Characterization of inositol lipids from *Leishmania donovani* promastigotes: identification of an inositol sphingophospholipid

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Abstract Inositol lipids account for 15% of the total cellular phospholipids of *Leishmania donovani* promastigotes. Four major inositol lipids were identified and characterized: phosphatidylinositol (PI), phosphatidylinositol phosphate (PI-P), phosphatidylinositol diphosphate (PI-P_2), and an inositol sphingophospholipid (InSL). Diacyl and alkyl acyl PI were identified. The major esterified fatty acids of PI, PI-P, and PI-P_2 were similar and unlike those of mammalian inositol glycerolipids. *Leishmania* inositol glycerolipids contained only trace amounts of arachidonic acid; the major species were C_{16} and C_{18} acids. The InSL comprised about 40% of the inositol lipids. The amide-linked fatty acids of InSL were mainly C_{16} and C_{18} acids. Differential hydrolysis and nuclear magnetic resonance spectrometry indicated that the InSL had a phosphoryl bond. The major long chain bases of the InSL were identified by gas–liquid chromatography and high resolution mass spectrometry as straight chain C_{16} and C_{18} sphingosines. The finding of InSL in *Leishmania* is of interest because InSL have previously been found only in plants and fungi. Metabolic radiolabeling experiments suggest that this lipid may be a precursor of an antigenic cell surface membrane lipophosphoglycan which is shed into the culture medium by the organism.—**Kaneshiro, E. S., K. Jayasimhulu, and R. L. Lester.** Characterization of inositol lipids from *Leishmania donovani* promastigotes: identification of an inositol sphingophospholipid. *J. Lipid Res.* 1986. **27:** 1294–1303.

Supplementary key words fatty acids • glucose • long chain bases • membrane lipophosphoglycan • polyphosphoinositides

Leishmania donovani, the causative protozoan agent of the human disease kala azar, or visceral leishmaniasis, can be grown axenically as the flagellated promastigote form. The major lipid classes and fatty acids of several *Leishmania* species grown in mass cultures have been characterized (1–3). Detailed analyses of the inositol lipids, however, have not yet been described. The present study was conducted to examine these lipids in promastigote cells of *L. donovani* grown in a chemically defined medium (4, 5). Inositol glycerolipids and an inositol sphingophospholipid (InSL) were identified and characterized.

Previous work on a membrane antigen that is released into the culture medium by *L. donovani* was described and was shown to exist as an integral component of the cell's surface membrane (4). This cell surface component exhibits immunochemical identity with excreted factor (4) and appears to have chemical properties similar to the *L. major* glycoconjugate receptor for macrophages described by Handman and Golding (6). In this report the results of metabolic radiolabeling experiments are described that suggest that this antigenic substance is a lipophosphoglycan that contains an InSL domain.

MATERIALS AND METHODS

Culture

Promastigotes of *Leishmania donovani,* MHOM/SD/62/1S (obtained from Dennis M. Dwyer, National Institutes of Health, Bethesda, MD) were grown in a modified (4) defined medium of Steiger and Steiger (5) lacking bovine serum albumin (RE III − BSA). Cultures were inoculated with 1/10 vol of day 4 cultures and were grown to late log or early stationary phase (4 days) at 25–26°C. In some experiments cells were radiolabeled by the addition of 3–67 nCi of [U-^{14}C]myo-inositol per ml, 67 nCi of [U-^{14}C]D-glucose per ml, 33 nCi of [1-^{14}C]deoxyglucose per ml, 50 nCi of [1-^{14}C]stearic acid per ml, or 0.1–1.0 μCi of [^{32}P]H_3PO_4 (New England Nuclear, Boston, MA,

Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; DS, DS-55 data system; EDTA, ethylenediaminetetraacetate; FAB, fast atom bombardment; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; IEP, immunoelectrophoresis; IgG, immunoglobulin G; InSL, inositol sphingophospholipid; LCB, long chain base; LPG, lipophosphonoglycan; LPPG, lipopeptidophosphoglycan; MeOH, methanol; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCSP, promastigote cell surface preparation; PI, phosphatidylinositol; PI-P, phosphatidylinositol phosphate; PI-P_2, phosphatidylinositol diphosphate; TEAE, triethylaminoethyl; TLC, thin-layer chromatography.

or Amersham Corp., Arlington Heights, IL) to the culture medium. Cells were concentrated by centrifugation at 5,000 *g* for 30 min at 4°C. Lipids were extracted from cell pellets either after centrifugation, after freezing at −70°C, or after lyophilization.

Extraction and fractionation of lipids

Total lipids were extracted with $CHCl_3$–methanol (MeOH)–conc. HCl 100:200:1 (v/v/v) acidic Bligh and Dyer (7), then purified by partitioning according to Folch, Lees, and Sloane Stanley (8). Alternatively, lyophilized promastigotes were extracted with $CHCl_3$–MeOH 2:1 (v/v). Lipids were fractionated by triethylaminoethyl (TEAE) cellulose column chromatography (9) and then the last two fractions were pooled and the salts were removed (8). This was designated the inositol lipid fraction. That all inositol lipids were in these two TEAE column fractions was verified by autoradiography of one-dimensional (1-D) thin-layer chromatograms (TLC) of each fraction off TEAE columns that were used to separate lipids from cells grown with ^{14}Cinositol (see below).

Inositol lipid classes

Total lipids or the inositol lipid fraction off TEAE columns were analyzed by one- or two-dimensional (2-D) chromatography employing silica gel-impregnated paper (Whatman SG 81) pretreated with ethylenediaminetetraacetate (EDTA) according to Steiner and Lester (10). Radiolabeled lipids separated by this procedure were subjected to autoradiography (NS-2T X-ray film, Kodak, Rochester, NY). The individual inositol lipids corresponding to spots on autoradiograms were cut out of the paper and the material was eluted with $CHCl_3$–MeOH 2:1 (v/v). Lipids were also analyzed by 1- or 2-D TLC using silica gel H or G and the solvent systems of Turner and Rouser (9). The TLC plates were exposed to I_2 vapor, or sprayed with "Phospray" (Supelco, Inc., Bellefonte, PA) for detection of P, and α-naphthol, diphenylamine (Supelco), or anthrone (11) for sugars. Purified phosphatidylinositol (PI) and the inositol sphingophospholipid (InSL) were isolated from the inositol lipid fraction off TEAE columns by preparative 1-D TLC on silica gel H or G with $CHCl_3$–MeOH–28–30% NH_4OH–H_2O 25:20:1.6:3.9 (v/v/v/v). Lipids were visualized by water or I_2 vapor, the bands were scraped off the plates with a razor blade, and the material was eluted from the silica gel with $CHCl_3$–MeOH 1:2 (v/v).

Lipids labeled with [^{14}C]inositol were subjected to mild alkali hydrolysis according to the procedures of Smith and Lester (12); they were then analyzed by 2-D SG 81 chromatography (10) and autoradiography. These autoradiograms were compared with those of unhydrolyzed material.

Diacyl and alkyl acyl species in the PI fraction obtained by neutral $CHCl_3$–MeOH extraction were analyzed after phospholipase C digestion (13) followed by acetylation (14) and separation by TLC on silica gel H plates (15). The *Bacillus cereus* enzyme (Sigma Chemical Co., St. Louis, MO) was used by procedures previously described (13). Complete digestion of PI by this enzyme was monitored by using *Leishmania* PI labeled with [^{14}C]inositol. Within 30 min almost all of the radioactivity was rendered water-soluble (**Fig. 1**). The relative amounts of diglyceride acetates were quantified by densitometry of plates after staining with phosphomolybdate (16). To obtain esterified fatty acids, diglyceride acetates were eluted from the silica gel that was scraped off unstained plates. Alkenyl acyl glycerides from plasmalogens were not detected by these methods, and aldehydes that would have been released from plasmalogens were similarly not detected by gas–liquid chromatography (GLC) in material eluted from regions of TLC plates where aldehydes were expected to be present (15).

Fatty acids

Phosphatidylinositol phosphate (PI-P) and phosphatidylinositol diphosphate (PI-P_2) were isolated by 1-D separations employing silica gel-impregnated paper ($CHCl_3$–MeOH–4 N NH_4OH 9:7:2, v/v/v) (9). The bands containing these lipids were cut and subjected directly to alkaline hydrolysis by the micromethod of MacGee and Allen (17). The fatty acid methyl esters (FAME) produced

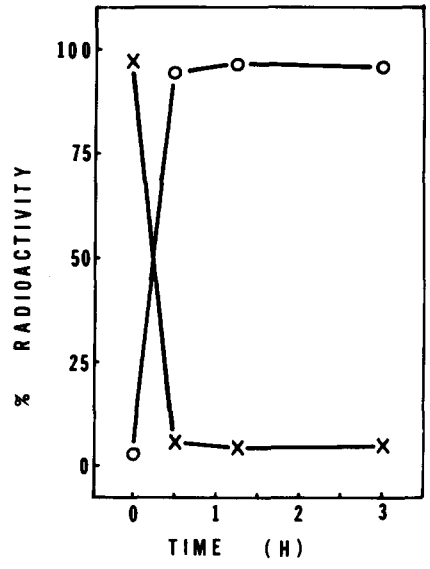


Fig. 1. *Leishmania donovani* PI metabolically prelabeled with [U-^{14}C]inositol was subjected to *Bacillus cereus* phospholipase C digestion. Samples were removed at various times and radioactivity in the organic (×) and the aqueous (○) phases was determined. Hydrolysis was complete within 30 min as indicated by the appearance of radioactivity in the water-soluble polar head group product.

were quantified by GLC on a 10% EGSS-X column as previously described (18).

Amide-linked fatty acids, long chain bases, and water-soluble products from the InSL were released by acid hydrolysis using conc. HCl–MeOH 1:5 (v/v) in sealed ampoules for 48 hr or in MeOH–H_2O-HCl 11:2.6:1 (v/v/v) overnight at 80°C as described by Carter and Gaver (19). Sphingolipid fatty acids were quantified by GLC as above. Alternatively, fatty acid methyl esters (FAME) were identified and quantified by capillary column GLC–mass spectrometry analyses. A Carlo Erba series 4160 Fractovap gas chromatograph fitted with a fused silica column (60 m × 0.32 mm i.d.) coated with SE-54 (SPB-5, Supelco) and a cooled on-column injection system was used to separate the FAME. Material from the GLC system was introduced into a high resolution mass spectrometer (MS) system (Kratos, MS-80, Manchester, England) through a jet separator. Samples were injected into the gas chromatograph at 60°C. Initial oven temperature was maintained for 2 min at 150°C to permit venting of the solvent, after which the valve to the mass spectrometer was opened. The oven temperature was increased by 6°C/min to 280°C. Analyses were started 1 min after the opening of the valve allowing material from the gas chromatograph to enter the MS. Electron impact mass spectra data were continuously collected and processed on a Data General NOVA/4C computer with a DS-55 data system (DS).

Long chain base

The long chain bases (LCB) released by acid hydrolysis of the InSL were converted to their N-trifluoroacetyl derivatives by the addition of trifluoroacetylmethyl ester and adjustment of the pH to 8.4 with triethylamine (20). The reaction was allowed to proceed overnight at room temperature. The solvent was then evaporated under a stream of N_2 and the samples were then placed under a vacuum for 2–3 min to ensure complete removal of triethylamine. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was then added to the N-trifluoroacetyl LCB and the mixture was heated for 30 min at 60°C. Samples were dried under a stream of N_2, then dissolved in chloroform before analyses by GLC–MS–DS.

Samples were injected into the GLC at 60°C. After 1 min the oven temperature was raised to 220°C, then programmed to increase by 2°C/min to 260°C. The GLC–MS data on the LCB derivatives were continuously collected 3 min after sample injection and 1 min after opening of the gas chromatograph to the MS valve.

Fast atom bombardment MS (FABMS) analyses were done on free ceramides released from the InSL by phospholipase C digestion. Sorbitol was applied to the tip of the FAB probe as described previously (21, 22). One to two μl of the solution of ceramides dissolved in chloroform was mixed in the sorbitol matrix. The FAB probe was introduced through the direct inlet system. The FAB gun and power supply were from Phrasor (Phrasor Scientific Inc., Duarte, CA).

Polar head groups

Polar head groups were isolated by subjecting individually isolated inositol lipids, prelabeled with [^{14}C]glucose, [^{32}P]H_3PO_4, or [^{14}C]inositol, to phospholipase C digestion (see above). Water-soluble head groups were subjected to 1-D TLC on either cellulose plates (Eastman 13255, Eastman Chemicals, Rochester, NY) or SG 81 paper (see above) using the solvent system phenol saturated with water–ethanol–acetic acid 50:5:6 (v/v/v) (11). The plates were

TABLE 1. Inositol phospholipid fatty acids of *Leishmania donovani* promastigotes

Fatty acid	PI	Diacyl PI	Alkyl Acyl PI	PI-P	PI-P_2	InSL
			weight % ± SEM			
14:0	4.0 ± 0.5	0.8 ± 0.3	0.7	6.3 ± 1.4	6.3 ± 0.9	0.0 ± 0.0
15:0	trace	0.3 ± 0.1	0.3	3.1 ± 1.3	1.7 ± 0.3	0.0 ± 0.0
16:0	5.3 ± 1.6	7.7 ± 5.2	6.4	12.0 ± 2.5	25.0 ± 5.1	6.1 ± 1.4
16:1	0.5 ± 0.3	0.9 ± 0.6	0.8	1.9 ± 0.2	6.7 ± 2.6	0.0 ± 0.0
17:0	trace	0.2 ± 0.1	0.4	1.1 ± 0.4	1.7 ± 0.7	0.0 ± 0.0
18:0	31.8 ± 0.9	17.1 ± 12.9	41.8	25.1 ± 1.9	23.0 ± 3.2	86.5 ± 4.8
18:1	45.8 ± 1.1	44.1 ± 5.1	35.3	29.7 ± 3.6	24.0 ± 5.1	7.0 ± 3.6
18:2	6.8 ± 0.5	8.6 ± 3.5	3.7	6.0 ± 1.3	3.0 ± 1.0	0.0 ± 0.0
20:2	1.5 ± 0.3	2.6 ± 0.5	1.5	2.1 ± 0.4	1.3 ± 0.3	0.0 ± 0.0
Others[a]	6.3 ± 2.4	17.8 ± 4.2	8.9	12.6 ± 2.3	7.3 ± 0.3	0.0 ± 0.0
No. preparations	4	3[b]	1[c]	9	3	4

[a] Others are the sums of FAME present in <0.5%.
[b] One preparation was a pooled sample.
[c] A single determination was done on a pooled sample.

then exposed to X-ray film and the migrations of the radiolabeled head groups, as visualized on autoradiograms, were compared.

Phosphorus

Phosphoryl and phosphonyl linkages in inositol lipids were determined by *1*) the differential hydrolysis procedures of Ferguson et al. (23) or *2*) ^{31}P nuclear magnetic resonance (NMR). ^{31}P NMR spectra were recorded at 121.5 MHz on a Bruker WM 300 NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) using phosphoric acid as the reference standard.

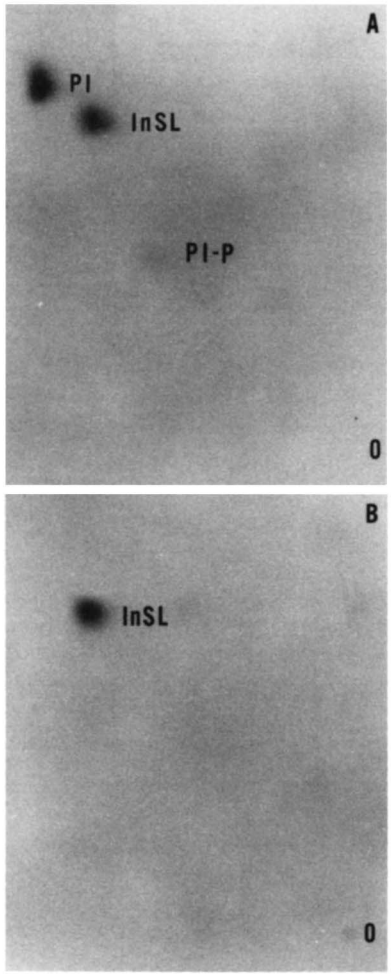


Fig. 2. Autoradiograms of two-dimensional chromatographic analyses of total lipids from *Leishmania donovani* metabolically prelabeled with [U-^{14}C]inositol before (A) and after (B) alkaline hydrolysis. After hydrolysis, only one detectable radioactive component, InSL, remained. Chromatography was done on silica gel-impregnated paper pretreated with EDTA. The solvent system used for separations in the first dimension was $CHCl_3$–MeOH–28–30% NH_4OH–H_2O 25:20:1.6:3.9 (v/v/v/v). Separations in the second dimension employed $CHCl_3$–MeOH–glacial acetic acid–H_2O 60:24:16:6.4 (v/v/v/v).

Cell surface membrane and shed antigens

Cell-free culture media in which *Leishmania* had been grown in the presence of a variety of radiolabeled precursors (see above) were used as a source of "shed" antigens (4). Culture media were concentrated and analyzed by immunoelectrophoresis (IEP) as previously described (4). The immunoglobulin G (IgG) fraction from rabbit polyvalent antisera used in these analyses was directed against promastigote cell surface preparations (PCSP) (4). Antigens were extracted from radiolabeled promastigotes by first extracting lipids with $CHCl_3$–MeOH 2:1 (v/v) and then with acetone. The antigens were then extracted by the "water saturated with butanol" method described for the isolation of cell surface membrane lipophosphonoglycans from *Acanthamoeba* by Korn, Dearborn, and Wright (24). Immunoelectrophoresis gels were stained with Coomassie brilliant blue (4) and subjected to autoradiography.

RESULTS

Inositol lipid classes

Inositol lipids from promastigotes were quantified by ^{32}P incorporated into phospholipids under isotope equilibration conditions by growth of the cells for 4 days with the radioisotope. Radioactivity in these lipids relative to that in total phospholipids was compared. The radioactivity in PI-P was divided by two and the radioactivity in PI-P_2 was divided by three. Total inositol lipids comprised 15% of the cellular phospholipids.

Cells grown with [^{14}C]inositol retained 4.3% of the incorporated radiolabel after the lipids were extracted. When the extracted lipids were purified by solvent partitioning, 89.4% of the radiolabel incorporated into cells was found in the lower organic phase and 6.2% in the upper phase. Of the inositol lipids, five classes were identified and quantified by radioactivity incorporated from [^{14}C]inositol into *Leishmania* lipids, which were subsequently purified by TEAE column chromatography and TLC. The inositol lipid composition of *L. donovani*, 1S, was PI, 45.1%; InSL, 37.9%; PI-P, 13.0%; PI-P_2, 2.2%; and lyso PI, 2.6% (n = 3).

Phosphatidylinositol contained both diacyl (90%) and alkyl acyl (10%) molecular species. There was no evidence for the presence of plasmalogens (alkenyl glycerols) in PI as judged by the TLC or GLC methods used in this study.

Inositol lipid head groups

Water-soluble polar head groups were released by phospholipase C digestion of *1*) total cellular lipids and

isolated InSL and PI metabolically labeled with [^{14}C]inositol, and 2) isolated InSL and PI metabolically labeled with [^{32}P]H_3PO_4. Analyses by cellulose TLC and autoradiography showed that all head groups had R_f values of 0.23–0.27. Only one radioactive band was detected in the sample of [^{14}C]inositol-labeled total lipids. Thus all inositol lipids had similar polar head groups.

When the defined medium for *Leishmania* was formulated (5), it was not determined whether or not inositol was an absolute growth requirement of the organism. In the earlier nutritional studies, glucose was found not to be an absolute requirement (5). In the present studies, inositol was not shown to be required for growth of *Leishmania donovani*, 1S, cultures. After six subcultures in RE III-BSA that lacked inositol, there was no decrease in the density of cultures. Unexpectedly, glucose was found to be an absolute requirement for growth of this strain of *L. donovani*. By two or three subcultures, after 5 days, all cells were dead in the absence of added glucose, or in the absence of both glucose and inositol. Addition of 10× or 100× inositol, in the absence of added glucose, did not prevent cultures from dying by day 5 of the third subculture.

[U-^{14}C]Glucose was added to promastigote cultures and PI and InSL were isolated from the cells. These inositol lipids were hydrolyzed and their water-soluble head groups were found to be radioactive as determined by TLC and autoradiography. Therefore, glucose can serve as a precursor for synthesis of inositol lipid head groups. These results indicate that this strain of *Leishmania* apparently has changed since the earlier nutritional studies were conducted (5); it now has a stringent glucose requirement for growth.

Analyses of P bonds established that *Leishmania* PI and InSL were in phosphoryl linkages. Differential hydrolysis followed by P_i assays indicated that 93% of PI and 113% of InSL were phospholipids (means of three or four replicate samples). That InSL contained only phosphoryl P was further confirmed by ^{31}P-NMR. A single peak corresponding to that of the phosphoric acid reference standard was present in the spectrum. No peak was detected at ca. −23 ppm, the chemical shift characteristic of phosphonyl bonds.

Fatty acyl groups

The fatty acyl groups of *Leishmania* inositol glycerolipids were not like those commonly found in most mammalian cells, i.e., 18:0 at C-1 and 20:4 at C-2. In contrast, the *Leishmania* inositol lipids contained only trace amounts of arachidonate although stearate was found in high concentrations (**Table 1**). Although the major fatty acids in PI, PI-P, and PI-P_2 were the same, there were relative increases in C_{16} acids and relative decreases in C_{18} acids with increases in phosphate groups of these lipids.

The fatty acyl groups of diacyl PI and alkyl acyl PI were analyzed after digestion with phospholipase C (Fig. 1) and separation by TLC. Their compositions were not dramatically different from those observed in the mixture when total PI fatty acids were analyzed (Table 1).

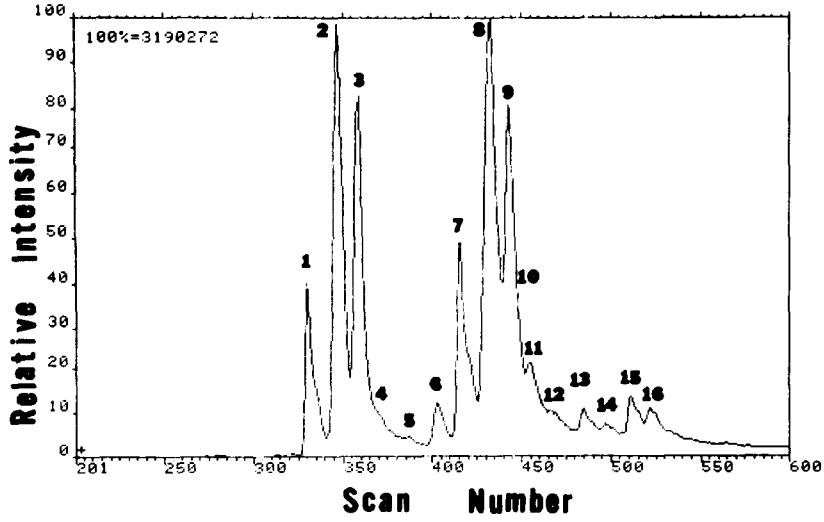


Fig. 3. Tracing of the total ion chromatograph of the LCB derivatives of the InSL from *Leishmania* promastigotes separated by capillary column GLC. Peaks 1 and 2 are C_{16} sphingosines, peak 3 is C_{16} dihydrosphingosine, peaks 4 and 5 are C_{17} sphingosines, peaks 7 and 8 are C_{18} sphingosines, peak 9 is C_{18} dihydrosphingosine, peaks 10 and 11 are C_{19} sphingosines, peak 12 is C_{19} dihydrosphingosine, peaks 14 and 15 are C_{20} sphingosines, and peak 16 is C_{20} dihydrosphingosine. Peaks 6 and 13 were not identified in this study.

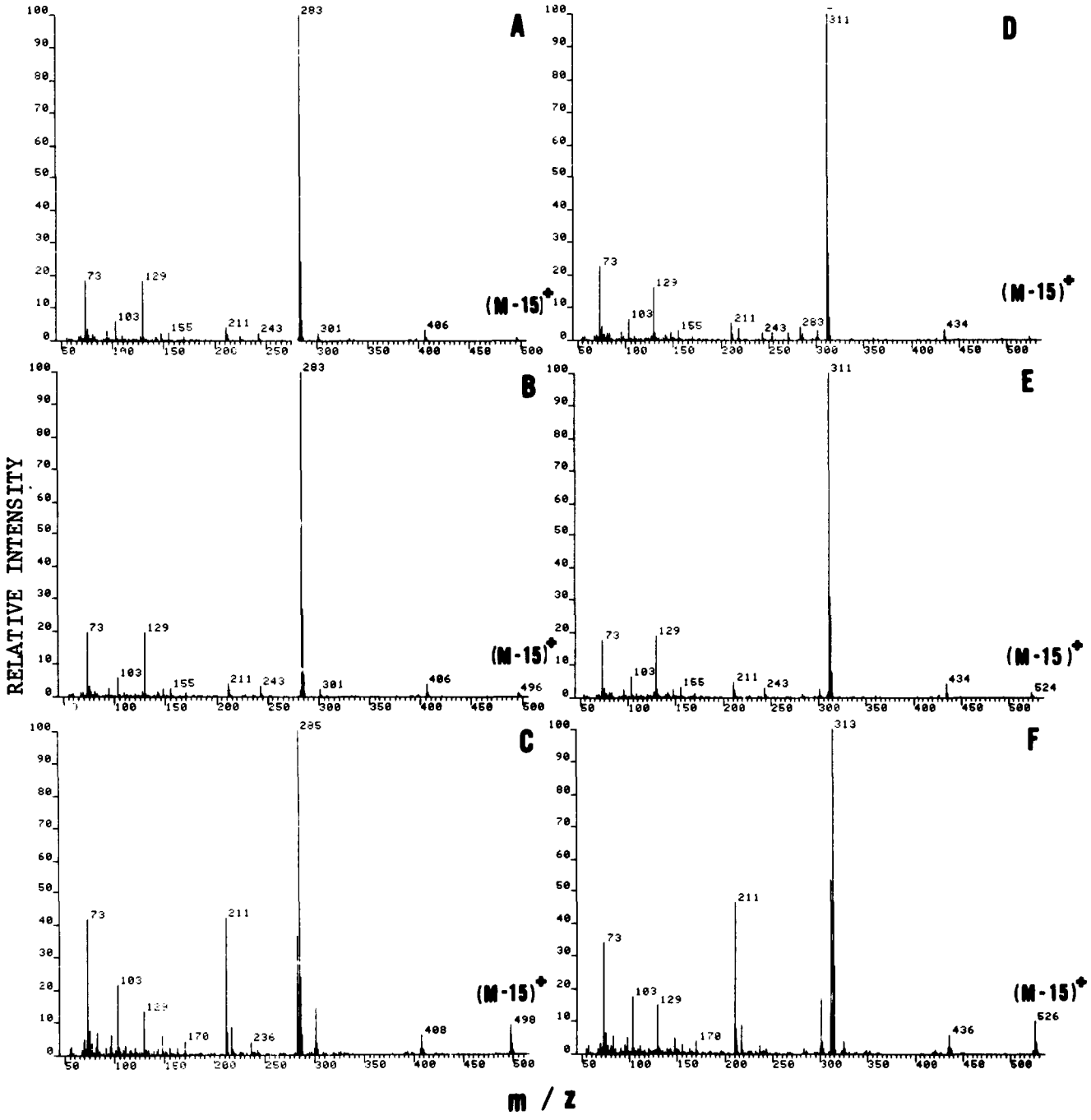


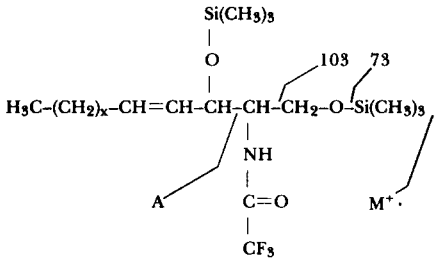
Fig. 4. Mass spectra of the six major LCB in *Leishmania donovani* InSL. A and B are spectra of C_{16} sphingosines (peaks 1 and 2 in Fig. 3); C is C_{16} dihydrosphingosine (peak 3 in Fig. 3); D and E are C_{18} sphingosines (peaks 7 and 8 in Fig. 3); F is C_{18} dihydrosphingosine (peak 9 in Fig. 3). Peaks indicated by $(M-15)^+$ represent the loss of a methyl group from the molecular ions which is commonly observed in silylated derivatives.

The inositol phosphosphingolipid

The InSL was identified as a sphingolipid by its resistance to alkaline hydrolysis. **Fig. 2** shows autoradiograms of total cellular lipids extracted from cells grown with [^{14}C]inositol and separated by TLC, before (A) and after (B) alkaline hydrolysis. After hydrolysis, only the InSL was left intact and it migrated to the same distance in 2-D chromatograms. The InSL was negative for sugars as tested by staining of TLC plates with α-naphthol, anthrone, or diphenylamine. [^{14}C]Deoxyglucose was not incorporated in detectable amounts into InSL.

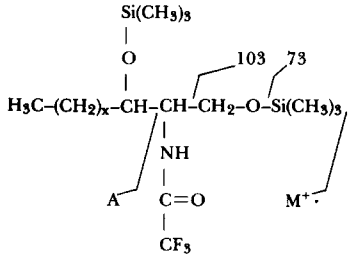
TABLE 2. Mass spectral analyses of the major long chain bases of the inositol sphingophospholipids of *Leishmania donovani* promastigotes

N-trifluoroacetyl, O-silyl sphingosines



	m/z	Relative Intensity	Elemental Composition
x = 12			
$(M^+ \cdot - 15)$	524	1.2	$C_{25}H_{49}NO_3Si_2F_3$
$(M^+ \cdot - 105)$	434	3.4	$C_{22}H_{39}NO_2SiF_3$
A	311	100.0	$C_{19}H_{39}OSi$
	301	2.9	$C_{10}H_{22}NO_2SiF_3$
	211	5.9	$C_7H_{12}NOSiF_3$
	129	22.6	$C_6H_{13}OSi$
	103	16.0	$C_4H_{11}OSi$
	73	28.6	C_3H_9Si
x = 10			
$(M^+ \cdot - 15)$	496	1.3	$C_{23}H_{45}NO_3Si_2F_3$
$(M^+ \cdot - 105)$	406	3.8	$C_{20}H_{35}NO_2SiF_3$
A	283	100.0	$C_{17}H_{35}OSi$
	301	2.1	$C_{10}H_{22}NO_2SiF_3$
	211	4.6	$C_7H_{12}NOSiF_3$
	129	20.7	$C_6H_{13}OSi$
	103	7.7	$C_4H_{11}OSi$
	73	24.0	C_3H_9Si

N-trifluoroacetyl, O-silyl dihydrosphingosines



	m/z	Relative Intensity	Elemental Composition
x = 14			
$(M^+ \cdot - 15)$	526	11.5	$C_{25}H_{51}NO_3Si_2F_3$
$(M^+ \cdot - 105)$	436	6.2	$C_{22}H_{41}NO_2SiF_3$
A	313	100.0	$C_{19}H_{41}OSi$
	301	18.5	$C_{10}H_{22}NO_2Si_2F_3$
	211	54.4	$C_7H_{12}NOSiF_3$
	129	20.8	$C_6H_{13}OSi$
	103	23.4	$C_4H_{11}OSi$
	73	44.1	C_3H_9Si
x = 12			
$(M^+ \cdot - 15)$	498	9.2	$C_{23}H_{47}NO_3Si_2F_3$
$(M^+ \cdot = 105)$	408	6.3	$C_{20}H_{37}NOSiF_3$
A	285	100.0	$C_{17}H_{34}OSi$
	301	14.4	$C_{10}H_{22}NO_2SiF_3$
	211	46.9	$C_7H_{12}NOSiF_3$
	129	13.8	$C_6H_{13}OSi$
	103	18.7	$C_4H_{11}OSi$
	73	36.9	C_3H_9Si

Stearate was the predominant amide-linked fatty acid of InSL (Table 1). Palmitate and oleate were the only other fatty acids that were detected in the InSL.

Gas–liquid chromatography–high resolution MS of the InSL LCB indicated that several species were present: isomers of C_{16} sphingosines (33.5%) and C_{18} sphingosines (34.8%), C_{20} sphingosine (2.8%), C_{16} dihydrosphingosine (13.7%), C_{18} dihydrosphingosine (12.5%), and other minor LCB. The sum of minor components present in less than 0.5% was 2.7% (n = 2, **Fig. 3**). All LCB contained straight chains. Mass spectra of the six major peaks are shown in **Fig. 4.** Elemental analyses obtained from high resolution data on the major ions in the spectra are shown in **Table 2.** The LCB were identified by the elemental analyses and their fragmentation patterns.

The ceramides released from phospholipase C digestion of the InSL were analyzed as a mixture by FABMS. The ions obtained were identified as C_{16}, C_{18}, and C_{19} sphingosine-containing ceramides and C_{16} and C_{18} dihydrosphingosine-containing ceramides. The molecular species observed in all cases were $(M+1 - H_2O)^+$ species. In FAB analyses, there is a loss of one molecule of water from the M+1 ion.

Cell surface membrane and shed antigens

Promastigotes were grown with either $[^{32}P]H_3PO_4$, [^{14}C]inositol, or [^{14}C]stearate. Autoradiograms of IEP gel analyses of cell surface antigens shed into the culture medium by the organisms, or extracted from promastigotes (4), showed that radiolabel from these compounds was incorporated into the slower migrating component of the major antigen (**Fig. 5**). This was detected by precipitin lines formed in these gels with polyvalent rabbit anti-PCSP IgG. The presence of radioactivity in precipitin lines was confirmed by comparison of autoradiograms and Coomassie blue-stained gels. However, the faster-migrating antigenic material that had the same antigenic determinants as the slower-migrating component (as indicated by contiguous precipitin lines) did not contain detectable radioactivity from [^{14}C]inositol or [^{14}C]stearate. Information presented in this and previous reports (4, 25) suggests that this cell surface membrane antigen is a lipophosphoglycan. The faster, anodic-migrating component may represent a breakdown product of the slower-migrating component since it is present in relatively greater amounts in the culture medium as compared to the antigen isolated from cells (Fig. 5).

DISCUSSION

This is the first report of a detailed analysis of the inositol lipids of a species of *Leishmania.* Surveys of lipids of *Leishmania* have been done by earlier workers (1–3, 26–

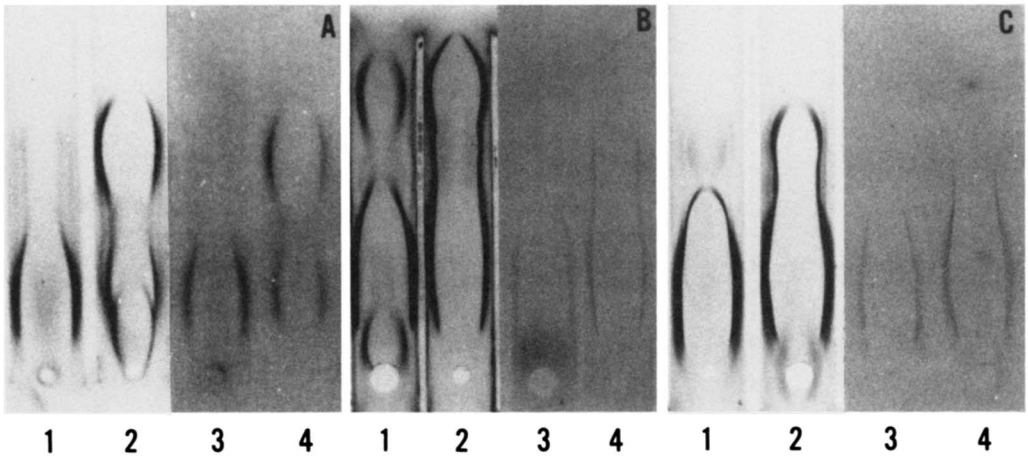


Fig. 5. Immunoelectrophoretic analyses of *Leishmania donovani* antigens isolated from cells (lanes 1) and in culture media (lanes 2). Antigens were metabolically prelabeled with [^{32}P]H_3PO_4 (A), [U-^{14}C]inositol (B), or [1-^{14}C]stearic acid (C). The sample origin wells for electrophoresis are at the bottom of the figure. Immunoprecipitation was accomplished by filling side troughs with rabbit polyvalent anti-promastigote cell surface preparations of IgG. Gels were stained with Coomassie brilliant blue (lanes 1 and 2) then the radioactivity present in the immunoprecipitin lines was detected by autoradiography (lanes 3 and 4). The slower-migrating component had radioactivity when cells were grown with either of the three radioisotopes. The faster-migrating component contained ^{32}P (A) but did not contain detectable radioactivity when cells were grown with either [^{14}C]inositol or [^{14}C]stearic acid.

30). Thorough characterizations of PI in a variety of *Leishmania* species were done by Beach, Holz, and Anekwe (2), but PI-P, PI-P_2, and InSL were not studied. The fatty acid composition of *L. donovani* PI determined in this study agreed with that of the earlier report (2). Phosphatidylinositol was identified in the kinetoplastid flagellate, *Trypanosoma cruzi,* the causative agent of Chagas' disease (31). Polyphosphoinositides and PI were identified in another kinetoplastid flagellate, *Crithidia* (32–35), a parasite of insects. In those studies, InSL was not detected in the lipids of those protozoans. Inositol sphingolipids are abundant in some yeast cells where they are usually present as glycosphingolipids (10, 12). In the present study, InSL of *Leishmania* was found not to be glycosylated, however, preliminary observations of [^{14}C]inositol-labeled lipids suggest that there are minor lipids present that may be similar to the mannose-containing inositol sphingolipids from yeast (10, 12). Radioactive spots, as well as diphenylamine-positive material on two-dimensional chromatograms with low R_f values like those of inositol glycosphingolipids with a hydroxy fatty acid and phytosphingosine, were detected in the present study but they were not further characterized because they represented only a small percentage of the total radioactive material present.

Earlier studies have shown that *Leishmania* lipids, including PI, contain alkyl acyl as well as alkenyl acyl lipids (1–3, 26–30). Also, metabolic studies on the relative rates of incorporation of radiolabeled precursors such as acetate, octadecanol, and 1-O-octadecyl-*sn*-glycerol into the alkenyl, alkyl, and acyl chains of PI and other glycerolipids have been reported (27–30); however, definitive chemical structures of the alkyl chains present were not established. Beach et al. (2) have identified 1-hexadec-9-enyl and 1-octadec-9-enyl as the major glyceryl ethers in the phosphatidylethanolamines of eight *Leishmania* species. Alkyl acyl PI was only partially characterized in this study; glyceryl ethers were not identified.

The previous finding of inositol, ceramides, fatty acids, and phosphorus in major cell membrane components of two protozoans may be relevant to the identification of InSL in the lipids of *Leishmania.* A lipopeptidophosphoglycan (LPPG) with a glycophosphoceramide structure was isolated from *T. cruzi* (36–38). The plasma membrane localization of LPPG was also identified by studies on isolated cell membranes from this kinetoplastid flagellate (39). A lipophosphonoglycan (LPG) from the soil amoeba, *Acanthamoeba castellanii* was also established as forming an integral part of the cell surface membrane (23, 40–44). Unlike the LPPG from *T. cruzi,* the LPG from *Acanthamoeba* did not contain a peptide moiety and the P was in phosphonyl rather than phosphoryl linkages (24). Our observations on a major cell surface antigen of *Leishmania* suggest that it contains sugars, P, fatty acids, and inositol. After the chemical compositions and structures of the *Leishmania* lipophosphoglycan and the other cell surface components of the other species that contain inositol lipid moieties have been elucidated, comparisons should reveal the similarities and differences. Such comparisons would provide information on the topological relationship of these molecules with the surface membrane of the cells.

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