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Binding of *Yersinia enterocolitica* to Rabbit Intestinal Brush Border Membranes, Mucus, and Mucin

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Mucus and its gel-forming glycoprotein component, mucin, are thought to protect the gastrointestinal tract from enteric pathogens by inhibiting their attachment to enterocytes. In this study, we investigated interactions between *Yersinia enterocolitica* (isogenic strains of virulent and nonvirulent organisms) and crude mucus, highly purified mucin, and brush border membranes (BBMs) isolated from the upper, mid-, and distal small intestine and the proximal colon of the rabbit. Adherence of radiolabeled bacteria was assessed to BBMs, mucus, and mucin immobilized in polystyrene microtiter plate wells. Virulent *Y. enterocolitica* showed saturable binding to mucus, mucin, and BBMs from all four regions of the intestinal tract, although adherence to BBMs was appreciably greater than that to mucus or mucin. Maximal binding of bacteria was higher to BBMs from the distal small intestine and the proximal colon than to those from the upper and mid-small intestine, which may in part explain why the organism localizes to the ileo-caecal regions of the gut. Adherence of virulent *Y. enterocolitica* to BBMs was significantly reduced in the presence of homologous mucus or mucin preparations. Binding of virulent bacteria appears to depend on plasmid-encoded proteins located on the outer surface membrane, since (i) the isogenic strain lacking the virulence plasmid showed markedly less binding to all BBM, mucus, and mucin preparations; (ii) growth of the virulent strain at 25°C, which inactivates its plasmid, significantly diminished binding to BBMs, mucus, and mucin; and (iii) mild proteolysis substantially decreased adherence of virulent bacteria to BBMs. Compared with rabbit intestinal and colonic mucins, binding of virulent *Y. enterocolitica* was significantly greater to purified human intestinal mucin and significantly less to rat intestinal mucin. These findings provide support for the role of mucus and mucin in host defense by preventing adherence of virulent *Y. enterocolitica* to epithelial cell membranes.

The mammalian gastrointestinal tract is lined with a continuous layer of mucus which is thought to form a physical barrier between the underlying mucosa and potentially harmful substances in the lumen and to act as a lubricant preventing physical injury of the mucosal surface (5, 33). The principal component of mucus is a complex, high-molecular-weight ($>2 \times 10^6$) glycoprotein (mucin) that is responsible for the characteristic visco-elastic properties of the secretion (33). It has been suggested that mucus or mucin itself may protect against enteric pathogens by entrapping them in the gut lumen, inhibiting their attachment to epithelial membranes, and assisting in their removal from the body. This suggestion is based largely on the observation that pathogenic organisms (16, 20, 28) and their secretory products (particularly enterotoxins [9, 31]) cause a marked increase in mucin secretion.

Recently, we investigated the effects of *Yersinia enterocolitica*, a common cause of infectious diarrhea in humans, on the production of intestinal and colonic mucin in the rabbit (25). Infection of rabbits with *Y. enterocolitica* is known to result in disease that resembles human yersiniosis both clinically and morphologically (34, 36). While synthesis and secretion of mucin were increased throughout the intestinal tract by day 6 postinfection, the greatest changes occurred in the distal small intestine and the proximal colon, where the morphologic impact of the disease is most severe (34, 36).

The mechanism by which *Y. enterocolitica* colonizes the gut is unknown. In contrast to nonpathogenic environmental isolates, pathogenic strains of *Y. enterocolitica* contain a 42- to 50-megadalton plasmid (10). While the virulence-associated plasmid is required for the pathogenesis of disease, its precise role is not clear. The plasmid codes for 16 to 20 proteins (38, 40). A number of these are located on the outer membrane of the bacterium and form fibrillae (14, 38) that significantly increase the surface charge and hydrophobicity of the organism (17, 39). Among the virulent properties of *Y. enterocolitica* is the ability to adhere to, invade, and detach a variety of epithelial cells (HeLa, Henle, HEp-2, and Vero cells) in tissue culture (11, 35, 37, 39, 41, 42). However, the virulence plasmid may not be responsible for tissue invasion, since this property is restricted to certain serotypes and biotypes and strains lacking the plasmid can also invade tissue culture cells (11, 35, 37, 39, 41). Recently, two chromosomal loci, *inv* and *ail*, were identified in a virulent strain of *Y. enterocolitica* which individually conferred an invasive phenotype on noninvasive *Escherichia coli* HB101 (29). The *inv* gene is present in all strains of *Y. enterocolitica*, but the *ail* gene was only found in pathogenic strains that invaded tissue culture cells (30). Although invasion appears to be controlled largely by the presence of particular chromosomal genes, a number of studies have suggested that the adhesive properties of *Y. enterocolitica* may be primarily determined by the virulence plasmid (11, 39, 41).

The adherent properties of *Y. enterocolitica* have been assessed largely from experiments carried out with epithelial cell cultures. These cells are undifferentiated, may not have the same surface properties as intestinal epithelial cells in vivo, and may lack the usual defense mechanisms against bacterial infections. Furthermore, the effects of mucus or mucin on *Y. enterocolitica* adherence have not been examined previously. The aims of this study were to investigate

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whether *Y. enterocolitica* is capable of binding to mucus, purified mucin, or brush border membranes (BBMs) from different regions of the rabbit intestinal tract and to determine whether preferential binding plays a role in localization of the organism to the distal small intestine and the proximal colon.

MATERIALS AND METHODS

Preparation of mucus and mucin. After an overnight fast, male New Zealand White rabbits (weighing ~1 kg) were sacrificed by a lethal dose of Euthanyl (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The entire small intestine and proximal colon were rapidly removed, and the small intestine was sectioned into upper, mid-, and distal regions. Each section of the gut was treated identically but separately. The mucosa was gently scraped with a rubber spatula to remove the adherent mucus layer. Care was taken to avoid damaging the underlying epithelium. Mucus scrapings were collected in ice-cold phosphate-buffered saline (PBS; 0.1 M NaCl, 0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.4) containing proteolytic inhibitors (5 mM disodium EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10 mM *N*-ethylmaleimide) and homogenized briefly with a Polytron tissue solubilizer to disperse the gel. The concentration of the solution was adjusted to 5 mg of protein per ml, as determined by the method of Lowry et al. (22), except in the case of mucus from the proximal colon, which was adjusted to a final concentration of 3 mg of protein per ml. The concentration of mucin in these crude mucus preparations was measured by a highly specific enzyme-linked immunoassay (24).

Mucin was purified in the presence of proteolytic inhibitors from the upper, mid-, and distal small intestine and the proximal colon of the rabbit as described previously (24). Mucosal scrapings were homogenized, and mucin was isolated by equilibrium density gradient centrifugation in CsCl (twice) followed by gel filtration on Sepharose 2B. Completely purified mucin, harvested from the void volume fractions of the column, was dialyzed and stored lyophilized at −80°C. The chemical compositions of the four rabbit mucins and their polymeric structures were documented in earlier studies (24). In addition, human and rat intestinal mucins were isolated by the same procedure from the entire length of the small bowel. Human mucin was prepared from tissue obtained 4.5 h postmortem from a patient (H35) with no history of gastrointestinal disease (23). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, all mucin preparations produced a diffuse, silver-staining band in the stacking gel; no other bands were observed in the separating gel, indicating the absence of noncovalently bound contaminating protein (23, 24). The failure to detect DNA (<2 μg of glycoprotein per ml), mannose, glucose, or uronic acid (by gas-liquid chromatography) confirmed the purity of all mucin preparations (23, 24). For binding assays, purified mucins were dissolved in PBS at a final concentration of 1 mg (dry weight)/ml.

Preparation of BBMs. Right-side-out BBM vesicles were prepared from the upper, mid-, and distal small intestine and the proximal colon of rabbits by the method of Forstner et al. (8). BBMs were suspended in PBS to a final protein concentration of 1 mg/ml, unless otherwise specified. Preparation of BBMs does not result in the copurification of mucin: when assessed by our mucin-specific immunoassay (24), BBM preparations of 1 mg of protein per ml contained <5 μg of mucin per ml.

For some experiments, rabbits were given an intraperitoneal injection of 100 μCi of D-[1-^{14}C]glucosamine hydrochloride (specific activity, 58.7 mCi/mmol; Dupont Canada Inc. (NEN Research Products), Lachine, Quebec, Canada) 3 h before sacrifice to label BBMs (7), which were then isolated as above.

Bacteria and growth conditions. Two isogenic strains of *Y. enterocolitica* serotype O:3, biotype 4, MCH700S (plasmid bearing, P^+) and MCH700L (plasmidless, P^-), were used. Virulence in experimental infection and the plasmid profiles of these strains have been described previously (21, 35, 36). Samples from frozen stock cultures of both strains were inoculated into minimum essential medium (GIBCO Diagnostics, Madison, Wis.) without methionine and grown overnight at 25°C in a rotary shaker (150 rpm). Fresh medium was inoculated with a 10% inoculum, and the culture was incubated at 37°C for a total of 3 h. After 30 min, 100 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham Canada, Oakville, Ontario, Canada) was added to the culture medium, followed 1 h later by 5 mg of cold methionine. In some experiments, the virulent strain of *Y. enterocolitica* P^+ was not shifted to 37°C but was maintained throughout at 25°C, even for radiolabeling. These organisms are designated *Y. enterocolitica* P^+ (25°C). At the end of the incubation period, bacteria were pelleted by centrifugation, washed twice with sterile PBS to remove unbound radiolabel, and finally suspended in sterile PBS at a concentration of ~10^{10}/ml. To assess whether the binding properties of *Y. enterocolitica* involved proteins on the outer membrane surface, separate bacterial cultures were digested with 1 mg of pronase (type XIV from *Streptomyces griseus*; Sigma Chemical Co., St. Louis, Mo.) per ml in PBS at 37°C for 1 h (21, 26) and were then washed and suspended. Control cultures were incubated concurrently but without pronase. Control and digested bacteria were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before and after pronase treatment as described by Lian and Pai (21). Viable counts of bacteria were obtained by plating 0.1-ml samples of serial 10-fold dilutions onto salmonella-shigella agar. To determine the radioactivity incorporated per bacterium, a 50-μl sample of the final suspension was counted by scintillation spectrometry.

Binding assay. The method used to assess binding of *Y. enterocolitica* to rabbit intestinal BBM, mucus, and mucin preparations was a modification of that described by Laux et al. (19). Duplicate assays were performed in 96-well round-bottomed polystyrene tissue culture plates (Flow Laboratories, Inc., McLean, Va.). Each substrate (50 μl) was added to the wells of the microtiter plate and incubated overnight at 4°C. Unbound material was removed by three washes with PBS. Residual binding sites were blocked by addition of 200 μl of skim milk (20 mg/ml in PBS) and incubation at room temperature for 2 h. Unbound material was again removed by three washes with PBS. Serial dilutions of freshly prepared radiolabeled bacteria (50 μl) were added to the wells and incubated for 2 h at 25°C. Preliminary experiments indicated that 2 h was sufficient time to achieve maximum binding of *Y. enterocolitica* strains to BBM, mucus, or mucin. After thorough washing with PBS (four times), plates were drained dry and wells were cut out and counted directly by scintillation spectrometry. Counting efficiency and quenching were unaffected by the presence of the well and were constant among experiments. The number of *Y. enterocolitica* bound to each well was calculated based on the specific activity of the bacterial preparation used for that particular experiment. Control wells containing skim milk but no BBM, mucus, or mucin were included in each

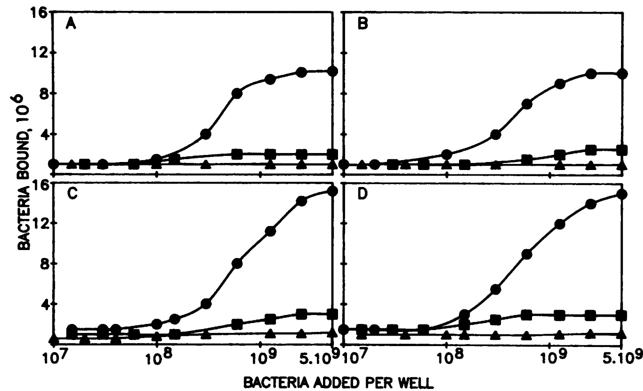

FIG. 1. Adherence of *Y. enterocolitica* to BBMs from the upper (A), mid- (B), and distal (C) small intestine and the proximal colon (D) of the rabbit. Symbols: ●, virulent *Y. enterocolitica* P^+; ■, *Y. enterocolitica* P^+(25°C); ▲, nonvirulent *Y. enterocolitica* P^-. BBMs (50 μl of 1 mg of protein per ml) were immobilized on polystyrene microtiter plate wells, and remaining binding sites in the wells were blocked with skim milk (200 μl of 20 mg/ml) before addition of bacteria. Each assay was performed at least three times in duplicate. The graphs presented are composites, compiled from data from all experiments.

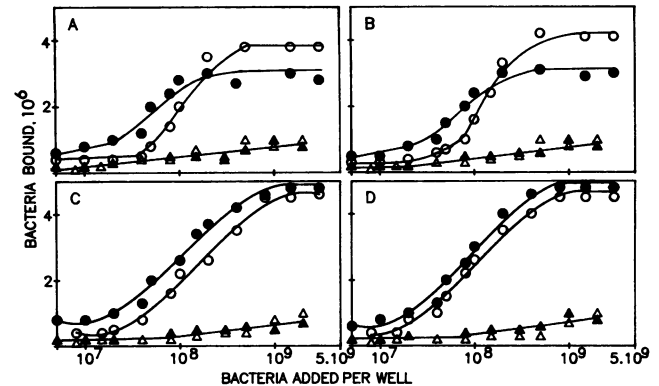

FIG. 2. Adherence of *Y. enterocolitica* to crude mucus and purified mucin from the upper (A), mid- (B), and distal (C) small intestine and the proximal colon (D) of the rabbit. Symbols: ● and ○, binding of *Y. enterocolitica* P^+ to mucus and mucin, respectively; ▲ and △, binding of *Y. enterocolitica* P^- to mucus and mucin, respectively. Mucus (50 μl of 5 mg of protein per ml [A, B, and C] or 3 mg of protein per ml [D]) or mucin (50 μl of 1 mg [dry weight]/ml) was immobilized on polystyrene microtiter plate wells, and remaining binding sites in the wells were blocked with skim milk (200 μl of 20 mg/ml) before addition of bacteria. Each assay was performed three times in duplicate. The graphs presented are composites, compiled from data from all experiments.

experiment to ensure that background binding of *Y. enterocolitica* did not exceed 10% of maximum binding.

To evaluate the inhibitory effects of homologous mucus or mucin preparations on the binding of *Y. enterocolitica* to BBMs, microtiter plate wells were initially coated overnight at 4°C with BBMs. After washing the wells and blocking with skim milk, dilutions (in 50 μl) of mucus or mucin from the same region of the gut as the BBM preparation were placed in the wells and then a constant amount of radiolabeled *Y. enterocolitica* (in 50 μl) was added. Following a 2-h incubation, wells were washed and counted as described above.

When possible, values are presented as means ± standard errors, and means were compared by Student's *t* test. Since the amount of radiolabel incorporated by bacteria and the actual number of bacteria added per well varied somewhat from experiment to experiment, it was not always possible to calculate standard errors for each datum point. In these cases, the results from all experiments were plotted on a single composite graph.

RESULTS

Binding of *Y. enterocolitica* to BBMs, mucus, and mucin. Adherence of isogenic strains of virulent and nonvirulent *Y. enterocolitica* to BBMs from the upper, mid-, and distal small intestine and the proximal colon of the rabbit was examined by adding increasing numbers of bacteria to microtiter plate wells coated with a constant amount of BBMs. Virulent *Y. enterocolitica* P^+ bound to all BBM preparations in progressively increasing numbers, reaching saturation at 2 $\times 10^9$ to 3 $\times 10^9$ bacteria added per well (Fig. 1). The slope of the binding curve was the same for BBMs from the four regions of the gut, but maximum binding was greatest to BBMs from the distal small intestine and the proximal colon. To confirm that this finding did not reflect differences in BBM binding to microtiter plate wells, radiolabeled BBMs were used to determine how much material actually bound to the wells. Labeled BBMs (of known specific activity) from the upper, mid-, and distal small intestine and the proximal colon were incubated in microtiter plate wells under standard assay conditions, but no bacteria were added. In all cases, essentially the same amount of protein (~8 μg) bound to the wells. Compared with virulent organisms, nonvirulent *Y. enterocolitica* P^- showed significantly lower binding to all BBM preparations ($P < 0.001$) (Fig. 1). When *Y. enterocolitica* P^+ was cultured at 25°C, a condition which inhibits the expression of plasmid-encoded proteins, binding to BBM preparations was markedly reduced (Fig. 1). Adherence of *Y. enterocolitica* P^+(25°C) was essentially the same as that of *Y. enterocolitica* P^-, and in both cases maximum binding was similar in the four intestinal regions.

Adherence of *Y. enterocolitica* P^+ and P^- to crude mucus and purified mucin preparations from the small intestine and proximal colon is shown in Fig. 2. As in the case of BBMs, *Y. enterocolitica* P^+ showed saturable binding to mucus and mucin derived from all four regions of the gut. Binding curves (slopes and maxima) were essentially the same for mucus and mucin isolated from the four intestinal regions. However, maximum binding and the slope of the binding curves to both mucus and mucin were appreciably lower than that seen with BBMs ($P < 0.001$). Compared with *Y. enterocolitica* P^+, adherence of nonvirulent *Y. enterocolitica* P^- and P^+(25°C) (latter data not shown) was significantly lower to all mucus and mucin preparations ($P < 0.005$ at maximum binding).

To assess whether binding of *Y. enterocolitica* to mucus was due to the mucin present, microtiter plate wells were coated with either mucus or an equivalent amount of mucin and a constant number of bacteria were added. In the case of *Y. enterocolitica* P^+, binding to mucin only accounted for 35 to 45% of the binding to mucus from all four regions of the intestinal tract (Fig. 3). In contrast, adherence of *Y. enterocolitica* P^- and P^+(25°C) to mucin accounted for the majority of the binding to mucus in all regions of the gut.

To investigate whether outer membrane proteins were involved in binding, bacteria were pretreated with pronase under conditions known to degrade plasmid-mediated cell surface proteins (21, 26) and adherence to BBMs was measured. Following proteolysis, binding of *Y. enteroco-*

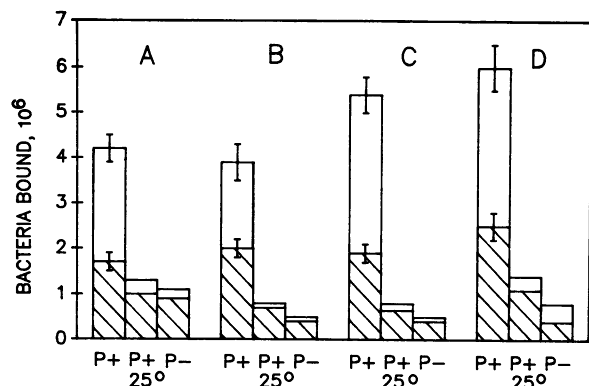

FIG. 3. Binding of *Y. enterocolitica* to mucus and mucin. Mucus (50 μl, containing 20 μg of mucin) or purified mucin (20 μg in 50 μl) from the upper (A), mid- (B), and distal (C) small intestine and the proximal colon (D) of the rabbit was immobilized on polystyrene microtiter plate wells, and remaining binding sites in the wells were blocked with skim milk (200 μl of 20 mg/ml) before addition of bacteria (6×10^8 to 8×10^8). P^+, Virulent *Y. enterocolitica* P^+; P^+/25°, *Y. enterocolitica* P^+(25°C); P^-, nonvirulent *Y. enterocolitica* P^-. Symbols: ▭, binding to mucus; ▨, binding to mucin. Each experiment was performed three times in duplicate. Binding of *Y. enterocolitica* P^+ to mucus was significantly greater than to a comparable amount of mucin ($P < 0.005$). Binding of *Y. enterocolitica* P^+(25°C) and P^- was essentially the same to both mucus and mucin.

litica P^+ to BBMs was reduced by ~40% compared with control organisms incubated in the absence of pronase (Fig. 4). (It should be noted that binding of control [nondigested] bacteria in these experiments did not reach the maximum levels shown in Fig. 1 since only $\sim 4 \times 10^8$ organisms were added to the wells.) Proteolysis had essentially no effect on adherence of *Y. enterocolitica* P^- or P^+(25°C) to BBMs; the reduction in binding after digestion was <10%. To confirm the effects of pronase on membrane proteins, bacteria were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before and after digestion. On Coomassie

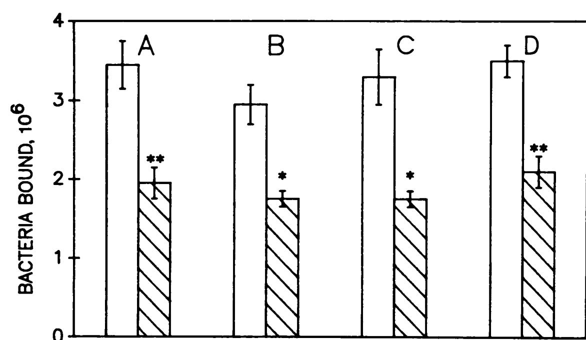

FIG. 4. Effects of pronase digestion on binding of *Y. enterocolitica* P^+ to BBMs from the upper (A), mid- (B), and distal (C) small intestine and the proximal colon (D) of the rabbit. *Y. enterocolitica* P^+ was digested with pronase (1 mg/ml) for 1 h at 37°C. Control bacteria were incubated simultaneously but without enzyme. Digested and control bacteria were washed thoroughly, and $\sim 4 \times 10^8$ organisms were added to microtiter plate wells previously coated with BBMs (50 μl of 1 mg/ml) and blocked with skim milk (200 μl of 20 mg/ml). Symbols: ▭, control *Y. enterocolitica* P^+; ▨, pronase-digested *Y. enterocolitica* P^+. Each experiment was performed twice in quadruplicate. Proteolysis significantly reduced binding of *Y. enterocolitica* P^+ to BBMs (*, $P < 0.02$; **, $P < 0.005$).

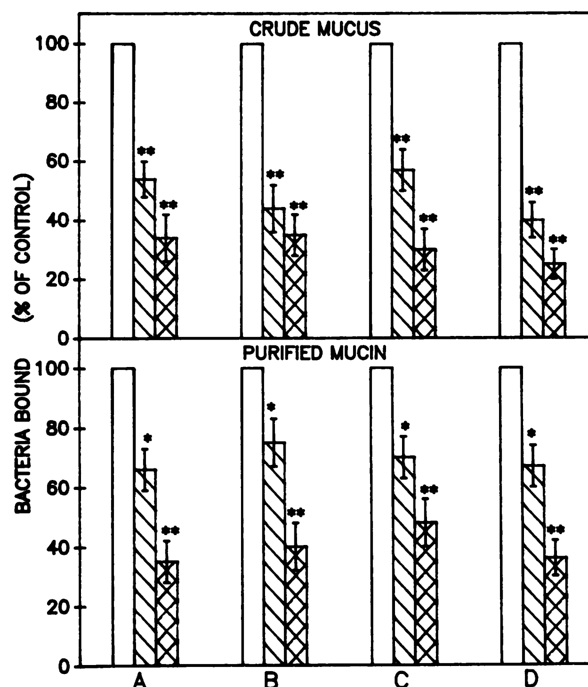

FIG. 5. Inhibition of binding of *Y. enterocolitica* P^+ to BBMs from the upper (A), mid- (B), and distal (C) small intestine and the proximal colon (D) of the rabbit by homologous crude mucus and purified mucin. Microtiter plate wells were coated with BBMs (50 μl of 5 mg/ml), and remaining binding sites in the wells were blocked with skim milk (200 μl of 20 mg/ml). (Upper) *Y. enterocolitica* P^+ (6×10^8 to 8×10^8 organisms) was added to the wells in the presence of PBS (□) or crude mucus (5 μg of protein containing 0.4 μg of mucin per well [▧]) or 50 μg of protein containing 4 μg of mucin per well [▩]). (Lower) *Y. enterocolitica* P^+ (6×10^8 to 8×10^8 organisms) was added to the wells in the presence of PBS (□) or purified mucin (5 μg of mucin per well [▧] or 50 μg of mucin per well [▩]). Each experiment was performed five times in duplicate. Binding of *Y. enterocolitica* P^+ was significantly reduced in the presence of both crude mucus and purified mucin (*, $P < 0.05$; **, $P < 0.005$).

blue-stained gels, the protein profiles of *Y. enterocolitica* P^- and P^+(25°C) were essentially the same, while *Y. enterocolitica* P^+ contained a prominent extra band of 200,000 molecular weight. Pronase digestion had no effect on the protein profiles of *Y. enterocolitica* P^- and P^+(25°C), but the 200-kilodalton protein of *Y. enterocolitica* P^+ was destroyed (data not shown). These results are identical to those shown in earlier studies by Lian and Pai (21) on the same strains of *Y. enterocolitica*.

Inhibition of binding of *Y. enterocolitica* to BBMs by mucus and mucin. In the presence of mucus or mucin, binding of *Y. enterocolitica* P^+ to BBMs from all four regions of the intestinal tract was markedly reduced (Fig. 5). Increasing concentrations of mucus or mucin increased the degree of inhibition. Binding of *Y. enterocolitica* P^+ to BBMs could be inhibited with as little as 1 μg of mucus (protein) and 2 μg of mucin; at levels below this, there was no detectable inhibition. The inhibitory effect of crude mucus was greater than could be explained by its mucin content. Inhibition with crude mucus containing 4 μg of mucin (Fig. 5, upper panel) was significantly greater ($P < 0.05$) than that seen with 5 μg of purified mucin (Fig. 5, lower panel). Since binding of *Y. enterocolitica* P^- and P^+(25°C) to BBMs from the four regions of the gut was initially very low, it was difficult to

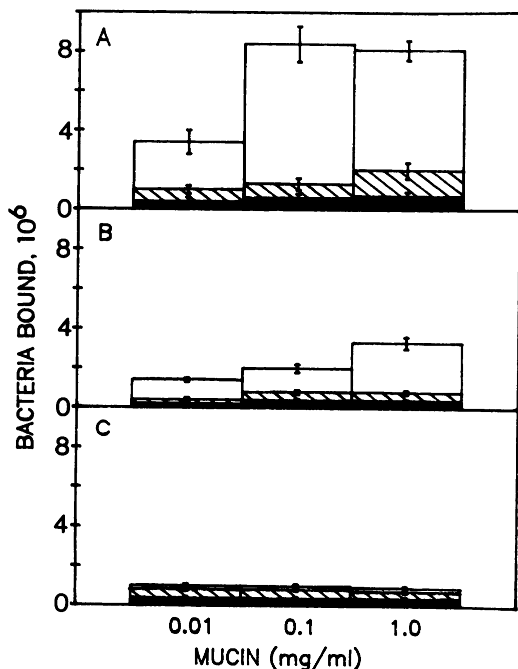

FIG. 6. Adherence of *Y. enterocolitica* to purified intestinal mucins from humans (A), rabbits (B), and rats (C). Microtiter plate wells were coated with purified mucin (in 50 μl), remaining binding sites were blocked with skim milk (200 μl of 20 mg/ml), and $\sim 10^9$ bacteria were added to each well. Symbols: □, *Y. enterocolitica* P^+; ▧, *Y. enterocolicia* P^+(25°C); ■, *Y. enterocolitica* P^-. Data shown in panel B indicate binding to mucin from the rabbit mid-small intestine. Adherence to rabbit intestinal mucin was measured three times in duplicate, while binding to human and rat intestinal mucin was assessed twice in duplicate. Compared with rabbit intestinal mucin, adherence of *Y. enterocolitica* P^+ was significantly greater to human intestinal mucin ($P < 0.001$) and significantly less to rat intestinal mucin ($P < 0.02$).

demonstrate reliably any inhibition of adherence by either mucus or mucin.

Binding of *Y. enterocolitica* to human and rat intestinal mucin. To assess whether binding of *Y. enterocolitica* to intestinal mucin was specific to the rabbit, we investigated adherence of the organism to purified intestinal mucins from humans and rats (Fig. 6). *Y. enterocolitica* P^+ showed saturable binding to human intestinal mucin, but the slope of the binding curve and maximum adherence were considerably higher than those observed to mucin from the rabbit small or large intestine ($P < 0.001$). In contrast, binding of *Y. enterocolitica* P^+ to rat intestinal mucin was significantly lower than that to rabbit intestinal mucins ($P < 0.02$). As in the case of rabbit mucins, nonvirulent *Y. enterocolitica* P^- or P^+(25°C) showed limited adherence to either human or rat intestinal mucin compared with virulent *Y. enterocolitica* P^+.

DISCUSSION

It has often been suggested that the mucus layer covering the epithelial surface may protect against colonization of the intestinal tract by enteric pathogens by inhibiting their attachment to enterocytes, an essential early step in the processes that lead to disease (1). To investigate this hypothesis, the present studies were designed with three objectives in mind: (i) to examine whether *Y. enterocolitica*, an enteroinvasive organism, was capable of binding to mucus, mucin (the gel-forming glycoprotein component of mucus), and BBMs from the rabbit intestine; (ii) to establish whether binding was greater in those regions of the gut most affected during yersiniosis, namely, the distal small intestine and the proximal colon (34, 36); and (iii) to determine whether mucus and mucin prevented adherence of the organisms to BBMs.

The virulent strain of *Y. enterocolitica* used in these experiments, P^+, showed saturable binding to mucus, mucin, and BBMs isolated from the upper, mid-, and distal small intestine and the proximal colon of rabbits, although adherence to BBMs was appreciably greater than that to mucus and mucin. Of particular interest was the observation that maximal binding of *Y. enterocolitica* P^+ was higher to BBMs from the distal small intestine and the proximal colon than to those from the upper and mid-small intestine. These findings suggest that the number of receptors for the organism is greater in cell membranes in the ileo-cecal regions of the gut. It is possible that enhanced binding of *Y. enterocolitica* P^+ to BBMs may at least in part explain why the organism localizes to, and causes the most damage in, the distal small intestine and the proximal colon. Similar results were obtained in previous studies on rabbit diarrheagenic *E. coli* RDEC-1, when adherence was found to be significantly greater to rabbit ileal BBMs than to jejunal BBMs (3). Again, the in vitro adherence of RDEC-1 to host cell membranes correlated positively with in vivo infectivity and colonization of the intestinal tract.

Binding of *Y. enterocolitica* P^+ to BBMs could not be attributed to the presence of mucin since our membrane preparations were essentially devoid of mucin. Similarly, it is unlikely that bacterial binding to mucin was due to the presence of BBM components since our mucins were highly purified. Binding curves indicated that the affinity of *Y. enterocolitica* P^+ for BBMs exceeded that for mucin, perhaps suggesting that the binding site in the BBM receptor differs somewhat from the one in mucin. Adherence of *Y. enterocolitica* P^+ to mucus could not be attributed entirely to the mucin present. Therefore, it is likely that the organism also binds to other components present in the crude mucus preparations, possibly fragments of membrane derived from sloughed epithelial cells or even secretions such as immunoglobulin A.

Inhibition experiments demonstrated that both mucus and mucin were capable of decreasing attachment of *Y. enterocolitica* to enterocyte BBMs. Perhaps not surprisingly, in view of the relative binding to mucus and mucin, mucus was a better inhibitor of bacterial attachment to BBMs than mucin. Thus, both crude mucus and its purified mucin component could provide mucosal protection in vivo against adherence and invasion by *Y. enterocolitica* by limiting interaction of the organism with epithelial cell membranes.

Adherence of *Y. enterocolitica* P^+ to BBMs, mucus, and mucin appears to depend on the presence of its virulence plasmid since the isogenic nonvirulent strain lacking the plasmid, *Y. enterocolitica* P^-, showed markedly reduced binding to all substrates from the four regions of the gut. In addition, growth of *Y. enterocolitica* P^+ at 25°C, which inactivates the plasmid, also significantly decreased binding of the organism to BBM, mucus, and mucin preparations, suggesting that adherence requires plasmid expression. Since mild proteolytic digestion, using conditions known to degrade exposed surface proteins (21, 26), substantially diminished (by ~40%) binding of *Y. enterocolitica* P^+ cultured at 37°C to BBMs, it would appear that the plasmid-

encoded proteins involved in adhesion are possibly located on the outer surface of the bacterial membrane. These results are in agreement with previous studies indicating a role for the outer membrane proteins of virulent *Y. enterocolitica* in its adherence properties (11, 39, 40).

It is not clear whether the receptor(s) or binding site(s) for *Y. enterocolitica* in mucus, mucin, and BBMs are the same in the four regions of the gut. Since the affinity of the organism for BBMs (as assessed from the slopes of the binding curves) did not change down the length of the intestinal tract, it would appear likely that the receptor (and its binding site) is the same in each region of the gut. Similarly, the binding sites in mucus and mucin did not appear to differ in the four regions of the intestinal tract.

Compared with rabbit intestinal and colonic mucins, binding of *Y. enterocolitica* P^+ was significantly greater to purified human intestinal mucin and significantly less to rat intestinal mucin. These findings illustrate that intestinal mucins from the three species do not contain the same bacterial binding sites, despite considerable similarity in their overall chemical compositions and physical structures. Since neither the peptide cores nor the oligosaccharide side chains of human, rat, and rabbit intestinal mucins have been sequenced, it is not yet possible to speculate whether the differences in bacterial binding are related to differences in the protein or the carbohydrate moeities of the mucins. Both the number and the affinity of the binding sites for *Y. enterocolitica* P^+ were found to increase in the order of rat intestinal mucin $<$ rabbit intestinal and colonic mucins $<$ human intestinal mucin. The observation of differential binding to human, rabbit, and rat intestinal mucins was particularly interesting in view of the fact that the strain of *Y. enterocolitica* P^+ used in the present studies was initially isolated from a patient with diarrhea and is known to be virulent in rabbits (25, 34, 36) but not in rats. This suggests that the species specificity of the organism may be related to its ability to carry out the initial step of the colonization or invasion process, namely, adherence to the intestinal mucus layer.

Previous studies have suggested an interaction between mucus and enteric organisms. For example, a nonpathogenic human fecal isolate of *E. coli* F-18, which rapidly colonizes the mouse large intestine, adheres to mouse colonic mucus, apparently through a specific interaction between the bacterial lipopolysaccharide and a high-molecular-weight glycoprotein of colonic mucus (4). Laux et al. (19) isolated porcine enterotoxigenic *E. coli* containing K88 and K99 mannose-resistant pili and found that these organisms adhere to crude mucus preparations from the small and large intestines of mice. Similarly, Mouricourt and Julien (32) demonstrated that enterotoxigenic *E. coli* strains, capable of causing diarrhea in calves, bind to calf intestinal mucus and that adhesion requires particular bacterial pili (K99, FY, and F41) and a glycoprotein receptor in mucus. Noninvasive RDEC-1 also adheres to rabbit intestinal mucus and mucin (as well as BBMs), again by means of pili (6). *Vibrio cholerae* O1 (43), *Campylobacter jejuni* (27), the fungal pathogen *Candida albicans* (15), and the protozoan *Entamoeba histolytica* (2) have all been reported to associate with mucus. Most of the above studies assessed adherence by using crude mucus preparations or partially purified mucins, isolated by gel filtration, which does not completely remove noncovalently attached material from the mucin. Therefore, it is not clear from these studies whether the organisms truly interact with the mucin component of mucus. In two cases, however, RDEC-1 (6) and *Entamoeba histolytica* (2), specific binding of the organism to thoroughly purified mucin was demonstrated. As in the present study with *Y. enterocolitica*, attachment of RDEC-1 and *Entamoeba histolytica* to rabbit intestinal BBMs and rat colonic epithelial cells, respectively, was significantly inhibited by the presence of homologous, highly purified mucin (2, 6). Thus, there is considerable support for the role of mucin in host defense by preventing adherence of enteric pathogens to epithelial cells.

The bacterial protein(s) responsible for attachment of *Y. enterocolitica* P^+ to BBMs, mucus, and mucin has not yet been identified. The virulence plasmid of *Y. enterocolitica* is extremely complex, coding for 16 to 20 polypeptides, some of which are located on the outer surface membrane (38, 40). Heesemann and Gruter (12) showed that attachment of *Y. enterocolitica* to cultured epithelial cells is mediated by the plasmid-encoded, 220-kilodalton outer membrane protein 1. Surface fibrillae, thought to be composed of outer membrane protein 1, are known to affect the charge and hydrophobicity of the organism significantly and appear to be required for autoagglutination and hemagglutination (13, 14, 18). In agreement with earlier studies by Lian and Pai (21), we found only one protein band of 200 kilodaltons on Coomassie blue-stained polyacrylamide gels of *Y. enterocolitica* P^+ that was not present in *Y. enterocolitica* P^- and P^+(25°C). This protein was destroyed by mild proteolysis, which also markedly decreased binding of the organisms to BBMs. Although it appears from these results that the adhesin of *Y. enterocolitica* is its plasmid-encoded outer membrane protein 1, it should be noted that Coomassie blue staining of polyacrylamide gels is a relatively insensitive technique and other proteins, present in only small quantities and therefore not detected on our gels, may be involved with the binding of *Y. enterocolitica* to BBMs, mucus, and mucin.

While mucus and mucin may protect the intestinal mucosa against enteric pathogens, it is also possible that the ability to bind to mucus components may actually facilitate colonization of the intestinal tract by immobilizing the bacteria in the gel. However, the mucus gel is continually eroded by enzymes (some of which are produced by the bacteria themselves) and by mechanical forces (passage of digesta). This erosion is likely to wash adherent bacteria into the lumen of the digestive tract. Thus, to achieve successful colonization, the replication rate of bacteria attached to mucus must be equal to the rate of erosion of the mucus layer itself. In all likelihood, the stability of the indigenous bacterial population of the gut, which appears to be limited largely to the mucus gel layer (5), is maintained as a result of this balance between replication and erosion. Since many enteric pathogens require access to the epithelium to mediate disease, the organism's virulence factors must overcome the host's defense system; that is, the pathogen must be able to resist turnover with mucus and cross the mucus gel barrier to the epithelium. The mechanisms of binding of *Y. enterocolitica* to epithelial membranes and mucin, including the nature of the bacterial adhesin and the mucin-epithelial receptor, and the means by which the organism traverses the mucus gel require further investigation.

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