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## Presence of K88-Specific Receptors in Porcine Ileal Mucus Is Age Dependent

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Ileal mucus and epithelial cells were isolated from newborn piglets that had never been fed and 35-day-old unweaned piglets. Both newborn and 35-day-old piglet mucus preparations supported growth of *Escherichia coli* Bd 1107/75 08, a K88-fimbriated porcine enterotoxigenic strain, equally well (i.e., generation times of 28 min were observed in both cases). Adhesion of *E. coli* Bd 1107/75 08 to 35-day-old piglet ileal epithelial cells was, at most, 2 times that of the same strain to newborn piglet ileal epithelial cells; however, adhesion of *E. coli* Bd 1107/75 08 to 35-day-old piglet ileal mucus was 16 times that of the same strain to newborn piglet ileal mucus. The receptor in 35-day-old piglet ileal mucus was K88 specific, since it could be removed by purified K88ab fimbriae. Furthermore, adhesion of *E. coli* Bd 1107/75 08 to 35-day-old piglet ileal mucus was blocked by PAB10, a K88ab-, K88ac-, K88ad-specific monoclonal antibody. Although *E. coli* Bd 1107/75 08 traversed both newborn and 35-day-old piglet ileal mucus about equally well in vitro and bound well to underlying ileal epithelial cells after passing through newborn ileal mucus, it did not bind to ileal epithelial cells after passing through 35-day-old piglet ileal mucus. The data are discussed with respect to the role that K88-specific receptors present in newborn and ileal mucus might play in the pathogenesis of porcine enterotoxigenic *E. coli* strains which bear K88 fimbriae.

Enterotoxigenic *Escherichia coli* strains bearing K88 fimbriae bind to the small intestine mucosa of piglets and cause diarrhea (9, 25, 26). Neonatal piglets are extremely sensitive to infection but can be protected by specific anti-K88 antibody present in the colostrum of vaccinated dams (21, 22). Upon weaning, however, piglets again become extremely sensitive to infection by K88-bearing enterotoxigenic strains (27).

It is well documented that *E. coli* strains bearing K88 fimbriae bind specifically to small intestine brush border membranes in vivo (3) and in vitro (10, 30) and that the ability to bind enhances virulence (9, 25, 26). Furthermore, it has been shown that piglets are resistant to infection if they are genetically defective in the ability to make K88-specific brush border receptors (23).

The epithelial cells in the mammalian small intestine are covered by a layer of mucus, secreted by specialized goblet cells (2, 17), which consists of mucin, a 2,000-kilodalton glycoprotein responsible for the viscosity of mucus, and many smaller proteins, glycoproteins, lipids, and glycolipids (1, 8, 11, 19, 20, 24, 29). Although it is clear that in order to bind to the underlying epithelial cells, K88-bearing *E. coli* strains must pass through the mucus layer, the role mucus plays in the pathogenic process is unclear. Therefore, in this study, we examined the ability of ileal mucus isolated from both neonatal and 35-day-old piglets to serve as a growth medium for *E. coli* Bd 1107/75 08, a K88-positive pig pathogen. We also examined these mucus preparations for the presence of K88-specific receptors and for their relative abilities to inhibit *E. coli* Bd 1107/75 08 binding to piglet ileal epithelial cells in vitro.

### MATERIALS AND METHODS

**Bacteria.** *E. coli* Bd 1107/75 08, hereinafter called *E. coli* 1107, was kindly provided and serotyped as K88ab by O. Soderlind, National Veterinary Institute, Uppsala, Sweden. *E. coli* 1107 is motile and naturally resistant to streptomycin sulfate (1 mg/ml). *E. coli* K-12 (K88ab) (13) was also used in this study.

**Animals.** Three newborn piglets that had never been fed and three 35-day-old unweaned piglets, all from different sows, were sacrificed as described previously (4). All piglets were healthy and were obtained from a large farm in Sweden. The piglets used in this study were of the K88-susceptible phenotype, that is, when interaction of K88-bearing *E. coli* 1107 with piglet ileal epithelial cells was observed microscopically, as described by Wilson and Hohmann (30), it was found that several bacteria bound, in each case, to the brush border membranes. Few, if any, bacteria were found bound to the epithelial cell proper of any of the piglets. The ileum of each animal, collected from about 20 cm above the colon, was divided into two equal parts. Mucus was collected from the lower ileum, and epithelial cells were collected from the upper ileum of each animal.

**Mucus isolation.** Mucus was isolated from the ileal walls by gentle scraping into HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) plus Hanks balanced salt solution (HEPES-Hanks buffer, pH 7.4). Epithelial cells and large cellular components were removed by centrifugation once at 11,000 × *g* for 10 min and once at 26,000 × *g* for 15 min, as described previously (4). The protein concentration of mucus preparations was determined by the Bio-Rad method.

**Epithelial cell isolation.** The upper ileum of each piglet was cut into approximately 1-m pieces. Each piece was sequentially washed 10 times in 20-ml portions of buffer, and the epithelial cells were harvested from rinses 3 to 10 as described previously (6). This procedure yielded essentially mucus-free viable epithelial cells, as judged microscopically.

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Epithelial cells were suspended in the same buffer containing 30% NCTC 135 (GIBCO tissue culture medium) and 20% glycerol at  $4 \times 10^6$  cells per ml and frozen at  $-20^\circ\text{C}$  until used in adhesion assays.

**Radioactive labeling of *E. coli* 1107.** *E. coli* 1107 was grown overnight as standing cultures at  $37^\circ\text{C}$  in culture tubes containing 5 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.). Cultures were diluted 1:20 in 10 ml of fresh tryptic soy broth containing 5  $\mu\text{Ci}$  of [*methyl*-1,2- $^3\text{H}$ ]thymidine (117 Ci/mmol; Amersham International, London, England) per ml. Cells were grown as standing cultures at  $37^\circ\text{C}$  to an  $A_{600}$  of 0.5 ( $3.5 \times 10^8$  CFU/ml), centrifuged for 5 min at  $3,000 \times g$  at  $5^\circ\text{C}$ , washed once in 10 ml of room temperature HEPES-Hanks buffer (pH 7.4), centrifuged as described above, and suspended in 10 ml of HEPES-Hanks buffer (pH 7.4) at room temperature.

**Mucus and epithelial cell immobilization.** Ileal mucus (0.5 mg of protein per ml) and epithelial cells ( $4 \times 10^6$  cells per ml) were immobilized in 24-well Nunclon polystyrene tissue culture plates (Nunc Inter Med, Roskilde, Denmark) as described previously (13).

**Adhesion assay.** The assay for adhesion to immobilized mucus and epithelial cells employed in this study has been described previously in detail (4, 13). All assays were performed in triplicate. Briefly, [ $^3\text{H}$ ]thymidine-labeled *E. coli* 1107 cells (0.25 ml) were added to polystyrene tissue culture wells containing immobilized ileal mucus or ileal epithelial cells. The tissue culture plates were incubated for 1 h at  $37^\circ\text{C}$ , and the wells were then washed twice with 0.5 ml of HEPES-Hanks buffer, pH 7.4, to remove unbound bacteria. Adhering bacteria were released by adding 0.5 ml of 5% sodium dodecyl sulfate (SDS) to each well and then incubating the plates for 1 h at  $60^\circ\text{C}$ . SDS was collected from each well, and the level of radioactivity was determined by scintillation counting.

**In vitro penetration of *E. coli* 1107 through ileal mucus.** The in vitro penetration assay has been described previously (18). Briefly, sets of polystyrene wells containing immobilized epithelial cells from one 35-day-old piglet were overlaid with ileal mucus (0.5 ml, 3 mg of protein per ml) from each of the newborn and 35-day-old piglets, thereby forming distinct layers. Samples of  $^3\text{H}$ -labeled *E. coli* 1107 (0.2 ml,  $1.0 \times 10^6$  to  $3.5 \times 10^6$  CFU per ml, depending on the experiment) were then carefully layered atop the mucus in the wells. The wells were then incubated at  $37^\circ\text{C}$  for 1, 3, and 5 h. At each time, a set of two wells containing mucus from each piglet was aspirated once to remove bacteria still in the mucus layer but leave any bacteria that had penetrated through and reached the mucus-epithelial cell interface. A second set of two wells containing mucus from each piglet was aspirated as described above and washed twice with 1 ml of HEPES-Hanks buffer, pH 7.4, to remove all bacteria which had penetrated the mucus but were not firmly bound to the underlying epithelial cells. The bacteria remaining in each set of wells were then collected in SDS and counted as described above.

**Growth of *E. coli* 1107 in newborn and 35-day-old piglet ileal mucus as the sole source of carbon.** Ileal mucus (3 mg of protein per ml) from each of the three newborn and three 35-day-old piglets was diluted to 1 mg of protein per ml in Davis broth minimal salts (DBMS; Difco). *E. coli* 1107 isolates grown in TSB overnight, as described above, were diluted  $10^4$ -fold into DBMS and then 1:100, i.e., to about  $10^3$  CFU/ml in each of the ileal mucus preparations in DBMS described above. The cultures were incubated at a standing temperature of  $37^\circ\text{C}$ , and at 0, 4, 7, and 24 h, samples from

each mucus preparation were diluted and plated on MacConkey agar (Difco) containing 100  $\mu\text{g}$  of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml. Plates were incubated for 24 h at  $37^\circ\text{C}$  prior to counting.

**Purification of K88ab fimbriae.** *E. coli* K-12 (K88ab) was grown to mid-exponential phase ( $A_{600}$  of 0.6) in brain heart infusion broth (Oxoid Ltd., Basingstoke, England). One hundred brain heart infusion agar plates were then each spread with 0.35 ml of the brain heart infusion broth-grown culture and incubated for 18 h at  $37^\circ\text{C}$ . K88ab fimbriae were then purified essentially as described by Stirn et al. (28), except that the final centrifugation was at  $230,000 \times g$  for 4 h. The pellet was taken up in HEPES-Hanks buffer, pH 7.4, and the fimbriae were adjusted to a concentration of 4 mg of protein per ml. SDS-polyacrylamide gel electrophoresis of purified K88ab fimbriae revealed only one Coomassie blue-stained band at 27,000 daltons, as expected for pure K88ab fimbriae (14).

**Coupling of purified K88ab fimbriae to cyanogen bromide-activated Sepharose 4B.** Cyanogen bromide-activated Sepharose 4B (4 g; Pharmacia, Uppsala, Sweden) was coupled to 15 mg of purified K88ab fimbriae (hereinafter called K88ab-Sepharose), according to accompanying instructions. As a control, 4 grams of cyanogen bromide-activated Sepharose 4B (hereinafter called Sepharose) was put through the same coupling procedure in the absence of purified K88ab fimbriae.

**Absorption of the K88-specific ileal mucus receptor from 35-day-old piglet ileal mucus with K88ab-Sepharose.** One milliliter of ileal mucus (0.5 mg of protein per ml) was absorbed with 20  $\mu\text{l}$  of K88ab-Sepharose for 20 min at  $4^\circ\text{C}$  with constant rotary mixing. The mixture was then centrifuged for 5 min at room temperature at  $9,000 \times g$  to sediment the K88ab-Sepharose, the supernatant was removed, and the process was repeated 9 times, for a total of 10 absorptions. As a control, 1 ml of the same ileal mucus was absorbed identically 10 times with Sepharose. Both the K88ab-Sepharose and the Sepharose-absorbed ileal mucus preparations were immobilized and tested for their abilities to bind  $^3\text{H}$ -labeled *E. coli* 1107 as described above.

**Inhibition of *E. coli* 1107 adhesion to ileal mucus by a K88ab-, K88ac-, K88ad-specific monoclonal antibody.** PAB10, a monoclonal antibody which interacts with K88ab, K88ac, and K88ad fimbriae, was kindly provided by Nils T. Foged, State Veterinary Serum Laboratory, Copenhagen, Denmark. PAB10 was diluted 1:10 in a suspension of  $^3\text{H}$ -labeled *E. coli* 1107 in HEPES-Hanks buffer, pH 7.4. The mixture was then incubated at  $37^\circ\text{C}$  for 30 min. As a control, an identical suspension of  $^3\text{H}$ -labeled *E. coli* 1107 without added PAB10 was incubated at  $37^\circ\text{C}$  for 30 min. The ability of each of these  $^3\text{H}$ -labeled *E. coli* 1107 suspensions to bind to immobilized 35-day-old piglet ileal mucus was then tested as described above.

**Centrifugation of the K88-specific receptor from 35-day-old piglet ileal mucus.** One ml of ileal mucus (0.5 mg of protein per ml) was centrifuged at  $26,000 \times g$  for 9 h at  $4^\circ\text{C}$ . The supernatant was removed, the pellet was suspended in 1 ml of HEPES-Hanks buffer (pH 7.4), and both the supernatant and pellet were immobilized and tested for their abilities to bind  $^3\text{H}$ -labeled *E. coli* 1107 as described above.

**Chemicals.** All chemicals were reagent grade.

**Statistics.** Where indicated in the text, means derived from triplicate samples were compared by Student's *t* test.

## RESULTS

**Growth of *E. coli* 1107 in ileal mucus in vitro.** Ileal mucus preparations (1 mg of protein per ml in DBMS) isolated from three neonatal and three 35-day-old piglets were inoculated with  $10^5$  CFU per ml of *E. coli* 1107 and incubated at 37°C. The bacteria grew equally well in all six preparations, with a generation time of about 28 min and a final yield of about  $3 \times 10^8$  CFU per ml.

**Adhesion of *E. coli* 1107 to immobilized ileal mucus and ileal epithelial cells.** Ileal mucus preparations and ileal epithelial cells from three neonatal and three 35-day-old piglets were immobilized in polystyrene tissue culture wells and tested for their abilities to bind *E. coli* 1107. Data obtained from all six animals showed that adhesion of  $^3\text{H}$ -labeled *E. coli* 1107 to 35-day-old piglet mucus preparations was 16 times greater than to neonatal mucus preparations ( $41,037 \pm 7,338$  cpm versus  $2,618 \pm 1,344$  cpm;  $P < 0.001$ ), whereas adhesion to 35-day-old piglet ileal epithelial cells was, at most, twice that to neonatal ileal epithelial cells ( $13,910 \pm 1,630$  cpm versus  $6,991 \pm 1,630$  cpm;  $P < 0.10$ ). Adhesion to bovine serum albumin was only  $210 \pm 65$  cpm.

Adhesion of *E. coli* 1107 to ileal mucus isolated from 35-day-old piglets was K88 specific in two ways. First, PAB10, a monoclonal antibody which interacts with K88ab, K88ac, and K88ad fimbriae, inhibited adhesion of  $^3\text{H}$ -labeled *E. coli* 1107 to 35-day-old piglet ileal mucus by greater than 90% (e.g.,  $100,337 \pm 3,967$  cpm, bound in the absence of PAB10, versus  $6,696 \pm 259$  cpm, bound in the presence of PAB10;  $P < 0.001$ ). Second, about 75% of the *E. coli* 1107 binding ability to 35-day-old piglet ileal mucus could be removed by incubating the mucus with purified K88 fimbriae bound to Sepharose. As an example, in one experiment, adhesion of *E. coli* 1107 to mucus adsorbed with Sepharose was  $50,380 \pm 2,626$  cpm, whereas adhesion to mucus adsorbed with K88ab-Sepharose was only  $12,475 \pm 1,818$  cpm.

**Penetration of *E. coli* 1107 through ileal mucus preparations in vitro.** Ileal epithelial cells isolated from one 35-day-old piglet were immobilized in polystyrene tissue culture wells. Sets of two wells were then overlaid with mucus preparations from neonatal and 35-day-old piglets, and the ability of *E. coli* 1107 to penetrate through the mucus layers to the mucus-epithelial cell interface and to bind to the epithelial cells was assayed (see Materials and Methods). The ability of *E. coli* 1107 to traverse the neonatal and 35-day-old piglet mucus layers to the mucus-epithelial cell interface varied from piglet to piglet (Fig. 1). However, after *E. coli* 1107 traversed the mucus layer of the 35-day-old piglets, it did not bind to any great extent to the underlying epithelial cells, relative to the binding observed after traversing neonatal mucus. Two typical experiments are illustrated in Fig. 1.

**Centrifugation of the K88-specific mucus receptor from 35-day-old piglet ileal mucus.** Ileal mucus preparations from 35-day-old piglets were centrifuged at  $26,000 \times g$  for 9 h, immobilized, and tested for their ability to bind *E. coli* 1107. In each case, about 50% of the K88-specific receptors were removed from the mucus by centrifugation. As an example, in one experiment, adhesion of  $^3\text{H}$ -labeled *E. coli* 1107 to uncentrifuged mucus was  $53,676 \pm 9,184$  cpm; to the supernatant of centrifuged mucus, it was  $26,730 \pm 3,308$  cpm; and to the mucus pellet, resuspended to its original volume in HEPES-Hanks buffer (pH 7.4), it was  $29,674 \pm 4,321$  cpm.

## DISCUSSION

The data presented here show that ileal mucus isolated from newborn piglets that had never been fed contains only

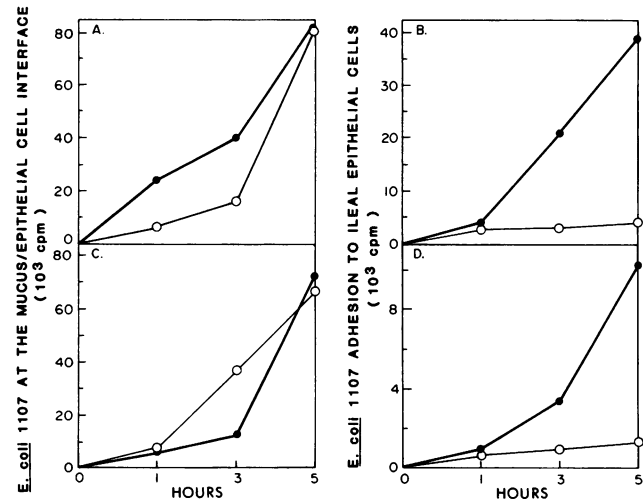


FIG. 1. Penetration of *E. coli* 1107 through newborn (●) and 35-day-old (○) piglet ileal mucus in vitro. (A and C) Penetration of *E. coli* 1107 to the mucus-epithelial cell interface; (B and D) adhesion to the epithelial cell layer. In panels A and B, layers of newborn piglet no. 1 mucus and 35-day-old piglet no. 1 mucus were used; in panels C and D, layers of newborn piglet no. 2 mucus and 35-day-old piglet no. 2 mucus were used. Data are presented as the means of duplicate samples. In each case, the counts per minute in duplicate assays was no greater than a 10% deviation from the mean of those assays.

1/16 of the amount of K88-specific receptor per milligram of protein found in ileal mucus isolated from 35-day-old unweaned piglets. Moreover, it appears that the K88-specific receptor present in 35-day-old piglet ileal mucus (at 3 mg of protein per ml) is concentrated enough to bind to *E. coli* 1107 K88-fimbriated cells and prevent the strain from binding to ileal epithelial cells (Fig. 1), whereas the amount of K88 receptor present in newborn ileal mucus (at 3 mg of protein per ml) is insufficient to prevent adhesion (Fig. 1). Since the piglets used in this study were of the K88-susceptible phenotype (see Materials and Methods), these data suggest the possibility that newborn piglets are more sensitive to enterotoxigenic strains of *E. coli* bearing K88 fimbriae than the older piglets, because of the lower amount of K88-specific receptor present in their ileal mucus layers.

It should be emphasized that while the amount of K88-specific receptor present in newborn piglet ileal mucus is small relative to that present in 35-day-old piglet ileal mucus, it still contains considerable K88-specific receptor activity relative to that of bovine serum albumin. This suggests that enterotoxigenic *E. coli* strains expressing K88 fimbriae could still bind specifically to the ileal mucus of newborn piglets, replicate rapidly, traverse the mucus layer, bind specifically to the underlying epithelial cells, release toxin, and thereby initiate the severe diarrhea observed in such animals. In other words, the relatively small amount of K88-specific receptor present in newborn piglet ileal mucus could actually contribute to the disease state by allowing the initial adhesion of the enterotoxigenic strain to the wall of the ileum. In contrast, while the relatively large amount of K88-specific receptor in 35-day-old piglet ileal mucus would also allow the adhesion and subsequent growth of enterotoxigenic K88-bearing *E. coli* strains in ileal mucus, it would also be in sufficient quantity to bind to the K88 fimbriae, prevent their adhesion to underlying epithelial cells, and thereby protect the animals.

We do not know the source of the K88 receptors in ileal mucus; however, it is clear that of the K88 receptor activity present in 35-day-old piglet ileal mucus, about 50% is contained in a very large component, i.e., it is sedimentable by centrifugation at  $26,000 \times g$  for 9 h. Clearly, further studies are necessary to learn the source and structure of the K88-specific receptors in piglet ileal mucus.

The generally accepted dogma regarding enterotoxigenic *E. coli* infections is that fimbriae are necessary to anchor the bacteria to intestinal epithelial cells such that they can resist washout caused by the peristaltic action of the small intestine. In support of this view, it is known that piglets which are genetically incapable of making K88-specific brush border receptors and therefore do not bind enterotoxigenic K88-bearing *E. coli* strains to their ileal epithelial cells are resistant to infection (23). Furthermore, *E. coli* K88-negative strains derived from a K88-positive pig pathogen which are still toxigenic are far less infectious than their plasmid-containing parent (9). The data presented here do not alter the idea that disease is initiated by adhesion to epithelial cells but suggest that the initial adhesion may be to the ileal mucus layer which overlies the epithelial cells. Once bound, the enterotoxigenic *E. coli* strain could then resist washout as long as its replication rate in ileal mucus exceeds the rate at which mucus is sloughed into the lumen of the intestine. Indeed, as reported here, both neonatal and 35-day-old piglet ileal mucus support a doubling time for *E. coli* 1107 of only 28 min in vitro.

It should also be noted that it is becoming increasingly clear that intestinal mucus contains receptors specific to the adhesins of several other intestinal *E. coli* pathogens, that is, rabbit ileal mucus contains receptors specific to the *E. coli* RDEC-1 AF/R1 fimbriae (7), calf small intestine mucus and mouse small and large intestine mucus contain receptors specific to K99 fimbriae (12, 15), and pig small intestine mucus appears to have receptors specific to 987P fimbriated *E. coli* (5). It should be of great interest to determine whether the amounts of receptor in intestinal mucus specific to these *E. coli* adhesins also vary with age.

Finally, if K88-specific receptors in mucus are protective when present in high concentrations, it may eventually be possible to feed piglets synthetic receptors which may, as they become entrapped in ileal mucus in vivo, help to protect piglets against infection by enterotoxigenic K88-bearing *E. coli* strains. In support of this view, it has recently been shown that glycoprotein glycans that inhibit adhesion of a calf enterotoxigenic K99-bearing *E. coli* strain in vitro protect colostrum-deprived newborn calves against lethal doses of the same microorganism (16).

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