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Inhibition of Attachment of *Escherichia coli* RDEC-1 to Intestinal Microvillus Membranes by Rabbit Ileal Mucus and Mucin In Vitro

BRENDAN DRUMM, ANTHONY M. ROBERTON,† AND PHILIP M. SHERMAN*

Division of Gastroenterology, Department of Paediatrics, Research Institute, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8

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Intestinal mucus is postulated to play a role in preventing colonization of the gastrointestinal tract by microbial pathogens. To evaluate the ability of both crude mucus and purified mucin, a glycoprotein of goblet cell origin, to inhibit mucosal adherence of enteric pathogens, we examined whether mucus and mucin derived from rabbit ileum interact with the rabbit enteropathogen *Escherichia coli* RDEC-1. We examined the manner in which mucus and mucin inhibited adherence of bacteria to rabbit ileal microvillus membranes (MVMs) in vitro. The purity of the mucin preparation was demonstrated by polyacrylamide gel electrophoresis before and after reduction and by showing that an antiserum raised to the mucin localized to goblet cells in rabbit intestine. Using radioactive labeling of bacteria, we quantitated attachment of RDEC-1 to MVMs, mucus, and mucin that had been immobilized on polystyrene microtiter wells. Binding of RDEC-1 to MVMs was also determined after preincubation of organisms with crude ileal mucus and purified mucin. RDEC-1 bound to both crude mucus and purified mucin when they expressed lectinlike adhesins, previously designated attachment factor rabbit 1 pili. Adherence of piliated RDEC-1 to MVMs, mucus, and mucin was significantly greater than when the bacteria were nonpiliated. Binding of piliated RDEC-1 to MVMs was decreased by preincubation of bacteria with both crude mucus (45.6 ± 4.2% of control) and purified mucin (50.2 ± 5.8%). These data indicate that the *E. coli* enteropathogen RDEC-1 can bind to purified glycoproteins of goblet cell origin and that adherence of these bacteria to mucin is mediated by expression of pili. The findings also support a role for intestinal mucus and its principal organic constituent, mucin, in preventing adherence of a known *E. coli* enteric pathogen to apical MVMs of enterocytes.

One of the major functional properties attributed to mucus is that it acts as a barrier which protects a host against colonization by microbial pathogens. Trapping of organisms in mucus is considered important because it may prevent adherence of bacteria to intestinal epithelial cells (9, 10). However, despite widespread acceptance of such a role for gastrointestinal mucus, there is scant evidence to support this hypothesis. The gel-like properties of mucus are due to its principal organic constituent, high-molecular-weight (>2 × 10^6) glycoproteins that are variously referred to as mucus glycoprotein and mucin (9, 10, 13). Mucin may also prevent adherence of bacteria to surface epithelial cells in the gastrointestinal tract.

We examined the binding of a noninvasive, nonenterotoxigenic rabbit enteropathogen, *Escherichia coli* RDEC-1 (3), to crude mucus and highly purified mucin of rabbit ileum. Under electron microscopy, RDEC-1 adheres to rabbit intestinal cells in vivo (24) in a manner identical to the intimate attachment of enteropathogenic *E. coli* to enterocytes in human infants (26). Adherence of RDEC-1 to enterocytes is characterized by focal destruction of apical microvillus membranes (MVMs) and pedestal formation of the plasma cell membrane at points of bacterial attachment (24). The rabbit enteropathogen adheres to and colonizes mucosa in the ileum, cecum, and colon (21). We have previously shown that, after RDEC-1 infection of rabbits, the organisms are also found in association with mucus in the lumen of the intestine (22).

The aims of this study were to determine whether RDEC-1 binds to purified mucin of goblet cell origin and to examine whether mucus and mucin inhibit binding of RDEC-1 to rabbit ileal MVMs in vitro.

MATERIALS AND METHODS

Bacteria and growth conditions. Stock cultures of RDEC-1 (serotype O15:H−) were held on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar slants at 4°C. To promote expression of mannose-resistant pili, previously designated AF/R1 (4), organisms were grown in static, nonaerated Penassay broth (Difco Laboratories, Detroit, Mich.) (4, 23). Expression of pili was confirmed by slide agglutination of bacteria by using an AF/R1 pilus antibody as previously described (4, 23). To suppress all pilus expression, organisms were grown from slants in brain heart infusion broth (Difco) (4). RDEC-1 was grown in 10 ml of sterile broth (Penassay broth or brain heart infusion broth) to which 100 μCi of sterile [^{3}H]thymidine (specific activity, 15.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added at the time of bacterial inoculation. After overnight incubation at 37°C, bacteria were harvested by centrifugation at 2,500 × *g* and washed twice in sterile phosphate-buffered saline (PBS), pH 7.4, at 25°C to remove the unbound radiolabel. Bacteria were suspended in PBS to a concentration of 5 × 10^9/ml. Viable counts of bacteria were obtained by serial 10-fold dilutions that were plated onto bile salt agar plates.

Mucus preparations. One-kilogram specific-pathogen-free male New Zealand White rabbits (Reimans Fur Ranch, Toronto, Ontario, Canada) were fasted overnight and sacrificed by an intravenous overdose of pentobarbital, the abdominal cavity was opened, and 30 cm of ileum proximal to the appendix was removed. The ileum was opened along

* Corresponding author.
† Present Address: Department of Biochemistry, University of Auckland, Auckland, New Zealand.

its length and washed with normal saline at 4°C. Mucosal scrapings were obtained by gently scraping the ileal surface with glass microscope slides. Scrapings were collected into a solution (1 g [wet weight]/100 ml) containing 5 mM EDTA, 2 mM phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, Mo.), and 5 mM *N*-ethylmaleimide (Sigma) to minimize proteolytic degradation of mucin (8). Scrapings were then homogenized in a Waring blender for 30 s. The homogenate was centrifuged at 30,000 × *g* for 30 min to remove particulate and cellular matter. The soluble supernatant was classified, as in previous experiments (22), as crude ileal mucus.

Mucin was purified from crude ileal mucus by isopyknic ultracentrifugation in cesium chloride as described by Mantle and Allen (18). Briefly, crude ileal mucus was suspended in a solution of cesium chloride (Boehringer GmbH, Mannheim, Federal Republic of Germany), adjusted to a final density of 1.42 g/ml, and centrifuged at 105,000 × *g* for 48 h at 4°C. The gradient was then separated into 21 fractions, and each fraction was dialyzed extensively against sterile distilled water at 4°C. Total protein in each fraction was determined by the method of Lowry et al. (16), and glycoprotein content was determined by a periodic acid-Schiff assay as previously described (17). Fractions which demonstrated high glycoprotein contents in comparison with total protein contents were pooled and subjected to a second ultracentrifugation in cesium chloride. After Lowry and periodic acid-Schiff assays on each of 21 collected fractions, those fractions with high glycoprotein contents were pooled and represented purified ileal mucin, which was used in all subsequent experiments. All steps in the preparation of mucus and mucin were performed at 4°C in the presence of proteinase inhibitors.

Mucin antibody. Polyvalent antiserum was produced by subcutaneous injection of a 900-g male Hartley guinea pig with the purified rabbit mucin preparation. An initial 30 μg of protein of mucin antigen was mixed in Freund complete adjuvant (Difco), and this was followed 2 and 6 weeks later by injections of mucin mixed in Freund incomplete adjuvant as previously described (20). Antiserum was obtained from the guinea pig by venous catheterization under general anesthesia 2 weeks after the final subcutaneous antigen challenge.

Fluorescent-antibody studies. Sections of normal rabbit ileum were quick-frozen in liquid nitrogen and stored at −70°C. Sections were cut into 5-μm-thick slices in a microtome and fixed to microscope slides in acetate. Slides were incubated for 30 min at room temperature with a 1-in-20 dilution of guinea pig immune serum. The slides were then washed three times in PBS. Subsequently, a 1-in-20 dilution of fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin G antibody (Miles Scientific, Div. Miles Laboratories, Inc.; Naperville, Ill.) was added and incubated on the slides for 30 min in darkness. Control slides were stained with fluorescein isothiocyanate conjugate alone, preimmune guinea pig serum, or heterologous antibody (11).

Analytical gel electrophoresis. Gel electrophoresis of intact, nonreduced rabbit mucin was performed as described by Laemmli (14), by using a vertical slab gel system (1.5 mm thick by 16 cm by 20 cm; Bio-Rad Laboratories, Richmond, Calif.). The separation gel contained 10% acrylamide, and the stacking gel contained 4.8% acrylamide. Mucin samples (20 μg of protein) were mixed in 1% sodium dodecyl sulfate (SDS) buffer, applied, and electrophoresed through both stacking and separating gels at 6 mA of constant current until a dye front of bromophenol blue reached the bottom of the gel. The gel was then stained with Coomassie brilliant blue.

Mucin was reduced by placing it in sample buffer containing 2.5% mercaptoethanol and boiling it for 3 min, followed by alkylation with 0.5 M iodoacetamide as described by Fahim et al. (8). Composite agarose-acrylamide gel electrophoresis was then performed, and the gel was stained with silver as previously described (8).

Immunoblots. Dot blot immunoassays were performed as described previously by Towbin and Gordon (25). Briefly, 10 μg of purified mucin was slowly dropped onto nitrocellulose paper and allowed to be absorbed by the nitrocellulose. After 1 h, the paper was incubated with 3% bovine serum albumin in 10 mM Tris hydrochloride–0.9% NaCl at pH 7.4 for 1 h at 40°C and subsequently washed with Tris-saline. Guinea pig antiserum raised to mucin was added at a 1-in-50 dilution and allowed to incubate on the paper overnight at 4°C. After six washes in Tris-saline, a 1-in-5,000 dilution of protein A conjugated to horseradish peroxidase (Bio-Rad) was added and incubated for 1 h at 25°C. Horseradish peroxidase color development reagent containing 4-chloro-1-naphthol (Bio-Rad) was added to develop any positive reaction.

MVM preparations. Right-side-out MVM vesicles were prepared from the ilea of one-kilogram male New Zealand White rabbits by the calcium chloride precipitation method of Kessler et al. (12). MVMs were suspended in PBS to a final protein concentration of 1 mg/ml. Preparation of MVMs does not result in copurification of immunoreactive glycoprotein of goblet cell origin (22).

Adhesion assay. The method used to study adherence of RDEC-1 to rabbit MVMs, mucus, and mucin was based on an assay first described by Laux et al. (15). Assays were performed by using 96-well polystyrene tissue culture plates (Flow Laboratories, Inc., McLean, Va.). Each of the mucosal preparations (0.4 mg in 0.4 ml) were incubated overnight in wells at 4°C. Unbound substrate was removed by three washes of PBS. To block residual binding sites, 0.4 ml of 1% bovine serum albumin (Fraction V; Sigma) was added to each well and incubated overnight at 4°C. Preliminary experiments indicated that bovine serum albumin concentrations of up to 20% did not influence the results. Unbound bovine serum albumin was then removed by washing the wells three times with PBS. Various numbers of radioactively labeled bacteria in volumes of 0.4 ml were added to the wells and incubated for 1 h at 37°C. The wells were subsequently washed three times with PBS to remove nonadherent bacteria. Adherent bacteria were removed from the wells by addition of 0.4 ml of 1% SDS to each well and incubation at 37°C for 3 h as previously described (15). Samples were then removed from each of the wells and placed into 10 ml of scintillation fluid (Aquasol-2; Du Pont NEN Research Products, Boston, Mass.), and the levels of radioactivity (disintegrations per minute) were determined by counting in a beta scintillation counter (Model LS7500; Beckman Instruments, Inc., Palo Alto, Calif.). Quenching was constant among samples. To determine incorporated radioactivity per bacterium, 5×10^9 bacteria in a 0.1-ml volume were also measured for radioactivity during each assay.

To evaluate the inhibitory effects of various substrates on the adhesion of organisms to MVMs, 10^9 bacteria were mixed with various concentrations of mucus, mucin, AF/R1 pilus antiserum, or preimmune serum and preincubated for 1 h at 37°C. The mixtures were added to microtiter wells which had previously been coated with MVMs. The assay then proceeded exactly as described above. Unless otherwise

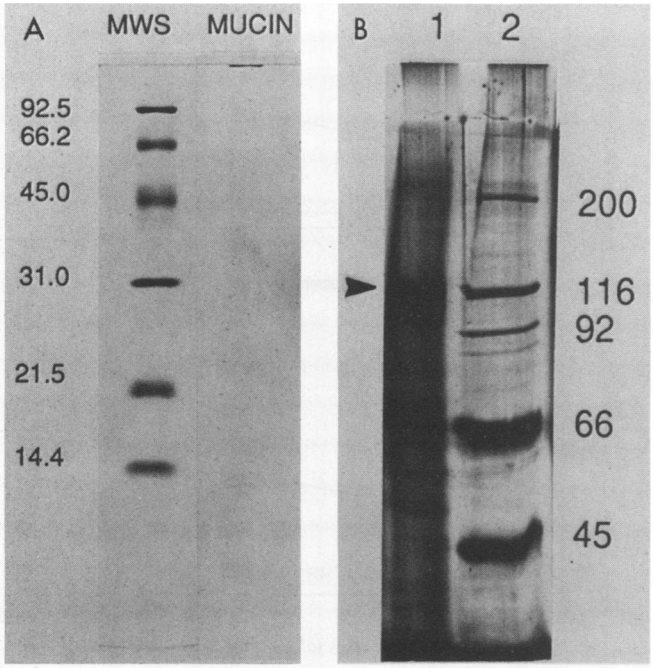

FIG. 1. (A) SDS-polyacrylamide gel electrophoresis showing molecular weight standards (MWS; 10^3; lane 1) and purified rabbit mucus glycoprotein (lane 2). No bands entered into the gel in lane 2, indicating the absence of low-molecular-weight contaminating proteins (13). (B) Silver stain of agarose-acrylamide gel after electrophoresis of rabbit mucus glycoprotein after reduction and alkylation (lane 1). A 118K band is present in the rabbit mucus preparation (arrowhead), a finding consistent with the presence of a putative link peptide described previously in other purified intestinal mucus glycoproteins (8). Lane 2 contains MWS (10^3).

stated, all assays were performed in triplicate and repeated on at least three separate occasions.

Statistics. Results are expressed as means ± the standard errors. Analysis between groups was performed by using the two-tailed, nonpaired Student *t* test (6).

RESULTS

Purification of rabbit ileal mucin. When 20 μg of mucin, prepared as described in Materials and Methods, was electrophoresed by using an SDS–10% polyacrylamide gel, no bands were detected to have entered into the gel (Fig. 1A), a finding consistent with the presence of high-molecular-weight mucin (mucus glycoprotein) in the sample (13). The gel profile supports the absence of contamination by lower-molecular-weight proteins and glycoproteins in the purified mucin sample. After reduction of intestinal mucin, electrophoresis revealed the presence of a 118,000-molecular-weight band (118K band; Fig. 1B), a finding that is consistent with the size of the putative link peptide previously demonstrated in purified intestinal mucins of other animal species (8).

A dot blot immunoassay performed by using the rabbit mucin preparation as the antigen and guinea pig antiserum showed that an antibody to this mucin preparation was present in the antiserum (data not shown). Indirect immunofluorescence demonstrated that the antibody bound to goblet cells in the rabbit ileum (Fig. 2a). In contrast, sections of rabbit ileum stained with fluorescein isothiocyanate, preimmune serum (Fig. 2b), or a heterologous antibody did not demonstrate fluorescence of goblet cells.

Adherence of bacteria to rabbit ileal MVMs, mucus, and mucin. Binding of AF/R1 piliated and nonpiliated RDEC-1 to MVMs that were adherent to polystyrene wells was exam-

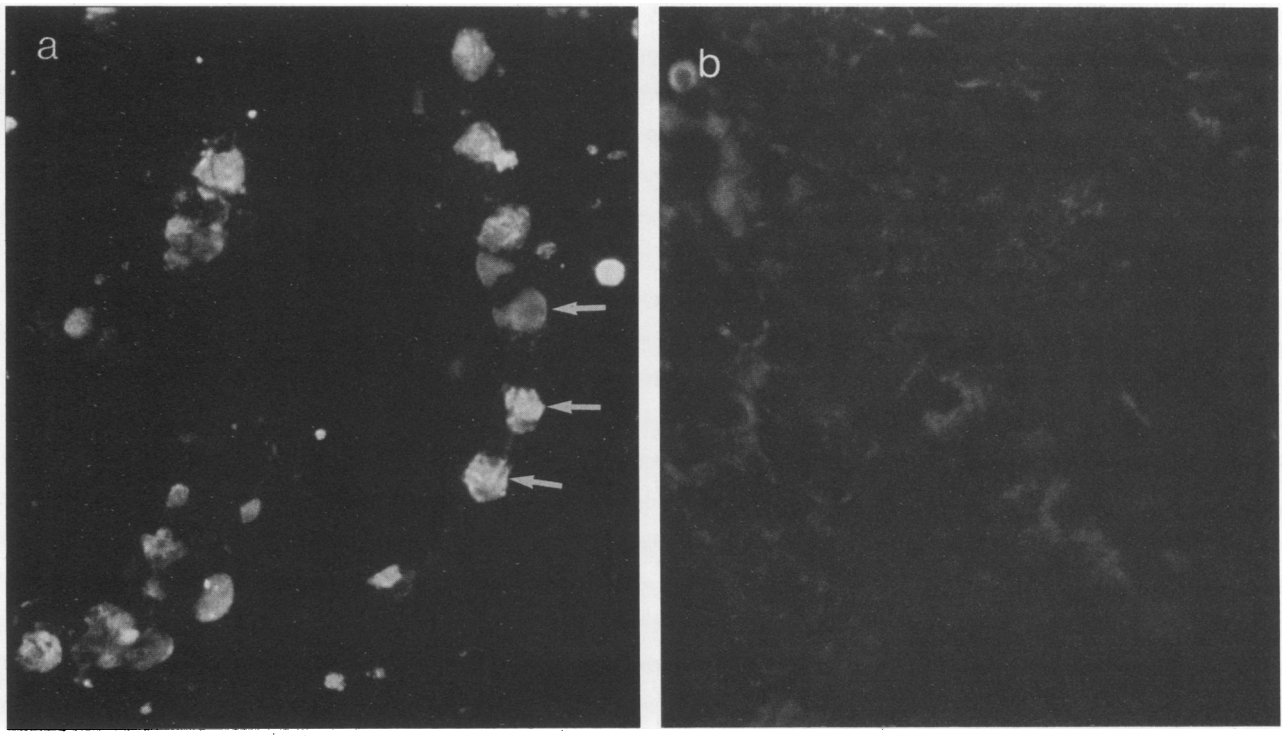

FIG. 2. (A) Indirect immunofluorescence demonstrates localization of guinea pig antiserum to goblet cells (arrows) in rabbit ileum. (B) Indirect immunofluorescence with preimmune guinea pig serum does not show localization to ileal goblet cells.

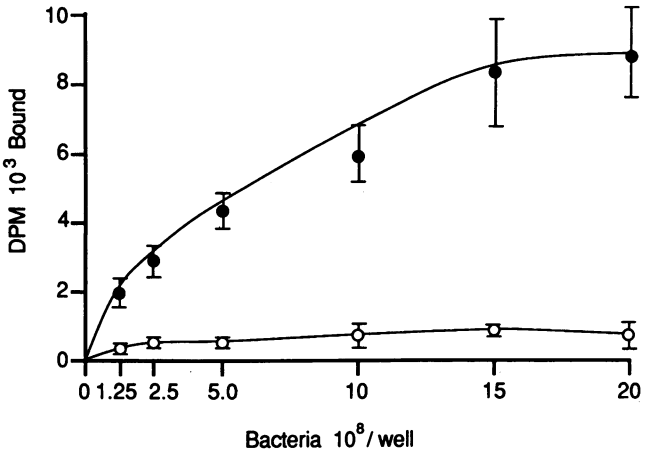

FIG. 3. Adherence of RDEC-1 (symbols: ●, AF/R1 piliated organisms; ○, organisms grown to suppress expression of pili) to rabbit ileal MVMs immobilized on polystyrene microtiter wells. Results are expressed as mean disintegrations per minute per well ± the standard error. Each assay was performed in triplicate and performed six times for piliated organisms and four times for nonpiliated bacteria.

ined by using increasing numbers of bacteria. RDEC-1 expressing AF/R1 pili bound to the ileal membrane preparations in progressively increasing numbers, up to 1.5×10^9 bacteria added per well, with saturation at increasing numbers of bacteria (Fig. 3). In contrast, nonpiliated RDEC-1 showed about 10 times less binding to MVMs, irrespective of the number of organisms added, consistent with previous observations that expression of pili is an important factor mediating attachment of RDEC-1 to rabbit ileal brush borders (4) and MVMs (23) in vitro.

To further support the role of AF/R1 pili in mediating attachment of RDEC-1 to MVMs, we preincubated piliated organisms with a 1-in-100 dilution of AF/R1 pilus-specific antiserum. Preincubation with pilus antiserum resulted in a reduction of binding of piliated bacteria to only 24 ± 1.2% of the original levels of adherence ($P < 0.001$).

Binding of piliated and nonpiliated RDEC-1 to mucus of rabbit ileum is illustrated in Fig. 4A. As shown for MVMs, piliated RDEC-1 bound to crude mucus. However, binding of AF/R1 piliated RDEC-1 to crude mucus was about 60% lower than adherence of piliated organisms to MVMs. Binding of RDEC-1 expressing AF/R1 pili to the purified mucin preparation (Fig. 4B) was not significantly different from that shown with crude mucus. As when MVMs were used as the substrate, binding of nonpiliated organisms to both mucus and mucin was significantly less than adherence of piliated RDEC-1.

Inhibition of binding of RDEC-1 to MVMs by crude mucus and purified mucin. When RDEC-1 was preincubated with crude mucus and purified mucin, inhibition of binding of AF/R1 piliated organisms to MVMs was demonstrated ($P < 0.001$). Inhibition of binding of bacteria to MVMs when piliated organisms were preincubated with mucus (1.2 μg of protein per well) and mucin (1.2 μg of protein per well) is illustrated in Fig. 5. Various levels of crude mucus, from 0.6 to 400 μg of protein, demonstrated similar degrees of inhibition of binding of piliated RDEC-1 to MVMs. At levels of mucus below 0.6 μg of protein, inhibition of binding of RDEC-1 to MVMs was progressively less. At 0.15 μg of protein, there was no further inhibition by mucus of bacterial binding to MVMs. Similarly, mucin inhibited AF/R1 piliated

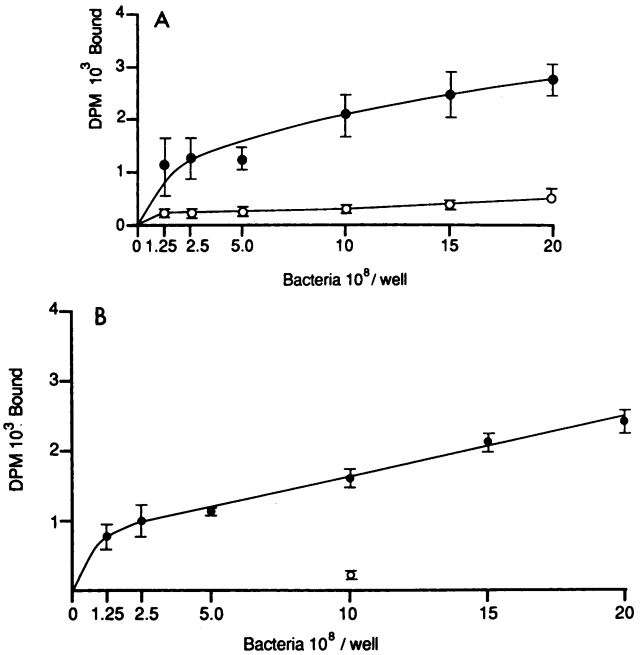

FIG. 4. Adherence of RDEC-1 (symbols: ●, AF/R1 piliated bacteria; ○, nonpiliated organisms) to rabbit ileal mucus (A) and purified mucus glycoprotein (B) immobilized on polystyrene microtiter wells. Assays were performed in triplicate and repeated in four separate assays for both piliated and nonpiliated organisms. Results are expressed as mean disintegrations per minute ± the standard error.

RDEC-1 attachment to MVMs when preincubated with bacteria at protein levels above 0.15 μg per well.

DISCUSSION

This study demonstrated that both crude mucus and purified intestinal mucus glycoprotein, also referred to as mucin, inhibited binding of an enteric pathogen to enterocyte

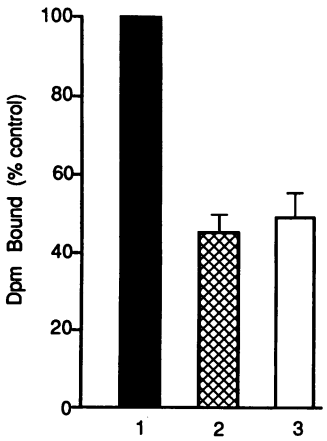

FIG. 5. Inhibition of binding of RDEC-1 to rabbit ileal MVMs by mucus and purified mucus glycoprotein. A total of 10^9 organisms were added to triplicate wells in the presence of saline (column 1), crude mucus (1.2 μg per well; column 2), and mucus glycoprotein (1.2 μg per well; column 3). Binding of RDEC-1 was significantly reduced in the presence of both crude mucus ($P < 0.001$) and highly purified mucin ($P < 0.001$).

apical MVMs. Theoretically, mucus and mucin could have potentiated binding of enteroadherent bacteria to mucosal membranes (7). However, in this study we showed that rabbit ileal mucus and goblet cell-derived mucin inhibited, rather than promoted, binding of bacteria to MVMs in vitro. In addition, the findings demonstrate binding of RDEC-1 to both crude mucus and purified mucin immobilized on the surface of polystyrene microtiter wells. Binding of bacteria was dependent on expression of mannose-resistant pili by the organisms. When pili were not expressed, binding of RDEC-1 to mucus, mucin, and enterocyte MVMs was minimal. Therefore, in addition to pili functioning as adhesins that mediate attachment of RDEC-1 to intestinal MVMs (23), these pili also promote bacterial binding to constituents of the surface mucous gel.

Mucus is a viscoelastic gel that covers the mucosal surface of the gastrointestinal tract. Mucus is composed primarily of water, with functional properties attributed to its major organic constituent, glycoproteins synthesized in the cytoplasm and then secreted from vacuoles of intestinal goblet cells. These glycoproteins are variously termed mucus glycoprotein and mucin (9, 10, 13). Because of high molecular weight and polydispersity, there was for many years great difficulty in preparing mucins in a pure form. More recently, highly purified mucins have been prepared from intestines (13). On the basis of three separate criteria, the rabbit ileal mucin prepared by us for these experiments was highly purified. (i) SDS-gel electrophoresis did not show evidence of low-molecular-weight contaminants (Fig. 1A) (13). (ii) The separation of a 118K band from the glycoprotein molecule after reduction (Fig. 1B) is consistent with the putative link component shown previously in other highly purified intestinal mucins (8). (iii) By indirect immunofluorescence (Fig. 2a), an antibody raised to ileal mucin was shown to localize to goblet cells in normal rabbit intestine (11).

Three major functions have previously been attributed to gastrointestinal mucus: protection of the underlying mucosa from chemical and physical injury, lubrication of the mucosal surface, and provision of a barrier against enteroadherence of pathogenic organisms (9, 10). Adherence of bacteria to surface enterocytes, in addition to enterotoxin production and tissue invasion, is an essential primary step for many pathogenic bacteria to mediate net fluid and electrolyte secretion into the intestinal lumen, which results in diarrheal disease (2). Although mucus has been postulated to protect against adherence by enteric pathogens, very little experimental evidence has previously been provided (1). The present study indicates that at least one *E. coli* enteropathogen, in addition to binding to crude mucus, adheres to highly purified intestinal mucin. It has not been previously demonstrated that goblet cell-derived mucin can also inhibit binding of an enteric pathogen to enterocyte apical membranes.

However, several recent publications have proposed that intestinal mucus contains specific receptors to which certain enteric bacteria can adhere. For example, Laux et al. examined the interaction of *E. coli* enteropathogens of porcine origin with a crude mucus preparation derived from intestines of mice (15). They showed that these enterotoxigenic *E. coli* strains, which expressed mannose-resistant pilus adhesins designated K88 and K99, adhere to crude mucus prepared from both the small bowels and the large intestines of mice.

Mouricout and Julien demonstrated binding of an enterotoxigenic *E. coli* strain expressing K99 pilus antigen to mucus prepared from calf small intestine (19). Similar to our findings, binding of bacteria to calf intestinal mucus was mediated by pili (19). However, mucus glycoprotein was isolated by column chromatography, a method that is not likely to yield only mucin derived from goblet cells. Further information that could be used to evaluate the purity of the calf mucus glycoprotein was not provided.

Binding of piliated RDEC-1 to mucus and mucin of rabbit ileum is unlikely related to the presence of associated secretory immunoglobulin A. Mucus and mucin were prepared from specific-pathogen-free rabbits that had not previously been exposed to the *E. coli* enteropathogen RDEC-1. In addition, recent findings indicate that secretory immunoglobulin A does not bind to intestinal mucin (R. Crowther, S. Lichtman, J. Forstner, and G. Forstner, Fed. Proc. **44**:691A, 1985).

There is a positive correlation between the ability of a wide variety of microorganisms to adhere to epithelial cells in vitro and their infectivity in vivo (2). Inhibition of binding of RDEC-1 to rabbit ileal MVMs in vitro by mucus and mucin may be important because there is also a correlation between in vitro adherence of this organism to MVMs and its infectivity in rabbits in vivo (5). Future work will evaluate the mechanisms of pilus-mediated binding by RDEC-1 to purified glycoproteins of goblet cell origin.

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