Vol. 157, No. 3, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 30, 1988 Pages 1239-1246

DI-O-ALKYLGLYCEROL, MONO-O-ALKYLGLYCEROL AND CERAMIDE INOSITOL PHOSPHATES OF LEISHMANIA MEXICANA MEXICANA PROMASTIGOTES

B.N. Singh, C.E. Costello*, D.H. Beach and G.G. Holz, Jr.

Department of Microbiology & Immunology, SUNY HSC, Syracuse, NY
*Department of Chemistry, Mass Spectrometry Facility, MIT, Cambridge, MA

Received October 31, 1988

Three acidic unsaponifiable lipid fractions were isolated by chromatographic methods from sandfly vector stages (promastigotes) of a protozoan parasite of man, Leishmania mexicana mexicana, cultured in vitro. Fast atom bombardment mass spectrometry, fast atom bombardment collision induced tandem mass spectrometry and metabolic labeling were used to characterize these lipids as di-O-alkylphosphatidyl-inositols, lyso-1-O-alkylphosphatidylinositols and inositol phosphosphingolipids. Molecular species of the dialkyl forms, new to natural product biochemistry, had a 20:0 substituent and either 17:1 or 18:1. The monoalkyl forms had either 17:0 or 18:0. The predominant ceramide had the 16:1 base and the lesser component the 16:0 base. In both, the N-acyl group was 18:0.

The leishmanial parasites of man have a motile life-cycle stage, the promastigote in the gut of their sandfly vectors and a non-motile stage, the amastigote in the macrophages of their animal reservoirs and man. Leishmaniasis is responsible for morbidity and mortality in many temperate and tropical regions worldwide. Chemotherapy is unsatisfactory and immunoprophylaxis and immunotherapy are at the research and development stage; consequently, a search continues for new modes of chemotherapy. One target is the inositol phosphate-containing membrane anchors [1,2] of glycoconjugates important to the host-parasite relationships of leishmanias. Diacylphosphatidylinositol anchors promastigote glycoprotein proteases [3] and lyso-1-O-alkylphosphatidylinositol anchors a lipophosphoglycan [4] that may protect the amastigote from the macrophage oxidative burst [5].

In an effort to identify potential membrane anchors for other leishmanial glycoconjugates, or intermediates in the metabolism of membrane anchors, we have

0006-291X/88 $1.50

isolated and characterized inositol phosphate-containing, unsaponifiable lipids of cultured promastigotes of a strain of Leishmania mexicana mexicana isolated from a human skin lesion.

MATERIALS AND METHODS

Culture, metabolic labeling and lipid extractions of promastigotes. Promastigotes of L. mexicana mexicana strain MHOM/BZ/79/Woods were grown to the late logarithmic phase at 25°C in 2600-ml low-form Erlenmeyer flasks; in 1-liter batches of filter-sterilized (Millipore 0.22 μm) RE III medium [6] (chemically-defined) supplemented with additional hemin (5 mg/liter). Cell populations at inoculation and harvest were enumerated with a Coulter Counter (Model Z_F, Coulter Electronics). In some experiments exponentially-propagating promastigotes (4-8 x 10^{10} in 100 ml culture medium) were metabolically labeled for 20 hr. with 50-300 μCi myo-[2-^{3}H]inositol (19 Ci/mmol), [9,10-^{3}H]palmitic acid (40 Ci/mmol) (Amersham) or $H_3{}^{32}PO_4$ (carrier-free) (New England Nuclear). Harvest in all experiments was by centrifugation and cells were washed twice with phosphate-buffered saline.

Promastigotes were extracted with 20 vol. of chloroform/methanol/H_2O (30:60:8, proportions by volume), chloroform/methanol (1:1) and chloroform/methanol (2:1), twice with each solvent, and the extracts were pooled and the solvents removed by flash evaporation. Then, the extracted material was taken up in a small volume of chloroform/methanol (2:1) and centrifuged at 10,000 g for 15 min, and the clarified extract was collected and saponified.

Separation, collection and purification of inositol phosphate-containing, unsaponifiable lipids. Extracted lipid was mixed with 0.35 M KOH in methanol by sonication. The mixture was incubated for 3 hr. at 40°C, neutralized with 10% acetic acid, then placed on a Sephadex A-25 (Pharmacia) column and the unsaponifiable lipids eluted with chloroform/methanol/H_2O (120:60:9). Next, the lipid eluate was reduced in volume by flash evaporation, and separated into a neutral fraction and a charged (acidic fraction) by DEAE-Sephadex A-25 (Pharmacia) column chromatography [7]. The charged lipids, eluted with chloroform/methanol/0.8 M sodium acetate (30:60:8) were taken to dryness by flash evaporation and incubated with 0.1 N NaOH in methanol at 40°C for 2 hr. [8]. Then, the mixture was dried in the same manner, redissolved in distilled H_2O/0.4 M EDTA (tetrasodium salt) (9:1) and dialyzed against changes of distilled H_2O for 60 hr. at 4°C in Spectrapor tubing (molecular weight cut-off 2000; Spectrum Medical Ind.). The resulting undialyzables were lyophilized, dissolved in chloroform/methanol (85:15) and applied to an Iatrobead (6RS-8060; Iatron Lab.) silicic acid column [9]. Inositol phosphate-containing, unsaponifiable lipids were eluted with chloroform/methanol (1:2), as judged by monitoring of the column eluate by TLC and TLC-autoradiography on pre-coated Silica Gel 60 plates (200 μm thick, E. Merck). Solvent systems were: chloroform/methanol/H_2O (60:35:8) and chloroform/methanol/28% NH_4OH/H_2O (50:40:3:7). Phosphorus-containing components were recognized with the Dittmer-Lester spray reagent [10]; and by ^{32}P autoradiography with Kodak X-Omat AR film directly exposed to TLC plates. Tritiated components were detected by fluorography at -75°C, with the film exposed to plates presprayed with EN^{3}HANCE (New England Nuclear).

The inositol phosphate-containing lipids were subfractionated, purified and collected by HPLC, with a Waters system (Millipore) equipped with a 4.6 mm x 150 mm Iatrobead column (6RS-8010). The column was equilibrated and eluted isocratically with chloroform/methanol/H_2O (75:24:1). Flow rate was 1 ml/min and fractions were collected at 1 min intervals for 25 min. Their composition and radioactivity were monitored by TLC, and by liquid scintillation spectrometry in Aqueous Counting Scintillant (Amersham). Fractions with common properties were pooled and yielded

three major inositol phosphate-containing lipids whose proportions were determined by TLC-densitometry.

Fast Atom Bombardment Mass Spectrometry. Samples for MS analysis were dissolved in chloroform/methanol (3:1) and this solution was mixed with an equal amount of the FAB matrix, which was glycerol for positive ion studies and triethanolamine for negative ion studies. About 0.3 μl of the mixture was used for each analysis. FABMS was carried out using the first (MS-1) of the two mass spectrometers of a tandem high resolution mass spectrometer (JEOL HX110/HX110) at +/- 10 kV accelerating voltage and 1:1000 resolution, with 100 Hz filtering with -/+ 18 kV postacceleration at the detector. For calibration, $(CsI)_nCs^+$ and $(CsI)_n^-$ cluster ions were used for the positive and negative ions modes, respectively. Single scans were acquired by scanning the magnet from m/z 100 to m/z 1500 in about 2.0 min. The JEOL FAB gun was operated at 6 kV with xenon as the FAB gas. Spectra were recorded with a JEOL DA5000 data system.

Tandem Mass Spectrometry. FAB CID MS/MS was carried out by using all four sectors of the JEOL HX110/HX110 spectrometer, an instrument of E_1B_1-E_2B_2 configuration. Collision-induced fragmentation (CID) took place in the third field-free region, thus operating both MS-1 (E_1B_1) and MS-2 (E_2B_2) as double-focusing instruments. Helium was used as the collision gas at a pressure sufficient to reduce the precursor ion signal by 80%. The FAB MS/MS spectra (linked scans of MS-2 at constant B/E ratio) were recorded with 30-Hz filtering, at the same scan rate as used for FABMS. MS-2 was operated at 1:1000 resolution, also with -/+ 18 kV postacceleration at the detector. MS-2 was calibrated with a mixture of CsI, NaI, KI and LiCl in the positive ion mode and a solution of CsI in glycerol in the negative ion mode [11]. FAB MS/MS spectra shown are profile data of two or three accumulated scans.

RESULTS

In an experiment representative of five others of like design, 10 l of L. mexicana mexicana culture produced 29 x 10^{10} promastigotes, which upon extraction yielded 0.48 g of chloroform/methanol-soluble lipids. Saponification and Sephadex A25 and DEAE Sephadex A25 column chromatography of the saponificate generated an acidic unsaponifiable fraction that, after a second saponification, and purification by dialysis and Iatrobead column chromatography, was subfractionated by HPLC (Iatrobead) into three component fractions; designated LM-1 (22% of total), LM-2 (56%), LM-3 (22%). Silica gel G TLC of those lipid fractions with the solvent system chloroform/methanol/H_2O (60:35:8) showed them to have the R_f values LM-1 = 0.34, LM-2 = 0.41, LM-3 = 0.5. TLC autoradiography of the fractions, from promastigotes labeled with myo-[2-^{3}H]inositol, [9,10-^{3}H]palmitic acid or $H_3{}^{32}PO_4$, revealed that each of them was labeled by the three lipid precursors.

Each of the fractions was found to be a mixture of closely-related compounds (Table I) whose molecular weights were determined on the basis of positive and

Table I

FABMS molecular weight determinations of Leishmania lipids

Fraction %	M_r	(+)FABMS	%	(-)FABMS	%
LM-1	572.3	573.3(M+H)	9	571.3(M-H)	19
		595.3(M+Na)[a]	27		
	586.4	587.4(M+H)	19	585.4(M-H)	81
		609.3(M+Na)[b]	45		
LM-2	779.5	780.5(M+H)[c]	31	778.5(M-H)	77
		802.5(M+Na)	35		
	781.5	782.5(M+H)	16	780.5(M-H)	23
		804.5(M+Na)	18		
LM-3	850.6	851.6(M+H)	8	849.6(M-H)	45
		873.6(M+Na)	31		
	864.7	865.7(M+H)	6	863.7(M-H)	55
		887.7(M+Na)	55		

[a]Obs. m/z 595.3233 (calc., $C_{26}H_{53}O_{11}PNa$, m/z 595.3223).
[b]Obs. m/z 609.3372 (calc., $C_{27}H_{55}O_{11}PNa$, m/z 609.3380).
[c]Obs. m/z 780.5407 (calc., $C_{40}H_{79}O_{11}P$, m/z 780.5391).

negative ion FAB mass spectra. The detailed structures of the lipids were determined from the CID FAB mass spectra measured for the $(M+H)^+$, $(M+Na)^-$ ions and for major fragment ions. The hexose was assumed to be inositol.

The base peak of the CID spectrum of LM-1 $(M+H)^+$, m/z 587.4, was m/z 261 (inositol phosphate + H), water-loss, m/z 243 (13%). Loss of inositol from the $(M+H)^+$ precursor led to the product ion at m/z 425 (30%), and by further loss of phosphate, to the ion at m/z 327 (18%). Product ions observed at m/z 335 (15%), m/z 173 (12%) and m/z 99 (16%) correspond to protonated glyceryl inositol phosphate, glyceryl phosphate and phosphoric acid, respectively. The product ion at m/z 303 (14%) corresponds to cleavage of the glycerol C(1)-C(2) bond with loss of the R group, indicating that the substituent is on C(1) rather than C(2), if this loss occurs as a single step. In the CID spectrum of $(M+H)^+$, m/z 573.3, the product ions at m/z 425 and 327 were shifted to m/z 411 and 313, respectively, but all others remained the same, indicating that the difference was in the chain length of the single R group. The assignment of the R groups as C18:0 (M_r 586) or C17:0 (M_r 572) alkyl (rather than

esters) was made on the basis of the observed resistance to alkaline hydrolysis and the elemental compositions determined for the sodiated molecular ions (Table I). The base peak, m/z 259, of the CID spectrum of LM-1 $(M-H)^-$, m/z 585.4, also indicated the presence of inositol phosphate, and was accompanied by a water-loss peak, m/z 241 (47%). Ohashi has reported observation of the inositol phosphate species at m/z 259 in the negative-ion FAB mass spectrum of 1(2)-palmitoyl-2(1)-oleoyl phosphatidylinositol [12]. Cleavage on either side of the glycosidic oxygen led to product ions at m/z 423 (66%) and 405 (73%). The R group was lost as the alkane, to produce the ion at m/z 331 (16%). Cleavage of the C(1)-C(2) bond and hydrogen loss led to the product ion at m/z 299 (24%).

The CID spectrum of the $(M-H)^-$ species of LM-3, m/z 849.6 and m/z 863.6, both showed consecutive losses of 282 u (C20 alkane) and 162 u (inositol) to produce ions at m/z 567 (95%) and 405 (100%) from the lower homolog, and m/z 581 (95%) and 419 (34%) from the higher homolog. Loss of 162 u directly from the molecular ions was also observed, at m/z 687 (34%) and 701 (66%), respectively. Product ions in each spectrum at m/z 259 and 241 could be attributed to inositol phosphate, but m/z 259 had only low abundance. Cleavage of the C(1)-C(2) bond and loss of the C(2) alkyl group led to the product ion at m/z 299 from either precursor. These assignments indicate that the second alkyl chain must be C17:1 for the lower homolog and C18:1 for the higher homolog. The C20 alkyl is tentatively assigned to the C(1) position and the unsaturated C17:1 or C18:1 to the C(2) position, but more data on authentic compounds is necessary for definite positional assignment of these ether-linked groups.

The molecular weights and elemental compositions of LM-2 indicate that the compounds contain nitrogen and phosphorus (Table I). The exact masses of the abundant fragments at m/z 520 and 522 were determined to be m/z 520.5092 (calc. for $C_{34}H_{66}NO_2$, m/z 520.5094) and m/z 522.5256 (calc. for $C_{34}H_{68}NO_2$, m/z 522.5250). The CID spectra of LM-2 were distinctly different from those of LM-1 and LM-3, but were analogous to those of glycosphingolipids [13]. The CID spectrum of the $(M+H)^+$,

m/z 780.6 showed facile loss of inositol phosphate (260 u) to produce the base peak, m/z 520. Losses of water to give m/z 762 (60%) and of inositol to yield m/z 600 (13%) also occurred. Observation of product ions at m/z 236, 254 and 284 in the CID spectra of m/z 780 and m/z 520 indicates that the base is C16:1 and the N-acyl group is C18:0. Similarly, product ions at m/z 238, 256 and 284 in the CID spectra of m/z 782 and 522 indicate that the base is C16:0 and the N-acyl group is C18:0.

DISCUSSION

Leishmanias have been reported to form a variety of phosphoinositol-containing lipid types including phosphatidylinositol, phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate, inositol phosphosphingolipid, 1-O-alkyl-2-acyl-phosphatidylinositol and lyso-1-O-alkylphosphatidylinositol-phosphoglycan (lipophosphoglycan) [4,14-16]. The diacyl forms of phosphatidylinositol are assumed to be involved in the early stages of cell surface signal transduction [17]. The inositol sphingophospholipids, found heretofore only in fungi and higher plants [18,19], were reported to be components of an antigen shed by L. donovani [16]. In the present FAB MS study the molecular species of inositol phosphosphingolipids found in L. mexicana mexicana were among those inferred by the results of the degradative analysis of the inositol phosphosphingolipids found in L. donovani [16].

The lyso-1-O-alkylphosphatidylinositols detected in L. mexicana mexicana had a 17:0 or 18:0 alkyl substituent and it is probable that they represent 1-O-alkyl-2-acylphosphatidylinositols deacylated by saponification in the course of isolation. Certainly, they are unlike the lyso-1-O-alkylphosphatidylinositol anchors of the lipophosphoglycans of L. donovani and L. major whose major alkyl groups were 24:0 and 26:0 [4,20]. The 1-O-alkyl-2-acylphosphatidylinositols recognized earlier [14-16] had not been completely characterized as to the nature of their alkyl and acyl moieties; however, the major fatty acyl groups of the 1-O-alkyl-2-acylphosphatidylinositols of L. donovani were 18:0 and 18:1 (total 77%) [16]. 1-O-alkyl-2-acylphosphatidylinositols appear to anchor a variety of proteins; e.g., bovine and human erythrocyte acetylcholinesterases

[21] and decay accelerating factor of human erythrocytes [22]. Also included in this category may be one or more proteins of Trypanosoma cruzi trypomastigotes [23]. Finally, the insulin-sensitive glycophospholipid of H35 hepatoma cells contains a 1-O-alkyl-2-acylglyceryl moiety [24].

The di-O-alkylphosphatidylinositols of L. mexicana mexicana promastigotes are the first dialkylglyceryl lipids reported from eucaryotic microbes; however, they have also been found in T. cruzi epimastigotes (Singh, Costello, Beach, and Holz; unpublished observation). Dialkylphosphatidylcholines have been described from bovine heart muscle [25], and dialkylglycerols from the anaerobic thermophilic bacterium, Thermodesulfotobacterium commune [26].

The knowledge that leishmanias form dialkyl, monoalkylacyl and lysomonoalkylphosphatidylinositols raises the possibility of a chemotherapy of leishmaniasis based upon impairment of functions performed by alkylether lipids. For example, a synthetic diether analog of 2-lysophosphatidylcholine with tumoristatic activity, 1-O-octadecyl-2-O-methylglycero-3-phosphocholine, strongly inhibits the growth of L. donovani promastigotes [27].

ACKNOWLEDGEMENTS

The authors are pleased to acknowledge the assistance of Dr. Roger A. Laine in the FABMS analysis of inositol phosphosphingolipids; also the technical participation of Ms. B. Seaton. This work was supported in part by U.S. Public Health Service Grants AI-05802 from the National Institute of Allergy and Infectious Diseases (to G.G.H.) and RR00317 from the Division of Research Resources (to K. Biemann).

REFERENCES

1. Low, M.G., and Saltiel, A.R. (1988) Science 239, 268-275.
2. Ferguson, M.A.J., and Williams, A.F. (1988) Ann. Rev. Biochem. 57, 285-320.
3. Bordier, C., Etges, R., Bouvier, J., Ward, J., Turner, M.J., and Cordosa de Almeida, M.L. (1987) In: Host-Parasite Cellular and Molecular Interactions in Protozoal Infections (K.-P. Chang and D. Snary, Eds.), NATO ASI Series, Vol. H11, pp. 157-163. Springer-Verlag, Berlin Heidelberg.
4. Orlandi, P., Jr., and Turco, S.J. (1987) J. Biol. Chem. 262, 10384-10391.

5. McNeely, T.B., and Turco, S.J. (1987) Biochem. Biophys. Res. Commun. 148, 653-657.
6. Steiger, R.F., and Steiger, E. (1977) J. Protozool. 24, 437-441.
7. Kundu, S.K. (1981) Methods Enzymol. 72, 174-185.
8. Ledeen, R.W., and Yu, R.K. (1982) Methods Enzymol. 83, 139-191.
9. Byrne, M.C., Agler, S.M., Aquino, D.A., Sclafani, J.R., and Ledeen R.W. (1985) Anal. Biochem. 148, 163-173.
10. Dittmer, J.C., and Lester, R.L. (1964) J. Lipid Res. 5, 126-127.
11. Sato, K., Asada, T., Kunihiro, F., Kammei, Y., Kubota, E., Costello, C.E., Martin, S.A., Scoble, H.A., and Biemann, K. (1987) Anal. Chem. 59, 1652-1659.
12. Ohashi, Y. (1984) Biomed. Mass Spectrom. 11, 383-385.
13. Domon, B., and Costello, C.E. (1988) Biochemistry 27, 1534-1542.
14. Gercken, G., Hintze, U., and Ahrens, K. (1976) Behring Inst. Mitt. 60, 58-64.
15. Beach, D.H., Holz, G.G., Jr., and Anekwe, G.E. (1979) J. Parasitol. 65, 203-216.
16. Kaneshiro, E.S., Jayasimhulu, K., and Lester, R.L. (1986) J. Lipid Res. 27, 1294-1303.
17. Nishizuka, Y. (1984) Nature 308, 693-698.
18. Smith, S.W., and Lester, R.L. (1974) J. Biol. Chem. 249, 3395-3405.
19. Carter, H.E., Strobach, D.R., and Hawthorne, J.N. (1969) Biochemistry 8, 383-388.
20. McConville, M.J., Bacic, A., Mitchell, G.F., and Handman, E. (1987) Proc. Natl. Acad. Sci. USA 84, 8941-8945.
21. Roberts, W.L., Kim, B.H., and Rosenberry, T.L. (1987) Proc. Natl. Acad. Sci. USA 84, 7817-7821.
22. Medof, M.E., Walter, E.I., Roberts, W.L., Haas, R., and Rosenberry, T.L. (1986) Biochemistry 25, 6740-6747.
23. Schenkman, S., Yoshida, N., and Cardoso de Almeida, M.L. (1988) Mol. Biochem. Parasitol. 29, 141-152.
24. Mato, J.M., Kelly, K.L., Abler, A., and Jarett, L. (1987) J. Biol. Chem. 262, 2131-2137.
25. Pugh, E.L., Kates, M., and Hanahan, D.J. (1977) J. Lipid Res. 18, 710-716.
26. Langworthy, T.A., Holzer, G., Zeikus, J.G., and Tornabene, T.G. (1983) System Appl. Microbiol. 4, 1-17.
27. Achterberg, V., and Gercken, G. (1987) Mol. Biochem. Parasitol. 23, 117-122.