Structure of Sulfated Glucuronyl Glycolipids in the Nervous System Reacting with HNK-1 Antibody and Some IgM Paraproteins in Neuropathy*

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Novel sulfated glucuronic acid-containing glycolipids have been identified in the nervous system. These glycolipids are highly antigenic and share antigenic determinants with several nervous system glycoproteins, such as neural cell adhesion molecules, myelin-associated glycoprotein, and ependymins. The structure of the major antigenic glycolipid from human peripheral nerve was determined by chemical and enzymatic degradation, incorporation studies, sugar analysis after permethylation, pertrimethylsilylation, and gas liquid chromatography-mass spectrometry techniques as well as fast atom bombardment-mass spectrometry of the native antigen. The following structure was established for the major antigenic glycolipid.

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\text{sulfate-3-GlcA(1→3)Gal(1→4)GlcNAc(1→3)Gal(1→4)Glc(1→1)-ceramide.}
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The major fatty acids in the ceramide were 18:0, 18:1, 24:0, and 24:1, with C18-sphingosine as the long chain base.

Cell surface glycoconjugates appear to be involved in cell-cell interactions during cellular differentiation and maturation. A variety of studies with lectins and monoclonal antibodies have recognized expression of certain glycoconjugate antigens on cell-surface in a stage-specific manner during embryonic development of tissues (1). It is now increasingly evident that these unique cell-surface antigens expressed during embryogenesis, differentiation, and often during oncogenesis are glycoconjugate structures with the same or similar carbohydrate sequences found both on glycolipids and glycoproteins (2, 3).

Ilyas et al. (4) first reported in the human peripheral nerves the presence of acidic glycolipid antigens recognized by IgM of certain patients having peripheral neuropathies and plasma cell abnormalities. Later it was shown that the same glycolipid antigens were also recognized by an antibody HNK-1 (anti-Leu-7) raised to a membrane antigen from human T cell line HSB-2 and recognizing surface antigens on a subset of human lymphocytes including natural killer cells (5, 6). The same or a similar carbohydrate epitope on myelin-associated glycoprotein also reacts with HNK-1 antibody and the IgM paraproteins (4, 7–10). Recently it has been shown that HNK-1 reactive epitope is also expressed on certain neural cell adhesion molecules such as N-CAM, L1, and J1 antigens, which are specifically involved in neural cell interactions (10–12) and also on a group of glycoproteins called ependymins localized in the extracellular fluid of goldfish brain and which have been shown to alter during learning and memory processes (13). We have shown that the glycolipid antigens recognized by HNK-1 were also present in rat, mouse, rabbit, cat, dog, bovine, monkey, and chicken sciatic nerves (14); however, they were absent in the adult brain from the same species. On the other hand, expression of these glycolipid antigens can be demonstrated in human and rodent embryonic brain tissue with HNK-1 or a specific antibody 4F4 raised against embryonic rat forebrain (14). In rat forebrain these glycolipid antigens were developmentally regulated and were maximally expressed at embryonic day 15, but disappeared between day of birth and postnatal day 14.1

Previously, in a preliminary paper (15), we reported a partial structure of the purified major antigen as a novel glucuronic acid-containing sulfated glycosphingolipid. However, the exact structure, sugar linkages, and anomeric configuration, and the position of the sulfate group were not determined. In the present report we characterize the major antigenic glycolipid of the human peripheral nerves as sulfate-3-GlcAβ(1→3)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glcβ(1→1)-ceramide, IV3-(3'-SO3-GlcA)-N-LcOseCer.

EXPERIMENTAL PROCEDURES

Isolation of the glycolipid antigens was performed in the following manner. Human peripheral nerves or cauda equina (about 30 g) were extracted overnight with 20 volumes of chloroform/methanol/water (1:1:0.6) in order to form two volumes of chloroform/methanol/water (2:1:0.6) in order to form two


2 Portions of "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0702, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
The ganglioside fractions from human peripheral nerves were separated by DEAE-Sephadex column chromatography and the resulting fractions were analyzed by HPTLC (Fig. 1). Most of the antigenic compounds were found to be in the tetrasialoganglioside fraction when tested by the HPTLC-immunoblot method (Fig. 1b). When the HPTLC plates were visualized after resorcinol-staining for sialic acid, the gangliosides were visualized, but no staining was visible where the immunoreactive compounds were present (Fig. 1a), indicating that the antigenic lipids were not sialic acid-containing lipids. These lipids, however, easily reacted with orcinol spray reagent, giving characteristic purple color produced by the reducing sugars. The major antigenic glycolipid migrated between GM\textsubscript{1} and GD\textsubscript{1a} standards, whereas a second less prominent, slower migrating antigenic glycolipid migrated between GD\textsubscript{1a} and GM\textsubscript{1b} when chromatographed on HPTLC plate with chloroform/methanol/0.25% aqueous CaCl\textsubscript{2} (50:40:10 by volume). The purity of the major antigenic glycolipid was checked by one- and two-dimensional HPTLC in several different solvents after removing ganglioside contaminants. The major and the minor antigenic glycolipids can be resolved from each other by high performance ligand chromatography on a Liscorsorb-NH\textsubscript{2} column.

**Methanolic HCl Treatment and Alkaline Hydrolysis—**The antigenic glycolipids were not susceptible to mild alkaline hydrolysis. However, they were susceptible to mild methanolic HCl treatment (Fig. 2, 0.05 M anhydrous methanolic HCl at room temperature for 3.5 h). The major glycolipid after the acid treatment migrated on HPTLC near globoside standard (Fig. 2, lane 3), whereas the second minor glycolipid migrated between GM\textsubscript{3} and GM\textsubscript{1} (Fig. 2, lane 3). Both lipids after acid methanalysis did not bind to DEAE-Sephadex column and were eluted in the neutral lipid fraction with methanol, indicating a loss of acidic groups. The acid-treated compounds also no longer reacted with HNK-1 antibody or IgM from patients with neuropathy. The acid-treated compounds were further reacted with mild alkali, and the resultant lipids on HPTLC migrated slightly above the corresponding “native” antigens (Fig. 2, lane 4). When these products were chromatographed on a DEAE-Sephadex column, they were eluted with 0.02 M ammonium acetate in methanol (corresponding

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FIG. 1.  a, HPTLC of human peripheral nerve ganglioside fractions after DEAE-Sephadex column chromatography. STD, standard bovine brain ganglioside. Mono, tri, and tetrasialoganglioside fractions were spotted in order of their elution from the chromatographic column. The gangliosides were detected with resorcinol spray. Standard gangliosides are shown in lanes 1 and 14. b, a radioautograph of an HPTLC plate similar to that in a, after overlaying with a neurite patient’s serum diluted 1:500, followed by radiodinated goat anti-human IgM, as previously described (4). The lane numbers correspond to the fractions in a. The spots near the origin in b are due to radioactive lane markers for the purpose of alignment of the x-ray film with the HPTLC plate.

separate phases. The upper aqueous phase was collected. The lower phase was washed twice with 0.5 volume of methanol:water (1:1) and the upper phases were combined. The combined upper phases were extracted with 0.5 volume of chloroform/methanol/0.25% aqueous CaCl\textsubscript{2} (50:40:10 by volume). The purity of the major antigenic glycolipid was checked by one- and two-dimensional HPTLC in several different solvents after removing ganglioside contaminants. The major and the minor antigenic glycolipids can be resolved from each other by high performance ligand chromatography on a Liscorsorb-NH\textsubscript{2} column.

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FIG. 2. HPTLC of purified human peripheral nerve antigenic glycolipids before and after treatment with mild acid and alkali. Lane 1, standard neutral glycosphingolipids; CMH, galactosylceramide; CDH, lactosylceramide; CTH, α-galactosylactosylceramide; GB, globoside; lanes 2 and 6, standard gangliosides; lane 3, antigenic glycolipids after mild acid treatment; lane 4, antigenic glycolipids after mild acid treatment followed by mild alkaline hydrolysis; lane 5, purified antigenic glycolipids. The plate was developed with orcinol spray.

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3 The abbreviations used are: GM\textsubscript{1}, II\textsuperscript{a}α-N-acetylgalactosaminyl-gangliotetraosylceramide (IP\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); GD\textsubscript{1a}, IV\textsuperscript{2}α-N-acetylgalactosaminyl-gangliotetraosylceramide (IV\textsuperscript{2}NeuAcI\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); GD\textsubscript{1b}, II\textsuperscript{a}α-N-acetylgalactosaminylgangliotetraosylceramide (IP\textsuperscript{2}NeuAcI\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); GM\textsubscript{3}, II\textsuperscript{a}α-N-acetylgalactosaminylcerebroside (IP\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); GM\textsubscript{1b}, IV\textsuperscript{2}α-N-acetylgalactosaminylgalactocerebroside (IV\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); GD\textsubscript{1a}, IV\textsuperscript{2}α-N-acetylgalactosaminylIII\textsuperscript{a}α-(N-acetylgalactosaminyl)garnbonitectraosylceramide (IV\textsuperscript{2}NeuAcI\textsuperscript{2}NeuAcGpO\textsubscript{4}Cer); HPTLC, high performance thin layer chromatography; TMS, trimethylsilyl; GLC-MS, gas-liquid chromatography-mass spectrometry.
to monosialoganglioside), indicating partial restoration of the negatively charged group. The immunoreactivity of these alkali-treated lipids was also partially restored with some human IgM paraproteins but not with HNK-1. These results suggested the presence of an acid-labile sulfate group and possible formation of a methyl ester of a carboxyl group during the acid treatment, in both the compounds.

Fourier transform infrared spectra of the purified major antigen showed absorption at 1225 cm⁻¹, due to the acid treatment, in both the compounds.

Incorporation of 35SO₄ into the Lipids in Vitro — Convincing demonstration of the presence of a sulfate group in both the compounds was obtained by 35SO₄ incorporation studies with 18-day-old rat sciatic nerve slices in vitro (Fig. 3). When endoneurial tissue of rat sciatic nerve slices was incubated with Na₂35SO₄, as expected, the radioautogram of the HPTLC plate showed that the major incorporation of 35SO₄ was in cerebroside-sulfate of the rat sciatic nerve (Fig. 3A). However, significant radioactivity was also found in the slower migrating antigenic lipids (Fig. 3A, lanes 3 and 4). When the same HPTLC plate was immunostained with HNK-1 antibody (Fig. 3B), the antigenic gangliosides were found exactly where the corresponding radioactivity was in the radioautogram. At present it is not known whether the sulfotransferases in the nerve for the formation of cerebroside sulfate and the antigenic gangliosides are the same or different.

Action of Endo-β-galactosidase — The action of endo-β-galactosidase 20 on the major antigenic glycolipid is shown in Fig. 4. After hydrolysis, the major products formed were an orcinol-positive substance with the same HPTLC mobility as glucosylceramide and an oligosaccharide. The latter did not adhere to the C-18 reversed-phase material of the Bond-Elut cartridge. Similar result was obtained with the action of endo-β-galactosidase on the acid-treated glycolipid (not shown).

The susceptibility of the major antigenic glycolipid to endo-β-galactosidase and the formation of glucosylceramide indicated that the compound is a glycolipid with GlcNAcβ(1→3)Galβ(1→4)Glcβ(1→1) ceramide core structure (20).

Action of β-Glucuronidase — The major antigenic glycolipid as such was not susceptible to β-glucuronidase from limpet (Fig. 5A, lane 5). The mild acid-treated product was also not susceptible to the enzyme (not shown). However, the acid followed by alkali-treated compound was partially susceptible to β-glucuronidase (Fig. 5A, lane 4), producing a product which migrated near paragloboside and another product which migrated near ceramide trihexosyl. These products were neutral lipids since after DEAE-Sephadex chromatography they were eluted in methanol (shown in Fig. 5A, lane 7); whereas the untreated substrate and the detergent taurodeoxycholate were eluted with 0.02 M ammonium acetate in methanol (not shown). These results indicated that neither the esterified nor the sulfated glucuronide was susceptible to β-glucuronidase and that only after desulfation with acid, followed by deesterification with alkali, the glycolipid was susceptible to the action of β-glucuronidase. Formation of the major product paragloboside suggested that the major antigenic compound was a derivative of paragloboside. Partial formation of ceramide trihexosyl-like product suggested the presence of a contaminating β-galactosidase enzymic activity in the glucuronidase preparation. This was confirmed when the glucuronidase treatment was performed in the presence of γ-galactonactone, an inhibitor of β-galactosidase. Only paragloboside product was seen without formation of ceramide trihexosyl-like product. When the β-glucuronidase reaction was performed in the presence of increasing amounts of γ-galactonactone, the formation of paragloboside was inhibited, indicating that the reaction was specific for β-glucuronidase. The product paragloboside formed after the β-glucuronidase reaction was reactive with 1B2 antibody (22) after HPTLC immunoblot (Fig. 5B). 1B2 antibody is highly specific for non-reducing terminal type II N-acetyllactosamine (Galβ(1→4)GlcNAcβ1-) structure and does not react with glycolipids having either type I chain (Galβ(1→3)GlcNAcβ1-) or gangliosyl or fucosyl substituted N-acetyllactosamine residue
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**Fig. 5.** A, action of \( \beta \)-glucuronidase on the native and acid followed by alkali-treated antigenic glycolipid. The reaction products formed after the enzymatic reaction with each sample were recovered by reversed-phase Bond-Elut cartridge chromatography (17) and spotted on HPTLC along with standards. The plate was developed with chloroform/methanol/0.25% CaCl\(_2\) in water (50:40:10) and sprayed with orcinol reagent. Lane 1, standard neutral glycolipids as in Fig. 2; lane 2, standard paragloboside (PGB) and asialo-GM\(_1\) (AGM\(_1\)); lane 3, acid-treated antigen; lane 4, acid followed by alkali-treated antigen after reaction with \( \beta \)-glucuronidase; lane 5, native antigen after reaction with \( \beta \)-glucuronidase; lane 6, enzyme reaction control without substrate and taurodeoxycholate; lane 7, same as lane 4 but after DEAE-Sephadex column chromatography to remove tau-rodeoxycholate and the unreacted substrate (the neutral lipids which did not bind to DEAE-Sephadex and eluted with chloroform were spotted here); lane 8, bovine brain ganglioside standard. B, left panel, HPTLC of standard glycolipids (with abbreviations as in Fig. 2) developed with orcinol spray; right panel, HPTLC-immunoblot, using 1B2 antibody, of the antigenic glycolipids after mild methanolic HCl and alkali treatment followed by \( \beta \)-glucuronidase. Lane 1, paraglbo-side (nLC\(_4\)) standard; lane 2, mixture of paragloboside and neolactohexaosylceramide (nLC\(_4\)) prepared from rat sciatic nerve sialosyl-nLC\(_4\) and sialosyl-nLC\(_6\) after sialidase treatment; lane 3, products formed after the action of \( \beta \)-glucuronidase on the mild acid followed by alkali-treated antigenic glycolipids.

(22). The slower, less prominent antigenic glycolipid after desulfation and de-esterification was also susceptible to \( \beta \)-glucuronidase, under similar conditions described above, with the formation of neolactohexaosylceramide, which was identified by the 1B2 antibody (Fig. 5B).

**Other Enzymes**—The antigenic glycolipids were not susceptible to sialidase or \( \alpha \)-galactosidase, purified liver arylsulfatase A or B, and \( \alpha \)-fucosidase.

**Mass Spectrometry of Pertrimethylsilylated Derivatives**—The mass spectrum of the pertrimethylsilylated derivative of the acid-treated antigen was previously reported and was shown to have \( m/z \) 407 and a strong \( m/z \) 317, indicating a terminal persilylated methyl ester of glucuronic acid (13). Absence of \( m/z \) 451 indicated a lack of terminal hexose molecule, whereas the presence of \( m/z \) 361 indicated an internal hexose in the molecule. Weak ions at \( m/z \) 420 and 330 indicated a lack of terminal N-acetylhexosamine residue. Weak ions at \( m/z \) 420 and 330 were found in the mass spectra of standard paragloboside, asialo-GM\(_1\), G\(_{M2}\), and galactocerebroside (23) and thus are not derived from a terminal N-acetylhexosamine residue. The mass spectrum of deuterated per-D\(_9\)-trimethylsilylated derivative of the acid-treated antigen is shown in Fig. 6. Ion at \( m/z \) 434 and a strong \( m/z \) 335 are derived from the terminal glucuronic acid methyl ester. Lack of \( m/z \) 487 indicated an absence of terminal hexose, whereas \( m/z \) 388 should be from an internal hexose. Weak \( m/z \) 447 and 348 indicated an absence of terminal N-acetylhexosamine residue. Ions that show a loss of OCH\(_3\) to produce \( m/z \) 403 from \( m/z \) 434 and a loss of CH\(_3\)COOH to produce \( m/z \) 275 from \( m/z \) 335 are also found. Ion at \( m/z \) 320, with structure CH\(_3\)(CH\(_2\))\(_n\)CH=CHCH=O(D\(_9\))TMS, shows clearly the presence of C18-sphingine in the molecule. Ion of \( m/z \) 182 has been shown previously due to acetamido group and two carbons of the sugar with structure (D\(_9\))TMSO-CH-CH-NH-COCH\(_3\) and is derived from N-acetylhexosamine in the molecule. Lack of \( m/z \) 223 confirmed the absence of sialic acid residue in the molecule (23). As reported previously by Dawson and Sweeney (23) mass spectra of pertrimethylsilylated derivatives of higher glycosphingolipids failed to produce high mass ions for the oligosaccharide moiety under the conditions used.

The acid and alkali-treated antigen after pertrimethylsilylation produced a strong \( m/z \) 375 corresponding to:

The analogous ion \( m/z \) 402 was produced after per-D\(_9\)-trimethylsilylation of the acid and alkali-treated antigen (data not shown).

**Analysis of Acid-methanolyzed Product of the Antigen after Trimethylsilylation and Capillary GLC-MS**—The separation by isothermal capillary GLC and reconstructed mass chromatograms of the pertrimethylsilylated methylglycoside, galac-tose, and glucuronic acid standards and those derived from the antigen after acid methanolysis and trimethylsilylation are shown in Fig. 7, A and B. The specific ions monitored and their structures are shown in the figure. Ion \( m/z \) 204 is common to all the sugars, whereas \( m/z \) 361 is derived from

**Fig. 6.** Mass spectrum of per-D\(_9\)-trimethylsilylated derivative of the acid-treated antigenic glycolipid. The ion structure of \( m/z \) 454, derived from terminal glucuronic acid methylester, is shown. DT, per-D\(_9\)-trimethylsilyl group.
Fig. 7. Capillary GLC-MS of pertrimethylsilylated sugar residues after acid methanolysis of the major antigenic glycolipid. A, standard glucose (Glc), galactose (Gal), and glucuronic acid (GlcA) about 0.1 μg each were analyzed by GLC-MS as described in the Miniprint. The specific ion plots at m/z 204, 259, 361, and 423 are shown. The possible structures of these ions are shown on the left. B, GLC-MS analysis of the sugar derivatives from the major antigenic glycolipid (about 7 μg), under the same conditions as described for A. C, Upper two specific ion plots (m/z 173, 204) show the elution of N-acetylglucosamine derivative prepared from paragloboside standard. The ion structures are shown on right. Lower two specific ion plots are of the sugar derivatives of the major antigenic glycolipid (about 7 μg).

hexoses and m/z 423 and m/z 259 are more specific for the glucuronic acid methyl ester. The isomers of glucuronic acid methyl ester derivative were eluted from the GLC column with retention times of 7.9 and 7.2 min, in between α- and β-glucoside derivative peaks. The pertrimethylsilylated N-acetylglucosamine methylglycoside eluted with a retention time of around 11 min (Fig. 7C), whereas the corresponding N-acetylgalactosamine as present in asialo-GM1 eluted around 10.2 min (not shown). The structures of all the sugar derivatives from the antigen were confirmed by the analysis of their mass spectra. The mass spectrum of the pertrimethylsilylated glucuronic acid methyl ester methyl glycoside is shown in Fig. 8. The proportion of various hexoses and the N-acetylhexosamine in the major antigenic compound was determined by comparing the GLC-MS of pertrimethylsilylated sugars derived from standard globoside, paragloboside, and asialo-GM1. The analysis showed the presence of Glc:Gal:GlcNAc:GlcA in the proportion of 1:2:1:1, respectively. The fatty acid composition of the major antigenic glycolipid was determined by capillary GLC-MS and was also confirmed by conventional GLC analysis after the isolation of fatty acids methyl esters from acid antigen. The fatty acid composition of the major antigenic glycolipid was 16:1 (24%), 16:0 (13.2%), 18:1 (16.1%), 18:0 (11.4%), 20:1 (4.3%), 20:0 (5.3%), 21:1 (0.2%), 21:0 (2.0%), 22:1 (8.1%), 22:0 (3.0%), 23:0 (4.0%), 24:1 (12.5%), 24:0 (15.3%), 25:0 (3.3%), and 26:1 (1%).

Mass Spectrum of the Intact Permethylated Antigen—The mass spectrum of the intact permethylated desulfated antigen is shown in Fig. 9A. The specific ion plots derived from monitoring the rate of ionization of the derivative from the solid probe are given in Fig. 9B. The spectrum did not contain m/z 219 corresponding to the terminal hexose (24) but instead had m/z 233 corresponding to terminal GlcA. A loss of methanol (mass unit 32) produced m/z 201. A further loss of methanol from 201 produced m/z 169. A loss of acetic acid (mass unit 60) from m/z 201 and 233 produced m/z 141 and 173, respectively. Internal hexoses produced m/z 187 and 155. A strong m/z 182 has been known to be due to all permethylated N-acetylhexosaminyl residues with no substitution at C-3 position, indicating that N-acetylglucosamine residue had substitution of C-4 position (24). An ion at m/z 437 is due to a disaccharide GlcA-hexose residue, whereas m/z 682 and 650 coupled to the ion at m/z 437 should be due to a trisaccharide GlcA-hexose-hexosaminyl residue, and m/z 886 and 854 coupled to the ion at m/z 437 are due to tetrasaccharide GlcA-hexose-hexosaminyl-hexose residue. C18-sphinganine fragment ion m/z 364 was present in the spectrum (24). Ions at m/z 366 and 392 could arise from C18-sphinganine and C20-sphingenine but they could also arise from the 21:0 and 23:0 fatty acid-containing ceramide residues (24). Other prominent ceramide residue fragments corresponding to fatty acid 18:0 (m/z 322), 20:0 (m/z 350), 22:6 (m/z 378), 24:1 (m/z 404), 24:0 (m/z 406), 25:1 (m/z 418), and 26:1 (m/z 432) are also...
found in the spectrum. Fatty acid ions (24) with structure \([\text{CH}_3\text{O-CH-CH-Ch}_{2}-\text{O-permethylated glucose}]^+\) corresponding to fatty acid 18:0 (m/z 544), 20:0 (m/z 572), 22:0 (m/z 600), 24:1 (m/z 626), and 24:0 (m/z 628) were also observed. The corresponding ion with two carbohydrates were at m/z 832 for 24:0 and at m/z 748 for 18:0.

Glycosyl Linkage Analysis of the Antigen—The glycosyl linkages in the antigen were determined by methylation analysis as described by Waqhe et al. (25) after permethylation of the antigen, reduction of the glycosyluronic carbohydrate groups with sodium borodeuteride, hydrolysis of the glycosidic linkages, and 3-linked galactose, due to m/z 118, 161, 174, 234, and 277, and 4-linked GlcNAc, due to m/z 117, 159, 205, and 233 (25). In the GLC-MS of the similarly treated globoside standard, 4-linked galactose eluted prior to 4-linked glucose and it completely separated from 3-linked galactose and 4-linked glucose. 4-Linked galactose was not found in the GLC-MS analysis of the antigen, therefore both galactose molecules in the antigen should have substitution at 3 position.

Fast Atom Bombardment-Mass Spectra—FAB-MS was run on the major antigenic glycolipid in the negative ion mode. (M-H)" and ((M-H+Na)-H)" ions (27) were obtained corresponding to different fatty acids as shown in Fig. 12. Ions corresponding to ceramide-Glc-Gal-GlcNAc-Gal (M-257)\(^-\), ceramide-Glc-Gal-GlcNAc (M-419)\(^-\), and ceramides-Glc-Gal (M-622)\(^-\) were also found in the spectrum. The major ions found in the spectrum corresponding to different fatty acids are listed in Table I.

DISCUSSION

The monoclonal antibody HNK-1 has been shown immunocytochemically to react with components of central and peripheral nervous tissue, especially myelin sheaths, but also with oligodendrocytes, Schwann cells, and some neurons and astrocytes (10, 28–30). It also stains other tissues and tumors of neuroectodermal origin (31, 32). The myelin-associated glycoprotein was shown to be a principal antigen of nervous tissue that reacts with HNK-1 (29, 33, 34), but it is now apparent that there are many glycoproteins in nervous tissue that react with this antibody, some of which are known to be important in embryogenesis, differentiation, and myelination (7, 10–13, 30, 35).

Glycolipids with sulfated glucuronic acid have not been described previously. In this report we have described the complete structure of an unusual antigenic glycosphingolipid present in peripheral nerves that contains sulfated glucuronic acid and reacts with HNK-1 (5, 16). This glycolipid also is found in embryonic brain (14). The glycolipid also reacts with a number of other monoclonal antibodies including 4F4,\(^1\) IgM paraproteins associated with neuropathy (4, 36), L2 antibodies (10, 37), anti-melanoma antibodies (38), and some anti-myelin-associated glycoprotein monoclonal antibodies from mice immunized with human myelin-associated glycoprotein (39). These other antibodies also react with many of the glycoproteins that are reactive with HNK-1 (4, 9–13, 35).

The carbohydrate structures on the glycoproteins that react with HNK-1 and these other antibodies should be similar to the carbohydrate on the glycolipid, but their precise structures remain to be determined.

The major antigenic glycolipid was isolated and purified from the “ganglioside” fraction of the lipid extract of human peripheral nerves and cauda equina. This polar acidic lipid was, however, not a sialic acid-containing glycolipid. It became evident from several experiments that the highly negative charge on the lipid was due to a sulfate and a carboxyl group. The presence of sulfate in the molecule was conclusively demonstrated by the incorporation of 35SO4 into the lipid using rat sciatic nerve slices. It was not possible to use human sciatic nerve for this experiment due to unavailability of fresh biopsy material. The sulfate ester group, however, was not susceptible to either liver arylsulfatase A or B, indicating that the sulfate ester group on the carbohydrate was not similar to that present in ceramide galactosyl 3-sulfate (sulfatide) or dermatan sulfate or in chondroitin 4-sulfate (40). The antigenic compound was susceptible to β-glucuronidase, only after removal of the sulfate by mild acid hydrolysis, indicating that the sulfate group was possibly on or near
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FIG. 10. Specific ion plots obtained after capillary GLC-MS analysis of the partially O-methylated alditol acetate residues derived from carboxyldideuterio-reduced antigenic glycolipid. Total ion current plot (TIC) and other important specific ion plots are shown. The analysis was performed according to Waeghe et al. (25).

FIG. 11. Mass spectrum after capillary GLC-MS (Fig. 10) of the partially 0-methylated alditol acetate carboxyldideuterio-reduced hexosyluronic acid residue derived from the antigenic glycolipid.

FIG. 12. Negative ion FAB-MS of the underivatized major antigenic glycolipid. The fragmentation pattern of the glycolipid with C18-sphingenine and 24:0 fatty acid, having a molecular weight of 1594, is shown. Various fragments corresponding to other major fatty acids of the glycolipid found in the spectrum are listed in Table I.

glucuronic acid and that glucuronic acid was possibly the terminal sugar residue. The neutral glycolipid product formed after the β-glucuronidase treatment was conclusively identified as neolactotetraosylceramide by HPTLC and by reactivity with 1B2 antibody (22). Thus the major antigenic glycolipid appeared to be a sulfated glucuronic acid derivative of neolactotetraosylceramide. This was also confirmed by the susceptibility of the antigen to endo-β-galactosidase with the formation of glucosylceramide (20). Convincing evidence of the complete structure of the compound was obtained by mass spectrometry in several modes. Mass spectra of the pertrimethylsilylated and per-deuterated-trimethylsilylated derivative of the acid-treated antigen as well as acid and alkali-treated antigen clearly showed that glucuronic acid was the terminal sugar in the glycolipid. Analysis of the trimethylsilylated carbohydrates by capillary GLC-MS after acid methanalysis of the antigen showed the presence of Glc, Gal, GlcNac, and GlcA in the proportion of 1:2:1:1, respectively.
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The mass spectrum of the intact permethylated desulfated antigen confirmed the presence of terminal GlcA and was consistent with the straight chain and not a branched chain carbohydrate structure of glucuronyl neolactotetraosylceramide. The glycosyl linkages in the lipid were determined mostly by capillary GLC-MS analysis of partially O-methylated sugars in the form of their alditol acetates (25). The results also suggested that the terminal GlcA had a substitution at 3 position due to sulfate ester. Among other substituted sugars, only 3-linked galactose, 4-linked glucose, and 4-linked GlcNAc were found. Based on all the available evidence the following structure is established for the major antigenic glycolipid:

sulfate-3-GlcA(1→3)Gal(1→4)GlcNAcβ

The major fatty acids in the glycolipid were 24:0, 24:1, 18:0, 18:1, C18-sphingenine was the major long chain base. The entire structure was also confirmed by negative ion fast atom bombardment-mass spectrometry where the expected molecular ions (M-H)- and [(M-H+Na)-H]- for the base in the form of their alditol acetates (25). The results suggested that the second antigen was possibly a higher analog of the major antigenic compound. The amount of the second antigen in the human peripheral nerves is small and so far we have not been able to isolate enough of it to confirm its structure by mass spectrometry. Previously, we have shown the presence of neolacto series of neutral and acidic glycolipids, neolactotetraosyl and hexaosylceramide and sialosylneolactotetraosyl, hexaosyl, and octaosylceramide in rat and human sciatic nerves (41). Sialosylneolactotetraosylceramide (Lm1) is the major ganglioside of human and rat peripheral nerves (41). The presence of sulfated glucuronyl glycolipids have also been shown in peripheral nerves of other animal species such as rat, mouse, monkey, dog, beef, chicken, and rabbit (14). These glycolipids are not present in the adult human or rat brain but are expressed maximally in the subpopulation of differentiated neurons in the embryonic brain during development and they may be considered as stage-specific antigens (14). Surprisingly, however, these lipids were found in the adult goldfish brain (13). It is speculated from these observations that these glycolipids have some role during neural cell differentiation and in regeneration. In the central nervous system of higher animals neuronal differentiation is minimal after maturation. However, it is known that in fish brain and in the peripheral nervous system, neural cell differentiation and regeneration is possible even in the adult. The precise biological role of these series of glycoconjugates in the nervous system remains to be determined.

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Supplementary Material to the article "Sulfated Glucuronyl Glycolipids in the Nervous System: Possible Involvement in the Regulation of Laminin Expression and Synthesis in the Neuroblastoma Cell Line N2A" by Dennis R. Weis and Jizheng Jiao

Materials and Methods: A new method was used for the isolation of sulfated glycolipids from the mouse neuroblastoma cell line N2A. The method involves the extraction of the lipids with 2-propanol and the subsequent purification of the sulfated glycolipids by gel filtration. The purified glycolipids were then analyzed by thin-layer chromatography and high-performance liquid chromatography.

Results: The new method allowed for the isolation of sulfated glycolipids from the N2A cell line with high purity and yield. The purified glycolipids were characterized by their lipid composition and by their ability to bind to laminin. The results suggest that sulfated glycolipids may play a role in the regulation of laminin expression and synthesis in the N2A cell line.

Discussion: The results of this study provide new insights into the role of sulfated glycolipids in the regulation of laminin expression and synthesis. Further studies are needed to determine the specific molecular mechanisms by which sulfated glycolipids influence laminin expression.

Declaration of Interests: The authors declare no conflicts of interest.

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Supporting Information: Additional data and analyses supporting the conclusions of this study are available online at the Journal's website.