Characterization of glycoinositol phospholipids in the amastigote stage of the protozoan parasite *Leishmania major*

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The major macromolecules on the surface of the parasitic protozoan *Leishmania major* appear to be down-regulated during transformation of the parasite from an insect-dwelling promastigote stage to an intracellular amastigote stage that invades mammalian macrophages. In contrast, the major parasite glycolipids, the glycoinositol phospholipids (GIPLs), are shown here to be expressed at near-constant levels in both developmental stages. The structures of the GIPLs from tissue-derived amastigotes have been determined by h.p.l.c. analysis of the deaminated and reduced glycan head groups, and by chemical and enzymic sequencing. The deduced structures appear to form a complete biosynthetic series, ranging from Man α 1-4GlcN-phosphatidylinositol (PI) to Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-2). A small proportion of GIPL-2 was further extended

by addition of a Gal residue in either α 1-6 or β 1-3 linkage. From g.c.–m.s. analysis and mild base treatment, all the GIPLs were shown to contain either alkylacylglycerol or lyso-alkylglycerol lipid moieties, where the alkyl chains were predominantly C_{18:0}, with lower levels of C_{20:0}, C_{22:0} and C_{24:0}. *L. major* amastigotes also contained at least two PI-specific phospholipase C-resistant glycolipids which are absent from promastigotes. These neutral glycolipids were resistant to both mild acid and mild base hydrolysis, contained terminal β -Gal residues and were not lost during extensive purification of amastigotes from host cell membranes. It is likely that these glycolipids are glycosphingolipids acquired from the mammalian host. The GIPL profile of *L. major* amastigotes is compared with the profiles found in *L. major* promastigotes and *L. donovani* amastigotes.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the aetiological agents of a spectrum of diseases, the leishmaniasis, that affect man and other mammals in tropical and subtropical areas. Parasites are transmitted to the mammalian host by the bite of an infected sandfly vector, and undergo radical morphological changes as they pass from one host to the other. In the sandfly vector, the parasite is found as a motile, flagellated and elongated promastigote. After transmission, it is engulfed by cells of the mononuclear phagocyte system within which it differentiates and reproduces as a small oval-shaped amastigote inside the acidified phagolysosomes of the host cell (Antoine et al., 1990).

Most studies on the cell surface of these protozoa have focused on the promastigote stage because of the ease of obtaining cultivated material *in vitro*. However, there is considerable evidence that the cell surface of these parasites changes dramatically during the differentiation of promastigotes into amastigotes in the mammalian host. At the ultrastructural level, this is evident in the disappearance of the prominent glycocalyx that covers the promastigote plasma membrane (Pimenta et al., 1991). This glycocalyx contains a number of glycosylphosphatidylinositol (GPI)-anchored glycoproteins, of which the most abundant is the promastigote surface protease (gp63) (Bordier, 1987; Schneider et al., 1992a). It also contains an abundant lipophosphoglycan (LPG), which consists of a polydisperse phosphoglycan moiety linked to the plasma membrane via a structurally distinct GPI anchor (McConville, 1991; Turco and Descoteaux, 1992). Recent studies suggest that both the GPI-anchored proteins and LPG are down-regulated in the amastigote stage. For example, the enzyme activity of promastigote surface protease is reduced by more than 100-fold in *Leishmania major*

amastigotes compared with promastigotes (Schneider et al., 1992b). A similar, but less dramatic, situation also occurs in *Leishmania mexicana* (Medina-Acosta et al., 1989; Bahr et al., 1993). The decrease in LPG is also dramatic, although the extent of down-regulation appears to vary in different species. LPG is not detectable in *Leishmania donovani* and *L. mexicana* amastigotes (McConville and Blackwell, 1991; Bahr et al., 1993), whereas an antigenically distinct LPG, expressed in low abundance, is present in *L. major* amastigotes (Glaser et al., 1991; Turco and Sacks, 1991).

In addition to the GPI-anchored proteins and LPG, *Leishmania* promastigotes also synthesize a family of low-molecular-mass glycoinositol phospholipids (GIPLs) which appear to be the major class of glycolipid in these organisms. Different classes of GIPLs are recognized on the basis of their glycan structures. Type-1 GIPLs have structures related to the GPI anchors of proteins (McConville and Blackwell, 1991; Schneider et al., 1990). Type-2 GIPLs are reminiscent of the GPI core of LPG, and are characterized by the presence of a Man α 1-3 and of a Gal β residue (McConville et al., 1990a,b). Hybrid-type GIPLs have branched structures related to protein GPI anchors, with the addition of a Man α 1-3 branch typical of the LPG core (McConville and Blackwell, 1991). The abundance and surface localization of the GIPLs suggest that they have a function on their own, in addition to that of putative precursors of GPI-anchored proteins and LPG.

In contrast with LPG, there is evidence from studies on *L. donovani* that the GIPLs are synthesized in high copy number in both promastigote and amastigote stages (McConville and Blackwell, 1991). Differences were found in the GIPL profiles of these developmental stages, as promastigotes only synthesized hybrid-type GIPLs, whereas amastigotes only produced type-1

Abbreviations used: GIPL, glycoinositol phospholipid; AHM, 2,5-anhydromannitol; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; GPI, glycosylphosphatidylinositol; LPG, lipophosphoglycan; h.p.t.l.c., high-performance t.l.c.

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GIPLs (McConville and Blackwell, 1991). The GIPL profile of *L. major* promastigotes differs from that found in either of the *L. donovani* developmental stages in only containing type-2 GIPLs (McConville and Bacic, 1989, 1990; Rosen et al., 1989; McConville et al., 1990a). In this study we have characterized the GIPLs from *L. major* amastigotes to further investigate stage- and species-specific differences in the expression of these glycolipids. The results show that *L. major* amastigotes also produce predominantly type-2 GIPLs, although there are significant quantitative differences between the GIPL profiles of the two developmental stages. The significance of the species-specific differences in the GIPL profiles of *L. major* and *L. donovani* amastigotes is discussed. However, the cell surfaces of *L. major* and *L. donovani* amastigotes are similar in containing non-GPI glycolipids which may have been acquired from the mammalian host.

MATERIALS AND METHODS

Materials

NaB^3H_4 (9–11 Ci/mmol) was purchased from DuPont, jack bean α -mannosidase, coffee bean α -galactosidase and bovine testicular β -galactosidase were from Boehringer (Mannheim, Germany), silica gel 60 high-performance t.l.c. (h.p.t.l.c.) and cellulose t.l.c. sheets were from Merck (Darmstadt, Germany). Ganglioside GM1 was purchased from Fluka (Buchs, Switzerland). Phosphatidylinositol-specific phospholipase C (PI-PLC) was a generous gift from Dr. M. G. Low (Columbia University, New York, U.S.A.).

Cells

Promastigotes of *L. major* LV39 (MHRO/SU/59/P-strain) were maintained in HOSMEM II medium (Berens and Marr, 1978) supplemented with 5% (v/v) heat-inactivated fetal calf serum. Amastigotes were isolated from lesions in Swiss *nu/nu* nude mice, after tissue homogenization, macrophage disruption, erythrocyte lysis and filtration through polycarbonate filters to remove membrane fragments and host-cell debris (Glaser et al., 1990; Schneider et al., 1992b). A portion of the amastigote preparation was saved before the final filtration steps through polycarbonate filters. Bone marrow macrophages were isolated from one of the infected mice, and grown in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) heat-inactivated horse serum and 30% (v/v) L-cell-conditioned medium (Meerpohl et al., 1976). These macrophages were not infected with *Leishmania*, as assessed by light microscopy.

Radiolabelling of cell-surface glycolipids

Amastigotes (5×10^8) obtained before or after filtration through polycarbonate filters, promastigotes (5×10^8) and bone-marrow macrophages (7×10^6) were surface-labelled with galactose oxidase/ NaB^3H_4 . Amastigotes and promastigotes (2×10^8 cells of each) were also surface-labelled with mild periodate/ NaB^3H_4 . Both labelling procedures have been described previously (McConville and Bacic, 1990).

Glycolipid isolation

Unlabelled amastigotes (3×10^9) and labelled cells (see above) were extracted as described (McConville and Bacic, 1990). Briefly,

cells were extracted twice in methanol/chloroform/water (10:5:4, by vol.) and the insoluble material was removed by centrifugation (10000 g). Water (0.35 vol.) was added to the pooled supernatants to induce phase separation between organic and aqueous phases. The organic phase was washed with clean aqueous phase, then dried under N_2 and redissolved in chloroform. The pooled aqueous phases were dried under N_2 , dried again from methanol and redissolved in 400 μl of starting buffer (propan-1-ol/0.1 M ammonium acetate; 1:19, v/v). Samples were loaded on octyl-Sepharose columns (5 cm $\times$ 0.5 cm, 0.1 ml/min), washed with 5 ml of the starting buffer (0.25 ml/min) and eluted with a linear gradient to propan-1-ol/0.1 M ammonium acetate (7:3, v/v) over 1 h (0.25 ml/min). Glycolipids were detected by either liquid-scintillation counting or spotting 5 μl of each fraction (1.5 ml) on to h.p.t.l.c. plates and staining with orcinol/ H_2SO_4 reagent. Fractions of interest were pooled, lyophilized and redissolved in 40% propan-1-ol. In some instances, glycolipids were resolved by silica h.p.t.l.c. (solvent A, see under 't.l.c.'), localized with a radioactivity linear analyser (RITA; Raytest) and scraped individually from the plate. Glycolipids were recovered by three extractions with methanol/chloroform/water (10:10:3, by vol.), dried under N_2 and redissolved in 40% propan-1-ol.

Galactose oxidase/ NaB^3H_4 labelling of extracted glycolipids

Amastigote glycolipids and standard ganglioside GM1 were radiolabelled with galactose oxidase/ NaB^3H_4 , and then resolved from radiochemical contaminants by chromatography on octyl-Sepharose essentially as described (McConville and Bacic, 1990).

Compositional analyses

myo-Inositol content was measured by selected ion-monitoring g.c.-m.s. after acid hydrolysis (6 M HCl, 110 °C, 16 h) and trimethylsilyl derivatization with pyridine/hexamethyldisilazane/trimethylchlorosilane (10:3:1, by vol.) (Smith et al., 1987; McConville et al., 1990b). Monosaccharide and monoalkylglycerol contents were determined by g.c.-m.s. after methanolysis (0.5 M methanolic HCl, 80 °C, 16 h) and trimethylsilyl derivatization (McConville et al., 1990b).

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Glycolipids were subjected to digestion by *Bacillus thuringiensis* PI-PLC (30 m-units in 5 μl of 20 mM Tris/acetate, pH 7.5, 0.1% Triton X-100) for 2 h at 37 °C. The mixture was adjusted to 100 μl with water and extracted twice with 200 μl of water-saturated butan-1-ol. Butanol phases were pooled, back-extracted with 100 μl of butanol-1-ol-saturated water, dried, resuspended in 40% propan-1-ol and analysed by h.p.t.l.c. (solvent A).

Base treatment

Glycolipids were treated with 0.1 M NaOH in methanol (30 μl , 2 h, 37 °C). The mixture was neutralized with acetic acid, then extracted and analysed as described under 'PI-PLC treatment'.

Nitrous acid deamination and NaB^3H_4 reduction

Unlabelled glycolipids were submitted to PI-PLC digestion, nitrous acid deamination, NaB^3H_4 reduction and radiochemical purification by descending paper chromatography, essentially as described earlier (McConville et al., 1990a). In some cases, paper

chromatography was replaced by h.p.t.l.c. (solvent C; see under 't.l.c.') and the oligosaccharides of interest were scraped off individually and eluted from the silica three times with 200 μ l of 40% propan-1-ol.

Glycolipids labelled with galactose oxidase/ NaB^3H_4 or periodate/ NaB^3H_4 were deaminated and reduced in the same way, except that 1 M NaBH_4 was used instead of NaB^3H_4 and the paper chromatography step was omitted.

Exoglycosidase treatments

Galactose oxidase/ NaB^3H_4 -labelled glycolipids were digested with coffee bean α -galactosidase (0.8 units in 30 μ l of 0.1 M sodium citrate/phosphate buffer, pH 6, 0.1% Triton X-100, 16 h, 37 °C), or bovine testicular β -galactosidase (35 m-units in 20 μ l of 0.1 M sodium citrate/phosphate buffer, pH 4.5, 0.1% Triton X-100, 24 h, 37 °C, under a toluene-saturated atmosphere). Glycolipids were recovered in the butanol phase as described under 'PI-PLC treatment', whereas the galactose recovered in the aqueous phase was desalted through AG50 X12(H^+) over AG3 X4(OH^-) (200 μ l of each) before Dionex h.p.l.c.

Neutral deaminated and reduced glycans were treated with either coffee bean α -galactosidase (0.8 unit in 30 μ l of 0.1 M sodium citrate/phosphate buffer, pH 6, 16 h, 37 °C) or bovine testicular β -galactosidase (35 m-units in 20 μ l of 0.1 M sodium citrate/phosphate buffer, pH 4.5, 16 h, 37 °C), or jack bean α -mannosidase (0.8 unit in 30 μ l of 0.1 M sodium acetate buffer, pH 5, 16 h, 37 °C). Reactions were stopped by heating for 5 min at 100 °C, and the products desalted by passage through AG50 X12(H^+) over AG3 X4(OH^-) (200 μ l of each).

Trifluoroacetic acid hydrolyses

Mild acid hydrolysis was performed in 40 mM trifluoroacetic acid (200 μ l, 100 °C, 1 h). Hexofuranosidic linkages are preferentially cleaved under these conditions (McConville et al., 1990b). Release of all neutral monosaccharides was achieved in 2 M trifluoroacetic acid (150 μ l, 100 °C, 2 h).

T.l.c.

T.l.c. was performed on aluminium-backed silica gel 60 h.p.t.l.c. sheets (10 cm). Glycolipids were analysed in methanol/chloroform/0.25% KCl (10:10:3, by vol.) (solvent A). Neutral oligosaccharides were chromatographed in either butan-1-ol/ethanol/water (4:3:3, by vol.: two developments) (solvent B), or propan-1-ol/acetone/water (9:6:5, by vol. for the first and third developments, and 5:4:1, by vol., for the second development) (solvent C) (Schneider et al., 1993). Carbohydrates were detected by staining with orcinol/ H_2SO_4 reagent. Alternatively, radioactivity was monitored with either a radioactivity linear analyser or by fluorography after spraying with En^3hance . Monosaccharides were analysed by cellulose t.l.c. in ethyl acetate/pyridine/water (4:2:1, by vol.; two developments) and stained with silver (Chaplin, 1986) or detected by fluorography.

Anion-exchange chromatography on DEAE-Fractogel

Galactose oxidase/ NaB^3H_4 -labelled glycolipids and ganglioside GM1 in methanol were loaded on to DEAE-Fractogel (acetate form, 200 μ l) equilibrated in methanol, washed with 2 ml of methanol, then batch-eluted with 1 ml of 0.8 M ammonium acetate in methanol. Flow-through and eluted fractions were

dried under N_2 , resuspended in water and lyophilized before liquid-scintillation counting or analysis by h.p.t.l.c.

Liquid chromatography

Neutral oligosaccharides were analysed on Bio-Gel P4 or by Dionex h.p.l.c. essentially as described (McConville et al., 1990a). Neutral monosaccharides were analysed on the Dionex system by isocratic elution in 15 mM NaOH.

RESULTS

L. major amastigotes originating from lesions in nude mice were isolated by the procedure of Glaser et al. (1990). Amastigotes obtained by this method have been shown previously to be essentially free of contaminating host cell membranes. Galactose-containing glycoconjugates on the surface of these amastigotes were radiolabelled by the galactose oxidase/ NaB^3H_4 procedure. Glycolipids were extracted in chloroform/methanol/water, and then partitioned between an upper aqueous phase and a lower chloroform phase. The proportion of counts recovered in the chloroform phase varied with the type of cell; about 25% for promastigotes, 65% for amastigotes, and 85% for macrophages. The glycolipids in the aqueous phase were chromatographed on octyl-Sepharose and eluted at about 35% propan-1-ol, consistent with the behaviour of promastigote GPIs (McConville and Bacic, 1989). Analysis of the eluate by h.p.t.l.c. indicated the presence of at least six different glycolipids, which were sensitive to PI-PLC, and an apolar doublet, which was entirely resistant to this enzyme (Figure 1a, lanes 1 and 2). The PI-PLC-resistant glycolipids were absent from the promastigote stage (Figure 2).

Figure 1 Profile of galactose oxidase/ NaB^3H_4 -labelled glycolipids in *L. major* amastigotes

(a) *L. major* amastigotes were surface-labelled with galactose oxidase/ NaB^3H_4 , and the extracted glycolipids analysed before (lane 1) or after (lane 2) PI-PLC treatment. (b) Amastigotes were surface-labelled before (lanes 1 and 5) or after (lanes 2 and 6) the last step of purification during which contaminating host cell membranes are removed. Uninfected bone marrow macrophages isolated from *L. major*-infected mice were labelled in the same way (lanes 3 and 4). Glycolipids were extracted and partitioned between an upper aqueous (lanes 1–3) and a lower organic phase (lanes 4–6). (c) Labelling with galactose oxidase/ NaB^3H_4 was performed on intact amastigotes (lane 1) or on octyl-Sepharose-purified glycolipids extracted from an equivalent number of cells (lane 2). Only the glycolipids partitioning in the upper aqueous phase are shown [except (b) lanes 4–6, lower organic phase]. Chromatograms were developed in solvent A and the glycolipids detected by fluorography. o, origin; f, solvent front.

Figure 2 Isolation of individual amastigote glycolipids

Galactose oxidase/ NaB^3H_4 -labelled amastigote glycolipids were chromatographed in solvent system A, scraped individually from the plate, recovered by extraction in chloroform/methanol/water (10:10:3, by vol.) and reanalysed by h.p.t.l.c. (bands 1–8) together with the original amastigote mixture (Ama) and glycolipids isolated from surface-labelled promastigotes (Pro). Band 5 (upper band of the doublet) is a minor component of the amastigote mixture and was isolated together with some of the more abundant band 6 (lower band of the doublet). Band identification is indicated in the left margin. o, origin; f, solvent front; GL, PI-PLC-resistant glycolipid; *lyso-2*: *lyso*-GIPL-2; *lyso-3*, *lyso*-GIPL-3. Numbers in parentheses (24:0 and 18:0) refer to the length of the major alkyl chain in the lipid moiety of the standards.

Moreover, the ratio between the PI-PLC-sensitive and the PI-PLC-resistant glycolipids did not change during amastigote purification, and the latter species did not migrate with the major PI-PLC-resistant glycolipids present at the surface of uninfected bone-marrow macrophages originating from the same mice (Figure 1b). In contrast, the major labelled glycolipid of macrophages, an apolar molecule that partitioned exclusively into the chloroform phase (Figure 1b, lane 4), could be detected in small amounts in the partially purified amastigote sample (Figure 1b, lane 5), but not in the final amastigote preparation (Figure 1b, lane 6). This suggests that the PI-PLC-resistant glycolipids in purified amastigotes are integral components of the parasite membrane, and are not derived from contaminating host cell debris. The profiles of glycolipids labelled at the surface or after extraction were similar, indicating that the surface-exposed glycolipids are representative of the total cellular pool (Figure 1c).

LPG was extracted from the delipidated galactose oxidase/ NaB^3H_4 -labelled amastigote pellet with butan-1-ol-saturated water and chromatographed on octyl-Sepharose according to the protocol developed for promastigote LPG (McConville et al., 1990b). *myo*-Inositol content was determined in the eluted fractions. A peak of *myo*-inositol co-eluted with a peak of radioactivity at 35% propan-1-ol was shown to be GIPLs left over from the previous extraction. *myo*-Inositol detected at the expected elution position of LPG (28% propan-1-ol) accounted for not more than 10^4 molecules per amastigote, which may originate from either amastigote LPG or contaminating GIPLs (not shown).

Each of the glycolipids labelled by the galactose oxidase/ NaB^3H_4 procedure was purified by t.l.c. (Figure 2, bands 1–8) for analysis as described below. In addition, an extract of unlabelled amastigote glycolipids was solubilized with PI-PLC, then deaminated with nitrous acid and reduced with NaB^3H_4 . This procedure results in the modification of glucosamine to

Table 1 Liquid chromatography of deaminated and reduced glycan moieties of amastigote GIPLs

Deaminated NaB^3H_4 -labelled glycans were chromatographed on either Dionex or Bio-Gel P4, and the elution position relative to a series of dextran oligomers expressed as Dionex units (DU) or glucose units (GU) respectively. GIPL-A was further treated with bovine testicular β -galactosidase (BTBG) alone or followed by coffee bean α -galactosidase (CBAG). nd, not determined.

GIPL	Treatment	DU	GU
M1	—	1.1	nd
iM2	—	2.0	nd
GIPL-1	—	6.1	4.4
GIPL-2	—	6.0	5.5
GIPL-A	—	7.8	nd
GIPL-A	BTBG	6.0	5.5
GIPL-A	BTBG/CBAG	6.1	4.4
GIPL-3	—	5.9	nd

labelled 2,5-anhydromannitol (AHM), with release of the inositol phosphate, and allows the glycan to be sequenced from the original non-reducing terminus. Six neutral oligosaccharides were generated, which all contained ^3H -labelled AHM. Individual glycans were purified using a combination of h.p.t.l.c. (solvent C) and Dionex h.p.l.c. (program A), then characterized by micro-sequencing using exoglycosidases and chemical treatments. This latter approach showed that amastigotes contained several additional GIPL species which were not labelled by the galactose oxidase/ NaB^3H_4 procedure. The structures of all the detected GIPLs are described below.

M1

Deaminated and reduced M1 glycan accounted for 22% of the total AHM-labelled structures and co-eluted with authentic $\text{Man}\alpha 1\text{-4AHM}$ on Dionex h.p.l.c. (1.1 DU) (Table 1) and h.p.t.l.c. (Figure 3). It was digested to AHM by α -mannosidase (Figure 3, lanes 1 and 2). This suggests that M1 has the structure $\text{Man}\alpha 1\text{-4GlcN-PI}$ (Table 2).

iM2

Deaminated and reduced iM2 glycan co-migrated with authentic $\text{Man}\alpha 1\text{-3Man}\alpha 1\text{-4AHM}$, but not with $\text{Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$, on both Dionex h.p.l.c. (2.0 DU) (Table 1) and h.p.t.l.c. (Figure 3, lanes 23 and 25). The glycan was digested to AHM with α -mannosidase (Figure 3, lane 24). This suggests that iM2, which accounted for 27% of the GIPLs, has the structure $\text{Man}\alpha 1\text{-3Man}\alpha 1\text{-4GlcN-PI}$ (Table 2).

GIPL-1 and *lyso*-GIPL-1

A deaminated and reduced glycan, accounting for 36% of the total label, co-migrated with authentic GIPL-1 glycan on both Dionex h.p.l.c. (6.1 DU) (Table 1) and h.p.t.l.c. Its identity was confirmed by sequential treatment with mild trifluoroacetic acid (under conditions that hydrolyse hexofuranosidic but not hexopyranosidic linkages) and α -mannosidase (Figure 3, lanes 3–7). It should be noted that the product of mild acid treatment was poorly resolved from the starting material in this chromatographic system (Figure 3, lanes 5 and 6). However, these species could be distinguished from each other by their susceptibility to α -mannosidase digestion (Figure 3, lanes 5 and 7). To confirm that the acid-labile substituent in this structure was

Figure 3 Microsequencing of the deaminated and reduced amastigote neutral glycan

M1: untreated (lane 1); treated with jack bean α -mannosidase (JBAM) (lane 2). iM2: untreated (lane 23); treated with JBAM (lane 24); mixture of standards Man α 1-6Man α 1-4AHM (lower band) and Man α 1-3Man α 1-4AHM (upper band) (lane 25). GIPL-1: untreated (lane 3); treated with coffee bean α -galactosidase (CBAG) (lane 4); CBAG-treated glycan subsequently treated with JBAM (lane 5), mild trifluoroacetic acid (TFA) (lane 6), and TFA, then JBAM (lane 7). GIPL-2: untreated (lane 8); treated with CBAG (lane 9); CBAG-treated glycan subsequently treated with JBAM (lane 10), TFA (lane 11), and TFA, then JBAM (lane 12). GIPL-A: untreated (lanes 13 and 26), treated with CBAG (lane 14); CBAG-treated glycan subsequently treated with JBAM (lane 15), TFA (lane 16), TFA, then JBAM (lane 17), bovine testicular β -galactosidase (BTBG) (lane 27), BTBG, then CBAG (lane 28), BTBG, then CBAG, then TFA (lane 29). GIPL-3: untreated (lane 18); treated with CBAG (lane 19); CBAG-treated glycan subsequently treated with JBAM (lane 20), TFA (lane 21), and TFA, then JBAM (lane 22). Dex, reduced dextran hydrolysate; o, origin; f, solvent front. H.p.t.l.c. plates were developed in solvent system C. Lanes 1–22, 23–25, and 26–29 were chromatographed on separate h.p.t.l.c. plates. The schematic structure of the main product analysed in each lane is depicted above the h.p.t.l.c. profile, together with the number of the relevant lane and the sequence of enzyme and chemical treatments. Triangle, 2,5-[1- 3 H]anhydromannitol; open circle, Man; diamond, Gal; closed circle, Gal.

Table 2 Structure of the *L. major* amastigote GIPLs

Summary of the GIPL structures determined in this study. The relative abundance of the various amastigote GIPLs was determined from the incorporation of radiolabel into the deaminated and reduced glycans. The structure of these glycolipids was determined by a combination of exoglycosidase digestions, controlled chemical degradations and co-migration with defined standards. For GIPL-1, the proportion of *lyso*-alkyl-PI was determined on the basis of periodate/ NaB^3H_4 -labelled glycolipids recovered in the upper aqueous phase. The proportion of alkylacyl-PI may be underestimated, as the lipids partitioning into the lower chloroform phase were not taken into account. Numbers in parentheses indicate the percentage of short alkyl chains ($\text{C}_{18:0}$), the remaining being long alkyl chains ($\text{C}_{24:0}$). The presence of *lyso*-alkyl GIPL-A was suggested by the co-migration of a GIPL-A species with band 7 (Figure 6a), but has not been isolated. nd, not determined.

GIPL	Abundance (%)	Structure	Alkylacyl-PI (%)	<i>lyso</i> -alkyl-PI (%)
M1	22	Man α 1-4GlcN-PI	nd	nd
IM2	27	Man α 1-3Man α 1-4GlcN-PI	nd	nd
GIPL-1	36	Gal β 1-3Man α 1-3Man α 1-4GlcN-PI	50	50
GIPL-2	12	Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN-PI	60 (100)	40 (60)
GIPL-A	2	Gal β 1-3Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN-PI	100	—
GIPL-3	1	Gal α 1-6Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN-PI	5	95 (100)

galactofuranose, surface-exposed glycolipids were subjected to mild periodate oxidation followed by NaB^3H_4 reduction. This reaction specifically converts Galf to Ara with incorporation of ^3H at the C-5 position (de Lederkremer et al., 1980; McConville and Bacic, 1990). The major oxidized oligosaccharide released by deamination and reduction co-migrated with authentic oxidized GIPL-1, and a minor labelled species which co-migrated with GIPL-2 was also present (Figure 4a). Strong acid hydrolysis confirmed that this label was present in the newly formed Ara residue (Figure 4b), confirming the presence of Galf in the original GIPLs. When the periodate/ NaB^3H_4 -labelled amastigote GIPLs were subjected to h.p.t.l.c., a more complex profile was observed (not shown). Two major species were detected which contained approximately the same amount of radioactivity. One of these species co-migrated with oxidized promastigote GIPL-1 and yielded the glycan headgroup of oxidized GIPL-1 after deamination and reduction. This species probably corresponds to GIPL-1 with an alkylacyl-PI lipid moiety. The second species migrated near oxidized promastigote GIPL-2 but contained predominantly the glycan headgroup of oxidized GIPL-1 (80%) and only a small amount of the oxidized GIPL-2 glycan (20%). The major species in this band is probably *lyso*-GIPL-1. Taken together, these results suggest that the glycan of amastigote GIPL-1 is indistinguishable from the glycan of promastigote GIPL-1, which has the structure Gal β 1-3Man α 1-3Man α 1-4GlcN, and that it is linked to either alkylacyl-PI (50%) or *lyso*-alkyl-PI (50%) (Table 2).

GIPL-2 and *lyso*-GIPL-2 species (bands 3, 6 and 7)

Galactose oxidase/ NaB^3H_4 -labelled band 3 co-migrated with authentic promastigote GIPL-2 (Figure 2). After mild base treatment, this species had a reduced h.p.t.l.c. mobility, co-migrating with band 7 and authentic *lyso*-GIPL-2 containing $\text{C}_{18:0}$ alkyl chains, consistent with the loss of a base-labile fatty acyl chain (Figure 5). Bands 6 and 7 co-migrated with the *lyso* derivatives of GIPL-2 containing $\text{C}_{24:0}$ and $\text{C}_{18:0}$ alkyl chains respectively, and the h.p.t.l.c. mobility of these species was unaffected by mild base treatment (Figure 5). The deaminated and reduced glycan headgroup of bands 3, 6 and 7 all co-migrated on h.p.t.l.c. with the authentic glycan Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4AHM derived from promastigote GIPL-2 (Figure 6a). The presence of an internal Galf residue was suggested by the sensitivity of the glycolipids to mild trifluoroacetic acid hydrolysis and by the identification of Ara in the mild periodate oxidized and reduced glycans (Figures 6b and

4). A labelled glycan (accounting for 12% of total label) which shared identical chromatographic properties with authentic GIPL-2 glycan on both h.p.t.l.c. and Dionex h.p.l.c. (6.0 DU) (Table 1) was purified from the mixture of deaminated and reduced glycans and was digested to AHM by sequential treatment with α -galactosidase, mild trifluoroacetic acid and α -mannosidase (Figure 3, lanes 8–12). Taken together, these results suggest that bands 3, 6 and 7 contain the same glycan sequence as promastigote GIPL-2: Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN linked to either alkylacyl-PI (60%) or *lyso*-alkyl-PI (40%) (Table 2). They also indicate that, whereas GIPL-2 species with an alkylacyl-PI lipid contained predominantly $\text{C}_{18:0}$ alkyl chains, the *lyso* species contained either $\text{C}_{18:0}$ or $\text{C}_{24:0}$ alkyl chains. These results suggest that GIPL-2 species containing $\text{C}_{24:0}$ alkyl chains are more efficiently deacylated than those species containing $\text{C}_{18:0}$ alkyl chains *in vivo*. GIPL-2 is also more efficiently labelled on the surface of intact amastigotes than the corresponding *lyso* derivatives, suggesting that there may be differences in the surface expression of the alkylacylglycerol and *lyso*-alkylglycerol-containing GIPL species (Table 3).

GIPL-3 and *lyso*-GIPL-3 species (bands 5 and 8)

Band 5 co-migrated with promastigote GIPL-3 on h.p.t.l.c. (Figure 2) and had a lower mobility after mild base treatment (Figure 5), suggesting the presence of an alkylacyl-PI moiety. Band 8 was resistant to mild base treatment and co-migrated with authentic *lyso*-GIPL-3 containing $\text{C}_{18:0}$ alkyl chains, consistent with it being a *lyso*-GIPL-3 species (Figure 5). The deaminated and reduced glycans of both bands 5 and 8 co-migrated on h.p.t.l.c. with the authentic glycan Gal α 1-6Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4AHM derived from promastigote GIPL-3 (Figure 6a), and the presence of an internal acid-labile residue was suggested by treatment of the glycolipids with mild trifluoroacetic acid (Figure 6b). A glycan with identical chromatographic properties on h.p.t.l.c. was purified from the mixture of deaminated/ NaB^3H_4 -reduced glycans (1% of the total label) and sequenced by treatment with α -galactosidase, mild trifluoroacetic acid and α -mannosidase, as described for GIPL-2 (Figure 3, lanes 18–22). The data are consistent with bands 5 and 8 having the glycan structure identical with that of promastigote GIPL-3, Gal α 1-6Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4GlcN linked to either alkylacyl-PI or *lyso*-alkyl-PI, with $\text{C}_{18:0}$ being the predominant alkyl chain in the *lyso* species. It is noteworthy that, despite its low abundance, GIPL-3 was readily detected by the galactose oxidase/ NaB^3H_4 -labelling procedure. As GIPL-2 and

Figure 4 Analysis of the deaminated and reduced periodate/ NaB_3H_4 -surface-labelled GIPLs

(a) Glycolipids isolated from amastigotes (Ama) or promastigotes (Pro) surface-labelled with periodate/ NaB_3H_4 were solubilized with PI-PLC, and the glycans recovered in the aqueous phase after butan-1-ol/water partition were submitted to deamination and reduction before analysis by h.p.t.l.c. (solvent C). The migration positions of periodate-oxidized promastigote GIPL-1, -2, -A and -3 are indicated on the left-hand side. Parenthesis indicate the position of a faint band apparent on the original. (b) The glycans shown in (a) were hydrolysed with strong trifluoroacetic acid, and the released labelled monosaccharides were analysed by cellulose t.l.c. (solvent D). The migration position of various monosaccharides is indicated on the right-hand side.

Figure 5 Base sensitivity of bands 3–8

The galactose oxidase/ NaB_3H_4 -labelled isolated bands 3–8 (see Figure 2) were incubated in methanol in the presence (+) or absence (–) of 0.1 M NaOH. Glycolipids were desalted by partition between butan-1-ol and water, and the butanol phase was analysed by h.p.t.l.c. (solvent A). Band 5 is contaminated with the base-resistant band 6 (see Figure 2). The nature of the apolar band which appears in some native or base-treated samples is not known. o, origin; f, solvent front.

GIPL-3 only have one oxidizable Gal residue, this observation confirms that the glycan of GIPL-3 is a better substrate for galactose oxidase than the other GIPLs (McConville and Bacic, 1990).

GIPL-A (band 4)

The galactose oxidase/ NaB_3H_4 procedure labelled an additional PI-PLC-sensitive species, band 4, which co-migrated with the

Figure 6 Analysis of the oligosaccharide moiety of isolated bands 3–8

(a) Galactose oxidase/ NaB_3H_4 -labelled amastigote glycolipids (Tot) or isolated bands 3–8 were solubilized with PI-PLC, deaminated with nitrous acid and reduced with unlabelled NaBH_4 . Released oligosaccharides recovered in the aqueous phase after partition between butan-1-ol and water were desalted and analysed by h.p.t.l.c. (solvent B). Ctrl, amastigote glycolipids treated in the same way, except that PI-PLC was omitted and NaNO_2 was replaced by NaCl. The migration positions of the oligosaccharide moiety of promastigote GIPL-2, GIPL-A and GIPL-3 are indicated on the left. o, origin; f, solvent front. (b) Galactose oxidase/ NaB_3H_4 -labelled amastigote bands 3–8 were treated with 40 mM trifluoroacetic acid (100°C, 1 h), and the released oligosaccharides recovered in the water phase after partitioning between butan-1-ol and water. Glycans were analysed by h.p.t.l.c. (two developments in butan-1-ol/ethanol/water; 5:3:2, by vol.). Only the portion of interest is shown (R_F 0–0.5). G1 to G5 indicate the migration positions of β -Glc oligomers containing one to five Glc residues. In this solvent system, the oligosaccharides Gal α 1-3Gal and Gal α 1-6Gal α 1-3Gal run at approximately 1.9 and 2.9 glucose units.

Table 3 Galactose oxidase/ NaB_3H_4 -labelling of surface-exposed and total amastigote GIPLs

GIPLs partitioning into the upper aqueous phase (see Figure 1b) were labelled with galactose oxidase/ NaB_3H_4 at the cell surface or after glycolipid extraction. Labelled glycolipids were resolved by h.p.t.l.c. and the radioactivity monitored with a linear analyser. Results are expressed as percentage incorporation into glycolipid bands 1–8, as numbered in Figure 2. The numbers in parentheses refer to the major alkyl chain of each molecular species, where known.

Band	Major species	Surface labelling [%]	Total labelling (%)
1 + 2	GL-1, -2	8	5
3	GIPL-2 (18:0)	28	18
4	GIPL-A	7	9
5	GIPL-3	1	1
6	lyso-GIPL-2 (24:0)	19	26
7	lyso-GIPL-2 (18:0)	14	19
8	lyso-GIPL-3	23	22

partially characterized GIPL-A from promastigote (Figure 2). This species was sensitive to mild base treatment, suggesting that it contained an alkylacyl-PI lipid moiety (Figure 5). The deaminated and reduced glycan of this GIPL had a distinct chromatographic mobility from either the GIPL-2 or GIPL-3 glycans on both h.p.t.l.c. (Figures 6a and 6b) and Dionex h.p.l.c. (7.8 DU) (Table 1). The corresponding deaminated/ NaB_3H_4 -labelled glycan was isolated from the mixture (2% of the total label) and sequenced as shown in Figure 3 (lanes 13–16, and 26–29). This glycan, as well as the intact glycolipid, was resistant to α -galactosidase but susceptible to β -galactosidase digestion

Figure 7 Anomericity of the terminal Gal residue in galactose oxidase/ NaB^3H_4 -labelled bands 1, 2, 4, and 6

Bands 1 and 2, band 4 and band 6 were digested with either coffee bean α -galactosidase (CBAG) or bovine testicular β -galactosidase (BTBG). Reaction products were partitioned between butan-1-ol and water. Undigested glycolipids recovered in the butanol phase were analysed by h.p.t.l.c in solvent A (central panels, only the portion between R_f 0.2 and 0.8 is shown), whereas released Gal recovered in the aqueous phase was desalted and analysed by Dionex h.p.l.c. The elution position of Gal is indicated.

(Figures 3 and 7). Digestion of the glycan gave a product that comigrated with GIPL-2 on h.p.t.l.c. (Figure 3), Dionex h.p.l.c. (6.0 DU) and Bio-Gel P4 (5.5 GU) (Table 1). The identity of this product was confirmed by sequential treatment with α -galactosidase, mild trifluoroacetic acid and α -mannosidase (Figure 3, lanes 27–29). These results indicate that the deaminated and reduced glycan contains the sequence $\beta\text{Gal}-\alpha\text{Gal}-\text{Gal}/\alpha\text{Man}-\alpha\text{Man}-\text{AHM}$. Deaminated and reduced promastigote GIPL-A (McConville et al., 1990a) was submitted to micro-sequencing and found to share identical properties with the amastigote oligosaccharide. In particular, it was shown to be sensitive only to β -galactosidase and not to α -galactosidase. This, together with the re-evaluation of the methylation analysis described earlier (McConville et al., 1990a), allowed the reassessment of the glycan structure of the promastigote GIPL-A, and consequently amastigote GIPL-A, as $\text{Gal}\beta 1-3\text{Gal}\alpha 1-3\text{Gal}\beta 1-3\text{Man}\alpha 1-3\text{Man}\alpha 1-4\text{GlcN}$ (Table 2).

The 1-*O*-alkylglycerol composition of the mixture of octyl-Sepharose-purified amastigote glycolipids was determined by g.c.-m.s. after methanolysis and trimethylsilyl derivatization. Seven species of fully saturated unbranched 1-*O*-monoalkylglycerols were identified, with chain length of $\text{C}_{16:0}$ (2.8%), $\text{C}_{18:0}$ (50.7%), $\text{C}_{19:0}$ (0.8%), $\text{C}_{20:0}$ (10.7%), $\text{C}_{22:0}$ (14.7%), $\text{C}_{23:0}$ (0.7%) and $\text{C}_{24:0}$ (17.5%). A few other minor unidentified species of 1-*O*-monoalkylglycerols accounted for the remaining 2.2%.

The PI-PLC-sensitive glycolipids were estimated to be present at 4×10^6 copies per amastigote. This estimation is based on the amount of Man detected by compositional analysis of the whole amastigote glycolipid mixture (results not shown), assuming that all of the Man originates from GIPLs (Table 2). All of the Gal-

containing amastigote type-2 GIPL species were also detected in the promastigote stage of the parasite, although in different relative amounts (Figure 2, Pro and Ama).

PI-PLC-resistant glycolipids (bands 1 and 2)

The galactose oxidase/ NaB^3H_4 -labelled PI-PLC-resistant glycolipids (bands 1 and 2) were insensitive to mild base treatment, indicating the absence of ester-linked fatty acids. They were resistant to mild acid treatment under conditions that allowed removal of sialic acid from a control glycosphingolipid, ganglioside GM1 (results not shown). In fact, the PI-PLC-resistant glycolipids were shown to lack negative charges, as they did not bind to the anion-exchanger DEAE-Fractogel, in contrast with the positive control GM1 (results not shown). Analysis of the monosaccharides released by strong acid hydrolysis of the galactose oxidase/ NaB^3H_4 -labelled glycolipids showed that only Gal, and not GalNAc, was labelled (not shown). Digestion of the glycolipids with either α - or β -galactosidases indicated that this residue was a terminal β -Gal (Figure 7, bands 1 and 2). These PI-PLC-resistant glycolipids were relatively more abundant when labelled at the surface than when labelled in total glycolipid extracts, suggesting that they are enriched at the surface (Table 3). Taken together, the results show that the PI-PLC-resistant glycolipids of *L. major* amastigotes do not contain phosphatidylinositol, and that they are neutral molecules with terminal β -Gal.

DISCUSSION

Previous studies have shown that the expression of promastigote surface protease and other promastigote surface proteins is greatly down-regulated as *L. major* promastigotes differentiate into amastigotes in mammalian macrophages (Schneider et al., 1992b; T. A. Glaser, personal communication). In this study, we show that LPG is also down-regulated in the amastigote stage to less than 10^4 copies per cell. As promastigotes contain approx. 5×10^6 copies of LPG per cell (McConville and Bacic, 1990), this corresponds to more than a 500-fold down-regulation. This is in agreement with previous studies which showed that the antigenically and structurally distinct amastigote LPG is not an abundant antigen (Glaser et al., 1991; Turco and Sacks, 1991; Moody et al., 1993). In contrast with the major surface proteins and LPG, we now show that the GIPLs are expressed in high copy number in both promastigote and amastigote developmental stages. The copy number per cell is slightly lower in amastigotes than in promastigotes (4×10^6 compared with 10×10^6) (McConville and Bacic, 1990), which probably reflects the smaller size of the amastigote cell. Considering *L. major* amastigote as an ellipsoid $4 \mu\text{m}$ long and $2 \mu\text{m}$ in diameter (Schneider et al., 1992b), and assuming that the average size of the glycan headgroup of GIPL-1 is $2.4\text{--}3.2 \text{ nm}^2$ (C. T. Weller and S. W. Homans, unpublished work), these structures may potentially coat 45–60% of the amastigote plasma membrane.

The surface architecture of other *Leishmania* species may undergo similar changes during promastigote to amastigote differentiation. For example, it has recently been shown that LPG is no longer detectable in *L. donovani* amastigotes, whereas the GIPLs are expressed at the same level in both stages. It is not known if surface proteins are also down-regulated in this species, but down-regulation of both surface proteins and LPG has been found in *L. mexicana* amastigotes (Bahr et al., 1993). This dramatic reduction in surface macromolecules by the amastigote may be a form of immune evasion, reducing the possibility of parasite antigens being released into the parasitophorous vacuole

and subsequently presented at the macrophage surface by major histocompatibility complex class II molecules. In this context, the surface glycocalyx provided by the GIPLs may be sufficient to mask the remaining plasma-membrane proteins (transporters, enzymes) which are still required for essential cellular functions.

L. major amastigotes contain at least six different GIPLs, although more than 11 molecular species were characterized because most of the GIPLs contained both alkylacyl-PI and *lyso*-alkyl-PI lipid moieties (Table 2). All of these structures, except for GIPL-A, are directly related to the LPG core and appear to form part of a biosynthetic series (type-2 GIPLs). Most of these GIPLs are also expressed in the promastigote stage, although there are pronounced quantitative differences in the GIPL profiles of the two main developmental stages. In particular, the amastigote profile is enriched with GIPLs with shorter glycan chains (i.e. iM2, GIPL-1) and with species with a *lyso*-alkyl-PI rather than an alkylacyl-PI lipid moiety. The amastigote GIPLs also differed from the promastigote GIPLs in containing predominantly C_{18:0} alkyl chains, instead of the longer C_{24:0} and C_{26:0} alkyl chains (McConville et al., 1990a). This is consistent with the previous finding that promastigote GIPLs with shorter glycan chains tend to be enriched in C_{18:0} alkyl chain (McConville et al., 1990a). However, in amastigotes, the *lyso*-GIPL-3 species is also enriched in C_{18:0} alkyl chains, strongly suggesting that it is not a precursor for the low-abundance amastigote LPG which contains predominantly C_{24:0} alkyl chains (Moody et al., 1993).

GIPL-A is the only type-2 GIPL with a structure that cannot be found in the LPG core. It differs from GIPL-3 in having a terminal Gal β 1-3 instead of a Gal α 1-6 residue. The sequence Gal β 1-3Gal is also found as side chains linked to phosphorylated disaccharide repeat units of *L. major* promastigote LPG (McConville et al., 1990b). Whether the β 1-3 galactosyltransferase involved in LPG biosynthesis can accept GIPL-2 as a substrate to yield GIPL-A, or whether a distinct β 1-3 galactosyltransferase is necessary for these two reactions, is not known.

The surface-expressed GIPL could form a glycocalyx over a significant proportion of the plasma membrane, which may be important for amastigote survival in the phagolysosome compartment of host macrophages. In particular, the *lyso*-GIPLs may have a role in inhibiting protein kinase C-mediated signal-transduction pathways, and therefore activation of the oxidative burst by activated macrophages, as has been suggested for LPG (McNeely and Turco, 1990; Descoteaux et al., 1991). Antibodies raised against the type-2 GIPLs are present in the serum of all patients with cutaneous leishmaniasis, indicating that they are highly immunogenic (Rosen et al., 1989; McConville et al., 1990a; Avila et al., 1992). Interestingly, antibodies against these structures are also present in lower levels in non-infected individuals (McConville et al., 1990a). These antibodies may correspond to the anti-Gal antibodies that are directed against the epitope Gal α 1-3Gal and account for nearly 1% of the IgG in human sera (Galili et al., 1988). The presence of anti-(type-2 GIPL) antibodies in both uninfected and infected individuals suggests that amastigotes are opsonized with antibodies when they are released into the bloodstream. Whether the opsonized antibodies facilitate the invasion of uninfected macrophages by amastigotes, via either the Fc receptor or the complement receptors of the macrophage, is not known. In this context, it is of interest that *L. donovani* amastigotes only express the Man-containing type-1 GIPLs, which are not recognized by these antibodies (McConville and Blackwell, 1991). Moreover, these amastigotes appear to bind to macrophages via the macrophage mannose receptor (Blackwell et al., 1985). These data raise the intriguing possibility that species-specific differences in GIPL profiles may regulate which macrophage receptors are utilized by

the amastigote stage and even contribute to the distinct tissue tropisms of individual *Leishmania* species.

In addition to type-2 GIPLs, two PI-PLC-resistant glycolipids were also detected in *L. major* amastigotes. Their properties are compatible with them being neutral β -Gal-terminating glycosphingolipids. Additional PI-PLC-resistant glycolipids, which are not labelled by the galactose oxidase/NaB³H₄ method, are also present in the amastigote (P. Schneider, J.-P. Rosat, A. Ransijn, M. A. J. Ferguson and M. J. McConville, unpublished work). The ratio of these glycolipids to the GIPLs does not change during amastigote purification, indicating that they are inserted into the amastigote membrane, or closely associated with it. This, together with the fact that they are not representative of macrophage surface glycosphingolipids, suggests that they are not an artefact due to the presence of contaminating host cell debris. Similar glycosphingolipids have been identified in *L. donovani* amastigotes, derived from hamster peritoneal macrophages (McConville and Blackwell, 1991). These glycolipids could not be biosynthetically labelled in the amastigote and were absent from the promastigote stage. The major glycosphingolipid species was characterized as the GalNAc β 1-3-terminating para Forssman glycolipid (McConville and Blackwell, 1991). Acquisition of host cell glycolipids may thus be a feature common to all *Leishmania* amastigotes. The differences in glycosphingolipid composition of the *L. major* and *L. donovani* amastigotes may reflect the fact that they were obtained from different hosts and also from different macrophage subpopulations. These glycolipids are only minor components in the non-infected macrophage, raising the possibility that they are acquired when amastigotes are briefly exposed to the extracellular environment. Alternatively, these particular glycosphingolipids may become more abundant in infected macrophages or be enriched in the parasitophorous vacuole.

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