Characterization of new gangliosides of the lactotetraose series in murine xenografts of a human glioma cell line

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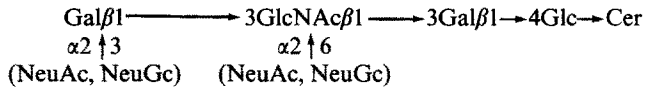
Received 4 April 1986

The major mono- and disialogangliosides of the extensively characterized established human glioma line D54MG were isolated and purified from subcutaneous solid xenografts grown in athymic (nu/nu) mice. Structural determination showed that they belonged to the lactotetraosylceramide series. The sialyllactotetraosylceramide contained 90% *N*-glycolyl- and 10% *N*-acetylneuraminic acid linked in an α2–3 linkage (IV^3NeuGc-$LcOse_4$Cer, IV^3NeuAc-$LcOse_4$Cer). The disialogangliosides had a previously undescribed type of structure with sialic acids linked to the terminal galactose in an α2–3 linkage and to *N*-acetylglucosamine in an α2–6 linkage. Not only did species with NeuAc or NeuGc occur, but also species with mixtures of the two sialic acids, e.g. NeuAC and NeuGc. The schematic structures of the new disialogangliosides are



Tumor ganglioside *Glioma-associated ganglioside* *Monosialoganglioside* *Disialoganglioside*
Lactotetraose series

1. INTRODUCTION

Neoplastic transformation of cells is accompanied by alteration of the ganglioside pattern [1] and quantitative or qualitatively distinctive tumor-associated gangliosides often appear. These gangliosides are suitable as immunogens for the production of specific antibodies with reactivities against tumor cells [2].

Primary brain tumors, which are often highly infiltrative lesions with ill-defined borders relative to normal brain, pose particular problems with regard to the isolation of their gangliosides. In malignant glioma tissue, tumor cell-associated gangliosides are often minor components because of the large number and amount of normal brain gangliosides that contaminate the infiltrative glioma tissue. Established glioma cell lines might therefore be a more suitable source for the isolation of pure tumor-associated gangliosides. In the present study we have determined the structure of the following glycolipids isolated from D54MG [3] murine xenografts: (i) the major monosialoganglioside; (ii) its corresponding neutral glycolipid; and (iii) its major disialoganglioside. The backbone structure of the monosialoganglioside is lactotetraosylceramide to which *N*-glycolyl- or *N*-acetylneuraminic acid is bound in an α2–3 linkage. The major disialoganglioside is also a lactotetraosylceramide to which sialic acid is bound to the terminal galactose in an α2–3 linkage and to *N*-acetylglucosamine in an α2–6 linkage. To our knowledge this is the first time disialogangliosides with such a structure have been described.

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00145793/86/$3.50

2. MATERIALS AND METHODS

2.1. *Materials*

D54MG is the Duke University subline of A172, a permanent cell line originally established from a human glioblastoma multiforme [4]. This line was established as a serially transplantable glioma line in Balb-c nu/nu athymic mice. Its growth, characteristics, morphology, karyotype and reactivity with intracellular, surface, and extracellular matrix monoclonal and polyclonal antibodies have been extensively described [3,5]. Tumors were harvested by removal of the subcutaneous tumors and immediate freezing by immersion in liquid nitrogen. Gangliosides and neutral glycolipids used as standards and references were all isolated at the Department of Psychiatry and Neurochemistry, Gothenburg University, Sweden.

2.2. *Isolation of gangliosides*

Neutral glycolipids, and mono- and disialogangliosides were extracted from 130 g tumor tissue and separated by anion-exchange chromatography as in [6,7]. Individual glycolipids were isolated by preparative thin-layer chromatography (TLC) on HPTLC plates.

2.3. *Analytical methods*

Ganglioside sialic acid was determined with the resorcinol assay [8]. *N*-Acetyl- and *N*-glycolylneuraminic acids were analysed by gas-liquid chromatography (GLC) as the trimethylsilyl derivative of the methyl ester methyl glycoside [9]. Sphingosine was assayed by a modification of the methyl orange method of Lauter and Trams [10]. The quantitative composition of the carbohydrate moiety was determined as alditol acetates by GLC [11]. Hydrolysis with *Vibrio cholerae* (EC 3.2.1.18, Behringwerke, Marburg-Lahn, FRG) sialidase was performed in 0.01 M Tris-maleate buffer (pH 6.5) containing 4 mM $CaCl_2$, with or without 0.5% (w/v) Triton X-100.

Permethylation of the gangliosides and neutral glycolipids and analyses of the partially methylated alditol acetates were carried out as described by Månsson et al. [12]. Fast atom bombardment-mass spectrometry (FAB-MS) was performed on a VG 7070E mass spectrometer equipped with a fast atom gun. The permethylated samples were dissolved in chloroform-methanol (1:5, v/v) to give a concentration of 5 nmol/μl. The stainless-steel target was coated with one drop of thioglycerol-glycerol (1:1, v/v) [13] or thioglycerol [14]. A solution of the permethylated sample (1 μl) was then added to the target. The target was bombarded with xenon atoms having kinetic energy equivalent to ~8 keV. Spectra were recorded in the positive ion mode by downfield mass controlled linear scans of 100–300 s duration. The resolution of the instrument was set to 300 ppm. In the mass range 300–800 amu the matrix derived background was subtracted.

3. RESULTS AND DISCUSSION

The D54MG tumor grown in nude mice contained 170 nmol ganglioside sialic acid per g wet wt tissue, of which 2/3 was in the mono- and 1/3 in the oligosialoganglioside fraction. Fig.1 shows the thin-layer chromatogram of the two ganglioside fractions separated in an alkaline solvent. The major band was approx. 80% of the total monosialoganglioside fraction and the major band in the oligosialoganglioside fraction approx. 70%. The central regions of these two ganglioside bands and the fastest migrating oligosialoganglioside

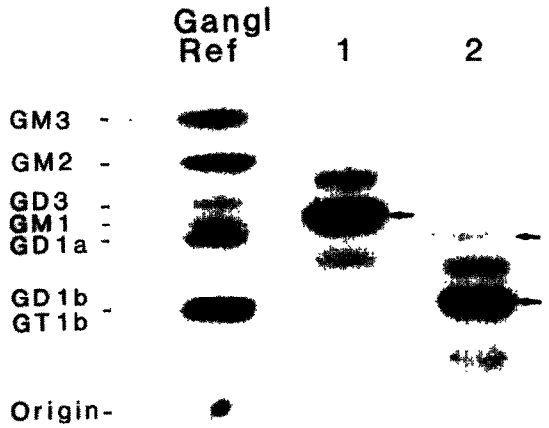


Fig.1. Thin-layer chromatographic separation of gangliosides from D54MG murine xenografts. Isolation and separation of the gangliosides are described in section 2. Chromatographic solvent was chloroform/methanol/2.5 M aqueous NH_3 (50:40:10, by vol.). The arrows indicate the ganglioside bands subjected to structural analyses. (1) Monosialo- and (2) oligosialoganglioside fraction.

(fraction 2, fig.1) were subjected to structural analyses.

The isolated monosialoganglioside contained sphingosine, sialic acid, glucose, galactose and *N*-acetylglucosamine in a molar ratio of 1:1:1:2:1. The ratio between *N*-glycolylneuraminic acid and *N*-acetylneuraminic acid was 9:1. Hydrolysis with sialidase or weak acid yielded a tetraglycosylceramide with TLC migration similar to that of globotetraosylceramide. Prolonged acid hydrolysis produced compounds with the same mobility on TLC as lactosylceramide and glucosylceramide with long-chain (C_{22}–C_{24}) fatty acids. The results of the permethylation analysis of the intact monosialoganglioside are shown in table 1. The finding of 4,6-Me_2-GlcNAcMe as the only amino sugar showed that the ganglioside belonged to the lactotetraose series. The appearance of 2,3,4,6-Me_4-Gal, in the desialylated compound suggests that the sialic acid was linked α2–3 to terminal galactose.

The FAB mass spectra of the permethylated ganglioside (fig.2) showed ions at *m/z* 406 and 374 (406 – 32) indicating *N*-glycolylneuraminic acid, and at *m/z* 376 and 344 (376 – 32) indicating *N*-acetylneuraminic acid. Sequence ions at *m/z* 855 and 1059 represent NeuGc-Hex-HexNAc and NeuGc-Hex-HexNAc-Hex, and at *m/z* 825 and

Table 1

Results of the permethylation analyses of D54MG murine xenograft gangliosides and neutral glycolipids

	Monosialo-ganglioside	Desialylated monosialo-ganglioside	Disialo-ganglioside fraction 1	Disialo-ganglioside fraction 2	Neutral glycolipid fraction
2,3,6-Me_3-Glc	1.00	1.00	1.00	1.00	1.00
2,3,4,6-Me_4-Gal	–	0.93	–	–	0.32
2,4,6-Me_3-Gal	2.03	0.93	1.69	1.45	0.90
2,3,6-Me_3-Gal	–	–	–	–	0.31
2,6-Me_2-Gal	–	–	–	0.53	–
3,4,6-Me_3-GalNAcMe	–	–	–	–	0.55
4,6-Me_2-GalNAcMe	–	–	–	0.36	–
4,6-Me_2-GlcNAcMe	0.88	0.83	–	–	0.36
4-Me-GlcNAcMe	–	–	0.71	0.51	–

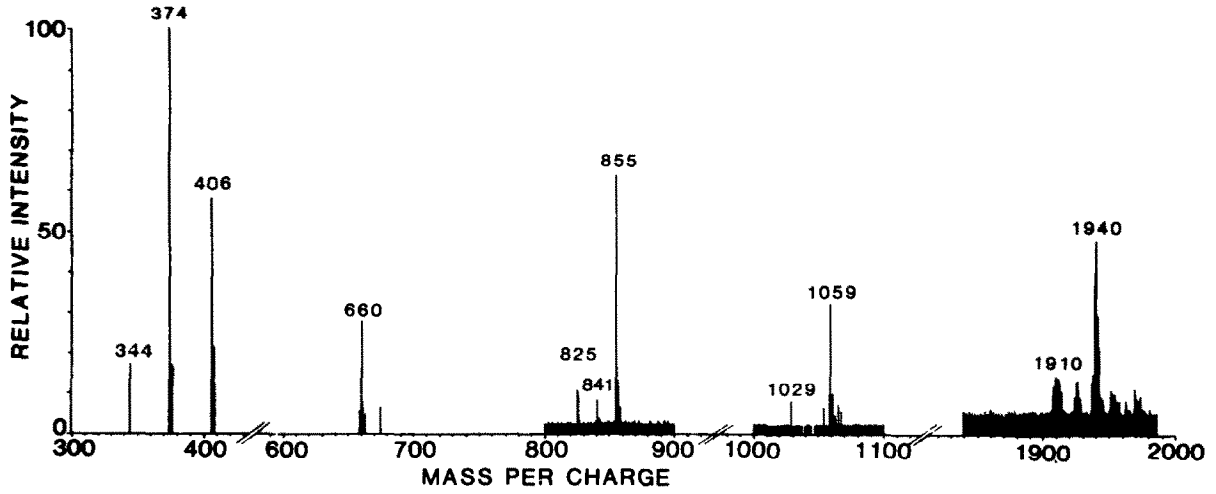


Fig.2. Positive ion FAB-MS of the permethylated monosialoganglioside. The matrix derived background was subtracted in the mass range 300–800 amu.

1029 the corresponding fragments containing NeuAc. The ceramide portion was represented mainly by ions at *m/z* 660, which corresponds to $C_{24:0}$ fatty acid and 4-sphingenine.

In the molecular ion region the ion at *m/z* 1940 (M + H) corresponds to NeuGc-Hex_3-HexNAc-Cer with $C_{24:0}$ fatty acid and 4-sphingenine. A small ion at *m/z* 1910 represents the NeuAc-containing ganglioside. The FAB mass spectra of the permethylated desialylated compound (fig.3) showed a dominating molecular ion at *m/z* 1549 (M + H) corresponding to HexNAc-Hex_3-Cer with $C_{24:0}$ fatty acid. Also, ions with low intensities were found at *m/z* 1535 and 1521, which corresponds to the same carbohydrate composition with $C_{23:0}$ and $C_{22:0}$ fatty acids. The intense ion at *m/z* 1199 may be derived from M + H − acyl chain (1549 − 350). Ions at *m/z* 464 and 432 (464 − 32) correspond to the terminal sequence Hex-HexNAc and the ion at *m/z* 668 to sequence Hex-HexNAc-Hex. Ions at *m/z* 660, 646 and 632 correspond to a ceramide portion with C_{24} to C_{22} fatty acids with 4-sphingenine. These results show that the structure of the major monosialoganglioside is IV^3NeuGc-$LcOse_4$Cer (NeuGcα2−3Galβ1−3GlcNAcβ1−3Galβ1−4Glcβ1−1Cer).

Sialidase or weak acid hydrolysis of the two oligosialoganglioside fractions 1 and 2 resulted in a neutral glycolipid with the same TLC migration as that of the glycolipid obtained from the monosialoganglioside. The slower migrating fraction 2 gave a sialidase-resistant monosialoganglioside with a migration slightly slower than that of GM1. The FAB mass spectra of the faster migrating oligosialoganglioside showed a weak ion at *m/z* 2271 (M + H) corresponding to $NeuAc_2$-Hex_3-HexNAc-Cer with $C_{24:0}$ fatty acid and 4-sphingenine, a ceramide composition verified by the ions at *m/z* 1921 (M + H − acyl chain) and 660. There were no ions representing a disialyl group. However, sequence ions were found at *m/z* 1186 and 1390 which correspond to $NeuAc_2$-Hex-HexNAc and $NeuAc_2$-Hex_2-HexNAc, respectively. In addition, the analysis of the partially methylated alditol acetates (table 1) revealed the occurrence of a disubstituted *N*-acetylhexosamine (4-Me-GlcNAcMe). Taken together these findings indicate the structure to be IV^3NeuAc, III^6NeuAc-$LcOse_4$Cer(NeuAcα2−3Galβ1−3(NeuAcα2−6)GlcNAcβ1−3Galβ1−4Glcβ1−1Cer).

The second, slightly slower migrating and major oligosialoganglioside fraction gave, on FAB mass spectral analysis, a dominating molecular ion at *m/z* 2331 (M + H) corresponding to $NeuGc_2$-Hex_3-HexNAc-Cer with $C_{24:0}$ fatty acid and 4-sphingenine. In addition, a weak molecular ion at *m/z* 2301 corresponding to NeuAc-NeuGc-Hex_3-HexNAc-$Cer_{24:0}$ was detected. The relative proportion of NeuGc, represented by ions at *m/z* 406 and 374, and NeuAc, represented by ions at

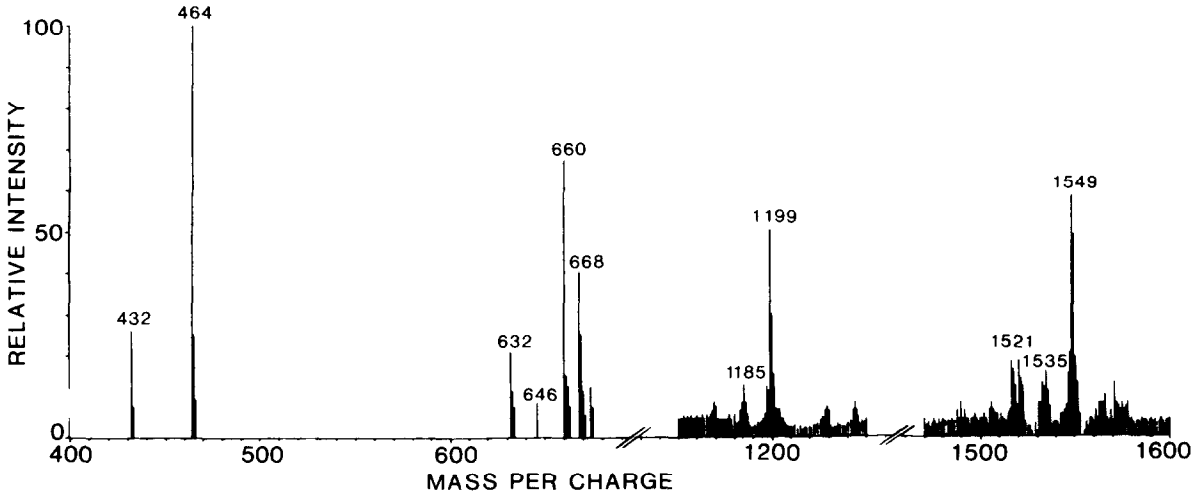
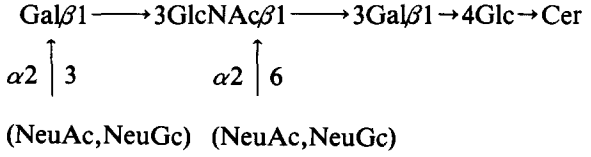


Fig.3. Positive ion FAB-MS of the permethylated desialylated monosialoganglioside. The matrix derived background was subtracted in the mass range 400–800 amu.

m/z 376 and 344 was 2:1. A group of sequence ions were found at m/z 1186, 1216, 1246 (the dominating one) and 1450 corresponding to $NeuAc_2$-HexNAc-Hex, NeuGc-NeuAc-HexNAc-Hex, $NeuGc_2$-Hex-HexNAc and $NeuGc_2$-Hex_2-HexNAc, respectively. Finally, the ion at m/z 855 revealed the occurrence of NeuGc-Hex-HexNAc. Analysis of the partially methylated alditol acetates showed 4-Me-GlcNAcMe, 4,6-Me_2-GalNAcMe and 2,6-Me_2-Gal. These results indicate that the analysed oligosialoganglioside fraction 2 contained two major structures IV^3-α-SA, III^6-α-SA,$LcOse_4Cer$ and IV^3-α-SA,II^3-α-SA, $GgOse_4Cer$.

A neutral glycolipid with a TLC migration similar to globotetraosylceramide was isolated from the solid tumor. It constituted less than 1% of the neutral glycolipid fraction. FAB-MS analyses showed the major molecular ion at m/z 1549 which corresponds to Hex_3-HexNAc-Cer with $C_{24:0}$ fatty acid and 4-sphingenine. The ion at m/z 1199 represents M + H − acyl chain. A terminal HexNAc is revealed by the ion at m/z 260, while the ions detected at m/z 464 and 668 correspond to Hex-HexNAc and Hex_2-HexNAc, respectively. These results combined with the permethylation analyses (table 1) suggest that the fraction is a mixture of globotetraosylceramide and lactotetraosylceramide (GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer and Galβ1−3GlcNAcβ1−3Galβ1−4Glcβ1−1Cer).

Our results suggest that the major gangliosides of D54MG solid glioma xenografts grown in nude mice belong to the lactotetraosylceramide series. $C_{24:0}$ fatty acid and 4-sphingenine are major components of the ceramide. The sialyllactotetraosylceramide contained 90% *N*-glycolyl- and 10% *N*-acetylneuraminic acid. Species of the new disialoganglioside with *N*-acetyl- and *N*-glycolylneuraminic acid only were identified, and the mass spectrometric data also indicated species with both *N*-acetyl- and *N*-glycolylneuraminic acid. The schematic structures of the new disialogangliosides might then be drawn:



ACKNOWLEDGEMENTS

This study was supported by research grants from the Swedish Medical Research Council (project no. B86-03X-07462-01 and 03X-627), National Swedish Board for Technical Development (project no.84-4667), the National Institutes of Health (KO7 NS00958-01, 1PO1 CA32672-05, 1PO1 NS20023-01, RO1-CA11898-15) and American Cancer Society Junior Clinical Faculty Fellowship, an Association for Brain Tumor Research Fellowship in memory of Bruce Davis.

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