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Identification of the Lipid Moiety and Further Characterization of the Novel Lipophosphoglycan-like Glycoconjugates of *Trichomonas vaginalis* and *Trichomonas foetus*

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The lipid moiety of the lipophosphoglycan (LPG)-like glycoconjugates of *Trichomonas vaginalis* and *Trichomonas foetus*, parasites of the urogenital tract of human and cattle, respectively, has been isolated and characterized by a combination of enzymatic and chemical degradation, chromatography, and mass spectrometry. The carbohydrate composition of the glycan inositol lipid core is also reported. The glycan inositol core of trichomonad glycoconjugates is unique in having more than one GlcN and is significantly larger than any other glycan core reported so far. *T. vaginalis* glycoconjugate binds strongly to the lectin RCA-I, which suggest that the macromolecule possesses terminal β1,4-linked galactosyl residues. The binding of *T. foetus* glycoconjugate to the lectin UEA-I suggests the presence of terminal α1,2-linked fucose. Acid hydrolysis of deaminated and reduced LPG products yields a [^{3}H]anhydromannitol-containing product, indicating the presence of unacetylated glucosamine in the trichomonad LPGs. Reductive radiomethylation has been applied to label free amino groups in the hexosamine or other free amine-containing residues of the trichomonad glycoconjugates. Treatment of the LPGs with phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* liberates a ceramide substituent. Treatment of LPGs with nitrous acid releases a phospholipid moiety containing *myo*-inositol and ceramide, implying that the LPGs are anchored in the membrane via an inositol–phosphate–ceramide. Structural characterization of the ceramide by gas–liquid chromatography (GC) and GC–mass spectrometry indicated the presence of the major long-chain base sphinganine (d 18:0 dihydrosphingosine) and a C 16:0 *N*-acyl group. Lipophosphoglycans from both parasites contain ceramide as their only lipid moiety. These results suggest that *T. vaginalis* and *T. foetus* anchor their LPG-like glycoconjugates on the cell surface via inositol–phosphoceramide and also the glycan inositol core of the macromolecule appears to be unique in nature.

The trichomonad parasites *T. vaginalis* and *T. foetus* are the causative agents of trichomoniasis, an infection of the genitourinary tract in human and cattle, respectively. *T. vaginalis* is a common sexually transmitted parasite that causes vaginitis and urethritis in humans. The parasites reside on the surface of the epithelium of the urogenital tract where they acquire many of their nutrients from the host. Trichomonads are fatty acid and sterol auxotrophs (1–3) but they appear to synthesize glycolipids and glycophosphosphingolipids *de novo* (4). Until recently, information regarding lipid metabolism in trichomonads has been scarce, and the roles of lipids and the involvement of glycoconjugates in parasite survival in a hostile environment remains unclear (2–4). How the trichomonad parasites attach to the surface of the urogenital tract and avoid destruction by the host is not well understood.

Cell surface glycoconjugates have been shown to be crucial for a parasite's survival in the hostile immune environment of the host. The presence of novel glycosylated inositol phosphosphingolipids from *T. foetus* and *T. vaginalis* has been previously reported (4, 5). Recent studies have shown the existence of a novel cell surface lipophosphoglycan (LPG)[2]-like glycoconjugate in *T. vaginalis*

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[2] Abbreviations used: LPG, lipophosphoglycan; PBS, phosphate-buf-

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and *T. foetus* which appears to be linked to an inositol phospholipid anchor (6). This paper extends the information on trichomonad LPG characterization by identifying their lipid moieties and the study of the glycan inositol core portion of the macromolecule. In addition, we have also provided information on the number of LPG molecules present on the cell surface of a trichomonad parasite which may have important implications in host/parasite interactions.

MATERIALS AND METHODS

Cell cultures. *T. vaginalis* strain CD-C85 (ATCC 50143) and *T. foetus* strain KV1 (ATCC 30924) were grown in Diamond's medium (7) with 10% heat-inactivated fetal calf serum (Hyclone Labs, Inc.) at 37°C in screw-capped 100- and 500-ml serum bottles. The initial pH for *T. vaginalis* was 6.2 and for *T. foetus* 7.2 and the inoculum was 10^6 ml^{-1}. Organisms were counted at 24 h (Coulter Counter, Model Z_f, Coulter Electronics), harvested by centrifugation (6000*g*), and washed with cold phosphate-buffered saline (pH 7.4).

Extraction and purification of LPG. Trichomonad LPGs were isolated and purified as previously described (6) except for the following modification. Solvent E (H_2O/ethanol/diethyl ether/pyridine/NH_4OH (15/15/5/1/0.017)) extracts were treated with pronase prior to LPG purification, as described earlier (6), throughout this investigation.

SDS-PAGE. SDS-PAGE was performed under reducing conditions according to Laemmli (8) using a 12% separating gel and a stacking gel (4%). Following electrophoresis LPG was detected by periodic acid-Schiff (9) and silver staining (Pierce Chemical Co.).

Nitrous acid deamination of LPG. An aliquot of purified LPG (1.5–2 mg) was resuspended in 0.5 ml of 0.1 M sodium acetate buffer (pH 3.5) containing 0.01% Zwittergent, followed by sequential additions of 150 μl of 1 M $NaNO_2$ and 3 μl of 6 N HCl. The reaction mixture was incubated for 4 h at 50°C or 48 h at room temperature. Following deamination the phospholipid product was removed by extraction with water-saturated *n*-butanol. The butanol extract was dried under N_2 and further purified on a C_{18} Sep-Pak cartridge (Waters) (4). The resulting phosphorylated inositol containing lipid product was hydrolyzed with strong acid (6 N HCl containing 3 μmol of Tris-HCl) (4) in order to confirm the presence of inositol by gas-liquid chromatography (GLC). Another aliquot of the phosphatidyl inositol containing lipid product was subjected to acid methanolysis (described in a subsequent section) in order to characterize the lipid moiety attached to the inositol residue.

In a separate set of experiments the purified LPG-like glycoconjugate was first treated with PI-PLC (as described in the preceding section) and the resulting oligosaccharide–inositol–phosphate core portion in the aqueous phase was deaminated as described earlier without detergent. The resulting nitrous acid deaminated product in the aqueous phase was reduced with $NaB[^3H]_4$ for 3 h, followed by addition of excess reductant for 16 h as described earlier (10) in order to convert the glycan GlcN residue into [^{3}H]2,5-anhydromannitol. Following acidification with acetic acid, the reaction mixture was desalted by AG50 × 10 (H^+) and dried by repeated evaporation with methanol. The resulting radioactive saccharide products were separated from radiochemical impurities by descending paper (Whatman 3 MM) chromatography for 14–16 h in *n*-butanol/ethanol/water (4/1/1) as the solvent system (10). The samples were eluted from the origin with water, filtered through a 0.2-μm Teflon filter, and dried under N_2. An aliquot of the resulting radiolabeled products was subjected to acid hydrolysis (2.5 N trifluoracetic acid, 3 h at 100°C), followed by HPAE-PAD to detect the presence of 2,5-anhydromannitol. To identify anhydromannitol, samples were applied to a Carbo Pac PA-1 column equilibrated in 100 mM NaOH. After 6 min, a linear gradient (40 min, 1 ml/min) of 0 to 100 mM NaOAC in 100 mM NaOH was started.

Preparation of the glycan-inositol core. Purified LPG was subjected to mild acid hydrolysis (20 mM HCl, 100°C 15 min) under N_2 to cleave the phosphorylated saccharides from LPG-like glycoconjugates (11). The resulting hydrolysate was dried under N_2 with benzene/ethanol (1/1), resuspended in 0.5 ml of 0.1 M NaCl and 0.1 N acetic acid, and applied to a phenyl-coupled Sepharose column (1 × 3 cm) as described earlier (6, 11). The phosphorylated saccharides from LPG did not bind to the hydrophobic support and eluted (6 ml) in the breakthrough fractions. The column was then sequentially washed with 0.1 N acetic acid (3 ml), water (4 ml), and solvent E (6 ml). The glycan–inositol–P-lipid core was eluted with solvent E.

Sugar compositional analysis of the glycan-inositol-P-lipid core. Aliquots of the native LPG (300 μg) and the resulting glycan–inositol–lipid core (isolated from 300 μg of LPG) were hydrolyzed with 2.5 N trifluoracetic acid at 100°C for 3.5 h. The hydrolysate was dried under N_2 with addition of toluene (2–3 times) and treated with alkaline phosphatase (20 units/ml in 0.1 M ammonium bicarbonate buffer pH 8.0) for 18 h at 37°C (6, 10). The reaction mixture was dried, resuspended in H_2O, and subjected to HPLC directly. The monosaccharide composition was determined by HPAE on a Dionex HPLC equipped with pulsed amperometric detector and CarboPac-PA1 column using 15 mM NaOH as the eluent (12). Another aliquot of the LPG and glycan core was subjected to strong acid hydrolysis for determination of inositol and amino sugars as their alditol acetates by GLC as described earlier (6).

Radiomethylation of LPG-like glycoconjugates. Reductive radiomethylation of free amino groups in GlcN or GalN residues of the LPG macromolecules was performed essentially as described earlier for acetylcholinesterase GPI anchor (13, 14) with a slight modification. Purified LPG samples (1–2 mg) were reductively radiomethylated with [^{14}C]HCHO (10–20 μCi, 57 mCi/m mole) and 62 mM $NaCNBH_3$ containing 10 mM unlabeled HCHO at 37°C for 1 h. The resulting radiomethylated glycoconjugate was dialyzed (Spectrapor, cutoff MW 3000) extensively against 0.1 M ammonium acetate buffer (pH 7.0), dried, and further purified on a phenyl-Sepharose column chromatography (6, 15). Radiolabeled LPG-like glycoconjugates were eluted with solvent E (6).

Lectin affinity chromatography. Galactose oxidase/$NaB[^3H]_4$-labeled and [^{14}C]radiomethylated LPG were used for lectin affinity chromatography. Cell surface radiolabeling of both trichomonads by the galactose oxidase/$NaB[^3H]_4$ was carried out as described earlier (6). Aliquots of radiolabeled LPG were chromatographed on columns (1 × 2 cm) of RCA-I (*Ricinus communis* I) and UEA-I (*Ulex europaéus*) covalently linked to agarose beads (EY Labs, Inc.) equilibrated with PBS, pH 7.4. The columns were first washed with PBS, and 1.2-ml fractions were collected. LPGs bound to the column were first eluted with 0.2 M lactose for RCA-I and 0.05 M α-fucose for UEA-I after PBS wash, followed by PBS and PBS containing 0.1% Triton X-100. Fractions (1.2 ml) were collected and measured for radioactivity.

Acidic methanolysis of LPGs for fatty acids and alkyl or acyl glycerol analysis. To a dried LPG sample (0.5 mg) was added 1 M anhydrous methanolic HCl (1 ml), and the reaction mixture was heated at 75°C for 16 h under N_2, cooled, and extracted with methylene chloride (2 ml) and water (0.75 ml). The lower organic phase (containing lipids) was recovered. The aqueous phase was reextracted with methylene chloride (1 ml), and the combined organic extracts were evaporated to dryness under N_2. An aliquot was subjected to GLC analysis. Another portion of the organic extract was acetylated with acetic anhydride/pyridine (1/1, 0.5 ml) for 1 h at 80°C, dried with toluene under N_2, resuspended in methylene chloride, and examined by GLC.

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fered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GLC, gas-liquid chromatography; GC/MS, gas chromatography/mass spectrometry; PAS, periodic acid-Schiff; PI-PLC, phosphatidylinositol-specific phospholipase C; TMS, trimethylsilyl; SIM, selective ion monitoring; LPPG, lipopeptidophosphoglycan; P-ceramide, phosphoceramide; HPAE-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection.

Methanolic HCl hydrolysis: Release of lipid moiety from trichomonad LPGs. Trichomonad LPG (1.2 mg) samples were also hydrolyzed with HCl in the presence of methanol and H_2O (3/27/6) at 75°C for 16 h under N_2. Each hydrolysate was dried, taken up in chloroform, and placed on a Bio-Sil A column (1 × 3 cm). Free fatty acids and fatty acid methyl esters were eluted with chloroform, reacted with diazomethane, and the reaction products chromatographed on Unisil columns (16). Samples of the fatty acid methyl ester fractions eluted with pentane/diethyl ether (95/5) were examined by GLC and gas chromatography/mass spectrometry (GC/MS).

The long chain bases (sphingosine) were eluted from the Bio-Sil A column with methanol, and each sample was taken to dryness by flash evaporation, redissolved, and purified by solvent extractions as described previously (17). A similar protocol was used to identify a lipid moiety from the *n*-butanol lipid extract obtained from nitrous acid deamination of LPGs. The resulting sphingosine products were acetylated and silylated as described previously (16). The GLC of fatty acid methyl esters and silylated long chain bases was performed as reported earlier (16). Identification of fatty acid methyl esters and sphingosine bases were made by comparisons of their retention characteristics and mass spectral fragmentation pattern with those of standards.

PIPLC digestion of native LPG and glycan inositol core: Release of lipid moiety. The native LPG and glycan inositol core were hydrolysed with PI-PLC from *Bacillus thuringiensis* as reported previously (6). Lipids were extracted with chloroform/methanol (2/1, 2 ml), dried, and subsequently hydrolyzed with HCl in the presence of methanol and H_2O (3/27/6) as described earlier. Fatty acids and long chain bases were determined as described in the above section.

Gas-liquid chromatography-mass spectrometry. The identity of fatty acid methyl esters and *N*-acetyl trimethylsilyl derivatives of sphingosine bases were verified by GC/MS. Fatty acid methyl esters were analyzed by GC/MS in the scan mode and *N*-acetyl-TMS derivatives of long chain bases were analyzed both in scan and selective ion monitoring (SIM) modes. Analysis was performed on a Hewlett-Packard. HP5970 (MSD) with a capillary-direct interface to an HP 5890A GC. The fused silica column was a HP-5 (30 m × 0.2 mm) and helium carrier gas flow rate was 1 ml/min. For fatty acid methyl esters temperature was maintained at 150°C for 2 min and then increased from 150 to 240°C at a rate of 4°C/min. For *N*-acetyl-TMS derivatives of long chain bases the temperature was maintained at 220°C for 2 min and then increased from 220 to 260°C at a rate of 5°C/min. The spectra were scanned from m/z 50 to 500 in 2 s.

RESULTS

Purification of LPG to apparent homogeneity was achieved by gel filtration (Sepharose 4 CL-B) and hydrophobic (octyl-Sepharose) chromatography followed by precipitation with cold methanol. LPG is the only PAS-positive species present in the precipitate when analyzed by SDS–PAGE. The same LPG band can also be visualized by silver staining. The LPG of *T. vaginalis* migrated as a broad heterogeneous band (40–68 kDa) and the LPG of *T. foetus* migrated as a homogeneous band (58 kDa) upon SDS–PAGE (Fig. 1). SDS–PAGE analysis of glycan inositol core showed a slightly higher mobility (30–58 kDa in *T. vaginalis* and 15–50 kDa in *T. foetus*) than the native LPG and appeared as a polydisperse band (Fig. 1). One liter of *T. vaginalis* (total cells 4.8×10^9) and 1 liter of *T. foetus* (1.7×10^{10} cells) parasites yielded 1.1 mg and 2.25 mg of LPGs, respectively.

Evidence for presence of myo-inositol in the lipid moiety of LPG and anhydromannitol in LPG. One of the universal features of glycosylated inositol–phospholipid anchors is the presence of nonacetylated glucosamine. Treatment of these molecules with nitrous acid deaminates the glucosamine and cleaves the glycosidic bond to inositol (18). The presence of *myo*-inositol in the organic extract following deamination of LPG was demonstrated by strong acid hydrolysis and GLC of the alditol acetates (data not shown). These results are consistent with the suggestion that an unacetylated hexosamine is linked to an inositol phospholipid anchor. Deamination of LPGs and subsequent reduction of the delipidated products with $NaB[^3H]_4$ followed by acid hydrolysis resulted in the formation of one radioactive [^{3}H] anhydromannitol peak that migrated similar to authentic 2,5-anhydromannitol (Sigma Co.) standard (4.05 min) when analyzed by Dionex HPLC (data not shown). This result confirms the presence of unacetylated glucosamine residue in the glycoconjugates.

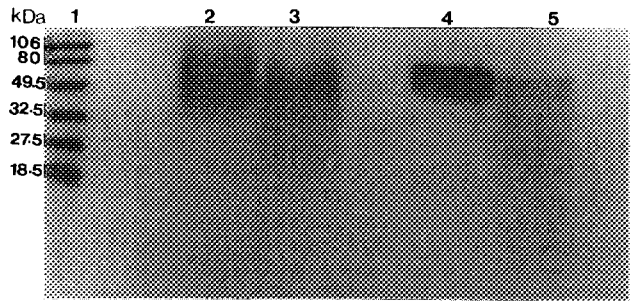

FIG. 1. SDS–PAGE analysis of native LPG and its glycan–inositol core from *T. vaginalis* and *T. foetus*. Glycan–inositol core was prepared after mild acid hydrolysis (20 mM HCl, 15 min, 100°C) of LPG followed by phenyl–Sepharose chromatography as described under Materials and Methods. The gel was stained with PAS. Lane designations: 1, prestain standards; 2, *T. vaginalis* LPG; 3, *T. vaginalis* glycan–inositol core; 4, *T. foetus* LPG; 5, *T. foetus* glycan–inositol core.

Quantification of the sugar components in the glycan inositol core of LPG-like glycoconjugates. The LPG-like glycoconjugates from the trichomonads are labile to mild acid treatment (6). This property was used to liberate the glycan inositol core from LPG, which was then purified by phenyl–Sepharose chromatography. The nanomole amounts of monosaccharide components in the glycan–inositol core of LPG compared with the amounts of sugars in the native LPG macromolecules are shown in Table I. The monosaccharide compositions of glycan–inositol core of *T. vaginalis* LPG are found in an approximate molar ratio of GlcN/Gal/Glc/Man/GalN/Inos of 27/17/4.5/3.5/1.3/1, as compared with molar ratios of 33/26/6/5/1.6/1 in the native glycoconjugate molecule (Table I). Analyses of the *T. foetus* glycan inositol core shows an approximate molar ratios of Fuc/Man/Gal/GlcN/Glc/GalN/Inos of 11/8/7/6/1.4/1/1, as compared with molar ratios of 33/28/11/18/2.7/1.3/1 in the native LPG macromolecule (Table I). Molar ratios are normalized to inositol which was determined by GLC. Such a high proportion of GlcN

TABLE I

Monosaccharide Composition of Native LPG-like Glycoconjugates and Glycan-Inositol-Lipid Core of *T. vaginalis* and *T. foetus*[a]

	T. vaginalis		*T. foetus*	
	Native glycoconjugate	Glycan-inositol core	Native glycoconjugate	Glycan-inositol core
Inos	7.8	6.3	7.5	6.7
Fuc	—	—	246	74.4
GalN	12.8	8.5	10	6
GlcN	260	168	135	42.4
Gal	202	107	83.5	48.44
Glc	49	28.5	20.5	9.5
Man	42	22	211	54.8

[a] Values are given in nanomoles, obtained from 300 μg of native LPG. An equal amount of LPG was treated with mild acid (20 mM HCl, 100°C, 15 min) in order to generate glycan-inositol-lipid core by phenyl-Sepharose chromatography as described under Materials and Methods.

and a GalN in the glycan core of LPGs are unique to trichomonad parasites.

Evidence of free amino groups in the glycan-inositol core of LPG and native LPG by reductive radiomethylation of LPG. The presence of free amino groups in the glycan core of trichomonad LPGs was confirmed by reductive radiomethylation of LPG-glycoconjugates. Radiomethylation has been used to radiolabel glucosamine and ethanolamine components in the GPI anchors of several mammalian proteins (14, 19). Mild acid (20 mM HCl, 15 min at 100°C) hydrolysis of [^{14}C]-radiomethylated LPG from *T. vaginalis* and subsequent hydrophobic chromatography on a phenyl–Sepharose column revealed that the majority of the radioactive counts (68%) are present in the glycan–inositol–lipid core of the molecule, indicating a presence of more than one free amino group (eluted with solvent E) and a lesser amount (32%) in the void volume (Fig. 2B). In *T. foetus* the reverse pattern is observed with the majority of [^{14}C]-labeled material, eluting in the void volume and small amounts of [^{14}C] labels are also present in the glycan–inositol–lipid core of LPG (Fig. 2C). This small amount of radioactivity in solvent E fraction may be associated with radiomethylation of an unacetylated GlcN linked to the inositol–phospholipid anchor of *T. foetus* LPG (discussed earlier). The reductive radiomethylation of an LPG-like glycoconjugate from both trichomonads provides evidence for the presence of more than one free amino group(s) (e.g., unacetylated GlcN and perhaps ethanol amine) in the glycan-inositol core of *T. vaginalis* and in the saccharide portion of *T. foetus* LPGs. Detailed structural studies of these glycan inositol cores and the native LPG molecules should reveal the identities of these free amino group(s) containing components.

Lectin affinity column chromatography. The trichomonad LPGs radiolabeled with either galactose oxidase/NaB[^{3}H]$_4$ or [^{14}C]radiomethylation provided identical results in lectin binding studies. The *T. vaginalis* glycoconjugate binds strongly to immobilized lectin ricin RCA-I that can be eluted with 0.2 M lactose (Fig. 3A). *T. foetus* glycoconjugate does not bind to lectin ricin. Previous studies have shown that the [^{3}H] counts from galactose oxidase/NaB [^{3}H]$_4$-labeled *T. vaginalis* are present in galactose rather than in galactosamine as in *T. foetus* (6). The strong binding of glycoconjugate to the lectin RCA-I suggests that the macromolecule possesses terminal β1,4-linked galactosyl residues (20).

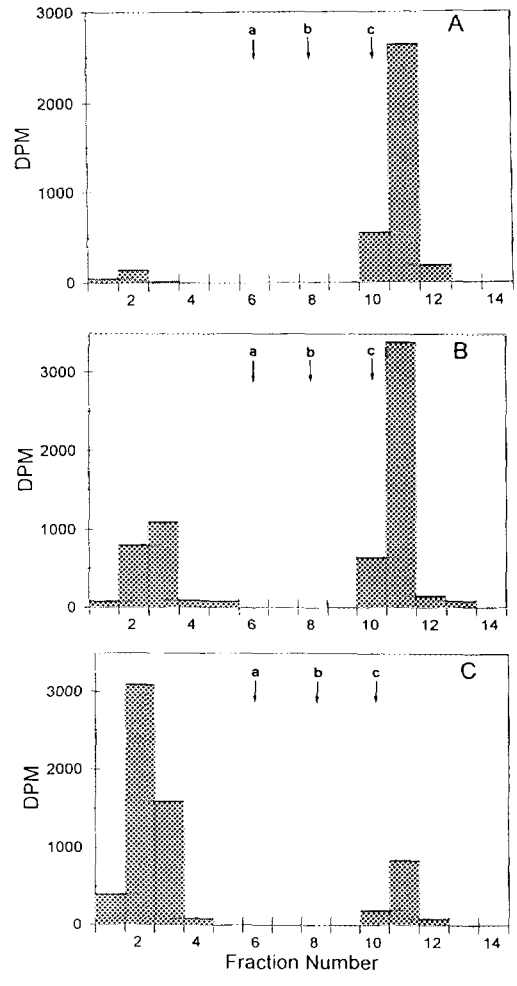

FIG. 2. Hydrophobic chromatography of the [^{14}C]-radiomethylated LPG-like glycoconjugates of *T. vaginalis* and *T. foetus* treated with 20 mM HCl for 15 min at 100°C. Samples were dried, resuspended in 0.1 N acetic acid and 0.1 M NaCl, and applied to a phenyl–Sepharose column (1 × 2 cm) equilibrated in 0.1 N acetic acid and 0.1 M NaCl. The column was washed first with 5 ml of 0.1 N acetic acid and 0.1 M NaCl. The column solvent was changed as indicated in the figure as follows: (a) 0.1 N acetic acid (2 ml); (b) water (2 ml); and (c) solvent E (5 ml). (A) untreated LPG of *T. vaginalis* (untreated LPG of *T. foetus* gave the identical results); (B) *T. vaginalis* LPG pretreated with 20 mM HCl; (C) *T. foetus* LPG pretreated with 20 mM HCl.

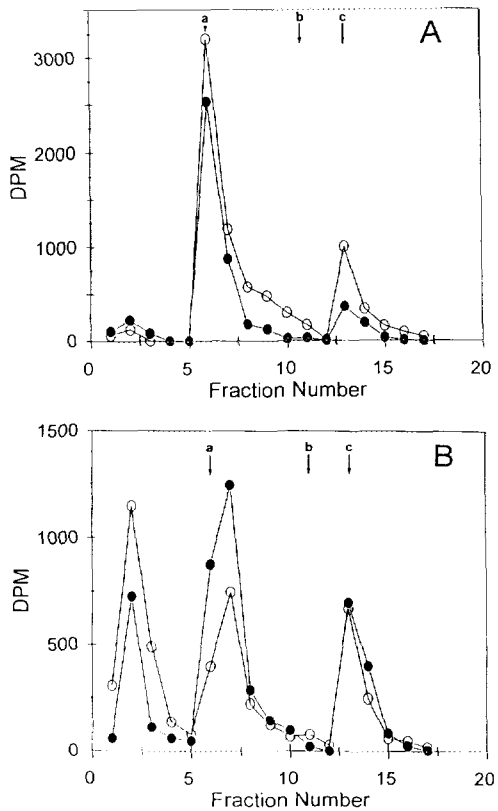

FIG. 3. Chromatography of *T. vaginalis* LPG on ricin (RCA-I) agglutinin-coupled agarose (A) and *T. foetus* LPG on UEA-I (*Ulex europaeus*) agglutinin-coupled agarose (B). Aliquots of radiolabeled LPGs were applied to the immobilized lectin columns (1 × 2 cm) equilibrated in PBS; 1.2-ml fractions were collected and measured for radioactivity. Column buffer was changed as indicated in the figure: (a) PBS containing 0.2 M lactose (A) and PBS containing 0.05 M α-fucose, (B); (b) PBS and (c) PBS containing 0.1% Triton X-100. (●) Radiomethylated LPG; (○) galactose oxidase/NaB $[^3H]_4$-labeled LPG.

The *T. foetus* glycoconjugate binds to the immobilized lectin UEA-I which can be eluted with 0.05 M α-fucose (Fig. 3B). Compositional analysis of the glycoconjugate demonstrated the presence of large amounts of fucose in the macromolecule. However, 40–60% of the labeled LPG elute in the breakthrough fraction, significant amounts of labeled LPG elute from the column with 0.05 M α-fucose, and the remainder elute with 0.1% Triton X-100 in PBS buffer. The binding of glycoconjugate to the lectin UEA-I may suggest that the LPG possess terminal α 1,2-linked fucosyl residue(s).

The binding of these LPG-like glycoconjugates to lectin is important and could be useful in studying the biosynthesis and function of these macromolecules. The nature of the glycosidic linkages between individual carbohydrate components and the position of the phosphate group as well as the identities of the acid labile (nitrous and mild acids) components remains to be established.

GLC and GC/MS analysis of the lipid products released from acid methanolysis. GLC and GC/MS analyses of fatty acid methyl esters obtained after acid methanolysis (1 N HCl in CH_3OH or aqueous methanolic HCl reagent) of LPG showed palmitic acid as the major fatty acid and stearic acid as a minor fatty acid (Fig. 4). The retention times and fragmentation patterns of fatty acid methyl esters from both LPGs are identical to palmitic and stearic acid methyl ester standards (Fig. 4). The GC/MS spectra of palmitic acid methyl ester showed the typical fragment ions at *m/z* 74, 87, 143, 227 (M-43), 239 (M-31) attributed to loss of the methoxyl group from the methyl ester) and an abundant molecular ion at *m/z* 270 (M^+). Stearic acid methyl esters showed fragment ions at *m/z* 74, 87, 143, 199, 255 (M-43), 267 (M-31) and had *m/z* 298 (M^+).

GLC analyses of the *N*-acetyl-O-TMS derivatives of the sphingolipid and bases of *T. vaginalis* and *T. foetus* LPGs showed mainly sphinganine (d18:0 dihydrosphingosine) (Fig. 5). Furthermore, GLC analyses of the lipid product released from PI-PLC digestion and nitrous acid deamination of trichomonad LPGs provided similar results (sphinganine as a sphingolipid base and palmitic acid as a major fatty acid). The lipid products released by PI-PLC treatment were resistant to mild base hydrolysis. This result further suggested the presence of a ceramide-containing lipid.

GC–MS analysis of sphingosine bases. GC–MS analysis in SIM mode of the *N*-acetyl-*O*-TMS derivative of the long chain base fraction from both LPGs showed one major peak in the total ion current (Fig. 6). The retention time and the fragmentation patterns of the *N*-acetyl-*O*-TMS derivative of the long chain base from both LPGs are identical to authentic *N*-acetyl-*O*-TMS derivative of the sphinganine (d18:0 dihydrosphingosine) standard (Fig. 6). The molecular ion peak is absent but the molecular weight is clearly indicated by the presence of a prominent peak at *m/z* 472 $(M\text{-}CH_3)^+$. Each spectrum has an ion at *m/z* 384 $(M\text{-}CH_2OTMS)^+$. The derivatized long chain base produces an abundant ion at *m/z* 73 $[(CH_3)_3Si]^+$ and *m/z* 174 which derived from cleavage between C2 and C3 to yield the ion $[CH\text{-}(NHCOCH_3)\text{-}CH_2OSiOTMS]^+$ found in the mass spectra of all the known sphingolipid bases. The ion at *m/z* 174 has only low abundance. A peak at *m/z* 157 was more abundant and is indicative of a saturated base. The ion at *m/z* 247 is attributed to cleavage at the C2–C3 bond with transfer of the TMS group from the oxygen on C3 to the nitrogen on C2. These two ions are prominent in the mass spectra of all the derivatives. The presence of an abundant ion at *m/z* 313 (M-174) represents cleavage of the C2–C3 bond with charge retention on the other portion of the molecule, indicating a molecular weight of 487 for dihydrosphinganine (d18:0) (21).

DISCUSSION

Trichomonad parasites express a novel cell surface LPG-like glycoconjugate (6) which may be involved in

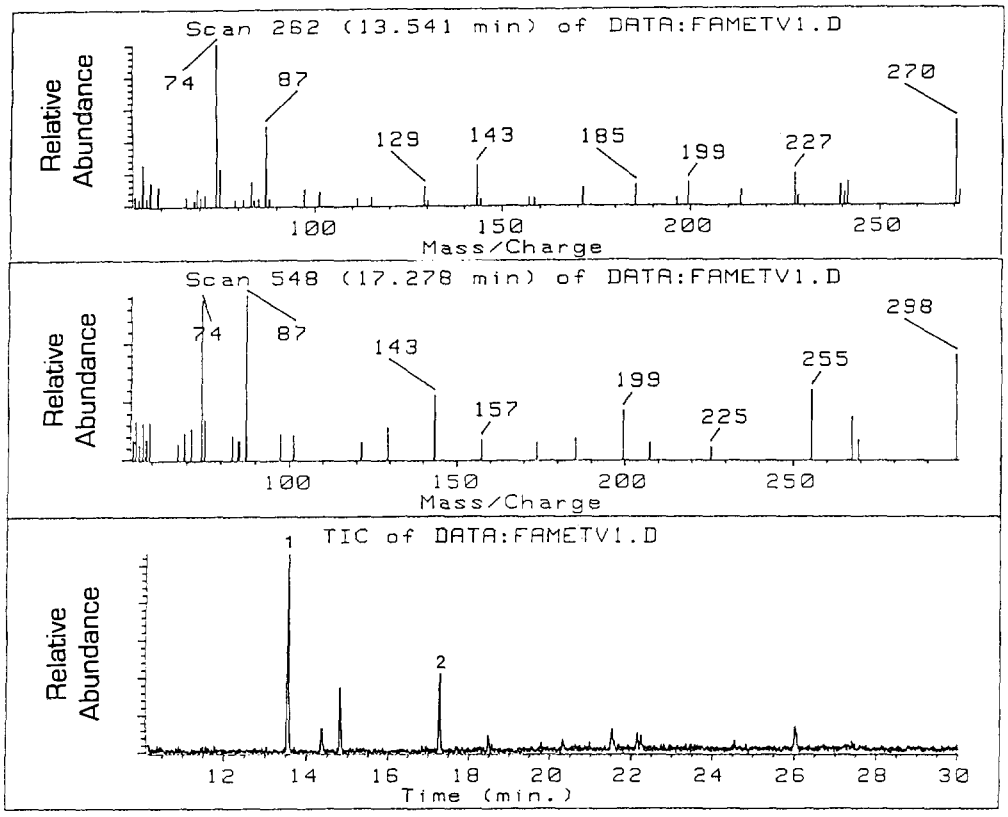

FIG. 4. GC/MS of the fatty acid methyl esters resulting from acid hydrolysis of *T. foetus* LPG followed by Bio-Sil A chromatography and methylation as described under Materials and Methods. (Bottom) Total ion current; (top) mass spectrum of peak 1 in the total ion current; and (middle) mass spectrum of Peak 2 in the total ion current. The other minor peaks in the total ion current were not related to fatty acid methyl and which probably came from diazomethane impurities during methylation. GC/MS of the fatty acid methyl esters of *T. vaginalis* LPG showed identical results.

parasite survival, attachment, infectivity, and host-cell recognition. A knowledge of their primary structure is essential to study the structure/function relationships and also to interpreting the biosynthetic pathways responsible for their makeup. The results presented here clearly indicate that the lipid component of the cell surface LPG-like glycoconjugates of *T. vaginalis* and *T. foetus* is a ceramide moiety. This is unlike the lipid moieties present in the lipophosphoglycans of *Leishmania* (lysoalkylglycerol) and other GPI–protein anchor molecules (alkylacylglycerol or diacylglycerol). The *T. foetus* and *T. vaginalis* ceramide moieties contain sphinganine as the long chain base (d 18:0, dihydrosphingosine) and palmitate (C 16:0) as the predominant *N*-acyl fatty acid. The identification and characterization of the lipid moieties was made by using hydrophobic and silicic acid chromatography and enzymatic and chemical degradation followed by GLC and GC/MS techniques.

In addition, GLC analyses of the organic lipid extracts obtained after PI-PLC treatment of LPGs or glycan inositol cores; and nitrous acid deamination from *T. vaginalis* and *T. foetus* revealed the presence of the long chain base sphinganine and palmitate as the major *N*-acyl group. The phospholipid product resulting from nitrous acid deamination of *T. foetus* and *T. vaginalis* LPG also showed the presence of a *myo*-inositol. These results strongly indicate that the inositol is linked to ceramide through a phosphodiester linkage as reported in TF_1 and TV_1 glycolipids of *T. foetus* and *T. vaginalis,* respectively.

There is considerable structural diversity among the reported glycolipid membrane anchors. Not only have a variety of glycan modifications been observed, but the lipid moieties can also be rather different. For example, there is evidence that certain protozoans have glycoconjugates anchored uniquely by a ceramide moiety. This was recently reported for *Dictyostelium* adhesion proteins (22), lipopeptidophosphoglycan (LPPG) from *T. cruzi* (23) and lipophosphoglycan from *Acanthamoeba castellani* (24). We have also reported the presence of inositol–P-ceramides (in both trichomonads) and novel glycophosphosphingolipids, TF_1 from *T. foetus,* and TV_1 from *T. vaginalis* (4, 5). It is of interest that the major long chain base in both is sphinganine (dihydrosphingosine, d 18:0) and the major *N*-acyl group is 16:0. These similarities

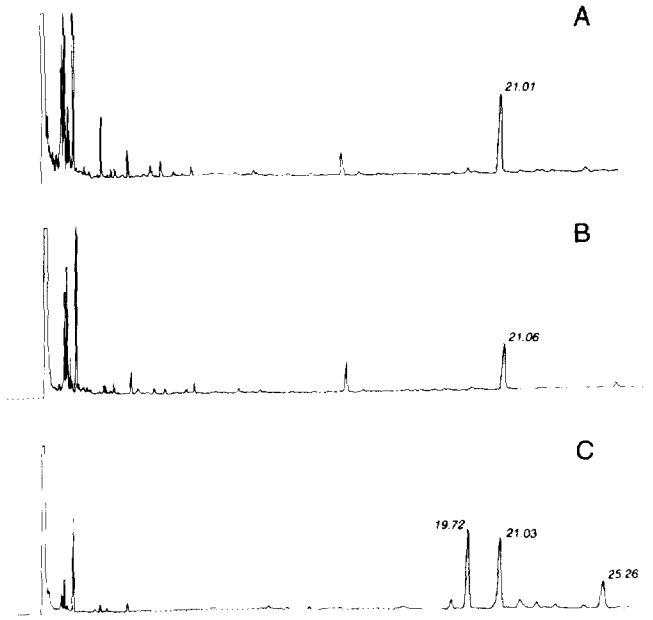

FIG. 5. Capillary column (SPB-5) GLC analysis of *N*-acetyl-*O*-trimethylsilyl ethers of sphingosine bases derived from acid hydrolysis of *T. vaginalis* and *T. foetus* LPG-like glycoconjugates as described under Materials and Methods. (A) *T. vaginalis* LPG; (B) *T. foetus* LPG; and (C) standard sphingosine bases, 4-E sphingenine (19.72 min), sphinganine (d 18:0 dihydrosphingosine, 21.03 min), and phytosphingosine (25.26 min).

lend support to our hypothesis that these inositol-P-ceramides and novel glycolipids TF_1 (from *T. foetus*) and TV_1 (from *T. vaginalis*) may possibly be precursors or intermediates in the biosynthesis of the complex LPG-like glycoconjugates present on the cell surface of trichomonads. There are several reports indicating the importance of ceramide lipid in regulating protein kinase-C (25–27); whether such sphinganine lipid in trichomonads plays a similar role remains to be investigated.

One of the novel features of the macromolecules is the presence of large amounts of GlcN in LPGs from both *T. foetus* and *T. vaginalis* and the presence of fucose in *T. foetus* LPG. The quantitative estimation of carbohydrates in the glycan–inositol core of LPGs provides evidence of the size of the glycan core of *T. vaginalis* and *T. foetus* glycoconjugates. The results suggest that the glycan inositol core has approximately 53 monosaccharide residues in *T. vaginalis* and 35 in *T. foetus* LPG, significantly larger than the core of *Leishmania* LPG (a hexasaccharide). The unique feature of the glycan inositol core is the presence of large amounts of GlcN (about 27 GlcN in *T. vaginalis* and 6 GlcN in *T. foetus*) and a GalN in both trichomonad LPGs.

The cellular copy number for the LPG-like glycoconjugates can be estimated based on the molar ratio of inositol per LPG macromolecule. The extraction of 1 liter of *T. vaginalis* ($\sim 4.8 \times 10^9$ cells) and *T. foetus* ($\sim 1.7 \times 10^{10}$ cells) yields approximately 22.5 and 50.1 nmol of total inositol, respectively. These results indicate a cellular copy number of 2.7×10^6 molecules of LPG-like glycoconjugate per cell of *T. vaginalis* and 2.0×10^6 molecules of LPG-like glycoconjugate per cell of *T. foetus* parasites. Such a high copy number suggests that the LPGs play an important role in the biology of trichomonad parasites and the infections they cause. In contrast to the trichomonads, *Leishmania* parasites contain approximately $1.2–5 \times 10^6$

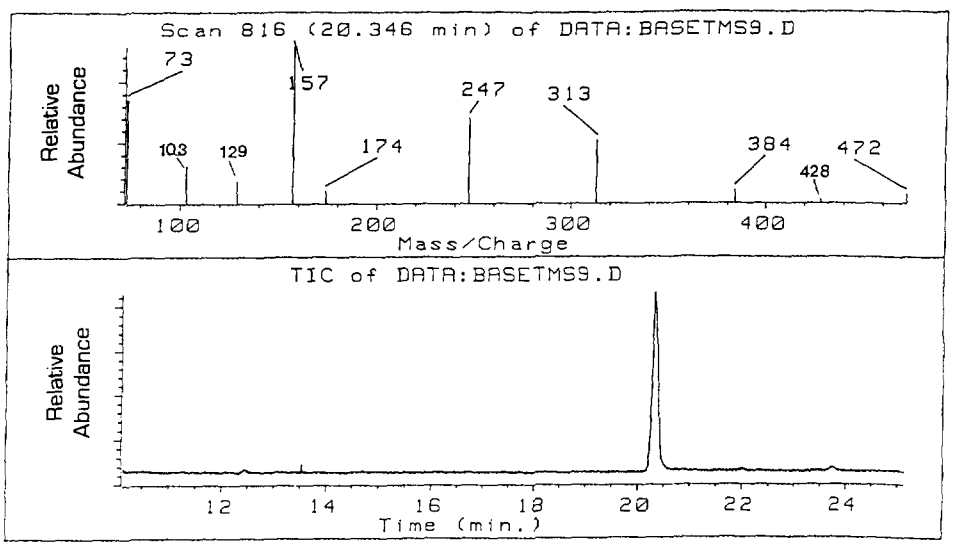

FIG. 6. GC/MS of the *N*-acetyl-*O*-trimethylsilyl ethers of sphingosine bases of *T. vaginalis* LPGs derived from acid hydrolysis. Bio-Sil A chromatography followed by acetylation and subsequent TMS derivatization were carried out as described under Materials and Methods. (Bottom) Total ion current; (top) mass spectrum of the peak indicated in the total ion current. Data was acquired by selective ion monitoring (SIM). GC/MS of the *N*-acetyl-*O*-TMS derivative of sphingosine base of *T. foetus* LPG showed identical results.

copies of LPG per cell (28). Activities and functional aspects of *Leishmania* LPG have been studied in great detail and several of the functions are believed to involve attachment and entry of the parasites into the host cell (28, 29). Whether or not trichomonad LPG is involved in the attachment of parasites to the vaginal epithelium or in protecting the parasites within the hostile environment of the host remains to be established.

T. vaginalis and *T. foetus* possess novel LPG-like glycoconjugate anchored on the cell surface via inositol–phosphoceramide. The architectural elements in the glycan inositol core of LPGs are unique to these parasites in having more than one GlcN and a GalN in both organisms and fucose in the *T. foetus* LPG core. The glycan core is significantly larger than any other glycan inositol core reported so far. Future detailed studies of these LPGs should undoubtedly reveal the structural and functional aspects of these unique glycoconjugates and should provide insights into the mechanism of action of these macromolecules, including mechanisms of host–parasite interaction.

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