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Ganglio-*N*-tetraosylceramide (GA1) of bovine and human brain

Molecular characterization and presence in myelin

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During our studies of bovine brain neutral glycosphingolipids (Ngsl's), we have purified a compound that co-migrates on thin-layer chromatogram with standard GA1 (purified by acid hydrolysis of GM1) and close to penta- (nLc_5Cer) glycosylceramide from bovine erythrocytes. The structure of the purified Ngsl from brain has been established by permethylation and by stepwise exoglycosidase hydrolysis. 600 MHz ^1H NMR spectroscopy of the oligosaccharide obtained from the Ngsl after endoglycoceramidase hydrolysis confirms the structure as ganglio-*N*-tetraosylceramide ($GgOse_4Cer$ or GA1) as Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer. We have identified GA1 in bovine, rat and human brain and myelin by TLC-immunostaining with monospecific anti-GA1 antiserum.

Bovine brain; Myelin; Neutral glycosphingolipids; NMR

1. INTRODUCTION

The majority of the brain gangliosides are of the ganglio-series, with GM4, GM3, GD3 and neolacto-series compounds minor constituents. The gangliosides GM1, GD1a, GD1b, GT1 and GQ1 contain ganglio-*N*-tetraosylceramide (asialo GM1 or GA1) as the neutral glycolipid core structure. Appreciable amounts of the globo- (Gb_3Cer), ganglio- (Gg_3Cer) and neolacto-series glycolipids have been characterized in normal human fetal brain [1]. III^3Fuc-nLc_4Cer, a minor constituent of normal human brain [2] has been reported in an appreciable amount (10%) in normal fetal brain [1] and is considered a stage-specific antigen. Mouse NK cells contain a high concentration of GA1 [3] that has been shown to be a precursor for GM1b synthesis [4,5]. The immunological and chemical identification of GA1 in mouse myelin [6] and characterization of GM1b in normal human brain [7] led us to purify and characterize GA1 in mammalian brain. The present study describes the purification and characterization of GA1 in bovine brain and its immunochemical identification in rat and human brain and CNS myelin.

Abbreviations: TLC, thin-layer chromatography; Ngsl, neutral glycosphingolipid; GM1, $II^3NeuAcGgOse_4Cer$; GM1b, $IV^3NeuAcGgOse_4Cer$; GA1 or $GgOse_4Cer$, III^3GalGg_3Cer; Gb_3Cer, Galα1→4Galβ1→4GlcCer; Gg_3Cer, GalNAcβ1→4Galβ1→4GlcCer; LacCer, Galβ1→4GlcCer; nLc_4Cer, Galβ1→3GlcNAcβ→4Galβ1→4GlcCer; nLc_5Cer, $IV^3GalnLc_4Cer$. Gangliosides are designated following Svennerholm, L. (1963) J. Neurochem. 10, 613–623.

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2. MATERIALS AND METHODS

Bovine brain was collected at a local abattoir, human brain was obtained immediately after autopsy following the accidental death of a previously healthy man, and rat brain was obtained from our laboratory after decapitation. Materials and methods were those previously used in our laboratory [8].

Glycolipids were extracted and Ngsl's were separated from gangliosides by DEAE-Sephadex A50 (acetate form). Individual Ngsl's were purified through biosil A (Bio-Rad) columns using chloroform:methanol:water and chloroform:methanol:2.5 N ammonia solvent systems, and collected in fractions that were checked by tlc. The purified fractions were pooled, and the purity of the compound was determined by tlc using two different solvent systems. Approximately 300 μg of the purified Ngsl was permethylated and acetylated [9,10] and the partially methylated alditol acetates were analyzed on a DB-1 column on a Hewlett-Packard 5980 chromatograph–mass spectrometer. The fatty acids and long-chain bases were analyzed as methyl ester and trimethylsilyl derivatives. Sequential carbohydrate residues were determined by specific exoglycosidase (200 m/unit) digestion of the Ngsl (20 μg) in 50 mM citrate buffer (pH 4.2) containing 0.1% taurocholate.

For NMR spectroscopy, the Ngsl was cleaved by endoglycoceramidase (2 m unit) in 50 mM acetate buffer (pH 6.0) containing 0.05% sodium taurocholate. The reaction was terminated by the addition of chloroform:methanol 2:1 (v/v) and the free oligosaccharide was purified from the upper phase through a biogel P2 column eluting with distilled water [11]. Both the Ngsl and the oligosaccharide released from the Ngsl by endoglycoceramidase were analyzed by ^1H NMR spectroscopy at 600 MHz. Approximately 300 μg of the glycosphingolipid sample [12] and 250 μg of the oligosaccharide sample [8,13] were prepared for NMR analysis. 600 MHz ^1H NMR spectroscopy was performed on a Bruker AMX-600 spectrometer interfaced with an Aspect X32 computer. The probe temperature was kept at 23°C. Further experimental details are essentially as described [13]. Chemi-

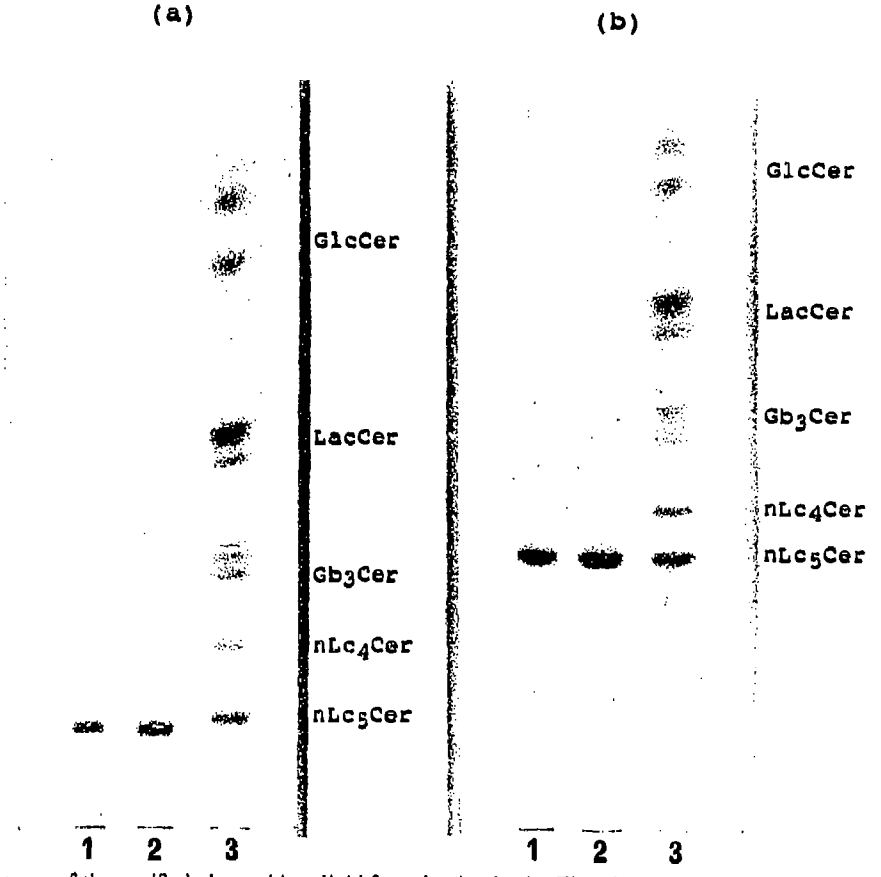
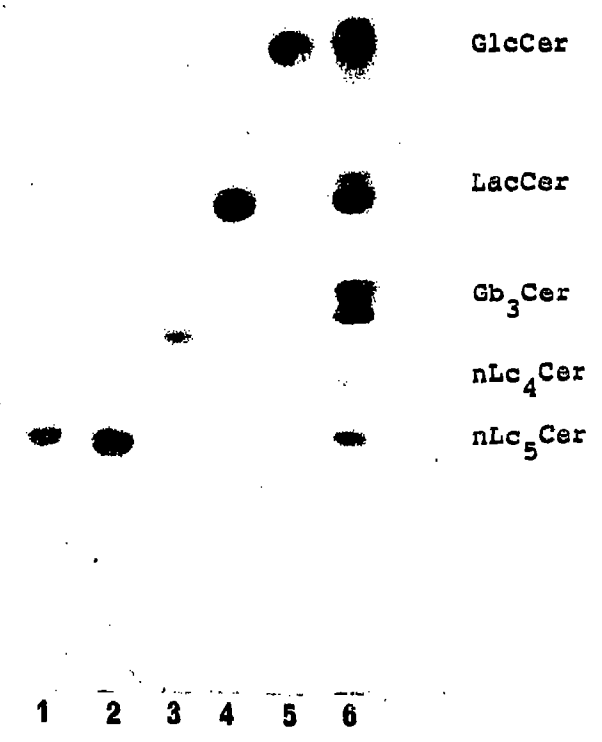


Fig. 1. Thin layer chromatogram of the purified glycosphingolipid from bovine brain. The plates were developed in (a) chloroform:methanol:2.5 N ammonia (60:40:9 v/v) and (b) chloroform:methanol:water (60:40:9 v/v) and visualized after DPA-spray. (Lane 1) standard GA1 ($GgOse_4Cer$); (lane 2) purified glycosphingolipid; (lane 3) standard neutral glycosphingolipid mixture from bovine erythrocytes.



cal shifts (δ) for the glycolipid are expressed in ppm downfield from TMS, measured by reference to internal DMSO-d_5 at δ 2.490; those for the oligosaccharide are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, measured by reference to internal acetate (δ 1.908 in D_2O at 23°C) with an accuracy of 0.002 ppm.

Myelin was prepared according to Norton and Poduslo [14] by repeated sucrose (0.32 M and 0.8 M) density gradient centrifugation and washing of the interphase with distilled water. The glycolipid was extracted and the purified Ngsl's were acetylated (pyridine:acetic anhydride, 3:2) and resolved on a florisil column [15] with dichloroethane:acetone (1:1 v/v). The acetylated Ngsl's were dialyzed and lyophilized after deacetylation with sodium methoxide. TLC-immunostaining was performed according to Kusunoki et al. [6] using 1% BSA in PBS, goat anti-rabbit IgG peroxidase (secondary antibody) and monospecific anti-GA1 antisera (primary antibody) prepared by absorption of anti-GA1-IgG on asialo-GM1-coated latex beads and eluting with 1 M NaSCN [16,17].

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Fig. 2. Stepwise specific exoglycosidase hydrolysis of the purified glycolipid. The plate was developed in chloroform:methanol:water (60:40:9 v/v) and visualized after DPA-spray. (Lane 1) standard $GgOse_4Cer$; (lane 2) purified glycolipid; (lane 3) lane 2 + β-galactosidase; (lane 4) lane 3 + β-hexosaminidase; (lane 5) lane 4 + β-galactosidase; (lane 6) standard neutral glycolipid mixture.

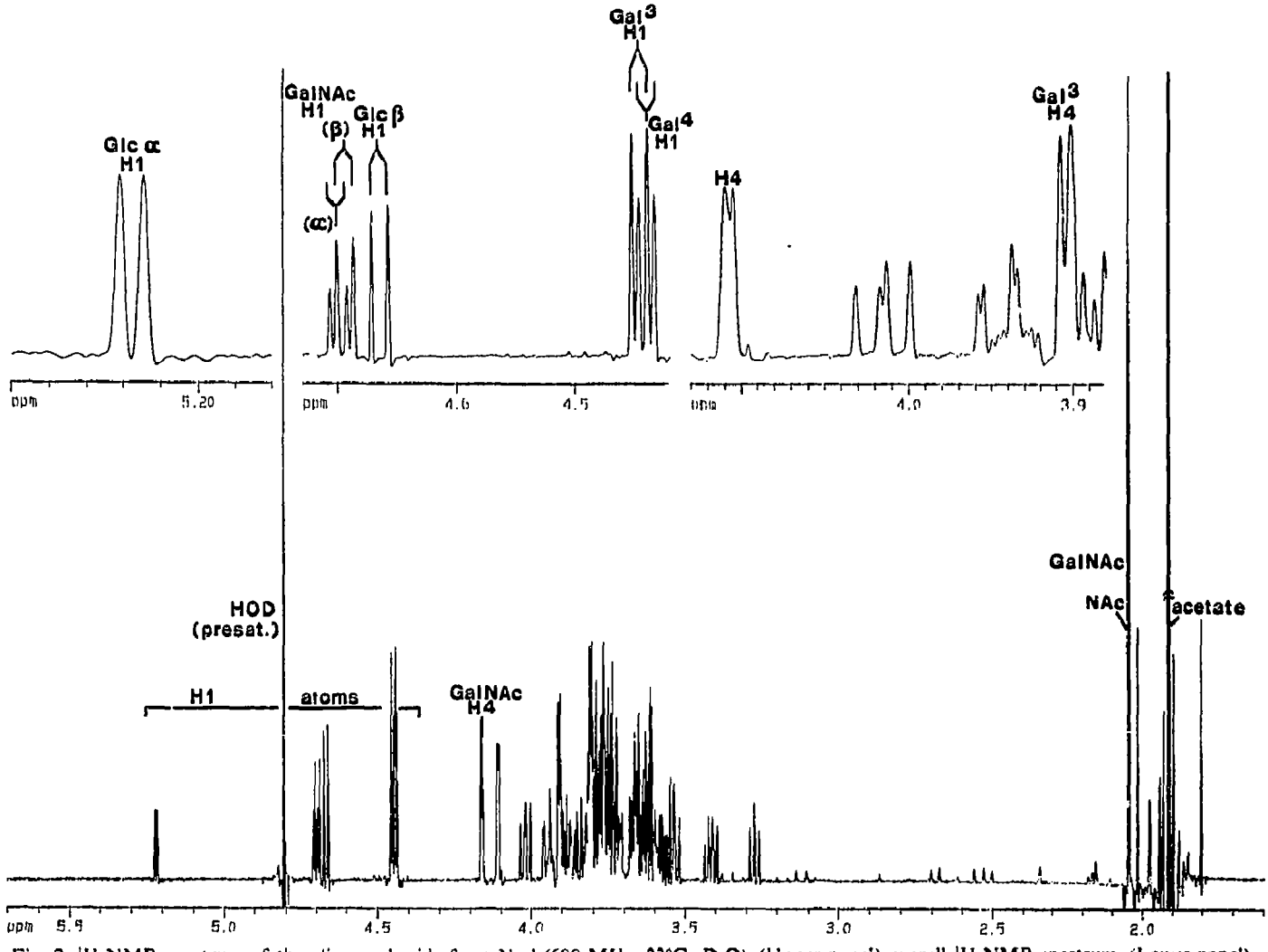

Fig. 3. ^{1}H NMR spectrum of the oligosaccharide from Ngsl (600 MHz, 23°C, D_2O). (Upper panel) overall ^{1}H NMR spectrum. (Lower panel) expanded regions showing the anomeric and non-anomeric structural reporter group signals indicative of the structure: Galβ(1—3)GalNAcβ(1—4)Galβ(1—4)Glc. Superscripts are used in Gal^3 and Gal^4 to differentiate between the Galβ(1—3) and Galβ(1—4) residues, respectively.

3. RESULTS AND DISCUSSION

The Ngsl (yield approximately 1 mg/40 g of acetone-dried brain) was homogeneous by TLC with two different solvent systems (Fig. 1). Its compositional analysis indicated Gal:GalNAc:Glc in a molar ratio of 1.95:0.8:1.0 and sphingenine (C18:1) as the only base. The acyl groups were mainly stearic with lesser amounts of palmitic and oleic acids (C18:0 86,5%; C16:0 8.0%; C18:1 5.5%). The four peaks of the partially methylated hexitol acetate derivatives were identified by GC-MS as 2,3,4,6-tetra-*O*-methylgalactitol-1,5-diacetates, 2,3,6-tri-*O*-methylgalactitol-1,4,5-triacetates, 2,3,6-tri-*O*-methylglucitol-1,4,5-triacetates and 4,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidogalactitol-1,3,5-triacetates. The parent glycolipid was hydrolysed by β-galactosidase (and not by α-galactosidase or β-hexosaminidase) to a trihexaosylceramide that migrated close to the R_F of standard Gb_3Cer. The product was hydrolysed further to di- and monohexaosylceramide by β-hexosaminidase and β-galactosidase (Fig. 2). 600 MHz NMR spectroscopy of the oligosaccharide (Fig. 3) confirmed the structure of the Ngsl as $GgOse_4Cer$ (GA1 or asialo-GM1). The ^{1}H NMR spectrum (Fig. 3) of the oligosaccharide released from the Ngsl showed the presence of a tetrasaccharide with the structure:

Galβ(1→3)GalNAcβ(1→4)Galβ(1→4)Glc

The chemical shifts of the structural reporter groups (marked in Fig. 3: H1 of Gal^3 at δ 4.448; H1 of GalNAc at δ 4.706/4.701; H1 of GA1^4 at 4.443/4.441; H1 of Glcα at 5.219; H1 of Glcβ at 4.665) are virtually identical to those reported [16] for $GgOse_4$ at 500 MHz and 27°C. 600 MHz ^{1}H NMR spectroscopic analysis of the intact Ngsl in DMSO-d_6 confirmed this structure; the chemical shifts of the structural reporter groups are essentially

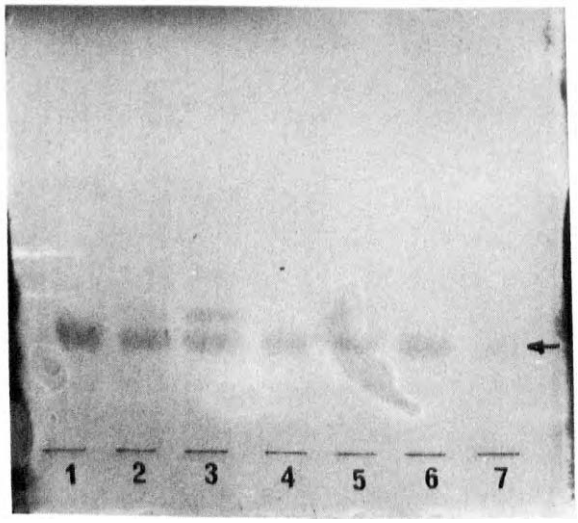

Fig. 4. Thin-layer chromatography staining of the Ngsl's from brain and myelin. The plate was developed in chloroform:methanol:water (60:40:9 v/v) and immuno-stained with anti-GA1 antisera. (Lane 1) bovine brain; (lane 2) normal human brain; (lane 3) rat brain; (lane 4) standard $GgOSe_4Cer$; (lane 5) bovine myelin; (lane 6) human myelin; (lane 7) rat myelin.

the same as those reported for GA1 by Koerner et al. [10] (compound 5, analyzed at 500 MHz, 30°C). Glycolipids of the globo-series are the major class in visceral organs, while fucolipids of the lacto- and neolacto-series are abundant in the ephithelial tissues of the digestive tract. Although the major glycolipids of normal adult brain consists of galactosylceramide, sulfatide, and gangliosides of the ganglio-series [1], the accumulation of some *neutral* glycolipids of the ganglio-, globo- and neolacto-series in brains from patients with inherited sphingolipidoses cannot be explained directly by the relevant glycosidase deficiencies (e.g. β-*N*-acetylhexosaminidase in GM2 gangliosidosis, β-galactosidase in GM1 gangliosidosis and galactosylceramide-β-galactosidase in Krabbe's disease). Gangliotetraosylceramide is one of the main glycolipids accumulating in GM1 gangliosidosis brain [17], but is barely detectable in normal brain [3]. It has also been reported in a few studies of juvenile [18,19] and fetal human brain [1] (identified by TLC and hexaosyl molar composition).

GA1 has been identified in adult mouse myelin by immunohistochemical staining, although its occurrence in other subcellular brain fractions was not excluded. The molar composition was that of GA1 [6], but a detailed chemical analysis was not done.

We have purified and analytically characterized GA1 from bovine brain and identified it in adult rat and normal human brain and in CNS myelin (Fig. 4). As shown in Fig. 4 two immunoreactive bands could be observed, particularly in rat brain. These bands were separated, purified by column chromatography and chemically characterized. Both of them are GA1 species with the same oligosaccharide but a different fatty acid composition.

It is noteworthy that experimental autoimmune encephalomyelitis can be induced in guinea pigs by GA1 [22] suggesting a possible role of GA1 in demyelination. Though its importance in myelination and demyelination is unknown, the careful study of the topographical distribution of GA1 during normal brain development may clarify its involvement in myelinogenesis.

To our knowledge, this is the first report of analytical characterization of GA1 and its presence on adult normal human myelin.

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