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Differential expression of surface glycoconjugates on *Entamoeba histolytica* and *Entamoeba dispar*

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ABSTRACT

The human large intestine can harbor two morphologically similar amoebae; the invasive *Entamoeba histolytica* and the non-invasive *Entamoeba dispar*. Whereas *E. histolytica* can produce intestinal and extra-intestinal lesions, *E. dispar* is present in non-symptomatic carriers. Although biochemical, genetic and proteomic studies have identified clear differences between these *Entamoebae*, it has become clear that several molecules, once assumed to be involved in tissue destruction, exist in both the virulent and the avirulent species. As surface molecules may play a role in invasion and could therefore determine which amoebae are invasive, we analyzed the glycoconjugate composition of *E. histolytica* and *E. dispar* using lectins. There was a significant difference between *E. histolytica* and *E. dispar* in the expression of glycoconjugates containing D-mannose and N-acetyl-α-D-galactosamine residues, but not between virulent and avirulent strains of *E. histolytica*. N-glycoconjugates with terminal α (1–3)-linked mannose residues participate in the adhesion and subsequent cytotoxicity of *E. histolytica* to cultured hamster hepatocytes. One of them probably is the Gal/GalNAc lectin.

1. Introduction

E. histolytica produces invasive amoebiasis (dysentery and liver abscesses), while *E. dispar* is a common, non-invasive organism identified in asymptomatic infections. Although the invasive and non-invasive amoebae are practically indistinguishable morphologically, they are genetically distinct [1]. Sargeaunt et al. [2] initially identified species-specific isoenzyme patterns, while Espinosa-Cantellano et al. [3] revealed ultrastructural differences in the surface coats of the species, as well as a weaker cytopathic effect and a lower phagocytic capacity of *E. dispar*. The various strains of *E. histolytica* also differ from one another genetically and vary in their capacities for virulence [4–6].

To identify potential virulence factors, the prototype virulent *E. histolytica* HM-1:IMSS strain and a less virulent *E. histolytica* strain have been compared by in vitro and in vivo analyses. These strains differ in their expression of several proteins, including peroxiredoxin, superoxide dismutase, KERP1, and the cysteine proteinases, as well as in levels of surface lipophosphoglycans [6–11]. Furthermore, studies have shown that the cell surfaces of *E. histolytica* and *E. dispar* are distinct [3,12]. Pathogenic strains of *E. histolytica* are more easily agglutinated with concanavalin A (Con A) than strains isolated from human asymptomatic carriers [13].

The pathogenic effects *E. histolytica* require direct contact with mammalian cells [14,15], therefore information on the surface properties of *E. histolytica* and *E. dispar* and their role in host–parasite interactions is essential for a proper understanding of the early mechanisms of tissue damage. Since one of the primary molecules involved in the adhesion event, the Gal/GalNAc lectin, is present in both the invasive *E. histolytica* and the non-invasive *E. dispar* [16], there must be additional molecules on the surface of *E. histolytica* and *E. dispar* that are responsible for the differences in their pathogenicity.

The aim of the present study was to examine the possible involvement of surface molecules in the interaction of these amoebae with their target cells. We therefore analyzed the presence of cell glycoconjugates on avirulent and virulent *E. histolytica* strains, as well as on *E. dispar*, using several biotinylated lectins. Avirulent and virulent

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E. histolytica presented more glycoconjugates containing D-mannose and N-acetyl-α-D-galactosamine residues than *E. dispar*. In cytotoxicity assays, N-glycoconjugates with terminal α (1–3)-linked mannose residues on virulent *E. histolytica* participated more efficiently in the adhesion and destruction of cultured hamster hepatocytes.

2. Materials and methods

2.1. Amoebae cultures

Virulent and avirulent *E. histolytica* cultures derived from strain HM-1:IMSS, originally isolated in 1967 from a patient with amoebic colitis in Mexico, were grown under axenic conditions at 35 °C in trypticase-yeast extract-iron medium (TYI-S-33), containing 15% (v/v) heat-inactivated adult bovine serum (BSA) [17]. The virulent strain of *E. histolytica* had been serially passed through hamster livers to preserve its virulence, while the avirulent *E. histolytica* strain (wild-type) had been maintained in culture, without passing through livers. *E. dispar* trophozoites from strain SAW 760 RR, clone A, isolated from a human adult male in England in 1979, were cultured in complete YI-S medium [18], and incubated at 36.5 °C.

2.2. Entamoeba strain virulence

Virulence of the three strains was determined by two methods [19,20]: In vivo, virulence was determined by grading the amoebic liver abscess development in hamsters after intraportal inoculation of live trophozoites. In vitro, virulence was determined by a cytopathic assay using primary cultures of hamster hepatocytes. Protocols for animal handling were previously approved by the Institutional Committee for Animal Care (IACUC, ID number 244/05).

2.3. Flow cytometry (FACS) analysis of carbohydrate residues of Entamoeba glycoconjugates

E. histolytica and *E. dispar* trophozoites were fixed in 2% (w/v) paraformaldehyde (Polysciences, Warrington, PA, USA) in PBS for 1 h at room temperature (RT). Amoebae (2.5×10^5) were incubated with the following biotinylated lectins: *Helix pomatia* (HPA, specific for N-acetyl-α-D-galactosamine), *Phaseolus vulgaris* (PHA, specific for α-D-galactose), *Tetragonolobus purpurea* (TPA, specific for α-L-fucose), *Canavalia ensiformis* (Con A, specific for α-D-mannose and α-D-glucose), *Pisum sativum* (PSA, specific for α-D-mannose and α-D-glucose), *Galanthus nivalis* (GNL, specific for α-D-mannose) (Sigma-Aldrich, St. Louis, MO, USA), for 1 h at RT. This procedure identified the major groups of glycoconjugates present in protozoa, such as glycoproteins, glycopeptides, peptidoglycans, and glycolipids [21]. Lectins were diluted in PBS at final concentrations of 1, 4.7, 3.2, 0.5, 25.6, and 6.3 μg mL^{-1}, respectively. They were incubated with streptavidin–fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO, USA), and diluted 1:1000 in PBS, for 1 h. The control amoebae were incubated without any lectin. The inhibition of lectin binding to trophozoites was studied by incubating the biotinylated lectins with the following specific sugar inhibitors for 15 min before incubation with the parasites: 200 mM D-mannose for Con A, PSA, and GNL; 200 mM N-acetyl-D-galactosamine for HPA; and 200 mM L-fucose for TP. Finally, they were evaluated by flow cytometry analysis (FACS Calibur).

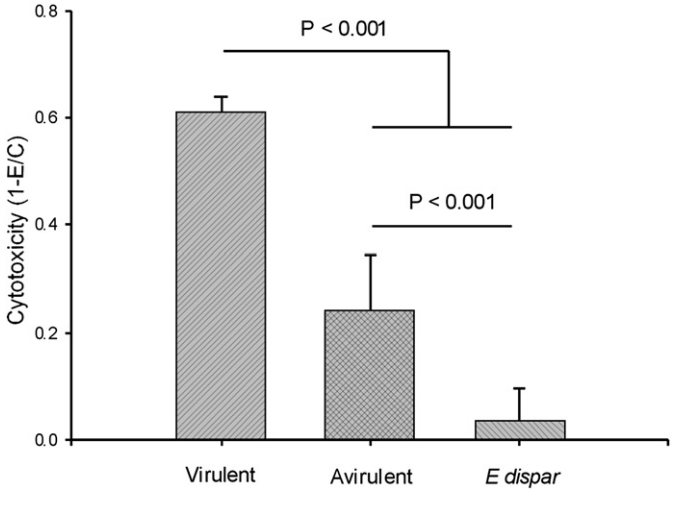

Fig. 1. Effect of virulent and avirulent *E. histolytica* and *E. dispar* trophozoites on hamster hepatocyte monolayers. Amoebae interacted with hepatocytes; cytotoxicity was measured spectrophotometrically. The cytotoxic effect of *E. histolytica* was significantly higher with virulent trophozoites than avirulent trophozoites and *E. dispar*. Data are presented as the mean ± SD ($P<0.001$, Bonferroni *t*-test).

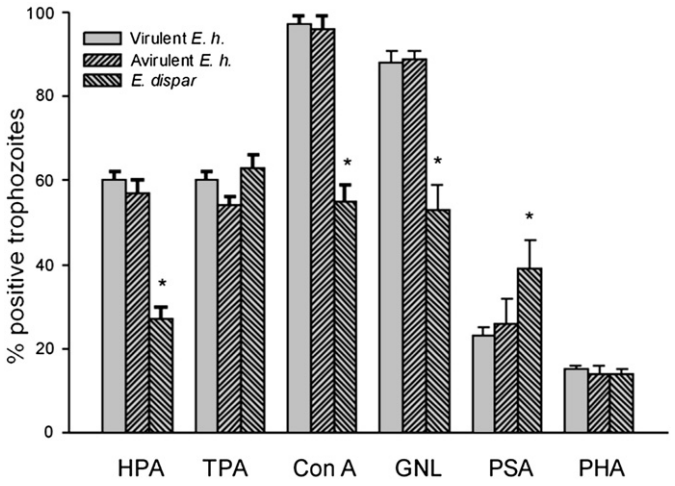

Fig. 2. FACS of surface carbohydrate residues in virulent and avirulent *E. histolytica* and *E. dispar*. The percentage of FITC-labeled parasites was significantly higher in both *E. histolytica* strains than in *E. dispar*, when HPA, Con A, and GNL were used (*$P<0.05$). In contrast, the percentage of trophozoites labeled with PSA was significantly higher in *E. dispar* than in both *E. histolytica* strains (*$P<0.05$). No significant difference between virulent and avirulent trophozoites of *E. histolytica* was observed ($P>0.05$).

2.4. Cytotoxicity assay

Livers were perfused through the portal vein and dissected. Hepatocytes were isolated and cultured using a modified collagenase perfusion method [22]. Suspensions of *E. histolytica* and *E. dispar* trophozoites were placed over the monolayer of cultured hepatocytes at a ratio of one trophozoite:eight hepatocytes. Interactions were carried out for 30 min. Adhesion mediated by lectins was inhibited by preincubating amoebae with Con A and GNL lectins at concentrations of 1, 10, and 100 μg for 5 min at RT. Trophozoites were centrifuged and then added to the hepatocyte monolayer. The cytotoxic effect on the hepatocytes was measured as a function of lactate dehydrogenase release, using the 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique. After 30 min of interaction, 20 μL of MTT stock solution (5 mg mL^{-1}) was added and the samples were incubated for 30 min at 37 °C. Dimethyl sulfoxide (DMSO) was then added and the optical densities of the samples were read at 540 nm. To carbohydrate inhibition of amoebic adherence and killing to hepatocytes the following sugars (Sigma-Aldrich, St. Louis, MO, USA) were evaluated: lactose, D (+) galactose, D-mannose, and N-acetyl glucosamine (GlcNAc). The solutions with the carbohydrate at a final concentration of 50, 100 and 200 mM were used. *E. histolytica* trophozoites in TYI were added to a confluent hepatocyte monolayer in DMEM medium and incubated 30 min at 36.8 °C. Control interactions were made in the absence of carbohydrates. Cytotoxicity was evaluated as describe above.

2.5. Affinity chromatography on immobilized GNL

A membrane fraction of *E. histolytica* was prepared according to the procedure reported by Texeira and Huston [23]. Trophozoites of *E. histolytica* strain HM-1:IMSS were washed with phosphate-buffered saline (PBS) and resuspended in 10 mM sodium phosphate buffer (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 3 mM iodoacetamide, and 3 mM N-ethylmaleimide (Sigma-Aldrich, St. Louis, MO, USA). Cells were sonicated and the cell lysates were centrifuged at 50,000 ×*g* (1 h, 4 °C). The pellet was then resuspended in 10 mM sodium phosphate buffer and centrifuged at 100,000 ×*g* (1 h, 4 °C) to separate the membrane fraction. Membranes were solubilized in 3% octylglucoside and clarified at 100,000 ×*g* (1 h, 4 °C) immediately before loading onto the column. An aliquot of the membrane amoebic protein solution (2 mg mL^{-1} in phosphate-buffered saline) was applied to a column of GNL lectin coupled to Sepharose-4B, which had been equilibrated with TBS–TCa^{2+} buffer (50 mM Tris/150 mM NaCl/0.05% (v/v) Tween 20/20 mM $CaCl_2$, pH 7.5). After incubating for 1 h at RT, the column was sequentially washed with TBS–TCa^{2+}, and then with TBS–TEDTA (TBSTCa^{2+} in which 20 mM $CaCl_2$ was replaced with 10 mM EDTA). The bound membrane proteins were eluted with EDTA in the same buffer. The

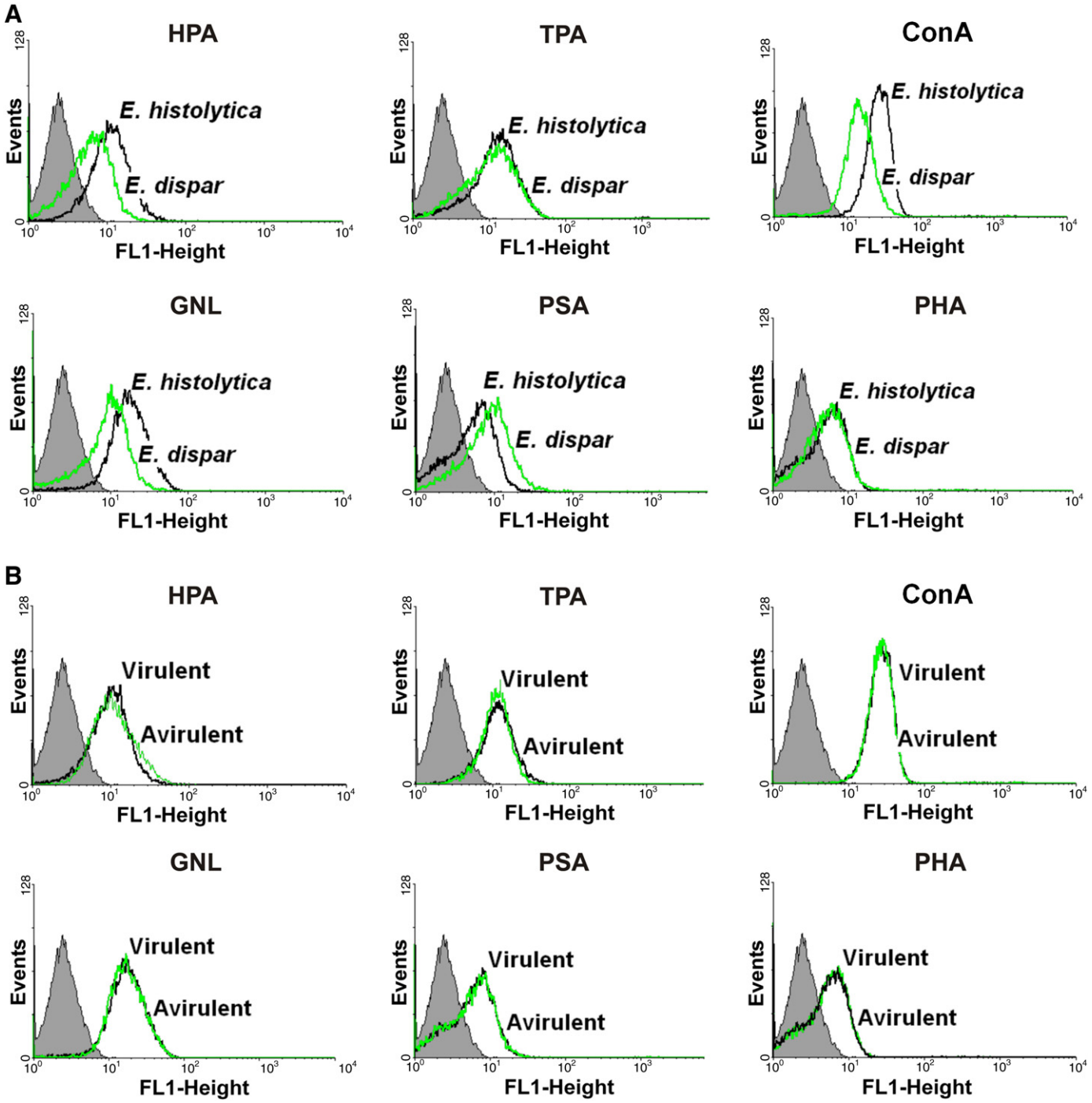

Fig. 3. Fluorescence intensities in *Entamoeba* trophozoites. A) Trophozoites from *E. histolytica* and *E. dispar* were stained with different biotinylated lectins, and analyzed by flow fluorometry. Two peaks of different fluorescence intensities can be seen with HPA, Con A, GNL, and PSA. The means of the fluorescence intensities were significantly different ($P<0.05$, Bonferroni *t*-test). In contrast, the fluorescence intensities were similar with TPA and PHA. B) The fluorescence intensities were similar between virulent and avirulent *E. histolytica* ($P>0.05$) with HPA, TPA, Con A, GNL, PSA, and PHA.

elute was monitored continuously at 280 nm and electroblotted as described by Towbin et al. [24]. For membrane amoebic protein detection we used an amoebic antiserum diluted 1:1000; then the membranes were incubated with streptavidin-bound horseradish peroxidase (Pierce, Rockford, IL, USA). Enzyme activities were detected with the substrate solution (0.1% (v/v) H_2O_2, 17.5% (v/v) methanol, 0.15% (w/v) 4-chloro-α-napthol, 82.5% (v/v) PBS) and incubated for 15 min. Finally, the amoebic proteins were identified on the nitrocellulose strips, and their molecular weights determined.

2.6. Statistical analysis

Data are presented as the mean ± SD. Comparisons between two groups were analyzed using Student's unpaired, two-tailed *t*-tests. For comparisons among more than two groups, one-way ANOVA was performed, and if a significant main effect or association was identified ($P<0.05$), the respective group means were compared using the Bonferroni *t*-test. All analyses were performed using the SigmaStat software (2.03 versions for Windows) (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Pathogenicity of Entamoeba trophozoites

The virulent culture of *E. histolytica* produced extensive lesions in the hamster liver, occupying nearly 40% of the whole liver weight, whereas the avirulent *E. histolytica* produced small lesions that healed in 48 h, and *E. dispar* produced no liver lesions. In vitro, *E. histolytica* trophozoites of the virulent strain significantly affected the viability of hepatocyte monolayers (ANOVA; $F_{2,35}=229$; $P<0.001$, Fig. 1) and destroyed more hepatocytes than the avirulent *E. histolytica* and *E. dispar* ($P<0.001$, Bonferroni *t*-test). Meanwhile, avirulent *E. histolytica* had a slightly higher cytopathic activity than *E. dispar*, which showed no significant effect on hepatocyte cultures ($P<0.001$, Bonferroni *t*-test).

3.2. Cell membrane glycoconjugates

Carbohydrate or glycan moieties attached to proteins and lipids (glycoconjugates) on the surface of trophozoites of *E. histolytica* and *E. dispar* were identified by FACS using a panel of biotinylated lectins (Fig. 2). The binding pattern of lectins on the surface of trophozoites from the three strains of amoebae was heterogeneous, showing a broad range of binding percentages, and their recognition patterns were distinct, depending on the strain and species. The percentage of FITC-labeled parasites was significantly higher in *E. histolytica* than in *E. dispar*, when HPA, Con A, and GNL were used (*$P<0.001$, Bonferroni *t*-test). In contrast, the percentage of FITC-labeled parasites was significantly lower in *E. histolytica* (23%) than in *E. dispar* (38%) when PSA was used (*$P<0.014$, Bonferroni *t*-test). With TPA and PHA, there was no difference between *E. histolytica* and *E. dispar*. In summary, our results show clear differences in the distribution of lectin-specific carbohydrates between the invasive *E. histolytica* and non-invasive *E. dispar*, and they indicated that the membrane glycoconjugates containing terminal α-D-mannosyl, α-D-glucosyl, and N-acetyl-α-D-galactosaminyl residues were more abundant on *E. histolytica* than *E. dispar*. In contrast, membrane glycoconjugates containing an α-1,6-linked fucose moiety were more abundant on *E. dispar*.

A specific binding sugar was used to perform inhibition studies for each lectin in order to evaluate the specificity of the lectin binding. D-mannose, N-acetyl-D-galactosamine, and L-fucose were used. N-acetyl-D-galactosamine inhibited HPA binding to the amoebae by almost 95%. Unexpectedly, L-fucose inhibited the binding of the TPA lectin to virulent *E. histolytica* and *E. dispar* more efficiently than to avirulent *E. histolytica*. While D-mannose blocked the binding of Con A to both pathogenic and non-pathogenic trophozoites, it blocked the binding of PSA and GNL lectin to virulent *E. histolytica* and *E. dispar* more efficiently than to avirulent *E. histolytica*. Overall, the results showed that monosaccharides inhibited lectin binding more strongly in the pathogenic *E. histolytica* than in the non-pathogenic *E. dispar* (data not shown).

3.3. FACS histograms of lectin binding

The surface binding of HPA, Con A, and GNL was greater on the virulent strain of *E. histolytica* than on *E. dispar* (Fig. 3A). The means of the fluorescence intensities of *E. histolytica* and *E. dispar* were different for HPA, Con A, and GNL (Table 1; $P<0.05$). In contrast, the surface binding of PSA was greater on *E. dispar* than on the virulent strain of *E. histolytica* ($P<0.05$). However, the surface binding of TPA and PHA was similar (overlapping) on *E. histolytica* and *E. dispar* ($P>0.05$). These results indicate that membrane glycoconjugates containing N-acetyl-α-D-galactosaminyl, α-D-glucosyl, and terminal α-D-mannosyl residues were more abundant on *E. histolytica* than *E. dispar*. In contrast, the glycoconjugates containing D-mannosyl residues and fucose binding sites, recognized by PSA, were more abundant on *E. dispar* than on *E. histolytica*.

To determine whether the content of glycoconjugates on the surface of the virulent and avirulent strains of *E. histolytica* was different, trophozoites were stained with the same panel of FITC-labeled lectins (Fig. 3B, Table 1). The surface density distribution of the glycoconjugates on virulent and avirulent *E. histolytica* was similar, since the fluorescence intensities in the two strains were equivalent for all the lectins ($P>0.05$).

3.4. Role of GNL and Con A lectins in the cytotoxicity of E. histolytica

To test the role of mannose-containing glycoconjugates in the adherence and damage to hepatocytes in primary cultures, we conducted a competitive cytotoxic inhibition assay that quantified the in vitro hepatocyte release of lactate dehydrogenase, measured using the MTT technique. We analyzed the cytotoxic effect of parasites on the hepatocyte monolayer in the presence of different concentrations (1, 10, and 100 μg) of the Con A and GNL lectins, which have affinity for D-mannose (Fig. 4). Whereas the cytotoxic effect of the virulent *E. histolytica* was only significantly inhibited by GNL at a concentration of 100 μg ($P<0.001$, Bonferroni *t*-test), the cytotoxic effect of the avirulent *E. histolytica* was significantly inhibited by GNL at both 10 and 100 μg ($P<0.01$, Bonferroni *t*-test). In contrast, Con A did not reduce *E. histolytica* parasite binding or cytotoxicity. A probable explanation for this apparently contradictory result is that although Con A and GNL have a common affinity for D-mannose, they bind glycans very differently [25].

To better analyze the direct role of the mannose termini glycoconjugates on the surface of *E. histolytica* during the adhesion to hepatocytes, we performed carbohydrate inhibition assays. Adherence of *E. histolytica*

Table 1
Fluorescence intensities in *Entamoeba* trophozoites labeled with lectins.

Trophozoites			
Lectin	*E. histolytica* (virulent)	*E. histolytica* (avirulent)	*E. dispar*
HPA	19 ± 1	18 ± 1	13 ± 1[a]
TPA	31 ± 4	27 ± 4	26 ± 3
Con A	39 ± 3	41 ± 5	27 ± 2[a]
GNL	26 ± 1	25 ± 1	15 ± 1[a]
PSA	15 ± 1	16 ± 1	22 ± 2[a]
PHA	10 ± 1	10 ± 1	10 ± 1

Fixed trophozoites from *E. histolytica* and *E. dispar* were stained with different biotinylated lectins, and analyzed by flow fluorometry. The data are presented as the mean ± standard deviation of the fluorescence intensities from three independent experiments.

[a] Differences in intensity of fluorescence between virulent *E. histolytica* and *E. dispar* were significant ($P<0.05$) as determined by Bonferroni *t*-test. There were no differences between virulent and avirulent trophozoites of *E. histolytica* ($P>0.05$).

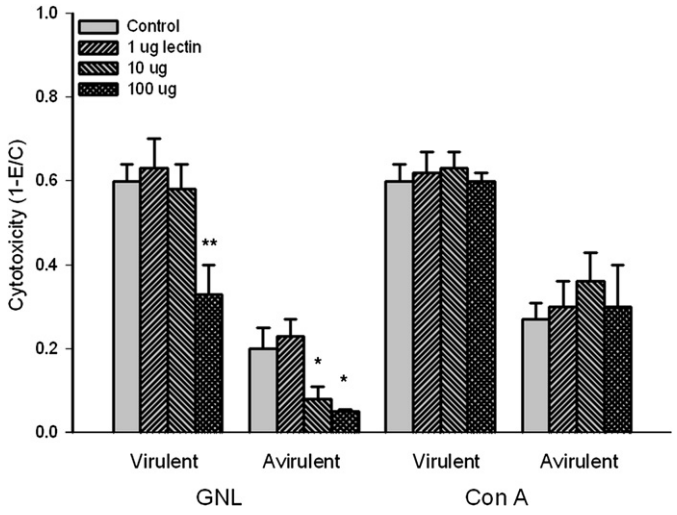

Fig. 4. GNL inhibits the cytotoxic effect of virulent and avirulent *E. histolytica* on hepatocyte monolayers. Different concentrations of lectins (1, 10, 100 μg) were used. GNL (100 μg) significantly inhibited monolayer destruction by virulent *E. histolytica* (**$P<0.001$). Also, 100 and 10 μg of GNL reduced the cytotoxic effect of the avirulent strain of *E. histolytica* on hepatocytes (*$P<0.01$). No cytotoxic effect was observed with Con A ($P>0.05$).

trophozoites and consequently damage to hepatocytes monolayers was significantly inhibited by lactose at 100 and 200 mM, and by D (+) galactose at 200 mM (*$P<0.01$, **$P<0.001$, *$P<0.05$, Fig. 5).

In summary, the data show that *E. histolytica* adherence to hepatocytes is mediated by glycoconjugates containing terminal D-mannose residues recognizable by GNL, and that these glycoconjugates play a role in the adherence to and consequent damage of hepatocytes. Thus, glycoconjugates recognized by GNL may contribute to the pathogenesis of amoebiasis.

3.5. Binding of GNL to a 170 kDa glycoprotein

To characterize the mannose-containing glycoproteins that are involved in adherence to and cytotoxicity of hepatocytes, the glycoconjugates bound by GNL were purified by affinity chromatography using a GNL-Sepharose column. The western blot analysis of the eluted molecules showed six main glycoconjugates recognizable by antibodies to *E. histolytica* (Fig. 6). One of them has a Mr of 170 kDa, which could be the heavy subunit of Gal/GalNAc specific lectin. The other molecules have a Mr of 150, 61, 57, 32 and 27 kDa.

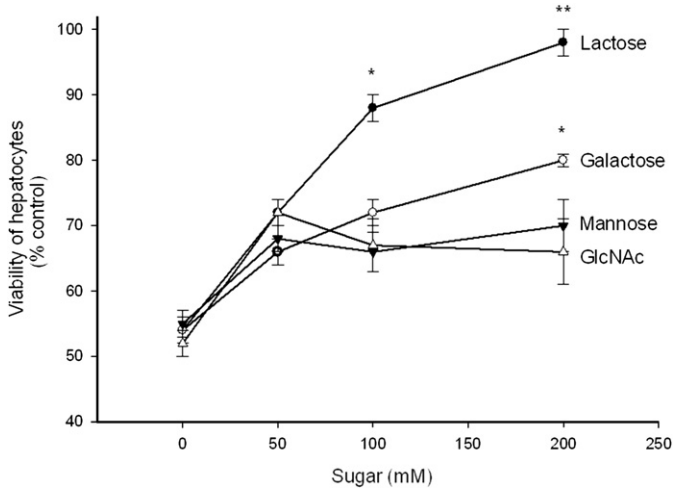

Fig. 5. Carbohydrate inhibition of the cytotoxic effect of virulent *E. histolytica* on hepatocyte monolayers. Different concentrations of carbohydrate (50, 100, 200 mM) were used. Data are expressed as the mean ± SD of the percentage of viability of hepatocytes with respect to the control. Lactose (100 and 200 mM) and D (+) galactose (200 mM) significantly inhibited monolayer destruction by virulent *E. histolytica* compared to the control, with lactose (*$P<0.01$, **$P<0.001$) being more effective than D (+) galactose (*$P<0.05$). Contrarily, there was no inhibition of cytotoxicity by D-mannose and GlcNAc ($P>0.05$).

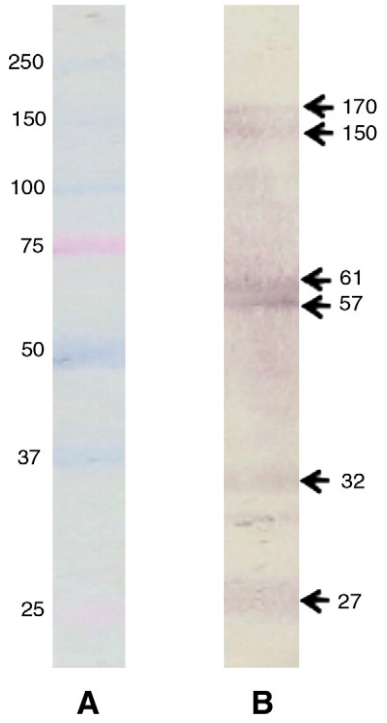

Fig. 6. Analysis by western blotting of membrane proteins eluted from a GNL-Sepharose column. A) Molecular weight marker. B) *E. histolytica* proteins. Glycoproteins of *E. histolytica* were eluted from the GNL-Sepharose column with EDTA, then subjected to SDS-PAGE and transferred to nitrocellulose. The proteins were revealed by using an antiserum to amoebic proteins. Glycoproteins of 170, 150, 61, 57, 32 and 27 kDa are indicated by arrows.

4. Discussion

Our study is the first to report an analysis of the glycoconjugates of *Entamoeba* trophozoites using a variety of lectins. It is also the first to recognize differential surface sugar molecule expression. Additionally, we analyzed the participation of glycoconjugates in the adhesion of amoebae to hepatocytes in culture.

FACS using a panel of plant lectins allowed us to detect the presence of cell membrane glycoconjugates on the surface of *Entamoeba* trophozoites. The reason for using various mannose-specific lectins (Con A, PSA and GNL) was based on the fact that the mannose-binding sites for each lectin are different, and consequently their specificities for glycoconjugates may vary [26,27]. The binding of HPA, TPA, Con A, PSA, and GNL lectins to the two species of *Entamoeba* demonstrated the presence of glycoconjugates containing N-acetyl-α-D-galactosaminyl, α-L-fucosyl, α-D-glucosyl, and α-D-mannosyl residues, as well as an α-1,6-linked fucose moiety. However, some of these glycoconjugates were more abundant in *E. histolytica* than in *E. dispar*, whereas others were more abundant in *E. dispar*.

Glycoconjugates with N-acetyl-α-D-galactosaminyl residues were found in only 27% of *E. dispar* compared to 60% of *E. histolytica* trophozoites. Glycoconjugates with L-fucosyl residues were present in *Entamoeba* trophozoites, confirming the presence of fucosyl residues on the membrane of *E. histolytica* [28], and *E. dispar*. Glycoconjugates with α-D-mannosyl and α-D-glucosyl residues were expressed more in *E. histolytica* (97%) than in *E. dispar* trophozoites (60%). Similarly, glycoconjugates with terminal α-D-mannosyl residues (labeled with

GNL) were expressed more in *E. histolytica* (88%) than in *E. dispar* trophozoites (53%). The differences in the expression of glycoconjugates containing D-mannosyl residues might be related to differences in the activity of mannosyl transferases in strains of *E. histolytica*.

Because GNL recognizes exclusively terminal D-mannosyl residues, especially those possessing Man (α1–3) Man units [29], our results suggest that *E. histolytica* expresses more glycoconjugates that carry high mannose-type glycan chains than *E. dispar*. In contrast, glycoconjugates recognized by PSA were expressed more in *E. dispar* (38%) than *E. histolytica* trophozoites (23%). Although Con A and PSA have α-glucosyl- and α-mannosyl-binding specificity, they also possess the ability to recognize different saccharide sequences, even on the same glycan structure. For example, Con A shows a great affinity for the trimannoside core substituted by two N-acetyl-P-glucosaminyl residues (oligosaccharide S-6a), whereas PSA has high affinity for oligosaccharides containing an α-1,6-linked fucose moiety [30–32]. Thus, it is probable that *E. dispar* expresses more glycoconjugates with fucose binding sites than *E. histolytica*.

The histogram analysis showed that fluorescence intensity differed for *E. histolytica* and *E. dispar* trophozoites labeled with HPA, Con A, GNL, and PSA. Trophozoites of the pathogenic strain were more intensively labeled with HPA, Con A, and GNL, whereas *E. dispar* trophozoites were more intensively labeled with PSA. This could reflect differences in the number of lectin binding sites, the binding constant, or the motility of the lectin binding sites on the membrane. In contrast, the fluorescence intensity was similar in virulent and avirulent strains labeled with the aforementioned lectins. Our results confirm several previous studies that have shown that the molecules on the surface coat of *E. histolytica* and *E. dispar* differ [10,33].

Because attachment to target cells is the key to the virulence of amoebae [15], we analyzed the cytotoxic effect of *E. histolytica* on hepatocytes in the presence of Con A and GNL lectins, which showed the highest affinity for the virulent amoebae. That only GNL, which exclusively binds to terminal D-mannose residues in N-linked glycans [29], inhibited the adherence and cytotoxicity of *E. histolytica* suggests that N-linked glycoconjugates with terminal D-mannosyl residues on the surface of *E. histolytica* play an important role in the interaction of virulent *E. histolytica* with hepatocytes. However, the inability of GNL to completely abrogate the cytotoxicity of the *E. histolytica* strain to hepatocytes indicates that other molecules may also be involved in adherence and cytotoxicity [34–36].

Studies have demonstrated that *E. histolytica* trophozoites attach to hepatocytes either by using their membrane-associated lectin specific for Gal/GalNAc, or by their mannose-containing glycoconjugates serving as a ligand for mannose-binding receptors [37] or asialoglycoprotein receptor [38] on the surface of the hepatocytes. To exclude the participation of these receptors, we made cytotoxic assays in presence of D-mannose. Whereas D-mannose and GlcNAc did not block the adherence of *E. histolytica* to hepatocytes ($P>0.05$), lactose and D (+) galactose did blocked this binding (**$P<0.001$, *$P<0.01$, *$P<0.05$, Fig. 5). Therefore a direct role for recognition of mannose termini on the surface of both hepatocytes and amoebae was excluded.

The strong binding of GNL to a 170 kDa molecule, demonstrated by western blot analysis of the membrane amoebic lysates as well as of the glycoproteins eluted from the GNL-sepharose column, indicates that this molecule probably corresponds to the *E. histolytica* Gal/GalNAc lectin. Therefore, these results support the alternative idea that GNL binds to terminal α(1–3)-linked mannose residues in the carbohydrate side chains of the well-defined Gal/GalNAc-specific lectin, and by steric hindrance inhibits the adherence to and cytotoxicity to hepatocytes. GNL but not Con A inhibited the adherence of *E. histolytica* to hepatocytes, in spite of the fact that both lectins bind mannose-containing structures and have similar Mr (~50 kDa), and potentially could mask the lectin binding site by steric hindrance. That only GNL, which exclusively binds to terminal α(1–3)-linked mannose residues in N-linked glycans [29,39], which inhibited the adherence and cytotoxicity of *E. histolytica*, suggests that the amoebic lectin contains N-glycans with terminal α(1–3)-linked mannose residues near to the carbohydrate recognition domain. A recent study reported that N-glycans of amoeba contain terminal α(1–3)-linked mannose residues [40]. Furthermore, N-linked glycans in the heavy chain of the galactose-specific lectin play a key role in the adherence of *E. histolytica* to host cells [41].

Finally, the binding of GNL to a glycoconjugate on the amoeba, together with its strict and exclusive specificity for Man (α1–3) Man units has two implications. First, GNL might be used to block binding of *E. histolytica* to the host intestinal epithelium, because the target cells lack terminal mannose residues and GNL is not toxic for epithelial cells [42]. Several studies have shown that GNL selectively inhibits a wide variety of HIV-1 and HIV-2 strains in different cell types by binding to the viral glycoproteins [42–44]. Second, GNL might be used to purify epitopes in amoebic mannose-containing lectins that are involved in adherence, with the aim to design new vaccines. For instance, the outer envelope glycoprotein (gp120) from HIV-1, HIV-2, and SIV purified using a specific high mannose-binding lectin, GNL agglutinin have been used to induce specific immune responses [45].

In summary, our data reveal differences in the expression patterns of surface coat glycoconjugates between *E. histolytica* and *E. dispar*, but not between avirulent and virulent *E. histolytica* trophozoites. They also demonstrate that adherence of *E. histolytica* to hepatocytes is mediated by glycoconjugates containing terminal α(1–3)-linked mannose residues that are recognizable by GNL, and that these glycoconjugates play a role in the adherence to and consequent damage of hepatocytes. One of these glycoconjugates probably corresponds to *E. histolytica* Gal/GalNAc lectin.

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