Role of Escherichia coli Type 1 Pilus in Colonization of Porcine Ileum and Its Protective Nature as a Vaccine Antigen in Controlling Colibacillosis

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This study was designed to evaluate the role of Escherichia coli type 1 pili in adherence of the organism to porcine small intestines and the efficacy of pili as a vaccine antigen in controlling neonatal colibacillosis. Our results demonstrated that an E. coli phase cloned to express type 1 pili readily attached to the small intestines of colostrum-deprived newborn pigs. Immunofluorescent staining of intestine sections revealed the presence of E. coli expressing type 1 pili only on the brush border, suggesting involvement of type 1 pili in the colonization process. Administration of anti-type 1 serum to newborn pigs prior to challenge reduced the level of gut-associated E. coli sixfold compared with controls. Purified type 1 pilus vaccine induced significant protection against colibacillosis in newborn pigs following challenge with E. coli expressing type 1 pili. Pigs born to vaccinated gilts scoured less and gained more weight than pigs born to control gilts. Our results demonstrate that type 1 pili are a virulence factor, as well as an effective vaccine antigen.

Neonatal diarrhea is an important disease which affects swine worldwide. There are several etiological agents known to cause diarrhea; the major cause is enterotoxigenic Escherichia coli. Following ingestion, E. coli cells colonize and proliferate in the small intestine and release one or both types of enterotoxins (heat labile or heat stable or both), resulting in diarrhea. Colonization of the intestinal epithelium is mediated by specific surface adhesives known as pili. Four pilus types (types K88, K99, 987P, and F41) have been implicated in the colonization process. Each of these pilus types has been purified and characterized (5, 17, 20, 22).

Pathogenic and nonpathogenic E. coli isolates have been reported to produce type 1 pili (14). It has been shown that 42% of the E. coli strains isolated from human diarrhea patients have type 1 pili and produce enterotoxins (13). E. coli strains expressing type 1 pili have also been isolated from patients with urinary tract infections (11, 15). E. coli isolates possessing type 1 pili have been shown to adhere to isolated buccal epithelial cells from humans (19), bovine mammary glands (7), and porcine intestinal epithelium (9). Colonization of E. coli expressing type 1 pili to oropharyngeal cells has been demonstrated in rats (6). In vitro studies have demonstrated that attachment to porcine intestinal epithelium (9) and attachment to human buccal epithelial cells (1, 6, 25) are effectively blocked by type 1 antiserum. Furthermore, type 1 antiserum has been shown to protect mice against pylonephritis (21). Brinton et al. (4) showed that 81% of the E. coli strains which they isolated from neonatal pig diarrhea expressed type 1 pili in addition to other known pili. Efforts to protect piglets against colibacillosis by immunization of pregnant dams with a variety of immunogens have been made. The best results were achieved with preparations containing purified pili, such as type K99 or 987P pili (8, 17, 18).

The present study was initiated to demonstrate the colonization of E. coli expressing type 1 pili on porcine intestinal epithelium and to evaluate the efficacy of a type 1 pilus vaccine in controlling colibacillosis in neonatal pigs.

MATERIALS AND METHODS

Antiserum. Rabbit antiserum to type 1, F41, K99, K88, and 987P pili were kindly provided by C. C. Brinton, Jr., University of Pittsburgh, Pittsburgh, Pa.

Animals. A total of 19 colostrum-deprived, newborn pigs were utilized in the attachment test: 20 ICR, Sprague-Dawley female mice (20 g) and 11 bred gilts were utilized for serological and efficacy tests, respectively.

Culture conditions. A 30-ml portion of Z medium was inoculated with E. coli A/001 and cultured at 37°C in a fermentor (New Brunswick Scientific Co., Inc., Edison, N. J.). Z medium consists of 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 8 g of NaCl, 1 g of dextrose, and 1 g of yeast extract (Difco) in 1 liter of distilled water (pH 7.2). E. coli E/001 was cultured in a semisynthetic medium containing 1.7 g of KH2PO4, 5.5 g of Na2HPO4, 1 g of dextrose, 1 g of Casamino Acids (Difco), 1 g of yeast extract (Difco), and 1 ml of trace salt solution in 1 liter of distilled water (pH 7.2). The trace salt solution consisted of 8 g of MgSO4·7H2O, 0.5 g of MnCl2·4H2O, 0.1 g of FeSO4·7H2O, 0.2 g of CaCl2·2H2O, and 1 g of NH4Cl dissolved in 1 liter of distilled water.

Organisms. E. coli A/001 (O148:H28) and E. coli E/001 (heat stable+/heat labile-) (O101:K30:H1+) express type 1 pili which are serologically similar. These strains were kindly provided by C. C. Brinton, Jr. Both cultures demonstrated mannose-sensitive hemagglutination of guinea pig erythrocytes.

Purification of type 1 pili. Type 1 pili were purified as described by Brinton (3). E. coli A/001, which was isolated from a case of human diarrhea, was utilized as the source of type 1 pili. Colonies expressing type 1 pili only were grown in Z medium (see above). The pili were sheared off by blending in an Omni-mixer (Du Pont Co., Wilmington, Del.) for 10 to 15 min. The bacteria were removed by centrifugation at 17,000 × g for 1 h at 4°C. The pili were crystallized from the supernatant by adding 0.1 M MgCl2. They were

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then removed by centrifugation at 17,000 × g for 1 h at 4°C and suspended in potassium phosphate buffer (pH 7). Contaminating proteins were removed by dialysis against sodium acetate buffer (pH 3.2). The protein concentration of the pili was determined by the method of Lowry et al. (16).

**Immunodiffusion.** An Ouchterlony type of immunodiffusion assay was performed in 0.9% agarose (FMC Corp., Rockland, Maine) poured onto a glass microscope slide. Wells were cut in the agar and filled with sample, and the preparations were incubated at room temperature for 24 to 48 h in a humid chamber. The antisera used are described above; the purified *E. coli* type 1 pilus used are also described above.

**Determination of gut-associated *E. coli***. Ileal segments 8 cm long were removed from each pig. Each segment was cut open, washed four times with phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 7 mM Na2HPO4, 1.5 mMKH2PO4, pH 7.2) at 4°C, and homogenized in an Omni-mixer (Du Pont); 10-fold serial dilutions of the resulting suspension were made in phosphate-buffered saline and cultured at 37°C for 18 to 24 h on MacConkey agar (Diffco) to determine the total bacterial count.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in a 10% gel by a modification of the procedure of Laemmli (12). The following samples were examined: sodium dodecyl sulfate-polyacrylamide gel electrophoresis low-molecular-weight standards (Bio-Rad Laboratories, Richmond, Calif.) and *E. coli* type 1 pili. The pilus preparation was adjusted to a pH value of less than 2 by adding 5 N HCl and heated to 100°C for 5 min. It was cooled to 25°C, and the pH was adjusted to 7 by adding 5 N NaOH. The samples were run reduced (3.8% 2-mercaptoethanol) and unheated. The gel (thickness, 1.5 mm) was stained with 0.2% Coomassie brilliant blue R-250 overnight and was destained in a solution containing 40% methanol and 7% acetic acid.

**Determination of lipopolysaccharide.** The presence of lipopolysaccharide in the purified pilus preparation was determined by using the Limulus amebocyte lysate test (Associates of Cape Code, Inc., Woods Hole, Mass.) (23).

**Immunofluorescent staining.** Porcine ileal segments were prepared as frozen sections to determine the involvement of type 1 pili in the colonization process. This was accomplished by indirect immunofluorescent staining as described by Bertschinger et al. (2). Frozen sections (thickness, 5 μm) were made and mounted on microscope slides. Several sections were fixed with 100% acetone and treated with rabbit antisera to type 1, K99, K88, and 987P pilus. After three washes with phosphate-buffered saline, the sections were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.). The slides were washed three times with phosphate-buffered saline and observed with a fluorescent microscope (Zeiss, Carl, West Germany).

**Vaccine.** Each dose of vaccine contained 5 mg of purified type 1 pili in aluminum hydroxide adjuvant.

**Attachment assay.** The 19 colostrum-deprived newborn pigs were divided into three groups. Each animal in the first group, which consisted of seven pigs, received 10 ml of normal rabbit serum orally. Each animal in the second group, which consisted of eight pigs, received 10 ml of rabbit anti-type 1 serum orally. The animals in the third group, which consisted of four pigs, were held as unchallenged controls. After 45 min each pig was challenged with 2 × 10⁹ *E. coli* A/001 cells intragastrically. The pigs were isolated individually following challenge. At 9 h postchallenge, the pigs were sacrificed, and ileal segments were removed aseptically for determination of gut-associated bacteria and for immunofluorescent staining.

**Vaccine trial.** To determine the serological response to the vaccine, 20 mice were immunized subcutaneously with 0.1 ml of the vaccine. The mice were bled 21 days later, and the sera were evaluated by using the agglutination test (26). Cultures expressing type K88, K99, 987P, or 1 pilus only were used as the antigens; this was achieved by growing the cultures on minimal glucose agar plates. The colonies which expressed particular pilus types were determined by their agglutination reactions with pilus-specific antisera. They were selected and streaked onto minimal glucose agar plates for preparation of antigen.

The 11 bred gilts were divided into two groups and used to determine the efficacy of the vaccine as described above. The first group consisted of five gilts. Each animal received two subcutaneous immunizations. The first was at 6 to 7 weeks prior to farrowing, and the second was given 3 weeks later. The second group consisted of six gilts which were held as unvaccinated controls. When the gilts farrowed, each newborn pig was deprived of colostrum and challenged within 6 to 12 h intragastrically with 2 × 10⁸ *E. coli* E/001 cells. Each pig was observed for 7 days following the challenge. The pigs were monitored to determine the duration and severity of scour, which were scored as follows: 0, no scour; 1, mild scours with no dehydration; 2, moderate scour with dehydration; 3, severe scour with dehydration, depression, and mortality. Blood samples were collected from each gilt prior to vaccination, at the time the booster was administered, and just after farrowing. Each sample was titrated against type 1 pilus antigen by using the agglutination test (26). Colostrum samples from three vaccinated animals and three controls were titrated against piliated and nonpiliated phenotypes.

**RESULTS**

As shown in Fig. 1, 6.6 × 10⁹ organisms were recovered from ileal segments of pigs which were challenged with *E. coli* A/001 following administration of normal serum. Sixfold fewer organisms (P < 0.05) (1.1 × 10⁹ cells) were recovered from ileal segments of pigs which received type 1 pilus antiserum prior to challenge. The organisms which were recovered from the ileal segments demonstrated mannose-sensitive hemagglutination of guinea pig erythrocytes. Fig-
ure 2a shows the immunofluorescent staining of ileal segments from pigs that received normal serum. Specific fluorescence of the brush border was observed when the section was stained with type 1 pilus antiserum. Fluorescence was not observed when similar sections were stained with antisera prepared against type K88, K99, 987P, or F41 pili. Similarly, sections from unchallenged, control pigs did not show any fluorescence (Fig. 2b).

The purified type 1 pili produced one band at a molecular weight of 18,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparations (Fig. 3). The immunodiffusion assay revealed a single precipitin line between the wells containing type 1 pili and type 1 pilus antiserum. No precipitin lines formed between type 1 pili and antisera to type K88, K99, 987P, and F41 pili (Fig. 4). The pilus preparation was found to be free of lipopolysaccharide, as determined by the Limulus amoebocyte lysate assay.

Serum from mice immunized with the type 1 pilus vaccine showed a strong agglutination reaction with E. coli expressing type 1 pili only. No agglutination occurred with E. coli expressing type K88, K99, or 987P pili only (Table 1).

Of 27 pigs born to gilts vaccinated with the type 1 pilus vaccine, 1 (3.7%) died, whereas 15 of 41 (37%) (P < 0.05) of the pigs in the control group died following challenge (Fig. 5); 21 of 27 (78%) of the pigs born to the vaccinated gilts and all of the pigs born to the control gilts developed scours. However, pigs in the vaccinated group developed mild scours, whereas control pigs developed severe scours (Fig. 6). Scours lasted for a significantly shorter time (P < 0.001) in the vaccinated group (1.9 days) than in the control group (4.2 days). As shown in Fig. 6, pigs born to the vaccinated group developed significantly less scours (P < 0.001) (scours score, 1.6) than pigs born to control groups (scours score, 2.6). Pigs born to vaccinated gilts gained significantly more weight (P < 0.01) (average, 2.4 pounds [1.09 kg] per pig) than pigs born to control gilts (average, 1.2 pounds [0.54 kg] per pig) in the 7 days following challenge (Fig. 6). As shown

FIG. 2. Immunofluorescent staining. (a) Ileal section from pig challenged with E. coli A/001 following staining with E. coli type 1 antiserum. (b) Ileal section from unchallenged control pig following staining with E. coli type 1 antiserum.

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of E. coli type 1 pili. Lane 1 contained type 1 pili (15 μg). Lane 2 contained the following low-molecular-weight standards from Bio-Rad Laboratories: phosphorylase b (molecular weight, 92,500 [92.5K]), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

FIG. 4. Immunodiffusion analysis of type 1 pili and type 1, K88, K99, 987P, and F41 antisera. Well 1, type 1 antiserum; well 2, K88 antiserum; well 3, K99 antiserum; well 4, 987P antiserum; well 5, F41 antiserum; well 6, normal rabbit serum; well 7, purified type 1 pili.
TABLE 1. Agglutination reaction of E. coli with pilus-specific antiserum

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>K88 (rabbit)</th>
<th>K99 (rabbit)</th>
<th>987P (rabbit)</th>
<th>Type 1 (mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K88 (O138:k81:K88ac)</td>
<td>++++</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>K99 (O9:K35:K99)</td>
<td>--</td>
<td>++++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>987P (O9:K103:NM)</td>
<td>--</td>
<td>--</td>
<td>++++</td>
<td>--</td>
</tr>
<tr>
<td>Type 1 (O148:H28)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>++++</td>
</tr>
</tbody>
</table>

* Formalin-inactivated whole cells were used. The optical density at 530 nm was adjusted to 0.8 by using a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

+ ++++, Strong agglutination; --, no agglutination.

in Table 2, sera and colostrum samples from vaccinated gilts showed significant increases \((P < 0.001)\) in agglutination titer to type 1 pili compared with sera from control gilts. However, colostrum samples from both vaccinated and control gilts showed no titer to a nonpiliated phenotype. Samples from both groups had titers of less than 1:8.

**DISCUSSION**

Pigs less than 1 week old are highly susceptible to colibacillosis. Vaccination of pregnant dams provides passive protection to newborn pigs through colostrum. In this study we found that E. coli expressing type 1 pili can adhere to the ileal epithelia of colostrum-deprived newborn pigs. Frozen sections of ileal segments following challenge with E. coli A/001 fluoresced on the brush border when the sections were treated with type 1 pilus antiserum but not when they were treated with antiserum prepared against type K99, K88, and 987P pili. Our results clearly suggest a role for type 1 pili in the colonization of epithelial cells of pig intestines. In vitro colonization of isolated epithelial cells of pig intestines has also been shown previously with the same strain used in this study (9).

E. coli strains possessing type 1 pili have been shown to colonize bladder epithelial cells of mice, and mutants defective in type 1 pili failed to colonize bladder epithelial cells, suggesting a role for type 1 pili in the colonization process (10). Similarly, E. coli strains expressing type 1 pili have been shown to be responsible for oral colonization of neonatal rats (6).

We found that the administration of type 1 pilus antiserum to pigs prior to challenge reduced the gut-associated bacterial count sixfold. These results suggest that the type 1 pilus antibody can block the colonization of E. coli bearing type 1 pili. Similarly, in vitro blocking of colonization of isolated porcine intestinal epithelial cells by type 1 antiserum has been demonstrated previously (9). Furthermore, attachment of E. coli cells to human buccal cells was blocked by type 1 pilus antibodies (1, 6).

This study was the first study to demonstrate that purified type 1 pilus protein (molecular weight, 18,000) is an effective antigen for inducing immunity in piglets being nursed by vaccinated dams. Our results indicate that the protection is mediated by type 1 antibodies in the colostrum of vaccinated sows. Colostrum from vaccinated sows showed a significant increase in the titer for the pilated phenotype but not in the titer for the nonpiliated phenotype.

Attempts by other investigators have failed to demonstrate the protective nature of type 1 pilus, whole-cell vaccine. These workers vaccinated sows with a strain known to express both type K88 and type 1 pili and challenged with a strain expressing type K99 and type 1 pili (24). Failure to demonstrate protection could have been due to the fact that

**FIG. 5.** Levels of mortality of pigs born to vaccinated and control gilts after challenge with E. coli E/001. Open bar, pigs born to vaccinated gilts; solid bar, pigs born to control gilts.

**FIG. 6.** Pigs showing clinical signs of colibacillosis following challenge with E. coli A/001. Open bars, pigs born to vaccinated gilts; solid bars, pigs born to control gilts.

**TABLE 2.** Serum and colostrum agglutination titers to E. coli type 1 pilus antigen in control gilts and in gilts vaccinated with type 1 pilus vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>Gilt no.</th>
<th>Serum titer</th>
<th>Agglutination titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prevac-</td>
<td>Preboost-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cination</td>
<td>ing</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>1324</td>
<td>128</td>
<td>4.096</td>
</tr>
<tr>
<td>animals</td>
<td>1325</td>
<td>64</td>
<td>4.096</td>
</tr>
<tr>
<td></td>
<td>1991</td>
<td>256</td>
<td>16,384</td>
</tr>
<tr>
<td></td>
<td>1994</td>
<td>128</td>
<td>4.096</td>
</tr>
<tr>
<td></td>
<td>1993</td>
<td>256</td>
<td>16,384</td>
</tr>
<tr>
<td>Mean</td>
<td>166</td>
<td>9.011</td>
<td>21.299</td>
</tr>
<tr>
<td>Controls</td>
<td>1322</td>
<td>128</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1323</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>1329</td>
<td>1,024</td>
<td>ND</td>
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<tr>
<td></td>
<td>1488</td>
<td>256</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1990</td>
<td>64</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1992</td>
<td>128</td>
<td>ND</td>
</tr>
<tr>
<td>Mean</td>
<td>288</td>
<td>341</td>
<td>597</td>
</tr>
</tbody>
</table>

* E. coli A/001 was used in agglutination assay.

b ND, Not done.
the vaccine strain and the challenge strain expressed different pili, although both carried type 1 pili. It has been demonstrated that type K99 pili cause attachment to intestines and that protection cannot be achieved with a pilus vaccine against a challenge strain expressing pili different from the vaccine (17).

Structural and serological differences within pilus families are of primary importance in vaccine development because of the diverse antigenicity of pilus determinants (S. W. Wood et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B104, p. 35). Type 1 pili occur on most of the E. coli strains isolated from humans, animals, birds, and the environment (3). However, human isolates are significantly different from swine isolates in expressing several type 1 pilus serotypes rather than a single serotype, as all swine isolates do (Fusco et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984). These findings indicate that type 1 pili are a functionally heterologous group and that the type 1 pili on some E. coli strains do contribute to in vivo adherence and are important protective antigens.

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LITERATURE CITED