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Mucin Isolated from Rabbit Colon Inhibits In Vitro Binding of *Escherichia coli* RDEC-1

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The rabbit enteric pathogen *Escherichia coli* RDEC-1 (serotype O15:H−) mediates attaching and effacing binding to colonic epithelium in a manner morphologically identical to that observed in both human enteropathogenic *E. coli* and enterohemorrhagic *E. coli* infections. The aim of this study was to determine if colonic mucus and its constituents, including mucin derived from goblet cells, inhibited RDEC-1 adherence in vitro. Crude mucus was prepared from mucosal scrapings of rabbit colon and separated by buoyant density into eight fractions. Purified mucin was characterized by gel electrophoresis, dot immunoblotting, indirect immunofluorescence, and amino acid composition. RDEC-1 bacteria were grown to promote and suppress the expression of mannose-resistant, hydrophobic pili. A nonpiliated mutant, strain M34, was also used as a negative control. Binding of radiolabeled RDEC-1 expressing pili was quantitated in the presence of crude mucus, purified mucin, and nonmucin fractions. Binding of piliated RDEC-1 to hydrophobic polystyrene wells was greater than for both nonpiliated RDEC-1 and strain M34 ($P < 0.05$). Both crude mucus and purified mucin mediated a concentration-dependent inhibition of piliated-RDEC-1 binding. Fractions of mucus without immunoreactive mucin did not inhibit the binding of RDEC-1 expressing hydrophobic pili. We conclude that colonic goblet cell-derived mucin mediates inhibition of piliated RDEC-1 attachment in vitro. Inhibition of bacterial adherence could prevent access of attaching and effacing *E. coli* enteric pathogens to the colonic mucosa in vivo.

Mucin is a high-molecular-weight glycoprotein synthesized and secreted by specialized epithelial cells (i.e., mucous cells) present within a number of body organ systems including the gastrointestinal tract. Mucin is characterized by its large size (0.5×10^6 to 2.0×10^6 Da), high content of carbohydrates (>70% [wt/wt]), and O-glycosidic bonds between *N*-acetylgalactosamine and either serine or threonine residues in the peptide backbone (29). Mucin is the major organic constituent of mucus, and its concentration is of primary importance in determining the physical and gel-forming properties of mucus (26, 29).

Mucus covers most of the surface of the gastrointestinal tract. Thus, it is interpositioned between the luminal contents of the intestine and the epithelial cells of the host. Mucus is proposed to have a number of functions including cytoprotection of the stomach (1), lubrication of intestinal contents (14), and protection against infection (29). Mucus may protect the underlying mucosa by a number of mechanisms, including prevention of adherence and invasion by enteric pathogens. Adherence of bacteria to epithelial cells is a complex process involving both specific and nonspecific interactions (6) that are crucial for development of toxin delivery, colonization, and subsequent invasion (3).

We have previously shown that after infection of rabbits with a noninvasive, nonenterotoxigenic rabbit enteropathogen, *Escherichia coli* RDEC-1 (serotype O15:H−) (7), the organisms are present in the mucous layer overlying surface epithelial cells of the small intestine (34). RDEC-1 expressing pili also bind to crude mucus and purified mucin of rabbit ileum which have been immobilized on polystyrene wells (13). Although RDEC-1 adheres to enterocytes and colonizes mucosa in the ileum, these organisms colonize the surface mucosa of cecum and colon to a greater extent (35). Therefore, in this study we examined the inhibition of in vitro binding of RDEC-1 by colonic mucus and its constituents, including purified mucin.

MATERIALS AND METHODS

Preparation of soluble rabbit colonic mucus. Colonic mucus was obtained from male New Zealand White rabbits (Reimans Fur Ranch, Toronto, Canada) weighing approximately 3 kg. Animals were sacrificed using intracardiac Euthanyl (MTC Pharmaceuticals, Cambridge, Canada) after intramuscular anesthesia with ketamine and xylazine. A midline abdominal incision was made to open the abdominal cavity, and the colon was removed. The excised colon was washed with normal saline at 4°C and opened along the longitudinal axis. Mucosal scrapings were obtained by gently scraping the luminal surface with a glass slide. The scrapings were immediately weighed and placed into a solution of proteinase inhibitors (100 ml/g [wet weight]) to minimize proteolytic degradation of native mucins (26). The proteinase inhibitor solution contained 5 mM EDTA (Sigma Chemical Co., St. Louis, Mo.), 5 mM *N*-ethylmaleimide (Sigma), 2 mM phenylmethylsulfonyl fluoride (Sigma), and 0.01% sodium azide (Sigma). Scrapings were then homogenized in a Waring blender for 30 s, and the homogenate was centrifuged at $30,000 \times g$ for 30 min at 4°C to remove pelleted cellular and particulate debris. The soluble supernatant, defined in this study as the crude colonic mucous preparation, was stored at −70°C until use in subsequent assays.

Separation of crude mucous components. Crude colonic mucus was subdivided into its components by buoyant density using isopycnic ultracentrifugation in cesium chloride, as described by Mantle and Allen (25). Crude mucus was suspended in a solution of cesium chloride (Toronto

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Research Chemicals, Toronto, Canada) to a starting density of 1.41 g/ml. The suspension was placed into polyallomer Quick Seal centrifuge tubes (25 by 89 mm; Beckman Instruments, Inc., Palo Alto, Calif.) and centrifuged in a 50.2 Ti rotor at 105,000 × *g* for 48 h at 4°C. After centrifugation, a needle was inserted to the bottom of the centrifuge tube and eight fractions of equal volume were collected by using a peristaltic pump (Pharmacia Canada, Inc., Baie D'Urfé, Canada). The eight fractions were analyzed with a refractometer (Abbe 3L; Milton Roy Co., Rochester, N.Y.) to determine buoyant density. Fractions were placed into wetted cellulose dialysis tubing (50,000-Da exclusion; Spectrum Medical Instruments, Inc., Los Angeles, Calif.) and dialyzed against deionized water for 48 h at 4°C. Total protein in each fraction was determined by the method of Lowry et al. (22). Glycoprotein content of each fraction was determined by a periodic acid-Schiff assay, as previously described (24). The low-density fractions containing nonmucin proteins, which have a high protein-to-glycoprotein ratio, and the high-density fractions with DNA material, which have both low protein and low glycoprotein contents, were stored at −70°C for later use. Samples which demonstrated high glycoprotein content in comparison to total protein were contained in the middle-density fractions. These fractions were pooled and subjected to a second ultracentrifugation in cesium chloride. Those fractions with a high glycoprotein-to-protein ratio after the second ultracentrifugation were pooled for use in this study as purified colonic mucin. All steps in the preparation of crude mucus and its components were performed at 4°C. Ileal mucin was prepared in an identical manner with the distal 20 cm of rabbit small intestine, as described elsewhere (13). Purified colonic mucin was reduced by being boiled for 3 min in 2.5% mercaptoethanol and alkylated by subsequent incubation in 0.5% iodoacetamide, as described previously (12).

Compositional analysis of rabbit colonic mucin. Amino acid analysis was performed at the Amino Acid Analysis Facility at The Hospital for Sick Children, using the Waters Pico-Tag System (Waters Associates, Milford, Mass.), which employs a Pico-Tag column (3.9 mm by 15 cm). Briefly, purified goblet cell-derived colonic mucin was hydrolyzed by a vapor-phase reaction using 6 M hydrochloric acid with 1% phenol at 110°C for 24 h. After hydrolysis, excess HCl was removed from the reaction vial under vacuum and the sample was redried with a solution consisting of methanol, water, triethylamine, and phenylisothiocyanate (PITC) (7:1:1:1). The derivatizing solution was then removed under vacuum, and the sample was redried to remove any traces of PITC (5, 17). The derivatized sample was dissolved in a fixed volume of sample diluent (pH 7.4) and placed onto a reverse-phase high-pressure liquid chromatograph (Waters) at 39.5°C. Amino acids were detected at 254 nm at an absorbance unit full-scale range of 0.01.

Antibody to rabbit colonic mucin. Polyvalent antiserum was produced by the subcutaneous injection of a 900-g male Hartley guinea pig with purified colonic mucin (30 μg of protein) together with Freund's complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.). Subcutaneous injections of the same antigen (30 μg of protein) with Freund's incomplete adjuvant (GIBCO) were repeated at 2- and 6-week intervals thereafter. Immune serum was obtained by venous catheterization under general anesthesia 2 weeks after the final antigenic challenge. Antibody to goblet cell-derived colonic mucin was semipurified from serum by precipitation in ammonium sulfate, dialysis, and centrifugation (16).

The reactivity of antibody in guinea pig immune serum was determined by dot blot immunoassay, as described by Towbin and Gordon (38). Briefly, 2.5 μg of mucin protein was applied as a spot onto nitrocellulose paper and allowed to air dry for 1 h. The paper was then incubated with 3% bovine serum albumin (Sigma) in 10 mM Trizma base (Sigma) with 0.9% saline at pH 7.4 for 1 h at 37°C. After being washed with Tris-saline, guinea pig antiserum was added in serial dilutions ranging from 1:100 to 1:100,000 and incubated on the paper overnight at 4°C. Guinea pig preimmune serum at a dilution of 1:500 was used as a negative control. After being washed with Tris-saline at room temperature to remove unbound antibody, a 1:5,000 dilution of protein A-peroxidase (Sigma) was added to the nitrocellulose strips and incubated for 1 h at room temperature. Horseradish peroxidase color development reagent containing 4-chloro-1-naphthol (Bio-Rad, Richmond, Calif.) was then added to develop any positive reaction. Dot blot immunoassay was performed in an identical fashion for crude mucous components; i.e., 2.5 μg of protein from each fraction was applied as a spot onto nitrocellulose paper and air dried. For these experiments, guinea pig immune serum was added at a dilution of 1:1,000.

Indirect immunofluorescence. Segments of rabbit colon were quick frozen in liquid nitrogen and stored at −70°C. Sections were cut 5 μm thick with a cold microtome and fixed onto microscope slides by using acetate. After being warmed to room temperature and washed with phosphate-buffered saline (PBS), the microscope slides were incubated for 30 min at room temperature in a 1:20 dilution of guinea pig anti-rabbit colonic mucin serum. The microscope slides were again washed with PBS, and a 1:20 dilution of fluorescein isothiocyanate conjugated to rabbit anti-guinea pig immunoglobulin G antibody (Miles Scientific, Naperville, Ill.) was added. The microscope slides were then incubated in darkness for 30 min at room temperature, washed with PBS, and examined by fluorescence microscopy. Control slides were stained with equal dilutions of fluorescein isothiocyanate conjugate alone, preimmune guinea pig serum, or heterologous antibody (15).

ELISA for rabbit mucin. Immunogenicity of rabbit colonic mucin was examined by using a sandwich-type enzyme-linked immunosorbent assay (ELISA). Briefly, wells of a 96-well Immulon-2 microtiter plate (Dynatech Laboratories, Inc., Alexandra, Va.) were coated by overnight incubation at 4°C with 0.5 μg of protein from colonic mucin antibody in 0.1 ml of coating buffer (0.015 M sodium carbonate anhydrous [Fisher Scientific Corp., Fairlawn, N.J.] and 0.035 M sodium bicarbonate [Sigma; pH 9.6]). Wells were emptied and washed three times with 0.2 ml of PBS (pH 7.6) containing 0.05% (vol/vol) Tween 20 (Sigma) (PBS-T). Remaining binding sites were then blocked by incubating each well with 0.2 ml of 5% bovine serum albumin (98 to 99% pure; Sigma) in PBS-T for 1 h at room temperature. After wells were washed with PBS-T, purified goblet cell-derived small-intestine and colonic mucins were diluted in PBS-T, and 0.1 ml of the diluted mucins was added to each well. Mucin, as antigen, was incubated for 2 h at room temperature. After the wells were again washed three times with PBS-T, 0.1 ml of a 1:500 dilution of mucin antibody-horseradish peroxidase conjugate in PBS-T was added to each well and incubated for 2 h at room temperature. Antimucin antibody was conjugated to horseradish peroxidase by periodate oxidation (2). Unbound antibody conjugate was removed from wells by washing them four times with PBS-T. The amount of antibody-horseradish peroxidase conjugate bound to mucin antigen in each well, which was a measure of

the amount of mucin bound to the coating antibody, was determined by colorimetric estimation of the horseradish peroxidase concentration with an automated microplate reader (MR600; Dynatech Laboratories, Inc., Chantilly, Va.) at a wavelength of 490 nm. All assays were read against control wells in which PBS-T replaced rabbit colonic mucin during the incubation of antigen. The ELISA was run in triplicate wells for each sample and for standards.

Polyacrylamide gel electrophoresis of rabbit colonic mucin. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of colonic mucin was performed under nonreducing conditions by the method of Laemmli (20) with a vertical slab gel system (1.5 mm by 16 cm by 20 cm; Bio-Rad). The separation gel contained 7.5% acrylamide, and the stacking gel contained 4% acrylamide. Mucin samples (10 μg of protein) were mixed in an equal volume of 10% SDS buffer and electrophoresed through both stacking and separating gels at 6 mA of constant current for 15 h. The gel was stained with silver (Bio-Rad silver stain kit) by the method of Merril et al. (27). Gels were calibrated with known high-molecular-weight standards (Bio-Rad).

Bacteria and growth conditions. Stock cultures of RDEC-1 (serotype O15:H−) were held on Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.) at 4°C. Organisms were grown in static, nonaerated Penassay broth (Difco Laboratories, Detroit, Mich.) to promote the expression of mannose-resistant pili, previously designated AF/R1 (4). Expression of pili was confirmed by slide agglutination of bacteria using AF/R1 pilus-specific antiserum (36) and by transmission electron microscopy after negative staining of organisms with 2% phosphotungstic acid (12, 33). RDEC-1 was grown in brain heart infusion broth to suppress AF/R1 pilus expression (10). Strain M34 (kindly provided by M. Wolf and E. Boedeker, Walter Reed Army Institute of Research, Washington, D.C.) is a mutant of RDEC-1 in which Tn*5* insertions into the plasmid coding for AF/R1 pili result in loss of pilus expression (41). Outer membrane profiles of piliated RDEC-1 and M34 are similar after growth of organisms in Penassay broth in both the presence and absence of an iron chelator (12). Therefore, in this study, M34 was grown in Penassay broth.

Bacteria were radiolabeled by supplementing 10-ml broth cultures with 150 μCi of sterile [^{3}H]thymidine (specific activity, 15.7 Ci/mmol; New England Nuclear, Boston, Mass.). Broth cultures were incubated overnight at 37°C and harvested by centrifugation at 2,500 × *g* for 15 min. After two washes in sterile PBS (pH 7.6) at 25°C to remove nonincorporated isotope, bacteria were resuspended in PBS at a concentration of 10^{10}/ml.

Bacterial adherence assay. Adherence of RDEC-1 and M34 was determined by using a modification of the in vitro method first described by Laux et al. (21). Various amounts of radiolabeled bacteria suspended in 0.35 ml of PBS were added to wells of a 96-well polystyrene tissue culture plate (Immulon-2; Dynatech). After incubation for 1 h at 37°C, nonadherent bacteria were removed by three washings with sterile PBS. To remove adherent bacteria, 0.35 ml of 1% SDS (Bio-Rad) was added to the wells and incubated for 3 h at 37°C. Samples were then removed and placed in 10 ml of scintillation fluid (Aquasol-2; New England Nuclear), and extracted radioactivity was determined by counting disintegrations per minute in a beta scintillation counter (model LS7500; Beckman). To determine incorporated radioactivity per bacterium, 10^9 bacteria in 0.1 ml were also placed in scintillation fluid and measured for radioactivity during each assay. In preliminary experiments, we compared the radioactivity in intact wells with results obtained using detergent extraction. SDS consistently removed 80% of radioactivity bound to the polystyrene wells (12). All samples were run in triplicate, and quenching was constant among samples.

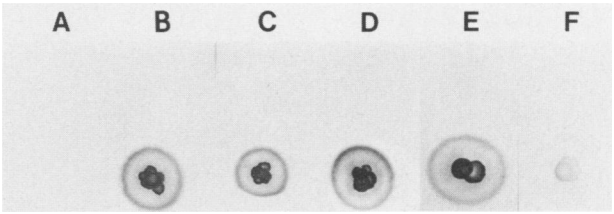

FIG. 1. Dot blot immunoassay of immune serum against purified rabbit colonic mucin. Purified colonic mucin protein (2.5 μg) was blotted onto nitrocellulose. A 1:500 dilution of guinea pig preimmune serum was added to panel A. Guinea pig immune serum was added in dilutions of 1:100 (B), 1:500 (C), 1:1,000 (D), 1:10,000 (E), and 1:100,000 (F). After washings, bound antibody was detected as described in Materials and Methods.

To examine the inhibitory effects of crude colonic mucus and its constituents on bacterial adherence, the preparations were preincubated for 5 min at 25°C with radiolabeled bacteria, and 0.35 ml of the mixture was then added to the microtiter wells. The adherence assay then proceeded exactly as described above.

Statistics. Results are expressed as means ± standard errors unless otherwise indicated. Comparisons between multiple groups were done by using the Fisher protected least-significant-difference one-factor analysis of variance (ANOVA) at 95% confidence intervals (28, 40).

RESULTS

Components of crude mucus. The density gradient obtained after the first ultracentrifugation ranged from 1.244 to 1.588 g/ml. There was a similar gradient after the second ultracentrifugation in cesium chloride (range, 1.248 to 1.580 g/ml). The densest fractions (fractions 1 and 2) were low in both protein and glycoprotein content. The least dense fractions (fractions 6, 7, and 8) showed a high protein-to-glycoprotein ratio. In contrast, the middle-density fractions (fractions 3, 4, and 5) had an elevated glycoprotein-to-protein ratio. These middle-density fractions (density around 1.41 g/ml) were pooled and underwent a second ultracentrifugation. The fractions with the highest glycoprotein-to-protein ratio after the second ultracentrifugation (fractions 3 and 4; data not shown) were pooled for use in this study as the purified colonic mucin preparation.

Purified rabbit colonic mucin. When purified colonic mucin (10 μg of protein) underwent SDS-polyacrylamide gel electrophoresis and the gel was stained with silver, none of the mucin sample entered the separating gel (data not shown). This finding is consistent with the presence of high-molecular-weight mucin and the absence of low-molecular-weight contaminants (19).

Dot blot immunoassay performed with rabbit colonic mucin as antigen and guinea pig antiserum showed that immune serum contained an antibody to mucin which was immunogenic even at a dilution of 1:100,000 (Fig. 1). Preimmune guinea pig serum was not immunogenic.

Indirect immunofluorescence demonstrated that guinea pig immune serum raised to purified mucin contained antibody that localized to vacuoles of goblet cells in rabbit colonic mucosa (Fig. 2A). In contrast, sections of rabbit

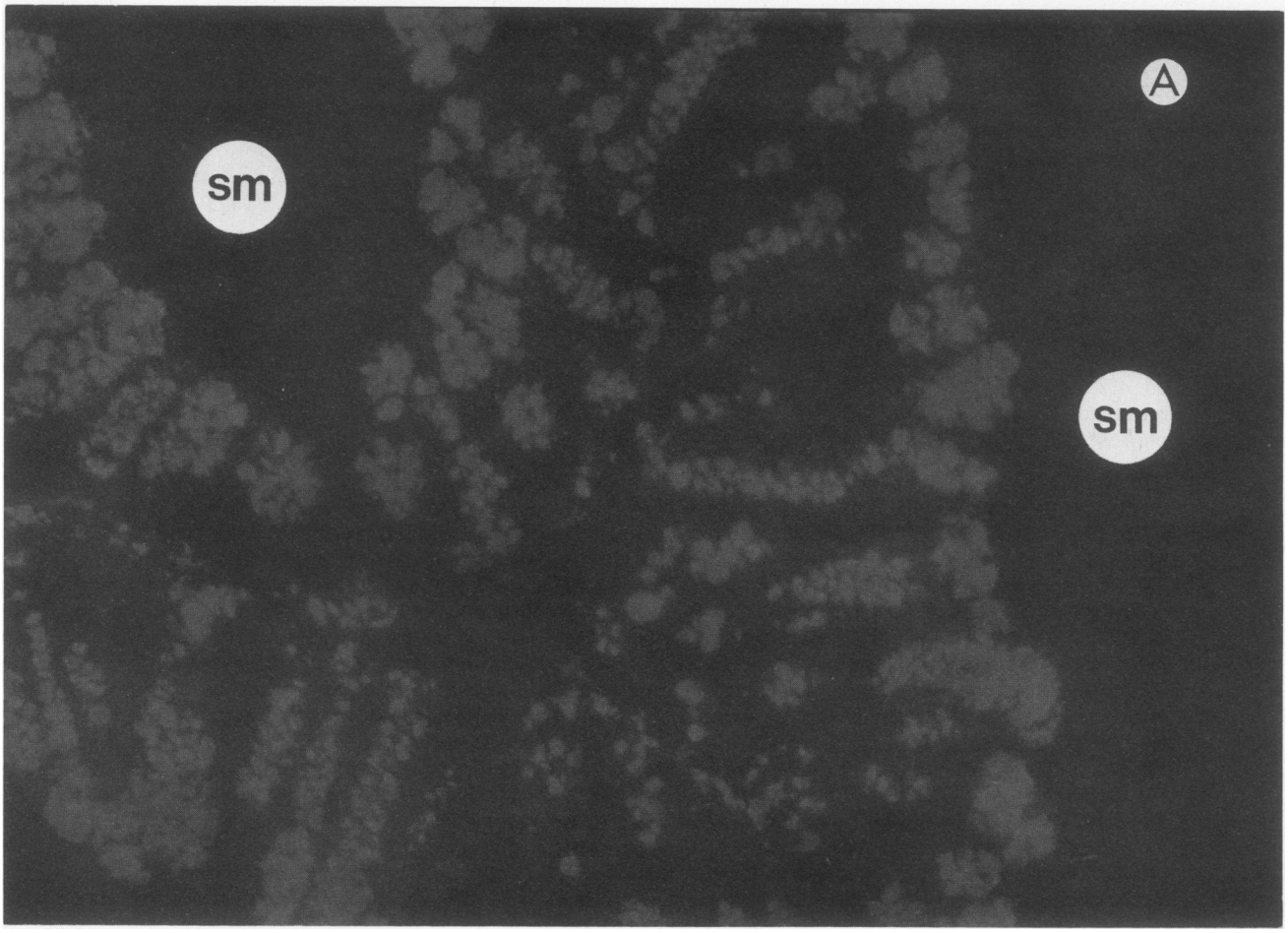

FIG. 2. Indirect immunofluorescence with colonic-mucin antibody on tissue sections of rabbit colon showed fluorescence in the vacuoles of goblet cells (A). No fluorescence was observed in the submucosa (sm). Preimmune guinea pig serum (B), heterologous antibody, and fluorescein isothiocyanate alone did not bind to goblet cell vacuoles.

colon stained with fluorescein isothiocyanate alone, preimmune serum (Fig. 2B), and heterologous antibody did not demonstrate fluorescence of intestinal goblet cells.

Using purified mucins as antigen in an ELISA showed that there was increased immunogenicity of the purified colonic mucin compared with purified rabbit ileal mucin (Fig. 3). This finding suggests that the colonic mucin preparation was specific for colonic mucosa.

The major peptide backbone region of mucin has an increased content of the amino acids serine, threonine, and proline (29). Two minor regions of the mucin peptide core have relatively increased amounts of either aspartic acid and glutamic acid or glycine and alanine (29). As shown in Table 1, amino acid compositions of both the major and minor regions of rabbit colonic mucin were comparable with mean values determined for colonic mucins in a number of other animal species. The amounts of amino sugars *N*-acetylgalactosamine (1.61 nmol/mg of protein) and *N*-acetylglucosamine (1.42 nmol/mg) in rabbit colonic mucin were also comparable with amounts in other mucins.

Bacterial adherence assay. Binding of RDEC-1, which was grown to both promote (i.e., Penassay broth) and suppress (i.e., brain heart infusion broth) phenotypic expression of AF/R1 pili, was compared with adherence of a nonpiliated mutant, strain M34, to polystyrene wells (Fig. 4). Increasing numbers of piliated RDEC-1 in the inoculum correlated with increased adherence of bacteria to polystyrene. In contrast, increased numbers of M34 bacteria in the inoculum did not result in greater binding of M34 to the polystyrene wells. RDEC-1 grown to suppress pilus expression showed an intermediate result for bacterial adherence. However, the phenotypic suppression of AF/R1 pili following growth in brain heart infusion broth is not complete (10) and thus accounts for the intermediate binding results. These findings are consistent with previous observations that expression of hydrophobic pili is an important factor mediating attachment of RDEC-1 in vitro (4, 10, 12, 34).

Progressive inhibition of piliated-RDEC-1 binding was observed when crude colonic mucus was used at concentrations of 10, 25, and 50 μg of protein per well (Fig. 5A). At a concentration of 50 μg per well, crude mucus completely inhibited bacterial adherence ($P < 0.001$). Similarly, preincubation of purified colonic mucin with various numbers of RDEC-1 also inhibited in vitro attachment of AF/R1-piliated RDEC-1 (Fig. 5B). With 15×10^8 bacteria per well in the inoculum, both 10 μg of purified colonic mucin (21,608 ± 1,593 dpm; $P < 0.05$) and 25 μg of purified colonic mucin (8,464 ± 4,245 dpm; $P < 0.05$) inhibited adherence of piliated RDEC-1 to polystyrene wells, which did not occur when piliated RDEC-1 were preincubated with either PBS alone or

FIG. 2—*Continued.*

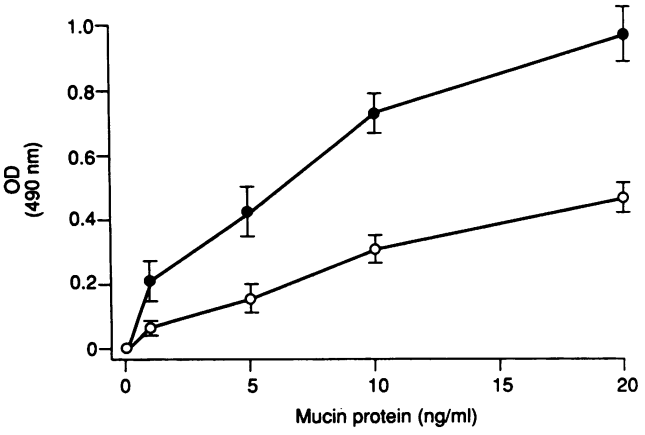

FIG. 3. ELISA with purified mucins of rabbit colon (●) and rabbit ileum (○) as antigens. Optical density (OD) readings at 5, 10, and 20 ng of mucin per ml were significantly different between ileal and colonic preparations (ANOVA, $P < 0.05$). Values at each point represent the mean ± standard error of the mean of four to six experiments.

5 μg of mucin protein per well. No visible agglutination of bacteria by mucus or mucin was observed during the preincubation period.

To determine which fractions of crude mucus inhibited binding of RDEC-1, equal amounts (10 μg of protein) of each of the crude mucous fractions separated after the first ultracentrifugation were preincubated with 15×10^8 piliated RDEC-1 per well. As shown in Fig. 6, the least-dense mucous fractions (i.e., high protein-to-glycoprotein ratio) did not inhibit binding of piliated RDEC-1. In contrast, the densest fractions demonstrated inhibition of RDEC-1 bind-

TABLE 1. Amino acid composition of mucins[a]

Mucin	Amt (mol%) of:		
	Ser + Thr + Pro[b]	Asp + Glu[c]	Gly + Ala + Val[c]
Purified rabbit colonic	38.4	23.1	19.8
Other colonic	41.4	17.2	21.6

[a] Results in the present study were comparable with the mean colonic-mucin amino acid profiles from previous investigations of a number of other animal species (reviewed in reference 29).

[b] The major peptide backbone region has a relatively high content of these amino acids.

[c] Minor peptide regions have a high content of these amino acids (29).

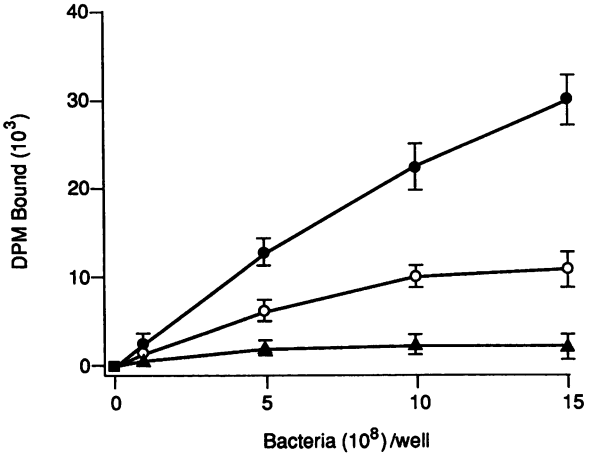

FIG. 4. Adherence of *E. coli* to polystyrene wells. When there were $>10^8$ bacteria in the inoculum, significantly more piliated RDEC-1 (n = 12) (●) (ANOVA, $P < 0.05$) bound to the wells than when both organisms were grown to suppress pili (n = 11) (○) and when a nonpiliated Tn*5* mutant, strain M34 (n = 6) (▲), was used.

ing that was equal to the inhibition when purified mucin was used (Fig. 6).

With a 1:1,000 dilution of antibody raised to purified colonic mucin, dot blot immunoassay revealed that immunogenic material was present in both of the high-density fractions (Fig. 7). In contrast, there was no reaction of antibody with antigens in the least-dense fractions. These findings provide evidence that only those fractions which contained mucin antigen were able to inhibit the in vitro adherence of RDEC-1 expressing AF/R1 pili.

Reduction and alkylation of purified colonic mucin (10 μg of Lowry protein per well) resulted in increased inhibition of binding of 15×10^8 piliated RDEC-1 (65.9% ± 0.4% of the PBS control) compared with levels with native mucin (36.8% ± 1.5%; $P < 0.001$).

DISCUSSION

Mucus is a viscoelastic gel which overlies the epithelial surface along the entire length of the gastrointestinal tract. Mucus is a complex mixture of water, electrolytes, various serum proteins and cellular macromolecules, and sloughed epithelial cells (29). The major organic constituent of mucus is a high-molecular-weight glycoprotein variously referred to as mucin and mucous glycoprotein (19, 29). A number of functions have been ascribed to both crude mucus and purified mucin. These include, for example, cytoprotection, lubrication, provision of a substrate for luminal proteases and glycosidases, behaving as a diffusion barrier for small molecules, and protection against infection (29). Protection against infection could involve a number of different mechanisms including prevention of adherence and colonization of colonic epithelium by pathogenic organisms.

Under transmission electron microscopy, RDEC-1 adheres to rabbit epithelial cells in vivo (37) in a manner identical to the intimate attachment of both enteropathogenic *E. coli* and enterohemorrhagic *E. coli* to enterocytes in humans (32, 39). The ability of microorganisms to adhere to epithelial cells in vitro is directly correlated with their infectivity in vivo (3). This relationship holds true for the adherence of RDEC-1 to epithelial cells in vitro and infectivity in rabbits in vivo (11).

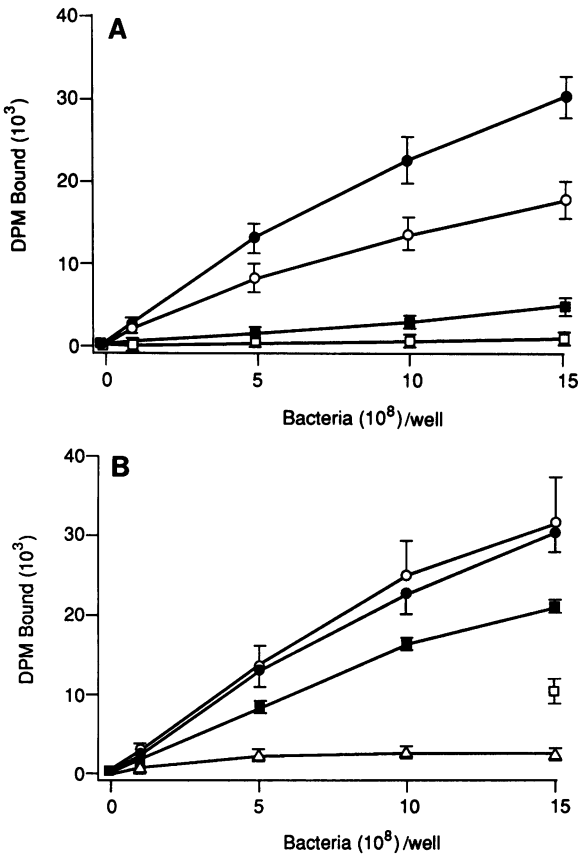

FIG. 5. (A) Inhibition of binding of piliated RDEC-1 by preincubation of organisms with crude colonic mucus. Increasing amounts of crude rabbit colonic mucus were incubated with various numbers of piliated RDEC-1. Symbols: ●, binding in the presence of PBS (n = 12); ○, preincubation of piliated RDEC-1 with 10 μg of mucous protein per well (n = 9); ■, 25 μg of mucous protein per well (n = 8); □, 50 μg of mucous protein per well (n = 7). For all bacterial inocula of $>10^8$ cells per well, each of the three mucus concentrations significantly inhibited binding of piliated RDEC-1 to polystyrene wells (ANOVA, $P < 0.05$). (B) Inhibition of binding of piliated RDEC-1 by preincubation of organisms with purified colonic mucin. Results for piliated RDEC-1 (●) and nonpiliated M34 (△) are shown for comparison as the positive and negative controls, respectively. Increasing amounts of purified rabbit colonic mucin were preincubated with piliated RDEC-1. Symbols: ○, 5 μg of mucin protein added to each well (n = 4); ■, 10 μg of mucin protein per well (n = 3); □, 25 μg of mucin protein per well (n = 5). There were no differences between piliated RDEC-1 incubated with either PBS or 5 μg of mucin (ANOVA, $P > 0.05$). With 15×10^8 bacteria in the inoculum, significantly fewer bacteria bound to wells in the presence of 10 and 25 μg of mucin (ANOVA, $P < 0.05$). There was no difference in the binding of M34 in PBS to polystyrene and the attachment of piliated RDEC-1 in the presence of 25 μg of mucin ($P > 0.05$).

Adherence of intestinal pathogens to mammalian cells is a complex process. Enteroadherence is mediated by both specific stereochemical interactions (i.e., receptor-ligand) and nonspecific physicochemical interactions (i.e., hydrophobicity, surface charge) (3, 6, 23). The AF/R1 pilus is an adhesin ligand expressed on the surface of RDEC-1 (10). AF/R1 pili mediate adherence of organisms to luminal glycoproteins (34), ileal mucins (13), M cells (18), and enterocytes (4, 10) of rabbit intestine. Infection of rabbits with

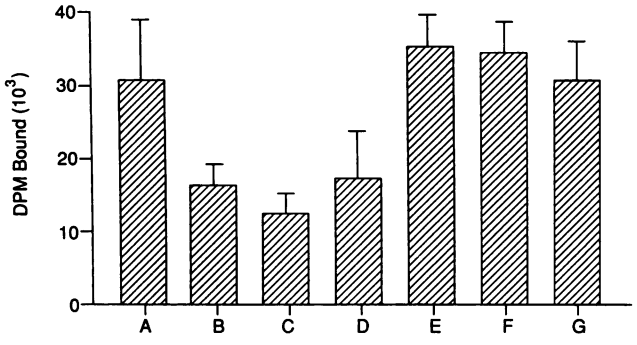

FIG. 6. Binding of piliated RDEC-1 in the presence of purified mucin and crude mucous fractions that were not employed for subsequent purification of colonic mucin. Piliated RDEC-1 (15×10^8 per well as inoculum) preincubated with PBS served as the positive control (column A). An equal protein content of 10 μg of protein per well for high-density fractions (i.e., fractions 1 and 2 [columns B and C, respectively]) inhibited binding of piliated RDEC-1 to the same degree as purified mucin (column D). In contrast, an equal protein content of low-density fractions (i.e., fractions 6, 7, and 8 [columns E, F, and G, respectively]) did not inhibit in vitro binding of RDEC-1. Each bar graph represents the mean ± standard deviation of 10 experiments, each run in triplicate.

AF/R1-piliated RDEC-1 results in greater morbidity (i.e., diarrhea) and mortality than infection with nonpiliated mutants (8, 41).

The adherence assay employed in the present study took advantage of the hydrophobic properties of piliated RDEC-1 (12). We confirmed that AF/R1 pili mediate attachment of organisms in vitro by showing that adherence of piliated organisms was greater than binding of both RDEC-1 grown in broth to inhibit the phenotypic expression of AF/R1 pili and a nonpiliated mutant, strain M34.

Density gradient ultracentrifugation of crude mucus provided a method to separate goblet cell-derived mucin from nonmucin proteins (i.e., less-dense fractions) and nucleic acids (i.e., densest fractions). A number of complementary methods were used to show that the middle-density fractions collected from the ultracentrifugations contain purified mucin. In contrast to a previous study with *Giardia lamblia*, in which the nonmucin protein fractions of small-intestine mucus were shown to enhance in vitro attachment of trophozoites (42), nonmucin fractions did not affect the in vitro adherence of RDEC-1 expressing hydrophobic pili. Only mucin-containing fractions inhibited binding of the rabbit enteric pathogen. There are both biochemical and histochemical differences between mucins derived from the small intestine and those derived from the colon (29). Colonic mucins are larger and more aggregated and contain more sulfate. The ELISA performed in the present study showed the heterogeneity of mucins, since antibody was more immunoreactive with colonic mucin than with ileal mucin.

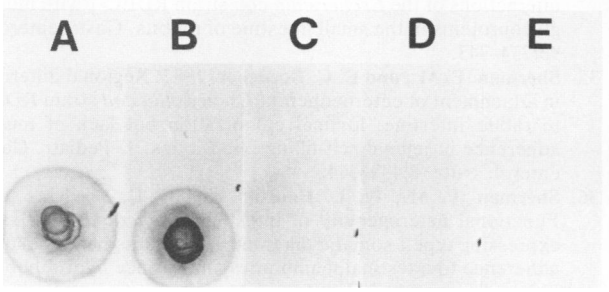

FIG. 7. Dot blot immunoassay of crude mucous fractions. At a 1:1,000 dilution of guinea pig anti-rabbit colonic mucin immune serum, immunoreactive antigens were present in high-density fractions (fractions 1 and 2) of crude mucus (A and B, respectively). In contrast, immunoreactive mucin was not present when equal amounts (2.5 μg of protein) of low-density fractions (fractions 6, 7, and 8) were blotted onto nitrocellulose paper (C, D, and E, respectively).

Although this study was not designed to identify the factor(s) responsible for colonic-mucin inhibition of RDEC-1 attachment, the biochemical structure of mucin does suggest a number of possibilities. These include, for example, competitive inhibition of bacterial adherence by mimicry of receptors on the apical surface of enterocytes. Chadee et al. (9) reported the presence of Gal-GalNAc in both rat and human mucins that mimic the cellular receptor to which *Entamoeba histolytica* adheres. Oligomannosyl receptors have also been demonstrated in N-linked oligosaccharides of the 118-kDa "link" glycopeptide component of mucin which serve as binding sites for type 1 pili expressed by an *E. coli* strain of serotype O157:H7 (31).

Alternatively, nonspecific interactions may occur between a part of the mucin molecule and bacterial ligands such as AF/R1 pili. AF/R1-piliated RDEC-1 is more hydrophobic than the nonpiliated mutant (12). The 118-kDa link glycopeptide of mucin is richer in hydrophobic amino acid residues than are the high-molecular-mass glycoprotein subunits of mucin (31). The link glycopeptide of mucin has been found in the mucin molecule from a number of intestinal sites in a variety of animal species (30). Interactions between hydrophobic adhesins, such as AF/R1 pili, and hydrophobic regions of the mucin molecule including the link glycopeptide could prevent the interaction of bacterial ligands with receptors on epithelial cells. The observation in this study that reduction and alkylation of colonic mucin increased inhibition of binding of RDEC-1 expressing AF/R1 pili suggests that the link peptide may be involved, since this constituent of mucin is separated from the native mucin and exposed under these conditions (30). Sajjan and Forstner (31) used similar conditions to demonstrate that the link peptide mediated mucin binding to type 1 pili expressed by *E. coli* CL-49 of serotype O157:H7.

Thus, inhibition of binding of pathogenic bacteria to epithelial cells in the intestinal tract may be on the basis of either specific stereochemical interactions between bacterial ligands and mucins or nonspecific physicochemical interactions between bacteria and goblet cell-derived glycoproteins. However, inhibition of binding is most likely related to these general properties acting in concert (23).

In summary, this study demonstrated that both crude mucus and purified mucin derived from rabbit colonic mucosa mediate inhibition of binding of AF/R1-piliated RDEC-1 to polystyrene wells. In contrast, non-mucin-containing fractions of colonic mucus did not inhibit in vitro binding of the attaching and effacing rabbit diarrheal pathogen. Future studies will examine whether inhibition of piliated-RDEC-1 binding in vitro is altered under conditions of mucosal inflammation when both the intestinal mucosa and the goblet cell-derived mucins are altered.

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