma was positive although faint when compared to the surrounding rat cerebrum (Fig. 4). Therefore, the  $C_6$  cell may be classified primarily as an astrocyte, although such classifications may be too simplified. Possibly, the amount of GFAP depends on the number of passages in culture as shown for CNP (Parker et al. 1980). The relatively decreased GFAP might reflect an alteration in gene regulation related to oncogenesis, altered cell-cell interactions within the glioma as compared to normal brain, or to passages in cultures. In a study of a human oligodendroglioma, the oligodendrocyte enzyme CNP was very low in the glioma as compared to oligodendrocytes by immunocy-

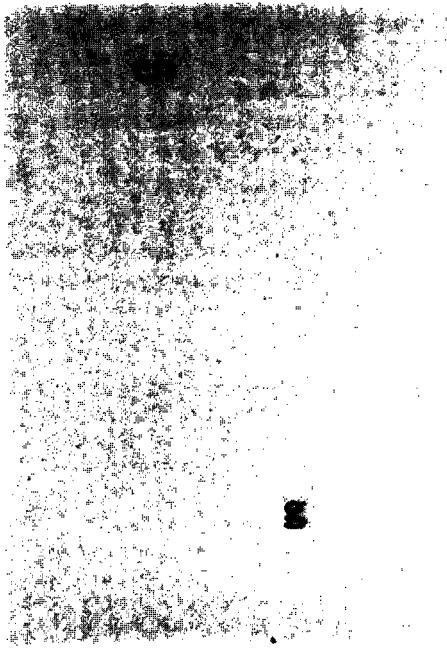


Fig. 6. Immunocytochemical control section of C<sub>6</sub> glioma in rat cerebrum. Replacing the rabbit immune serum with preimmune serum resulted in no immunostaining in the C<sub>6</sub> glioma (g) or the surrounding cerebrum. g = C<sub>6</sub> glioma; cn = caudate nucleus. Original magnification:  $\times 120$ .

tochemistry and enzyme assay (Sheedlo et al. 1985). In the latter case, cell culture and nitrosomethylurea are not factors. These are two examples of gliomas containing low levels of proteins specific for those supposed parent cell types. The reduction in these specific proteins may bear a biological relationship to the naturally occurring glioma. It seems to be part of the reversion to a state of less differentiation.

In conclusion, (Mg<sup>2+</sup> + Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity in homogenates of the cerebral C<sub>6</sub> gliomas was not significantly above Mg<sup>2+</sup>-baseline. (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity of C<sub>6</sub> glioma implanted in rat cerebrum was slightly less than, while Mg<sup>2+</sup>-ATPase activity was more than double that of C<sub>6</sub> glioma cells in culture. Immunoreactivity for (Na<sup>+</sup> + K<sup>+</sup>)-ATPase was almost absent in C<sub>6</sub> glioma in vivo and in vitro, consistent with the greatly reduced enzyme activity. Therefore, the enzyme appears to be expressed little in this cell line under in vitro or in vivo conditions. In addition, the C<sub>6</sub> cell line exhibits very low levels of GFAP immunostaining but none of MBP staining on paraffin sections.

#### Acknowledgements

The authors thank Yukari G. Shichi for secretarial assistance. This research was supported by NIH grants 15935, 07222 and 07838.

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