Absence of lipophosphoglycan-like glycoconjugates in *Entamoeba dispar*

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SUMMARY

Invasive amoebiasis is the result of infection of *Entamoeba histolytica*. The closely related *Entamoeba dispar* can colonize the human gut but does not cause invasive disease. In this study, *E. dispar* was analysed for the presence of the lipophosphoglycan-like (LPG) glycoconjugate known to be present on the cell surface of *E. histolytica*. *E. dispar* cells were radio-isotope labelled with [^{3}H]galactose or [^{3}H]inositol. The acidic glycoconjugates were extracted and analysed by hydrophobic chromatography over phenyl–Sepharose and by sodium dodecyl sulphate polyacrylamide gel electrophoresis. No LPG-like molecules could be identified in *E. dispar* in contrast to *E. histolytica*, suggesting that these molecules may be absent in the non-pathogenic species.

Key words: lipophosphoglycan, glycoconjugate, *Entamoeba dispar*, cell surface.

INTRODUCTION

Entamoeba histolytica is the causative agent of amoebiasis. *Entamoeba dispar* is a distinct species and, although its cysts are morphologically indistinguishable from *E. histolytica* and it colonizes the human intestine, it does not cause invasive disease. Biochemical and genetic markers distinguishing the two species have been reported (Diamond & Clark, 1993). However, studies to date show that most suspected virulence proteins, such as the pore-forming peptide amoebapore (Leippe *et al*. 1993), galactose/*N*-acetyl galactosamine inhibitable lectin (Mann *et al*. 1997) and cysteine proteinases (Bruchhaus *et al*. 1996), are also found in *E. dispar*. In the case of cysteine proteinase genes the number of homologues is different in the two species, 6 in *E. histolytica* and 4 in *E. dispar* (Bruchhaus *et al*. 1996). One of the cysteine proteinases (Ehcp5) found only in *E. histolytica* is associated with the cell surface (Jacobs *et al*. 1998) and is thought to be involved in pathogenesis. However, blocking its expression by antisense RNA did not inhibit cytopathic or haemolytic activity (Ankri, Stolarsky & Mirelman, 1998). The expression level of cysteine proteinases also differs in the two species, but it is difficult to correlate pathogenicity with the presence and/or level of expression of cysteine proteinase genes alone.

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The cell surface glycoconjugate lipophosphoglycan (LPG) was originally isolated and characterized from *Leishmania donovani* (Turco, Wilkerson & Clawson, 1984). Later it was found to be present in almost all species of *Leishmania*, *Trypanosoma cruzi* (Singh *et al*. 1994), *Trichomonas vaginalis* (Singh, 1993), *Tritrichomonas foetus* (Singh, 1993) and *E. histolytica* (Bhattacharya, Prasad & Sacks, 1992). The organization and structure of LPG in *Leishmania* varies with the developmental stage of the parasite and there is a definite correlation between the structure of LPG and virulence of the parasite (McConville *et al*. 1992). In *E. histolytica* the LPG-like molecule has been demonstrated in cells grown under both axenic and xenic conditions (Bhattacharya *et al*. 1992; Moody *et al*. 1997). LPG has been found in all *E. histolytica* isolates tested using specific monoclonal antibodies and biochemical analysis (Bhattacharya *et al*. 1990*b*; Srivastava *et al*. 1995) but not *Entamoeba invadens* or *Entamoeba moshkovskii*. A previous study has suggested that monoxenic *E. dispar* may contain either very low or no detectable level of LPG; instead there may be low levels or a modified form of a related but distinct molecule lipophosphopeptidoglycan (LPPG) (Moody *et al*. 1997). Since there is always the possibility that the glycoconjugate isolated under such conditions may be derived from, or the expression regulated by the associated organisms (bacteria, *Crithidia*), we have re-examined this issue by attempting to isolate LPG-like molecules from axenized *E. dispar* and from *E. dispar* cells associated with a bacterial flora or *Crithidia fasciculata*. Our results suggest that no LPG-like glycoconjugate can

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be isolated from *E. dispar* using the conditions under which such molecules were found in *E. histolytica*.

MATERIALS AND METHODS

E. histolytica strain HM-1 : IMSS was grown axenically in TYI-S-33 medium (Diamond, Harlow & Cunnick, 1978). Two strains of *E. dispar* were grown : strain SAW 760 axenically or monoxenically in the presence of *Crithidia* (Clark, 1995) and strain 135 xenically in the presence of a bacterial flora (Robinson, 1968). Strain 135 was isolated in the Hospital for Tropical Diseases, London and has been shown to be *E. dispar* by isoenzyme characteristics and amplification of a specific fragment of DNA by polymerase chain reaction (J. P. Ackers, unpublished observations). *E. dispar* SAW 760 and *C. fasciculata* were cultured in YI-S medium (Diamond, Clark & Cunnick, 1995).

Two different methods were used for extraction of LPG-like molecules (McConville *et al.* 1987; Orlandi & Turco, 1987). Prior to extraction, *E. dispar* cells were radio-isotope labelled with the precursor [^{3}H]galactose or [^{3}H]inositol as described (Bhattacharya *et al.* 1990*a*). The extracted material (1–10 μg of carbohydrate) was further purified over a column of phenyl–Sepharose (0·5–1·0 ml bed volume) and eluted with 'solvent E' (ethanol : diethyl ether : pyridine : ammonia : water (15 : 15 : 5 : 1 : 0·017)) or eluted with a gradient of 1-propanol. The amount of glycoconjugate in each fraction was determined by measuring the amount of radioactivity or by a spot test using an orcinol–sulfuric acid spray (Redman *et al.* 1995). Some of the eluted material was concentrated and analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The radioactive glycoconjugates were visualized by fluorography (Laskey & Mills, 1975).

RESULTS

LPG-like glycoconjugates can be eluted from a phenyl–Sepharose column either by 'solvent E' or by a gradient of 1-propanol. In general, these molecules from different species including *E. histolytica* elute at about 25–30 % 1-propanol (Bhattacharya *et al.* 1992). It has been observed that about 40–60 % of the [^{3}H]galactose-labelled material of *E. histolytica* elutes from the phenyl–Sepharose column as LPG-like molecules. A typical elution profile with 'Solvent E' is shown in Fig. 1A and the SDS–PAGE pattern of the eluted material is shown in Fig. 2, lane 1. These glycoconjugates migrate as diffuse bands centred around 100 kDa and 45 kDa relative to protein molecular weight standards. [^{3}H]Galactose-labelled axenic *E. dispar* cells were extracted and subjected to phenyl–Sepharose chromatography as described by Turco *et al.* (1984). The elution profile is shown in Fig. 1A and the SDS–PAGE pattern of the eluted material is shown in Fig. 2, lane 2. Though there was some glycoconjugate eluted from the hydrophobic column by the polar organic 'solvent E', the gel pattern did not show the diffuse band expected of a highly glycosylated compound as observed with *E. histolytica*. The presence of a rather sharp band may be due to a glycosylated protein. There was no radioactive glycoconjugate eluted from the phenyl–Sepharose column with propanol concentrations greater than 10 % (Fig. 1B). A bar on top of the figure indicates the fractions where LPG-like molecules of *E. histolytica* eluted under similar conditions (between 27 and 33 % propanol). In order to check if there were any LPG-like glycoconjugates in the material eluted at 5–10 % propanol, SDS–PAGE analysis was carried out and the result did not show any diffuse band typical of LPG-like molecules (Fig. 2, lane 3). It is possible that the *E. dispar* glycoconjugates did not get labelled with [^{3}H]galactose. Metabolic labelling was therefore carried out with [^{3}H]inositol and the extracted labelled material eluted at about 30 % propanol as for LPG (Fig. 1C). SDS–PAGE analysis of the material did not reveal any LPG-like molecules (Fig. 2, lane 4) suggesting that there may be inositol labelling of other lipid molecules.

Similar studies were repeated with monoxenic *E. dispar* cells growing with *C. fasciculata* and xenic *E. dispar* cells with an undetermined bacterial flora. Radio-isotope labelled glycoconjugate from cells growing with *C. fasciculata* was fractionated on a phenyl–Sepharose column and the bound fraction eluted with increasing concentrations of 1-propanol. Unlike axenic *E. dispar* cells, a small fraction of the radioactivity eluted at about 27·5 % propanol (330 cpm which is not visible due to the scale used) which is characteristic of LPG-like molecules (Fig. 1D). In order to determine whether *C. fasciculata* may itself have similar molecules, these cells were also metabolically labelled with [^{3}H]galactose and the glycoconjugates extracted and analysed as described. The phenyl–Sepharose chromatographic profile is shown in Fig. 1E. It is clear from the pattern that there are significant amounts of [^{3}H]-galactose-labelled material eluting at about 30 % propanol. Since monoxenic cultures are grown in the presence of *Crithidia* it is likely that the small amount of glycoconjugates from monoxenic cultures which were observed to elute in about 30 % propanol may have been contributed by *Crithidia*. Absence of typical LPG-like molecules was also confirmed by analysing the glycoconjugates eluted at 27·5 % propanol in SDS–PAGE (Fig. 2, lane 5). No LPG-like bands were observed in *C. fasciculata*. In the same way [^{3}H]galactose-labelled *E. dispar* isolate 135 cells, grown xenically, were also analysed and the phenyl–Sepharose pattern is shown in Fig. 1F. No radio-isotope labelled material was observed to elute between 25 and 30 % propanol.

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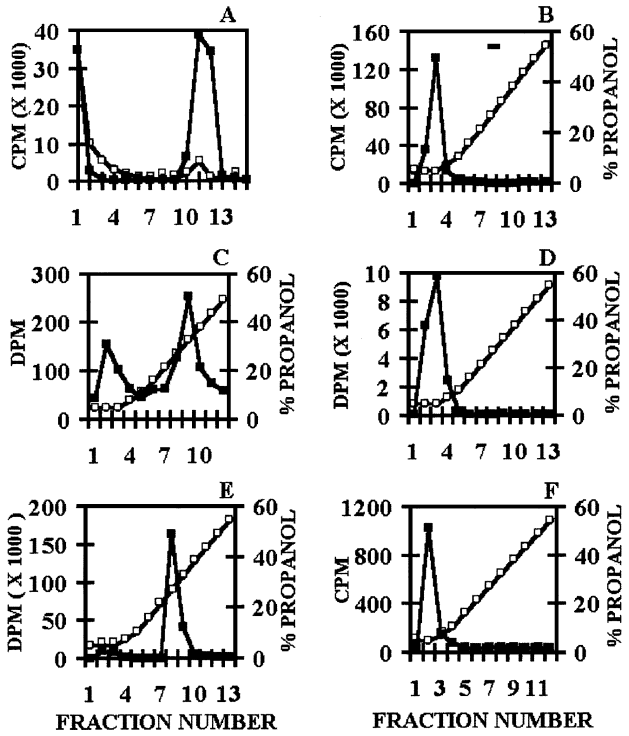

Fig. 1. Phenyl–Sepharose elution profiles of LPG-like glycoconjugates using solvent E (A) and propanol gradient (B–F). (A) [^{3}H]galactose-labelled HM-1:IMSS strain of *Entamoeba histolytica* (■) and [^{3}H]galactose-labelled axenic *E. dispar* SAW 760 (□). (B) [^{3}H]galactose-labelled axenic *E. dispar* SAW 760. (C) [^{3}H]inositol-labelled axenic *E. dispar*. (D) [^{3}H]galactose-labelled monoxenic *E. dispar* (in the presence of *Crithidia fasciculata*). (E) [^{3}H]galactose-labelled *C. fasciculata*. (F) [^{3}H]galactose-labelled xenic *E. dispar* strain 135. The bar in (B) indicates the position where LPG-like molecules of *E. histolytica* eluted as determined by staining with orcinol–sulfuric acid. (B–F) ■ CPM/DPM; □ % PROPANOL.

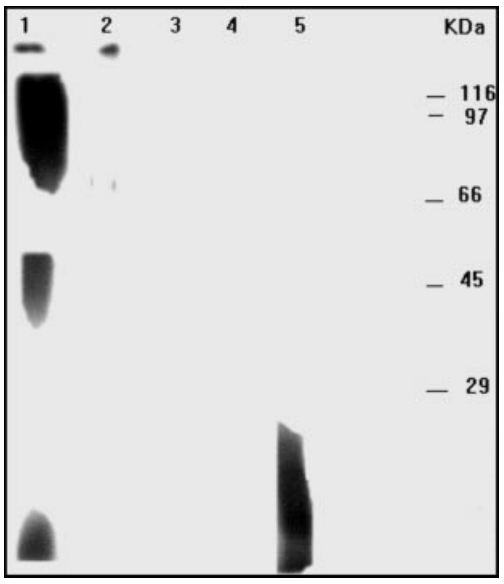

Fig. 2. Analysis of LPG-like glycoconjugates on SDS–PAGE. Lane 1: [^{3}H]galactose-labelled, solvent E-extracted LPG-like molecules from *Entamoeba histolytica*; lane 2: [^{3}H]galactose-labelled, solvent E-eluted fraction from axenic *E. dispar*; lane 3: [^{3}H]galactose-labelled $<10\%$ propanol fraction from axenic *E. dispar*; lane 4: [^{3}H]inositol-labelled axenic *E. dispar*; lane 5: [^{3}H]galactose-labelled *Crithidia fasciculata*. The amount of radioactivity loaded in each lane varied between 30000 and $\sim$50000 cpm. Some of the lanes were fluorographed for 4–5 weeks in order to observe weak signals.

DISCUSSION

The data presented here using different methods of extraction and analysis of glycoconjugates clearly show that LPG-like molecules are neither synthesized nor present in *E. dispar* cells grown under axenic, monoxenic and xenic conditions or in different isolates. Since *E. dispar* cells are normally cultivated in association with other organisms, such as bacteria or *Crithidia*, it is likely that some of the glycoconjugates previously extracted from these cells may be derived from the associated organisms. This may be particularly relevant in a situation when the amount of the desired glycoconjugate appears to be very small, as in the case of monoxenic cells and the associated organism *Crithidia* displaying molecules with similar elution profiles. *Crithidia* also make glycoconjugates, as revealed by [^{3}H]galactose incorporation but these molecules must be different from the typical LPG-like molecules observed in *E. histolytica* as revealed by SDS–PAGE analysis. In an earlier study, Moody *et al.* (1997) did not observe LPG-like molecules in 3 isolates (SAW 760 RR clA, SAW 1734 R cl AR, MAV-1) of monoxenic *E. dispar* cultures by extraction and immunological methods but suggested the presence of another molecule, lipopeptidophosphoglycan (LPPG), in small amounts. In this study there has not been any indication of the presence of LPPG-like molecules (based on the migration pattern in SDS–PAGE gels). Neither monoclonal (Marinets *et al.* 1997) nor polyclonal antibodies (A. Bhattacharya & R. Arya, unpublished observations) specific for *E. histolytica* LPG-like molecules recognize *E. dispar*. Since all isolates of *E. histolytica* tested have LPG-like molecules, it is significant that the presence of these molecules cannot be demonstrated in any of the isolates of *E. dispar*.

The surface coat (glycocalyx layer) in *Leishmania* spp. is composed of the surface LPG molecules and the nature of this layer is dependent on the structure of LPG molecules (Pimenta *et al.* 1994). LPG-like negatively charged glycoconjugates make up the major portion of the glycocalyx layer of *Leishmania*. Two independent studies have shown that the cell surface structure of *E. dispar* and *E. histolytica* is different (Pimenta & Diamond, 1997; Espinosa-Cantellano *et al.* 1998). While *E. histolytica* has a thick uniform glycocalyx layer, no such structure is observed in *E. dispar*. Early observations indicated

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that negatively charged glycoconjugates make up the glycocalyx layer of *Entamoeba* (Lushbaugh & Miller, 1974). Since LPG-like molecules are the main negatively charged glycoconjugates present on the cell surface of *E. histolytica*, these may be the main constituent of the glycocalyx. Therefore, lack of a glycocalyx layer also supports the absence of LPG-like molecules in *E. dispar*.

All the suspected virulence-associated proteins – the pore-forming peptide amoebapore (Leippe *et al.* 1993), galactose/*N*-acetyl galactosamine inhibitable lectin (Mann *et al.* 1997) and cysteine proteinases (Bruchhaus *et al.* 1996) – are present in both the *E. histolytica* and *E. dispar* species. It appears that LPG-like glycoconjugates may be the only class of molecules present only in *E. histolytica* and therefore may play an important role in pathogenesis. In *Leishmania*, LPG is known to protect cells against complement and attack by oxygen radicals (McNeely & Turco, 1990). Apart from its involvement in attachment of *E. histolytica* to target cells (Stanley, Huizenga & Li, 1992), LPG may also be forming a protective surface coat during invasion of tissue. Therefore, LPG-like molecules may be a good target for novel therapeutics and vaccines. This is supported by a recent experiment where anti-LPG antibodies protected SCID mice against *E. histolytica* infection (Marinets *et al.* 1997).

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