Experimental Infection of Newborn Pigs with an Attaching and Effacing Escherichia coli O45:K"E65" Strain

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The ability of a nonenterotoxigenic, K88-negative porcine Escherichia coli strain of serogroup O45:K"E65" to induce attaching-effacing lesions was investigated in newborn pigs. Typical attaching-effacing lesions, characterized by intimate adherence of bacteria to mature enterocyte brush borders with effacement of the microvilli, were observed on light and electron microscopy. Bacteria were also seen in intracytoplasmic vacuoles of mature enterocytes and, in areas of heavier colonization, in the lamina propria of the intestinal mucosa. A moderate inflammatory response with mild focal ulceration of the intestinal mucosa was observed. In a sequential study, we observed that the attaching-effacing lesions were well established in the duodenum, jejunum, and ileum by 12 h postinoculation but did not develop in the cecum and colon until 24 to 48 h postinoculation. Initially, bacteria were very intimately attached, with an irregular arrangement on the enterocyte apical cell membrane, and subsequently reoriented to form a typical palisade arrangement with a narrow regular gap between the bacterial cell wall and the enterocyte apical cell membrane. This phenomenon of early intimate attachment of irregularly disposed bacteria has not been reported for human enteropathogenic attaching and effacing E. coli and could represent a new and different mechanism of attachment and effacement to intestinal epithelial cells.

Escherichia coli is a major cause of enteric disease in humans and other animal species (29). Diarrheagenic E. coli have been grouped into four major classes: (i) enterotoxigenic E. coli, (ii) enteroinvasive E. coli, (iii) enteropathogenic E. coli (EPEC), and (iv) enterohemorrhagic E. coli (14). Enterotoxigenic E. coli strains colonize the small intestinal mucosa by means of fimbriae and produce enterotoxins (heat-stable enterotoxin a [STa], STb and a heat-labile enterotoxin) that cause hypersecretory diarrhea (19, 29). This category of strains is the most important cause of E. coli diarrhea in pigs and calves (19). Enteroinvasive E. coli strains invade and multiply in mature enterocytes, eventually causing cell death, and have a particular tropism for the colonic mucosa (14); they have not been reported in domestic animals (10, 23). EPEC strains do not produce classical enterotoxins and are not enteroinvasive (14, 27). Certain EPEC strains adhere intimately to the intestinal mucosa, where they cause effacement of the microvilli by mechanisms still not well defined (14, 30); the term attaching and effacing E. coli (AEEC) is applied to E. coli that cause these lesions (16). Most enteropathogenic AEEC strains possess the EPEC adherence factor (EAF) genes which are necessary for localized adherence of bacteria to HEp-2 or HeLa cells in tissue culture (21). However, the plasmid-mediated EAF is not necessary for production of attaching and effacing lesions in experimentally infected gnotobiotic pigs, although its presence facilitates bacterial adherence in the small intestine (30). In addition, an EPEC strain lacking the EAF plasmid still induced attaching and effacing lesions in human intestinal tissue culture (13). More recently, TnphoA mutagenesis studies have demonstrated that the genes affecting EPEC adherence may be located on both a plasmid and the chromosome (4). The process of attachment and effacement of EPEC involves close attachment of bacteria to intestinal epithelial cells, loss of microvilli, formation of pedestals by epithelial cells, and disorganization of the underlying cytoskeleton of the epithelial cell with a proliferation of actin (13, 16, 30). Knutton et al. (13) have demonstrated an initial attachment of EPEC to the intact brush border of cultured human intestinal mucosal cells and proposed a two-stage adherence involving an initial nonintimate, possibly fimbrially mediated adherence of bacteria to an intact brush border, followed by a later stage of intimate bacterial adherence with a regular 10-nm gap between bacteria and the cell membrane. Nevertheless, the early events in the establishment of bacterial adherence in vivo have not been clearly defined. In addition, most in vivo studies of the pathogenesis of EPEC infection to date have involved inoculation of gnotobiotic pigs with human EPEC strains. Enterohemorrhagic E. coli strains are also AEEC, but in contrast to enteropathogenic AEEC, they cause little or no leukocytic exocytosis in the lamina propria, generally involve only the cecum and colon, and produce more-severe, hemorrhagic lesions in natural infection of humans and experimental infection of newborn pigs (14, 30). AEEC strains are a major cause of diarrhea in rabbits and induce lesions similar to those of human enteropathogenic AEEC strains (23, 24).

Recently, lesions suggesting AEEC have been demonstrated in natural cases of porcine diarrhea (10). We have isolated strains of E. coli belonging to the O45:K"E65" serogroup in field cases of postweaning diarrhea. These strains do not possess any known fimbriae (K88, K99, 987P, and F41), produce neither classical enterotoxins (STa, STb, and the heat-labile enterotoxin) nor Shiga-like toxins, and in some cases have been associated with lesions suggesting AEEC. Strains of the O45:K"E65" serogroup have been associated with edema disease and postweaning diarrhea and do not generally produce enterotoxins or Shiga-like toxins (19, 29). Thus, the objectives of this study were to investigate the attaching and effacing activity of one of these strains

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and to study the initial stages of adherence of this strain to the intestinal mucosa in experimentally infected newborn pigs.

MATERIALS AND METHODS

Natural occurrence of attaching and effacing lesions in pigs. Of 1,350 E. coli strains isolated from diarrheic pigs submitted to the Faculté de Médecine Vétérinaire from 1979 to 1989, 17 (1.3%) belonged to serogroup O45:K"E65" (Table 1). A higher proportion (3.4%) of strains isolated from weaned pigs (age, 29 to 60 days) compared with that (0.6%) from unweaned pigs (age, 0 to 28 days) was of the O45:K"E65" serogroup. All O45:K"E65" strains originated from different farms and were isolated in pure culture or as the predominant serogroup. In a retrospective study, intestinal segments from O45:K"E65"-positive pigs were reexamined for the presence of attaching-effacing lesions. Although not all intestinal segments were available for each animal, typical attaching and effacing lesions were present in at least four (36.4%) of the O45:K"E65"-positive weaned pigs. No attaching and effacing lesions were observed in the O45:K"E65"-positive unweaned pigs. Multifocal colonization of the brush border of mature enterocytes by gram-negative coccobacilli arranged in palisades with effacement of the microvilli, enterocyte degeneration, and light to moderate inflammation in the lamina propria was observed, mostly in the ileum of affected pigs.

Bacterial strain. E. coli 4725 (O45:K"E65"), isolated from the ileum of a weaned, 4-week-old diarrheic pig for which lesions suggesting AEEC were observed on light microscopic examination of the small intestine, was selected for further studies. The strain was stored on Dorset agar until the study began. It was serotyped with rabbit antisera for the O and K antigens (3). This strain possessed a high-molecular-mass plasmid of about 80 MDa but did not contain the EAF genes (9a) and adhered strongly to HeLa cells in a diffuse manner.

Detection of surface antigens in vitro. Strain 4725 was grown in the appropriate culture conditions and examined for production of fimbrial antigens F4 (K88), F5 (K99), F6 (987P), F41, and F165 by the indirect fluorescent antibody technique (IFAT) (3, 6, 7).

Detection of enterotoxins and cytotoxins. Strain 4725 was tested for enterotoxigenicity by using the gut-loop technique in 5- to 6-week-old pigs (3). The strain was examined for production of the enterotoxins STa, STb, and the heat-labile enterotoxin by using the infant mouse test, the gut-loop test in 5- to 6-week-old weaned pigs, and tissue culture assays with Y1, CHO, and Vero cells, respectively (3, 11). Shiga-like toxin and cytotoxic distending toxin production were determined in Vero and CHO cells (11). Production of hemolysin was detected by culture on blood agar base (Difco Laboratories, Detroit, Mich.) plus 5% (vol/vol) calf blood at 37°C overnight.

Infection of pigs. Strain 4725 was tested for attaching and effacing activity by experimental infection of colostrum-deprived newborn pigs (5). Pigs were collected aseptically by hysterotomy and deprived of colostrum. They were given intragastrically via stomach tube 10 ml of an overnight tryptic soy broth culture of E. coli (approximately 10^9 CFU) with 10 ml of 0.1% peptone water. The pigs were kept in sterile cages at 30 to 32°C, placed in an isolated room, and fed an artificial-milk formula. In a preliminary experiment, two pigs from a first litter were infected when 6 h old and killed at 12 and 72 h postinoculation (p.i.). An additional pig from the same litter was given 20 ml of 0.1% peptone water and kept as a control. Subsequently, seven pigs from a second litter were infected with strain 4725 when they were 48 h old. They were euthanatized at 12, 24, 48, 72, 96, and 108 (two pigs) h p.i. An additional two pigs from the same litter were given 20 ml of 0.1% peptone water and kept as controls. Pigs from a third litter were infected with strain 4725 when they were 24 h old and euthanatized at 3, 6, 12, 24, and 48 h p.i. Specimens were collected for light and electron microscopy, immunofluorescence, virology, and bacteriology studies.

Light microscopy. Samples from the stomach; duodenum; proximal, middle (two), and distal jejunum; ileum; cecum; and proximal and distal colon were fixed in 10% buffered Formalin and processed for paraffin sectioning according to standard techniques. Sections were stained with hematoxylin, phloxine, and safranine (HPS) (18); Gram stains were also done on all sections.

Electron microscopy. Samples from the duodenum, jejunum, ileum, cecum, and proximal colon were fixed in cacodylate buffer (0.1 M, pH 7.0) containing 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, and dehydrated through a series of acetone washes. After dehydration in propylene oxide, samples were embedded in Spurr low-viscosity embedding resin. Thin sections were stained with uranyl acetate and lead citrate and examined on a Philips 201 electron microscope operating at 60 kV (5).

Virology. Segments from the duodenum, jejunum, and ileum were examined for the presence of rotavirus (types A and C) and coronavirus (transmissible gastroenteritis) by using indirect immunofluorescence (17). Anti-coronavirus and -rotavirus type A sera were obtained from Institut Armand-Frappier, Laval-des-Rapides, Quebec, Canada. Anti-rotavirus type C serum was obtained from K. W. Theil, Ohio Agricultural Research and Development Center, Wooster, Ohio.

Bacteriology. Segments from the jejunum, ileum, cecum, and proximal colon were cultured on blood agar base (Difco) plus 5% sheep blood agar and on MacConkey agar. All E. coli isolates were serotyped by using nine colonies per isolate. Frozen sections from the duodenum, jejunum, ileum, cecum, and proximal colon were examined by indirect immunofluorescence by using a specific O45:K"E65" antiserum as described previously (2).

RESULTS

Bacterial strain characterization. E. coli 4725 was alpha-hemolytic; did not possess any of the fimbriae K88, K99, 987P, F41, or F165; and produced neither enterotoxins (STa, STb, and the heat-labile enterotoxin) nor cytotoxins (Shiga-like and cytotoxic distending toxins).

Nature of lesions. In a preliminary experiment to evaluate
the pathogenicity of strain 4725, one pig inoculated with this strain was sacrificed at 12 h p.i., a second pig was sacrificed at 72 h p.i., and a control piglet was sacrificed at 96 h after inoculation with only tryptic soy broth. Only the second pig developed diarrhea, at about 66 h p.i. At 12 h p.i., multifocal colonization by gram-negative rods of the brush borders of mature enterocytes and the apical surfaces of goblet cells was observed in the duodenum and proximal jejunum. The bacteria were eosinophilic with HPS staining and were often arranged in palisades. There were many neutrophils in the villous capillaries, but there was no significant exocytosis in the lamina propria. E. coli was isolated in pure culture and in large numbers from the ileum but not from the cecum and colon and was identified as O45:K"E65" by slide agglutination.

At 72 h p.i., a multifocal, focally extensive colonization of mature enterocyte brush borders and apical surfaces of goblet cells by gram-negative rods was observed in all intestinal segments except the distal colon (Fig. 1A). In the cecum and the colon, colonization of the epithelium of the crypts was also observed (Fig. 1B). Colonization was most intense in the duodenum and the cecum. The bacteria were basophilic with HPS staining, had a characteristic palisade arrangement, and appeared to be in close apposition with the cellular apical membrane. Bacteria were also seen in intracytoplasmic vacuoles in some mature enterocytes and, in areas of heavier colonization, in the lamina propria in which they were sometimes in lacteals (Fig. 2). There was a moderate neutrophilic exocytosis in the lamina propria. Epithelial degeneration was characterized by increased cytoplasmic eosinophilia typically starting at the cellular apex, followed by condensation of the cell, and eventually desquamation of individual or small clusters of cells (Fig. 2); this loss of surface epithelium was not severe, and there was no extensive ulceration. A light to moderate villous atrophy was observed in the small intestine and seemed to be correlated with the intensity of the inflammatory reaction in the lamina propria. Bacteria adhering to the enterocytes or found in vacuoles or in the lamina propria were identified as E. coli O45:K"E65" by the IFAT (Fig. 3). E. coli were isolated in pure culture and in large numbers from the ileum, cecum, and colon and were identified as O45:K"E65" by slide agglutination. In both pigs, examination for rotavirus and coronavirus by immunofluorescence was negative.

In the pig examined at 72 h p.i., transmission electron microscopy (TEM) revealed numerous rod-shaped bacteria intimately attached to the apical cell membrane of mature enterocytes, with effacement of adjacent microvilli (Fig. 4A). Bacteria were arranged in regular palisades, parallel to the microvilli. The bacterial cell wall and the apical cell membrane of the enterocyte were constantly separated by a narrow, regular gap of about 10 nm (Fig. 4A, inset). Typical cups, pedestals, and apical electron-dense regions were seen at attachment sites. Bacteria were also seen in intracytoplasm-
mic vacuoles (Fig. 4A). Many of the colonized enterocytes were degenerating, whereas noncolonized enterocytes appeared normal.

The control piglet remained clinically normal. On necropsy, no macroscopic or microscopic lesions or evidence of bacterial intestinal colonization was observed.

Development of bacterial adherence. Pigs from two additional litters were inoculated with the same strain in order to study the early development of bacterial adherence and of the attaching and effacing lesions in the intestine. No diarrhea was observed in any of the inoculated pigs, although slightly soft colonic contents were observed at necropsy in two pigs killed at 72 and 96 h p.i. In general, attaching and effacing lesions similar to those of the pigs in the preliminary experiment were observed. The lesions were generally more scattered and less extensive than in the preliminary experiment, especially in the cecum and colon. In all cases, the attaching-effacing bacteria were shown to be E. coli O45: K"E65" by the IFAT. E. coli isolated from the ileum, cecum, and colon in all inoculated pigs were identified as O45: K"E65" by slide agglutination. Examination for rotavirus and coronavirus by immunofluorescence was negative for all pigs.

At 3, 6, and 12 h p.i., the duodenum and proximal jejunum were colonized by bacteria which were generally amphophilic with HPS staining (Table 2) and a mild to moderate neutrophilic exocytosis was observed in the lamina propria of these intestinal segments. Similar but much more scattered lesions were observed in the distal jejunum and the ileum. TEM revealed many typical, well-established attaching and effacing lesions similar to those already described. In contrast, some more electron-dense bacteria were tightly attached to the apical cell membrane with no visible gap (Fig. 4B). These bacteria were not aligned in palisades but were disposed irregularly on the apical cell membrane. E. coli of serogroup O45: K"E65" had already colonized the cecal epithelium as shown by IFAT but were not visible on light microscopy.

At 24 h p.i., lesions in the small intestine were similar but more severe; bacteria were amphophilic or basophilic with HPS staining. Neutrophilic exocytosis in the lamina propria was more intense, light to moderate degenerative changes in enterocytes were present, and there was light villous atrophy. Many irregularly dispersed bacteria tightly attached to the apical cell membrane with no visible gap were still observed on TEM. E. coli of serogroup O45: K"E65" had colonized the cecal and colonic epithelium as shown by IFAT, although they were not visible on light microscopy.

At 48 h p.i., degenerative changes were observed in mature enterocytes of the small intestine; they were condensed, eosinophilic, and occasionally desquamating. The cecum and colon showed scattered bacterial colonization of mature enterocytes and crypt cells on light microscopy. All
attached bacteria now had the typical palisade arrangement and were separated from the apical cell membrane by a regular gap of about 10 nm (Fig. 4C). At 72 h p.i., lesions in the cecum and colon were more severe and degenerative changes were apparent. At 96 and 108 h p.i., the lesions were essentially the same as those observed at 72 h p.i.; in addition, a few lesions similar to those observed at 12 and 24 h p.i. (irregularly dispersed, tightly attached bacteria) were seen and interpreted as new colonization foci.

In the two control pigs, sacrificed at 108 h, no significant lesions were observed and E. coli of the O45:K’E65’ serogroup was neither isolated from the intestine nor observed on IFAT.

**DISCUSSION**

We have demonstrated that at least one porcine E. coli strain belonging to the O45:K’E65’ serogroup is attaching and effacing in experimentally infected pigs; furthermore, this strain produces lesions similar to human enteropathogenic AEEC strains (14). Thus, we studied the poorly understood early stages of attaching and effacing of E. coli to intestinal epithelial cells in vivo in newborn pigs experimentally infected with this porcine strain. Attaching and effacing lesions were well established in the proximal small intestine 12 h p.i. Lesions in the cecum and colon were not observed until 48 h p.i., even though bacterial colonization was demonstrated in these sites 12 and 24 h p.i., respectively, by IFAT. Our TEM observations in the proximal small intestine suggest that even though the attaching and effacing process is rapid, there is an evolution of the bacterial adherence. Initially, irregularly disposed bacteria tightly adhere; they then subsequently reorient to form palisades and develop a narrow, regular gap (10 nm) between the bacterial cell wall and the cell membrane of the enterocyte. Thus, there could be an intermediate stabilizing step between the initial non-intimate adherence of bacteria to intact microvilli and the later intimate adherence of bacteria to the enterocyte cell membrane, with the 10-nm gap described by Knutton et al. (13). This is to our knowledge the first description of this phenomenon which could represent a new and different mechanism of attachment and effacement. Indeed, strain 4725 does not contain the genes encoding for the EAF of human EPEC strains, although a similar plasmid-encoded adhesin may be present. On the other hand, few other studies have examined the development of lesions immediately after infection of piglets with human AEEC strains, and this initial attachment step may have been present but not observed in these studies. Further studies with this strain might help elucidate one of the mechanisms of attachment-effacement.

The newborn pig is a good experimental model for the study of the attaching and effacing activity of human, calf, and rabbit strains (8, 9, 16, 30–32). Natural AEEC infection has been described in humans, calves, pigs, lambs, dogs, cats, and rabbits (2, 10, 20, 26, 33). Most AEEC infections in animals, with the exception of those in calves, produce enteropathogenic AEEC-like lesions. In the latter species, AEEC strain S102-9 produces enterohemorrhagic E. coli-like lesions similar to those observed for E. coli serogroup O157:H7 in humans (9, 20). The rabbit AEEC strain RDEC-1 and certain O157:H7 strains possess plasmid-encoded fimbriae (12, 22), but EPEC adherence to the intestinal epithelium is probably due in part to the plasmid-encoded EAF (21, 30). Distribution and extent of lesions vary between and within species in natural and experimental AEEC infections, although the ileum and colon seem to be the most frequent sites (10, 16). In the present study, porcine AEEC strain 4725 colonized mainly the proximal small intestine (especially the duodenum) and the cecum, although all the intestinal tract except the distal colon was affected. The more-extensive lesions observed in the preliminary experiment could be explained by the relative immaturity of the pigs compared with those in the second experiment. Nonimmune host factors may influence the severity and extent of AEEC lesions (30). Furthermore, pigs in the second and third litters were inoculated later than those in the preliminary experiment and thus would have had a more developed intestinal microflora which may have influenced the extent of colonization (30). Bacteria have been observed in the intestinal lamina propria of pigs infected with an O55 strain (30). We
observed bacteria in the lamina propria in areas of heavier colonization; the bacteria appeared to be draining in lacteals and did not seem to cause any significant damage. Membrane-bound bacteria have already been reported in the apical cytoplasm of enterocytes in enterohemorrhagic and enteropathogenic AEEC-infected animals (28, 31, 32). This phenomenon has been studied in vitro (1, 15). Although its significance is not yet clear, it could be an endocytic process induced by AEEC strains (1, 32) and could be important in the attaching and effacing process (4).

Diarrhea developed in only one infected pig, in the preliminary experiment, at 66 h p.i. In other experimental infections with enteropathogenic AEEC strains, diarrhea was not always induced. When observed, its onset varied from 20 h p.i. in pigs infected with human enteropathogenic AEEC strains (30) to 10 days p.i. in rabbits infected with rabbit enteropathogenic AEEC strains (25). It has been suggested that the extent of lesions would determine whether diarrhea will occur or not (16, 30); this might explain why no significant diarrhea was observed in the second and third litters in which the pigs had less-extensive AEEC lesions.

In conclusion, we have demonstrated that a porcine E. coli strain belonging to serogroup O45:K665 causes attaching and effacing lesions similar to those of human enteropathogenic AEEC strains in experimentally inoculated newborn pigs. Lesions were well established in the proximal small intestine 12 h p.i. but were not seen in the cecum and colon until 48 h p.i., even though bacterial colonization was already present in these sites at 12 and 24 h p.i., respectively. In addition, our results suggest that contact between the bacteria and the epithelial cell membrane is initially very intimate and then evolves to form the narrow, regular gap typically observed in AEEC lesions. Further pig infection studies using this strain may elucidate the mechanisms involved in the development of the attachment of porcine AEEC to the intestinal epithelium.

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