Structural analysis and immunohistochemical localization of two acidic glycosphingolipids from the porcine, parasitic nematode, Ascaris suum

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The acidic glycolipid fraction (AF) of the porcine, parasitic nematode, Ascaris suum, consisted of two subfractions. The major component AF II reacted with orcinol-sulfuric acid and molybdate, while the minor component AF I gave a positive reaction with azure-A, a cationic dye specific for sulfatides. Sugar constituent analysis, methanalysis, methylation analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, liquid secondary-ion mass spectrometry, and gas-liquid chromatography/mass spectrometry specified AF II to be an unusual phosphoinositolglycosphinogolipid (Galz1-Ins-P-1ceramide) and the minor component AF I to be a 3-sulfogalactosylcerebroside (HSO₃⁻3Galβ1–1ceramide). The ceramide moiety of both components consisted of lignoceric (C24:0) and cerebronic (C24h:0) acids and mainly C17 iso-branched sphingosine. Immunohistochemical localization studies of the glycolipid-bound antigenic determinants with a polyclonal antiserum against AF II and an anti-sulfatide monoclonal antibody against AF I revealed the presence of the AF II-epitope in the intestine, whereas the AF I-epitope was found in the hypodermis, contractile zone of somatic muscle cells, and the external musculature of the uterus. To our knowledge, this is the first report of the presence of a sulfatide in an invertebrate.

Key words: Ascaris suum/glycosphingolipids/phosphoinositol/sulfatide/immunohistochemistry

Introduction

Glycosphingolipids as amphiphilic membrane components play an important role in various cell-surface phenomena, such as cell–cell adhesion, differentiation, cellular growth, neurological disorders and cancer, binding of enzymes, hormones and toxins, and host-parasite interactions (Hakomori and Igarashi, 1995). Sulfatides represent a class of acidic glycosphingolipids containing one or two sulfate esters as substituents on their oligosaccharide chain. To date, about 20 different structures isolated from various tissues of vertebrates, echinoderms, and microorganisms have been established (Matkina and Taniguchi, 1985). In the vertebrate nervous system the absence of sulfogalactosycerebroside leads to normal myelination, but with abnormal function and regional instability (Bosio et al., 1996; Coetzee et al., 1996). An accumulation was found in metachromatic leucodystrophy (Mehl and Jatzkewitz, 1965) and gargoylism (Austin et al., 1964). Next to nervous tissue, the highest concentrations of sulfatides occur in the kidney (Martensson, 1966; Slomiany and Slomiany, 1978; Tadano and Ishizuka, 1978a,b; Iida et al., 1989; Nagai et al., 1989; Tadano-Aritomi et al., 1992, 1994, 1996). Sulfatides are believed to be involved in the adaptation of kidney cells to conditions of high osmolarity (Niimura and Ishizuka, 1990, 1991), whereas in mucosal regions they protect cells exposed to acid (Natomi et al., 1988). Sulfatides on erythrocytes of humans (Hansson et al., 1978) and bovine origin (Kushi et al., 1996) may be involved in the mediation or regulation of cell adhesion systems, since these molecules were found to specifically interact with cell adhesion molecules, such as, laminin, thrombospondin, and von Willebrand factor (Roberts, 1987). Sulfated galactosylcerebrosides have been shown to avidly bind to selectin receptors blocking selectin-dependent inflammatory responses in vivo. Furthermore, selective binding has been demonstrated for galactosylcerebrosides sulfated in positions 2 and/or 3 of the carbohydrate ring (Marinier et al., 1997). In developing Xenopus laevis embryos sulfatide was found to be a marker of the vegetal hemisphere, vegetal blastomeres, and endodermal cells (Kubo et al., 1995).

To our knowledge, sulfatides have not so far been reported in invertebrates. We report here that 3-sulfogalactosylcerebroside also occurs in the prototypic, porcine parasite nematode Ascaris suum, where it is located in the hypodermis, contractile zone of somatic muscle cells, and the external musculature of the uterus.

Phosphoinositol-containing glycolipids (phyto- or mycolipids) have been found to be widespread in plants (Carter et al., 1954, 1969a,b; Hsieh et al., 1978; Kaul and Lester, 1978; Laine and Hsieh, 1987), yeast (Wagner and Zofcsik, 1966; Steiner et al., 1969; Smith and Lester, 1974; Törörojana et al., 1974; Hechtberger et al., 1994), protozoans (Costello et al., 1993; Singh et al., 1994; Azzouz et al., 1995), fungi (Brennan and Roe, 1975; Haynes et al., 1993; Levery et al., 1996), and the lugworm (Sugita et al., 1995). In human peripheral nerve tissue, a phosphoinositolgalactosylcerebroside (Ins2-P-3GalCer) is next to sulfatide the most abundant acidic glycolipid present (Mansson et al., 1991). The function of these phosphoinositol-containing glycolipids is at present unknown.

The structure of the major component of A.suum acidic glycosphingolipids, Galz1–2Ins1-P-1ceramide, was published recently (Sugita et al., 1996), and has been independently verified in this publication. Immunohistochemical localization revealed a specific distribution of this compound in the intestine.

Results

Isolation and purification of acidic-fraction glycosphingolipids

Glycosphingolipids were extracted from pulverized and lyophilized A.suum worms as described in the Materials and methods. Contaminant triacylglycerols were removed from the raw extract.
with an acetone-wash. Glycosphingolipids were separated into a neutral and acidic fraction by anion-exchange column chromatography. The acidic fraction was further purified by saponification and phase-partition, according to Svennerholm and Fredman (1980). Acidic glycosphingolipid species were separated by high-performance liquid chromatography (HPLC) on an Iatrobeads column yielding designated acidic fractions AF II and, a faster migrating minor component, AF I.

**Chemical characterization of AF I and AF II**

The acidic glycosphingolipids AF I and AF II were resolved by high-performance thin-layer chromatography (HPTLC) and stained with orcinol-sulfuric acid, Dittmer-Lester reagent, and azure-A (Figure 1). AF I, which showed a migration behavior similar to that of a sulfatide-standard, gave a positive reaction with both orcinol-sulfuric acid and azure-A indicating a sulfate-substituent, whereas AF II gave a positive reaction with Dittmer-Lester reagent (molybdate) indicating the presence of phosphate moieties. After methanolysis, AF I was degraded to a structure with a hydrophobicity compatible to that of a ceramide monohexoside (data not shown).

**Carbohydrate structural analysis of AF I**

The acidic glycolipid component AF I was studied by carbohydrate constituent analysis, methylation analysis, methanolysis, liquid secondary-ion mass spectrometry (LSIMS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Determination of the anomeric configuration of the hexose glycosidic linkage was achieved by chromium trioxide oxidation and subsequent carbohydrate constituent analysis. Cleavage induced by methanolysis was confirmed by HPTLC and MALDI-TOF-MS (data not shown).

Both negative ion-mode LSIMS (Figure 2a) and MALDI-TOF-MS of AF I revealed pseudomolecular ions ([M-H] -) at m/z 864, 879, 893, 907, 921, 923, and 925; this number of pseudomolecular ions implied heterogeneity in the lipid moiety of the molecule. After methanolysis, positive ion-mode MALDI-TOF-MS showed pseudomolecular ions ([M+Na]+) at m/z 809, 823, 837, 851, 865, 867, 869 (Figure 2b). The sensitivity towards methanolysis, the resulting loss of an decrement of 80 Da and the positive reaction with azure-A would indicate the presence of a sulfate-substituent. Carbohydrate constituent analysis established the presence of galactose and methylation analysis showed it to be a 3-substituted galactosyl residue. Chromium trioxide oxidation and subsequent carbohydrate constituent analysis indicated nearly complete loss of galactose, signifying its ß-anomeric linkage to the ceramide residue (data not shown).

**Ceramide analysis**

To study the heterogeneity of the fatty acids and sphingoid-bases of the ceramide moiety, the fatty acids were released from the glycosphingolipids by strong alkaline treatment. MALDI-TOF-MS analysis of lyso-AF I, after methanolysis, revealed pseudomolecular ions ([M+Na]+) at m/z 456, 470, 484, and 498,
indicating that C16-C19 sphingosine-bases may represent major ceramide constituents. For fatty acid analysis, total acidic glycosphingolipids were hydrolyzed and the resultant fatty acid methyl esters were investigated by gas-liquid chromatography/mass spectrometry (GLC/MS). Fatty acid methyl esters were identified, after chemical ionization with ammonia, as their pseudomolecular ions ([M+NH₄]⁺). Cerebroside acid (C24:0) comprised 73.7% of the two fatty acids present and tetracosanoic acid methyl esters were identified by their retention times and pseudomolecular ions ([M+NH₄]⁺) after chemical ionization with ammonia. Relative amounts are based on peak ratios of individual fatty acid derivatives normalized to 100%. C24:0, saturated fatty acid with 24 carbon atoms; C24h:0, saturated hydroxy fatty acid, etc. For C24h:0, the hydroxyl group was assigned to C2 with (R)-configuration. The absolute configuration was determined by the retention time of the corresponding (R)-phenylethylamide. Sphingoid-bases were determined by GLC/MS of the corresponding fatty acid methyl esters generated by peridate and periodate/permanganate oxidation. d16:0, 2-amino-1,3-hexadecanediol; d16:1, 2-amino-4-hexadecene-1,3-diol; t18:0, 2-amino-1,3,4-octadecanetriol; etc., iso-branched.

To chemically determine the structure of the sphingoid-base(s) present, acidic glycosphingolipids were hydrolyzed and the released sphingoid-bases were sequentially oxidized with periodic acid and periodate/permanganate, in order to generate their corresponding free fatty acids. After derivatization as their methyl esters with diazomethane, the fatty acids were analyzed by GLC/MS following chemical ionization with ammonia. Fatty acid methyl esters were identified by their retention times and pseudomolecular ions ([M+NH₄]⁺). The resulting fatty acid composition and derived sphingoid-base equivalents are summarized in Table IB.

**Structural analysis of AF II**

The structural data obtained in the case of AF II confirmed the structure recently published by Sugita and coworkers (Sugita et al., 1996). Since the data described here provide additional information on the sphingoid-base composition of this glycolipid, the complementary results are given in a condensed form.

Carbohydrate constituent analysis revealed the presence of galactose and (after HF-treatment) inositol in equimolar ratios. HF-treatment and strong alkaline treatment resulted in the liberation of the ceramide moiety, which demonstrated a direct phosphate-ceramide ester linkage. The fact, that inositol was only found after HF-treatment further indicated the identity of AF II as being an inositolphosphoceramide. Methylation analysis disclosed a terminal galactose residue. The stability of the hexose towards chromium trioxide oxidation implied an α-linked galactose residue. Negative ion LSIMS revealed pseudomolecular ions ([M-H]-) at m/z 1041, 1055, 1057, 1069, 1071, 1085, and 1087, as well as fragment ions due to the loss of a hexose residue ([M-Hex-H]-) at m/z: 879, 893, 895, 907, 909, 923, and 925 and the loss of Hex-Ins ([P-Cer]-) at m/z: 717, 731, 733, 745, 747, 761, and 763. After peracetylation, pseudomolecular ions ([M-H]-) at

![Fig. 2. Mass spectrometric analysis of acidic glycolipid component AF I before and after methanolsysis. (a) Glycosphingolipid AF I was analyzed by LSIMS in the negative ion-mode using triethanolamine as matrix, [M-H]- pseudomolecular ions registered are given in accurate mass values rounded to the nearest mass unit. (b) MALDI-TOF-MS of AF I after methanolsysis in positive-ion reflectron mode. Pseudomolecular ions [M+Na]+ are given in accurate mass values rounded to the nearest mass unit.](https://academic.oup.com/glycob/article-abstract/8/9/891/2912213/fig?target=AS18130)
Immunohistochemical analysis

Prior to immunohistochemistry, the anti-sulfatide antibody SNH.1 was tested for its specificity and sensitivity in enzyme-linked immunosorbent assay (ELISA) against dilutions of 5–250 ng of sulfatide, mono- and disialogangliosides, the neutral and zwitterionic fractions of _A. suum_ glycosphingolipids, and the acidic glycosphingolipid AF II of _A. suum_. The antibody showed positive reactions only with AF I and the sulfatide standard (data not shown). In parallel and by HPTLC-immunochemical staining, polyclonal rabbit anti-AF II antibodies were shown to react only with the AF II-compound and not with AF I (Figure 1d).

For the immunohistochemical localization of the phosphoinositol- and sulfatide-containing epitopes, cryosections (5 µm) of adult worms were incubated with the AF II-specific polyclonal antiserum and the AF I-specific monoclonal antibody (SNH.1), respectively. The staining patterns of both antibodies were defined by a restricted distribution of label within the organs and tissues of the adult worm. The yellowish tinge observed on positive, immunofluorescent staining, normally considered non-specific, resulted from a combination of the red autofluorescence induced by the Evans blue counterstain and the green fluorescence of fluorescein isothiocyanate (FITC; Figure 3a,d). To facilitate the anatomical interpretation of FITC-stained immunosections of adult _A. suum_, 1 cm segments were fixed, embedded in paraffin wax (60°C melting point), sectioned at 5 µm, and stained with hematoxylin-eosin according to standard procedures, prior to examination by light- (Figure 3g) and phase contrast-microscopy (Figure 3h). AF II-specific immunofluorescence was confined to the intestine. The elongated, cylindrical epithelial cells of the intestinal mucosa were positive, lacking a

m/z 1461, 1475, 1477, 1489, 1491, 1505, and 1507 were determined. The ceramide moiety of AF II was analyzed together with AF I, and showed the major fatty acid species to be C24:0 and C24:0 as well as C16–C19 for the sphingosine-bases (see Table I).
specific cytoplasmic or plasma membrane bias, while the microvilli of the brush border, basal lamina, and nuclei were negative (Figure 3a,b). AF I-specific immunofluorescence was not confined to a particular organ or tissue. The syncytial hypodermis was immunostained with a fibrillated appearance (Figure 3c). The overlying, somatic muscle cells were immunolabeled. The obliquely striated, coelomyarian muscle cells were positive in the sarcoplasm and myofibrils of the contractile portion, but not the surrounding basal lamina (Figure 3c). The cuticle, when present in cryosections, remained unstained. Within the female reproductive tract, the external musculature of the uterus was immunostained, whilst the basal lamina and large uterine epithelial cells, and the contained spermatozoa and cuticle-enclosed embryos remained unlabeled (Figure 3d). In addition, the proximal and distal regions of the ovarian growth zone were immunoreactive. In the former region, immunolabeling was restricted to discernible cytoplasmic granules of the developing oocytes, longitudinal striations of the ovarian epithelial cells and overlaid by apparently randomly distributed, large droplets, which did not correspond to any anatomically recognizable (cell) structure (Figure 3e). In the latter region, immunolabeling was restricted to the cytoplasmic granules and plasma membranes of developing oocytes, longitudinal striations of the ovarian epithelial cells and overlaid by apparently randomly distributed, large droplets, which did not correspond to any anatomically recognizable (cell) structure (Figure 3f).

Chloroform/methanol treatment abolished antibody binding in the intestine, hypodermis, contractile zone of the somatic muscle cells, and external musculature of the uterus, whereas in the ovaries epitope recognition was unaffected (Figure 3f). These results of treatment with organic solvent suggest that the distribution of phosphoinositol-containing- and sulfatide-epitopes in the tissues and organs of <i>A. suum</i> are glycolipid-bound, except for the sulfatide-dependent immunoreactivity of the ovaries, which might, in part, be glycoprotein-bound.

Discussion

The sulfatide described in this study represents a minor component of approximately 0.3 µg/g dry weight of <i>A. suum</i>. The structure of the sulfatide was established by carbohydrate constituent analysis, methylation analysis, LSIMS, MALDI-TOF-MS, methanolysis, chromium trioxide oxidation, and GLC/MS analysis of the ceramide constituents to be HSO₃–3Galβ1–1ceramide. The ceramide moiety was characterized by the fatty acid component as 2-(R)-hydroxy tetracosanoic acid, and sphingosine component as straight-chained and isobranched C16–C19 sphingoid-bases, with isobranched C17-sphingosine as the major component.

In human brain, sulfatides were first detected by Thudichum (1884) and identified by Blix (1933). Sulfatides are major lipid constituents of the brain, kidney and gastric mucosa, i.e., in
organisms characterized by cells with “high traffic membranes.” In the brain, sulfatides play an important role in myelination (Bosio et al., 1996; Coetzee et al., 1996). Other functions are the adaptation to conditions of high osmolarity (Nimura and Ishizuka, 1990, 1991), blood coagulation (Holt et al., 1989), and protection of the mucosa against self-digestion (Natomi et al., 1988).

The enriched occurrence of sulfatides in the gastric mucosa raises the question as to whether the sulfatide found in A. suum is of host origin, since mucosal cells are a source of nutrition for this intestinal parasite. Mucosal sulfatides are usually characterized by long-chain fatty acids, which are often hydroxylated (Suzuki et al., 1968; McKibbin, 1969; Natomi et al., 1988), and C18 sphingosine- and phytosphingosine-bases. A galactosylcerebroside as the putative biosynthetic precursor of this sulfatide could not be detected in A. suum (Lochnit et al., 1997). However, the composition of the ceramide moiety with cerebroside acid and C17 iso-branched sphingosine resembled that of the neutral and zwiterionic glycosphingolipid structures of A. suum (Lochnit et al., 1997, 1998), thus indicating an Ascaris-derived structure and not one originating from the host. Presumably, the sulfotransferase involved in the biosynthesis of this anionic glycosphingolipid catalyzes the sulfation of galactosylcerebroside so efficiently that the sulfatide precursor could not be detected. The sulfatide represents a third biosynthetic pathway for glycosphingolipids in A. suum leading to neutral and zwiterionic structures (Lochnit et al., 1997, 1998), and the two acidic structures Galα1-Ins-P-1ceramide and HSO₃⁻-3Galβ1–1ceramide.

The organic solvent-treatment of sectioned organs and tissues of adult A. suum suggested the existence of two different classes of molecule for the presentation of the sulfatide-epitope, i.e., glycolipid(s) and glycoprotein(s). The glycolipid-bound epitope was expressed in the previously described organs or organ-systems of the hypodermis (Fetterer and Wasiuta, 1987); the somatic musculature (Rosenbluth, 1965; Stretton, 1976) and the uterine external musculature (Ishii and Yanagisawa, 1954), unexpectedly without any definitive indication of a plasma membrane location. In contrast, the glycoprotein-bound epitope was expressed at a membrane location within the ovary, i.e., the plasma membrane of developing oocytes and the longitudinal striations of the spiral-shaped, epithelial cells (Ishii and Yanagisawa, 1954), as well as an assumed similar location within the oocytic, membrane-bound cytoplasmic granules. The simplest explanation for the presumed artefact of immunopositive droplets apparently overlying ovarial sections is the extrusion of cytoplasmic granules from the tissue during processing and their coalescence into the larger structures observed. The functional significance of the organ-specific distribution of the sulfatide in the hypodermis, the contractile zone of the somatic muscle cells, and the external musculature of the uterus is at present unknown.

Even less is known concerning the function(s) of the phosphoinositol-containing glycosphingolipids, although they are widely distributed in nature. It has been speculated that these structures may function as anchors for membrane proteins in a way similar to the description for glycosyl-phosphatidylinositol anchors (Assouz et al., 1995), but with a higher stability due to the increased rigidity of the ceramide moiety.

The organic solvent-treatment of sectioned organs and tissues of adult A. suum suggested the existence of a single class of molecule for the presentation of the phosphoinositol-containing epitope, i.e., glycolipid(s). The glycolipid-bound epitope was expressed only in the previously described intestine (Kessel et al., 1961; Sheffield, 1964), again, unexpectedly without any definitive indication of a plasma membrane location. According to the composition of its ceramide, the same interpretation as for the origin of AF I can be made for AF II. Both components are characterized by a nematode-type ceramide moiety of very long-chained, even-numbered, nonhydroxylated and hydroxylated fatty acids, and mostly branched sphingoid-bases with 16–19 C atoms, as opposed to the fatty acids and sphingoid-base species composition of the mammalian-type.

Since the phosphoinositol-containing glycosphingolipid has been found solely in the intestine of A. suum, it may be speculated that this sphingolipid plays a role in one or more functions of the intestinal tract, such as digestion, uptake of nutritive substances, or osmoregulation.

**Materials and methods**

**Materials**

Undamaged, washed adult male and female worms were collected at the local abattoir. Sulfatide standard, mono- and disialogangliosides were purchased from Sigma. The monoclonal anti-sulfatide antibody (SNH.1) was a generous gift from Prof. J. Johnson (Institute of Immunology, University of Munich, Germany). Acid-treated Salmonella minnesota R595 was prepared and kindly supplied by Dr. C. Galanos (Max-Planck-Institute for Immunobiology, Freiburg, Germany).

**Isolation of glycolipids**

Worms (800 g wet weight) were pulverized at -20°C in a precooled Waring blender and lyophilized. Glycolipids were isolated and purified as described previously (Lochnit et al., 1997). In short: glycolipids were extracted with chloroform/methanol/water (10:10:1; v/v/v), chloroform/methanol/800 mM aqueous sodium acetate (30:60:8; v/v/v) and 2-propanol/hexane/water (55:20:25; v/v/v), pooled, and evaporated to dryness. To remove most of the contaminating triacylglycerols the residue was treated with precooled acetone at 4°C. Neutral and acidic glycosphingolipid fractions were separated by DEAE-Sephadex column chromatography. After dialysis, the acidic fraction was saponified in 1 M methanolic sodium hydroxide for 2 h at 37°C. Lipid contaminants were removed after neutralization with acetic acid in methanol by phase-separation, according to Svennerholm and Fredman (1980). Individual acidic glycosphingolipid species were separated by HPLC on a porous silica gel column (Iatrobeads 6RS-8010, 10 μm, 4.6 ×500 mm, Machery-Nagel). Glycosphingolipids were eluted with a linear gradient from 100% eluant A (2-propanol/hexane/water, 55:44:1; v/v/v) to 100% eluant B (2-propanol/hexane/water, 55:35:10; v/v/v) in 30 min at a flow rate of 2 ml/min; 2 ml fractions were collected and monitored for glycosphingolipids by HPTLC. Corresponding fractions were pooled and rotary evaporated to dryness.

**HPTLC**

For HPTLC separation, HPTLC-silica gel 60 plates from Merck were used. Glycolipids were dissolved at 2 µg/µl in chloroform/methanol/water (65:25:4, v/v/v). For reproducibility, HPTLC was performed according to Nores et al. (1994). Chloroform/methanol/0.02% CaCl₂ (60:37:8, v/v/v) was used as the running solvent. Glycosphingolipids were visualized by spraying with orcinol/sulfuric acid, Dittmer-Lester reagent (molybdate) or azure-A (Kean, 1968).

**MALDI-TOF-MS**

MALDI-TOF-MS data were obtained using a Vision 2000 instrument (Finnigan MAT), operating in the positive- and
negative-ion reflectron and linear modes. Ions were produced by a pulsed ultraviolet laser beam (nitrogen laser, λ = 337 nm). The matrix used was 2,5-di-hydroxybenzoic acid (Sigma), 10 g/l, in 0.1% aqueous trifluoroacetic acid-acetonitrile (1:2, v/v).

**LSIMS**

LSIMS was carried out with a MAT 900 mass spectrometer (Finnigan MAT) equipped with a cesium gun, which was operated at an emission current of 2–3 µA. Mass spectra were recorded at an acceleration potential of 5 kV with a resolution of approximately 3000 and were acquired using a DEC 2100 data system. Spectra of native glycosphingolipids were recorded in the negative-ion mode using triethanolamine (Merck) as matrix.

**Methanolysis**

Sulfatides were dried in a stream of nitrogen and incubated for 16 h at room temperature in 1 ml 20 mM methanolic hydrochloric acid. Desulfated glycolipids were dried in a stream of nitrogen.

**Carbohydrate constituent analysis**

Carbohydrate constituent analyses were carried out as detailed previously (Geyer et al., 1982).

**Methylation analysis**

Glycosphingolipids were permethylated and hydrolyzed (Paz-Parente et al., 1985). The respective, partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by capillary GLC/MS using the instrumentation and microtechniques described previously (Geyer and Geyer, 1994).

**Chromium trioxide oxidation**

Peracetylated (Dell, 1990) glycosphingolipids were incubated for 1 h at 37°C in 100 µl glacial acetic acid containing 10 mg CrO₃. Glycosphingolipids were recovered by partition following the addition of 300 µl H₂O and 300 µl CHCl₃ as the organic phase (Hoffman et al., 1972).

**Preparation of lyso-glycosphingolipids**

Glycosphingolipids were dried and incubated for 20 h at 100°C with 1 M methanolic potassium hydroxide. The solution was neutralized with acetic acid and desalted on reverse-phase, silica gel cartridges (Williams and McCluer, 1980).

**Ceramide analysis**

For fatty acid analysis, glycosphingolipids were hydrolyzed according to Gaver and Sweeley (1965). The resultant fatty acid methyl esters were analyzed by capillary GLC/MS (Lochnit et al., 1997) using the instrumentation described previously (Geyer and Geyer, 1994). For the separation of fatty acid species, a fused-silica capillary column (DB1, 0.25 mm ID, 60 m; ICT) was used. The column temperature was increased from 80°C, at 7°C/min, to a final temperature of 320°C and held isothermally for 10 min. Spectra were recorded either after chemical ionization with ammonia or electron-impact ionization at an electron energy of 2.4033 × 10⁻¹⁷ J or 1.1215 × 10⁻¹⁷ J, respectively. For determination of the absolute configuration at C2 of the hydroxy-fatty acids, they were converted to the corresponding (R)-phenylethylamides, trifluoracetylated, and analyzed as described previously (Lochnit et al., 1997). Sphingoid-bases were characterized after conversion to their corresponding fatty acids by periodate and periodate/permanganate oxidation as methyl esters, as described previously (Lochnit et al., 1997).

**ELISA**

Glycolipids were dissolved in ethanol and adsorbed on to Polysorb microtiter plates (Nunc) by evaporation of the solvent at 37°C. Plates were blocked with 0.5% bovine serum albumin (BSA) in 25 mM Tris-buffered saline pH 7.5, 100 mM NaCl (TBS-B) for 1 h at 37°C (Katiuk et al., 1996). After washing the plates twice with 300 µl/well of 10-fold diluted TBS containing 0.05% Tween-20 (TBS-T10), the anti-sulfatide antibodies were added in TBS-10-B (100 ng/well) and incubated for 1 h at 37°C. Unbound antibodies were removed by 5 washings with TBS-T10. Peroxidase-coupled anti-mouse Ig (Dako) was used as second antibody (10 ng/well). After five washes with TBS-T10, 100 µl well of peroxidase substrate and indicator (H₂O₂ and tetramethylbenzidine, respectively; Sigma) was added. The reaction was stopped after 15 min by adding 100 µl/well 12.5% sulfuric acid. Absorption was measured at 450 nm with an ELISA-Reader (Bio-Rad).

**Generation of polyclonal AF II-specific antiseraum**

AF II (575 µg) was suspended in 500 µl H₂O by sonication and added to a 1 ml suspension of 500 µg acid-treated S. minnesota R595 (Galano et al., 1971). After lyophilization, the residue was resuspended in 750 µl H₂O and sonicated, and 250 µl aliquots were stored at -20°C. For immunization the aliquots were diluted to 400 µl and mixed with 400 µl complete Freund's adjuvant with vigorous vortexing for 15 min to form a stable emulsion. White female rabbits were immunized subcutaneously and boosted twice after 4 and 7 weeks, respectively. Three weeks after the last booster injection blood was taken from the ear vein and centrifuged, and the serum was stored at -20°C in 50 µl aliquots.

**Immunohistochemistry**

Segments (1–5 cm) obtained from various regions of frozen adult worms were embedded in Tissue-Tek OCT-Compound (Miles), and transverse cryosections (5 µm) were obtained with a Jung Frigocut 2800E cryotome (Leica) at -40°C. The cryosections were affixed to standard glass slides coated with a polylysine-solution (0.01%, Sigma), treated with acetone for 12 min at -40°C, and air-dried. The sections were washed for 5 min with 0.1 M phosphate-buffered saline (PBS), pH 7.4, blocked with 2% BSA in PBS for 30 min (the block steps were considered optional because of the unchanged distribution of specific immunostaining in its absence), and incubated for 30 min at 37°C with either polyclonal AF II-specific antiserum or the AF I-specific, anti-sulfatide monoclonal antibody (IgM, SNHL1; Schriever et al., 1989) diluted at 1:20, 1:50, and 1:100 with PBS, respectively. After washing three times with cold PBS and reblooming with 2% BSA in PBS for 30 min, they were incubated for 30 min at 37°C with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-mouse Ig (Dako, Hamburg) diluted with PBS at 1:20, respectively, and containing 0.01% Evans blue as counterstain. After washing the sections three times with PBS and mounting in glycerol-buffer (pH 8–9, containing 73 mg NaHCO₃, 16 mg Na₂CO₃ dissolved in 10 ml H₂O, and made up to 100 ml with glycerol), they were examined with a Diaplan fluorescence microscope (Leitz) and photographed.
To investigate the presence of lipid-bound antigens, the fixed sections were treated for 15 min with chloroform/methanol (1:1, v/v), followed by a PBS-wash, before incubation with the respective antibodies.

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Abbreviations

AF, acidic fraction; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorben assay; FTTC, fluorescein isothiocyanate; GLC/MS, gas-liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Ins, inositol; LSIMS, liquid secondary-ion mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PBS, phosphate-buffered saline; TBS, tris(hydroxymethyl)aminoethane-buffered saline; TTBS; TBS containing 0.05% Tween-20; TTBS-10, TTBS-10-fold diluted; TTBS-10-B, TTBS-10 containing 0.5% BSA.

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