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Identification and Characterization of Mouse Small Intestine Mucosal Receptors for *Escherichia coli* K-12(K88ab)

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Adhesion of ^{3}H-labeled *Escherichia coli* K-12(K88ab) to CD-1 mouse small intestine mucus and brush border preparations, immobilized on polystyrene, was studied. *E. coli* K12(K88ab) was shown to adhere readily to either crude mucus or brush border preparations, but not to bovine serum albumin. In contrast, the nearly isogenic *E. coli* K-12 strain, i.e., lacking the K88ab plasmid, did not bind well to either mucus, brush borders, or bovine serum albumin. The adhesion of *E. coli* K-12(K88ab) to both mucus and brush borders required pilus expression (i.e., growth at temperatures greater than 18°C) and was inhibited by pretreatment of either mucus or brush borders with trypsin, pronase, or sodium metaperiodate and by the presence of D-galactosamine. Crude mucus was fractionated by gel filtration, and the proteins in receptor-containing fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were Western blotted to nitrocellulose. Adhesion of $^{35}SO_4$-labeled *E. coli* K-12(K88ab) and $^{35}SO_4$-labeled *E. coli* K-12 to Western blots followed by autoradiography revealed two *E. coli* K-12(K88ab)-specific mucus receptor proteins (57 and 64 kilodaltons). Brush borders contained the same two receptor proteins present in mucus and an additional 91-kilodalton receptor protein.

Enterotoxigenic *Escherichia coli* (ETEC) which cause diarrhea in pigs contain the K88 antigen on their surfaces (15, 27). The K88 antigen, encoded on a plasmid (24, 26), initiates infection by binding pig-specific ETEC to the pig small intestine wall (15, 37, 38). The K88 antigen is contained in a fimbrial structure which is commonly known as the K88 adhesin (39). The K88 adhesin exists in at least three antigenic forms (K88ab, K88ac, and K88ad) which appear to have adhesive specificities for brush border membranes isolated from pigs of differing phenotype (3, 13, 28, 34).

Although K88 adhesin-bearing ETEC cause diarrhea almost exclusively in pigs, it has been reported that they kill suckling mice (19) and that K88-specific receptors exist in mouse and calf epithelial cells (31) and mouse, rat, and rabbit mucus (21) as well as in pig brush border membranes and mucus (11, 34).

The biochemical nature of K88 adhesin-specific receptors has not been clearly elucidated. Indirect evidence exists for both glycolipid and glycoprotein porcine brush border receptors, depending on the assay system employed (18, 33), and for the involvement of several different sugars in K88 adhesin-receptor binding (e.g., terminal β-D-galactosyl residues [11], *N*-acetylglucosamine and *N*-acetylgalactosamine [2], and D-galactosamine [33]), again depending on the assay system employed.

In the present investigation, we examined the nature of K88ab adhesin-specific receptors in CD-1 mouse small intestine crude mucus and in brush border membranes. We identified what appear to be two glycoprotein receptors (57 and 64 kilodaltons [kDa]) present in both mucus and brush border membranes and an additional 91-kDa glycoprotein receptor which resides exclusively in brush borders. Adhesions of *E. coli* K-12(K88ab) to these receptors was found to be periodate sensitive and inhibited predominantly by D-galactosamine.

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MATERIALS AND METHODS

***E. coli* strain.** *E. coli* K-12 (Gyles) and *E. coli* K-12 (Gyles)(K88ab) were supplied by Richard Wilson, *E. coli* Reference Center, Department of Veterinary Science, The Pennsylvania State University, University Park, Pa. The *E. coli* K-12 (Gyles) (K88ab) strain represents a strain of *E. coli* K-12 (Gyles) which has received the K88ab plasmid. Hereafter these strains will be referred to as *E. coli* K-12 and *E. coli* K-12(K88ab).

Mucus preparations. Crude mucus was prepared from the small intestines of 5- to 8-week-old CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) as previously described (21). Twenty-four hours before use the mice were deprived of food and given sterile water containing 0.5% (wt/wt) streptomycin sulfate. The following day the animals (usually four to six) were sacrificed, and the small intestines were removed and placed in sterile petri dishes containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Hanks buffer (pH 7.4). The individual intestines were pooled and cut into 2- to 3-cm lengths. Any feces and partially digested food present were expressed from each section with a rubber spatula. The sections were than transferred to a second set of petri dishes containing HEPES-Hanks buffer (pH 7.4) and split open with a scalpel. The split sections were agitated to remove any remaining debris and transferred to a third set of petri dishes. Each section was then gently scraped with a rubber spatula to remove the mucus layer covering the mucosal surface.

After the intestinal sections were discarded, the mucosal scrapings were centrifuged at 27,000 × *g* for 15 min to remove particulate and cellular material. The resulting supernatant, hereafter called crude mucus, was then analyzed for protein content (22) and employed in adhesion assays.

Preparation of brush borders. Intestinal cells from the mouse small intestine were prepared by the method of Weiser (42). Twenty-four hours before use the mice were deprived of food and given sterile water containing 0.5%

(wt/wt) streptomycin sulfate. Routinely 8 to 10 mice were employed. Sections of the small intestine were removed and flushed with 10 ml of 154 mM saline containing 1 mM dithiothreitol. Sections of intestine were then filled with 27 mM sodium citrate buffer (pH 7.3) and incubated for 15 min at 37°C. The intestines were then refilled with phosphate-buffered saline (PBS) (pH 7.3) containing 1.5 mM EDTA and 0.5 mM dithiothreitol and reincubated for 15 min at 37°C. The PBS eluent was collected and centrifuged at 900 × *g* for 5 min to recover intestinal cells. The cells were washed three times in PBS (pH 7.3) and suspended in HEPES-Hanks buffer, pH 7.4. Brush borders were prepared from freshly isolated intestinal cells by a modification of the method of Frantz et al. (10). Briefly, cells were suspended in a buffer of 300 mM mannitol, 12.5 mM HEPES, and 1 mM EDTA, pH 7.4. The cells were centrifuged at 200 × *g* for 10 min, resuspended to 10^6 cells per ml, and homogenized in a Dounce homogenizer. Homogenized cells were diluted threefold with cold distilled water, and solid magesium chloride was added to a concentration of 10 mM. The homogenate was stirred for 15 min at 0°C and centrifuged at 3,000 × *g* for 15 min to remove whole cells and precipitated nuclei. The supernatant was recentrifuged at 27,000 × *g* for 30 min, and the brush border pellet was suspended in HEPES-Hanks buffer, pH 7.4. Each preparation was then washed at least four times by recentrifugation at 27,000 × *g* and resuspension in 6 ml of HEPES-Hanks buffer, ph 7.4. To ensure that the brush borders were free of contaminating soluble mucosal components, a sample of each supernatant was immobilized and assayed for adhesion by the procedure described below. Typically, supernatants from the first several washes showed the presence of soluble receptors, i.e., were positive in the adhesion assay, whereas the supernatant from the final wash contained less than 10 μg of protein per ml and was negative in the adhesion assay.

Labeling of *E. coli* K-12(K88ab). *E. coli* K-12(K88ab) was grown on 10 ml of brain heart infusion agar supplemented with 0.01% sodium acetate and 0.01 mCi of sodium [^{3}H]acetate per ml (specific activity, 90 mCi/mmol; New England Nuclear Corp., Boston, Mass.). The plates containing sodium [^{3}H]acetate were routinely inoculated to confluence with 0.1 ml of a 3-h broth culture *E. coli* K-12(K88ab). The plates were incubated for 18 h at 37°C. After incubation the cells were harvested, washed once in HEPES-Hanks buffer (pH 7.4), and resuspended in HEPES-Hanks buffer (pH 7.4). The optical density of the culture was then determined at 600 nm and adjusted to an absorbance reading of 1.0. A sample of the suspension was removed, and the number of CFU present was determined by diluting and plating on appropriate medium. As required, *E. coli* K-12 was labeled in an identical manner.

Adhesion assay. All adhesion assays were performed in triplicate in multiwell polystyrene tissue culture plates (Linbro 24-well flat-bottom tissue culture plates; well diameter, 1.6 cm; Flow Laboratories, Inc., McLean, Va.). Crude mucus (0.25 ml) was added to each well and incubated overnight at 4°C. Unless otherwise indicated the crude mucus preparations employed were adjusted to 1.0 mg of protein per ml. Brush border preparations containing 0.4 to 1.0 mg of protein per ml were immobilized in a similar manner. Bovine serum albumin (BSA), 0.25 ml of a 10% solution, was added to control wells. After incubation, the wells were washed twice with HEPES-Hanks buffer (pH 7.4) to remove unbound protein.

Once crude mucus or brush border preparations were immobilized, 0.25 ml of ^{3}H-labeled *E. coli* was added to each well, and the plates were incubated for 1 h at 37°C. The wells were then washed twice with 1 ml of HEPES-Hanks buffer (pH 7.4) to remove unbound bacteria. Adherent bacteria were recovered by adding 0.5 ml of 0.5% sodium dodecyl sulfate (SDS) to each well and reincubating the plates for 3 h at 37°C. Samples were then removed, and the level of radioactivity was determined by scintillation counting. In each experiment both the total amount of radioactivity incorporated and the total number of cells added to each well were determined. These values were used to calculate the amount of [^{3}H]acetate incorporated per cell. This in turn was used to calculate the number of CFU per well. Usually the level of [^{3}H]acetate incorporated ranged from 10^{-3} to 10^{-4} cpm per bacterial cell. Control experiments showed that less than 30 cpm bound to mucus was due to label released by *E. coli* K-12(K88ab) during the time of the assay (19).

Proteolytic enzyme treatment. Trypsin or pronase (0.25 ml of solutions containing 800 μg of enzyme per ml) were added directly to wells containing immobilized crude mucus or brush borders, and the plates were incubated for 2 h at 37°C and overnight at 4°C. The plates were then washed with HEPES-Hanks buffer (pH 7.4), and the adhesion assay was carried out. Control experiments performed with BSA-treated, rather than enzyme-treated, mucosal preparations showed no inhibition of adhesion.

Sodium metaperiodate oxidation. Small intestine crude mucus and brush border preparations previously immobilized on tissue culture plates were subjected to sodium metaperiodate oxidation by the method of Izhar et al. (14). Briefly, 0.2 ml of 0.01 M sodium metaperiodate or 0.01 M sodium iodate in 0.2 M sodium acetate buffer (pH 4.5) was added to each well, and the plates were incubated in the dark at 4°C for 3 h. The wells were then washed with HEPES-Hanks buffer (pH 7.4) and assayed for bacterial adhesion.

Antisera. The ability of specific antisera to inhibit adhesion was assessed. Each well containing immobilized mucosal components received 0.05 ml of specific antiserum or normal rabbit serum. ^{3}H-labeled *E. coli* K-12(K88ab) cells were then added and assayed for adhesion. The antisera employed were prepared in rabbits against the *E. coli* K-12(K88) and *E. coli* K-12(K99) strains and were kindly supplied by Richard Wilson, *E. coli* Reference Center, Department of Veterinary Science, The Pennsylvania State University, University Park, Pa. Each antiserum was exhaustively absorbed with *E. coli* K-12. The resulting specific antisera were assayed for agglutinating activity against homologous and heterologous strains of *E. coli* K-12 and strains of K88- and K99-positive *E. coli*. In each instance anti-K88 antiserum was found to be capable of agglutinating known K88-positive strains of *E. coli*, but not *E. coli* K-12 or K99-positive *E. coli*. Conversely, anti-K99 antiserum was capable of agglutinating *E. coli* K99-positive strains, but not *E. coli* K-12 or K88-positive strains of *E. coli*.

Gel filtration. Crude mucus or brush borders isolated from the mouse small intestine were fractionated on Bio-Rad agarose gel A.5 columns (1.5 by 37 cm) equilibrated in HEPES-Hanks buffer, pH 7.4. Freshly prepared, undialyzed mucus samples (20 mg of protein) in HEPES-Hanks buffer or brush borders (5 mg of protein) were applied to the column, and 2.5-ml fractions were collected at a flow rate of 25 ml/h. Eluted protein was detected by reading the absorbance of each fraction at 280 nm. Crude mucus and brush borders were prepared from pooled small intestine sections from 4 to 6 or 8 to 10 mice, respectively.

The column void volume was determined before mucus fractionation with high-molecular-weight dextran (5 × 10^6 to

40×10^6; Sigma Chemical Co., St. Louis, Mo.). The eluted dextran was detected by the indole method (5).

Individual fractions were immobilized and assayed for *E. coli* K-12(K88ab) adhesion. Adhesion was detected by immobilizing 0.25 ml of a 1:4 dilution of each fraction of crude mucus or 0.25 ml of undiluted brush border fractions to tissue culture plates and carrying out the previously described adhesion assay.

Occasionally, large quantities of crude mucus (40 mg of protein) were applied to the column. Protein and adhesion peaks were determined, and fractions showing significant levels of adhesion were pooled. Pooled fractions were dialyzed extensively against distilled water and lyophilyzed. Recovered material was assayed for protein by the method of Lowry et al. (22), and carbohydrates were detected by gas-liquid chromatography (see below).

Carbohydrate analysis of neutral and amino sugars. Carbohydrate determinations were performed by gas chromatography with both arabinose and myoinositol used as the internal standards. Samples were prepared as described by Porter (30). Thermal decomposition of amino sugars was prevented by avoiding any heating after the hydrolysis step. A Varian 3700 gas chromatograph equipped with CDS-111 integrator-microprocessor and a 3% SP-2330 coated 100/120 Supelcoport glass column (6 ft [183 cm] by 2 mm) were used for the analysis. Temperature was programmed from 170 to 210°C over 16 min. Sialic acid was determined as described by Svennerholm (40).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 10% polyacrylamide gels as described previously (23). The standard proteins used were: phosphorylase *b*, 92.5 kDa; BSA, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. After electrophoretic separation the gels were stained with Coomassie blue or periodic acid-Schiff (PAS) stain (12).

Western blotting assay for identifying receptors. Samples were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) by the method of Towbin (41). Membranes were then incubated overnight at 4°C in 0.01 M PBS (pH 7.2) containing 1% BSA. Adhesion of *E. coli* K-12(K88ab) to receptors bound to nitrocellulose membranes was assayed by incubating the nitrocellulose membrane with $^{35}SO_4$-labeled *E. coli* K-12(K88ab) cells for 1 h at room temperature. The bacterial suspension employed was grown and labeled with $Na_2{}^{35}SO_4$ as described previously (4). After incubation the membranes were washed extensively in PBS containing 0.05% Tween 20. The nitrocellulose membrane was fixed with PBS containing 4% formaldehyde for 15 min at room temperature and then incubated for 20 min in NEF-974 Enlightening rapid autoradiography enhancer (New England Nuclear Research Products, Boston, Mass.). Membranes were autoradiographed for 24 h, and X-ray films were developed by the method of Laskey and Mills (20).

Chemicals. All chemicals were reagent grade.

RESULTS

The ability of *E. coli* strains expressing the K88 antigen to adhere to immobilized crude mucus has been previously described (21) and is demonstrated by the data presented in Table 1. *E. coli* K-12(K88ab), a strain which possesses the K88ab plasmid and expresses the K88 antigen, adhered readily to mouse small intestine crude mucus preparations immobilized on polystyrene but poorly to BSA, whereas the isogenic plasmidless *E. coli* K-12 strain adhered poorly to both the small intestinal preparation and BSA. The adhesion of *E. coli* K-12(K88ab) in this system has previously been shown to be mannose resistant but inhibited by the presence of specific anti-K88 antiserum or growth of the organisms at 18°C, a temperature which prevents pilus production (35).

TABLE 1. Adhesion of *E. coli* K-12(K88ab) and *E. coli* K-12 to immobilized crude mucus and brush border preparations

Expt no.	Prepn	Adhesion (cpm ± SE)[a]	
		E. coli K-12(K88ab)	*E. coli* K-12
1	Crude mucus[b]	2,855 ± 53	253 ± 20
	BSA	336 ± 28	204 ± 18
2	Brush borders[c]	2,652 ± 77	747 ± 16
	BSA	504 ± 80	610 ± 68

[a] All assays were performed in triplicate. In these and all subsequent experiments, bacterial suspensions were adjusted to an absorbance of 1.0 at 600 nm. This corresponds to a bacterial suspension containing about 10^9 CFU/ml.

[b] In this experiment, the specific activity of *E. coli* K-12(K88ab) was 1.2×10^{-4} cpm/CFU and that of *E. coli* K-12 was 1.7×10^{-4} cpm/CFU. Data were adjusted to the specific activity of *E. coli* K-12.

[c] In this experiment, the specific activity of *E. coli* K-12(K88ab) was 3.4×10^{-4}cpm/CFU and that of *E. coli* K-12 was 2.8×10^{-4} cpm/CFU. Data were adjusted to the specific activity of *E. coli* K-12(K88ab).

The data in Table 1 also demonstrate the ability of *E. coli* K-12(K88ab) to adhere to brush border preparations which have been immobilized on polystyrene. Once again *E. coli* K-12(K88ab) was observed to adhere to immobilized brush borders about fivefold more than to BSA. The isogenic *E. coli* K-12 demonstrated a low ability to adhere to brush borders, only slightly greater than to BSA. The adhesion of *E. coli* K-12(K88ab) to brush borders was also inhibited by specific anti-K88 antiserum or growth of the organisms at 18°C but not by anti-K99 antiserum or normal serum (Table 2).

Inhibition of adhesion by proteases and sodium metaperiodate. Crude mucus and brush borders were immobilized on polystyrene, and wells were subsequently treated with trypsin or pronase (Table 3). Treatment with either enzyme resulted in an extensive reduction of *E. coli* K-12(K88ab) adhesion. Immobilized crude mucus and brush border prep-

TABLE 2. Effect of specific antisera and growth temperature on adhesion of *E. coli* K-12(K88ab) to crude mucus and brush borders[a]

Serum[b]	Bacterial growth temp (°C)	Adhesion (cpm ± SE) to:	
		Crude mucus[c]	Brush borders[d]
None	37	3,627 ± 291	5,131 ± 485
None	18	618 ± 58	925 ± 82
Normal	37	4,358 ± 318	5,005 ± 189
Anti-K88	37	216 ± 22	757 ± 226
Anti-K99	37	3,766 ± 318	4,922 ± 227

[a] All assays were performed in triplicate at 37°C.

[b] Normal or immune rabbit sera were diluted 1/30 in HEPES-Hanks buffer (pH 7.6), and 0.05 ml was added to each well. The final dilution was 1/150.

[c] In this experiment, the specific activities of *E. coli* K-12(K88ab) grown at 37 and 18°C were 1.4×10^{-4} and 4.6×10^{-4} cpm/CFU, respectively. For all experimental groups, adhesion to BSA-treated control wells was between 203 and 233 cpm. Data were adjusted to the specific activity of the culture grown at 37°C.

[d] In this experiment, the specific activities of *E. coli* K-12(K88ab) grown at 37 and 18°C were 2.2×10^{-4} and 1.5×10^{-4} cpm/CFU, respectively. For all experimental groups, adhesion to BSA-treated control wells was between 637 and 728 cpm. Data were adjusted to the specific activity of the culture grown at 37°C.

TABLE 3. Inhibition of adhesion by pretreatment of immobilized crude mucus and brush borders with proteases and sodium metaperiodate[a]

Pretreatment (concn)	Adhesion (% of control)[d]	
	Crude mucus	Brush borders
Trypsin[b] (800 μg/ml)	20.9 ± 4.4	14.5 ± 2.8
Pronase[b] (800 μg/ml)	17.4 ± 2.8	11.4 ± 1.0
BSA[b] (800 μg/ml)	119.0 ± 3.2	112.2 ± 4.1
Sodium metaperiodate[c] (10 mM)	18.3 ± 1.9	23.4 ± 2.0
Sodium iodate[c] (10 mM)	77.6 ± 6.6	84.0 ± 5.2

[a] Assays were performed in triplicate.

[b] Pretreatments were conducted with 0.25 ml of enzyme or BSA (800 μg/ml) in HEPES-Hanks buffer (pH 7.4) for 2 h at 37°C and overnight at 4°C. After the wells were washed twice with 1 ml of HEPES-Hanks buffer (pH 7.4), adhesion assays were performed.

[c] Pretreatments were in 0.2 M sodium acetate buffer (pH 4.5) for 3 h at 4°C, before washing as in footnote *b* above.

[d] Adhesion of *E. coli* K-12(K88ab) to untreated mucus and brush borders was always between 3,000 and 5,000 cpm, depending on the experiment. Data are expressed as mean percentage of control ± standard error. Adhesion to BSA-treated control wells was between 12 and 22% of control values.

arations were also subjected to treatment with sodium metaperiodate, to oxidize sugars (Table 3). Control wells were treated with sodium iodate. Treatment of both crude mucus and brush border preparations with sodium metaperiodate resulted in a marked reduction in the level of *E. coli* K-12(K88ab) adhesion relative to that after identical treatment with sodium iodate (Table 3). Together these data suggested that the K88ab-specific receptors in crude mucus and brush borders were, at least in part, glycoprotein in nature.

Fractionation of crude mucus and brush border preparations by gel filtration. Crude mucus preparations were subjected to Bio-Rad A.5 gel filtration chromatography. The A_{280} of each fraction was determined, and alternate fractions were immobilized and assayed for the presence of K88ab-specific receptor activity. Figure 1 shows the results of two representative experiments. Absorbance readings were generally consistent from experiment to experiment, although some variation was observed in the amount of material associated with the very high-molecular-size (>5,000 kDa) void-volume fractions (Fig. 1). *E. coli* K-12 (K88ab) adhesion was routinely observed in association with the broad range of fractions representing components with molecular sizes ranging from 30 to 500 kDa. In some cases (e.g., Fig. 1A) adhesion was also observed in association with the void-volume fractions. Brush border preparations were also subjected to gel filtration chromatography on a Bio-Rad A.5 agarose column, and individual fractions were assessed for the presence of K88ab-specific receptor activity. As expected for membrane-containing preparations, all receptor activity was associated with the void-volume fractions (data not shown).

Sugar analysis of mucosal preparations and inhibition of adhesion by monosaccharides. Because of the apparent sensitivity of the mucosal receptors to sodium metaperiodate (Table 3) and previous reports which showed that K88ab-specific agglutination of erythrocytes (11) and adhesion to brush borders (2, 33) were inhibited by the presence of free sugars, the sugar contents of both of the pooled receptor-containing fractions obtained by gel filtration of crude mucus and brush borders were determined (Table 4). Each of the sugars listed in Table 4, as well as mannosamine and the *N*-acetyl forms of D-glucosamine, D-galactosamine, and D-mannosamine, which would be detected as nonacetylated sugars by gas-liquid chromatography, were assayed for their ability to inhibit *E. coli* K-12(K88ab) adhesion in vitro (Table 5. Adhesion to both brush borders and crude mucus was inhibited most consistently and to the greatest extent by the presence of galactosamine. Pretreatment of the immobilized mucus and brush border preparations with 0.1 M D-galactosamine for 1 h followed by washing with HEPES-Hanks buffer (pH 7.4) had no effect on subsequent *E. coli* K-12(K88ab) adhesion, suggesting that D-galactosamine inhibits adhesion by binding to *E. coli* K-12(K88ab) (data not shown). Significant inhibition of adhesion was also observed in the presence of other sugars; however, these inhibitory effects were far less pronounced than that of D-galactosamine (Table 5).

Identification of K88ab mucus and brush border receptor proteins. SDS-PAGE was conducted on brush borders and pooled receptor-containing fractions obtained by gel filtration chromatography. The pooled fractions employed corresponded to fractions 24 to 30 (the void volume), fractions 45 to 55, and fractions 56 to 64 of Fig. 1A. After electrophoretic

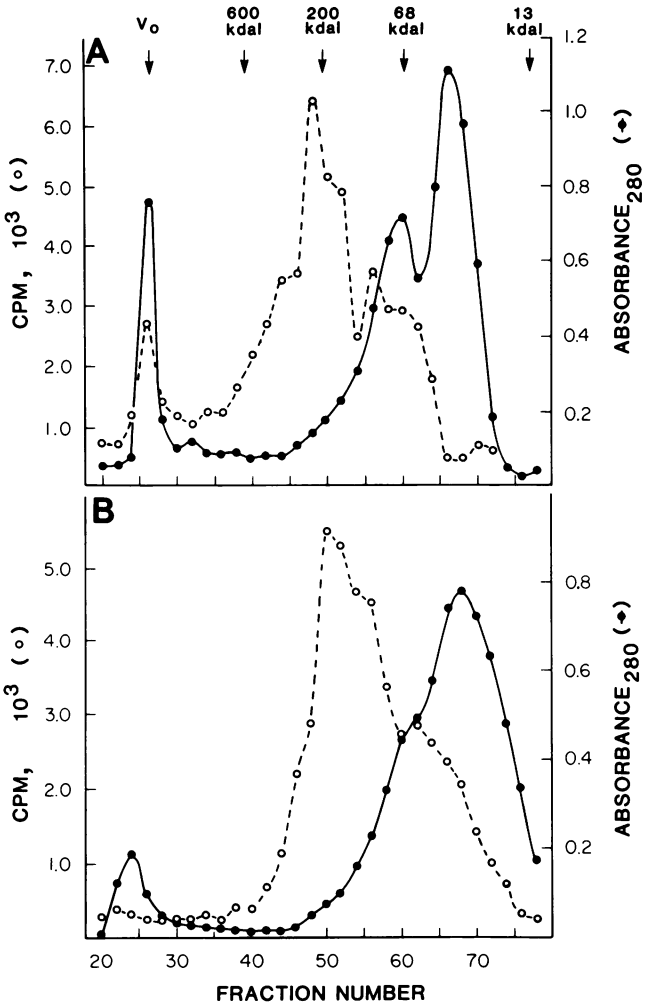

FIG. 1. Adhesion of *E. coli* K-12(K88ab) to fractionated crude mucus. Freshly prepared mouse small intestine crude mucus (20 mg of protein) was fractionated on a Bio-Rad A.5 agarose gel column (2.5 by 37 cm). Individual fractions were immobilized, and adhesion assays were performed. Panels A and B represent data from two separate experiments. V_o represents the void volume.

TABLE 4. Sugar analysis of mucosal preparations

Sugar	μg of sugar/mg of protein[a] (% of total)	
	Mucus receptor-containing fractions[b]	Brush borders
Glucosamine	38.0 (34.2)	7.2 (19.1)
Galactose	24.2 (21.6)	11.0 (28.4)
Glucose	20.0 (18.0)	10.5 (27.1)
Mannose	21.4 (18.9)	3.7 (3.5)
Galactosamine	7.0 (6.3)	4.9 (12.6)
Fucose	1.0 (0.9)	1.3 (3.3)
Sialic acid	ND[c]	ND

[a] The protein contents of the receptor-containing mucus and brush border preparations samples analyzed were 266 and 468 μg of protein, respectively.
[b] The receptor-containing fractions analyzed correspond to fractions 41 to 55 in Fig. 1A.
[c] ND, Not detected.

separation, duplicate gels were stained with Coomassie blue to visualize individual proteins or PAS stain to visualize glycoproteins (Fig. 2A).

To identify individual proteins capable of acting as receptors for the K88ab adhesin, proteins separated by SDS-PAGE were Western blotted to nitrocellulose, and the ability of $^{35}SO_4$-labeled *E. coli* K-12(K88ab) to adhere to individual proteins was assessed by autoradiography (Fig. 2B). All the mucus preparations employed produced autoradiographic patterns indicating K88ab-specific adhesion to two proteins with molecular sizes of 57 and 64 kDa, respectively. Brush border preparations and void-volume fractions, in addition to containing the 57- and 64-kDa receptor proteins, contained a major 91-kDa receptor protein. An identical Western blot analysis conducted with $^{35}SO_4$-labeled *E. coli* K-12 did not show evidence of adhesion to any proteins present in any of the preparations (data not included). Although PAS staining showed glycoproteins to be present near the receptor proteins, it could not be stated with certainty that the 57-, 64-, and 91-kDa receptor proteins were PAS positive. Similarly, although Fig. 2B indicates that the 57-, 64-, and 91-kDa components represent the major K88ab receptors present in these preparations, we cannot rule out the possible presence of additional minor lower-molecular-weight receptors. This is particularly true in the poorly defined void-volume fractions shown in lane 1.

TABLE 5. Sugar inhibition of *E. coli* K-12(K88ab) adhesion to crude mucus and brush border preparations

Inhibitor	IC_{50}[a]	
	Crude mucus	Brush borders
D-Fucose	>200	>200
L-Fucose	>200	>200
D-Galactose	150	200
D-Glucose	>500	>200
D-Mannose	>500	>200
D-Galactosamine	6	13
D-Glucosamine	>250	150
D-Mannosamine	>200	>200
N-Acetylgalactosamine	>200	>200
N-Acetylglucosamine	250	200
N-Acetylmannosamine	>250	200

[a] Expressed as the millimolar concentration of inhibitor required to reduce adhesion to 50% of the control values. All sugars were added immediately before the adhesion assay and were present during the entire assay period. Control values for adhesion of *E. coli* K-12(K88ab) to immobilized crude mucus or brush borders were between 4,000 and 8,800 cpm, depending on the experiment.

DISCUSSION

The adhesive properties of K88-bearing *E. coli* have been extensively investigated by using a variety of in vitro systems. *E. coli* cells which possess the K88 adhesin have previously been shown to adhere to intestinal epithelial cells and brush borders from a number of species, including pigs (3, 34) and mice (31). Similarly, *E. coli* cells which possess the K88 adhesin have been shown to hemagglutinate erythrocytes from a variety of sources (6, 16, 29, 39). Although it is clear from these studies that the K88 adhesin is capable of binding in a highly specific manner to membrane receptors, relatively little is known regarding the nature and identity of the receptors involved. The loss of adhesion which occurs after treatment of the receptors with sodium metaperiodate and the ability of terminal galactosyl residues (11), *N*-acetylglucosamine (2), *N*-acetylgalactosamine (2), and D-galactosamine (33), depending on the assay system employed, to inhibit adhesion or hemagglutination have led to general agreement that sugars are an important part of the structure of the receptor. However, specific membrane glycoprotein or glycolipid receptors have not been identified, although evidence suggesting the involvement of both types of molecules in bacterial adhesion to membranes has been reported (18, 33).

In a previous report from this laboratory (21), a number of K88ab adhesin-bearing strains of *E. coli* were shown to adhere in vitro to mouse small intestine crude mucus preparations which had been immobilized on polystyrene. In the present report these findings were extended to show the *E. coli* K-12(K88ab) cells are also capable of adhering to immobilized mouse small intestine brush border preparations. In both cases, adhesion is mannose resistant (Table 4), inhibited by the presence of specific antiserum (Table 2), and requires pilus expression (Table 2), i.e., growth of the organisms at temperatures greater than 18°C (16). Together, these studies indicate that the mouse small intestine contains both soluble and membrane-bound components capable of acting as receptors for the K88 adhesin. Indeed, the data suggest that mouse crude mucus preparations are a rich source of K88 adhesin-specific receptors.

The receptors responsible for adhesion in the present system appear to be glycoproteins which very likely contain D-galactosamine or D-galactosamine-like residues. That is, D-galactosamine, present in both crude mucus and brush border preparations (Table 4), inhibited *E. coli* K-12(K88ab) adhesion to both crude mucus and brush border preparations at far lower concentrations than any of the other sugars tested (Table 5). Furthermore, adhesion was periodate, trypsin, and pronase sensitive (Table 3). These results are in general agreement with those of Sellwood (33), who found D-galactosamine to be the best inhibitor of purified K88-adhesin binding to porcine brush borders. In this case as well, adhesion was found to be both trypsin and sodium metaperiodate sensitive.

It should be noted that although D-galactosamine is capable of inhibiting the binding of the K88ab adhesin, this does not necessarily indicate that D-galactosamine is actually a component of the receptors. It may be, for example, that the K88ab-binding site of the receptor contains a component which is stereochemically similar to D-galactosamine. In addition, on the basis of studies of other adhesin-receptor systems, notably the mannose-sensitive type I *E. coli* adhesin (7, 25, 32; I. Ørskov, S. Ferencz, and F. Ørskov, Letter,

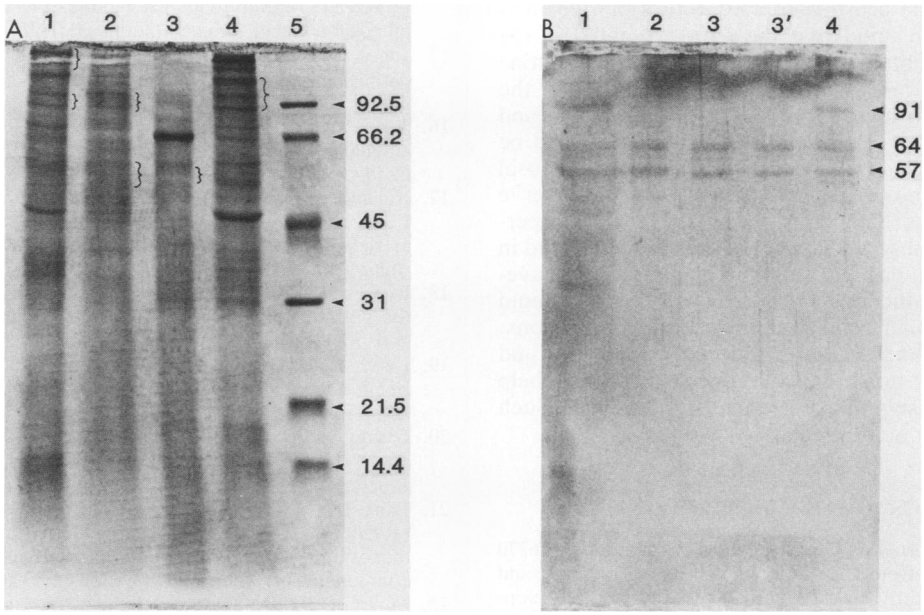

FIG. 2. Identification of mouse small intestine mucus and brush borders K88ab-specific receptors. (A) SDS-PAGE of fractionated mucus and brush borders. Lanes: 1, void-volume fractions 24 to 30 from Fig. 1A, 10 μg of protein; 2, mucus fractions 40 to 55 from Fig. 1A, 20 μg of protein; 3, mucus fractions 56 to 64 from Fig. 1A, 20 μg of protein; 4, brush borders, 30 μg of protein; 5, protein standards. Molecular sizes of the protein standards (kilodaltons) are indicated on the right. Brackets represent PAS-positive regions. (B) Autoradiogram of *E. coli* K-12(K88ab) adhesion to a Western blot of fractionated mucus and brush borders. Lanes: 1, void-volume fractions as in panel A, 15 μg of protein; 2, mucus fractions 40 to 55 as in panel A, 15 μg of protein; 3, mucus fractions 56 to 64 as in panel A, 15 μg of protein; 3', duplicate of lane 3; 4, brush borders, 15 μg of protein. Molecular sizes of the K88ab-specific receptors (kilodaltons) are indicated on the right. For further details see Materials and Methods.

Lancet i:887), the α-Gal-(1→4)-β-Gal binding adhesins of uropathogenic *E. coli* (17, 25), and the glycolipid-binding adhesins of *E. coli* K99 (36), the D-galactosamine-like component is almost certainly present as part of a larger, more complex carbohydrate moiety.

Three K88 adhesin-specific protein receptors were identified. The brush border preparations contained protein receptors with molecular sizes of 57, 64, and 91 kDa, while receptor activity in the soluble mucus preparations was associated primarily with 57- and 64-kDa protein receptors. Whether these proteins are glycoproteins as the sodium metaperiodate and D-galactosamine inhibition data suggest awaits their purification and sugar analysis.

While it is clear that receptors specific for the K88ab adhesin are present in both the soluble crude mucus preparations and the brush border preparations, the origin and function of these components in vivo are unclear. The 91-kDa protein receptor appears to represent a unique brush border membrane receptor, and it is likely that the presence of this receptor in the void-volume fraction of some soluble preparations was due to small contaminating membrane complexes not removed by centrifugation. The 57- and 64-kDa receptors may also represent brush border membrane glycoproteins. The presence of such membrane components in the soluble crude mucus preparations would not be unusual since brush border membrane proteins are known to be released into the gut lumen (43). Alternatively, the 57- and 64-kDa receptors in the brush border preparations may represent secreted mucus glycoproteins which are not removed by the washing procedures employed. It has been reported that mucus glycoproteins are often tenaciously bound to brush borders and are not easily removed by routine washing procedures (6, 9).

Although the origin of the 57- and 64-kDa receptors is unclear, these components appear to represent glycoproteins normally associated with the mucus layer of the intestine. This layer, which represents a major structural feature of the small intestine, is complex in composition and appears to consist of mucin molecules and large amounts of associated glycoproteins and glycolipids (35). Mucin itself is a large, heavily glycosylated molecule (2,000 kDa) composed of multiple subunits which may range in size from 250 to 500 kDa (1, 8). Although mucin appears to make up only a small percentage of the mucus layer, it aggregates readily and is responsible for the gel-like properties of the mucus layer (1, 8). Whether the crude mucus receptors identified in the present study represent partially degraded mucin subunits, as yet undefined glycoproteins associated with the mucus layer, or membrane-bound receptors which have become solubilized remains to be determined; however, their presence raises significant questions regarding the role of the mucus layer in the prevention or initiation of colonization by ETEC. On one hand, it is possible that the mucus layer could serve as an initial point of contact for ETEC adhesion and thus may be important in the colonization process. On the other hand, it is possible that soluble receptors present in mucus may normally serve to prevent colonization by interfering with the attachment of ETEC to the underlying epithelial cells. This in turn implies that some form of disruption of the mucus may be a prerequisite for colonization.

While it is interesting to speculate on the nature and role of

the K88ab mucus and brush border receptors in vivo, it is important to keep in mind that the data presented in the present study were obtained by using mouse small intestine preparations. Final resolution of the relationship of the mouse mucus and brush border receptors and the mucus and brush border receptors of the natural hosts remains to be determined. Nonetheless, the mouse small intestine mucosal preparations employed contain components which can serve as receptors for the K88ab adhesin, and the general properties of the adhesion observed are similar to those reported in porcine systems. In addition, the mouse model is a convenient one and, like the hemagglutination systems, should provide useful information on adhesin-receptor interactions. Further purification and characterization of both mouse and porcine mucus and brush border receptors should help resolve the relationship of the receptors involved. Such studies are currently in progress.

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