Structural Elucidation and Monokine-inducing Activity of Two Biologically Active Zwitterionic Glycosphingolipids Derived from the Porcine Parasitic Nematode *Ascaris suum*

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The isolated neutral glycosphingolipid fraction from the pig parasitic nematode, *Ascaris suum*, was fractionated by silica gel chromatography to yield a neutral and a zwitterionic glycosphingolipid fraction, the latter of which mainly contained two zwitterionic glycosphingolipids termed components A and C. Preliminary chemical characterization with hydrofluoric acid treatment and immunochemical characterization with a phosphocholine-specific monoclonal antibody indicated that both components contained phosphodiester substitutions: phosphocholine for component A, and phosphocholine and phosphoethanolamine for component C. Both components were biologically active in inducing human peripheral blood mononuclear cells to release the inflammatory monokines tumor necrosis factor α, interleukin 1, and interleukin 6. Component A was the more bioactive molecule, and its biological activity was abolished on removal of the phosphocholine substituent by hydrofluoric acid. The glycosphingolipid components were structurally analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, liquid secondary ion mass spectrometry, methylation analysis, ¹H NMR spectroscopy, exoglycosidase cleavage, and ceramide analysis. Their chemical structures were elucidated to be (see Structure I below),

![Structure I](image)

The carbohydrate moiety oligosaccharide core was characterized as belonging to the arthro series of protostomial glycosphingolipids. The ceramide moiety was distinguished by (R)-2-hydroxytetrasaccharic acid as the dominant fatty acid species and by the C17 iso-branched sphingosine and sphinganine bases, 15-methylhexadecasphing-4-enine and 15-methylhexadecasphinganine, respectively.

Analyses of the immunoreactivity between neutral fraction glycolipids derived from various species of parasitic nematodes have indicated a high degree of serological cross-reactivity (1, 2). The structural basis for this immunological cross-reactivity between parasitic nematodes at the level of glycolipids is at present unknown. Structural studies on the neutral fraction glycosphingolipids from adults of the porcine parasitic nematode *Ascaris suum* have revealed that the identified arthro series oligosaccharide chain was not immunogenic, i.e. did not exhibit immunoreactivity toward infection sera from *A. suum*-infected mice (2, 3). However, a zwitterionic glycosphingolipid fraction was also isolated from *A. suum* that demonstrated a phosphodiester sidechain as a structural modification of possibly phosphocholine (PC) and phosphoethanolamine (PE). In addition, these zwitterionic glycolipids were immunogenic/antigenic, i.e. exhibited immunoreactivity toward infection sera from *A. suum*-infected mice (2). PC-containing macromolecules have been regularly detected.

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1. The abbreviations used are: PC, phosphocholine; PE, phosphoethanolamine; CPH, ceramide pentahexoside; HPTLC, high-performance thin-layer chromatography; IL, interleukin; LPS, lipopolysaccharide; LSIIMS, liquid secondary-ion mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; N-glycolipid, neutral glycolipid; Nε-glycolipid, zwitterionic glycolipid; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; Hex, hexose; HexNAc, N-acetylhexosamine.
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in the extracts of numerous species of parasitic nematodes by immunological means (4–9). Structurally, this moiety has been found bound to N- and O-linked glycans of glycoproteins, although the exact structure of the PC-oligosaccharide linkage is at present unknown (10). The biological significance of PC glycans in the host parasite relationship revolves around their immunomodulatory activity (11) such that the frequent observation of host T-cell hypo-responsiveness to filarial nematode infection (12) may involve PC because of its ability to block T- and B-cell antigen-specific proliferation (13, 14).

Little is known as to the biological activity of glycolipids, in general, and parasitic helminth-derived glycolipids, in particular, as regards their putative modulation of the host immune response via the cytokine network. Gangliosides have been found to be inhibitory in terms of cytokine synthesis and release (15), whereas neutral glycosphingolipids of the cestode *Echinococcus multilocularis* inhibited the production of interleukin 2 (IL-2) (16). Because of the physico-chemical similarity between glycosphingolipids and lipopolysaccharides (LPS) of Gram-negative bacteria and the induction by the latter of bioactive protein mediators in the host, i.e. cytokines, responsible for the effects of endotoxemia (17), a comparative study was performed by Krziwon et al. (18) on the ability of the former to stimulate the production of inflammation-associated cytokines. An atypical, zwitterionic glycosphingolipid (as regards the linkage of the glucuronic acid residue to the ceramide moiety and the presence of nonacylated glucosamine) from the LPS-negative, Gram-negative bacterium *Sphingomonas paucimobilis* induced the synthesis and release of the human mononuclear cell-derived, inflammation-associated cytokines tumor necrosis factor α (TNF-α), IL-1, and IL-6 but with approximately 10,000-fold less activity than LPS, in this respect.

We report here on the structures and biological activity of two immunoreactive, zwitterionic fraction glycosphingolipids from *A. suum* in terms of their ability to stimulate the production of the human mononuclear cell-derived, inflammation-associated cytokines TNF-α, IL-1, and IL-6.

EXPERIMENTAL PROCEDURES

**Materials**—Undamaged, washed adult male and female worms were collected from the local abattoir and stored at ~70 °C until further use. LPS from *Salmonella fredenau* was kindly donated by H. Brade (Borstel Research Institute).

**Preparations**—Worms (800 g wet weight) were pulverized at ~20 °C in a precooled Waring blender and lyophilized. Glycolipids were isolated as previously described (3). In short, glycolipids were extracted with chloroform/methanol/water 10:10:1 (by vol), chloroform, methanol, 0.5 M sodium acetate 30:60:8 (by vol), and 2-propanol, n-hexane, water 55:20:25 (by vol) and evaporated to dryness. To remove most of the containing triglycerides, the residue was treated with acetone at 4 °C for 2 h. Neutral (N-Nz-) and acidic glycosphingolipids were separated by DEAE-Sephadex A-25 column chromatography (Pharmacia). The column was equilibrated with and the sample taken up in chloroform/methanol/water 30:60:8 (by vol). N-Nz- glycosphingolipids were obtained in the flow-through, and the acidic glycosphingolipids were eluted with chloroform, methanol, aqueous 0.5 M sodium acetate 30:60:8 (by vol). Neutral (N-) and zwitterionic (Nz-) glycolipid fractions were further fractionated on a silica gel column (Merck). Homogeneous, zwitterionic components A and C were obtained by isocratic elution with chloroform/methanol/water 10:10:2.5 (by vol) from a silica gel column (1 × 50 cm, 70–250 mesh; Merck).

**Bioassay Determination of Released Cytokines**—The isolated Nz-glycosphingolipids, component A and C, and ceramide pentahexoside (CPH) derived from component A by HF treatment (see below) were subjected to sterile distilled water dialysis to remove potential cell culture contaminants and traces of organic solvents. After Speed-Vac lyophilization, the glycosphingolipids were resuspended at 1 mg/ml in sterile distilled water, sonicated, and stored at ~20 °C until further use. As a positive control, S. fredenau-derived LPS was solubilized in pyrogen-free phosphate-buffered saline at 1 mg/ml, neutralized with triethylamine, sonicated, and stored at 4 °C until further use.

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated with Ficoll-Paque (Pharmacia) on density gradient centrifugation. The washed PBMC (10^6/ml) were cultured in U-form microtiter plates (Greiner, Nürtingen, Germany) at 200 μl in RPMI 1640 medium containing antibiotics, 10% heat-inactivated human serum, and the relevant glycolipid. After a 6-h incubation at 37 °C (5% CO2), the supernatants were centrifuged at 1200 rpm for 5 min and investigated for cytokine activity.

The supernatants of glycolipid-stimulated PBMC were analyzed by bioassay as to the cytokine activities of TNF-α, IL-1, and IL-6 (19). The cytokotic activity of TNF-α was determined with the TNF-sensitive L929 fibrosarcoma cell line (20). The proliferative capacity of IL-1 was assayed with the human dermal fibroblasts (21). The proliferation of IL-6-dependent murine B9.9–3A4 hybridoma cells was applied to determine IL-6 activity.

High Performance Thin-layer Chromatography (HPTLC)—For HPTLC separation, HPTLC silica gel plates (Merck) were used. Glycolipids were dissolved at 2 μg/μl in chloroform/methanol/water 10:10:3 (by vol). For reproducibility, HPTLC was performed according to Nöres et al. (22). Chloroform/methanol/water 10:10:3 (by vol) was used as the running solvent. For two-dimensional HPTLC, chloroform, methanol, 2% aqueous ammonia 10:10:3 (by vol) was employed as the second direction-running solvent. The glycosphingolipids were visualized using I2 vapor and/or by spraying the plates with orcinol/sulfuric acid (carbohydrate-positive compounds) or ninhydrin (free amino groups) and heating. For immunostaining, the developed HPTLC plates were fixed with polyvinyl alcohol/dimethylacrylate (Rehm & Haas, Darmstadt, Germany), blocked with phosphate-buffered saline and bovine serum albumin, and incubated with the phosphocholine-specific monoclonal antibody TEPC-15 (Sigma) overnight at 4 °C, as described elsewhere (1). Peroxidase-coupled anti-mouse Ig (Dako Diagnostics, Hamburg, Germany) was used as the secondary antibody.

**Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)**—MALDI-TOF-MS data were obtained using a Vision 2000 instrument (Finnigan MAT, Bremen, Germany) operating in the positive-ion reflectron and linear modes. Ions were formed by a pulsed, ultraviolet laser beam (nitrogen laser, λ = 337 nm). The matrix used was 2,5-dihydroxybenzoic acid (Sigma) at 10 g/liter in 0.1% aqueous trifluoroacetic acid/acetonitrile 1:2 (by vol).

**Liquid Secondary-ion Mass Spectrometry (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 3,000 and were acquired using a DEC 2100 data system. Spectra of native, peracetylated, or permethylated glycosphingolipids were recorded in the positive-ion mode using 3-nitrobenzyl alcohol (Aldrich) as matrix.

**NMR Spectroscopy**—The 1H NMR spectra were recorded as 333 K on a Bruker DRX 600 spectrometer with deuteronium-exchanged samples (0.9 mg each) for solutions in Me2SO with 50 milliunits of DNase, 166 milliunits of β-D-galactosidase, 166 milliunits of α-D-galactosidase, 166 milliunits of endoglycoceramidase (Sigma) and 0.1% aqueous trifluoroacetic acid/acetonitrile 1:2 (by vol).

**Endoglycoceramidase Cleaveage**—Nz-glycosphingolipids were resuspended in 100 μl of 50 mM sodium acetate buffer, pH 5.0, containing 1 g/liter sodium taurodeoxycholate, and 0.5 milliunits of endoglycoceramidase (Sigma) were added. After incubation at 37 °C for 24 h, another 0.5 milliunits of enzyme were added. The reaction was stopped after 48 h by adding 400 μl of H2O and 400 μl of water-saturated n-butanol for phase separation of the reaction products.

**Pyridylation—Nz-oligosaccharides** obtained by endoglycoceramide cleavage were reductively pyridylaminated (23) and the pyridylaminated oligosaccharides were separated by amino-phase HPLC, as described previously (24).

**Zymoglycosidase Treatment**—Pyridylaminated oligosaccharides were cleaved after obligatory HF treatment with either α- or β-galactosidase (EC 3.2.1.22) from coffee beans (Boehringer Mannheim), N-acetyl-β-D-hexosaminidase (EC 3.2.1.52) from jack beans, or β-D-mannosidase (EC 3.2.1.25) from Helix pomatia (Oxford Glycosystems, Abingdon, UK). For cleavage, the dried oligosaccharides were taken up in 50 μl of 50 mM sodium citrate, pH 4.0, and incubated at 37 °C for 24 h with 50 milliunits of α-D-galactosidase, 166 milliunits of β-N-acetylhex-
osaminidase, and 25 milliunits of \( \beta \)-mannosidase, respectively.

**Methylation Analysis**—\( \text{Nz-glycosphingolipids (20 \( \mu \)g) were permethylated both before or after HF treatment and hydrolyzed (25). Partially permethylated aldol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by capillary GLC/MS using the instrumentation and microtechniques described elsewhere (26).**

**Peracetylation**—\( \text{Nz-glycosphingolipids were peracetylated with acetic acid/trifluoroacetic anhydride 1:2 (by vol) for 10 min at room temperature (27).**

**Identification of Zwitterionic Substituents**—Phospholine was released by HF treatment of \( \text{Nz-glycosphingolipid components A and C. Liberated choline residues were derivatized with pentfluoropropionic acid anhydride (Supelco, Deisenhofen, Germany) and analyzed by LSIMS. Ethanolamine was identified as its 9-fluorenylmethoxycarbonyl-derivative by HPLC (28) after HF treatment of component C.**

**N-Methylation of Phosphoethanolamine**—\( \text{Nz-glycosphingolipid component A was treated with 200 \( \mu \)l of 750 mM aqueous sodium carbonate containing 20 \( \mu \)l of methyl iodide for 2 h at 50 °C (29, 30), and thereafter, desalted on a reverse-phase cartridge (31).**

**Ceramide Analysis**—For fatty acid analysis, \( \text{Nz-glycosphingolipids (1–10 nmol) were hydrolyzed according to Gaver and Sweeley (32). The resultant fatty acid methyl esters were analyzed by capillary GLC/MS using the instrumentation described previously (26). For the separation of fatty acid species, a fused silica capillary column (DB1, 0.25 mm internal diameter, 60 m, ICT, Bad Homburg, Germany) 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 (CI-MS) with ammonia or electron-impact ionization (EI-MS) at an electron energy of 2.4033 \( \text{eV (4 J or 1.1215 \times 10^{-17} J, respectively. For determination of the absolute configuration at C-2 of the contained hydroxy fatty acids, they were converted to the corresponding (R)-phenylethylamines and trifluoracetylated as described previously (3). Sphingoid bases were analyzed after conversion to the corresponding fatty acids by periodate/permanaganate oxidation as their methyl and picolinyl esters as described elsewhere (3).**}

**RESULTS**

**Isolation of Zwitterionic Components A and C**—Glycosphingolipids were separated into a neutral and acidic fraction by anion-exchange column chromatography. Two-dimensional HPTLC of the resultant neutral fraction indicated the presence of two groups of glycosphingolipids: N-neutral, \( \text{Nz-neutral zwitterionic glycosphingolipids (see Fig. 1). For isolation of the two main zwitterionic components A and C, the neutral fraction was subfractionated into a neutral and neutral zwitterionic fraction by silica gel column chromatography. A further silica gel column chromatography yielded four fractions designated as components A, B1, B2, and C. The fractions B1 and B2 represented nonhomogeneous, minor zwitterionic components and will not be discussed further in this publication.**

**Chemical and Immunochemical Characterization**—The two zwitterionic components A and C were separated on HPTLC by chloroform/methanol/water 10:10:3 (by vol) as running solvent. Both components gave positive reactions on incubation with iodine vapor, spraying with orcinol/sulfuric acid, and molybdate-reagent (organic phosphate groups). HPTLC-immuno-staining with the phosphocholine-specific monoclonal antibody TEPC-15 is shown in Fig. 2. Due to the approximately 50-fold higher sensitivity of HPTLC-immunostaining, additional, minor species of components A and C resulting from heterogeneities in their lipid moieties were also visualized. The component C also reacted with ninhydrin, indicating the presence of a free amino group. HF treatment of the zwitterionic compounds yielded glycosphingolipids with migration properties on HPTLC similar to CPH, which showed no reaction with TEPC-15 or ninhydrin. Choline was identified after HF treatment of component A and derivatization with pentfluoropropionic acid anhydride and analysis by LSIMS, yielding a molecular ion \([\text{M}]^{+}\) at m/z 250. Ethanolamine was identified after HF treatment of compound C as its 9-fluorenylmethoxycarbonyl derivative by HPLC and co-chromatography with the standard (data not shown).

**Zwitterionic Component A- and C-induced Monokine Production**—Since we consider \( A. suum \) merely as a model for the human parasitic nematode \( A. lumbricoides \), all in vitro procedures were performed with human and not porcine PBMC. The zwitterionic components A and C and the component A-derived CPH were assayed as to their biological activity in inducing the inflammatory monokines TNF-\( \alpha \), IL-1, and IL-6 because of the postulated similarities in physico-chemical properties and biological activity between glycosphingolipids and LPS. Components A and C, but not ceramide pentasaccharide, were shown to be bioactively active in terms of a dose-dependent response in the release of TNF-\( \alpha \), IL-1, and IL-6 (see Fig. 3). For IL-1 and IL-6, this dose dependence of cytokine release was evident up to and including 1000 ng/ml component A, with the apparent presumption that higher concentrations were inhibitory at the cellular level. Of the two zwitterionic glycolipids tested, component A was the more bioactive in inducing the monokines TNF-\( \alpha \) and IL-1; component A and to a lesser extent component C were also capable of inducing low levels of IL-6 activity as demonstrated in three separate experiments (data
The apparent inconsistency in concentration levels measured was due to the inherent between-experiment variability of the bioassay system with different human donors.

Structural Analysis of Zwitterionic Components A and C—For structural analysis, the zwitterionic components A and C were subjected to MALDI-TOF-MS, LSIMS, methylation analysis, and exoglycosidase digestion. The results of MALDI-TOF-MS and LSIMS are summarized in Table I and methylation data in Table II.

Positive-ion MALDI-TOF-MS analysis in the linear mode of the zwitterionic compounds A and C revealed pseudomolecular ions at $m/z$ 1732 ([M + Na]$^+$) and 1833 ([M + H]$^+$), respectively, whereas in the reflectron mode, pseudomolecular ions at $m/z$ 1669 ([M - 87] + 2Na + H)$^+$; loss of choline) and 1813 ([M - 45] + Na + 2H)$^+$, loss of ethanolamine) were respectively observed (see Fig. 4 and Table I), due to metastable decay. After HF treatment, a pseudomolecular ion ([M + Na]$^+$) at $m/z$ 1568 was measured in linear and reflectron mode for components A and C, indicating a homologous glycolipid backbone with the composition Hex$_3$HexNAc$_2$-Cer corresponding to component CPH of the neutral glycolipid fraction of *A. suum* (3).

Positive-ion LSIMS of the native zwitterionic compounds A and C revealed pseudomolecular ions at $m/z$ 1710, 1712 ([M + H]$^+$) and 1833 ([M + H]$^+$), respectively (see Table I). After permethylation, pseudomolecular ions at $m/z$ 1899 ([M - 87] + Na + 2H)$^+$; loss of choline) were observed for both compounds due to the loss of the phosphoethanolamine substituent in component C during the permethylation procedure; the addition of sodium acetate shifted the pseudomolecular ions ([M - 87] + 2Na + H)$^+$ to $m/z$ 1921, 1923. Pseudomolecular ions ([M + H]$^+$) at $m/z$ 2340, 2342 and $m/z$ 2463, 2465 were, respectively, observed after peracetylation of the two zwitterionic compounds (see Fig. 5), whereas pseudomolecular ions at $m/z$ 2298, 2300 and 2379, 2381 and 2421, 2423, respectively, are most likely due to incomplete acetylation and/or ketene elimination.

To locate the monosaccharide linkage and phosphodiester substitution positions, methylation analysis of the two zwitterionic compounds A and C was performed with the permethylation procedures both before or after HF treatment (Table II). If the permethylation procedure was performed after HF treatment, a pseudomolecular ion ([M + Na]$^+$) at $m/z$ 1568 was measured in linear and reflectron mode for components A and C, indicating a homologous glycolipid backbone with the composition Hex$_3$HexNAc$_2$-Cer corresponding to component CPH of the neutral glycolipid fraction of *A. suum* (3).

Positive-ion LSIMS of the native zwitterionic compounds A and C showed similar results with terminal galactose, 3-substituted mannose, 4-substituted glucose, 4-substituted N-acetylglucosamine, and 3-substituted N-acetylgalactosamine (Table II, columns A1 and C1). HF treat-
Glycolipids were analyzed in native, permethylated, or peracetylated form by MALDI-TOF-MS and LSIMS. For MALDI-TOF-MS, native compounds or permethylated, and peracetylated glycolipids were dissolved in chloroform/methanol/water 10:10:3 (by vol), and 2,5-dihydroxybenzoic acid was used as matrix. For LSIMS, native compounds were dissolved in chloroform/methanol/water 10:10:3 (by vol) or dichloromethane, respectively, and applied to a matrix of 3-nitrobenzyl alcohol.

Analysis of zwitterionic glycosphingolipids A and C from A. suum by MALDI-TOF-MS and LSIMS

| Component | MALDI-TOF-MS | LSIMS |
|-----------|--------------|-------|
|           | Linear | Reflecter | Calculated | Linear | Reflecter | Calculated | Calculated |
| A         | 1732<sup>a</sup> | 1732.0<sup>b</sup> | 1732.0<sup>b</sup> | 1658<sup>c</sup> | 1658.0<sup>c</sup> | 1658.0<sup>c</sup> | 1658.9<sup>c</sup> | 1658.9<sup>c</sup> |
|           | 1669<sup>d</sup> | 1669.0<sup>d</sup> | 1671.0<sup>d</sup> | 1652.9<sup>e</sup> | 1654.9<sup>e</sup> | 1654.9<sup>e</sup> |
| C         | 1833<sup>b</sup> | 1833.0<sup>b</sup> | 1833.0<sup>b</sup> | 1835.0<sup>d</sup> | 1835.0<sup>d</sup> | 1835.0<sup>d</sup> | 1835.0<sup>d</sup> | 1835.0<sup>d</sup> |
|           | 1813<sup>c</sup> | 1813.0<sup>c</sup> | 1813.0<sup>c</sup> | 1814.0<sup>d</sup> | 1814.0<sup>d</sup> |

<sup>a</sup>, [M + Na]<sup>+</sup>; <sup>b</sup>, [M + H]<sup>+</sup>; <sup>c</sup>, [M - 87 + 2Na + H]<sup>+</sup>; <sup>d</sup>, [M - 87 + Na + Li + H]<sup>+</sup>; <sup>e</sup>, [M - 87 + Na + 2H]<sup>+</sup>; <sup>f</sup>, permethylated; <sup>g</sup>, peracetylated; <sup>h</sup>, after the addition of sodium acetate.

**Table II**

Methylation analysis of zwitterionic glycosphingolipids A and C

| Linkage | A1 | A2 | C1 | C2 | C3 | Alditol acetate |
|---------|----|----|----|----|----|----------------|
| Gal1-   | 0.5| 0.6| 0.7| 0.5| 0.3| 2,3,4,6-GalOH |
| -3Man1- | 0.9| 1.0| 0.9| 0.8| 0.2| 2,4-ManOH     |
| -4Glc1- | 1.0| 1.0| 1.0| 1.0| 1.0| 2,3,6-GlcOH   |
| -3,6Man1- | - | - | - | 0.4| 0.5| 2,4-ManOH     |
| -4,6GlcNAc1- | - | + | - | + | + | 3-GlcN(Me)AcOH |
| -4,6GlcNAc1- | + | + | + | + | + | 3-GlcN(Me)AcOH |
| -3GalNAc1- | + | + | + | + | + | 4,6-GalN(Me)ArOH |

After the permethylation procedure revealed for compound A the presence of a 4,6-disubstituted N-acetylglucosamine (Table II, column A2) that indicated location of the phosphocholine substituent at the C-6 of N-acetylglucosamine. For compound C, a 4,6-disubstituted N-acetylglucosamine and a 3,6-disubstituted mannose were found along with 3-substituted mannose (Table II, column C2), the latter of which is formed due to the liability of the phosphoethanolamine substituent to the conditions of the permethylation procedure (33). If the phosphoethanolamine substituent was stabilized by methylation to choline before permethylation, the major mannose constituent was found to be 3,6-disubstituted mannose, indicating the localization of phosphoethanolamine at the C-6 of mannose (Table II, column C3).

The zwitterionic glycolipids were cleaved by endoglycoceramidase, and the liberated oligosaccharides were reductively pyridylaminated and isolated by amino-phase HPLC. For determination of the anomeric configurations of individual glycosidic bonds, pyridylaminated oligosaccharides, after HF-treatment, were sequentially incubated with α-galactosidase, β-N-acetylhexosaminidase, and β-mannosidase, resulting in the release of one galactosyl residue, two N-acetylhexosaminyl residues, and one mannosyl residue, as confirmed by amino-phase HPLC.

As a second method for structural confirmation, the anomeric linkages of the sugar residues in components A and C were further elucidated by 1H NMR spectroscopy as α-Gal<sup>Y</sup>, β-GalNAc<sup>Y</sup>, β-GlcNAc<sup>H</sup>, β-Man<sup>H</sup>, and β-Glc<sup>C</sup> (Table III). Chemical shift values and coupling constants (J<sub>1,2</sub>) of the anomeric protons were found to be very similar, indicating identical linkages and composition in both glycosphingolipids. With the exception of the terminal α-Gal residue, all sugars were identified to express β-anomeric linkage. The anomeric linkage of the Man<sup>H</sup> could not be determined by a one-dimensional 1H NMR experiment but was investigated following the connectivities of the spin system using two-dimensional correlation spectroscopy (COSY) and two- and three-step-related coherence transfer (RCT-1 and -2) (data not shown). All anomeric linkages determined by 1H NMR were found to be identical as compared with results obtained by enzymatic degradation reactions (see above).

In addition, glycosphingolipids A and C showed characteristic singlets (integral 9H) for the methyl protons of the choline residue [-N(CH<sub>3</sub>)<sub>3</sub>] (3.135 ppm, compound A; 3.156 ppm, compound C) originating from a phosphocholine residue being assigned by methylation analysis to position 6 of the GlcNAc<sup>III</sup> residue in both glycosphingolipids.

For ceramide analysis, the two zwitterionic glycolipids were subjected to acid hydrolysis according to Gaver and Sweeley
FIG. 4. MALDI-TOF-MS analysis of zwitterionic components A and C. Native zwitterionic glycosphingolipid components A (a–d) and C (e–g) were analyzed by MALDI-TOF-MS in positive-ion linear (b, d, and f) and reflectron (a, c, e, and g) modes either before (a, b, c, and e) or after HF-treatment (c, d, and g) with 2,5-dihydroxybenzoic acid as matrix. Pseudomolecular ions are given in accurate mass values rounded to the nearest mass unit. [M + Na]+; [M + H]+; [(M - 87) + 2Na + H]+; [(M - 87) + Na + Li + H]+; [(M - 45) + Na + 2H]+. Inset in a, after LiCl addition.
Fatty acids were extracted with n-hexane and analyzed by GLC/MS. In agreement with previous data on neutral A. suum glycosphingolipids (see Table III and Fig. 4 in Ref. 3), the results demonstrated the predominant presence of 2-hydroxytetracosanoic acid. The absolute configuration at C-2 was found to be (R) by GLC/MS analysis of the corresponding tri-fluoroacetylated (R)-phenylethylamide (data not shown).

Sphingoid bases were analyzed after periodate and periodate/permanganate oxidation as their methyl and picolinyl esters (data not shown). The results indicated the presence of C17 iso-branched sphingosine and sphinganine bases in agreement with Ref. 3.

**DISCUSSION**

A slowly emerging chemical characteristic of invertebrate glycoconjugates (glycolipids, glycoproteins) is their frequent substitution by electrically neutral but amphoteric moieties. The diversity of zwitterionic glycoconjugates among the various phyla of the Invertebrata would point to their biological importance, but as yet, unknown functional significance. A major post-translational modification of parasitic helminth antigens is apparently PC. This antigenic determinant has been detected in nematodes (5, 8, 34, 35), in trematodes, including Schistosoma mansoni (9), and in the cestode Bothriocephalus scorpii (36). In fact, the frequency of serological cross-reactivity between cestodes, trematodes and, in particular, nematodes (37) may be accounted for by the broad distribution of PC-bearing molecules. The (macro)molecular location of the PC moiety is in most cases unknown, but at least in the excretory/secretory product (ES-62) of the adult filarial nematode, Acanthocheilonema viteae, it is attached to the protein backbone via an N-linked glycan (38).

Zwitterionic glycosphingolipids have been structurally characterized from various members of the invertebrate phyla, including identification of the monosaccharide-amphoteric moiety in the Sarcomastigophora (Flagellata) as Man-phosphoethanolamine (39), in the Annelida as Gal-phosphocholine (40–44), in the Arthropoda (Crustacea) as Glc-phosphonoethanolamine (45), in the Arthropoda (Insecta) as GlcNAc-phosphoethanolamine (33, 46, 47), in the Mollusca (freshwater Bivalvia) as Man-phosphoethanolamine (48), in the Mollusca (marine Gastropoda) as Gal-phosphonoethanolamine (49, 50), and in the Nematoda (Ascaridida) as GlcNAc-phosphocholine and Man-phosphoethanolamine (Ref. 3 and this publication).

Localization of zwitterionic substituents such as phosphocholine or phosphoethanolamine was performed by HF treatment, both before or after permethylation and subsequent hydrolysis, reduction, and peracetylation (in the range of 10 mg of glycosphingolipid). The alkali instability of the phosphoethanolamine substituent, however, requires selective N-methylation before the permethylation procedure. MALDI-TOF-MS analysis of the zwitterionic glycolipids revealed a characteristic fragmentation in the reflectron mode, probably due to the loss of choline (M+H)- and ethanolamine (M−45), respectively, by metastable decay, which was not detectable in the linear mode. A similar fragmentation pattern has been observed in LSIMS after permethylation, whereas the peracetylated structures were found to be stable. This idiosyncratic fragmentation behavior may help to detect and identify zwitterionic substituents by mass spectrometry.

Structural elucidation of the two major, zwitterionic glycosphingolipids (components A and C) of the porcine, parasitic...
nematode *A. suum* has shown their common pentasaccharide core to belong to the arthro-carbohydrate series (as originally isolated from glycosphingolipids of the blowflies *Calliphora vicina* and *Lucilia caesar*). The amphoteric substituent PC is linked to C-6 of the third monosaccharide in the oligosaccharide chain, GlcNAc, of component A, and, uniquely, the amphoteric substituents PE and PC are simultaneously linked to C-6 of the second and the third monosaccharide in the oligosaccharide chain, Man and GlcNAc, respectively, in component C. Component C, therefore, represents the first member of the glycosphingolipids to carry two zwitterioner substituents. The carbohydrate and ceramide moieties of the two zwitterionic glycosphingolipids correspond to the recently elucidated arthropentaosyl ceramide of *A. suum*. Therefore, we have assumed that the biosynthetic pathway of the former involves zwitterioner substitution of the latter, either at the level of CPH or incomplete oligosaccharide cores.

The 3H-NMR data obtained for both glycosphingolipids A and C were found to be structurally closely related to that identified in a pentaglycosyl phosphoglycosphingolipid (NZ5a) isolated from the blowfly *C. vicina* Meigen (51). Comparing the glycosphingolipid NZ5a of the blowfly *C. vicina* Meigen and compound A described here, both belong to the arthro series, and only three structural differences were observed: (i) a terminal α-galNAcβ being α-galNAcβ instead of α-galNAcβ, (ii) the terminal sugar (α-galNAcβ) being (1→4)-linked in NZ5a and α-galNAcβ being (1→3)-linked in compound A, and (iii) 2-aminoethyl phosphate instead of a phosphocholine substituent in position 6 of GlcNAcβ. The biological properties of the glycosphingolipid NZ5a, however, were not investigated.

Glycosphingolipids have been shown to be immunomodulatory molecules that suppress cells of the immune system, both in vivo and in vitro. Thus, gangliosides inhibit the in vitro proliferative response of various classes of activated immune cells such as T- and B-lymphocytes, macrophages, and natural killer cells (52). However, the molecular mechanism(s) underlying the immunosuppressive activity of glycosphingolipids are incompletely understood but include the direct interaction of ganglioside micelles with IL-2 and IL-4 in the modulation of IL-2/IL-4-dependent processes (53) and the interference of monocytes at the level of antigen presentation (54). The immunomodulation of T-lymphocyte activation in vivo and in vitro, observed in the case of *Trypanosoma cruzi* ganglioside micelles with IL-2 and IL-4 in the modulation of IL-2/IL-4-dependent processes (53) and the interference of monocytes at the level of antigen presentation (54). The immunomodulation of T-lymphocyte activation in vivo and in vitro, observed in the case of *Trypanosoma cruzi* ganglioside micelles with IL-2 and IL-4 in the modulation of IL-2/IL-4-dependent processes (53) and the interference of monocytes at the level of antigen presentation (54).

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