Virulent and avirulent *Entamoeba histolytica* and *E. dispar* differ in their cell surface phosphorylated glycolipids

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SUMMARY

Virulent strains of *Entamoeba histolytica* have been reported to produce a mixture of phosphoglycoconjugates that share some structural features with the lipophosphoglycans (LPGs) of *Leishmania*. Purification of these glycoconjugates is essential to their precise structural characterization. In this study we have extracted 'LPG-like' molecules from various virulent *E. histolytica* strains and purified on the basis of charge differences, 2 apparently related glycoconjugates a 'LPG' and a 'lipophosphopeptidoglycan (LPPG)'. In marked contrast to the abundance of these 'LPG' and 'LPPG' molecules in the virulent strains, avirulent *E. histolytica* and *E. dispar* strains produce either very low, or no detectable levels of LPG, and either low levels or modified forms of 'LPPG'. Monospecific polyclonal antibodies prepared against that 'LPG' of the virulent strain HM-1:IMSS cl6 identified epitopes shared between both the 'LPG' and the 'LPPG' of this and other virulent strains, using Western blot analysis. Flow cytometric analysis of a range of strains using these antibodies identified a surface distribution of these molecules and confirmed a correlation between surface exposure of epitopes bound by these antibodies and parasite virulence.

Key words: *Entamoeba histolytica*. *Entamoeba dispar*, lipophosphoglycan, lipophosphopeptidoglycan, virulence.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* is the aetiological agent of amoebiasis which is a major cause of human sickness and death throughout the world (Walsh, 1986). An appropriate strategy for host protection may be to inactivate the virulence determinants of these parasites. To date, the best characterized virulence determinants of these parasites include the galactose/*N*-acetyl-galactosamine-inhibitable lectin (Kain & Ravdin, 1995; Petri & Schnaar, 1995) the amoebapore protein (Leippe *et al*. 1994) and a number of cysteine proteases (Scholze & Tannich, 1994). A family of amoebic glycoconjugates sharing some structural similarities with the lipophosphoglycans (LPGs) of *Leishmania* (McConville, 1991; Moody, 1993; Turco & Descoteaux, 1992) has also been recently identified in *E. histolytica* (Bhattacharya, Prasad & Sacks, 1992; Stanley, Huizenga & Li, 1992) and proposed to contribute to amoebic adhesion and cytotoxicity (Stanley *et al*. 1992). Preliminary studies indicated that this family of molecules showed considerable polymorphism between strains of *E. histolytica* (Srivastava *et al*. 1995).

In this study we have extracted these 'LPG-like' molecules from a range of virulent *E. histolytica* strains and purified from within the family, 2 apparently related molecules: a 'LPG' and a 'lipophosphopeptidoglycan (LPPG'). Avirulent strains from *E. histolytica* and *E. dispar* strains differ from their virulent counterparts as they contain either very low, or no detectable levels of 'LPG', and either low levels or modified forms of the 'LPPG'. The possible significance of these molecules to virulence is discussed.

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MATERIALS AND METHODS

Parasites

Entamoeba histolytica strains (also referred to as Pathogenic *E. histolytica*, and distinguished by characteristic zymodemes (Sargeaunt, Williams & Green, 1978) were grown axenically in TYI-S-33 medium (Diamond, Harlow & Cunnick, 1978) and harvested in the logarithmic phase of growth. *E. histolytica* strains examined include HM-1:IMSS clone 6 (cl6), HK-9, Rahman and BNI:0591 (isolated from a liver abscess of a patient by Dr G. Burchard, Bernhardt-Nocht Institute, Hamburg, Germany; cloned and axenized in our lab (Bracha, Nuchamowitz & Mirelman, 1995)). Isolates of BNI:0591 were also co-cultured with *Crithidia fasciculata* ($10^7/3 \times 10^6$ amoebae) or bacterial flora. The virulence status of these amoebic strains was assessed as their ability to cause lesions in hamster livers (Buchard & Mirelman, 1988). The avirulent variant BNI:0591^{Y} appears to be a spontaneous mutant from the virulent strain BNI:0591 that was recovered after a freeze–thaw process. *E. dispar* strains (also referred to as *E. histolytica* Non-

Pathogenic strains and distinguished by characteristic zymodemes (Sargeaunt *et al*. 1978) were grown monoxenically together with *C. fasciculata* in the above medium. The strains used were SAW 760RR clA, SAW 1734 R clAR and MAV-1 (obtained from Dr E. Orozco, CINESTAV, Mexico). *E. invadens* (strain IP-1 from Dr L. S. Diamond, NIH, Bethesda, MD) of reptilian origin was grown axenically in the same medium at room temperature. Amoebae were harvested by chilling 48–72 h cultures followed by low-speed centrifugation (5 min, 600 ***g***). The trophozoites were washed 3 times with phosphate-buffered saline (PBS; pH 7·4) and extracted immediately, or stored at −70 °C prior to use.

Isolation of 'LPG-like' molecules

Harvested amoebae trophozoites were extracted essentially as described for the purification of *Leishmania major* promastigote LPG (McConville *et al*. 1987). Briefly, amoebae (3×10^8 cells) were extracted twice with chloroform/ methanol/water (1:2:0·8, v/v) to remove neutral lipids, phospholipids, glycolipids and low molecular weight metabolites. The cell-pellet volume was assumed to be water in adjusting the proportions of solvents for the first extraction. 'LPG-like' molecules were isolated from the delipidated amoebae by repeated extraction with 1-butanol-saturated water (using 10 times the volume of the original cell pellet for each extraction). The extract was then chromatographed using octyl-Sepharose (Pharmacia; 10^8 amoebae equivalent per 5 ml column) equilibrated in 0·1 M ammonium acetate, containing 5% 1-propanol and eluted in a linear gradient of 1-propanol (5–65%) (McConville *et al*. 1987, 1990). The percentage of 1-propanol in each fraction was determined by refractometry. Carbohydrate content was determined using the method of Dubois *et al*. (1956) and protein content was assayed using the Pierce (USA) BCA (bicinchoninic acid) colorimetric protein assay. Separation of glycoconjugates that co-eluted over octyl-Sepaharose was achieved using anion-exchange chromatography (Fast-flow DEAE-Sepharose, Sigma) in the presence of 30% 1-propanol and eluting with an ammonium acetate gradient (20–600 mM). Mixed preparations of LPG and LPPG (octyl-Sepharose fractions) were also subjected to TX-114 solubilization and a subsequent temperature-induced phase partitioning to facilitate separation (Bordier, 1981).

Antibody production

Antibodies directed against purified 'LPG' of strain HM-1:IMSS cl6 were produced in guinea-pigs by injecting 50 μg of purified 'LPG' subcutaneously on 3 occasions at biweekly intervals. The antigen was introduced in the first instance with Freund's complete adjuvant, in the second with Freund's incomplete adjuvant and in the third without adjuvant. Antibodies directed against HM-1:IMSS cl6 'LPG' were purified by affinity chromatography on a Sepharose matrix conjugated to the corresponding antigen (Miron & Wilchek, 1987).

SDS–PAGE and Western blot analysis

Samples were boiled for 5 min in reducing or non-reducing SDS-sample buffer and separated using SDS–PAGE in 13% acrylamide (Laemmli, 1970). Gels were stained for carbohydrate using the method of Durbray & Bezard (1982) and stained for protein using the method of Blum, Beier & Gross (1987). Samples separated by SDS–PAGE were transferred to nitrocellulose for antibody probing (Towbin, Staehelin & Gordon, 1979). Horse-radish peroxidase (HRP)-conjugated antibodies were detected using the ECL kit (Boehringer-Mannheim, FRG). The monoclonal antibodies WIC 108·3, WIC 79·3 and 4A2-A2 directed against *Leishmania* LPG epitopes (Kelleher *et al*. 1994), a rabbit polyclonal antiserum directed against *Leishmania major* promastigote LPG, and a sample of *L. major* LPG were a kind gift of Dr Emanuela Handman. The horse-radish peroxidase-conjugated lectins Concanavalin A, *Arachis hypogaea* (peanut agglutinin), *Glycine max* (soyabean agglutinin), *Ricinus communis* and *Triticum vulgaris* (wheat germ agglutinin; Sigma) were used to identify the presence of specific saccharide structures.

Phosphatidylinositol-specific phospholipase C digestion

Treatment of the glycoconjugates with *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI–PLC; Oxford GlycoSystems Ltd, UK) was undertaken in 20 mM Tris-acetate, pH 7·4 (200 μl) 0·1% Triton X-100 (TX-100) (1U/50 μg glycoconjugate), 8 h at room temperature and 16 h at 37 °C, under a toluene atmosphere. The digest was extracted 3 times with 1 ml of toluene to remove the TX-100 and released glyceride (Ferguson, 1992). The aqueous phase was fractionated over a 1 ml octyl-Sepharose column with 5%, 35% and 65% cuts of 1-propanol in the presence of 0·1 M ammonium acetate. Elution in 5% 1-propanol was defined as the unbound fraction, elution in 35% and 65% 1-propanol was defined as the bound fraction. The carbohydrate content of each fraction was determined as described above using the method of Dubois *et al*. (1956).

LPG and LPPG depolymerization

Purified phosphorylated glycoconjugates were hydrolysed at hexose-1-phosphate linkages with

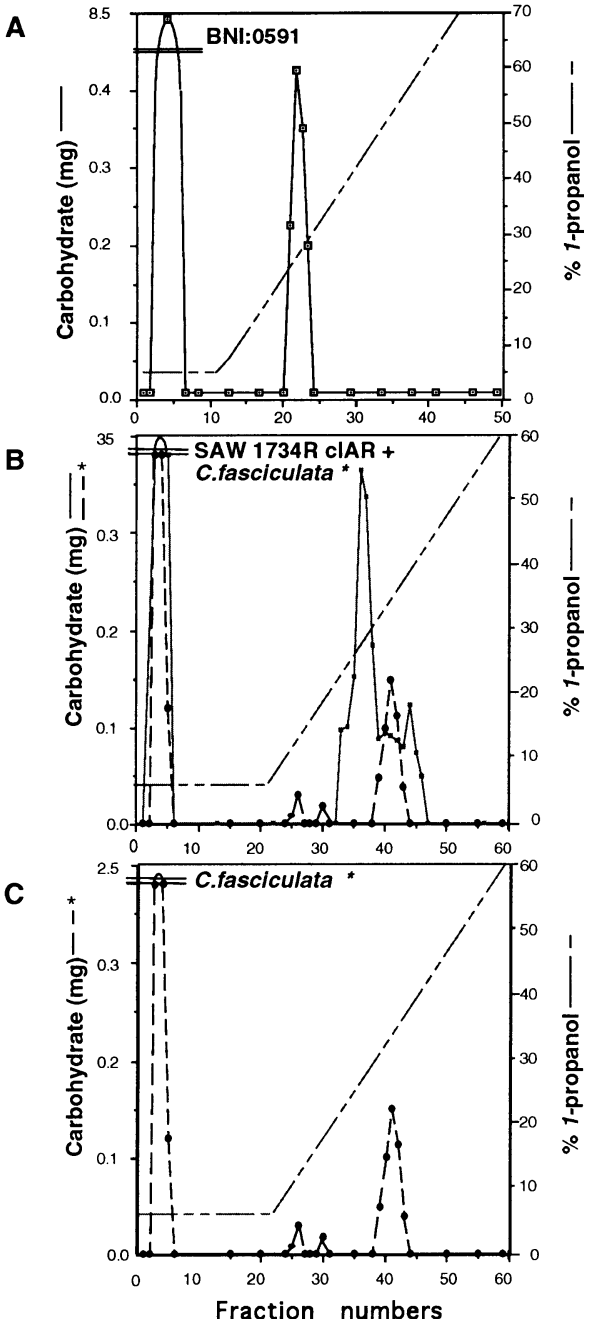

Fig. 1. Elution profiles of the glycoconjugates of *Entamoeba histolytica*, *E. dispar* and associated flora from the hydrophobic interaction chromatography matrix octyl-Sepharose using a linear gradient of 1-propanol (5–65 %) in 0·1 M ammonium acetate buffer. (A) BNI:0591 (also representative of the virulent strains HM-1:IMSS cl6, HK-9, the avirulent strains Rahman and BNI:0591^{V} and xenically cultured BNI:0591). (B) SAW 1734R clAR+ *Crithidia fasciculata* (also representative of the virulent *E. histolytica* strain BNI:0591 and the *E. dispar* strain SAW 760RR clA co-cultured with *C. fasciculata*) this profile is shown as an unbroken line and *C. fasciculata* alone is shown as a dashed line); (C) *C. fasciculata*.

mild acid (40 mM trifluoroacetic acid (TFA)), for 8 min at 100 °C (McConville *et al.* 1990). Products of 'LPG' and 'LPPG' hydrolysis were subjected to TX-114 solubilization and a subsequent temperature-induced phase partitioning to separate hydrophilic from hydrophobic components (Bordier, 1981).

Immunofluorescent staining

Freshly harvested trophozoites were fixed in 3 % paraformaldehyde in PBS for 1 h at room temperature, washed and incubated with 'LPG' monospecific guinea-pig antibodies ($0{\cdot}25 \times 10^6$ trophozoites in 600 μl containing 150 μg antibody) for 1 h with rotation at room temperature. The parasites were washed 3 times in 1 ml of PBS then incubated in the dark with fluorescein isothiocyanate (FITC) goat anti-guinea-pig antibodies (Jackson Immuno Research labs, USA) (1:50 dilution) for 1 h at room temperature. The parasites were again washed 3 times in 1 ml of PBS prior to examination using fluorescence microscopy (Olympus BH-2, Tokyo) or flow cytometry with a fluorescence-activated cell sorter (FACScan, Becton Dickinson).

Amino acid analysis

Amino acids were derivatized with *o*-phthalaldehyde (OPA) and analysed by reverse-phase chromatography on a Hewlett Packard Amino Quant system.

RESULTS

Isolation of the 'LPG-like' molecules of Entamoeba histolytica *and* E. dispar

The 'LPG-like' glycoconjugates of *E. histolytica* and *E. dispar* were solvent extracted and eluted from an octyl-Sepharose matrix at approximately 28 % 1-propanol, as reported for *Leishmania* LPG (McConville *et al.* 1987). The elution profiles of all the *E. histolytica* strains grown axenically or with bacteria resemble that of the virulent strain BNI:0591 (Fig. 1A). The elution profiles of *E. histolytica* and *E. dispar* strains grown monoxenically, in the presence of *C. fasciculata*, conform to the profile of *E. dispar* strain SAW 1734R c1AR (Fig. 1B). Glycoconjugates of *C. fasciculata* cultured axenically (10^9 cells, which is equivalent to the number of *C. fasciculata* co-cultured with 3×10^8 amoebae) eluted as minor peaks at 10 and 15 % 1-propanol and as a major peak above 30 % 1-propanol (Fig. 1C). No glycoconjugates were found to elute above 5 % 1-propanol from extracts of bacterial flora (equivalent numbers to that grown with 4×10^7 amoebae), or from trophozoites of *E. invadens* (data not shown).

The octyl-Sepharose eluted 'LPG-like' glycoconjugates from *E. histolytica* strains identified to be virulent in hamsters, comprised a mixture of 2 components that were separated according to charge

Table 1. Molecular characteristics of the glycoconjugates from *Entamoeba histolytica* and *E. dispar* strains that were eluted from octyl-Sepharose at 28% 1-propanol and subsequently fractionated using DEAE anion-exchange chromatography

		Weakly charged molecules ('LPG'*)		Highly charged molecules ('LPPG'*)	
Isolate	Strain	M_r (kDa)	Anion-exchange elution peak†	M_r (kDa)	Anion exchange elution peak†
E. hystolytica					
Virulent	HM-1:IMSS cl6	45	110 mM	160	250 mM
Virulent	BNI:0591 (xenic)	45	90 mM	160	250 mM
Virulent	BNI:0591	45	90 mM	160	250 mM
Virulent	HK-9	65	130 mM	125	250 mM
Avirulent	BNI:0591^{Y}	45	90 mM	160	250 mM
Avirulent	Rahman§	—	—	65	400 mM
E. dispar	MAV-1‡	37	N.A.	65	N.A.
	SAW 1734R clAR	—	—	76	500 mM
	SAW 760RR clA	—	—	93	N.A.

* Nomenclature assigned as described in the Results section. Molecular weights determined by SDS–PAGE migration.
† Buffers used were ammonium acetate in 30% 1-propanol.
‡ The glycoconjugates of MAV-1 were separated using TX-114 detergent separation.
§ An additional glycoconjugate was fractionated using anion-exchange from the octyl-Sepharose 28% 1-propanol fractions of Rahman: average M_r 93 kDa, eluting at 250 mM ammonium acetate.
N.A., Not attempted.

Table 2. Carbohydrate yields of the glycoconjugates from *Entamoeba* and *E. dispar* strains that were eluted from octyl-Sepharose at 28% 1-propanol and fractionated using DEAE anion-exchange chromatography

(The glycoconjugates of MAV-1 (85 μg/10^8 amoebae) were separated using TX-114 detergent separation, which does not allow total sample recovery. A rough approximation indicates that the 'LPG- and LPPG-like' molecules of these strains are present in equal quantity.)

Isolate	Strain	'LPG' (μg/10^8 trophozoites	'LPPG'* (μg/10^8 trophozoites)	Total† (μg/10^8 trophozoites)
E. hystolytica				
Virulent	HM-1:IMSS cl6	98	272	370
Virulent	BNI:0591 (xenic)	103	322	425
Virulent	BNI:0591	93	262	355
Virulent	HK-9	87	37	124
Avirulent	BNI:0591^{Y}	6	77	83
Avirulent	Rahman	—	385‡	424
E. dispar	SAW 1734R clAR	—	354	354
	SAW 760RR clA	—	150	150

* Nomenclature assigned as described in the Results section.
† Quantification represents an average of a minimum of 3 preparations except for BNI:0591 (xenic), where only single data sets were available.
‡ The remaining 39 μg represents an unidentified heterogeneous glycoconjugate (Fig. 3B, Lanes 6 and 8).

using anion-exchange chromatography. A degree of heterogeneity in molecular weight, charge (Table 1) and abundance (Table 2) was identified between the respective glycoconjugates of these strains. An important difference in composition between the 2 glycoconjugate components of virulent strains was suggested by carbohydrate and protein staining following SDS–PAGE. As exemplified by *E. histolytica* isolate BNI:0591 in Fig. 2A, the highly charged, high molecular weight population (160 kDa) stained for both carbohydrate and protein, while the weakly charged, low molecular weight population (45 kDa) stained for carbohydrate, but not for protein. This pattern is very similar to that observed for the strains HMI:IMSS cl6, BNI:0591-xenic and HK-9 (with some molecular weight variation; Table 1).

The *E. histolytica* strain BNI:0591^{Y} that was avirulent in hamsters, contained similar glycoconjugates to the virulent *E. histolytica* strains, but at

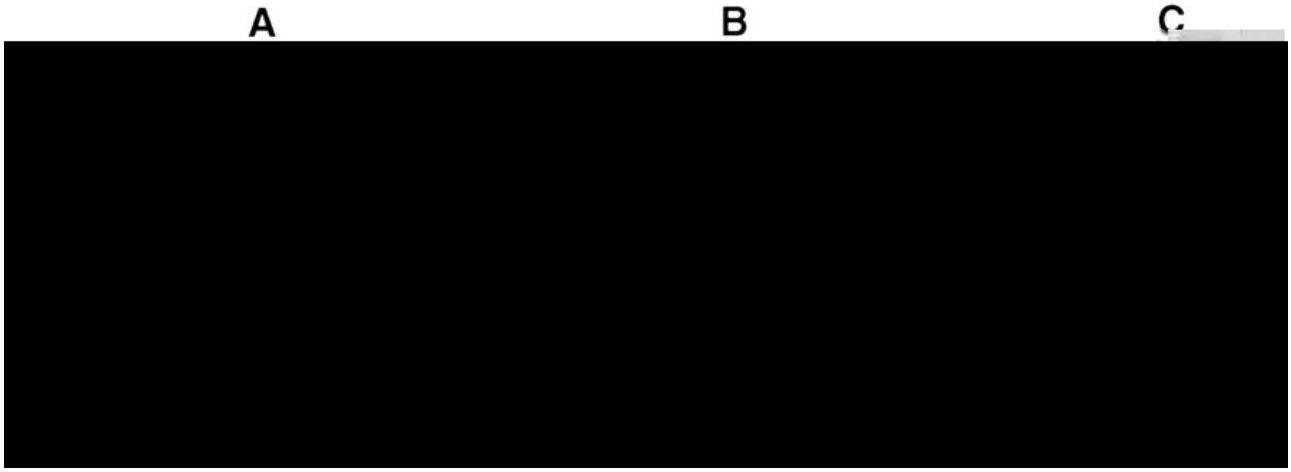

Fig. 2. SDS–PAGE separation of 'LPG' and 'LPPG' of *Entamoeba histolytica* and *E. dispar* strains. In all panels the left side are gels stained for carbohydrate and the right side are gels stained for protein. (A) Virulent *E. histolytica* strain BNI:0591 ('LPPG': Lanes 1, 3; 'LPG': Lanes 2, 4). (B) Avirulent *E. histolytica* strain Rahman ('LPPG': Lanes 5, 7; and an undefined glycoconjugate: Lanes 6, 8). (C) *E. dispar* strain SAW 1734R clAR ('LPPG': Lanes 9, 10).

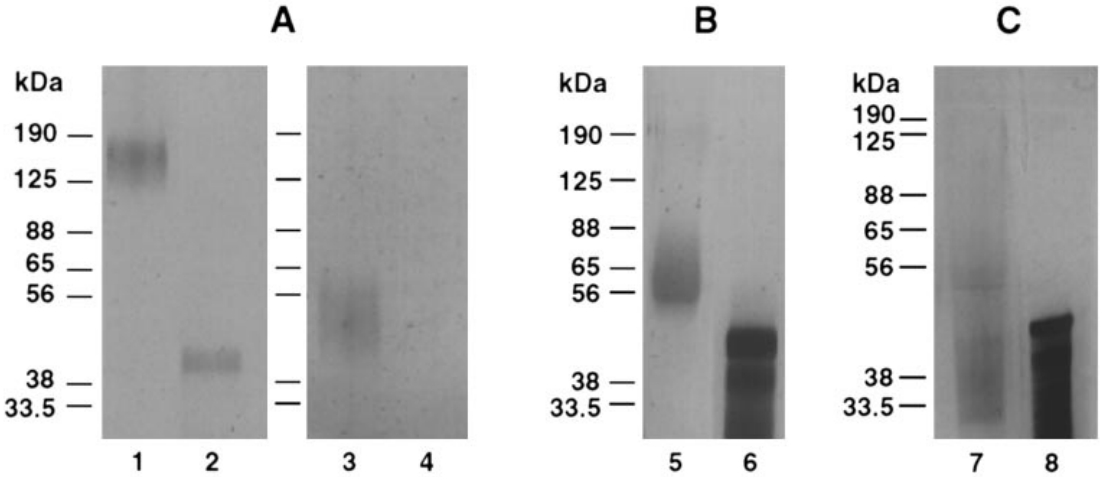

Fig. 3. SDS–PAGE of the mild acid hydrolysates of 'LPG' and 'LPPG' of *Entamoeba histolytica* and *E. dispar* strains. Gels are stained for carbohydrate. (A) Virulent strain BNI:0591 ('LPPG': Lanes 1, 2; 'LPG' and : Lanes 3, 4). (B) Avirulent *E. histolytica* strain Rahman, ('LPPG': Lanes 5, 6). (C) *E. dispar* strain SAW 1734R clAR ('LPPG': Lanes 7, 8). Lanes 1, 3, 5, 7 are the untreated glycoconjugates; Lanes 2, 4, 6, 8 are the glycoconjugates after hydrolysis.

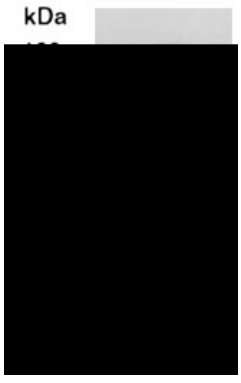

Fig. 4. Western blot analysis of the 'LPG' (Lane 1) and the 'LPPG' (Lane 2) of the virulent *Entamoeba histolytica* strain HM-1:IMSS cl6 reacted with a monospecific guinea-pig serum directed against the 'LPG' of the same strain.

very low levels (Table 2). The avirulent *E. histolytica* strain Rahman was distinguished by containing a single major, highly charged glycoconjugate that stained for both carbohydrate and protein (Fig. 2B). A minor glycoconjugate co-eluted from the hydrophobic interaction matrix in the 28% 1-propanol peak, and fractionated over the anion-exchange resin at high ionic strength (250 mM ammonium acetate); however, its low levels prevented further characterization. The *E. dispar* strain MAV-1 contained very low levels of 2 glycoconjugates that differed in molecular weight from the virulent *E. histolytica* counterparts. Two additional *E. dispar* strains SAW 1734R clAR, and SAW 760RR clA, contained clearly distinct glycoconjugate profiles, with apparently single, heterogeneous, molecular populations that stained for both carbohydrate and protein

Table 3. PI–PLC sensitivity of the 'LPG' and 'LPPG' from *Entamoeba histolytica* and *E. dispar* as monitored by a change in hydrophobicity

Classification	Strain	Glycoconjugate	+/− PI–PLC*	Octyl-Sepharose fractions (%)		Change in hydrophobicity (%)
				Unbound	Bound	
E. histolytica						
Virulent	HM-1:IMSS cl6	'LPG'	−	0	100	—
			+	67	33	67
		'LPPG'	−	19	81	—
			+	69	31	50
Avirulent	Rahman	'LPPG'	−	12	88	—
			+	73	27	61
E. dispar	SAW 1734R clAR	'LPPG'	−	27	73	—
			+	88	12	61
Control	*Leishmania major* promastigote	LPG	−	0	100	—
			+	67	33	67

* Before and after digestion with *Bacillus thuringiensis* phosphatidylinositol specific phospholipase C.

(shown for SAW 1734R clAR; Fig. 2C; Tables 1 and 2). The glycoconjugate of the SAW 1734R clAR strain was also identified to be highly charged.

Composition of the amoebic glycoconjugates

The weakly charged glycoconjugates of the virulent *E. histolytica* and *E. dispar* strains contained carbohydrate (Table 2), phosphate (as indicated by sensitivity to mild acid; Fig. 3) and a inositol-phosphate lipid-anchor (as indicated by sensitivity to PI–PLC; Table 3) and are subsequently referred to as a 'lipophosphoglycans (LPGs'). The highly charged glycoconjugates in addition to these components, also contained significant levels of amino acids (Table 4) and are subsequently referred to as a 'lipophosphopeptidoglycans (LPPGs'; nomenclature adapted from a report by Isibasi *et al.* (1982)).

Mild acid sensitivity of these glycoconjugates indicates that the phosphate is in a hexose-1-phosphate linkage. The 'LPG' and 'LPPG' molecules of all the *E. histolytica* and the *E. dispar* strains were sensitive to mild acid hydrolysis. 'LPG' was totally depolymerized by this treatment (shown for BNI:0591 in Fig. 3A, Lanes 3 and 4) which is typical of the LPGs of *Leishmania*. 'LPPG' in contrast, was hydrolysed from a heterogenous smear characteristic of a glycoconjugate to a series of discrete, lower molecular weight bands, where the major band was approximately 40 kDa (shown for *E. histolytica* virulent strain BNI:0591 in Fig. 3A, Lanes 1 and 2: avirulent *E. histolytica* strain Rahman in Fig. 3B, Lanes 5 and 6; and *E. dispar* strain SAW 1734R clAR in Fig. 3C, Lanes 7 and 8). These bands stained for both carbohydrate and protein, and phase separated into the detergent phase of a Triton X-114 extraction, indicative of a hydrophobic nature (not shown). These products of hydrolysis would suggest that there exists a carbohydrate-protein-containing hydrophobic core component common to the 'LPPGs' of the virulent and avirulent *E. histolytica* strains and the *E. dispar* strains.

The sensitivity of these 'LPG' and 'LPPG' molecules of PI–PLC hydrolysis is recorded in Table 3. Sensitivity to hydrolysis with this enzyme is consistent with the presence of an inositol-phosphate linkage as found in glycosyl phosphatidylinositol (GPI-) anchors. The 'LPGs' and 'LPPGs' of both virulent (HM-1:IMSS cl6 and BNI:0591) and avirulent strains (Rahman and SAW 1734R clAR) were sensitive to hydrolysis by this phospholipase to the same extent as the GPI-anchored LPGs of *Leishmania* (monitored as a change in the hydrophobicity in the range of 50–67%).

Amino acid analysis of the 'LPG' of the virulent *E. histolytica* strains (BNI:0591 and HM-1: IMSS cl6) identified a small protein component between 2 and 4%). Slight sensitivity of these molecules to the protease of *Streptomyces griseus* (data not shown) can not confirm whether the amino acids are present as a structural component of the 'LPGs' or are simply associated (as identified for the 'LPGs' of *Leishmania*; Bates, 1995). In contrast, the amino acid composition of the 'LPPG' molecules of the virulent *E. histolytica* strains (BNI:0591) and HM-1:IMSS cl6) are estimated at 8–15% wt/wt. The complete degradation of the 'LPPG' of the virulent *E. histolytica* strain HM-1:IMSS cl6 by the protease of *S. griseus* (data not shown) suggests that the protein is present as a structural component. The amino acid components of the glycoconjugates of the avirulent *E. histolytica* strain Rahman and the *E. dispar* strain SAW 1734R clAR are estimated to be 12–13% wt/wt. Strikingly, aspartate, glutamate, serine, proline and lysine are the dominant amino acids for 'LPPGs' of both the *E. histolytica* and

Table 4. Amino acid composition of the 'LPG's and 'LPPG's from *Entamoeba histolytica* and *E. dispar* strains

(All percentages were determined from component weights.)

Classification	Strain	Glycoconjugate	Most abundant amino acids (amino acid/total amino acid, %)						Other amino acids (amino acid/total amino acid, %)	Percentage amino acid/total glycoconjugate
			Asp	Glu	Ser	Thr	Lys	Pro		
E. histolytica										
Virulent	HM-1:IMSS cl6	'LPPG'	22	16	15	3	12	14	18	15
Virulent	BNI:0591	'LPPG'	20	13	12	2	10	28	15	8
Avirulent	Rahman	'LPPG'	30	11	18	1	17	14	9	12
E. dispar	SAW 1734R clAR	'LPPG'	29	18	18	1	17	7	10	13
E. histolytica										
Virulent	HM-1:IMSS cl6	'LPG'	35	14	13	5	29	3	1	4
Virulent	BNI:0591	'LPG'	22	11	13	1	8	25	20	2

E. dispar strains and they are present at similar levels in these molecules (Table 4). The abundance of aspartate and glutamate in these 'LPPG' molecules are likely to contribute to their high negative charge. This amino acid compositional data further supports the suggestion that a protein-containing component is common to the 'LPPGs' of both *E. histolytica* and *E. dispar* strains.

Antibody- and lectin-staining of E. hystolytica *strains*

A guinea-pig polyclonal antiserum raised against the 'LPG' of the virulent *E. histolytica* strain HM-1:IMSS cl6 was seen by Western blot analysis to bind both the 'LPG' and the 'LPPG' of this strain (Fig. 4, Lanes 1 and 2 respectively) and 'LPPGs' of a number of other virulent strains (BNI:0591, HK-9; data not shown). Certain epitopes therefore appear to be conserved not only between these glycoconjugates within a single strain, but also among *E. histolytica* strains. Western blot analysis of 'LPPG' of the avirulent *E. histolytica* strain Rahman and of the *E. dispar* isolate SAW 1734r clAR identified poor binding by this polyclonal serum (data not shown). The poor binding of these antibodies to the glycoconjugates of the avirulent amoebae strains indicates that different epitopes are accessible in the glycoconjugates of the virulent and avirulent strains.

The surface exposure of these 'LPG/LPPG' glyconjugates on fixed trophozoites was monitored using flow cytometry as analysed by fluorescence activated cell sorting (FACS; Fig. 5) and fluorescence microscopy (not shown). Antibody binding was clearly greater in the virulent strains (shown here for HM-1:IMSS cl6, BNI:0591 and HK-9: Fig. 5A) as compared to the avirulent *E. histolytica* strains (BNI:0591^{Y} and Rahman: Fig. 5B) and the *E. dispar* strains (SAW 760RR clA and SAW 1734R clAR: Fig. 5C).

The presence of terminal glucose or mannose in an α-configuration was indicated by Concanavalin A binding to both the 'LPG' and 'LPPG'. The presence of galactose was not identified by either RCA, PNA or SBA binding, despite Bhattacharya's (Bhattacharya *et al*. 1992) identification of incorporation of ^{3}[H]galactose into the glycoconjugates (and our own observations of ^{3}[H]galactose labelling of HM-1:IMSS cl6, data not shown). This would suggest that any galactose present is not linked in a terminal position. In addition, the failure of WGA to bind these molecules suggests that *N*-acetyl-glucosamine is absent. The saccharide components of these glycoconjugates were also examined using a number of the well-characterized antibodies directed toward epitopes of the 'LPGs' of *Leishmania*. None of these antibodies bound the glycoconjugates of *E. histolytica*, which would suggest that either these epitopes are absent or masked. In addition, the

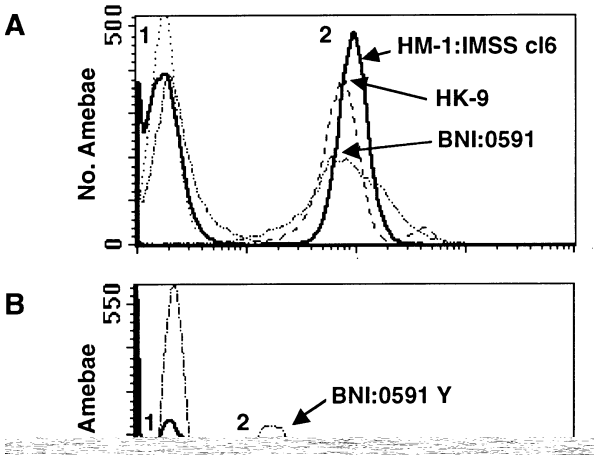

Fig. 5. Flow cytometric analysis of 'LPG/LPPG' in *Entamoeba histolytica* strains using a monospecific guinea-pig serum directed against the 'LPG' and of the virulent strain HM-1:IMSS cl6 (Peak 2). Binding of the secondary antibody alone to the amoebae are included as controls (Peak 1). (A) Virulent *E. histolytica* strains HM-1:IMSS cl6, BNI:0591 and HK-9. (B) Avirulent *E. histolytica* BNI:0591^{Y} and Rahman. (C) *E. dispar* strains SAW 1734R clAR and SAW 760RR clA.

guinea-pig serum raised against the 'LPG' of *E. histolytica* failed to recognize promastigote 'LPG' of *Leishmania major* (data not shown). These findings indicate that despite a number of structural features in common, the 'LPG-related' molecules of *E. histolytica* differ from their counterparts in *Leishmania*.

DISCUSSION

The 'LPG-like' glycoconjugates of virulent and avirulent *E. histolytica* are clearly distinct. These molecules were first described by Bhattacharya *et al.* (1992), who also reported molecular heterogeneity among the 'LPG-like' molecules from a range of *E. histolytica* strains (Srivastava *et al.* 1995). In this study we have identified that these 'LPG-like' molecules from virulent *E. histolytica* strains actually contain 2 distinct glycoconjugate components. We identified that these 2 glycoconjugates are charged differently and we exploited these differences using anion-exchange chromatography to separate these molecules. Purification of these molecules has enabled us to identify the unique characteristics of the individual molecular families. The name 'LPG' was assigned to molecules containing PI–PLC sensitive lipid anchors, phosphate and saccharide components. 'LPPG' molecules possess, in addition to the features of the 'LPG', a significant amino acid content.

We found that both 'LPG' and 'LPPG' molecules are abundant in the virulent *E. histolytica* strains that we tested. To ensure that trophozoite glycoconjugates were not an artifact of axenic cultivation, the virulent *E. histolytica* strain BNI:0591 was co-cultured with *C. fasciculata* or bacteria, and the high levels of 'LPG' and 'LPPG' production appeared to be little affected. Avirulent *E. histolytica* strains have clearly distinct glycoconjugate profiles from their virulent counterparts and conform to one of two categories. The archetype of the first category is the avirulent *E. histolytica* strain BNI:0591^{Y} which contains 'LPG' and 'LPPG' at approximately one fourth the level of its virulent counterparts. This strain is apparently a spontaneous avirulent mutant which was recovered after a freeze-thaw process, from the parent *E. histolytica* strain that we identified to be highly virulent in hamster livers (Bracha *et al.* 1995). Corresponding to the loss of virulence in hamsters was a simultaneous drop in 'LPG' and 'LPPG' levels. The second category, exemplified by strain Rahman, contains no detectable levels of 'LPG' and a relatively high level of modified 'LPPG' which exhibits a faster electrophoretic mobility in SDS–PAGE and a higher negative charge than the 'LPPGs' of virulent *E. histolytica* strains.

In this report we record the first observation of 'LPG-like' glycoconjugates in strains of *E. dispar*. Intriguingly, common trends in glycoconjugate status were identified for both *E. dispar* strains and avirulent *E. histolytica* strains. *E. dispar* strains, like the avirulent *E. histolytica* strains also conform to one of the two categories. The *E. dispar* strain MAV-1, which originated from the lab of Dr E. Orozco complies with the first category as it contains low levels of 'LPG' and 'LPPG' (of the same order of magnitude as the *E. histolytica* strain BNI:0591^{Y}, although of significantly lower molecular weight). The *E. dispar* strains SAW 760RR clA and SAW 1734R clAR conform to the second category of strains as they contain no detectable levels of 'LPG' and relatively high levels of modified 'LPPGs'.

Intriguingly, 'LPPG' (of various molecular weights) appear to be present in all the *E. histolytica* and *E. dispar* strains examined, suggesting that these molecules may perform a function important to their survival. 'LPG', in contrast, is present in significant amounts only in virulent *E. histolytica* strains. The presence of these glycoconjugates at the surface of

virulent *E. histolytica* strains is consistent with participation in mediating cell–cell interactions. The contribution of these molecules to amoebic adherence was suggested by Stanley's report (Stanley *et al*. 1992) of blocking amoebic-binding to chinese hamster ovary (CHO) cells using a monoclonal antibody directed to a heterogeneous glycoconjugate (apparently 'LPG/LPPG'). The apparent absence of 'LPG-like' molecules from an *E. invadens* isolate of reptilian origin (also observed by Srivasta *et al*. 1995) further supports the notion that these glycoconjugates may perform an important function in human host cell interactions.

In *Leishmania*, the LPGs are major virulence determinants that function to protect from complement attack, in host cell attachment, and to protect the phagocytosed parasite (Turco & Descoteaux, 1992). It is fascinating that virulent amoebae also contain molecules with some structural similarities to the LPGs of *Leishmania*. While we were unable to identify shared epitopes between the amoebic glycoconjugates and the LPGs of *Leishmania* using a range of monoclonal antibodies directed toward epitopes of LPG in *Leishmania*, the basic structural constituents of an inositol-phosphate linked lipid anchor and phosphate-linked sugars appear to be conserved in these amoebic molecules. With our ability to purify these molecules we are now in a position to determine their chemical structures, to examine directly their contribution to amoebic/host-cell interactions and to define epitopes that may mediate adherence.

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