INFECTION AND IMMUNITY, Apr. 1992, p. 1603–1612
0019-9567/92/041603-10$02.00/0

Vol. 60, No. 4

Biochemical Characterization of Rat Colonic Mucins Secreted in Response to *Entamoeba histolytica*

SIL-KING TSE AND KRIS CHADEE*

Institute of Parasitology of McGill University, 21,111 Lakeshore Road, Ste.-Anne-de-Bellevue, Quebec, Canada H9X 1C0

Received 21 October 1991/Accepted 14 January 1992

Invasion of the colonic mucosa by *Entamoeba histolytica* trophozoites is preceded by colonic mucus depletion. The aim of our studies was to determine whether *E. histolytica* caused a differential secretion of mucin species in a rat colonic loop model. Mucus secretion in response to amoebae was followed by release of acid-precipitable ^{3}H-glucosamine metabolically labelled glycoproteins and in vitro labelling of glycoprotein secretion with NaB^3H_4. The secretory response consisted of high-M_r goblet cell mucins and an increase in the secretion of low-M_r nonmucin glycoproteins as determined by Sepharose 4B column chromatography. High-M_r mucins subfractionated by Cellex-E (ECTEOLA) ion-exchange chromatography demonstrated a minor neutral and a major acidic mucin (>98%) species. Marked differences between the neutral and acidic mucin species were indicated by immunogenicity and amino acid compositions. Thin-section histochemistry of rat colons confirmed secretion of neutral and acidic mucins in response to *E. histolytica* and demonstrated secretory activity from goblet cells from both the crypts and interglandular epithelium. *E. histolytica* mucus secretagogue activity was generalized and may function to deplete the host's protective mucus layer, facilitating invasion by the parasites.

Colonic mucus glycoproteins or mucins are produced and secreted by goblet cells and are integral to the viscoelastic gel which overlies the mucosal surface (1, 23). Mucins are large macromolecules composed of a peptide core and oligosaccharide side chains linked via O-glycosidic bonds to serine and threonine residues (22, 32). The integrity of mucins within the gel structure is an essential factor in maintaining normal colonic function as well as resistance to disease.

Structural and immunological analyses performed by several investigators have shown that intestinal mucins can be subfractionated into neutral and acidic glycopeptide species with distinct amino acid and carbohydrate contents and immunogenicities (15, 19, 29, 39). More recently, studies employing cDNA probes (16, 17) also support the concept of the existence of at least two distinct mucin species, suggesting that specific polypeptide backbones which may be subject to differential regulation during the course of disease are synthesized.

A growing body of evidence has emerged to indicate that alterations in mucin composition occur in a variety of disease states (36). What remains unclear, however, is the physiologic relevance of this heterogeneity to the pathology of the intestinal disease process. Histochemical studies demonstrate well-characterized abnormalities in mucin during malignancy and cystic fibrosis and in chronic inflammatory bowel diseases, ulcerative colitis, and Crohn's disease (13, 23, 33, 40). Similar alterations in mucin content (increase in rates of mucin synthesis and qualitative changes in the histochemical composition) have been demonstrated during infection with the intestinal nematode parasite *Nippostrongylus brasiliensis* (18, 36).

Entamoeba histolytica is an enteric protozoan parasite which, through its ability to disrupt and invade the colonic mucosa, causes substantial morbidity and mortality in humans (38). The pathogenesis of invasive amoebiasis involves colonization of the colon, disruption or dissolution of the colonic mucus layer, adherence to and lysis of colonic epithelial cells, and parasite invasion into the mucosa, leading to dissemination of trophozoites to the liver or other organs to form abscesses (7). In experimental animal models of invasive amoebiasis, depletion of mucus in the colon preceded invasion (6, 8). We have recently shown (4) that *E. histolytica* trophozoites exhibit potent mucus secretagogue effects in a rat colonic loop model. The secretion of preformed and newly synthesized mucins was enhanced, and the synthesis of colonic glycoproteins was stimulated. It was suggested that *E. histolytica* mucus secretagogue activity may contribute to depletion or alteration of the protective mucus blanket, facilitating pathogenesis of invasive amoebiasis.

Amoebic adherence is an absolute prerequisite for parasitic cytolytic activity (30) and the initiation of amoebic invasion in the colon (8, 10). In vitro studies have established that adherence of *E. histolytica* to tissue culture cells and colonic epithelial cells is mediated by the 170-kDa heavy subunit of the 260-kDa heterodimer surface galactose–*N*-acetylgalactosamine (Gal-GalNAc) adherence lectin (10, 24). Purified rat and human colonic mucins were shown to bind with high affinity (dissociation constant, 8.20×10^{-11} M^{-1}) to the Gal-GalNAc lectin of *E. histolytica*, which was saturable and specifically inhibited by galactose or galactose-containing oligosaccharides (3). Colonic mucins may therefore function as an important host defense by binding to the amoebic adherence lectin and preventing attachment to host cells (3, 10).

The present study was designed to determine whether *E. histolytica* elicits a differential secretion of neutral and/or acidic mucins in rat colon. Findings which determine the nature of the secretory activity in response to *E. histolytica* may have direct consequences in the pathogenesis of invasive amoebiasis. We demonstrated that amoebae elicit a generalized secretory response consisting of a minor neutral and a major acidic species.

* Corresponding author.

MATERIALS AND METHODS

Cultivation and harvesting of *E. histolytica* trophozoites. The virulent axenic *E. histolytica* trophozoites (strain HM1-IMSS) originally provided by L. Diamond (National Institutes of Health, Bethesda, Md.) have been cultured in our laboratory and passaged twice through gerbil livers. Culture conditions and harvesting of amoebae for study were as previously described (5).

Experimental animals. Male Wistar rats (4 to 5 weeks old; Charles River, Quebec, Canada) each weighing approximately 240 g were used in this study. Laboratory chow and water were available ad libitum. All rats were fasted overnight before experimentation.

Preparation of crude solubilized rat colonic mucus. The following procedure was used to obtain control (unstimulated) mucus glycoprotein samples. Twenty rats were killed with CO_2, and the entire colon was removed and placed in cold Dulbecco's phosphate-buffered saline (PBS; pH 7.2). The colons, briefly lavaged with the same solution to remove the majority of fecal matter, were split open longitudinally, and the mucosal surfaces were scraped vigorously with a glass slide. All mucosal preparations were pooled and dispersed with a ground-glass homogenizer (Mandell Scientific Company Ltd, Guelph, Ontario, Canada) in cold PBS. The preparations were centrifuged (20,000 × *g*), and the supernatant was dialyzed (12,000- to 14,000-M_r exclusion) extensively against deionized water at 4°C. The protein concentrations were determined by the method of Bradford (2), with bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Mo.) as a standard. The dialyzed material was partially lyophilized to reduce the volume and stored at −20°C until further use.

Rat colonic loop studies and [^{3}H]glucosamine labelling of glycoproteins. Rats were injected intraperitoneally with 20 μCi of [^{3}H]glucosamine (specific activity, 40 Ci/mmol; ICN Biomedicals, Inc., Irvine, Calif.) in 0.5 ml of sterile PBS. After 3 h, the rats were anesthetized with ketamine (100 mg/kg) and acepromazine (2.5 mg/kg), and the abdominal cavity was opened. Colonic loops were surgically tied (<10 cm in length) with black silk thread (3-0; Ingram and Bell, Quebec, Canada), leaving the neurovascular supplies intact. The colon 1 cm distal to the cecum and the entire rectum were not included. Immediately following loop isolation, each loop was flushed with 10 ml of warm PBS to remove fecal debris and nonadherent mucus and inoculated with 1 ml of sterile PBS containing no addition (control), purified cholera toxin (CT; 80 μg; Sigma Chemical Co.; 23.1 limit of bluing per mg of protein), or logarithmic-growth-phase (60 to 72 h) *E. histolytica* trophozoites (3×10^6). Three rats were used for each condition in duplicate experiments.

Quantitation of in vivo-secreted mucus glycoproteins. After a 1-h incubation, each loop was separately excised and split open longitudinally. Secreted mucus and lightly adherent mucus were collected by gently scraping the luminal surface with a glass slide and vortexed at high speed (15 to 20 min) in cold PBS. The preparations were centrifuged twice to remove cell debris (1,000 × *g* for 5 min), and supernatant containing the radiolabelled glycoproteins was precipitated with equal volumes of 10% trichloroacetic acid and 1% phosphotungstic acid (10% TCA–1% PTA; Sigma Chemical Co.) for 30 min at 4°C. The acid pellets were harvested by centrifugation (2,000 × *g* for 10 min), solubilized with PBS (pH 7.2), and neutralized to pH 7.0 with 0.1 M NaOH.

In vitro labelling of colonic glycoproteins with NaB[^{3}H]$_4$. In parallel experiments, secreted unlabelled colonic glycoproteins were harvested from PBS-, CT-, and *E. histolytica*-exposed loops and labelled in vitro by sodium metaperiodate oxidation treatment (Sigma Chemical Co.) followed by reduction with NaB[^{3}H]$_4$ as previously described (9, 29). Crude mucus (100 to 200 μg) was suspended in 0.5 to 1.0 ml of PBS (pH 7.4) containing 10 mM sodium metaperiodate and incubated in the dark (0°C) for 1 h. Excess periodate was removed from the samples by dialyzing against PBS (12,000- to 14,000-M_r exclusion) for 12 h. The nondialyzable material then underwent reduction treatment by addition of 5 mCi of NaB^{3}H$_4$ (specific activity, >100 mCi/mmol; NEN, Mississauga, Ontario, Canada) at 37°C for 35 min. Radiolabelled mucins were isolated by Sepharose 4B (S4B) column chromatography (void volume [V_0] material) as described below. Purity of the mucins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7% polyacrylamide gels under reducing conditions as previously described (10), followed by fluorography using En^{3}Hance (NEN) and XAR-5 film (Kodak).

S4B column chromatography. S4B column chromatography separates high-M_r colonic mucins in the V_0 (10, 28). Radiolabelled (^{3}H-glucosamine or NaB^{3}H$_4$) mucus glycoproteins secreted in response to PBS, CT, or *E. histolytica* were subjected to S4B chromatography. Aliquots of ^{3}H activity (counts per minute) were applied to an S4B column (1.0 by 30 cm; Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated in 0.01 M Tris-HCl buffer (pH 8.0) containing 0.001% sodium azide (Sigma Chemical Co.). The column flow rate was 7 ml/h, and 0.5-ml fractions were collected. Five milliliters of scintillation fluid (ICN Biomedicals, Inc.) was added to 100-μl aliquots of each S4B fraction, and the ^{3}H activity was determined for the whole elution profile.

For the preparation of unlabelled mucins, 100 mg of a crude solubilized rat colonic mucus preparation was applied to an S4B column (2.5 by 50 cm), and 4-ml fractions were collected and monitored for protein (280 nm) and carbohydrates by the phenol-H_2SO_4 method (11). Mucins in the V_0 fractions were collected, dialyzed exhaustively against distilled water, and lyophilized. Protein concentration was determined by the method of Bradford (2) with BSA as the standard.

Cellex-E (ECTEOLA) ion-exchange column chromatography. TCA-PTA-precipitable ^{3}H-labelled S4B V_0 mucin was suspended in 0.5 ml of 4 mM sodium phosphate buffer (pH 6.4) and applied to a column (1.0 by 30 cm; Bio-Rad Laboratories) equilibrated in the application buffer at 4°C. The column was eluted with an increasing gradient of elution buffers of 0.05, 0.10, 0.50, and 2.0 M NaCl made in 4 mM sodium phosphate buffer (pH 6.4). Four-milliliter fractions were collected at a rate of 9 ml/h. Aliquots (1 ml) were added to scintillation fluid (5 ml), and the ^{3}H activity for each fraction was determined. To obtain unlabelled mucin species for further analysis, 10 mg of S4B V_0 mucins was applied to a Cellex-E column. Fractions (2 ml) were collected at a rate of 9 ml/h and monitored for protein at 280 nm and for carbohydrates with phenol-H_2SO_4 (11). A single neutral species (eluted with 0.05 M NaCl) and two acidic mucin species (eluted with 0.50 and 2.0 M NaCl) were pooled according to the classification of Wesley et al. (39) for further analysis.

Western blots. SDS-PAGE of the mucus glycoprotein fractions following S4B and Cellex-E ion-exchange chromatography was performed with a 7% separating and a 4% stacking gel under reducing conditions, as previously described (10). Proteins were transferred to a 45-μm-pore-size nitrocellulose membrane (Bio-Rad) by using a Transphore

electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) according to the Western blot (immunoblot) technique of Towbin et al. (35). Additional membrane protein-binding sites were blocked with 10% adult bovine serum (Hyclone Laboratories, Logan, Utah) in blocking buffer (10 mmol of Tris [pH 7.5] per liter, 400 mmol of glycine per liter, 150 mmol of NaCl per liter) overnight (4°C). Normal or immune rabbit anti-rat mucin serum (made against CsCl-purified mucin) diluted 1:1,000 in dilution buffer (50 mmol of Tris per liter, 200 mmol of NaCl per liter) was incubated with the nitrocellulose containing the transferred proteins for 2 h at 22°C. The membrane was then washed three times with washing buffer (10 mmol of Tris per liter, 140 mmol of NaCl per liter, 0.01% Tween 20) and incubated with biotinylated goat anti-rabbit immunoglobulin G (1:1,000 dilution; Bethesda Research Laboratories, Bethesda, Md.) for 1 h at 22°C. After extensive washing in washing buffer, the nitrocellulose was incubated with a 1:500 dilution of streptavidin-horseradish peroxidase (Amersham, Arlington Heights, Ill.) for 1 h, washed five times in washing buffer, and reacted with the color reagent 4-chloro-1-naphthol (Bethesda Research Laboratories) containing methanol and 3% hydrogen peroxide (Sigma) in dilution buffer. The biotinylated molecular weight standards used were the following (Bio-Rad): rabbit muscle myosin (200,000), *Escherichia coli* β-galactosidase (116,250), rabbit muscle phosphorylase *b* (97,000), BSA (66,200), and hen egg white ovalbumin (42,699).

Amino acid compositional analysis. Amino acid analysis was performed on the neutral and acidic mucin species obtained from Cellex-E ion-exchange chromatography as previously described (41). Samples were dissolved in a known volume of water and dried by speed vacuum centrifugation in acid hydrolysis tubes (previously pyrolyzed). The dried samples were hydrolyzed for 2 h at 150°C under an atmosphere of N_2-HCl-H_2O-phenol. The hydrolyzed samples were dried, dissolved in loading buffer (LKB/Pharmacia; sodium citrate, pH 2.2), and analyzed by ion-exchange chromatography–ninhydrin detection employing an Alpha-Plus amino acid analyzer (LKB/Pharmacia) and standard elution conditions.

Histochemical analysis of rat colonic mucin. Experiments for histochemical analysis of rat colonic mucin were conducted in parallel with the ^{3}H-glucosamine labelling studies; the initial step of injecting the isotope was omitted. After the studies were completed, colonic tissues (0.5 cm) were fixed in 10% buffered neutral formalin, processed in plastic and paraffin, sectioned at 1 to 2 μm, and stained with hematoxylin-eosin, periodic acid-Schiff (PAS) reagent, alcian blue (AB), or PAS-AB stain. Photomicrographs were taken with an Olympus BH-2 microscope. Acidic mucins show a blue color by PAS-AB stain; purple indicates a mixture of neutral and acidic mucins, whereas red indicates neutral mucins alone.

RESULTS

Characterization of the secreted mucus glycoproteins. Rats were injected intraperitoneally with ^{3}H-glucosamine, and after 3 h, closed colonic loops were injected with PBS; a known mucus secretagogue, CT (4); or live *E. histolytica* trophozoites. The glycoproteins secreted in response to these conditions for 1 h were quantitated by CsCl density gradient centrifugation, S4B and Cellex-E ion-exchange column chromatography, histochemical staining, immunoblotting using a polyclonal antimucin antibody, and amino acid analysis.

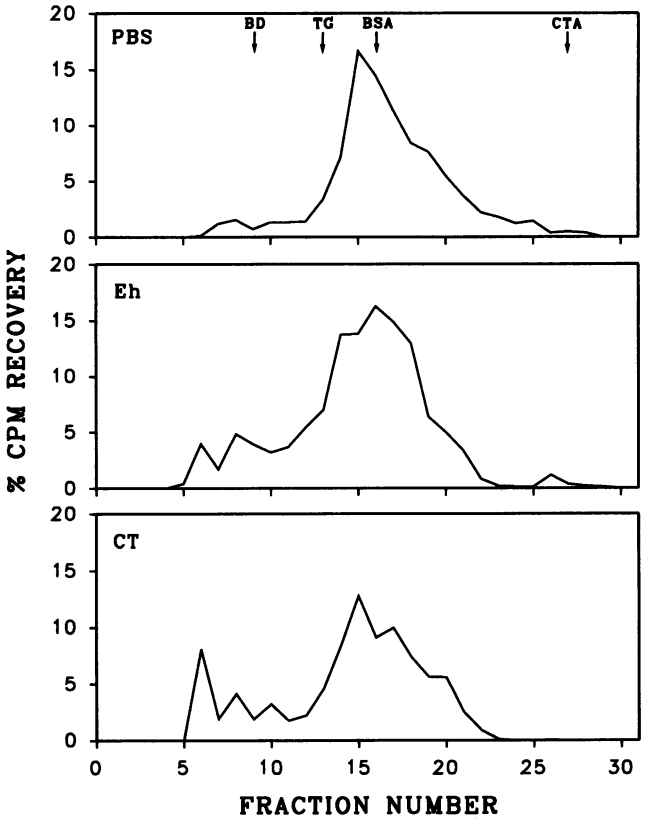

FIG. 1. S4B column chromatography of TCA-PTA-precipitated secreted ^{3}H-glucosamine-labelled rat colonic glycoproteins following saline (PBS), *E. histolytica* trophozoite (Eh), or CT stimulation. For each condition, 2,000 cpm was applied to the column, and total counts per minute were counted for each fraction as described in Materials and Methods. The V_0 of the column was determined with blue dextran (BD; $>2 \times 10^6$ M_r); other M_r markers include thyroglobulin (TG; 669,000), BSA (67,000), and chymotrypsinogen A (CTA; 25,000) (Pharmacia Fine Chemicals, Quebec, Canada) as indicated.

(i) CsCl density gradient centrifugation studies. A CsCl density gradient of acid-precipitated radiolabelled glycoproteins secreted in response to *E. histolytica* and CT compared with that of those secreted in response to the PBS control demonstrated that the majority of the radiolabelled glycoproteins were low-density, nonmucin glycoprotein components (<1.40 g/ml). The levels of mucins in the high-density fractions 6, 7, and 8 (>1.42 g/ml) were low and were insufficient to be used as starting material for further study. We therefore decided to label the crude soluble secreted glycoproteins in vitro by oxidation followed by reduction with NaB^{3}H$_4$. With CsCl density gradient, results obtained were similar to those obtained with in vivo labelling with ^{3}H-glucosamine in that the majority of the radiolabelled glycoproteins were low-density, nonmucin components. All subsequent studies were therefore performed using S4B-purified mucus preparations as the starting material.

(ii) S4B column chromatography. Figure 1 demonstrates a differential partitioning of the in vivo ^{3}H-glucosamine-labelled glycoproteins in the V_0 and included volumes upon S4B chromatography. The radiolabelled glycoproteins se-

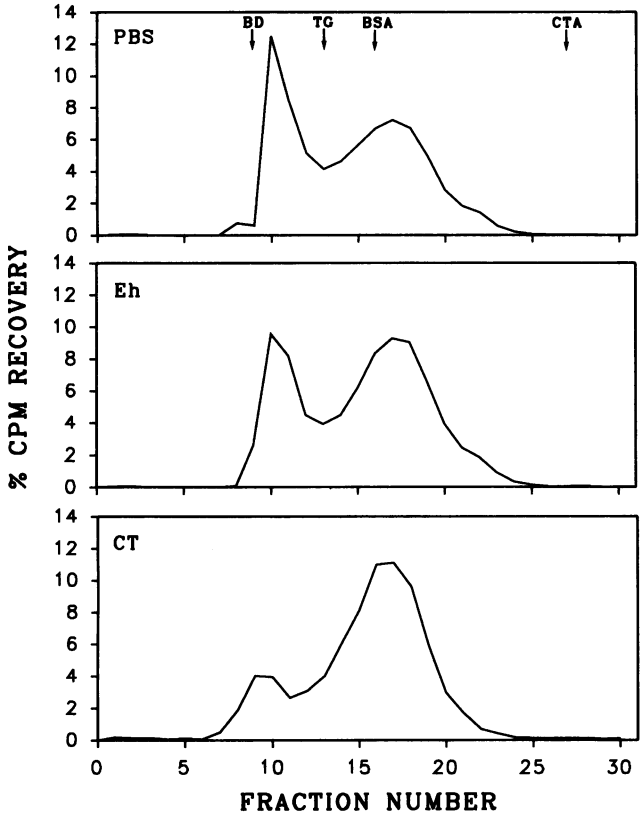

FIG. 2. S4B column chromatography of soluble luminal glycoprotein secretions labelled in vitro with NaB^3H_4 following stimulation by saline (PBS), *E. histolytica* trophozoites (Eh), or CT. For each condition, 500,000 cpm was applied to the column, and total counts per minute were counted for each fraction. M_r standards are as indicated for Fig. 1.

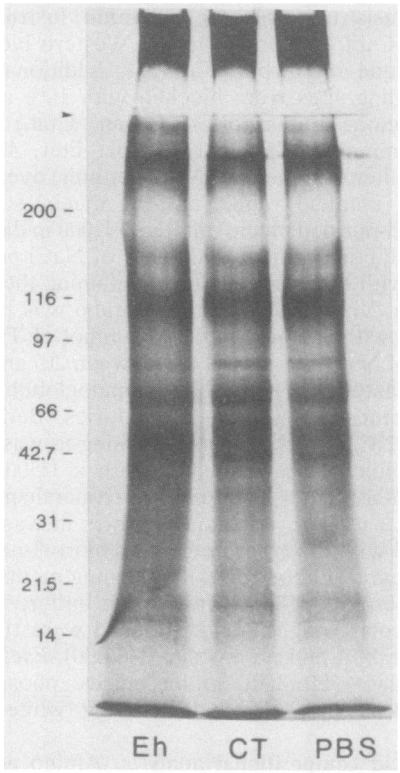

FIG. 3. Fluorograph of SDS-PAGE (7% acrylamide gel) of rat colonic glycoprotein secretions labelled in vitro. NaB^3H_4-labelled glycoproteins (120,000 cpm per lane) obtained from *E. histolytica* (Eh)-, CT-, or PBS-exposed loops were run under reducing conditions as described in the text. The arrowhead indicates the border of the stacking and separating gels. M_r standards used were myosin (200,000), *Escherichia coli* β-galactosidase (116,250), rabbit muscle phosphorylase *b* (97,400), BSA (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400) (Bio-Rad Laboratories).

creted in response to *E. histolytica* and CT contain both mucin (V_0) and nonmucin glycoprotein components, with greater V_0 mucins compared with those in response to PBS. For *E. histolytica*-exposed loops, ^{3}H-glucosamine in the included fractions (13 to 20) eluted as a broad shoulder (67,000 molecular weight).

S4B profiles for the in vitro-labelled glycoproteins with NaB^3H_4 are shown in Fig. 2. In contrast to the in vivo studies, the in vitro-labelled glycoproteins contained a greater amount of high-M_r mucins in the V_0. This was pronounced for PBS- and *E. histolytica*-exposed loops. In both the *E. histolytica*- and CT-exposed loops, the broad shoulder in the included fractions also suggests that a greater proportion of low-M_r glycoproteins are secreted as well. For all three conditions, radiolabelled high-M_r mucins (stacking gel) as well as nonmucin components (separating gel) were demonstrated by SDS-PAGE followed by fluorography (Fig. 3). There were no prominent low-M_r glycoproteins in the running gel for *E. histolytica*- and CT-exposed loops to account for the greater included fractions noted on S4B chromatography elution profiles.

(iii) Cellex-E (ECTEOLA) ion-exchange chromatography. Mucin isolated by S4B column chromatography is a polymer containing different glycoprotein subunits with a heterogeneous oligosaccharide composition. In human as well as rat colons, different mucin species demonstrating distinct amino acid compositions, oligosaccharides, and immunogenicities have been isolated by anion-exchange chromatography (15, 19, 29, 39). On the basis of these features, we wanted to determine whether *E. histolytica* caused a differential secretion of neutral and acidic mucins by using the mucin species classification proposed by Wesley et al. (39).

To determine whether rat colonic mucins can be separated into different mucin species, S4B V_0 mucins from normal rat colons (vigorously scraped) fractionated on a Cellex-E column demonstrated a major neutral (0.05 M NaCl) and two major acidic (0.50 and 2.0 M NaCl) protein peaks with high carbohydrate content (Fig. 4).

S4B V_0 mucins labelled in vivo with ^{3}H-glucosamine or in vitro with NaB^3H_4 were precipitated with TCA-PTA and loaded onto a Cellex-E column. Figure 5 shows the elution profile of ^{3}H-glucosamine-labelled mucin species. For both PBS- and *E. histolytica*-exposed loops, the majority of the mucins were acidic and were eluted with 0.50 and 2.0 M NaCl, respectively. The levels of neutral mucins eluted with 0.05 M NaCl were low in *E. histolytica*-exposed loops and undetectable in the PBS controls. A protein fraction nonadherent to the column initially eluted with the column buffer may be a neutral mucin species. A similar elution profile was noted for CT (data not shown). S4B V_0 mucins labelled in vitro with NaB^3H_4 and loaded onto a Cellex-E column also

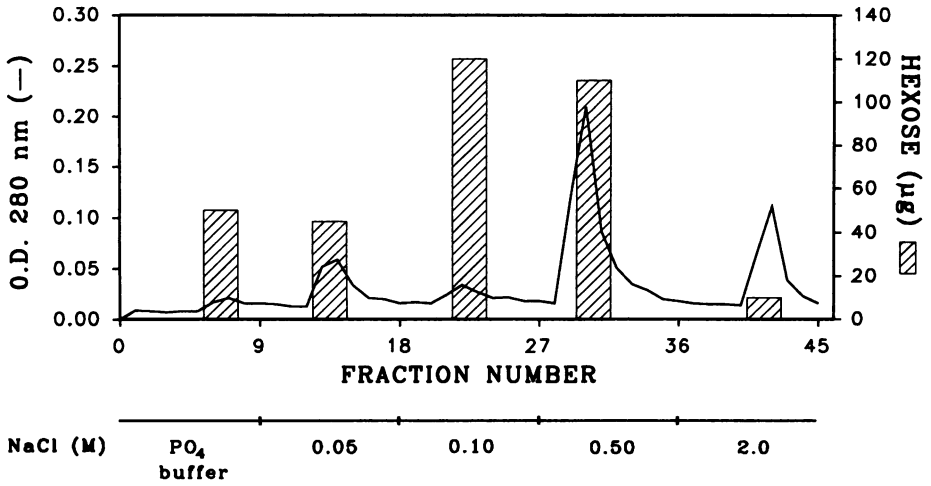

FIG. 4. Cellex-E (ECTEOLA) ion-exchange chromatography of S4B-purified rat colonic mucins. Fractions 1 to 9 were eluted with 4 mM sodium phosphate equilibrating buffer; fractions 10 to 18 were eluted with 0.05 M NaCl; fractions 19 to 27 were eluted with 0.10 M NaCl; fractions 28 to 36 were eluted with 0.50 M NaCl; and fractions 37 to 45 were eluted with 2.0 M NaCl and monitored for protein by measuring the A_{280}. Hexose content was determined by the phenol-H_2SO_4 assay for the following pooled protein peak fractions: 5 to 8, 12 to 15, 21 to 24, 29 to 32, and 40 to 43.

demonstrated that the majority of the mucins secreted in response to *E. histolytica* were acidic with a minor neutral peak (Fig. 6); these results are similar to the in vivo ^{3}H-glucosamine-labelled mucins.

To demonstrate that Cellex-E column chromatography separates distinct neutral and acidic mucin species, a Western blot analysis was performed and compared with that of S4B V_0 mucins. With an antibody made against CsCl-

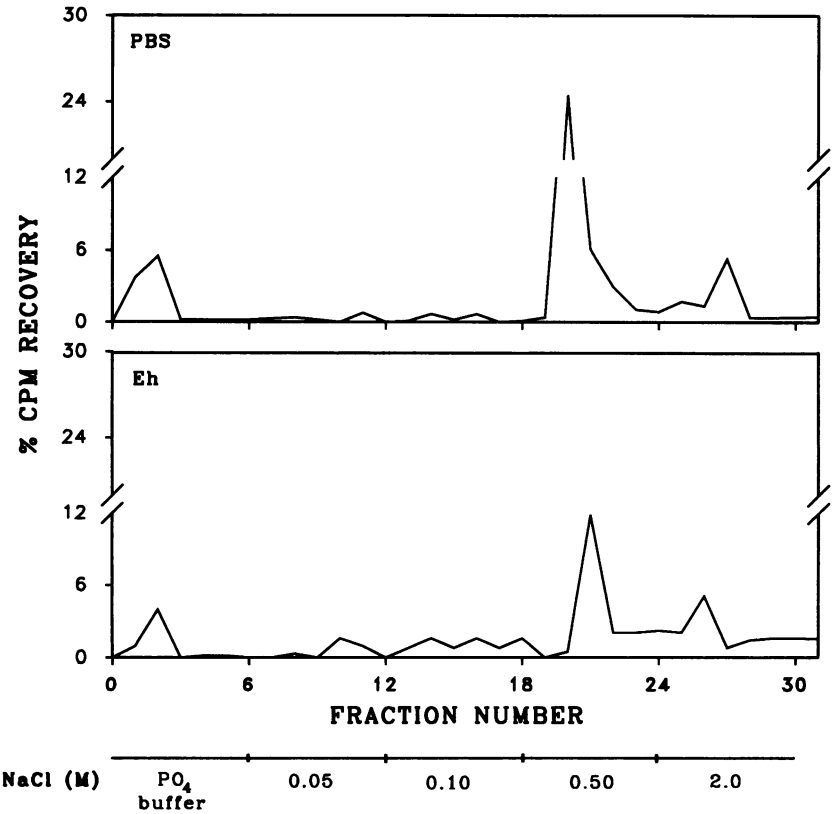

FIG. 5. Cellex-E ion-exchange column chromatography of S4B V_0 ^{3}H-glucosamine-labelled rat colonic mucins isolated from PBS- and *E. histolytica* (Eh)-exposed loops. The column (1.0 by 30 cm) was loaded with 2,000 cpm of TCA-PTA-precipitated mucins, and column fractions were eluted with increasing salt concentrations at a flow rate of 9 ml/h, collecting 4-ml fractions. ^{3}H-activity per fraction was counted by using liquid scintillation.

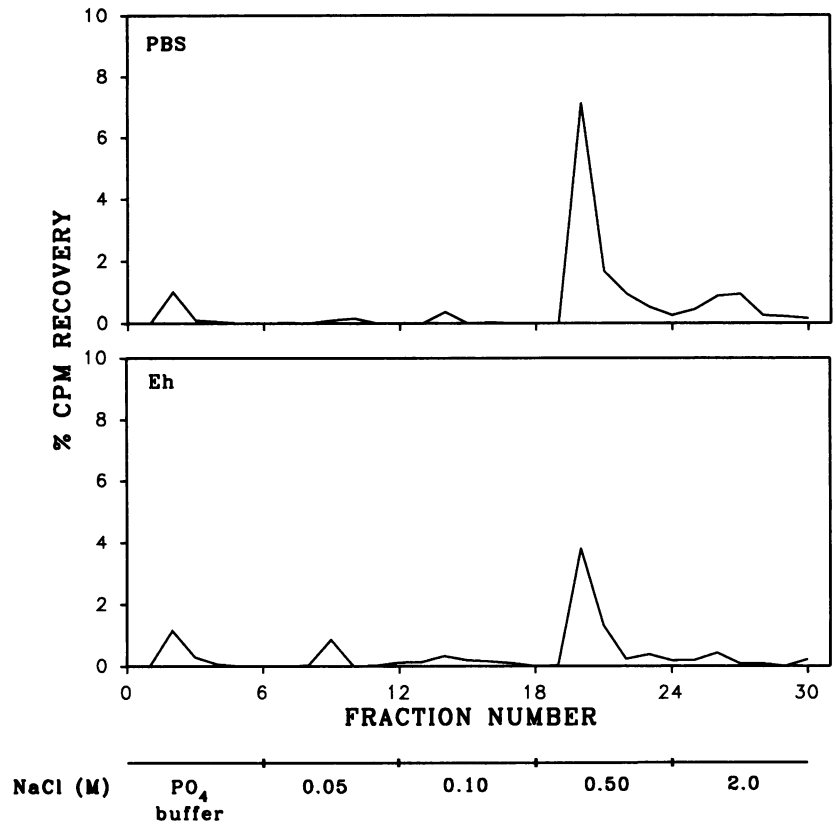

FIG. 6. Cellex-E ion-exchange column chromatography of S4B V_0 NaB^3H_4-labelled rat colonic mucins isolated from PBS- and *E. histolytica* (Eh)-exposed loops. The column was loaded with 20,000 cpm of TCA-PTA-precipitated mucins, and column fractions were eluted under standard conditions as described in the legend to Fig. 5. ^3H-activity was determined for each fraction by using liquid scintillation.

purified rat mucins, immunoreactive material in the stacking gel was noted for both the S4B V_0 and the acidic mucin species, whereas for the neutral mucin, no immunoreactive material was noted in the stacking gel (Fig. 7). However, present in all three lanes is the smear with an approximate molecular mass of 118 kDa, the proposed link peptide subunit of native mucin (31). As noted in earlier studies (12, 20, 31), a 2-mercaptoethanol artifact (false-positive band) is shown at approximately 60 to 65 kDa. Our results indicate that both neutral and acidic mucins are immunogenically distinct and may represent different species.

Amino acid analysis. To further explore the relationship of neutral and acidic mucins as two distinct species, the amino acid compositions were determined; the data are presented in Table 1. The amino acid compositions of the samples were typical of mucin-type glycoproteins, with relatively high concentrations of serine, threonine, glutamic acid, glycine, and aspartic acid (43 to 47%). The neutral species had a higher content of serine, alanine, and lysine, while the acidic species was higher in threonine, glutamine, proline, and valine, which supports the concept of two different mucin species.

Histochemical analysis of secreted mucins. By PAS and AB staining, neutral mucins (red by PAS staining) predominated the glandular (crypt) and interglandular (mucosal surface) epithelium, while acidic mucins (blue by AB) were abundant in the glandular regions of PBS-inoculated rat colons. In *E. histolytica*-exposed loops, mucus secretion was evident by the number of goblet cells with apical protrusions, releasing mucus, or cavitated. By AB staining, acidic mucin secretion was pronounced in the glandular epithelium (Fig. 8A) with numerous cavitated goblet cells and a thick mucus exudate in the crypts. In PAS-stained sections, neutral mucins were abundant in the majority of goblet cells, but secretion was evident in the crypt regions (Fig. 8B). When both AB and PAS stains were used, acidic and neutral mucins (purple) were evident in goblet cell secretions and in the mucus exudates (Fig. 8C), supporting the concept that both mucin types are secreted in response to *E. histolytica*.

DISCUSSION

The aim of the present study was to determine whether the enteric pathogen *E. histolytica* would cause a differential secretion of mucin species that may be important in the pathogenesis of intestinal amoebiasis. Colonic mucins act as high-affinity ligands for the Gal-GalNAc adherence lectin, inhibiting amoebic in vitro adherence and lysis of colonic epithelial cells (3, 10). Depletion or dissolution of colonic mucins is therefore necessary before the parasite can adhere to and invade the epithelial mucosa. How *E. histolytica* depletes or compromises the host's protective mucus layer prior to invasion is not known. Mucinase activity by amoebic proteinases and glycosidases has not been demonstrated; however, amoebae do secrete a heat-stable mucus secretatogue which may facilitate colonic invasion (4). In rat colonic loop studies, the secretory response toward *E. histolytica* trophozoites consisted of high-M_r goblet cell mucins and an increase in the secretion of low-M_r nonmucin glycoproteins (CsCl gradients and S4B profiles). This was

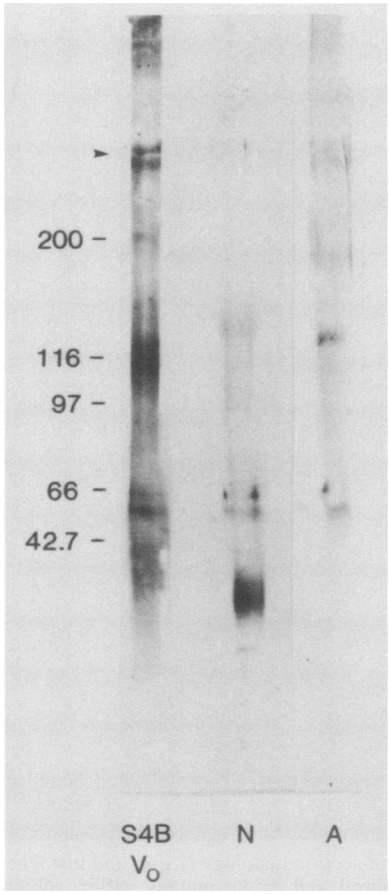

FIG. 7. Western blots of SDS-PAGE (7% acrylamide gel) of the pooled fractions of Cellex-E ion-exchange column chromatography (from Fig. 4) of neutral (0.05 M NaCl), acidic (0.5 M NaCl), and S4B V_0 mucins for comparison. Each lane was loaded with 1 μg of protein boiled in 1% SDS in the presence of β-mercaptoethanol and probed with rabbit anti-rat mucin antibodies (4). The arrowhead indicates the border of the stacking and separating gels. Note the prominent immunoreactive high-M_r mucins in the stacking gel for acidic and S4B V_0 mucins, as compared with neutral mucins.

TABLE 1. Amino acid composition of rat colonic mucins

Amino acid	Mean mol% ± SD[a]		
	In S4B V_0	Cellex-E ion-exchange chromatography of [b]:	
		Neutral species (0.05 M NaCl)	Acidic species (0.50 M NaCl)
Asp	9.59 ± 0.01	10.33 ± 0.45	10.79 ± 0.24
Thr	6.43 ± 0.55	6.62 ± 0.63	8.31 ± 0.11
Ser	7.38 ± 0.60	5.18 ± 0.29	3.82 ± 0.04
Glu	10.85 ± 0.31	10.92 ± 0.61	13.19 ± 0.19
Pro[c]	9.16 ± 0.30	6.73 ± 1.49	10.28 ± 0.18
Gly	8.66 ± 0.50	11.57 ± 0.38	11.25 ± 0.28
Ala	8.68 ± 0.86	12.77 ± 0.77	9.06 ± 0.60
Val	6.85 ± 0.40	6.66 ± 0.09	7.90 ± 0.19
Met	2.07 ± 0.28	1.20 ± 0.38	1.00 ± 0.33
Ile	5.15 ± 0.70	4.45 ± 0.02	4.37 ± 0.36
Leu	8.70 ± 0.42	7.52 ± 0.86	6.87 ± 0.18
Tyr	Nil	Nil	Nil
Phe	3.42 ± 1.27	3.66 ± 0.05	3.21 ± 0.15
His	2.60 ± 0.03	1.93 ± 0.03	1.77 ± 0.09
Lys	6.33 ± 0.14	6.02 ± 0.17	5.73 ± 0.04
Arg	4.02 ± 1.03	4.40 ± 0.11	2.41 ± 0.43

[a] $n = 2$ for each sample.
[b] Pooled neutral (fractions 13 to 17) and acidic (fractions 29 to 34) mucin species from Fig. 4.
[c] Cysteine coelutes with proline.

demonstrated in the secretion following in vivo metabolic labelling with ^{3}H-glucosamine and in vitro by labelling the soluble luminal glycoprotein secretions with NaB^3H_4. Analysis of the secreted ^{3}H-mucins (S4B V_0) by Cellex-E ion-exchange chromatography demonstrated a major acidic mucin (>98%) and a minor neutral mucin species. Both mucin species showed marked differences in immunogenicity and amino acid composition. A generalized secretion of both neutral and acidic mucins was further confirmed by thin-section histochemistry, demonstrating the secretion of mucus by goblet cells in the crypts and interglandular epithelia.

Contrary to earlier findings (14, 22), colonic mucins have been described as highly heterogeneous in nature. Rat colonic mucins purified by S4B and DEAE-cellulose ion-exchange chromatography are a mixture of two related acidic high-M_r mucins and several nonmucin fractions (membrane glycoproteins [19]). Both acidic fractions (IV and V) had similar compositions; however, the major fraction IV was a sulfated glycoprotein with high concentrations of serine and threonine. Gold et al. (15) have analyzed proteolytic digests (pronase) of human colonic mucins on S4B and obtained two components: a fucoacid peptide fraction (enriched in serine and alanine) and a sialoacid peptide fraction (enriched in threonine and proline). Human colonic mucins isolated by DEAE ion-exchange chromatography elute as six distinct species (28, 29). Each species was identifiable by hexose, hexosamine, sialic acid, and sulfate content, as well as blood group activities (25, 37). More recently (39), human small intestinal mucins fractionated on Cellex-E ion-exchange columns generated two separate species: a highly glycosylated neutral species (<1 mol% sialic acid) and a less heavily glycosylated acidic species (>10 mol% sialic acid). The neutral mucin was enriched in serine, alanine, and asparagine, whereas the acidic mucin contained a significant enrichment of threonine, proline, and glycine residues. Similar to the above studies, rat colonic neutral mucins contained more sugars (hexose) than the acidic mucins and were significantly enriched in lysine residues instead of asparagine, whereas the acidic mucin was enriched in glutamine and valine residues instead of glycine. However, similar to human colonic mucins (15, 39), rat neutral and acidic mucins had in common a greater amount of serine and alanine residues and threonine and proline residues, respectively. These results collectively suggest that the intestine produces unique species-specific glycopeptide determinants as well as shared determinants. Recent studies (27) have demonstrated that the human colonic mucosa contains distinct subpopulations of goblet cells that produce distinctive combinations of specific mucin glycoprotein species. Histochemical analysis of colonic sections exposed to *E. histolytica* was not sensitive enough to distinguish whether subpopulations of goblet cells produced only neutral or acidic or a combination of both mucin species.

Recently, molecular evidence involving complementary cDNAs encoding portions of the polypeptide cores of human intestinal mucins (16) revealed that all cDNA clones contained tandem repeats of 69 nucleotides which encoded a threonine- and proline-rich sequence. These repeat units expressed similarities to the acidic fraction of intestinal

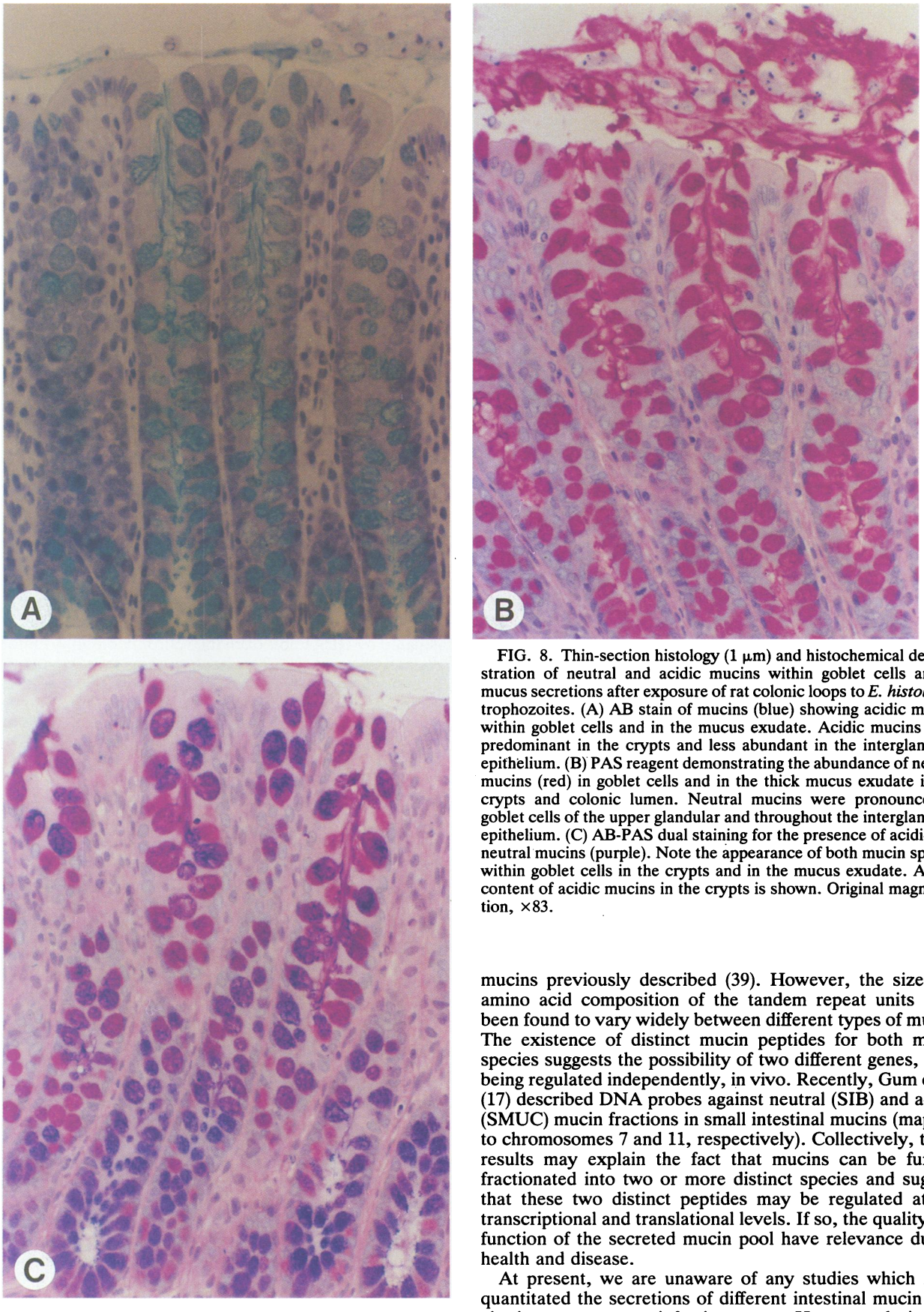

FIG. 8. Thin-section histology (1 μm) and histochemical demonstration of neutral and acidic mucins within goblet cells and in mucus secretions after exposure of rat colonic loops to *E. histolytica* trophozoites. (A) AB stain of mucins (blue) showing acidic mucins within goblet cells and in the mucus exudate. Acidic mucins were predominant in the crypts and less abundant in the interglandular epithelium. (B) PAS reagent demonstrating the abundance of neutral mucins (red) in goblet cells and in the thick mucus exudate in the crypts and colonic lumen. Neutral mucins were pronounced in goblet cells of the upper glandular and throughout the interglandular epithelium. (C) AB-PAS dual staining for the presence of acidic and neutral mucins (purple). Note the appearance of both mucin species within goblet cells in the crypts and in the mucus exudate. A high content of acidic mucins in the crypts is shown. Original magnification, ×83.

mucins previously described (39). However, the size and amino acid composition of the tandem repeat units have been found to vary widely between different types of mucin. The existence of distinct mucin peptides for both mucin species suggests the possibility of two different genes, each being regulated independently, in vivo. Recently, Gum et al. (17) described DNA probes against neutral (SIB) and acidic (SMUC) mucin fractions in small intestinal mucins (mapped to chromosomes 7 and 11, respectively). Collectively, these results may explain the fact that mucins can be further fractionated into two or more distinct species and suggest that these two distinct peptides may be regulated at the transcriptional and translational levels. If so, the quality and function of the secreted mucin pool have relevance during health and disease.

At present, we are unaware of any studies which have quantitated the secretions of different intestinal mucin species in response to an infectious agent. However, during the

course of an infection, neutral and acidic mucin secretion in response to the intestinal nematode parasite *N. brasiliensis* has been documented (18, 21). Intestinal expulsion of the parasites coincided with an increased rate of mucin synthesis (goblet cell hyperplasia) as well as qualitative changes in the histochemical composition of mucins in goblet cells (18). Goblet cells in hyperplastic crypts and villi were shown to contain a marked increase in neutral mucin at 10 days, whereas at 15 days postinfection, an increase in acidic mucins was observed. Quantitative and qualitative changes in mucin synthesis may function to interfere with the physiochemical properties of the mucus layer to render it more protective and aid in the expulsion of worms. However, it is not known whether the changes in mucin composition were a direct response toward the parasites or reflect a response initiated by inflammatory or immune reactions or repair to the tissues. Our studies measured the secretion of mucins at one point in time and showed no clear differences in the secretion of neutral and acidic mucin species based on the elution profiles of Cellex-E ion-exchange chromatography and histochemical analysis.

In comparison with PBS, *E. histolytica* consistently caused the secretion of a minor neutral mucin species eluted with 0.05 M NaCl (Fig. 5 and 6). However, in comparison with the histochemical staining, both neutral and acidic mucins are secreted by goblet cells. In normal unstimulated rat colons, following vigorous scraping of the tissues, a major neutral and acidic mucin species is present and represents total cellular mucin content. However, by ^{3}H-metabolic or in vitro labelling of glycoprotein secretions (mostly nonadherent mucus), only a major acidic mucin species is present. This suggests that neutral mucins may not be a major secretory mucin species or that they may not be a component of newly synthesized mucin. Alternatively, Cellex-E column chromatography may not be an efficient method to quantitate neutral and acidic mucin secretions. Though the significance of mucin heterogeneity remains to be clarified, the relevance of chromatographically distinct mucin species is demonstrated by the observation that the colonic mucosal content of one particular mucin species, species IV, is selectively diminished in specific association with ulcerative colitis (28, 33, 34). It was proposed that mucin species IV is differentially secreted in response to the pathological process rather than retained within the intracellular pools of the mucosa. However, a recent study evaluating colonic mucin composition in monozygotic twins with or without inflammatory bowel disease suggests that even though altered glycoproteins may be genetically determined, such alterations alone were not sufficient to initiate the disease process (37). Such changes in mucin content have not been described in patients with other inflammatory diseases, including Crohn's colitis (28). Abnormalities in intestinal mucus glycoproteins in association with cystic fibrosis include increased concentrations of acidic mucin species (23, 33, 40). As well, histochemical examinations of colonic tumors reveal abnormal patterns of mucus secretion (13, 23). Mucosa adjacent to carcinomas of the colon (transitional mucosa) is often characterized by an increase in sialomucins and a corresponding decrease in or absence of sulfomucins, which predominate in normal mucosa.

In summary, we have shown biochemically, histochemically, and immunologically that both neutral and acidic mucin species occur in the rat colon and that in response to *E. histolytica*, both mucin species are secreted, supporting the idea of a generalized secretory response toward the parasite. Further studies are needed to determine possible specific biochemical alterations during the course of the disease which may lead to imbalances of mucin components. This may have important consequences in the regulation of the physical mucus structure and host susceptibility to amoebic infections. *E. histolytica* mucus secretagogue activity may function to deplete the host's protective mucus lining by causing a generalized secretion of both neutral and acidic mucins.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada MA10187 to K. Chadee. Research at the Institute of Parasitology is partially funded by the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Québec (FCAR).

We thank W. Duguid from the Department of Pathology of the Montreal General Hospital for critical review of the manuscript and K. Keller for expert technical assistance.

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