Ganglioside Composition of an Experimental Mouse Brain Tumor

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ABSTRACT

The ganglioside composition of an experimental ependymoblastoma was examined in C57BL/6 mice. This tumor was produced by Dr. H. Zimmerman in 1949 from methylcholanthrene implantation in the brain and has been maintained in serial transplants through many generations. The influence of tumor environment on ganglioside composition was determined by studying the tumor growing intracerebrally and s.c. (over the skull and in the flank). The ganglioside composition of this tumor is markedly different from that of adult mouse brain. The total ganglioside sialic acid content (µg/100 mg dry weight) of the tumor growing in the cerebral, s.c. over the skull, and in the flank was 70.4 ± 3.8 (N = 3), 66.8 (N = 2), and 41.7 ± 0.7 (N = 3), respectively. These values are about 10-fold lower than the ganglioside content of normal mouse cerebrum. This tumor contains a significant amount of N-glycolyneuraminic acid (NGNA). Histological analysis revealed two basically different cell types. The predominant cell type is densely packed and poorly defined in shape, whereas the minor cell type is less densely packed and fibroblast-like in shape. GM3, which migrates as double bands on thin-layer chromatography, is the predominant ganglioside of this tumor in all three regions of growth. Also present in all regions are gangliosides NANA-GM3 and GM4. Significant amounts of GM1, GT1b, GT1d, and GG0 are present only in the cerebral tumor. These gangliosides therefore represent contaminants from normal brain tissue surrounding the tumor and are not native to the tumor. Ganglioside GD3, however, is a minor component of the tumor. Using a thin-layer chromatography-immunostaining method with anti-GM1 antibody, we found significant amounts of ganglioside with a Galβ1-3GalNAc backbone migrating near GM1 and a low total ganglioside content are also characteristics of this tumor. A preliminary report of these findings has appeared (8).

INTRODUCTION

Gangliosides are a family of sialic acid-containing glycosphingolipids that are enriched in the outer surface of plasma membranes and are most abundant in the central nervous system. Dramatic abnormalities in ganglioside composition are associated with the development of neural tumors in humans. These abnormalities generally involve reductions in total ganglioside concentration and elevations in gangliosides GM3 and GD3 (1–4). Although the relationship of these ganglioside changes to brain tumor formation is not yet clear, Hakamori and Kannagi (5) suggest that tumorigenic changes are closely associated with a loss of growth control and anchorage-dependent cell proliferation; the most common denominator of oncogenesis. A better understanding of these ganglioside changes may provide clues as to the role of gangliosides in neural cell neoplasia. Moreover, knowledge of tumor cell ganglioside composition can be useful for diagnostic and therapeutic purposes.

Little is known about the ganglioside composition of experimental mouse brain tumors. In a brief report, Stoolmiller et al. (6) found that GM1 and GD1 were the predominant gangliosides in four mouse glial tumors. In a series of ethynitrosourea-induced neural tumors in rats, Chou et al. (7) found elevated amounts of GM3. It therefore appears that elevations of GM3 are common to both murine and human neural tumors.

Our purpose was to characterize the ganglioside composition of an experimental ependymoblastoma growing intracerebrally and s.c. in C57BL/6 mice. In contrast to previous findings in other murine tumors, this tumor expresses a high content of gangliosides containing N-glycolyneuraminic acid. An abundance of GM3 and a low total ganglioside content are also characteristics of this tumor. A preliminary report of these findings has appeared (8).

MATERIALS AND METHODS

Mice. The experimental brain tumor used in these studies was obtained as a gift from Dr. Carl Sutton of the University of South Florida, Tampa. The tumor was produced originally by Dr. H. Zimmerman in 1949 from methylcholanthrene implantation into the cerebral cortex of a C57BL/6 (B6) mouse and was classified as an ependymoblastoma (9, 10). The tumor is now maintained in our laboratory through serial i.c. transplants in B6 mice. The procedures of Zimmerman and Arnold (11) were used for i.e. and s.c. transplantation. The B6 mice used for these transplants came from The Jackson Laboratory, Bar Harbor, ME. The animal husbandry conditions were the same as described previously (12).

Adult B6 mice, containing either i.e. or s.c. tumors, were killed by cervical dislocation. Mice containing the i.c. tumors were killed 15 to 25 days after tumor implantation, whereas mice containing the s.c. flank tumors were killed 2 to 3 months after implantation. The i.c. tumors were dissected away from normal surrounding brain tissue. The tumors growing s.c. in the flank were dissected away from connective tissue encapsulation. In addition to studying tumors growing s.c. in the flank, we also studied tumors growing s.c. over the skull. These arose from tumor expansion through the implantation burr-hole in the skull (Fig. 1).

Histological Studies. The procedures of Zimmerman and Arnold (11) were used for the histological examination of the tumor. Briefly, the brain and flank tumor tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin.

Ganglioside Studies. The tumor tissues used for these studies were frozen and then lyophilized to remove water. The gangliosides were then isolated and purified by our previously described methods (12–14). The ganglioside sialic acid content of the tumor was determined by the gas-liquid chromatographic method of Yu and Ledeen (15). This method is useful for detecting both NANA and NGNA. The total content of NANA and NGNA was expressed per 100 mg dry weight of tissue. The distribution of individual tumor gangliosides was analyzed using HPTLC plates (Silica gel 60; E. Merck, Darmstadt, Fed. Rep. Germany) according to the method of Ando et al. (16). The conditions of HPTLC development are described in Figs. 4 and 5. The presence of alkali-labile gangliosides was assessed through a comparison of base-treated (0.1 N NaOH at 37°C for 1 h) and nonbase-treated samples.

Preliminary structural characterization of the tumor gangliosides was performed using anti-GM1 immunostaining on HPTLC plates (17, 18). Briefly, an HPTLC plate containing gangliosides was developed as described in Fig. 4. The plate was then treated with a hexane solution containing 0.4% polysobutylmethacrylate as described (17, 18). Arthrobacter ureafaciens neuraminidase was used to remove sialic acid.

3 H. Zimmerman, personal communication.

2 To whom requests for reprints should be addressed.

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The abbreviations used are: i.e., intracranial; NANA, N-acetylneuraminic acid; NGNA, N-glycolyneuraminic acid; HPTLC, high-performance thin-layer chromatography.
EXPERIMENTAL MOUSE BRAIN TUMOR: GANGLIOSIDE COMPOSITION

Intracerebral Growth

Subcutaneous Growth

Fig. 1. Illustration of tumor grown in the mouse brain. A small piece of tumor tissue was implanted into the right cerebral hemisphere through a burr-hole in the skull. The tumor expended through this burr-hole and grew s.c. over the skull. The ganglioside composition in these growth locations were compared.

residues from the gangliosides. After incubation with anti-GA1 serum, the plate was incubated with 125I-staphylococal protein A. The plate was then exposed to X-ray film and autoradiographed for 48 h at —80°C. This treatment identifies only those gangliosides having an asialo-GM1 or GA1 backbone (glucose-galactose-N-acetylgalactosamine-galactose). After autoradiography, the plate was sprayed with the resorcinol reagent (19) to visualize all gangliosides (Fig. 6). A direct comparison can therefore be made between the same gangliosides visualized by autoradiography and the resorcinol spray. Gangliosides having a GA1 backbone will be visualized by both autoradiography and resorcinol, whereas gangliosides not having a GA1 backbone will be visualized only by the resorcinol reagent (Fig. 6).

RESULTS

The gross morphology and growth pattern of this experimental tumor is similar in brain and flank and consists of a solid, cohesive, nonhemorrhagic mass (Fig. 2, A and B). The tumor can be easily removed from the brain and leaves a well-defined crater. The histological profile is also similar in brain and flank and consists of two basically different cell types. The first cell type, which is the predominant type, is densely packed and varies markedly in size and shape (Fig. 3). These cells show abnormal mitotic figures and contain large irregularly shaped nuclei with complex chromatin networks. The second cell type, which comprises a much smaller percentage of the tumor, is less densely packed and has a long narrow fibroblast-like shape (Fig. 3). These cells show no mitotic activity and contain darkly stained nuclei with poorly defined chromatin structure. Thin strands of wispy blue staining material are observed surrounding these cells. No differences in the number of inflammatory cells were observed between the cerebral and flank tumors. Our preliminary results indicate that this tumor does not stain with labeled antibody to glial fibrillary acid protein.

The total ganglioside concentration of the flank tumor is very low (41.7 µg/100 mg dry weight, Table 1) relative to the concentration found in adult mouse brain (about 400–600 µg/100 mg dry weight). The tumor contains a higher ganglioside content when growing i.c. than when growing s.c. (Table 1). A remarkable feature of this tumor is the presence of N-glycolyl-neuraminic acid. Furthermore, GM3 (hematoside) is the predominant ganglioside of this tumor (Fig. 4) and consists of both GM3-NANA and GM3-NGNA (Fig. 5). The ammonia solvent system is especially useful for separating these different hematoside structures (Fig. 5). Both the NANA and the NGNA hematosides migrate as double bands on the HPTLC plates.

Polysialogangliosides (GD1a, GD1b, GT1a, and GQ1b) appear only in the tumor growing i.c. (Fig. 4, lanes 2 and 3; and Fig. 5, lane 2), and are absent from either of the s.c. growing tumors (Fig. 4, lanes 4–7; and Fig. 5, lanes 3 and 4). Ganglioside GD3, which will migrate above GM1 in the ammonia solvent system, is present in very low amounts (Fig. 5). A ganglioside migrating with GM2-NGNA appears more concentrated in the tumor growing s.c. in the flank than in the tumor growing intracerebrally or s.c. over the skull (Figs. 4 and 5).

Mild base treatment caused slight changes in the distribution of tumor gangliosides. The most noticeable effect was the removal of a minor alkali-labile ganglioside in the region of GM2-NGNA (Fig. 4). This treatment also increased the proportion of GQ1b in the i.c. tumor (Fig. 4, lane 2). The bands migrating above GM1 in Fig. 4 were also alkali labile, but did not stain positive with the resorcinol reagent. These bands may represent small amounts of contaminating phosphatides.

The HPTLC-autoradiogram of the flank tumor gangliosides, immunostained with anti-GA1 antibody, is compared directly with the resorcinol staining of the same gangliosides in Fig. 6. Only those gangliosides with a GA1 backbone will appear on the autoradiogram, whereas all gangliosides will appear after resorcinol staining. Those gangliosides in the mouse cerebral cortex having a GA1 backbone (GM1, GD1a, GT1a, GD1b, GT1b, and GQ1b) are clearly stained with the anti-GA1 antibody. The absence of immunostaining in the GM3 region of the chromatogram is consistent with the absence of a GA1 backbone in GM3.

Fig. 2. Histological appearance of the tumor growing in the brain (A) and s.c. in the flank (B). × 286 (A); × 263 (B).
The intense immunostaining in the brain tumor indicates the presence of \( G_{A1} \)-containing gangliosides. The immunostained double bands in the \( G_{M1} \) region of the chromatogram likely represents \( G_{M1} \) since these bands migrate with \( G_{M3} \) in the water and ammonia solvent systems (Figs. 4 and 5). The structures of the other positively stained \( G_{A1} \)-containing gangliosides are presently unknown. There was no immunostained bands in the flank tumor that migrated with known mouse brain polysialo-gangliosides, \( i.e., G_{D1s}, G_{D1b}, G_{T1b}, \) and \( G_{Q1b} \).

**DISCUSSION**

The neuropathology and growth characteristics of the Zimmerman experimental ependymoblastoma have been well characterized (9, 10, 20), but the classification of this tumor as an ependymoblastoma remains controversial (21–23). Although the histological profile of the tumor is similar in brain and flank, evidence of clear ependymal differentiation is difficult to see (Fig. 2) and (24). Moreover, the presence of two distinct cell populations (Fig. 3) indicates that the tumor is heterogeneous or mixed. The failure of the tumor to stain with antibody to glial fibrillary acid protein indicates that it is not of glial cell origin or lacks glial cell differentiation. We would therefore agree with Rubinstein (22) and Yates\(^5\) that this tumor is best classified as being poorly differentiated.

A remarkable feature of this tumor is its high concentration of NGNA-containing gangliosides. \( N\)-Glycolyneuraminic acid differs from \( N\)-acetyleneuraminic acid in having a glycolyl group instead of an acetyl group attached to the nitrogen on carbon 5 (25). The presence of NGNA-containing gangliosides has not been reported previously in mammalian brain tumors. The absolute amount of NGNA is relatively constant in the three regions of tumor growth (Table 1). Since \( G_{M3} \)-NGNA is also a prominent ganglioside in this tumor (Fig. 4), it likely represents a substantial proportion of the total tumor NGNA content.

The origin of the NGNA-containing gangliosides in this tumor is presently unknown. NGNA is not found in gangliosides of normal mouse brain,\(^6\) but is present in gangliosides of such nonneural tissues as liver (26) and erythrocytes (27). Because this tumor is mostly nonhemorrhagic, it is unlikely that the NGNA arises from erythrocyte contamination. Moreover, mouse erythrocytes contain \( G_{M4} \), which is not detectable in this tumor. Since the tumor does not show either glial or ependymal differentiation, we cannot rule out the possibility that the NGNA comes from nonneural cellular components. Support for this contention comes from our histological findings that the tumor contains a minor population of cells with a fibroblast-like morphology. This raises the possibility that the NANA and NGNA reflect the biochemical diversity of the two major cell types; with the NANA coming from the more abundant densely packed cells and the NGNA coming from the less abundant fibroblast-like cells.

On the other hand, the NGNA may arise from altered sialic metabolism as a consequence of malignant transformation. Support for this contention comes from findings of NGNA in HeLa cells (28), and of \( G_{M3} \)-NGNA in human colon carcinoma (29, 30). Because NGNA-containing gangliosides are not present in normal human tissues (30), the appearance of \( G_{M3} \)-NGNA in colon carcinoma may be associated with malignant transformation. In view of the potentially important role of \( N\)-glycolylation in cellular recognition, differentiation, and malignant transformation (25), further studies are needed on the composition and localization of NGNA-containing gangliosides in this and other murine brain tumors.

Another interesting feature of this tumor is its very low total ganglioside concentration. The concentration of NANA in the s.c. tumors (30–56 \( \mu \)g/100 mg dry weight) (Table 1) is about 10-fold lower than the concentration found in normal mouse cerebral cortex. Significantly reduced ganglioside concentrations are also observed in malignant human brain tumors (1, 3, 4). The presence of \( G_{D1s}, G_{D1b}, G_{T1b}, \) and \( G_{Q1b} \) in the i.c. tumor, but their absence from the s.c. tumor (Figs. 4 and 5), indicates that these gangliosides are not native to the tumor. Instead, they represent contaminants from normal brain tissue surrounding the tumor and likely contribute to the slightly elevated ganglioside concentration of i.c. tumor compared to the s.c. tumor (Table 1). Since NANA is the predominant sialic acid in mouse brain gangliosides,\(^7\) the difference of about 20 \( \mu \)g of NANA between the i.c. (63.1 \( \mu \)g) and the mean of the s.c. tumors (43 \( \mu \)g) (Table 1), indicates that about 32% of the i.c. tumor NANA content is due to contamination by mouse brain gangliosides. The presence of \( G_{M2} \)-NGNA in the s.c. flank tumor, but its absence in the i.c. tumor, also indicates that this ganglioside is not native to the tumor and may arise from connective tissue encapsulation.

\( G_{M3} \) is the predominant ganglioside in this tumor. This is also a major ganglioside in human neural tumors (1, 2, 4), in experimental murine neural tumors (6, 7), and in several cultured cell lines derived from human and murine neural tumors (4, 31–35). In addition to an abundance of \( G_{M3} \), most of these tumor tissues and cell lines have a low ganglioside concentration and reduced amounts of polysialo-gangliosides. Our findings are consistent with these observations. Although \( G_{M3} \) is a minor ganglioside in normal adult mammalian brain, it is a major

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\(^{5}\) A. J. Yates, personal communication.

\(^{6}\) T. N. Seyfried, personal observation.

\(^{7}\) T. N. Seyfried and R. K. Yu, unpublished data.
EXPERIMENTAL MOUSE BRAIN TUMOR: GANGLIOSIDE COMPOSITION

Fig. 4. Thin-layer chromatogram of ependymoblastoma gangliosides in C57BL/6 mice. Lane 1, adult cerebral cortex; lanes 2 and 3, gangliosides from tumor grown intracerebrally and not treated (lane 2) and treated (lane 3) with mild base (0.1 M NaOH); lanes 4 and 5, gangliosides from tumor grown s.c. over the skull (Fig. 1) and not treated (lane 4) and treated (lane 5) with mild base; lanes 6 and 7, gangliosides from tumor grown s.c. in the flank and not treated (lane 6) and treated (lane 7) with mild base; lane 8, GM3-NGNA standard purified from adult mouse liver. Approximately 1.5 µg of total sialic acid was spotted for each lane on an HPTLC plate. The plate was developed by one-ascending run with chloroform:methanol:water (55:45:10 by volume) that contained 0.02% CaCl₂·2H₂O. The bands were visualized by the resorcinol spray.

Fig. 5. Thin layer chromatogram of ependymoblastoma gangliosides in C57BL/6 mice. Lane 1, adult cerebral cortex; lanes 2-4, gangliosides from tumor grown intracerebrally, s.c. over the skull, and s.c. in the flank, respectively. All samples were treated with mild base. Approximately 1.5 µg of total sialic acid was spotted for each lane. The plate was developed in chloroform:methanol:5 M NH₄OH:0.4% CaCl₂·2H₂O (50:50:4:5 by volume), and the bands were visualized with the resorcinol spray.

Ganglioside in rapidly growing mammalian embryonic tissues (36, 37). It therefore appears that elevations of GM₃ and reductions of total and polysialogangliosides are common to several types of rapidly growing undifferentiated cells.

In addition to being the most abundant ganglioside in the tumor, GM₃ also migrates as double bands on HPTLC (Figs. 4 and 5). These double bands most likely arise from structural heterogeneity in the ceramide portion of the molecule. Chou et al. (7) found that the splitting of GM₃ in experimental neurinomas resulted primarily from differences in the fatty acid composition of the upper and lower bands; with the upper GM₃ band having a greater proportion of longer chain fatty acids than the lower GM₃ band. We also found similar differences in fatty acid composition between the upper and lower GM₃ bands in normal human brain (38) and in human liver (39). Similar differences in fatty acid composition between upper and lower bands may also account for the splitting of GM₃-NGNA in the tumor (Fig. 4). The reason for these differences in fatty acid composition is unclear.

The tumor contained very little GD₃. This contrasts markedly with findings in human malignant astrocytomas, where GD₃ is a major ganglioside (1-4) and can be considered a marker for poorly differentiated tumors of glial origin (40). Moreover, the concentration of GD₃ in human astrocytoma is positively correlated with the degree of malignancy and invasiveness (1, 3). Although Chou et al. (7) found relatively little GD₃ in ethylnitrosourea-induced neural tumors in rats, Stoolmiller et al. (6) reported elevated amounts of GD₃ in mouse glial tumors. The ganglioside bands that we found migrating below GM₁ in the chloroform:methanol:water solvent system (Fig. 4) cannot be GD₃ since these bands contained an asialo-GM₁ (A₁) oligosaccharide backbone (Fig. 6). This contention is supported further by finding a low amount of GD₃ in the chloroform:methanol:ammonia solvent system (Fig. 5), where GD₃ will migrate above GM₁ (39). The differences in GD₃ content among various
EXPERIMENTAL MOUSE BRAIN TUMOR: GANGLIOSIDE COMPOSITION

neutral tumors may reflect differences in cellular origin or tumorigenic transformation.

We conclude that the ganglioside composition of the Zimmerman experimental ependymoblastoma differs markedly from the composition in normal mouse brain. The tumor is similar to other murine and human neural tumors in having an elevated \( G_{M3} \) content and significant reductions in total ganglioside concentration and polysialogangliosides. The tumor is unique in having a high content of gangliosides containing NGNA. This may, however, be a consequence of cellular heterogeneity or of malignant transformation. It would be interesting to determine if differences in ganglioside composition among various murine neural tumors are associated with differences in tumor growth and cytological composition. Finally, an understanding of the basic biology and biochemistry of brain tumors may lead to more effective diagnosis and therapy.

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