Rat and Human Colonic Mucins Bind to and Inhibit Adherence Lectin of *Entamoeba histolytica*

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Abstract

Establishment of adherence by *Entamoeba histolytica* is mediated by a 170-kD Gal/GalNAc inhibitable lectin and is required for cytolysis and phagocytosis of mammalian target cells. We studied the biochemical mechanisms of the in vitro interaction between rat and human colonic mucins and axenic *E. histolytica* trophozoites. Crude mucus prevented amebic adherence to Chinese hamster ovary (CHO) cells by up to 70%. Purification of the colonic mucins by Sepharose 4B chromatography, nuclease digestion, and cesium chloride gradient centrifugation resulted in a 1,000-fold enrichment of the inhibitory mucins. Purified rat mucin inhibited amebic adherence to and cytolysis of homologous rat colonic epithelial cells. Oxidation and enzymatic cleavage of rat mucin Gal and GalNAc residues completely abrogated mucin inhibition of amebic adherence. The binding of rat ^{125}I-mucin to amebae was galactose specific, saturable, reversible, and pH dependent. A monoclonal antibody specific for the 170-kD amebic Gal/GalNAc lectin completely inhibited the binding of rat ^{125}I-mucin. Rat mucin bound to Affigel affinity purified the amebic lectin from conditioned medium. Colonic mucin glycoproteins act as an important host defense by binding to the parasite's adherence lectin, thus preventing amebic attachment to and cytolysis of host epithelial cells.

Introduction

Entamoeba histolytica is an enteric protozoan that causes substantial worldwide morbidity and mortality in humans due to its ability to disrupt and invade the colonic mucosa. Invasive intestinal infection may lead to dissemination of trophozoites to the liver or other organs, producing amebic abscesses. However, the vast majority of intestinal infections are asymptomatic and without evidence of tissue invasion (1).

The pathogenesis of amebiasis apparently requires the parasite to colonize the gut, overcome mucosal defense mechanisms, and adhere to and lyse mucosal cells and host leukocytes (2–6). In vitro studies have indicated that the cytopathogenic activity of *E. histolytica* is initiated by adherence to mammalian target cells via a 170-kD Gal/GalNAc[1] adherence lectin (2, 3, 5–7). After adherence, contact-dependent cytolytic mechanisms and amebic phagocytosis of the target cells occurs (3, 5, 8).

Sequential histopathology in a gerbil model of invasive intestinal amebiasis demonstrated that parasites first colonize the gut by attachment to the mucus layer. This is followed by amebic adherence to mucosal epithelial cells (9), depletion of intraepithelial and goblet cell mucin, amebic cytolysis of the surface epithelium, and finally trophozoite invasion into the lamina propria and crypts (9, 10). Studies by Leitch et al. (11) in rat colonic loops revealed that *E. histolytica* trophozoites were rapidly absorbed by mucus, immobilized, and sloughed. In human intestinal amebiasis depletion of mucin is evident in either diffuse mucosal lesions or in acute colitis (12, 13).

Crude colonic mucus contains large-molecular weight mucin glycoproteins and nonmucin components (14–17). Mucins are secreted by goblet cells (14, 15), account for a small percentage of the total mucus layer, and form an integral part of the viscoelastic gel coating the mucosal surface. The colonic mucin macromolecule contains a peptide core and is linked via *O*-glycosidic bonds to oligosaccharide chains rich in galactose and GalNAc (18, 19). Colonic mucus functions as a lubricant, protects the mucosal surface from potentially injurious agents, and supports growth of normal bacterial flora (14, 15). Fractionation of the mucin glycoproteins by sequential Sepharose 4B chromatography and subsequent purification by nuclease digestion and cesium chloride density gradient ultracentrifugation has been well established (15, 16). A homogeneous high-molecular weight (9.0×10^5 D) colonic mucin glycoprotein has been isolated from rats (18, 19).

Colonization of the colon by *E. histolytica* trophozoites is associated with adherence to the constantly renewing mucus blanket; the biochemical mechanisms responsible for this association are unknown. Colonic mucins may facilitate parasite colonization of the colon and also act as a protective barrier to prevent amebic adherence and destruction of colonic epithelial cells. Mucin is important in normal colonic function and defects in mucin content are associated with a variety of disease processes (20–23). Little is known, however, about the functional properties of colonic mucin glycoprotein and the mechanisms by which it serves as a first line of host defense against enteric pathogens.

To elucidate the biochemical mechanisms of interaction between colonic mucin glycoproteins and *E. histolytica*, we studied the effects of rat and human mucin glycoprotein preparations in an in vitro model of the disease and determined the

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; Vo, excluded volume.

interaction of purified colonic mucins with the parasite's adherence lectin. We demonstrated that by a highly specific binding to the Gal/GalNAc adherence lectin of *E. histolytica*, rat and human colonic mucins exert a protective effect inhibiting amebic adherence to and cytolysis of target epithelial cells.

Methods

Preparation of solubilized rat and human colonic mucin glycoproteins. Colonic mucin glycoproteins were prepared from 4–5-wk-old male Wistar rats (Hilltop Laboratories, Dublin, VA) that were fasted for 24 h. Animals were killed by cervical dislocation and the entire colon placed in cold phosphate buffered saline (PBS; pH 7.2). The colon was cut open longitudinally and the mucosal surface gently scraped with a glass slide. The mucosal preparations were pooled and the mucus glycoproteins solubilized by vigorous vortexing for 10 min in cold PBS. The preparation was centrifuged three times (30,000 *g*) at 4°C for 15 min and dialyzed (1,000 D exclusion) extensively against deionized water. The protein concentration was determined by the method of Bradford (24) using bovine serum albumin as standard. Crude mucus was lyophilized and stored at −60°C until further use.

Human colonic tissues (left and transverse colons) were obtained from patients requiring colectomy for localized colonic carcinoma. Mucosal scrapings were taken 5–10 cm on either side of the carcinomas, and histologic examination by Dr. Innes confirmed that only normal mucosa was utilitzed. Specimens were received within 60 min after removal and placed on ice. After vigorous scraping of the colonic mucosa in cold (4°C) PBS, the crude human mucus preparation was obtained as described.

Purification of colonic mucin glycoproteins by Sepharose 4B column chromatography, nuclease digestion, and cesium chloride (CsCl) equilibrium density-gradient centrifugation. Lyophilized crude mucus preparations (10–20 mg) were dissolved in 0.01 Tris HCl, 0.001% sodium azide (Sigma Chemical Co., St. Louis, MO) and applied to Sepharose 4B (1.5 × 30 cm column) (Bio-Rad Laboratories, Richmond, CA) equilibrated in the application buffer. The column flow rate was 20 ml/h; 1-ml fractions were collected and monitored for protein by absorbance at 280 nm and carbohydrate by the phenol-H_2SO_4 method (25) using galactose as standard. Material from the excluded volume (Vo) and included fractions (fractions 27–39, 45–57 for rat and 27–41 for human) were pooled, dialyzed exhaustively against deionized water (4°C), and lyophilized. For large scale preparation of the colonic mucins 90–100 mg of crude mucus was applied to a 2.5 × 50 cm Sepharose 4B column with a flow rate of 12 ml/h. The void volume of the Sepharose 4B column was determined with Blue Dextran ($> 2 \times 10^6$); other molecular weight markers included thyroglobulin (669,000), bovine serum albumin (67,000), and chymotrypsinogen (25,000) (Pharmacia Fine Chemicals, Piscataway, NJ).

The high-molecular-weight mucin glycoprotein (Vo) carbohydrate containing fractions from the Sepharose 4B column chromatography were digested with 100 μg/ml bovine DNase 1 and bovine RNase III (Sigma Chemical Co.) to remove nucleic acid contaminants (26). After 14-h digestion at room temperature in 5.0 ml PBS (pH 7.4, containing 0.02% (wt/vol) NaN_3 and 1 mM $MgSO_4$), the digest mixture was centrifuged at 15,000 *g* for 30 min to remove the resultant flocculent precipitate and the supernatant then dialyzed against PBS (4°C) for 24 h. The dialyzed material was then diluted with PBS and cesium chloride (Sigma Chemical Co.) added to make a 59% (wt/vol) solution (starting density, 1.42 g/ml). The sample (10 ml) was distributed in polyallomer centrifuge tubes (14 × 89 mm; Beckman Instruments, Inc., Palo Alto, CA) and ultracentrifuged in a SW41 rotor at 250,000 *g* for 48 h at 4°C. After centrifugation, the bottoms of the tubes were pierced and eight fractions of equal volume were collected in preweighed test tubes for density determination. Fractions were dialyzed (10,000 D exclusion) exhaustively against deionized water for 24 h and then assayed for protein (24), nucleic acid absorbance at 260 nm, and carbohydrate by the phenol-H_2SO_4 method (25).

Amino acid compositional analysis of rat colonic mucin. Purified mucin glycoprotein (CsCl fraction 6) was hydrolyzed in vacuo for 24 h at 110°C in 6 N HCL and the amino acids composition determined by reverse-phase high-performance liquid chromatography (Waters Associates, Milford, MA) by the method of Heinrikson and Meredith (27) in the Protein and Nucleic Acid Sequencing Center at the University of Virginia.

Cultivation and harvesting of E. histolytica and Chinese hamster ovary (CHO) cells. A cloned population of axenic *E. histolytica* trophozoites (HM1-IMSS) were originally provided by Dr. L. Diamond, National Institutes of Health, Bethesda, MD, and have been maintained in our laboratory for ~ 6 yr. The amebae were grown in TYI-S-33 (Biosate, iron, and serum) medium (28). (Gibco, Grand Island, NY) containing 100 U penicillin and 100 μg streptomycin sulfate/ml. Trophozoites were harvested during log phase growth after 48–72 h as previously described (8).

CHO cells were grown in F12 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 U penicillin and 100 μg streptomycin sulfate/ml. Cells were harvested from confluent cultures with 0.25% trypsin (Gibco), washed, and resuspended in the test medium.

Preparation of rat colonic epithelial cells. Rat colonic epithelial cells were prepared by a modified method of Weiser (29). The colons from fasted (24 h) Wistar rats (6 wk old) were immediately removed after cervical dislocation and flushed three times with a warm (37°C) 0.154 M NaCl, 1 mM dithiothreitol solution (Sigma Chemical Co.) to remove fecal contents and mucus. The colons were then filled with citrate buffer (5 ml) to dissociate the cells (KCl, 1.5 mM; NaCl, 96 mM; sodium citrate, 27 mM; KH_2PO_4, 8 mM; Na_2HPO_4, 5.6 mM, pH 7.3). After being tied at both ends the colons were immersed in PBS (Ca^{2+}, Mg^{2+}-free) (Gibco) solution at 37°C for 15 min. After the 15-min incubation citrate buffer was removed and the colon filled with PBS (Ca^{2+}, Mg^{2+}-free) containing 1.5 mM EDTA and 0.5 mM dithiothreitol at 37°C for 4 min. The PBS buffer containing the suspended epithelial cells was collected in plastic centrifuge tubes. Colons were filled with fresh buffer two to three times and after each 4-min incubation the cells were collected and pooled. Epithelial cells were washed twice in PBS (Ca^{2+}, Mg^{2+}-free) and passed through a pyrex wool column (Corning Glass Works, Corning, NY) to remove clumped and dead cells. Cells were suspended in test medium and kept at 4°C before use. Viability of epithelial cells as determined by trypan blue exclusion criteria was > 95%.

Adherence of amebae to CHO and rat colonic epithelial cells. Amebic adherence to target cells was performed as previously described (5). Amebae were washed in medium 199 (Gibco) containing 5.7 mM cysteine, 25 mM Hepes, and 0.5% BSA (Sigma Chemical Co.) at pH 6.8 (M199s), and 10^5 amebae/ml were incubated with or without mucin preparations for 1 h at 4°C. Amebae were washed to remove unbound mucins and amebae (10^4) and CHO or colonic cells (2×10^5) were suspended in fresh M199s, centrifuged at 150 *g* for 5 min, and incubated at 4°C for 2 h. Rosette formation was defined as the percentage of amebae with three or more adherent target cells, which was determined by counting > 100 amebae per tube (5).

Amebic killing of target rat colonic epithelial cells. Amebic killing of target rat colonic epithelial cells was performed in a pellet assay as previously described (5). Briefly, amebae (10^4) and colonic cells (2×10^5) were suspended in M199s containing 1% heat-inactivated fetal bovine serum with or without the mucin preparations. The cells were centrifuged at 150 *g* for 5 min and incubated at 37°C for 1 h. The viability of colonic cells was determined by trypan blue exclusion criteria and expressed as the percentage of the initial number of live colonic cells.

Oxidative and enzymatic degradation of rat colonic mucin. Treatment with neuraminadase (Sigma Chemical Co.; insoluble enzyme) was performed by suspending the purified rat colonic mucin (fraction 6 CsCl; 5 μg/1 U enzyme) in 0.05 M sodium acetate buffer (pH 5.5) at 37°C for 24 h (19) with constant agitation. The supernatant was dialyzed (10,000 D exclusion) exhaustively against deionized water for 24

h and lyophilized before all further treatments. Metaperiodate oxidation was performed by suspension of 5 μg of mucin in 5 mM sodium metaperiodate (Sigma Chemical Co.), PBS, pH 7.4, at 0°C in the dark for 60 min. Galactose oxidase (Sigma Chemical Co.) was added at 5 U enzyme per 5 μg mucin suspended in PBS and incubated at 37°C for 45 min (30). Enzymatic hydrolysis of the mucins was carried out by incubating the mucins at 37°C for 36 h with β-galactosidase (100 U), β-*N*-acetylhexosaminidase (1 U), and α-L-fucosidase (0.1 U) (all from Sigma Chemical Co.) in 0.5 M sodium citrate buffer, pH 4.0, with constant agitation. All mucin samples were dialyzed (10,000 D exclusion) exhaustively against deionized water for 24 h and lyophilized before use.

Mucin iodination and binding studies. Purified mucin glycoproteins (CsCl fraction 6 rat mucin) were radiolabeled with $Na^{125}I$ (New England Nuclear, Boston, MA) using iodo-beads (Pierce Chemical Co., Rockford, IL) (31) at room temperature for 15 min. The iodinated mucins were isolated by chromatography on Sephadex G25 columns (Isolab Inc., Akron, OH). TCA (Sigma Chemical Co.) (10%) precipitable ^{125}I counts were > 90%. Binding of colonic mucin to amebae was studied in M199s at 10^5 amebae/ml at 4°C. After incubation with the labeled mucin, amebae were sedimented (300 *g*, 5 min at 4°C), the supernatant discarded, and 100 μl M199s added. The cells were layered over oil (0.25 ml, four parts silicon oil [Accumetric Inc, Elizabeth, KY], one part mineral oil [Sigma Chemical Co.]) and microfuged for 1 min (9,000 *g*) (32). The tips of the tubes containing the cellular pellet were cut off with a scalpel and ^{125}I activity counted in a gamma counter (Beckman Instruments, Inc.). Specific binding of mucin to amebae was determined by adding 1,000-fold excess unlabeled rat Vo mucin or galactose (55 mM). In some studies, amebae were first exposed at 4°C for 60 min to an IgM monoclonal antibody (F-14) (1:10 dilution of tissue culture supernatant) specific for the Gal/GalNAc adherence lectin of the parasite or a nonrelevant control antibody supernatant (33).

Polyacrylamide gel electrophoresis of iodinated mucin bound to E. histolytica. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the high-molecular weight mucin glycoproteins were performed with a 10% polyacrylamide gel under reducing and nonreducing conditions according to the method of Laemmli (34). The gels were calibrated with the following prestained molecular weight standards (Diversified Biotech): myosin (200,000), phophorylase B (95,500), glutamate dehydrogenase (55,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), lactoglobulin (18,400), and cytochrome *c* (12,400).

To determine the specific mucin interaction with *E. histolytica,* 1 × 10^5 or 5 × 10^5 amebae were incubated with ^{125}I-mucin (CsCl fraction 6 rat mucin) for 1 h in M 199s at 4°C (50 ng of mucin, 1.5 × 10^5 cpm) with or without galactose (55 mM) or F-14 antibody. After incubation with the ^{125}I-mucin, the amebae were washed three times by centrifugation (300 *g*, 5 min at 4°C) in M199s and amebae were analyzed by SDS-PAGE (10% polyacrylamide gel) followed by autoradiography using Fluoro-Hance (Research Products International, Mt. Prospect, IL) and XAR-5 film (Eastman Kodak Co., Rochester, NY).

Affinity chromatography. Sepharose 4B void volume rat mucins were coupled to Affigel 15 (Bio-Rad Laboratories), following the manufacturer's instructions at a ratio of 1 mg mucin proteins to 2 ml of packed beads in 0.1 M bicarbonate buffer, pH 8.0, at 4°C. The percent of coupling of the Sepharose 4B void material to the beads was between 50 and 70% as determined by Bradford protein assay (24).

Amebic trophozoites (1–2 × 10^7/ml) were metabolically labeled with [^{35}S]methionine (100 μCi/ml) (New England Nuclear) for 4 h at 37°C in methionine-free Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated adult bovine serum (HyClone Laboratories, Logan, UT), 25 mM Hepes and 5.7 mM cysteine, pH 6.9. The culture supernatant (conditioned medium) was obtained by centrifugation at 150 *g* for 5 min at 4°C. The conditioned medium was centrifuged at 250,000 *g* for 30 min at 4°C, adjusted to pH 6.9, and applied to the mucin-Affigel affinity column at a rate of 5 ml/h. The column was washed extensively with 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.35, until no detectable radioactivity was washed from the column. The mucin-Affinity column was then incubated for 30 min with 0.5 M galactose in column buffer and further fractions collected. The galactose-eluted fractions were analyzed by SDS-PAGE followed by autoradiography using Fluoro-Hance (Research Products International) and XAR-5 film (Eastman Kodak Co.).

Statistics. Data are presented as means±standard error of the means. The results were analyzed by one-way analysis of variance and Student's *t* test.

Results

Effect of rat and human colonic mucus on amebic adherence to CHO cells. Adherence of *E. histolytica* trophozoites to CHO cells is mediated by the parasite's Gal/GalNAc lectin (3, 5, 7). Exposure of amebae (10^5) to crude rat (1–300 μg/ml) and human (50–300 μg/ml) colonic mucus preparations for 1 h at 4°C followed by washing resulted in inhibition of amebic adherence to CHO cells in M199s in a dose-dependent manner ($P < 0.05$; Fig. 1). Maximum inhibition of amebic adherence was 70 and 44% at 300 μg/ml of crude rat and human mucus, respectively (Fig. 1).

Fractionation of rat and human crude colonic mucus preparations by Sepharose 4B column chromatography resulted in a high-molecular weight carbohydrate-rich void volume; the lower molecular weight fractions were devoid of carbohydrate (Fig. 2). At 50 μg/ml per 10^5 amebae the high-molecular weight rat and human mucin glycoproteins (Vo fractions 13–18), and not the lower molecular weight included fractions, inhibited amebic adherence to CHO cells ($P < 0.001$; Table I). A dose response for the high-molecular weight colonic mucins demonstrated an ~ 10-fold enrichment of inhibitory activity compared with the crude mucus preparations (Fig. 3). In addition, rat colonic Vo mucin (100 μg/ml) was able to completely inhibit amebic adherence to CHO cells ($P < 0.001$; Fig. 3). Nuclease-treated Vo mucins were no different than untreated mucins in inhibiting amebic adherence to CHO cells ($n = 6$).

The rat and human high-molecular weight mucins obtained from Sepharose 4B column chromatography were purified by nuclease digestion and CsCl density equilibrium centrifugation, resulting in eight glycoprotein fractions with different densities, protein, carbohydrate, and nucleic acid content (Fig. 4). The colonic mucin glycoproteins that inhibited amebic adherence were found exclusively in the high-density, carbohydrate-rich (> 1.50 g/ml) fractions (Table II). The amino acid composition of the material obtained in CsCl gra-

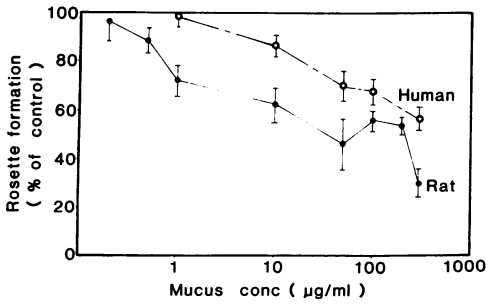

Figure 1. Effect of solubilized crude rat and human colonic mucus on amebic adherence to CHO cells. Amebae (10^5) were exposed to the mucin for 1 h at 4°C and washed before interaction with the CHO cells. Absolute value for rosette formation in control studies was 73±8.10%. At ≥ 10 μg/ml, $P < 0.05$ compared with control.

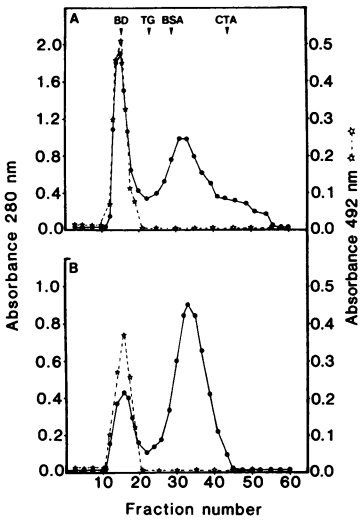

Figure 2. Sepharose 4B column chromatography of solubilized colonic mucus glycoproteins. (*A*) Lyophilized rate (20 mg) or (*B*) human (10 mg) crude mucus was applied to a Sepharose 4B column (1.5 × 30 cm) equilibrated in 0.01 M Tris-HCl, 0.001% sodium azide, pH 8.0, and developed at a rate of 20 ml/h collecting 1-ml fractions under continuous A280 monitoring. Total carbohydrates was determined by the phenol-H_2SO_4 method (25) using galactose as standard (492 nm). Material from the excluded volume (Vo, fractions 13–18) and included fractions (27–39, 43–57 in *A* nd 27–41 in *B*) were pooled and dialyzed exhaustively against deionized water and lyophilized. The column was calibrated with blue dextran (*BD*; $< 2 \times 10^6$ mol wt), thryoglobulin (*TG;* 669,000), bovine serum albumin (*BSA;* 67,000), and chymotrypsinogen A (*CTA;* 25,000) as molecular weight standards.

dient fraction 6 were characteristic of colonic mucin glycoproteins (18, 35) with high concentrations of threonine, serine, glycine, and proline (Table III); threonine and serine accounted for 31% of the total amino acids.

At 1 μg/ml, the purified rat colonic mucins in fractions 6 and 7 (purified mucin fractions) completely inhibited amebic adherence to CHO cells (Table II; $P < 0.001$). Inhibition of adherence was observed with human mucin in fractions 4–8, again with maximum inhibitory activity in fractions 6 and 7 (Table II; $P < 0.001$). A dose response for the effect of the purified rat and human colonic mucins from CsCl fraction 7 on amebic adherence to CHO cells (Fig. 5) indicated almost complete inhibition with ≥ 500 ng/ml, resulting in a 100-fold more inhibitory activity than the Sepharose 4B void volume

Table I. Effect on Amebic Adherence to CHO Cells of Pooled Fractions from Sepharose 4B Column Chromatography of Rat and Human Colonic Mucin Glycoproteins

	Rosette formation*	
Pooled fractions	50 μg/ml rat mucin	50 μg/ml human mucin
	% control	*% control*
13–18	5±0.64‡ (n = 10)	18±1.47‡ (n = 10)
27–39	85±2.46 (n = 20)	99±1.71§ (n = 10)
43–57	96±1.57 (n = 10)	—

* Absolute value for rosette formation in controls was 83±6.14%. ‡$P < 0.001$ compared with control. §Fractions 27–41 (see Fig. 2).

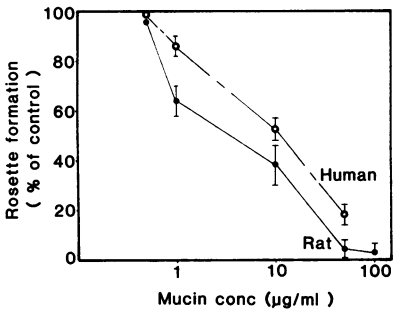

Figure 3. Effect of high-molecular weight rat and human mucin (Vo, Sepharose 4B) on amebic adherence to CHO cells. Absolute value for rosette formation in control studies was 83±6.91%. At 1.0 μg/ml, $P < 0.05$ for both rat and human mucin compared with control, at ≥ 10 μg/ml, $P < 0.001$ for each.

material and 1,000-fold greater activity compared with the crude mucus preparation.

These studies suggested that purified rat and human colonic mucins acted as a high-affinity ligand for the parasite's Gal/GalNAc-inhibitable adherence lectin. Galactose monomers at 55 mM completely inhibit amebic adherence to CHO cells (5). However, we found that ^{3}H-galactose (0.022 μM) (1.7×10^6 cpm/4×10^5 amebae) does not remain bound to amebae after washing three times and that exposure of amebae to galactose (55 mM) at 4°C followed by washing does not alter subsequent adherence of amebae to CHO cells (Table IV). When galactose (55 mM) was present during exposure of amebae to purified rat colonic mucins at 4°C, followed by washing, there was no subsequent inhibition of amebic adherence to CHO cells (Table IV). This finding indicates that ga-

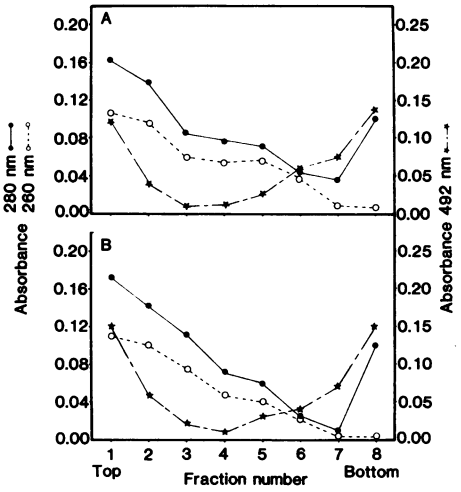

Figure 4. Cesium chloride equilibrium density gradient centrifugation of colonic mucin glycoproteins. (*A*) Lyophilized rat (4 mg) or (*B*) human (4 mg) mucins isolated from void volume Sepharose 4B column chromatography after nuclease digestion was dissolved in PBS containing 59% wt/vol CsCl (starting density of solution, 1.42 g/ml) and centrifuged at 250,000 *g* for 48 h at 4°C. After centrifugation, eight 1-ml fractions were collected. Density after centrifugation ranged from 1.27 g/ml (fraction 1) to 1.57 g/ml (fraction 8) for rat mucins (*A*) and 1.30 g/ml (fraction 1) to 1.60 g/ml (fraction 8) for human mucins (*B*). Fractions were dialyzed exhaustively against deionized water for 24 h and monitored for nucleic acid (260 nm), protein (280 nm), and carbohydrates (phenol-H_2SO_4 method, 492 nm).

Table II. Effect on Amebic Adherence to CHO Cells of Mucin Glycoproteins Obtained by Cesium Chloride Density Centrifugation

Fraction number from CsCl	Rosette formation (n = 6 for each)*	
	1 μg/ml rat mucin	1 μg/ml human mucin
	% control	*% control*
1	95±3.07	100±4.70
2	92±3.83	98±2.53
3	95±3.98	84±7.75
4	92±5.04	68±9.10‡
5	82±4.24	47±7.04‡
6	3±1.76§	12±3.32§
7	0.4±0.51§	10±4.92§
8	46±10.80‡	61±5.08‡

* Absolute value for rosette formation in controls was 80±4.19%. ‡$P < 0.05$. §$P < 0.01$ compared with control.

lactose abrogated binding of inhibitory rat colonic mucins to the amebae. As an osmotic control, glucose (55 mM) did not alter mucin-mediated inhibition of amebic adherence (Table IV).

Effect of rat colonic mucins on amebic adherence to and cytolysis of isolated rat colonic epithelial cells. To more directly study the protective effects of rat colonic mucins, we assayed amebic adherence to and cytolysis of isolated homologous rat colonic cells. Crude rat colonic mucus (300 μg/ml), the high-molecular weight Vo mucin from Sepharose 4B chromatography (100 μg/ml), and purified mucin fraction 7 (from CsCl, 500 ng/ml) inhibited amebic adherence to the rat colonic cells (Table V; $P < 0.01$). All preparations were equally effective at the concentrations studied in preventing cytolysis of rat colonic cells by *E. histolytica* trophozoites at 37°C

Table III. Amino Acid composition of Cesium Chloride Gradient Rat Fraction 6 Mucin Glycoprotein

Amino acid	Residues/1,000 amino acids
Asp	43
Thr	199
Ser	107
Glu	63
Pro	76
Gly	209
Ala	31
Val	37
Cys	30
Met	41
Ile	26
Leu	31
Tyr	17
Phe	19
Lys	33
His	14
Arg	23

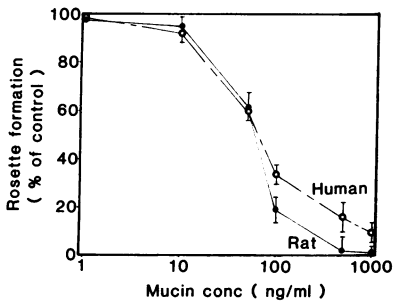

Figure 5. Inhibition of amebic adherence to CHO cells with purified rat and human colonic mucin glycoproteins (fraction 7) obtained from cesium chloride equilibrium centrifugation. Absolute values for rosette formation in control studies was 77±5.55%. At ≥ 50 ng/ml, there was inhibition of amebae adherence, $P < 0.001$ compared with control for each.

(Table V; $P < 0.001$). Pretreatment of the amebae with the mucin preparations at 4°C, followed by washing for removal of unbound mucins, abrogated the mucin-mediated inhibition of cytolysis during a 1-h incubation at 37°C.

Effect of oxidized and glycosidase-treated rat colonic mucin on amebic adherence to CHO cells. To confirm that the galactose-containing carbohydrate residues on the purified rat colonic mucin was responsible for inhibition of amebic adherence, we performed sequential oxidation of glycosidase digestion of the mucin glycoproteins. Neuraminidase treatment had no effect on the ability of rat colonic mucin to inhibit amebic adherence to CHO cells (Table VI). Neuraminidase treatment was the initial step for all further oxidative or glycosidase studies, as this has been demonstrated to make the mucin molecule more susceptible to enzymatic activity of other glycosidases (19, 30). Incubation with galactose oxidase or sodium metaperiodate substantially reduced the ability of neuraminidase-treated rat mucins to inhibit amebic adherence (Table VI; $P < 0.01$). Sequential sodium metaperiodate and galactose oxidase treatment completely abrogated the ability of neuraminidase-treated rat mucins to inhibit amebic adherence to CHO cells (Table VI; $P < 0.001$). Incubation of neuraminidase-treated mucin with β-galactosidase and α-*N*-acetylhexosaminidase also greatly reduced the inhibitory activity (Table VI; $P < 0.001$), whereas neuraminidase-treated mucin exposed to α-L-fucosidase was as effective in inhibiting amebic adherence to CHO cells as control or neuraminidase-treated rat colonic mucin (Table VI).

Table IV. Effect of Galactose on Rat Colonic Mucin-mediated Inhibition of Amebic Adherence to CHO Cells

Amebae pretreatment	Rosette formation after washing amebae (n = 8)*
	% control
Galactose (55 mM)	98±4.42
Mucin‡ (500 ng/ml)	7±3.31§
Mucin (500 ng/ml) + galactose (55 mM)	98±3.90
Glucose (55 mM)	96±3.54
Mucin (500 ng/ml) + glucose (55 mM)	8±2.32

* Absolute value for rosette formation in controls was 78±4.01%. ‡Rat mucin glycoproteins (fraction 7) obtained from cesium chloride equilibrium centrifugation. §$P < 0.001$ compared to galactose or glucose controls. Glucose also served as a control for osmotic effect.

Table V. Effect of Rat Colonic Mucins on Amebic Adherence and Cytolysis of Homologous Rat Colonic Epithelial Cells

Mucin preparations*	Rosette formation ($n = 8$)‡	Percent live colonic cells ($n = 8$)
	% *control*	% *control*
Control medium	100	55±3.72
Crude mucus (300 μg/ml)	18±15.10§	96±7.45§
Vo (100 μg/ml)	20±6.11§	95±6.58§
Fraction 7 CsCl (500 ng/ml)	3±2.00§	90±5.02§

* Mucin concentrations known to maximally inhibit amebic adherence to CHO cells were utilized. ‡Absolute value for rosette formation in controls was 65±5.71%. §$P < 0.001$ compared with control.

The binding of ^{125}I-rat colonic mucins to E. histolytica trophozoites. Specific binding of ^{125}I-rat colonic mucin glycoproteins (CsCl fraction 6) to amebae was studied at 4°C using 1,000-fold excess unlabeled rat colonic mucins (rat Vo) or galactose (55 mM). Galactose appeared to inhibit the binding of rat mucins to amebae as determined by the CHO cell adherence asay (Table IV). The unlabeled mucin ligand or galactose inhibited total binding of ^{125}I-mucins to *E. histolytica* trophozoites by ≥ 90%. Saturation of galactose specific binding occurred at 40 ng/ml of mucin with a total specific binding of 0.105 ng/10^5 trophozoites (Fig. 6 *A*). The time course for the galactose specific binding of ^{125}I-mucin (30 ng/ml) to amebae is illustrated in Fig. 6 *B*; maximum binding occurred by 60 min. The specific binding of ^{125}I-mucins to amebae was pH dependent, being maximal at pH 5.5–7.0 (Fig. 6 *C*). ^{125}I-Mucins did not bind to glutaraldehyde (1.0%) fixed amebae, a treatment known to inactivate amebic Gal/GalNAc lectin binding (2, 3, 33).

Table VI. Effect of Oxidative and Glycosidase Treatment on Rat Colonic Mucin-mediated Inhibition of Amebic Adherence to CHO Cells

Treatment (rat colonic mucins fraction CsCl, 1 μg/ml)	Rosette formation ($n = 5$–8)*
CsCl F6, untreated	4±0.81‡
Neuraminidase (1 U)	5±1.32‡
Neuraminidase plus:	
Galactose oxidase (5 U)	36±4.40§
Sodium metaperiodate (5 mM)	72±5.48‖
Sequential sodium metaperiodate and galactose oxidase	98±1.98‖
β-galactosidase (100 U)	68±2.98‖
β-*N*-Acetylhexosaminidase (1 U)	89±2.71‖
α-L-Fucosidase (0.1 U)	9±4.73‡

* In control studies, exposure of amebae to enzymes alone or enzymes plus the mucin preparation did not affect amebic adherence to CHO cells compared with absolute control study or control CsCl F6, respectively. Absolute value for rosette formation in controls was 84±6.81%. ‡$P < 0.01$ compared with control. §$P < 0.01$ compared with CsCl F6 untreated. ‖$P < 0.01$ compared with CsCl F6 untreated and $P < 0.01$ compared with galactose oxidase.

The ^{125}I-mucin glycoproteins that specifically bound to amebae were eluted off with galactose (55 mM in 1 ml M199s incubated for 5 min at 4°C). Sepharose 4B column chromatography of the eluted mucin revealed the labeled molecules to be exclusively in the high-molecular weight void volume as is characteristic of purified mucin molecules (15, 16) (Fig. 7). In addition, boiling with 1% SDS and 0.2 M β-mercaptoethanol for 5 min had no effect on the galactose-inhibitable binding of ^{125}I-mucins to amebae. Reduction of nonlabeled mucins with β-mercaptoethanol also had no effect on its ability to inhibit amebic adherence to CHO cells (Table VII).

Analysis of the ^{125}I-rat colonic mucin by SDS-PAGE followed by autoradiography under nonreducing conditions (mucins boiled in 1% SDS without β-mercaptoethanol) revealed that radiolabeled proteins did not enter into the separating gel (Fig. 8). However, after reduction with β-mercaptoethanol, a 150-kD iodinated band was evident; high-molecular weight labeled mucins were also present at the top of the gel (Fig. 8). Amebae exposed to the rat ^{125}I-mucins and analyzed under reducing conditions by SDS-PAGE demonstrated that binding of the labeled mucin molecules to amebae was specifically inhibited by galactose or the F-14 monoclonal antibody known to be directed exclusively against the amebic Gal/GalNAc adherence lectin (Fig. 8) (7, 33).

Affinity chromatography for identification of the amebic protein that binds to rat colonic mucins. An affinity chromatography column consisted of rat mucin (Vo from Sepharose 4B chromatography) linked to Affigel 15; 50–70% of the material bound to the Affigel by protein assay. In addition, after 2 h in the coupling buffer, 25% of ^{125}I-mucin (50 ng, 1 ml gel) bound to the Affigel, confirming linkage of purified mucin to affinity substrate. The mucin-Affigel column specifically bound a major 170-kD and two minor lower molecular weight (< 80 kD) ^{35}S metabolically labeled amebic *E. histolytica* proteins from an applied culture supernatant (Fig. 9). The 170-kD protein was not bound to a control Affigel column that did not contain bound colonic mucin. This 170-kD protein has been shown to be the Gal/GalNAc adherence lectin of *E. histolytica* (3, 7). Western blot analysis of the galactose eluate from the mucin affinity column with monoclonal antibody F-14 was positive for the 170-kD protein, confirming its identity as the parasite's adherence lectin.

Discussion

The results of this study demonstrate that purified rat and human colonic mucins bind specifically to the galactose-inhibitable adherences lectin of *E. histolytica.* The binding of colonic mucin proteins to the amebic lectin inhibited the parasite's in vitro adherence to and cytolysis of CHO cells and homologous rat colonic epithelial cells. This study demonstrates a specific biochemical and functional interaction between colonic mucins and an important worldwide enteric parasite.

Crude rat and human soluble colonic mucus inhibited amebic adherence to CHO cells by up to 70%. However, higher concentrations (400–1,000 μg/ml) of rat crude mucus preparations were needed to inhibit amebic destruction of CHO cell monolayers (up to 60%; data not shown). Separation by Sepharose 4B column chromatography demonstrated that the inhib-

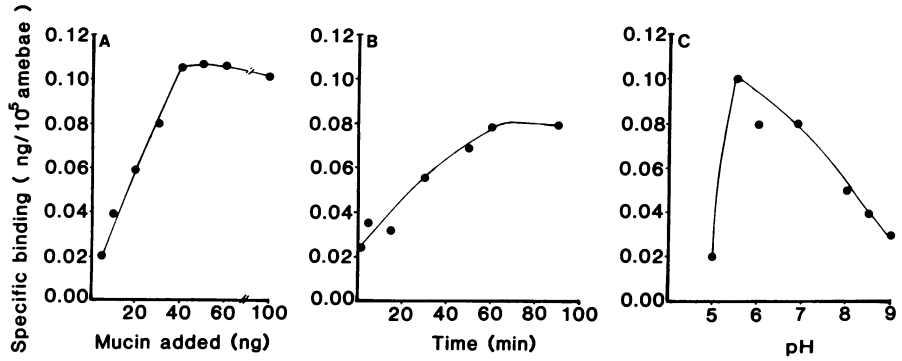

Figure 6. The specific binding of rat ^{125}I-mucin (fraction 7 from cesium chloride gradient) to *E. histolytica* trophozoites (10^5 in 1 ml of M199s) at 4°C. Specific binding was ≥ 90% as determined by addition of 1,000-fold Vo mucins or galactose (55 mM); galactose-specific binding is represented. (*A*) Concentration dependence of galactose-specific binding of rat ^{125}I-mucins at 60 min with saturation evident at ≥ 40 ng. (*B*) Time course of galactose specific binding (30 ng ^{125}I-mucin/ml), maximal binding was present at ≥ 60 min. (*C*) Effect of pH on galactose-specific binding of rat ^{125}I-mucins (30 ng mucin/ml), optimal binding was observed at pH 5.5–7.0.

itory mucin component was in the high-molecular weight void volume. These high-molecular weight proteins accounted for 10 and 1% of rat and human mucus, respectively, and contained > 80% carbohydrate per μg protein. Separation of the noncovalently bound mucin glycoproteins by CsCl equilibrium density gradient ultracentrifugation (36, 37) resulted in further enrichment of inhibitory mucins found predominantly in the high-density, carbohydrate-rich fractions that accounted for < 1% of the purified mucins.

Amino acid composition of CsCl gradient rat fraction 6 confirmed that purified mucin glycoproteins were under study. The high concentrations of threonine and serine (31% of total amino acids) are typical of rat colonic mucins (18, 35). Other workers (18, 35) have emphasized that high concentrations of aspartate and glutamate are characteristic of colonic mucin, and our results are in agreement with this. The high content of glycine in our preparation is noteworthy and is typical of the "linkage" peptides described for pig gastric and human small intestinal mucin glycoproteins (38). Both rat and human mucins purified by CsCl were 100-fold more potent than Sepharose 4B mucins in inhibiting amebic adherence to CHO or rat colonic cells. Crude, Sepharose 4B, and CsCl preparations of rat colonic mucus at correspondingly lower concentrations inhibited amebic lysis of homologous colonic epithelial cells, confirming the protective role of the mucin glycoproteins.

The binding of colonic mucins to amebae was specifically inhibited by galactose as first suggested by the ameba-CHO rosette assay and then demonstrated by studies of the binding to amebae of iodinated purified rat colonic mucins. We utilized this finding to characterize the specific binding of rat colonic mucins to amebae. Galactose-specific elution of bound iodinated mucins from amebae followed by Sepharose 4B chromatography confirmed that we successfully iodinated the high-molecular weight void volume mucin species and that no labeled lower molecular weight native mucus proteins were specifically bound to the amebae. We also identified by SDS-PAGE the labeled mucins that specifically bind to the parasite's surface. Under nonreducing conditions, no labeled proteins entered the gel. After reduction by boiling with β-mercaptoethanol, a 150-kD iodinated protein was recognized as well as labeled proteins not entering the gel. This may be a subunit of rat colonic mucin glycoproteins. It has been found that under nonreducing conditions mucin glycoproteins do not enter as low as 6% acrylamide gels under SDS-PAGE (39). The addition of β-mercaptoethanol to SDS-PAGE of human intestinal mucin glycoproteins results in the presence of lower molecular weight (118 kD) mucin species (38, 39).

The direct finding that galactose and the F-14 monoclonal antibody inhibit the binding of iodinated rat colonic mucins to *E. histolytica* trophozoites indicates that the parasite's Gal/

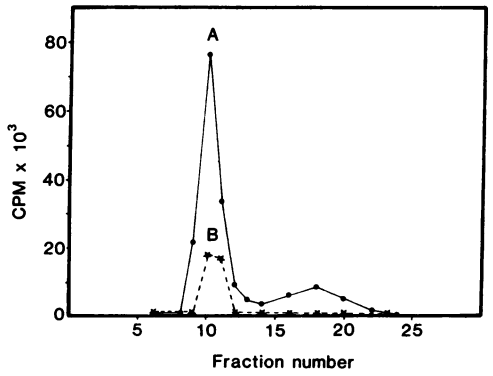

Figure 7. Sepharose 4B column chromatography of (*A*) rat ^{125}I-mucins (fraction 6 CsCl, 50 ng 1.5×10^5 cpm) and (*b*) rat ^{125}I-mucins eluted off from 10^6 *E. histolytica* trophozoites with 55 mM galactose (5 min at 4°C). ^{125}I-mucins bound/10^6 amebae was 1.51 ng (6.5×10^3 cpm); eluted labeled native mucins were found to be exclusively of a high-molecular weight species of Sepharose 4B chromatography.

Table VII. Effect of Reduction by β-Mercaptoethanol on Rat Colonic Mucin-mediated Inhibition of Amebic Adherence to CHO Cells

Rat colonic mucin preparations (fraction 6, CsCl, 500 ng/ml)*	Rosette formation (n = 8)
	% control
Baseline fraction 6	3±2.11
Boiled in 1% SDS for 5 min without BME	8±4.12
Boiled in 1% SDS for 5 min with 0.2 M BME	8±6.02

* Mucin preparations were dialyzed overnight (14 h) in M199s at room temperature after treatments before use. Absolute value for rosette formation in controls was 68±4.04%. BME, β-mercaptoethanol.

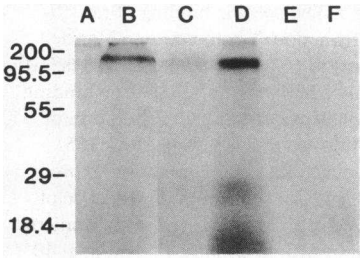

Figure 8. Autoradiograph of SDS-PAGE (10% acrylamide gel) of rat ^{125}I-mucins from CsCl fraction 6 and those which specifically bound to amebae. In lane *A*, ^{125}I-mucins (2 ng, 3 $\times$ 10^4 cpm) are run under nonreducing conditions (sample boiled in 1% SDS without β-mercaptoethanol) and do not enter the separating gel. Lane *B*, ^{125}I-mucins (2 ng, 3 $\times$ 10^4 cpm) are run under reducing conditions (sample boiled for 5 min with 1% SDS and 0.2 M β-mercaptoethanol) and a 150-kD labeled protein as well as the high-molecular weight species is noted. Lanes *C–F* are all run under reducing conditions. Lanes *C* (10^5 amebae exposed to 50 ng ^{125}I-mucins, 1.5 $\times$ 10^5 cpm) and *D* (5 $\times$ 10^5 amebae exposed to 50 ng ^{125}I-mucins) demonstrate binding of the labeled mucin glycoproteins to amebae. This ^{125}I-mucin binding (5 $\times$ 10^5 amebae exposed to 50 ng mucin) is absent if galactose is present (lane *E*) or if amebae were pretreated for 1 h at 4°C with the F-14 monoclonal antibody (lane *F*) which is specific for the amebic Gal/GalNAc adherence lectin (7, 33). Exposure of amebae to an antiamebic monoclonal antibody (G8) which does not recognize the Gal/GalNAc lectin (33) had no effect on mucin binding (not shown).

GalNAc adherence lectin (3, 7) is the surface receptor for the mucin glycoproteins. In addition, we found that affinity chromatography with rat colonic mucins purified the parasite's 170-kD adherence lectin from a culture supernate. Colonic mucins from rat and humans are rich in oligosaccharides that contain GalNAc and galactose (18–20, 40). Therefore, it is reasonable for the mucin molecule to serve as a high-affinity ligand for the Gal/GalNAc adherence lectin of *E. histolytica.* Oxidation and β-galactosidase treatment removed the inhibitory effects of the mucin glycoproteins, further supporting the importance of the mucin GalNAc and galactose residues in mediating binding to amebae. That purified colonic mucins specifically bind to the parasite's surface lectin in vitro is consistent with mucin preventing amebic adherence to and lysis of CHO and rat colonic epithelial cells. This finding strongly supports the conclusion that colonic mucins function in vivo to prevent parasite adherence to and lysis of the colonic epithelium.

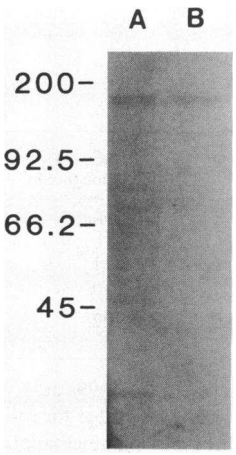

Figure 9. Affinity chromatography of conditioned medium from [^{35}S]-methionine labeled amebae applied to an Affigel 15 rat colonic mucin column. Lane *A*, conditioned medium applied to the column. Lane *B*, autoradiograph of SDS-PAGE of galactose eluate demonstrates the 170-kD adherence lectin (major protein) and two lower (< 80 kD) minor protein bands. The 170 kD amebic protein did not bind to a control Affigel 15 column (not shown).

Studies of the interaction between *E. histolytica* and colonic mucins in vivo have demonstrated that mucus depletion is essential for pathogenesis of invasive disease (12, 13). Binding and subsequent enzymatic degradation (mucinase activity) of the colonic mucin glycoproteins by *E. histolytica* might be a required virulence factor for the parasite. We have recently demonstrated that *E. histolytica* trophozoites induce secretion of rat colonic mucin glycoproteins (41). The protective effect of colonic mucins may account in part for the low frequency of invasive colitis in patients harboring *E. histolytica* (1, 42). However, colonic mucin glycoproteins may facilitate colonization by the parasite thus providing a selective evolutionary bias for amebae to develop a galactose-binding lectin. Colonic mucus could also serve as a food source for the parasite. Strains of *Escherichia coli* that utilize mucus as a source of carbon and nitrogen can also bind mucus proteins and are more effective in colonizing the intestine (43). Colonic mucin may also have a protective role in amebiasis due to its ability to bind released parasite products such as the amebic lectin (3, 7), cytotoxins, and enterotoxins (44–46). Mucins have been shown to bind bacterial toxins and whole bacteria as a protective mechanism against disease (47–49). Recently, a nonmucin component from human small intestine has been shown to increase attachment and survival of *Giardia lamblia* trophozoites in vitro and may serve as a colonizing factor in the gut (50). The interaction between intestinal mucus and other parasite systems requires further study.

As evident by the high specificity for the binding of colonic mucins to the parasite's adherence lectin, secretory IgA antibody directed against the amebic adherence lectin could also serve as a potent host defense against infection by *E. histolytica.* Noninvasive intestinal colonization by *E. histolytica* does not elicit a humoral antibody response nor effective host secretory immunity that eradicates the parasite (42, 51). Patients cured of invasive amebiasis develop a humoral (42) and cell-mediated immune response (42, 52) and are apparently resistant to recurrent colitis or liver abscess (53); the exact host mechanisms responsible for this resistance and the rate of subsequent reinfection (colonization) in immune patients are unknown. Possibly, colonic mucin glycoprotein secretion may be augmented upon reinfection in immune individuals, analogous to the mucus trapping and expulsion noted with helminth infections (54, 55).

In summary, this study demonstrates specific binding of colonic mucin glycoproteins to the Gal/GalNAc-inhibitable adherence lectin of *E. histolytica* trophozoites thus preventing parasite adherence and cytolysis of colonic epithelial cells. Colonic mucins may facilitate amebic colonization but may also absorb and neutralize amebic toxins and prevent parasite invasion.

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