

Characterization of Binding of *Escherichia coli* Strains Which Are Enteropathogens to Small-Bowel Mucin

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Before an enteropathogen binds to the small bowel, it must interact with the small-bowel mucus (SBM) layer. To determine whether this interaction involves specific binding of diarrheagenic *Escherichia coli*, we used a quantitative assay with labeled, purified rabbit SBM. Binding of SBM from an adult rabbit was significantly greater to strain 162, an agglutinating *E. coli* strain, than it was to RDEC-1, a rabbit pathogen, and was significantly greater to strain 2348/PMAR, an enteropathogenic *E. coli* strain, than it was to strains 1392⁺ and 1392⁻, which are enterotoxigenic *E. coli* strains with and without colonizing fimbriae, respectively. Binding of strains RDEC-1, 2348/PMAR, and 162-4 was significantly greater to SBM than to bovine serum albumin. Binding of all strains increased in a linear fashion with increasing amounts of SBM and was reproducible ($r = 0.85$). Binding was significantly greater at pH 5.7 than at pH 7.4 or 8.0 for all five strains. Temperature did not alter the binding of any strain. Strains 162-4 and RDEC-1 bound significantly more to proximal SBM than to rabbit distal SBM, while strains 1392⁺ and 1392⁻ bound significantly more to distal SBM. Oxidation of sugars from SBM significantly decreased the binding of all strains. Each pathogenic *E. coli* strain bound distinctively to SBM; the SBM sugars appeared to mediate this binding for all *E. coli* strains. Binding was also dependent on mucin characteristics, as binding varied by region of the gut (increased for proximal SBM for strains 162-4 and RDEC-1 and for distal SBM for strains 1392⁺ and 1392⁻). The developmental age of the gut significantly affected binding only of the rabbit pathogen RDEC-1.

Escherichia coli isolates act by a variety of mechanisms to disturb small-bowel physiology and produce diarrhea (22, 33). Virulence traits such as the heat-labile and heat-stable enterotoxins and the ability to attach to and efface small-bowel mucosa are among the most important of these mechanisms. The colonization of the small bowel by the toxigenic organisms or the attachment of enteropathogenic *E. coli* strains to the small bowel is important in the capability of these organisms to cause diarrheal disease, yet the steps leading to colonization or attachment are very poorly understood (14, 23, 32). The initial contact by a bacterium, after its entry into the small bowel from the stomach, is with the unstirred mucous layer of the small-bowel lumen. The specifics of the interaction between specific pathogens and this layer are largely unknown. Recent data, which were obtained by studying both crude and purified intestinal mucins immobilized on polystyrene, have suggested that various strains of *E. coli* attach specifically to mucin glycoproteins within this layer, probably by the association of colonizing fimbriae with specific mucin sugars (4, 6, 12, 13, 17). In this study we characterized the binding of various *E. coli* strains, which are enteric pathogens, to small-bowel mucin glycoproteins in solution rather than immobilized to plastic. In our study, some of the pathogenic *E. coli* strains that we characterized had colonizing fimbriae, and some of them had adherence traits which were not mediated by fimbriae.

In the normal host, it is likely that specific traits of the mucin glycoprotein itself, as well as those of *E. coli*, affect the ability of the bacteria and the mucus to interact. It is known that mucin varies from species to species (20) and

develops with the host (27) and that the relative concentration and proportion of the five mucin sugars alter with the region of the gut as well as with the development of the host (8, 20). It is suggested that these regional and developmental changes may affect the predisposition of a host for protection from colonization by or infection with pathogens. It is also recognized that the mucin glycoprotein is changed in hosts with certain disease states. Although this is better described for the respiratory tree than for the gastrointestinal tract, alterations in mucin have been noted in patients with gastrointestinal disease (9, 21). These alterations, such as those classically described for the respiratory mucin of children with cystic fibrosis, are assumed to predispose the child to respiratory infections by increasing the colonization of the respiratory tree with pathogenic bacteria (30, 31). We examined the bacterial interaction with mucin from animals at different stages of development and from specific regions of the small bowel with the hope that these data will then be useful in developing an understanding of the interactions between bacteria and mucin in the small bowels of children with malnutrition and diarrheal illness.

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MATERIALS AND METHODS

Bacterial strains. The following *E. coli* strains were used in this study: strain 162-4, an enteroadherent-aggregative *E. coli* strain, as identified by adherence to HEP-2 cells in tissue culture (18, 29); strain RDEC-1, a rabbit pathogen, which causes intestinal lesions in rabbits similar to those seen in children with enteropathogenic *E. coli* (25, 26) and which was kindly provided by Edgar Boedecker (Uniformed Serv-

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ices University of the Health Sciences, Bethesda, Md.); strain 2348/PMAR, an enteropathogenic *E. coli* strain that was kindly provided by the Center for Vaccine Development, University of Maryland, Baltimore (1, 19); and strains 1392⁺ and 1392⁻, an isogenic pair of *E. coli* strains which no longer contain plasmids for either heat-labile or heat-stable toxin, respectively. Strain 1392⁺ expresses colonizing factor antigen II, while 1392⁻ no longer contains that plasmid material. Both 1392⁺ and 1392⁻ were also provided by the Center for Vaccine Development.

E. coli strains were maintained on nutrient agar. They were grown in Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract broth for 18 h at 37°C with shaking for use in the mucin-binding assays. Prior to use in the binding assays, strains were washed three times in phosphate-buffered saline (PBS) and resuspended in PBS to a standardized optical density at a wavelength of 600 nm. Strains were also grown at 18°C for use in the binding assay.

Bacteria were also grown in tryptic soy broth (Difco) with or without the addition of 100 mg of crude mucin. Growth was determined by serial dilution and plating of the broth culture at 18 and 24 h.

Mucin glycoprotein. Mucin was prepared from the small bowels of rabbits (3). With the rabbits under general anesthesia, the ileum and jejunum were removed from fasted rabbits and placed in petri dishes on ice. All subsequent steps were performed on ice to prevent the degradation of the mucin glycoproteins. The bowel was rinsed with cold PBS (pH 7.4) and cut into 10-cm segments, keeping proximal small-bowel segments separate from distal small-bowel segments. Individual segments were slit lengthwise, and the mucosal surface was scraped with glass microscope slides. The mucosal scrapings were pooled and vortexed with a small amount of PBS until they were homogeneous, about 15 min total, with frequent cooling on ice. Mucosal scrapings were centrifuged at 15,000 × *g* at 4°C for 25 min, and the supernatant from that centrifugation was recentrifuged at the same speed and for the same duration. The initial pellet was suspended in phosphate buffer (pH 7.4) and centrifuged. These combined supernatants were pooled, centrifuged at 25,000 × *g* at 4°C for 20 min, and dialyzed at 4°C overnight against distilled water in a dialysis membrane with a 10,000 to 12,000 molecular weight cutoff. The dialyzed supernatant was lyophilized after determination of the amounts of protein (15). A total of 30 to 50 mg of this crude mucus was fractionated on a Sepharose 4B column (50 by 2.5 cm; Pharmacia LKB Products) with 0.01 M Tris buffer at pH 8. Protein peaks in the collected fractions were determined from the A₂₈₀ readings. Fractions containing proteins with similar molecular weights were pooled, dialyzed, and lyophilized after determination of the protein concentration (15).

Mucin was prepared for these studies from specific-pathogen-free New Zealand White rabbits (Millbrook Farm, Amherst, Mass.) of three different ages: postweaning rabbits (weight, 800 to 1,000 g), adolescent rabbits (weight, 1.5 kg), and fully adult rabbits (weight, 3 to 5 kg).

Adherence assay. Mucin that was partially purified by column chromatography was radioiodinated by the method of Markwell (16). A total of 100 to 400 μg of mucin was labeled with ¹²⁵I by incubation with Iodobeads (Pierce Chemical Co., Rockford, Ill.) in 0.2 M phosphate buffer at pH 7.4. Specific activity was determined by precipitation in 10% trichloroacetic acid and was expressed as gamma counts per microgram of protein. Iodinated mucin (10 μg) was incubated for 2 h (unless otherwise specified) in microcentrifuge tubes with a standardized number of each *E. coli*

strain in PBS (pH 7.4) under the conditions specified for each assay. After incubation, the solution of mucin and bacteria was washed three times by centrifugation (3,000 × *g* at 4°C), with resuspension in PBS with a change of microcentrifuge tubes after the second wash. Final washed bacterial pellets were counted with a gamma counter. Each experiment was done in quadruplicate, unless otherwise noted. As the assay depended on the solubility of mucin and the adherence of these glycoproteins to the *E. coli* strains which could be centrifuged out of solution, any gamma counts present in the final pellet could be translated into micrograms of mucin glycoprotein per CFU of *E. coli* (5). Results are given as the micrograms of mucin bound per 1.0 × 10⁹ CFU of *E. coli*. The negative control for these assays was bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) that was iodinated and incubated with *E. coli* in the same manner as described above for the mucin glycoproteins.

Manipulation of the adherence assay. The adherence assay was also performed in buffers at pH 5.7 and 8.0. Assays were routinely performed at 27°C, but assays were also performed at 4 and 37°C.

Manipulation of mucin glycoproteins. Mucin carbohydrates were nonspecifically oxidized from the partially purified mucin by incubation with sodium metaperiodate (100 U/ml; Sigma) in PBS (pH 7.4) at 4°C for 1 h in the dark. Oxidized mucin was then dialyzed against distilled water for 24 h, lyophilized, and used in the adherence assays. The total concentrations of carbohydrate in mucin samples both pre- and postoxidation were determined by the phenol-H₂SO₄ method (7). Results are expressed as the mean A₄₉₅ of four separate determinations and reflect the carbohydrate concentration per 100 μg of mucin (protein weight). For use in some adherence assays, mucin was boiled in 0.2 M phosphate buffer (pH 7.4) for 5 min prior to iodination. Mucin was also incubated with 0.5 mg of trypsin (Sigma) per 100 ml for 40 min prior to iodination and for use in the adherence assay.

Gel electrophoresis. Gel electrophoresis of fractionated rabbit mucin was performed by the method of Laemmli (11) on a vertical slab gel (1.5 by 16 by 20 cm; Aquabogues Equipment, Aquabogues, N.Y.). The separating gel contained 12% acrylamide and the stacking gel contained 5% acrylamide. A total of 20 μg (protein weight) of samples was applied in 1% sodium dodecyl sulfate buffer without boiling and was electrophoresed at 6 mA of constant current. Gels were stained with silver stain (Bio-Rad Laboratories, Richmond, Calif.). Mucins that were treated with sodium metaperiodate and trypsin and that were denatured by boiling were electrophoresed by the same method.

Statistics. Statistics were done by Student's *t* test.

RESULTS

Bacteria. Growth of all bacterial strains at 6 and 18 h was equivalent in broth with and without the addition of 100 mg of crude mucin. None of the bacterial strains in broth with mucin agglutinated, as determined by direct visualization under the microscope.

Mucin glycoprotein. Rabbit small-bowel mucin glycoprotein consistently eluted in a sharp peak from the Sepharose 4B column just before blue dextran and therefore has a molecular weight just greater than 2 × 10⁶ (Fig. 1). A total of 20 μg of protein from the pooled fractions of this peak that was run on a 12% sodium dodecyl sulfate gel under non-denaturing conditions revealed only faint bands at 67 and 90 kilodaltons, suggesting that there was relatively minor con-

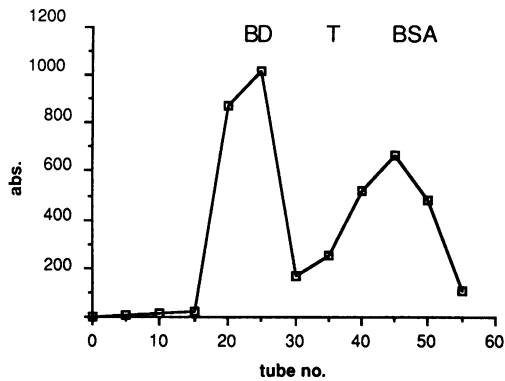


FIG. 1. Profile of rabbit small-bowel mucin after Sepharose 4B chromatography read at an A_{280} (abs., absorbance). Molecular weight standards were as follows: BD, blue dextran; T, thyroglobulin; BSA, bovine serum albumin.

tamination by lower-molecular-mass (nonmucin) proteins (6, 11).

Binding assay. The binding of *E. coli* 162-4 and RDEC-1 was significantly greater to small-bowel mucin ($P = 0.00$ and 0.001 , respectively) than to bovine serum albumin (Fig. 2). The binding of strains 1392⁺ and 1392⁻ to mucin was slightly but not significantly greater than the binding of these strains to bovine serum albumin. The binding of the various *E. coli* strains to small-bowel mucin from a 3.2-kg rabbit varied significantly as well (Fig. 2). Strain 162-4 bound significantly more small-bowel mucin than all other strains did ($P = 0.05$, 0.005 , 0.004 , and 0.004 , respectively, for strains RDEC-1, 2348/PMAR, 1392⁺, and 1392⁻). Strain RDEC-1 routinely bound slightly more (1.5 times) to small-bowel mucin than did strain 2348/PMAR, although this difference was not statistically significant. Strain RDEC-1 bound significantly more to small bowel mucin than did strains 1392⁺ and 1392⁻ ($P = 0.05$ and 0.04 , respectively). When the bacteria were grown at 18°C (to suppress expression of pili), strain RDEC-1 bound significantly more small-bowel mucin than it did when it was grown at 37°C (0.93 versus 0.28 $\mu\text{g}/1.0 \times 10^9$ CFU; $P = 0.05$). Strain 2348/PMAR also bound more small-bowel mucin when it was grown at 18°C (0.61 versus 0.36 μg ; $P = 0.04$). The binding of strains 1392⁺, 1392⁻, and 162-4 was not different when the bacteria were grown at 18°C as

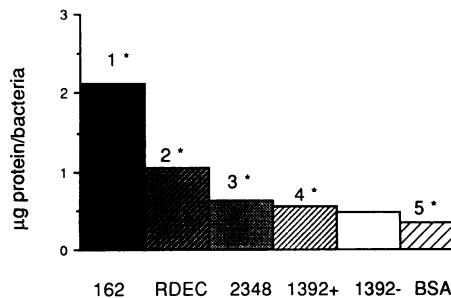


FIG. 2. Micrograms of bovine serum albumin (BSA) and mucin (mean) prepared from a 3.2-kg rabbit bound to 1.0×10^9 CFU of various *E. coli* strains: 1*, 162-4 > RDEC ($P = 0.06$) > 2348 ($P = 0.005$) > 1392⁺ ($P = 0.004$) > 1392⁻ ($P = 0.002$) > bovine serum albumin ($P = 0.0$); 2*, RDEC > 1392⁺ ($P = 0.09$) > 1392⁻ ($P = 0.04$) and bovine serum albumin ($P = 0.001$); 3*, 2348 > 1392⁺ ($P = 0.7$) > 1392⁻ ($P = 0.37$) > bovine serum albumin ($P = 0.04$); 4*, 1392⁺ > 1392⁻ ($P = 0.64$).

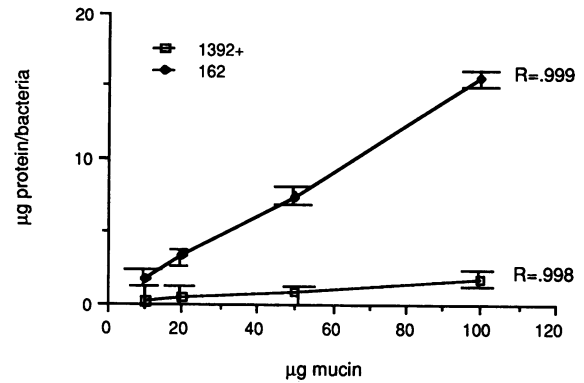


FIG. 3. Micrograms of mucin (mean \pm standard deviation) bound to 1.0×10^9 CFU of *E. coli* with increasing concentrations of mucin in the binding assay.

opposed to 37°C. When the binding assay was done in the presence of 1% mannose, the binding of RDEC-1 decreased from 0.39 to 0.1 $\mu\text{g}/1.0 \times 10^9$ CFU of bacteria ($P = 0.0$). RDEC-1 was the only strain evaluated that is known to have type 1 pili. There was no significant difference in the binding of strains 2348/PMAR, 1392⁺, and 1392⁻ to small-bowel mucin. The differences in binding were consistent from assay to assay, between various mucin batches prepared from the same rabbit, and between iodinations.

Bacterial binding of all *E. coli* strains to mucins was less at 15 min than at 1 h ($P = 0.02$, 0.02 , 0.04 , 0.05 , and 0.05 for strains 162-4, RDEC-1, 2348/PMAR, 1392⁺, and 1392⁻, respectively). Binding did not continue to increase over time and was unchanged at the 2- and 4-h determinations. The binding of all strains to small-bowel mucin increased in a linear fashion when the concentration of mucin in the assay was increased from 10 to 100 μg . The data shown in Fig. 3 are for strains that bound both the highest (162-4) and the lowest (1392⁺) levels. The binding of the other *E. coli* strains also increased in a linear fashion. When the temperature of the binding assay was varied from 4 to 37°C, the binding of none of the strains to small-bowel mucin was affected. However, the pH at which the assay was done significantly affected the binding of all strains (Fig. 4). The binding of strains 162-4, RDEC-1, 2348/PMAR, 1392⁺, and 1392⁻ to mucin from a 1.5-kg rabbit was significantly greater at pH 5.7

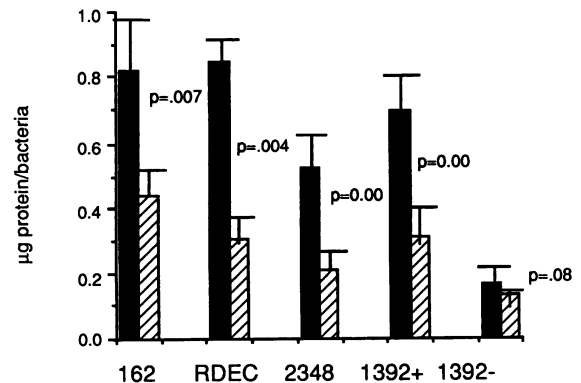


FIG. 4. Micrograms of mucin (mean \pm standard deviation), which was prepared from a 1.5-kg rabbit, that bound to 1.0×10^9 CFU of *E. coli* when the binding assay was performed at pH 5.7 (■) and 7.4 (▨).

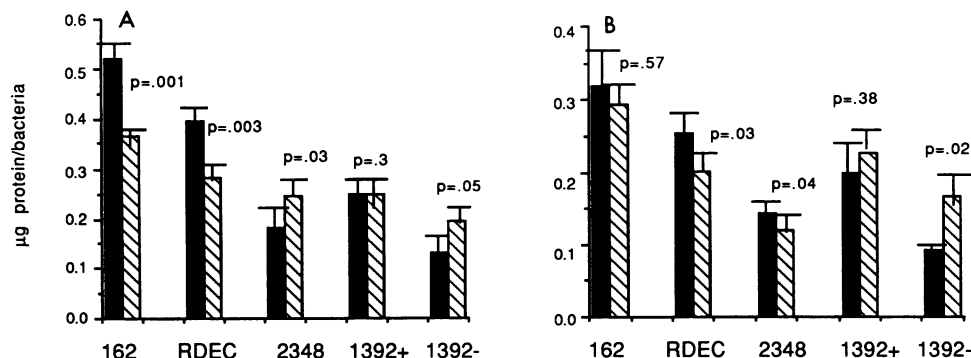


FIG. 5. (A) Micrograms of proximal (■) and distal (▨) small-bowel mucin (mean \pm standard deviation), which was prepared from a 1.5-kg rabbit, that bound to 1.0×10^9 CFU of *E. coli* at pH 5.7. (B) Micrograms of proximal (■) and distal (▨) small-bowel mucin (mean \pm standard deviation) that bound to 1.0×10^9 CFU of *E. coli* at pH 7.4.

than at pH 7.4. In addition, all of these strains except strain 1392+ bound significantly less small-bowel mucin at pH 8.0 than they did at pH 7.4 ($P = 0.0, 0.007, 0.006, 0.233,$ and 0.00 , respectively, for strains 162-4, RDEC-1, 2348/PMAR, 1392+, and 1392-). The binding of bovine serum albumin was not significantly altered by decreasing the pH of the binding assay to 5.7.

The regional differences in strain binding seen with mucin from proximal and distal small bowel at the pH expected in those regions (pH 5.7 in the proximal small bowel and pH 7.4 in the distal small bowel) are shown in Fig. 5. At pH 5.7, strains 162-4 and RDEC-1 bound significantly more proximal small-bowel mucin than distal small-bowel mucin. RDEC-1 bound significantly more proximal small-bowel mucin at pH 7.4 as well as at pH 5.7. The binding of strain 162-4 to proximal and distal small-bowel mucin was not significantly different at pH 7.4. Strain 2348/PMAR bound significantly more distal small-bowel mucin at pH 5.7, but bound more proximal small-bowel mucin ($P = 0.04$) at pH 7.4. Fimbriated strain 1392+ bound equivalent amounts of proximal and distal small-bowel mucin at both pH 5.7 and 7.4, but non-fimbriated strain 1392- bound significantly more distal small-bowel mucin at both pH 5.7 and 7.4.

Strain RDEC-1 bound significantly more small-bowel mucin harvested from postweanling rabbits (weight, 800 g; $0.332 \mu\text{g}/1.0 \times 10^9$ CFU of bacteria) than it did that harvested from an adolescent rabbit (weight, 1.5 kg; $0.132 \mu\text{g}/1.0 \times 10^9$ CFU of bacteria; $P = 0.05$). The binding of strains 162-4, 2348/PMAR, 1392+, and 1392- varied, but it did not vary significantly with mucins from rabbits of various ages. The total carbohydrate contents of the mucins from these rabbits increased with increasing age and size. The carbohydrate from 100 μg (protein weight) of the mucin from the 800-g rabbit had a mean A_{495} of 0.119 in the phenol- H_2SO_4 carbohydrate assay, as compared with values of 0.138 (32% increase) for the 1.5-kg rabbit and 0.156 for the 3.2-kg rabbit (45% increase). When the mucin from a 3.2-kg rabbit was prepared from proximal and distal gut segments, the carbohydrate content of 100 μg of the proximal mucin had a mean absorbance of 0.154 while the carbohydrate content of 100 μg of distal mucin had a mean absorbance of 0.175.

Manipulation of the mucin glycoprotein. The results of the binding assay when the mucin glycoproteins were oxidized by 100 U of sodium metaperiodate prior to use are shown in Fig. 6. Binding of all strains was significantly decreased after oxidative removal of carbohydrates. When carbohydrate concentrations were assessed after oxidation, the mean

absorbance of 100 μg of the oxidized proximal mucin preparation from the 3.2-kg rabbit was 0.018, an 89% decrease from the absorbance of 0.154 of 100 μg of the untreated mucin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mucin treated with sodium metaperiodate did not reveal low-molecular-weight protein bands. When the binding of bacteria to mucin treated with sodium metaperiodate was assessed over time, it behaved in the same fashion as untreated mucin, increasing from 15 min to 1 h. An increase in the concentration of mucin in these assays increased the binding of bacteria, although binding never approached the level seen with untreated mucin.

Similar results were seen when the mucin glycoprotein was incubated with 0.5% trypsin prior to use in the binding assay. Binding of all strains was significantly decreased in the trypsin-treated mucin (Fig. 7). Breakdown products were seen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of trypsin-treated mucins with broad bands at 45, 67, and 90 kilodaltons. Additionally, mucin binding to *E. coli* strains after the mucin was denatured by boiling it for 5 min was significantly decreased. Binding of strain 162 decreased from 0.95 μg with untreated mucin to 0.46 $\mu\text{g}/1 \times 10^9$ CFU ($P = 0.0$); for strain 1392-, binding decreased from 0.63 μg with untreated mucin to 0.38 μg with boiled mucin ($P = 0.04$). Differences in binding between denatured and trypsin-treated mucins were not significant for any strain. Boiling did not alter polyacrylamide gels of mucin, nor did it significantly alter the total carbohydrate concentration (the mean

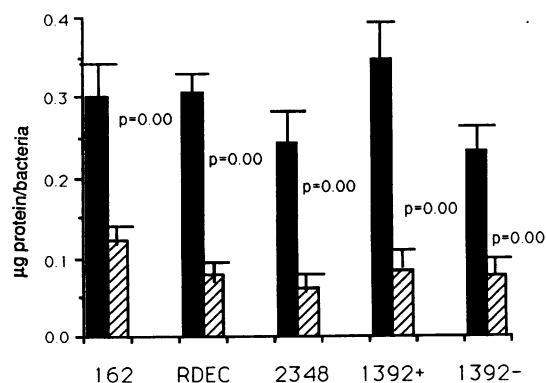


FIG. 6. Micrograms of proximal mucin (mean \pm standard deviation) that bound to 1.0×10^9 CFU of *E. coli* after oxidation of mucin by 100 U of sodium metaperiodate. The solid bars represent untreated mucin; the hatched bars represent oxidized mucin.

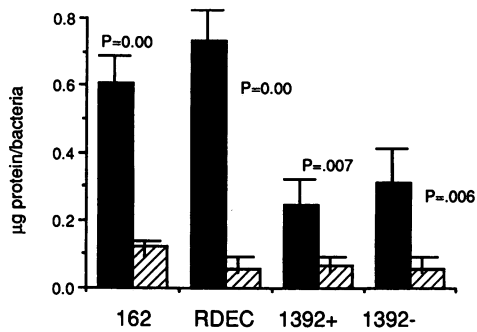


FIG. 7. Micrograms of proximal mucin (mean \pm standard deviation) that bound to 1.0×10^9 CFU of *E. coli* after incubation of mucin in 0.5% trypsin. The solid bars represent untreated mucin; the hatched bars represent trypsin treated mucin.

absorbance of untreated mucin was 0.154; that of boiled mucin was 0.148).

DISCUSSION

Little is known about the function of intestinal mucin in the pathogenesis of diarrheal disease. It has been assumed that the mucous layer of the small bowel traps potential pathogens and protects the microvillus-brush border from them, but this assumption has been based on very few hard data (6, 8, 24). Very recently, studies have begun to describe the interaction of *E. coli* that are part of the normal colonic flora and those that are pathogens with colonic and small-bowel mucin glycoproteins (4, 12, 13, 17). These studies have been based on an adherence assay in which mucin was immobilized on plastic and the number of radioactively labeled *E. coli* strains which adhere to that immobilized mucus is determined (12). One of these studies, that by Laux et al. (13), found that the presence of the K88 colonizing fimbria, a colonizing pilus that is usually associated with piglet enterotoxigenic strains, increased the binding of *E. coli* to mouse small-bowel mucin but not to bovine serum albumin. That binding decreased when mucin was treated with pronase, trypsin, or sodium metaperiodate. Binding was also specifically inhibited by D-galactosamine, and low-molecular-weight receptors were identified in brush border preparations as well as in mucin (13). Similarly, Laux et al. (13) found that *E. coli* strains with K88 or K99 fimbriae were adherent to mouse small- and large-bowel mucin, while *E. coli* strains containing human colonizing factor antigen II or nonpathogenic *E. coli* K-12 were not adherent. This adherence was mannose resistant. Similar findings were reported by Cohen et al. (4) for a normal flora *E. coli* strain, F-18, with colonic mucin. Mouricout and Julien (17) reported the specific adherence of *E. coli* strains with K99 and F41 colonizing fimbriae to immobilized small-bowel mucin from foals, while strains without colonizing fimbriae did not adhere (17). Drumm et al. (6) examined the binding of *E. coli* RDEC-1 with AF/R1 pili and type 1 pili to rabbit mucin. In those studies RDEC-1 bound significantly more to mucin when it was grown to promote the expression of pili than when it was grown to repress the growth of pili. Drumm et al. (6) also reported that the presence of mucin prevented the binding of RDEC-1 to microvillus membrane preparations, supporting the fact that normal mucin has a protective role.

In contrast, our studies were done with mucin and bacteria in solution, as first described by Cohen et al. (5), who also examined the ability of a colonization-positive *E. coli* strain

(F-18, normal human flora) to bind to mouse colonic mucin and reported that it was greater than that of the same *E. coli* strain without its colonizing plasmid. As a result of using the "in-solution" assay, we are confident that we are dealing with a difference in binding rather than a difference in bacterial growth in mucin during the incubation period of the adherence assay, as all strains grew equivalently in the presence of mucin.

We found that the binding of mucin and *E. coli* varied extensively by strain but that it was not necessarily dependent on the presence of colonizing fimbriae. The *E. coli* strain which consistently bound most extensively in our studies was strain 162-4, which exhibits aggregative adherence to tissue culture cells and has no pili by hemagglutination or examination by electron microscopy with negative staining (C. A. Wanke et al., submitted for publication). Binding of this strain did not change when strains were grown under conditions which suppressed pili. *E. coli* RDEC-1 and 2348/PMAR bound consistently large amounts of rabbit small-bowel mucin, and both strains appear to cause disease by attachment to and effacement of small-bowel mucosa. RDEC-1 adherence was decreased when the assay was done in the presence of 1% mannose, which suggests that type 1 pili may play a role in the adherence of this organism. RDEC-1 binding also increased after growth to suppress pili, which suggests that the AF/R1 pilus is not essential to binding, as has been suggested by an in vivo study with this organism, which demonstrated that nonpilated mutants still adhere to the small bowel and cause diarrhea (2). The previously toxigenic *E. coli* strain with colonizing fimbriae, 1392⁺, bound significantly less mucin than the other pathogens did and did not bind significantly more mucin than its nonfimbriated isogenic sibling strain (1392⁻) did. Interestingly, growth at 18°C to suppress pili did not change the binding of fimbriated strain 1392⁺. These data suggest either that the sensitivity of the adherence assay is not great enough to detect subtle differences in binding or that in mucin-*E. coli* interactions, bacterial surface structures other than pili are operative.

We found that the interaction of both fimbriated and nonfimbriated *E. coli* strains and small-bowel mucin was specific, suggesting, as have previous studies, that the adherence is receptor mediated. This binding was significantly greater than binding to bovine serum albumin by the nonfimbriated strains. It was pH dependent and temperature independent for all strains. We found that the binding increased with an increase in incubation time and in a linear fashion with increasing concentrations of mucin for fimbriated and nonfimbriated strains. We were not able to saturate the binding with increasing amounts of mucin, probably because the number of bacteria used in the assay was too large. We found that binding of all strains was dependent on the glycosylation of the mucin protein core and that the oxidative removal of the carbohydrates, without evidence of degradation of the protein core, decreased binding. This also agrees with the previously published findings for *E. coli* with colonizing fimbriae (17). We found that the protein structure, including the tertiary structure, of the mucin glycoprotein molecule is necessary for binding, as denaturation of the protein by boiling or degradation of the protein core by pretreatment with trypsin also decreased binding. Trypsin treatment of immobilized mucin has been shown to decrease binding in other studies (13, 17).

When mucin glycoproteins from proximal and distal small bowel were assayed independently for their ability to bind, we found that binding varied greatly by strain. At the

physiologic pH of the proximal small bowel, strains 162-4 and RDEC-1 bound significantly more to proximal mucins than to distal mucins. In contrast, strain 2348/PMAR bound more distal small-bowel mucin at pH 5.7 but more proximal small-bowel mucin at pH 7.4. This suggests that when the small-bowel pH is raised by malnutrition or alterations in gastric acid secretion (10), enteropathogenic *E. coli* might be more readily able to colonize the proximal small bowel. The fact that *E. coli* 1392⁺ and 1392⁻, with and without colonizing fimbriae, respectively, both bound more readily to distal small-bowel mucin may suggest that mucin serves to promote selective regional colonization of the small bowel by these organisms. It may also be possible that the toxins that these organisms ordinarily produce alter the ability of these strains to colonize other regions of the gut. Region-specific binding by pathogens may well prove to be important in the protection of the host or in the initiation of disease in the abnormal host. As the pH environment was able to significantly alter binding of all organisms, including 1392⁺, which did not otherwise bind significantly to mucin, it is easy to hypothesize that malnutrition or disease (including other gastrointestinal infections) which alters pH may alter mucin function sufficiently to affect its protective nature.

We found, as has been previously reported (20), that the carbohydrate concentration of the small-bowel mucin increased as the developmental age of the animal increased. Functionally, we found that this developmental difference was of significance only for the rabbit pathogen examined, RDEC-1. This pathogen bound more to the mucins from smaller, younger rabbits with less total carbohydrate. This suggests that the specific carbohydrate that is exposed is of more importance than is the total amount of carbohydrate on the mucin core. That the other strains did not exhibit significant differences in binding may reflect the differences in the carbohydrate specificity of the *E. coli* strains that we tested. The carbohydrate assay done in this study did not identify which of the five mucin sugars was in the terminal or exposed position. Studies to define the precise carbohydrate that is involved in binding of each bacterium are under way.

The *in vivo* function of the small-bowel mucous layer remains to be defined. Our study begins to demonstrate, as have others, that the interaction between this glycoprotein layer and *E. coli* strains that are enteric pathogens is specific and complex. Our studies additionally provide data that this specific interaction is not necessarily dependent on bacterial pili but is dependent on both the characteristics of the bacterial strain and the precise mucin glycoprotein that is examined. We have provided data that the function of this glycoprotein layer depends on the region of the gut, the age of the host, and the environment in which the interaction between bacteria and mucin takes place. Further studies to define more precisely the function of this barrier between bacteria and mucin in hosts with various conditions of disease and malnutrition are necessary to understand the role that the mucin layer plays in the protection of the host or its possible role in the initiation or promotion of diarrheal disease.

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LITERATURE CITED

- Baldini, M. D., J. B. Kasper, and M. M. Levine. 1983. Plasmid mediated adhesion in EPEC. *J. Pediatr. Gastroenterol. Nutr.* 2:534-538.
- Cantey, J. R., L. R. Inman, and R. K. Blake. 1989. Production of diarrhea in the rabbit by a mutant of *Escherichia coli* (RDEC-1) that does not express adherence (AF/R1) pili. *J. Infect. Dis.* 160:136-141.
- Chadee, K., W. A. Petrie, D. J. Innes, and J. I. Ravdin. 1987. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. *J. Clin. Invest.* 80:1245-1254.
- Cohen, P. S., J. C. Arruda, T. J. Williams, and D. C. Laux. 1985. Adhesion of a human fecal *Escherichia coli* strain to mouse colonic mucus. *Infect. Immun.* 48:139-145.
- Cohen, P. S., R. Rossoll, V. J. Cabelli, S.-L. Yang, and D. C. Laux. 1983. Relationship between the mouse colonizing ability of a human fecal *Escherichia coli* strain and its ability to bind a specific mouse colonic mucous gel protein. *Infect. Immun.* 40:62-69.
- Drumm, B., A. M. Robertson, and P. M. Sherman. 1988. Inhibition of attachment of *Escherichia coli* RDEC-1 to intestinal microvillus membranes by rabbit ileal mucus and mucin *in vitro*. *Infect. Immun.* 56:2437-2442.
- Dubois, J., K. A. Gilles, and J. K. Hamilton. 1956. Colorimetric method for determination of sugars and related substances. *Ann. Chem.* 28:350-356.
- Forstner, J. F. 1978. Intestinal mucins in health and disease. *Digestion* 17:234-263.
- Forstner, J. F., A. Wesley, M. Mantle, H. Kopelman, D. Man, and G. Forstner. 1984. Abnormal mucus: nominated but not yet elected. *J. Pediatr. Gastroenterol. Nutr.* 3(Suppl.):S67-S73.
- Gilman, R. H., R. Partanen, K. H. Brown, W. M. Spira, S. Kahanam, B. Greenberg, S. R. Bloom, and A. Ali. 1988. Decreased gastric acid secretion and bacterial colonization of stomach in severely malnourished Bangladeshi children. *Gastroenterology* 94:1308-1314.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Laux, D. C., E. F. McSweeney, and P. S. Cohen. 1984. Adhesion of enterotoxigenic *E. coli* to immobilized intestinal mucosal preparations: a model for adhesion to mucosal surface components. *J. Microbiol. Methods* 2:27-39.
- Laux, D. C., E. F. McSweeney, T. J. Williams, E. A. Wadolkowski, and P. S. Cohen. 1986. Identification and characterization of mouse small intestine mucosal receptors for *Escherichia coli* K-12(K88ab). *Infect. Immun.* 52:18-25.
- Levine, M. M., M. P. Rennels, and V. Daya. 1980. Hemagglutination and colonizing factor antigens in enterotoxigenic and enteropathogenic *E. coli* that cause diarrhea. *J. Infect. Dis.* 141:733-737.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Markwell, M. A. K. 1982. A new solid state reagent to iodinate proteins. *Anal. Biochem.* 125:427-432.
- Mouricout, M. A., and R. A. Julien. 1987. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf small intestinal mucins. *Infect. Immun.* 55:1216-1223.
- Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine. 1987. Patterns of adherence of diarrheagenic *E. coli* to HEP-2 cells. *J. Pediatr. Infect. Dis.* 6:829-831.
- Nataro, J. P., K. O. Maher, P. Mackie, and J. B. Kaper. 1987. Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. *Infect. Immun.* 55:2370-2377.
- Neutra, M. R., and J. F. Forstner. 1987. Gastrointestinal mucus. Synthesis, secretion, and function, p. 975-1004. *In* L. R. Johnson (ed.), *Physiology of the gastrointestinal tract*, 2nd ed. Raven Press, New York.
- Podolsky, D. K., and D. A. Fournier. 1988. Alterations in mucosal content of colonic glycoconjugates in inflammatory bowel disease by monoclonal antibodies. *Gastroenterology* 95:

- 379-387.
22. **Robins-Browne, R. M.** 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. *Rev. Infect. Dis.* **9**:28-53.
 23. **Satterwhite, T. K., D. G. Evans, H. L. Dupont, and D. J. Evans, Jr.** 1978. Role of *Escherichia coli* colonization factor antigen in acute diarrhea. *Lancet* **ii**:181-184.
 24. **Sherman, P., N. Fleming, J. Forstner, N. Roomi, and G. Forstner.** 1987. Bacterial and the mucus blanket in experimental small bowel bacterial overgrowth. *Am. J. Pathol.* **126**:527-534.
 25. **Sherman, P. M., and E. C. Boedecker.** 1987. Regional differences in attachment of enteroadherent *E. coli* strain RDEC-1 to rabbit intestine: liminal colonization but lack of mucosal adherence in jejunal self-filling blind loops. *J. Pediatr. Gastroenterol. Nutr.* **6**:439-444.
 26. **Sherman, P. M., and E. C. Boedecker.** 1987. Pilus mediated interactions of the *E. coli* strain RDEC-1 with mucosal glycoproteins in the small intestine of rabbits. *Gastroenterology* **93**:734-743.
 27. **Snyder, J. D., and W. A. Walker.** 1987. Structure and function of intestinal mucin: developmental aspects. *Int. Arch. Allergy Appl. Immunol.* **82**:351-356.
 28. **Ulshen, M. H., and J. L. Rollo.** 1980. Pathogenesis of *E. coli* gastroenteritis in man: another mechanism. *N. Engl. J. Med.* **302**:99-101.
 29. **Vial, P. A., R. Robins-Browne, H. Lior, V. Prado, J. B. Kaper, J. P. Nataro, D. Manwal, A. El-Sayed, and M. M. Levine.** 1988. Characterization of enteroadherent-aggregative *E. coli*, a putative agent of diarrheal disease. *J. Infect. Dis.* **158**:70-79.
 30. **Vishwanath, S., and R. Ramphal.** 1984. Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. *Infect. Immun.* **45**:197-202.
 31. **Vishwanath, S., R. Ramphal, C. M. Guay, D. DesJardins, and G. P. Pier.** 1987. Respiratory-mucin inhibition of the opsonophagocytic killing of *Pseudomonas aeruginosa*. *Infect. Immun.* **56**:2218-2222.
 32. **Wanke, C. A., and R. L. Guerrant.** 1987. Small-bowel colonization alone is a cause of diarrhea. *Infect. Immun.* **55**:1924-1926.
 33. **Wanke, C. A., and R. L. Guerrant.** 1988. Enterotoxigenic *E. coli*, p. 253-263. *In* M. G. J. Farthing and G. T. Keusch (ed.), *Enteric infection*. Chapman & Hall, Ltd., London.