Phosphocholine-containing, zwitterionic glycosphingolipids of adult *Onchocerca volvulus* as highly conserved antigenic structures of parasitic nematodes

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Human *Onchocerca volvulus* infection sera were found to recognize zwitterionic glycolipids of *O. volvulus* and to cross-react with those of other parasitic nematodes (*Ascaris suum*, *Setaria digitata* and *Litomosoides sigmodontis*). By the use of an epitope-specific monoclonal antibody, zwitterionic glycolipids of all these nematode species were observed to contain the antigenic determinant phosphocholine. A hyperimmune serum specific for arthro-series glycolipid structures reacted with the various neutral glycolipids of all these nematodes, which demonstrated that their oligosaccharide moieties belonged to the arthro-series of protostomial glycolipids. These results indicated that arthro-series glycosphingolipids carrying, in part, phosphocholine substituents, represent highly conserved, antigenic glycolipid markers of parasitic nematodes. Three glycolipid components of the *O. volvulus* zwitterionic fraction were structurally characterized by matrix-assisted laser-desorption/ionization time-of-flight MS, methylation analysis and exoglycosidase treatment. Their chemical structures were elucidated to be phosphocholine-6GlcNAc(β1-3)Man(β1-4)Glc(1-1)ceramide, GalNAc(β1-4)-[phosphocholine-6]GlcNAc(β1-3)Man(β1-4)Glc(1-1)ceramide and Gal(α1-3)GalNAc(β1-4)[phosphocholine-6]GlcNAc(β1-3)-Man(β1-4)Glc(1-1)ceramide for the zwitterionic ceramide tri-, tetra- and penta-hexosides respectively. The ceramide composition was found to be dominated by 2-hydroxylated docosanoic ($C_{22h:0}$), tricosanoic ($C_{23h:0}$) and tetracosanoic ($C_{24h:0}$) acids, and C_{17} sphingosine ($C_{d17:1}$) (where $_h$ is hydroxylated and $_d$ is dihydroxylated).

Key words: glycolipids, phosphocholine, on-target enzymic cleavage, parasitic filarial nematodes.

INTRODUCTION

The antigenic determinant phosphocholine (PC) has been found to be the structural basis for the immunological cross-reactivity of zwitterionic glycolipids from various parasitic nematodes [1–3]. Zwitterionic glycolipids of *Litomosoides sigmodontis* (= *carinii*) and *Ascaris suum* reacted with homologous and heterologous infection sera derived from animals experimentally infected with *A. suum*, *L. sigmodontis* and *Nippostrongylus brasiliensis* [2,3]. In addition, zwitterionic glycolipids from *L. sigmodontis*, which is a rodent laboratory model for filariases, were recognized by sera from humans infected with *Onchocerca volvulus*, *Brugia malayi*, *Loa loa*, and *Strongyloides stercoralis* [3]. Detailed structural analysis of the zwitterionic glycolipid antigens of parasitic nematodes has been performed for two components bearing the PC substituent, which were derived from the porcine parasite *A. suum*, that yielded the structures Gal(α1-3)-GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)Cer (component A) and Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)[PE-6]Man(β1-4)Glc(β1-1)Cer [component C; containing PC and phosphoethanolamine (PE) substituents] [1]. Both components were found to be biologically active in inducing human peripheral-blood mononuclear cells to release the proinflammatory monokines tumour necrosis factor α, interleukin 1 and interleukin 6, whilst the biological activity was dependent on the presence of the PC substituent [1].

Besides these immunological, biological and structural analyses of glycolipid-conjugated PC, macromolecules containing PC have been detected using monoclonal antibodies (mAbs) in extracts of numerous species of parasitic nematodes [4–7]. They have been localized to the pseudocoelomic cavity (inner cuticular surface and intestinal lining) in adult males and the reproductive tract (uterine endothelium and embryo membranes) in adult females of the human filarial nematode *B. malayi* [4].

The excretory/secretory PC-containing protein ES-62, secreted by *Acanthocheilonema viteae*, has been structurally analysed to show PC attached to N-linked glycans [8–11], comprising substoichiometrically fucosylated, truncated, complex-type structures ($Fuc_{0-1}Man_3GlcNAc_{3-6}$; [12]). The location of the substitution is likely to be to GlcNAc residues present as either mono-, di-, tri- or tetra-antennary, monomeric stubs [12] or as up to pentameric, (β1-4)-linked, chito-oligomeric antennae [13]. Both PC-substituted, N-glycan families have been shown to be similarly conserved in the human filarial nematode *O. volvulus* and its bovine analogue *Onchocerca gibsoni* [13]. The protein-bound PC determinant is not restricted to N-linked glycans, being predominantly present on O-glycans in *O. gibsoni* [5]. The longevity of filarial nematode infections and frequently associated modulation of the host's immune response have been correlated with the release of PC-containing excretory–secretory protein products, and related to their immunomodulatory/suppressive properties, i. e., the inhibition of polyclonal activation/pro-

Abbreviations used: CDH, ceramide dihexoside; CMH, ceramide monohexoside; CPH, ceramide pentahexoside; CTetH, ceramide tetrahexoside; CTH, ceramide trihexoside; GC–MS, gas chromatography–MS; HexNAc, *N*-acetylhexosamine; HPTLC, high-performance TLC; mAb, monoclonal antibody; Int., intensity; MALDI-TOF-MS, matrix-assisted laser-desorption/ionization time-of-flight MS; PC, phosphocholine; PE, phosphoethanolamine; TFA, trifluoroacetic acid; $_h$, $_d$ and $_t$, hydroxylated, dihydroxylated and trihydroxylated; ΔM, mass difference.

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liferation of human T- and murine B-lymphocytes *in vitro* [14–16]. Such PC-containing excretory–secretory products may play a role in the B- and T-lymphocyte hyporesponsiveness of human filariases. They have been implicated, following ligation, with selectively uncoupling the antigen–receptor and associated protein tyrosine kinase complex from signalling cascades responsible for the transduction of signals for activation, transcription and proliferation [17–19]. This immunomodulation of T- and B-cells has been considered to be an escape mechanism by which the filarial parasite avoids both cell-mediated and humoral immunity.

In the present study we have disclosed by immunostaining and detailed structural analysis that PC-containing glycosphingolipids are highly conserved among parasitic nematodes, which parallels the known presence of PC-containing glycoprotein glycans of different filarial nematodes [10,11,13].

Some of the results reported here have already been presented at the International GlycoBioTechnology Symposium, held in Braunschweig, Germany, on 3–8 May 1998.

EXPERIMENTAL

Isolation and purification of glycolipids

An adult *O. volvulus* worm homogenate was used for the preparation of worm-derived antigen, as described previously [20]. Freeze-dried worm pellets (350 mg) were stored at −20 °C until lipid extraction. Glycolipids were isolated by consecutive extractions as described elsewhere [21]. Raw extracts were saponified in 50 ml of methanolic 0.1 M NaOH for 2 h at 37 °C. Salts and hydrophilic contaminants were removed by reverse-phase chromatography (Chromabond $C18_{ec}$; Macherey–Nagel, Düren, Germany; [22]) and the glycolipids were fractionated on a DEAE-Sephadex column (10 mm × 50 mm, A-25, acetate form; Pharmacia, Freiburg, Germany) as outlined previously [22]. The neutral-fraction glycolipids were resolved on a silica-gel cartridge (Waters, Eschborn, Germany), as described elsewhere [2], and analysed by matrix-assisted laser-desorption/ionization time-of-flight MS (MALDI-TOF-MS), as well as high-performance TLC (HPTLC) and orcinol/H_2SO_4 staining. The fractions chloroform/methanol (19:1, v/v) to chloroform/methanol (1:1, v/v) contained neutral glycolipids and were pooled and purified by Florisil chromatography [22]. The fractions chloroform/methanol/water (60:35:8, by vol.) and (15:30:4, by vol.) contained glycolipids that migrated similarly to *A. suum* zwitterionic glycolipids on HPTLC [2] and gave similar MALDI-TOF-MS spectra [1], and were thus pooled and designated the 'zwitterionic fraction'. Glycolipids from adult *Setaria digitata* worms were extracted analogously, the crude extract was desalted by reverse-phase chromatography and used for HPTLC analyses without further purification. Glycolipids from adult *A. suum* worms, and *L. sigmodontis* microfilariae and adult females were isolated as described previously [2,3].

HPTLC

HPTLC, orcinol/H_2SO_4 staining and immunostaining were performed as described elsewhere [23]. On immunostaining, sera from four patients with an *O. volvulus* infection, an anti-arthro [30]-series rabbit hyperimmune serum (G. Lochnit, R. D. Dennis, S. Nispel and R. Geyer, unpublished work) and the PC-specific mouse mAb TEPC-15 (Sigma, Deisenhofen, Germany) were applied as primary antibodies. Goat horseradish peroxidase-conjugated anti-rabbit Ig and rabbit horseradish peroxidase-conjugated anti-mouse Ig (Dako Diagnostics, Hamburg, Germany), as well as rabbit horseradish peroxidase-conjugated anti-human Ig (Sigma), were used as secondary antibodies (diluted 1:1000). Staining was performed using a chloronaphthol/diethylphenylenediamine substrate mixture as described previously [23].

HF and exoglycosidase treatment

HF treatment of zwitterionic glycolipids from *O. volvulus* and *A. suum* was performed as described previously [1]. For exoglycosidase cleavage, HF-treated glycolipids were taken up in 50 μl of 50 mM sodium citrate buffer, pH 6.0, containing 1 g/litre sodium taurodeoxycholate and incubated at 37 °C for 24 h with 250 m-units of α-galactosidase from green coffee (*Coffea* sp.) beans (Roche Diagnostics, Mannheim, Germany). Enzyme addition was repeated after 12 h. The sample was purified by anion-exchange chromatography on a DEAE-Sephadex column [2] and the resultant neutral fraction analysed by HPTLC orcinol/H_2SO_4 staining, MALDI-TOF-MS and methylation analysis.

Carbohydrate composition and methylation analysis

Glycolipids were hydrolysed in aq. 4 M trifluoroacetic acid (TFA) for 4 h at 100 °C and analysed as their alditol acetates by gas chromatography (GC) [24]. For linkage analysis, glycolipids were permethylated, being either untreated, before or after HF treatment and hydrolysed (4 M TFA, 4 h, 100 °C). Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analysed by capillary GC–MS, using the instrumentation and microtechniques described elsewhere [25].

MALDI-TOF-MS and on-target enzymatic cleavage

MALDI-TOF-MS and on-target enzymatic cleavage was performed using the instrumentation and methods described previously [26]. For on-target enzymic cleavage, α-galactosidase from green coffee beans (Roche Diagnostics), β-*N*-acetylhexosaminidase from jack beans (*Canavalia ensiformis*; Sigma) and β-mannosidase from snail acetone-dried powder (Sigma) were used.

Ceramide analysis

The zwitterionic glycolipid fractions of *O. volvulus* and *A. suum* were treated with 200 μl of anhydrous 1 M methanolic HCl for 16 h at 80 °C. The released fatty acid methyl esters were separated from the remaining sphingoid bases and saccharide residues by a three-fold phase partition against n-hexane. The pooled n-hexane partitions were dried down under N_2. Fatty acid methyl esters were analysed by GC–MS (Finnigan, Sunnyvale, CA, U.S.A.; model 4500) using a fused-silica capillary column (Optima 210, 0.25 mm internal diameter, 30 m long; Macherey–Nagel). The temperature was increased from 100 °C, at 4 °C/min, to a final temperature of 280 °C. Spectra were recorded in the positive-ion mode after electron-impact ionization (70 eV; source temperature 190 °C). For sphingoid base analysis, as a modification of the method described by Zanetta et al. [27], the lower methanol partition was dried under N_2 and, after addition of 200 μl of acetonitrile and 25 μl of pentafluoropropionic anhydride (Supelco, Deisenhofen, Germany), the sample was heated for 30 min at 150 °C, dried under N_2 and taken up in acetonitrile. Samples were analysed by GC–MS using fused-silica capillary columns [Optima 5 (0.25 mm internal diameter, 30 m long) and Optima 210 (as described above; Macherey–Nagel)]. The injector temperature was 280 °C, and the column temperature was

increased from 130 °C, at 6 °C per min, to a final temperature of 250 °C. GC–MS was performed in the negative-ion mode after chemical ionization with ammonia at 150 eV and in the positive-ion mode after electron-impact ionization (70 eV, source temperature 190 °C). For identification, $C_{d18:1}$, $C_{d18:0}$ and $C_{t18:0}$ (where $_t$ is trihydroxylated) standards (Sigma) were used.

RESULTS

PC and the arthro-series motif as common determinants of parasitic nematode lipid-bound carbohydrate structures

Neutral and zwitterionic glycolipids from *O. volvulus* were compared immunochemically with the corresponding glycolipid fractions from *A. suum* adults [1,21], *S. digitata* adults and *L. sigmodontis* microfilariae and adult females (Figure 1). Neutral glycolipids were revealed by HPTLC–immunostaining using an anti-arthro-series hyperimmune serum raised against *A. suum* neutral-fraction glycolipids (Figure 1A). This serum detected co-migrating ceramide trihexoside (CTH) and ceramide pentahexoside (CPH) components in all four nematode species examined. Human *O. volvulus* infection serum revealed cross-reactive zwitterionic glycolipids in all four parasitic nematode species surveyed (Figure 1B). This cross-reactivity, at least in part, would appear to be due to the common antigenic determinant PC being present on all these glycolipids, as indicated by their immunoreactivity with the PC-specific mAb TEPC-15 (Figure 1C). All four nematode species exhibited a band with migration properties similar to those of component A, which has been structurally analysed ([1]; Figure 1C, lanes 2–6). When comparing the chemical and immunological staining of *A. suum* zwitterionic glycolipids (Figures 1B and 1C), component C, which was one of the chemically dominant glycolipids, was only weakly recognized immunologically by the *O. volvulus* infection serum (Figure 1B, lane 3) and mAb TEPC 15 (Figure 1C, lane 3). In contrast, the fastest-migrating *A. suum* zwitterionic glycolipid was strongly recognized by the mAb TEPC 15 (Figure 1C, lane 3); however, chemically it was found to be a minor component (Figure 1C, lane 1). The comparative immunochemical HPTLC staining (Figure 1) indicated that arthro-series glycosphingolipids carrying, in part, PC substituents, represented highly conserved antigens among parasitic nematodes.

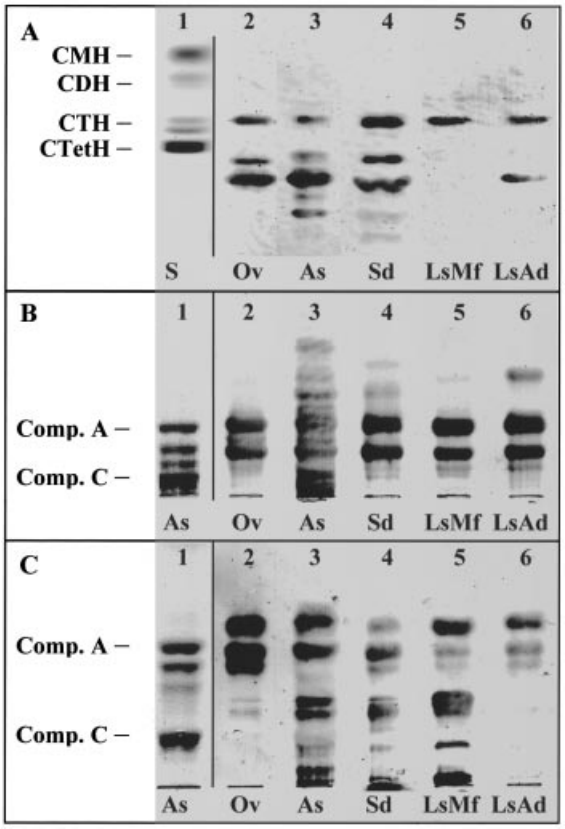

Figure 1 HPTLC–immunostaining of neutral and zwitterionic glycolipids from different, parasitic nematodes

(**A**) Neutral glycolipids were separated by HPTLC [running solvent chloroform/methanol/aq. 0.25% KCl (5:4:1, by vol.)] and revealed with anti-arthro-series hyperimmune serum (lanes 2–6; dilution 1:100). (**B**) Zwitterionic glycolipids were resolved by HPTLC (running solvent as in **A**) and revealed with a pool of four human *O. volvulus*-infection sera (lanes 2–6; dilution 1:100). (**C**) Zwitterionic glycolipids were separated by HPTLC [(running solvent chloroform/methanol/water (10:10:3, by vol.)] and revealed with the PC-specific mAb TEPC-15 (lanes 2–6; dilution 1:500). A globoside standard mixture containing CMH-CTetH (**A**, lane 1) and *A. suum* zwitterionic glycolipids (**B**, lane 1 and **C**, lane 1) were revealed by orcinol/H_2SO_4 staining. Comp. A, component A; Comp. C, component C as *A. suum* zwitterionic glycolipids of known structure. Glycolipid fractions were derived from: Ov, *O. volvulus* adults; As, *A. suum* adults; Sd, *S. digitata* adults; LsMf, *L. sigmodontis* microfilariae; LsAd, *L. sigmodontis* adult females.

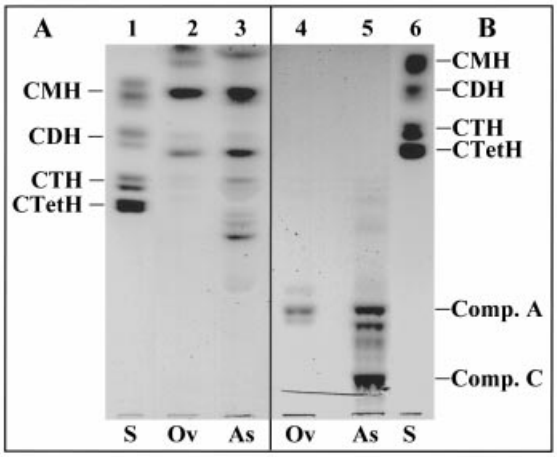

Figure 2 HPTLC–orcinol/H_2SO_4 staining of *O. volvulus* and *A. suum* neutral and zwitterionic glycolipids

(**A**) Neutral glycolipids of *O. volvulus* (lane 2) and *A. suum* (lane 3) were separated by HPTLC using the running solvent chloroform/methanol/aq. 0.25% KCl (5:4:1, by vol.). (**B**) Zwitterionic glycolipids of *O. volvulus* (lane 4) and *A. suum* (lane 5) were resolved by HPTLC using the running solvent chloroform/methanol/water (10:10:3, by vol.). Comp. A, component A; Comp. C, component C as *A. suum* zwitterionic glycolipids of known structure. The globoside standard (S) of CMH-CTetH corresponded to ceramide mono-, di-, tri- and tetra-saccharides respectively. Ov, *O. volvulus*; As, *A. suum*.

Neutral and zwitterionic glycolipids from *O. volvulus* were also compared by HPTLC and chemical staining with the equivalent fractions from *A. suum* adults. The *O. volvulus* neutral glycolipid fraction was further purified by Florisil chromatography and collated by HPTLC (Figure 2A). Both nematodes exhibited ceramide monohexoside (CMH) and ceramide dihexoside (CDH) species of identical migration properties. Although significant amounts of apparent CTH and CPH species were present in *A. suum* (Figure 2A, lane 3), for *O. volvulus* these components could not be detected (Figure 2A, lane 2). In parallel, *O. volvulus* zwitterionic glycolipids, without further purification, were collated by HPTLC (Figure 2B). The chemically predominant

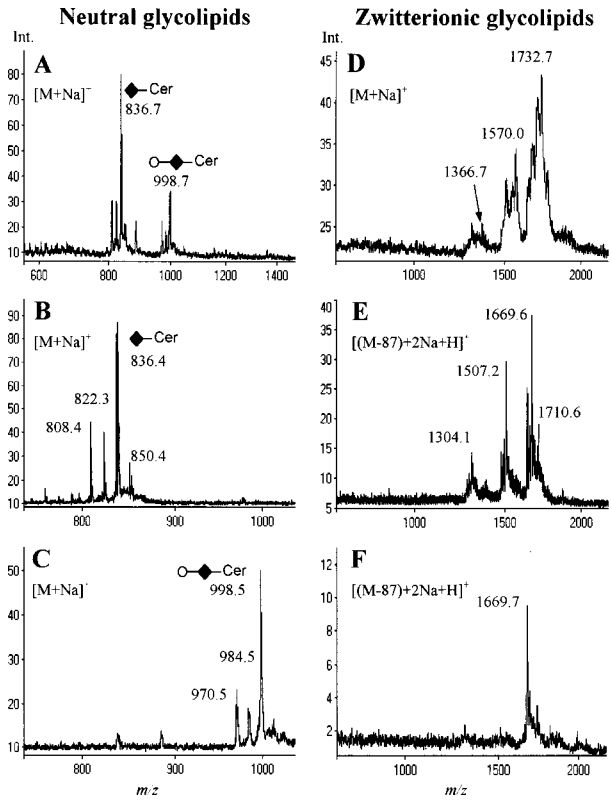

Figure 3 MALDI-TOF-MS analysis of *O. volvulus* neutral as well as *O. volvulus* and *A. suum* zwitterionic glycolipids

(**A**) *O. volvulus* total neutral glycolipids; (**B** and **C**) *O. volvulus* purified CMH and CDH compounds, respectively; (**D** and **E**) *O. volvulus* zwitterionic glycolipids; (**F**) *A. suum* zwitterionic glycolipid component A. Analyses were performed in the positive-ion reflectron (**A–C**, **E** and **F**) or linear (**D**) modes. Pseudomolecular ions are given in monoisotopic masses (reflectron mode) or average masses (linear mode) rounded to one decimal place. ○, Man; ◆, Glc; Cer, ceramide.

O. volvulus zwitterionic glycolipid component co-migrated with the *A. suum* zwitterionic glycolipid component A, whose structure has been previously elucidated [1].

Structural analysis of *O. volvulus* neutral glycolipids

MALDI-TOF-MS of the *O. volvulus* neutral glycolipid fraction (Figure 3A) revealed the pseudomolecular ions $[M+Na]^+$ for CMH and CDH only. Neutral glycolipids were fractionated on a silica-gel cartridge and the purified CMH and CDH species re-analysed by MALDI-TOF-MS. The CMH mass spectrum (Figure 3B) exhibited pseudomolecular ions $[M+Na]^+$ at m/z 808.4, 822.3, 836.4 and 850.4. The dominant Na^+ adduct at m/z 836.4 corresponded to a major pseudomolecular ion observed for *A. suum* CMH at m/z 837 [21]. The CDH mass spectrum (Figure 3C) revealed signals at m/z 970.5, 984.5 and 998.5. The mass difference (ΔM) between respective CMH and CDH ions of 162 Da corresponded to one hexose residue. Again, the dominant CDH pseudomolecular ion at m/z 998.5 coincided with the Na^+ adduct of a major *A. suum* CDH species at m/z 998 [21].

Methylation analysis of the total neutral glycolipid fraction revealed terminal Glc, 4-substituted Glc, terminal Man and terminal Gal in the relative amounts 1.0:0.45:0.3:0.05 respectively. Methylation analysis of isolated CDH resulted in the ratio of terminal Man to 4-substituted Glc of 0.5:1.0 respectively. On-target enzymic cleavage of CDH with β-mannosidase resulted in the loss of one hexose residue and shifted the CDH pseudomolecular ions ($[M+Na]^+$) from 998.5, 984.5 and 970.5 to 837.1, 823.1 and 809.1 respectively. Hence methylation analyses and on-target enzymic cleavage indicated CDH to be Man(β-4)-Glc(1-1)Cer and CMH to be Glc(1-1)Cer.

Structural analysis of *O. volvulus* zwitterionic glycolipids

MALDI-TOF-MS of *O. volvulus* zwitterionic fraction glycolipids revealed major pseudomolecular ions of characteristic PC fragmentation at m/z 1669.6, 1507.2 and 1304.1 in the positive-ion reflectron mode {$[(M-87)+2Na+H]^+$; loss of choline; Figure 3E} and corresponding ions at m/z 1732.7, 1570.0 and 1366.7 in the positive-ion linear mode ($[M+Na]^+$; Figure 3D). Again, the dominant ions at m/z 1669.6 (Figure 3E) and 1732.7 (Figure 3D) were in agreement with the *A. suum* zwitterionic glycolipid component A (Figure 3F; [1]). Carbohydrate composition analysis of the native *O. volvulus* zwitterionic glycolipid fraction revealed the monosaccharide molar ratios for Gal/Glc/Man/GalNAc to be 0.65:1.65:1.0:1.0 respectively. In order to confirm the presence of the arthro-series carbohydrate structure and to determine the position(s) of the phosphodiester sub-

Table 1 Methylation analysis of *O. volvulus* total zwitterionic glycolipid fraction

The partially methylated monosaccharide derivatives obtained after hydrolysis, reduction and peracetylation were analysed by GC–MS. Results are expressed as peak ratios of the alditol acetates found on the basis of 2,4,6 ManOH = 1.0. Gal(1- as linkage corresponded to 2,3,4,6-GalOH or 2,3,4,6-tetra-*O*-methylgalactitol as alditol acetate; GalNAc(1- as linkage corresponded to 3,4,6-GalN(Me)AcOH or 2-deoxy-2-(*N*-methyl)acetamido-3,4,6-tri-*O*-methylgalactitol as alditol acetate, etc. **Bold type** has been used to denote significant differences in composition after the various treatments. α-Gal'ase, α-galactosidase.

		Relative amount				
Linkage	Treatment ...	Native	Methylation preceded by HF treatment	Methylation followed by HF treatment	Methylation preceded by HF/α-Gal'ase treatments	Partially methylated alditol acetate
Gal(1-		0.5	0.3	0.65	–	2,3,4,6-GalOH
-4)Glc(1-		0.9	1.0	1.0	0.8	2,3,6-GlcOH
-3)Man(1-		1.0	1.0	1.0	1.0	2,4,6-ManOH
GlcNAc(1-		–	0.3	–	0.15	3,4,6-GlcN(Me)AcOH
GalNAc(1-		0.45	0.15	0.5	**0.80**	3,4,6-GalN(Me)AcOH
-3)GalNAc(1-		0.65	0.3	0.75	–	4,6-GalN(Me)AcOH
-4)GlcNAc(1-		–	**0.75**	–	**0.85**	3,6-GlcN(Me)AcOH
-4,6)GlcNAc(1-		–	–	**0.55**	–	3-GlcN(Me)AcOH

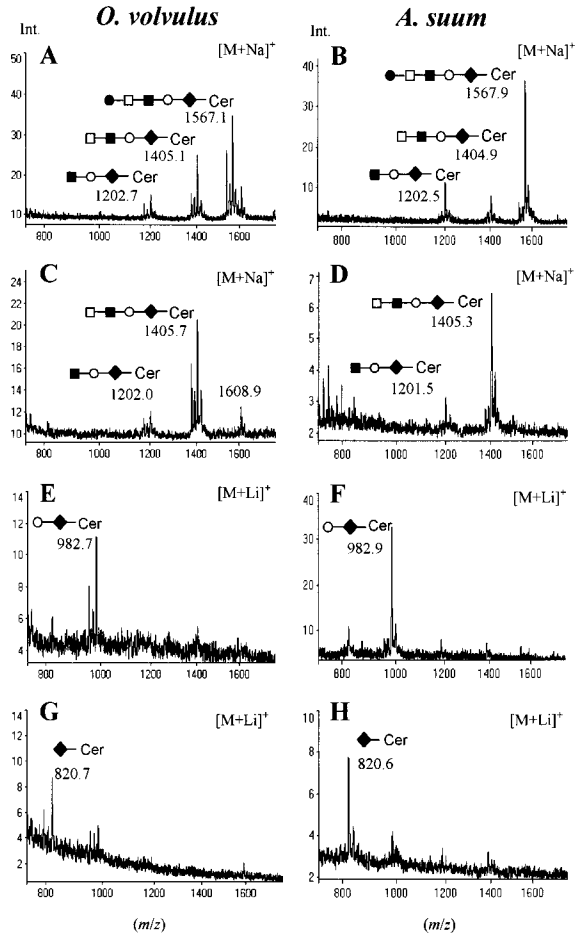

Figure 4 MALDI-TOF mass spectra of the products of on-target sequential exoglycosidase treatments of *O. volvulus* (left column) and *A. suum* (right column) HF-treated zwitterionic glycolipids

Enzymic cleavage of the glycolipids (**A** and **B**) was performed with 1.5 m-units of α-galactosidase from green coffee beans (6h; **C** and **D**), 80 m-units of β-*N*-acetylhexosaminidase from jack beans (overnight; **E** and **F**) and 3.5 m-units of β-mannosidase from snail (overnight; **G** and **H**). In some cases, 1 μl of a 4 mM LiCl solution was added to the analyte spot to produce $[M+\mathrm{Li}]^+$ pseudomolecular ions instead of the Na^+ adducts. Pseudomolecular ions are given in accurate mass units rounded to one decimal place. Measurements were performed in the positive-ion reflectron mode. ●, Gal; □, GalNAc; ■, GlcNAc; ○, Man; ◆, Glc; Cer, ceramide.

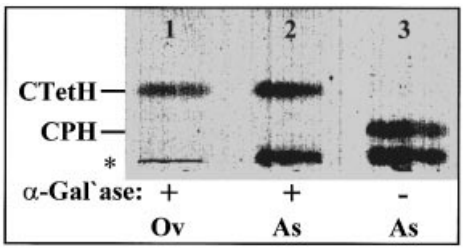

Figure 5 Cleavage of *O. volvulus* and *A. suum* HF-treated zwitterionic glycolipids with α-galactosidase

A. suum zwitterionic glycolipids yielded mainly CPH after HF treatment (lane 3). For *O. volvulus* and *A. suum* HF-treated zwitterionic glycolipids, cleavage with α-galactosidase (green coffee beans) resulted in a CTetH (lanes 1 and 2 respectively). Under the running solvent conditions employed [chloroform/methanol/water (65:25:4) by volume], more polar glycolipids of *A. suum* remained at the origin (indicated by asterisk; lanes 2 and 3). Glycolipids were revealed by orcinol/H_2SO_4 staining. Glycolipid fractions were derived from: Ov, *O. volvulus* adults; As, *A. suum* adults. α-Gal'ase, α-galactosidase treatment.

stitution, methylation analyses either without, before or after HF treatment were performed (Table 1). Native zwitterionic glycolipids yielded terminal Gal, 4-substituted Glc, 3-substituted Man, and 3-substituted and terminal GalNAc residues. HF treatment after permethylation revealed the additional presence of a 4,6-disubstituted GlcNAc residue. When HF treatment was performed before permethylation, in order to remove PC, the GlcNAc residue appeared to be 4-substituted. This indicated that PC was 6-linked to the 4,6-disubstituted GlcNAc residue. In the methylation analysis of the native zwitterionic glycolipid fraction, no GlcNAc species was observed, as PC had not been removed and the resultant derivative with PC still attached at the 6-position was not volatile and, therefore, not detected by GC–MS. In order to determine monosaccharide sequences and anomeric linkages, *O. volvulus* and *A. suum* zwitterionic glycolipids were analysed in parallel by sequential on-target enzymic cleavage with specific exoglycosidases and MALDI-TOF-MS. Following HF treatment, the mass spectra exhibited the characteristic signals for CTH, ceramide tetrahexoside (CTetH) and CPH (Figures 4A and 4B; [21]). Incubation with α-galactosidase cleaved one hexose residue from the dominant CPH components at 1567.1, 1553.1 and 1539.1 Da ($\Delta M = 162$ Da; Figures 4C and 4D). However, an additional minor CPH component appeared to be present in the *O. volvulus* sample with a pseudomolecular ion at 1608.9, which was not cleaved by α-galactosidase treatment (Figure 4C). This CPH component exhibited a ΔM of 203 Da to the CTetH species in Figure 4(C), which indicated the presence of a terminal HexNAc. Further degradation by β-*N*-acetylhexosaminidase treatment removed two HexNAc residues consecutively (Figures 4E and 4F). The observed mass shift of 422 Da resulted from the loss of two HexNAc residues ($\Delta M = 406$ Da) and the exchange of Na^+ by Li^+ ($\Delta M = 16$ Da). β-Mannosidase removed one hexose residue and resulted in CMH (Figures 4G and H). In addition, the zwitterionic glycolipids of *O. volvulus* and *A. suum*, after HF treatment, were incubated with α-galactosidase and the reaction products were analysed by HPTLC; they exhibited the migration properties of CTetH (Figure 5, lanes 1 and 2). Methylation analysis of *O. volvulus* zwitterionic glycolipids after HF and α-galactosidase treatments yielded terminal GalNAc, 4-substituted and terminal GlcNAc, 3-substituted Man and 4-substituted Glc (Table 1). This revealed terminal Gal to be α-linked to the 3-position of GalNAc and yielded the sequence Gal(α1-3)GalNAc(β1-4)-GlcNAc(β1- and not Gal(α1-4)GlcNAc(β1-3)GalNAc(β1- for the carbohydrate backbone.

Fatty acids were analysed by GC–MS using electron-impact ionization (Table 2). The results demonstrated the predominance of hydroxytetracosanoic acid ($C_{24h:0}$) (where $_h$ is hydroxylated). *O. volvulus* zwitterionic glycolipids were found to contain higher relative amounts of $C_{22h:0}$ and $C_{23h:0}$ than the corresponding *A. suum* glycolipids. The obtained fragment-ion patterns showed all hydroxy fatty acids to be 2-hydroxy derivatives, which is in agreement with previous data [1,21]. Analysis of the pentafluoropropionic acid-derivatized sphingoid base fraction by GC–MS in the positive-ion mode following electron-impact ionization allowed C_{17}-sphingosine ($C_{d17:1}$) (where $_d$ is dihydroxylated) to be identified as the main compound (results not shown), which is in accordance with the data obtained for *A. suum* zwitterionic glycolipids [1]. A characteristic ion at m/z 395 was observed, which corresponded to a C_{17}-sphingosine frag-

Table 2 Fatty acid analysis of *O. volvulus* and *A. suum* zwitterionic glycosphingolipid fractions

Fatty acid methyl esters were analysed by capillary GC–MS and identified by their retention times and molecular ($[M]^+$) as well as fragment ions after electron-impact ionization. Relative amounts are based on peak ratios of individual fatty acid derivatives normalized to 100%. $C_{24:0}$, saturated fatty acid with 24 carbon atoms; $C_{24h:0}$, saturated hydroxy fatty acid, etc.

Fatty acid methyl ester	Molecular ion $[M]^+$	Species ...	Relative amount (%)	
			O. volvulus	*A. suum*
$C_{16:0}$	270		3.0	1.6
$C_{18:0}$	298		4.7	5.7
$C_{20:0}$	326		0.5	–
$C_{22:0}$	354		1.7	1.4
$C_{22h:0}$	370		22.8	7.0
$C_{23h:0}$	384		13.7	3.3
$C_{24:0}$	382		1.2	10.9
$C_{24h:0}$	398		52.4	70.1

ment with only one pentafluoropropionic acid moiety remaining. The fragmentation pattern was analogous to that observed for the $C_{d18:1}$ standard. Detection in the negative-ion mode following chemical ionization resulted in a major ion at m/z 540 which coincided with an ion at m/z 554 found for the $C_{d18:1}$ standard (loss of HF and one pentafluoropropionic acid moiety). A similar fragmentation has been described by Zanetta et al. for sphingosines derivatized with heptafluorobutyric acid [27].

Taken together, the data allowed the structures of *O. volvulus* zwitterionic CTH, CTetH and CPH to be defined as PC-6GlcNAc(β1-3)Man(β1-4)Glc(1-1)Cer, GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(1-1)Cer and Gal(α1-3)GalNAc(β1-4)[PC-6]-GlcNAc(β1-3)Man(β1-4)Glc(1-1)Cer respectively.

DISCUSSION

The various serological and analytical procedures performed in the present study for the characterization of *O. volvulus* neutral and zwitterionic glycosphingolipid fractions varied in their specificities and detection limits. Immunochemically, neutral fraction CTH, CTetH and CPH were strongly immunoreactive with the anti-arthro-series hyperimmune serum, but chemically, HPTLC-orcinol/H_2SO_4 staining, carbohydrate composition analysis, methylation analysis and MALDI-TOF-MS revealed these glycosphingolipids to be only minor components in comparison with CMH and CDH. Thus these findings differ from those of a previous study of *Onchocerca* spp. glycolipids, from adult worms of the bovine filarial nematode *O. gibsoni*, which suggested the presence of neutral components (orcinol/H_2SO_4-spray reagent) and acidic components (sialic acid-specific, resorcinol-spray reagent) by HPTLC [28]. Similarly, the *O. volvulus* pooled infection sera and the PC-specific mAb showed different patterns of immunoreactivity with *O. volvulus* zwitterionic glycosphingolipids, manifesting other immunologically dominant compounds besides component A, yet HPTLC-orcinol/H_2SO_4-staining and methylation analysis demonstrated component A to be chemically the dominant zwitterionic glycolipid. While PC-substituted CTH and CTetH were easily detected by MALDI-TOF-MS, they were not detected by HPTLC–orcinol/H_2SO_4 staining owing to the lower sensitivity of this method (cf. Figures 2 and 3).

The phylogenetic structural conservation between the oligosaccharide backbone of neutral and zwitterionic glycosphingolipids from the Nematoda and Arthropoda is not so surprising in the light of recent data [29] showing the nematode lineage to be nearer to that of the arthropods than the Annelida. The oligosaccharide cores are based on the prototypic, arthro-series glycosphingolipid CTetH of the blowfly *Calliphora vicina*, i.e., GalNAc(β1-4)GlcNAc(β1-3)Man(β1-4)Glc(β1-1)Cer [30]. The predominant traits of the oligosaccharide moiety of *O. volvulus* neutral and zwitterionic glycosphingolipids were: (1) the neutral glycolipid fraction was represented by the components CMH and CDH (structurally analysed) and CTH-CPH (immunochemically analysed only); (2) the zwitterionic glycolipid fraction was represented by the components CTH, CTetH and CPH with the third residue in the carbohydrate chain, GlcNAc, substituted by PC at position C-6, that extended the homology of structural conservation with the porcine parasite *A. suum* [1,21]; and (3) the zwitterionic components belonged to a biosynthetic series in which elongation proceeded by the stepwise attachment of the specified monosaccharide. The zwitterionic CPH of *O. volvulus* (the present study), *A. suum* [1] and *Caenorhabditis elegans* [31] represents a modulation of the insect-derived, arthro-series glycosphingolipid, because the prototypic CTetH can be extended in one of three ways, i.e., the addition of an α-GalNAc residue to yield component 5A, an α-Gal residue to yield component 5B or a β-Gal residue to yield component 5C [30,32,33]. Insect glycosphingolipids are elongated from CPH component 5A [30], whilst nematode glycosphingolipids utilize CPH component 5B as either a termination point [1,21] or for extension [31]. Besides the structures of *O. volvulus* PC-containing CTH, CTetH and CPH defined in the present study, pseudomolecular ions have been detected in reflectron-mode MALDI-TOF-MS of the zwitterionic glycosphingolipid fraction before and after HF treatment (see, for example, signals at m/z 1710.6 in Figure 3E and at 1608.9 in Figure 4C), which indicated the presence of a second PC-substituted CPH component with the putative composition $PCHexNAc_3Hex_2Cer$. Its resistance to α-galactosidase treatment indicated that the terminal galactose present in component A has been replaced by a HexNAc residue. The carbohydrate structure of this second CPH species may be analogous to the CPH component 5A [GalNAc(α1-4)GalNAcβ(1-4)GlcNAc(β1-3)Man(β1-4)Glc(β1-1)Cer] found in *C. vicina* [30], which has been found to occur, in part, with a PE modification [33]. However, this proposal was not examined further in the present study owing to the extremely small amounts of available material.

The structures of PC-substituted *N*-linked glycans of excreted-secreted and somatic proteins from parasitic filarial nematodes [12,13] and the PC-substituted zwitterionic glycosphingolipids of parasitic and free-living nematodes ([1,31]; the present study) are highly conserved. The function(s) of these exogenous glycoproteins of the former has been related to their immunomodulatory properties and correlated with the longevity of filarial parasitism as survival factors in subverting the immune response of the host, a characteristic trait in the microfilaraemic state of infection [34]. The resultant cellular anergy is assumed to facilitate the persistence of the parasitic infection, but at the same time may protect the host against the development of parasite-induced immunopathogenesis, e.g., inflammation. The generation and regulation of protective or pathogenic immune responses in filariases have been investigated intensively with the focus on those antigens that may elicit and confer protective immunity, whether they are known to be substituted with PC [35] or not [36,37].

The finding of PC-substituted glycosphingolipids and proteins in the free-living nematode *C. elegans* [31], however, would argue for their function as endogenous factors in the worm itself. Nothing is known as to the biological significance of free-living

nematode-derived PC (macro)molecules, although, they have exhibited tissue- and stage-specific expression during ontogeny as potential markers for embryonic, hypodermal-seam cell differentiation and post-embryonic basement-membrane differentiation [31]. We have assumed that the highly conserved structures are an indication of their biological importance. The speculation is that the functional protein- and lipid-bound PC epitopes of the free-living nematode have been misappropriated by parasitic nematodes for additional roles relevant to the parasitic mode of existence, such as modulation of the host's immune response.

We acknowledge the expert technical assistance of Peter Kaese, Siegfried Kühnhardt, Werner Mink and Markus Schwinn. This study was supported by the German Research Council (SFB 535, Projects A8 and Z1).

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Received 1 February 2000/15 March 2000; accepted 28 March 2000