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Role of the K88 Antigen in the Pathogenesis of Neonatal Diarrhea Caused by *Escherichia coli* in Piglets

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The role of the K88 antigen of *Escherichia coli* in neonatal diarrhea of piglets was studied by comparing a K88-positive strain with three K88-negative strains derived from the K88-positive strain. K88 antigen was produced by the K88-positive strain in the intestinal tract of gnotobiotic piglets, whereas K88-negative strains did not regain the ability to synthesize K88 antigen. Synthesis of the antigen conferred different colonization characteristics on the four strains; K88-positive bacteria adhered to the mucosa of the small intestine, whereas K88-negative bacteria did not attach and were distributed throughout the lumen. Adhesion of K88-positive bacteria to tissue from the small intestine of gnotobiotic piglets was demonstrated in vitro and was inhibited by antisera that contained K88 antibodies. Attachment did not occur with bacteria grown at 18 C. Adhesion of cell-free K88 antigen was also demonstrated. The K88-positive strain and one of the K88-negative strains were equally virulent in gnotobiotic piglets. In contrast, the K88-positive strain killed 50% of conventionally reared piglets, whereas the K88-negative strain killed only 3%. Adhesion of the K88-positive strain, but not of the K88-negative strain, to the mucosa of the small intestine was demonstrated. Our results show that K88 antigen is responsible for attachment of K88-positive bacteria to the wall of the small intestine, and that adhesion is essential for the virulence of K88-positive bacteria in conventionally reared piglets.

Neonatal diarrhea in piglets is characterized by the proliferation of certain strains of *Escherichia coli* in the small intestine (41). Colonization of the anterior small intestine by these strains followed by their multiplication to reach large numbers (41) and production of enterotoxins (21, 28, 39) results in clinical disease. The comparatively small numbers of non-enteropathogenic strains of *E. coli* in the small intestine of apparently healthy piglets (24, 41) may be due, in part, to the removal of bacteria by gut motility (1, 11, 18). Adhesion of *E. coli* to the intestinal epithelium has been demonstrated (2, 12, 13, 14, 40, 44, 46, 50), and it is possible that attachment allows enteropathogenic strains to overcome gut motility and to reach large numbers in the small intestine.

Strains of enteropathogenic *E. coli* isolated from diseased piglets frequently possess K88 antigen (20, 45, 52). In contrast to the other K antigens, K88 is a protein component (48) that forms a substantial layer of fine filaments on the surface of the cell (47). Its synthesis is controlled by a plasmid that can be spontaneously lost (30). Addition of the K88 gene to the bacterial genome and its retention in this group of bacteria suggest that the antigen has a biological function that assists the survival of K88-positive bacteria as enteropathogens. In two laboratory strains of *E. coli*, K88 antigen caused mannose-resistant hemagglutination of guinea pig red blood cells (47). This has been confirmed and extended in a detailed investigation of K88-positive and K88-negative bacteria (Jones and Rutter, *in preparation*). Thus, it seemed reasonable to postulate that the hemagglutinating property of K88 antigen reflects an adhesive ability that assists bacterial colonization of the small intestine. Initially, it was necessary to demonstrate that K88 antigen was synthesized in the intestinal tract. In vivo and in vitro methods were then developed to determine whether K88 antigen was involved in the adhesion of K88-positive bacteria to the mucosa of the intestine. Finally, the virulence of K88-positive and K88-negative bacteria was compared in gnotobiotic and conventionally reared piglets.

MATERIALS AND METHODS

Culture media. Blood agar contained Hartley broth (9), 1% (w/v) agar, and 5% (v/v) citrated ox blood. Buffered glucose nutrient agar contained 1 liter of nutrient broth no. 2 (Oxoid Ltd., London), 0.45 g of KH_2PO_4, 0.81 g of Na_2HPO_4, and 8 g of agar (Difco Laboratories Ltd., West Molesey, England). A solution of glucose sterilized by membrane filtration was added to a final concentration of 0.1% immediately before the plates were poured.

Preparation of antisera. OK, O, and H antisera were prepared in rabbits (45). K88 antisera were prepared from cell-free K88 antigen preparations containing 2 to 5 mg of protein per ml. The antigen was emulsified in an equal volume of Freund complete adjuvant (Difco), and 1 ml of emulsion was inoculated subcutaneously into a rabbit on three occasions at weekly intervals (48). Antisera were absorbed by suspending 4 g (wet weight) of bacteria from 18-hr buffered glucose nutrient agar cultures in a final volume of 10 ml of antiserum diluted 1 to 5 in saline. The suspension was incubated for 2 hr at 37 C followed by 18 hr at 4 C. Bacteria were removed by centrifugation at 10,000 × *g* for 1 hr, and the antiserum was filtered through a 220-nm membrane filter. Absorbed and unabsorbed antisera were tested by slide and tube agglutination tests with live and boiled cultures of homologous and heterologous strains, and the results were interpreted according to accepted criteria (32, 33, 45).

Serological tests. Double diffusion (Ouchterlony) tests were done in an agar medium (48) with added 0.145 M NaCl. O, H, and K agglutinins were estimated by titration (35, 45) or by slide agglutination tests (45). For slide agglutination tests, antiserum was diluted 1 to 5 or 1 to 10 in saline. Normal rabbit serum and saline controls were included.

Strains of E. coli. A K88-positive strain (W1) with antigenic formula 0149:K91(B),K88ac(L):H10 was provided by W. J. Sojka (Central Veterinary Laboratory, Weybridge, Surrey). Three K88-negative strains designated W1(J2), W1(J35), and W1(J134) were derived from W1. Strain W1(J2) was obtained by ethidium bromide treatment (8), strain W1(J35) was isolated after ultraviolet irradiation, and strain W1(J134) was a spontaneous mutant. K88-negative bacteria were separated from K88-positive bacteria by plating the final cultures on Tergitol-7 medium (36). The K88-negative strains agglutinated with OK antisera prepared against cultures of the 0149:K91(B),K88ac(L):H10 serotype, but not with OK antisera prepared against other K88-positive bacteria. In O agglutination tests, boiled cultures of all four strains agglutinated with O antiserum prepared against the parent strain, but live cultures were inagglutinable. All of the strains agglutinated with H antiserum prepared against strain Bi623-42 (011:K10:H10), provided by Ida Ørskov, Statens Seruminstitut, Copenhagen. On the basis of these results, it was concluded that the four strains were antigenically similar, but that the parent strain synthesized K88 antigen. Precipitation tests confirmed this conclusion.

All of the strains were hemolytic on bovine blood agar plates. Similar volumes of fluid were produced by overnight broth cultures of each strain in ligated intestinal loops (38) prepared in four piglets, and it was concluded that all produced enterotoxin. All grew on minimal medium agar (9) and were of the same biotype. None of the strains produced mannose-sensitive (fimbrial) hemagglutination of red blood cells when tested in a procedure (J. P. Duguid, *personal communication* to G.W.J.) based on that described by Duguid and Gillies (15). In contrast, only the K88-positive parent strain produced mannose-resistant hemagglutination of guinea pig red blood cells at 4 C due to the presence of K88 antigen (Jones and Rutter, *in preparation*). Thus the K88-negative strains were apparently identical to the parent strain except that they did not synthesize K88 antigen. Other strains of porcine enteropathogenic *E. coli* were provided by W. J. Sojka, H. W. Smith (Houghton Poultry Research Station, near Huntingdon), A. Gush, and A. J. Woods (Veterinary Investigation Centre, Reading).

Extraction of K88 antigen. Bacteria were cultured on Tryptose glucose agar medium for 16 hr, harvested in 0.1 M phosphate buffer (*p*H 7.0), and homogenized for 1 min (M.S.E. Homogeniser, M.S.E. Ltd., London) to release K88 antigen. The bacteria were removed by centrifugation and the supernatant fluid was stored at 4 C for 3 days. The *p*H was adjusted to 5.3 by the addition of acetic acid, and the precipitate was collected, washed, and reprecipitated several times (48). Yields were approximately 10% in terms of the original concentration of K88 antigen.

Piglets. Gnotobiotic piglets were procured, reared in plastic isolators (49, 51), and infected orally at 1 to 2 days of age. Conventionally reared suckling piglets were infected at birth before receiving colostrum (35).

Necropsy procedures. The small intestine was divided into three equal parts. Lengths of intestine (5 cm) from the center of each portion were ligated, excised, and stored at −70 C for fluorescent-antibody (FA) studies. The apex of the spiral coil of the colon was treated in the same way. Viable counts of bacteria (35) were made on contents expressed from the intestine immediately anterior to the sections removed for FA studies. Rectal and intestinal swabs were cultured to enumerate the enteropathogenic strain (35) in other piglets.

Indirect FA technique. Tissue was supported in gelatin (27), and transverse sections (8–10 μm) were cut on a Pearse refrigerated microtome (Slee Medical Equipment Ltd., London) at −20 to −15 C. Sections mounted on glass slides were dried in air, fixed in methanol at room temperature for 20 min, and stained (29). Rabbit antiserum and sheep anti-rabbit globulin conjugated with fluorescein (Wellcome Laboratories Ltd., Beckenham, Kent) were diluted 1 to 10. OK antiserum prepared in rabbits against strain W1(J2) was used to locate the bacteria in the intestine. In the FA technique, this antiserum stained only cultures of the 0149:K91(B),K88ac(L):H10 serotype, including the K88-negative mutants of strain W1. K88 antiserum prepared against cell-free antigen from a strain of the 0147:K89(B),K88ac(L) group was used to detect K88 antigen. In the FA technique, this antiserum stained only K88-positive cultures grown at 37 C but not at 18 C. The specificity of staining reactions was evaluated with absorbed rabbit antisera (5, 29). All antisera

were also examined in agglutination and precipitation tests to confirm the presence of appropriate antibodies. Pooled normal rabbit serum was used as a control in each test.

In vitro adhesion of E. coli to piglet intestinal tissue. Gnotobiotic piglets were starved for 16 hr, killed, and the intestine was removed. The middle third of the small intestine was opened longitudinally and gently washed with complete phosphate-buffered saline (PBS; 16). Then discs of tissue (about 6 mm in diameter) were cut with a cork borer and placed in PBS.

Bacteria grown on buffered glucose nutrient agar at 37 C for 16 hr or at 18 C for 48 hr were harvested into PBS at 37 C to give a concentration of 10^9 colony-forming units (CFU) per ml. One disc of tissue was added to 1 ml of suspension and gently agitated at 30 rev/min on an orbital shaker (Luckham Ltd., Burgess Hill, England) for 30 min at 37 C. The surplus PBS was removed with blotting paper, and the disc was gently washed in two changes of PBS and homogenized in 5 ml of Ringer salt solution (Oxoid) in a glass homogenizer (Gallenkamp & Co. Ltd., London). Viable counts were made on the homogenate. Zero time counts were determined by exposing tissue to the bacterial suspension for approximately 1 sec. All tests were done in duplicate. In antiserum inhibition tests (see text) the bacterial suspension was first incubated for 30 min at 37 C with dilutions of rabbit antisera (inactivated at 56 C for 30 min), and the test was then performed in the usual manner.

Strains W1 and W1(J2) were included in all tests as positive and negative controls. Discs of tissue incubated in parallel were frozen in a dry ice-acetone slurry, mounted, sectioned, and stained by the FA technique.

Adhesive properties of cell-free K88 antigen. Discs of tissue were placed in a suspension of K88 antigen diluted in PBS and incubated at 37 C with gentle agitation for 30 to 60 min. The tissue was frozen, and sections were prepared for FA staining.

RESULTS

Colonization of the alimentary tract of gnotobiotic piglets by K88-positive or K88-negative strains. Eight piglets from four different litters were infected with the K88-positive strain, and four piglets were infected with each of the K88-negative strains. The piglets were given 3×10^8 CFU in 1 ml of Ringer salt solution. Severe diarrhea occurred 11 to 43 hr after infection, and the piglets were killed before they became severely dehydrated. Viable counts made on the contents of the small intestine of each piglet showed that the K88-positive strain and the three K88-negative strains colonized the whole of the small intestine (Table 1). There were no obvious differences in the colonizing abilities of the K88-positive and K88-negative strains. Counts in the large intestine were almost 1 $\log_{10}$ higher than in the small intestine.

Synthesis of K88 antigen in the small intestine of gnotobiotic piglets. Cryostat sections stained with K88 antiserum demonstrated the presence of fluorescent bacteria in the small intestine of piglets infected with the K88-positive strain. No fluorescence was observed if the antiserum was absorbed prior to staining with live cultures of different K88-positive bacteria or with cell-free K88 antigen. No fluorescence was observed when sections prepared from the intestine of piglets infected with the three K88-negative strains were stained with K88 antiserum. Fluorescent bacteria were observed when antiserum prepared against the W1(J2) K88-negative strain was used in the test. It was concluded that K88 antigen was produced by the K88-positive strain but not by the K88-negative strains in the alimentary tract. This was confirmed by precipitation tests with K88 antiserum and extracts of intestinal tissue from infected piglets.

Location of K88-positive bacteria in the small intestine of gnotobiotic piglets. The K88-positive bacteria were closely associated with the tissue surface and were generally absent from the lumen of the intestine. There was no reduction in the intensity of fluorescence after washing the lumen with Ringer solution. Occasionally, the bacteria were in microcolonies in a matrix of K88 antigen, in close association with the epithelial surface (Fig. 1). The bacteria appeared to have penetrated the mucous secretion coating the epithelium. In some areas, a confluent layer of K88 antigen was present on the mucosal surface, although individual bacteria were not distinguishable. Sections of

TABLE 1. *Viable counts of Escherichia coli in the small intestine of gnotobiotic piglets after oral infection with K88-positive and K88-negative strains*

Strains	Ranges of $\log_{10}$ viable counts		
	Anterior small intestine	Middle small intestine	Posterior small intestine
K88 positive	5.1–8.8 (Mean[a] 7.1)	6.3–9.0 (Mean 7.8)	6.5–9.3 (Mean 8.4)
K88 negative	6.4–8.3 (Mean[b] 7.5)	6.5–8.8 (Mean 7.9)	7.4–9.5 (Mean 8.4)

[a] Average of eight observations with strain W1.
[b] Average of 12 observations with strains W1(J2), W1(J35), and W1(J134).

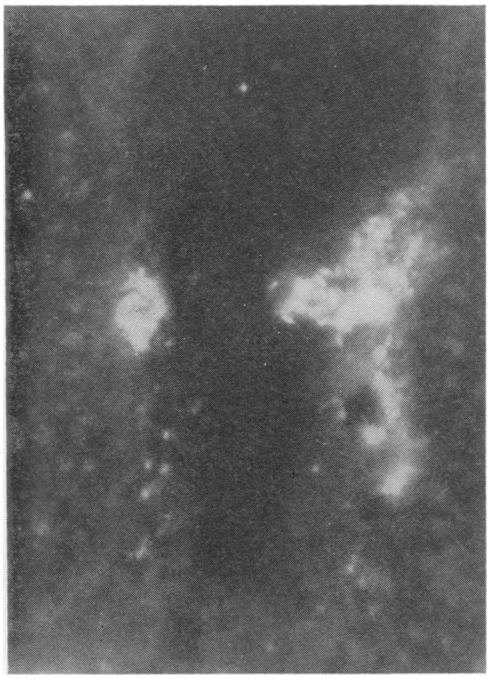

FIG. 1. *Microcolonies of fluorescent K88-positive bacteria of strain W1 on the surfaces of two adjacent villi. Tissue from the posterior small intestine of an infected gnotobiotic piglet; section stained by the FA technique with K88 antiserum* (×310).

tissue stained by the FA technique were subsequently overstained with the periodic acid-Schiff technique (10). The surface of the tissue and the spaces between the villi consisted of strongly periodic acid-Schiff-positive material, indicating that K88 antigen becomes closely associated with periodic acid-Schiff-positive material on the epithelial surface.

Fluorescent bacteria were present in the posterior small intestine on the sides of the villi in each of eight piglets and were absent from the villous tips. Penetration to the bases of villi occurred in five of eight piglets. A similar pattern of colonization was observed in the middle of the small intestine, except that penetration of bacteria to the bases of villi was restricted to three of eight piglets. In contrast, adherence of bacteria to the epithelium of the anterior small intestine occurred in only five of eight piglets. Adhesion occurred mainly at the tips and on the sides of villi and, in one animal, bacteria were present at the bases of villi. Although a few bacteria were closely associated with the mucosa of the anterior small intestine in the remaining three of eight animals, an accurate assessment of adhesion was not possible because too few organisms were present in the sections.

Location of the K88-negative strains in the small intestine of gnotobiotic piglets. In contrast to the results with the K88-positive strain, the three K88-negative strains were evenly distributed throughout the lumen of the intestine. Washing the lumen with Ringer solution removed most of the fluorescence, indicating that the bacteria were not attached to the tissue surface. Staining of the epithelium with K88 antiserum did not occur in the intestinal tissue from these piglets.

Synthesis of K88 antigen and location of K88-positive and K88-negative strains in the large intestine of gnotobiotic piglets. The K88-positive strain produced K88 antigen in the large intestine, but in the majority of the piglets there was no evidence of adhesion of K88-positive or K88-negative strains to the epithelial surface or of bacterial penetration into the crypts of Lieberkühn.

In vitro adhesion to tissue from the small intestine of gnotobiotic piglets; comparison of K88-positive and K88-negative strains. Adhesion of bacteria to discs of intestinal tissue from gnotobiotic piglets was studied by viable counts and by the FA technique.

In zero time (1 sec) tests with bacterial suspensions of W1 and the three K88-negative strains, there was a nonspecific carry-over of approximately 0.05 to 0.1% of bacteria on the washed discs of tissue. This occurred in tests with all of the four strains, and therefore could not be attributed to the presence of K88 antigen. There was an increase of 1.6 to 2.0 $\log_{10}$ in the viable counts on tissue incubated with bacterial suspensions of strain W1 for 30 min compared with 1 sec. This was statistically significant ($P < 0.001$) by the t test and could not be attributed to bacterial multiplication. Although there was a statistically significant increase in the viable counts on tissue incubated with the three K88-negative bacterial suspensions between 1 sec and 30 min, this was invariably less than 10-fold and was significantly less ($P < 0.001$) than the increase in tests with the K88-positive strain. An example of a typical test is shown in Table 2. Similar results were obtained in tests with tissue from the anterior third and posterior third of the small intestine of gnotobiotic piglets.

Comparable results were obtained in tests with 21 other strains of porcine enteropathogenic *E. coli* belonging to a number of OK groups. The $\log_{10}$ viable counts on tissue incubated with eight K88-positive strains increased by 1.4 to 2.1 (mean 1.8). In contrast, the $\log_{10}$ viable counts on tissue incubated with 13 K88-negative strains increased by 0.2 to 0.9 (mean 0.7). The difference between the two groups was statistically significant

TABLE 2. *In vitro adhesion of Escherichia coli to tissue from the small intestine of a gnotobiotic piglet*

Strains	Log_{10} viable counts[a] on tissue at		Increases of log_{10} viable counts[b]	Observations with fluorescent-antibody technique
	1 sec	30 min		
W1, K88 positive	5.7	7.3	1.6	Adhesion
W1(J2), K88 negative	6.2	6.6	0.4	No adhesion
W1(J35), K88 negative	5.6	6.4	0.8	No adhesion
W1(J134), K88 negative	5.4	6.3	0.9	No adhesion

[a] Mean for duplicate tests.
[b] Log_{10} viable count at 30 min minus log_{10} viable count at 1 sec.

($P < 0.001$). The K88-negative strains included five nonpathogenic strains from normal piglets and six strains of serotypes that are usually associated with diarrheal and edema disease of older pigs.

Cryostat sections of intestinal tissue incubated with the bacterial suspensions for 30 min were stained by the FA technique. In tests with the K88-negative strains, the bacteria were distributed in a random fashion between the villi and were not associated with the mucosal surface (Fig. 2). In contrast, the K88-positive strains adhered to the tissue surface in a layer that was frequently many cells thick (Fig. 3). These results show that the significant difference between the viable counts of K88-positive compared with K88-negative bacteria was due to adhesion of K88-positive bacteria to the tissue.

Inhibition of adhesion of K88-positive strains by culture at 18 C for 48 hr. K88-positive strains cultured at 18 C do not produce K88 antigen (31). The log_{10} viable counts on intestinal tissue incubated for 30 min at 37 C with suspensions of nine K88-positive strains cultured at 18 C increased by 0.7 to 0.9 (mean 0.8). The increases in control tests with the same strains cultured at 37 C were 1.6 to 2.0 (mean 1.8). The difference between the two groups was statistically significant ($P < 0.001$). In addition, the FA technique showed that bacteria cultured at 18 C do not adhere to intestinal tissue after 30 min of incubation at 37 C.

Inhibition of adhesion of the K88-positive strain (W1) by antisera. The increase in log_{10} viable counts on intestinal tissue incubated with strain W1 was significantly reduced ($P < 0.001$) by previous incubation of the bacterial suspension with rabbit antisera that contained antibodies to K88 antigen (Table 3). The FA technique confirmed that, in these tests, the bacteria did not adhere to the intestinal tissue. Although antisera prepared against other surface antigens caused bacterial agglutination, this did not significantly reduce adhesion.

Adhesion of cell-free K88 antigen to intestinal tissue from gnotobiotic piglets. Discs of intestinal tissue were incubated for 30 min at 37 C with pooled cell-free K88 antigen of strain W1 containing 500 μg of protein per ml. K88 antigen was detected with K88 antiserum as a fluorescent layer on the mucosa. The antigen appeared to adhere to a periodic acid-Schiff-positive layer on the tissue surface. The spaces between the villi were occluded with periodic acid-Schiff-positive material but showed only traces of fluorescence. Only prior absorption of the K88 antiserum to remove K88 antibody removed the staining properties of the antiserum. Staining occurred after absorbing the antiserum with intestinal mucus from gnotobiotic piglets or with homogenized Tryptose glucose agar medium, indicating that blood group substances are not involved in the staining reaction. Fluorescence also occurred with OK antisera prepared from other K88-positive bacteria, but not with OK antiserum prepared against W1(J2), or with O and H antiserum prepared against W1. There was no fluorescence if fluo-

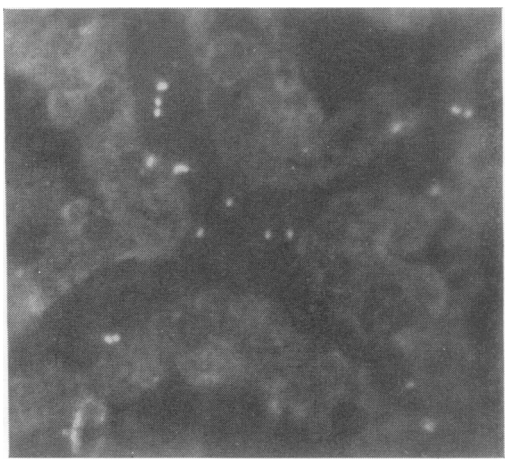

FIG. 2. *Fluorescent K88-negative bacteria distributed between the villi (in transverse section) of piglet intestine. Tissue incubated with a suspension of strain W1(J2); section stained by the FA technique with W1(J2) OK antiserum* (×625).

rescein-labeled sheep anti-rabbit serum was used alone.

Virulence of the K88-positive strain and one of the K88-negative strains in gnotobiotic piglets. Strain W1(J2) was selected to compare the virulence of a K88-negative mutant strain with the K88-positive parent strain W1 in piglets. Five of six gnotobiotic piglets from three different litters died within 36 hr of oral dosing with 1 ml of bacterial suspension containing 10^8 CFU of the K88-positive or K88-negative strain. Watery diarrhea and dehydration were generally evident prior to death, and the infecting strain was cultured from rectal swabs. At necropsy, pure cultures of the infecting strain were recovered from the anterior and posterior small intestine and from the large intestine. Thus, there was no difference in the virulence of the two strains in gnotobiotic piglets; both colonized the intestinal tract and caused severe diarrhea and death.

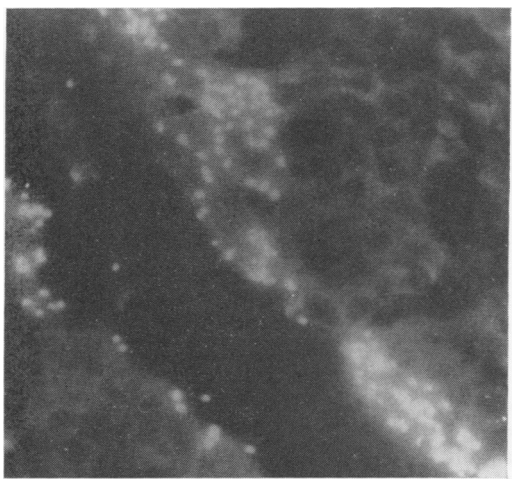

FIG. 3. *Fluorescent K88-positive bacteria on the surfaces of two adjacent villi of piglet intestine. Tissue incubated with a suspension of strain W1; section stained by the FA technique with W1(J2) OK antiserum* (×625).

Virulence of the K88-positive strain in conventionally reared piglets. The results of similar infection experiments with conventionally reared suckling piglets infected orally at birth with 10^8 CFU of strain W1 are shown in Table 4. The K88-positive strain killed 22 of 44 piglets from four different litters. Different litters varied in their susceptibility to the K88-positive strain (35). Moderately severe diarrhea occurred in many of the piglets for 24 to 48 hr. In the piglets that died, severe diarrhea commenced soon after infection and the piglets lost weight. This was associated with the recovery of almost pure cultures of strain W1 from rectal swabs. Deaths occurred within 48 hr of infection, and pure or almost pure cultures of strain W1 were recovered from swabs taken from the anterior small intestine. Tissue from the small intestine of recently dead piglets stained by the FA technique showed that K88-positive bacteria were closely associated with the mucosal surface of the small intestine (Fig. 4). The W1 strain was not detected in rectal swabs taken from the surviving piglets 48 hr after infection or in swabs taken from the intestinal tract at necropsy.

TABLE 3. *Inhibition by selected antisera of the attachment of strain W1 to piglet intestinal tissue*

Rabbit antiserum prepared against	Antiserum diluted 1 in	Increase in $\log_{10}$ viable counts between 1 sec and 30 min	Observation with fluorescent-antibody technique[b]	Reciprocal of serum agglutination titer with live organisms of strain W1
Cell-free K88 antigen of strain P110	10	0.4	−	512
[0147:K89(B), K88ac(L)][a]	100	0.7	−	
Live organisms of strain W1	10	0.4	−	1,024
[0149:K91(B), K88ac(L):H10][a]	100	0.6	−	
Live organisms of strain W1(J2)	10	1.7	+	8,192
[0149:K91(B):H10]	100	1.7	+	
Heated organisms of strain W1 [0149]	10	1.3	+	4
	100	1.7	+	
Live organisms of strain Bi623-42	10	1.4	+	2,048
[011:K10:H10]	100	1.8	+	
Normal rabbit serum	10	1.6	+	0
	100	1.6	+	
No serum		1.7	+	

[a] Only these antisera contained K88 antibody.
[b] Adhesion, +; no adhesion, −.

TABLE 4. *Mortality of conventionally reared piglets attributed to neonatal diarrhea after oral administration of Escherichia coli*

Litter no.	Mortality[a] attributed to K88-positive strain W1[b]	Litter no.	Mortality[a] attributed to K88-negative strain W1(J2)[c]
1	4/9	5	1/10
2	7/10	6	0/10
3	1/8	7	0/3
4	10/17	8	0/9

[a] No. that died out of no. challenged.
[b] Mortality attributed to K88-positive strain W1, 50%.
[c] Mortality attributed to K88-negative strain W1(J2), 3%.

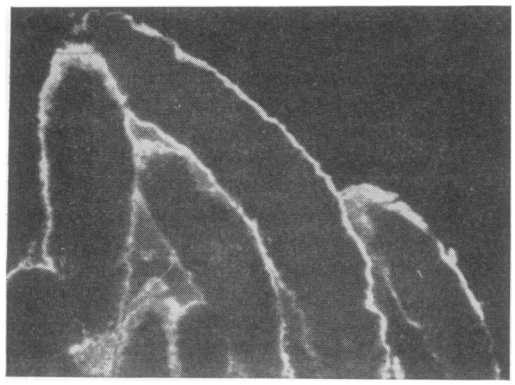

FIG. 4. *Fluorescent K88-positive bacteria of strain W1 on the surfaces of villi. Tissue from the anterior small intestine of an infected, conventionally reared piglet; section stained by the FA technique with K88 antiserum (×150).*

Virulence of the K88-negative strain in conventionally reared piglets. In contrast to the virulence of the K88-positive strain, only 1 of 32 piglets died after infection with the K88-negative strain (Table 4). Diarrhea was less severe and the piglets gained more weight, compared with the piglets infected with the parent W1 strain. On the basis of rectal swabs, the K88-negative strain persisted longer in the intestinal tract, compared with the K88-positive strain. In three of four litters, the K88-negative strain was not detected at necropsy 7 days after infection. However, in the litter in which the death of one piglet was attributed to the K88-negative strain, the organism was recovered in almost pure cultures from the anterior small intestine of three of seven surviving piglets. These three piglets had moderately severe diarrhea until slaughter at 7 days, but gained as much weight as their apparently healthy litter mates. The FA technique showed that the K88-negative strain was detectable only in the terminal small intestine of some piglets (Fig. 5), and that the strain showed no evidence of adhesion to the mucosal surface. Although the K88-negative strain was the predominant organism cultured from the anterior small intestine, there were probably too few organisms in the tissue sections to be detected in the FA technique. At least 10^6 organisms per g of contents must be present for adequate staining results.

DISCUSSION

The growth of pathogenic bacteria in vitro may lead to the acquisition of characters that are not present in vivo (37), and to support the hypothesis that K88 antigen assists colonization of the piglet intestinal tract by K88-positive strains of *E. coli*, it was necessary to demonstrate synthesis of the antigen by a K88-positive strain in the intestine of piglets. The intestinal environment of gnotobiotic piglets differs considerably from that of conventionally reared piglets. For example, the gut flora are absent, and the animals do not receive maternal antibodies. Despite these differences, gnotobiotic piglets were preferred to study colonization of the intestinal tract by K88-positive and K88-negative strains because: (i) they have defined gut flora, in contrast to early weaned or suckling, conventionally reared piglets; (ii) they are highly susceptible to monocontamination with strains of *E. coli* (25), and therefore it was unnecessary to administer a heavy challenge of bacteria that may be sufficient to populate the intestinal tract without further

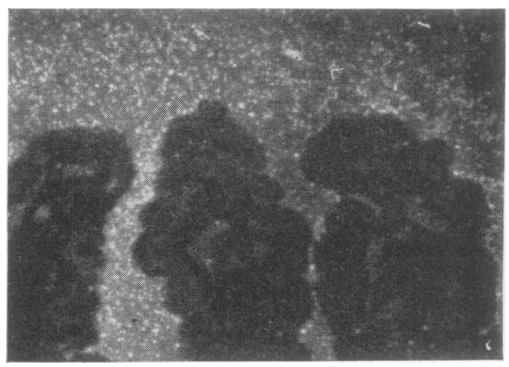

FIG. 5. *Fluorescent K88-negative bacteria of strain W1(J2) in the lumen of the terminal small intestine of an infected, conventionally reared piglet; section stained by the FA technique with W1(J2) OK antiserum (×150).*

proliferation; and (iii) it was possible to compare different strains in litter mates without the risk of cross-infection.

The piglets were infected with a K88-positive strain of *E. coli* and with three K88-negative strains derived from the K88-positive strain. The K88-negative strains were isolated by three different techniques to reduce the possibility that any one selection procedure resulted in the loss of other characters that may influence growth of the organism in vivo. The K88-positive strain synthesized K88 antigen in the piglet intestine, whereas none of the K88-negative strains regained the ability to do so. Synthesis of K88 antigen allowed the K88-positive bacteria to adhere to and colonize the epithelial surface of the small intestine, whereas the K88-negative strains were randomly distributed in the lumen. In contrast, both K88-positive and K88-negative strains colonized the lumen of the large intestine. Further support for the assumption that K88 antigen is responsible for attachment was provided by in vitro adhesion of K88-positive strains but not K88-negative strains to intestinal tissue from gnotobiotic piglets. Cell-free K88 antigen also showed a strong affinity for the mucosal surface of intestinal tissue. It is unlikely that fimbriae were involved in adhesion, as none of the four strains produced mannose-sensitive (fimbrial) hemagglutination.

During the present study, essentially similar in vivo results were reported with early weaned and starved piglets (2, 6). The strains and tissues were not examined for K88 antigen, but it seems likely that the antigen was responsible for adhesion. Although the present results show that K88 antigen is necessary for attachment of K88-positive bacteria to the mucosa of the small intestine, other strains of *E. coli* may possess different adhesive mechanisms. Wild-type K88-negative *E. coli* adhere to the wall of the small intestine (40, 46); however, it appears that K88-negative strains attach less effectively than K88-positive strains after oral dosing (6). In addition, a K88-negative strain attached to the mucosa of both the small and large intestine (12) and thus showed a different tissue specificity compared with K88-positive bacteria. In the present study, K88-negative strains belonging to the serotypes used by previous authors (12, 40) did not attach in vitro to discs of intestinal tissue from gnotobiotic piglets. The most interesting explanation for these results is that the strains produce adhesive factors only in vivo. However, clinical signs may reflect a similar host response rather than the presence of similar virulence factors.

Although adhesion may bring bacteria into closer contact with host defense mechanisms, it would allow organisms to overcome the disadvantages of living in a constantly moving environment (19). The epithelial surface is likely to provide a more stable environment than the lumen of the intestine, and the bacteria would be in close proximity to nutrients transported across the epithelial surface. Furthermore, *E. coli* may be able to utilize mucus. Evidence for the latter suggestion is provided by the survival of porcine enteropathogenic strains of *E. coli* in starved piglets (6, 26), in ligated loop experiments, and by the production of glycosidases by certain strains of *E. coli* (22). The ability of mucus to remove particles from the gut epithelial surface (17) suggests that bacteria must first penetrate the mucus to adhere to epithelial tissue. Although human enteropathogenic strains of *E. coli* possess stronger mucinolytic activity for ovomucin compared with non-enteropathogenic strains (34), a similar pattern of results was not obtained with porcine strains (3). It would now be relevant to use piglet intestinal mucus as the substrate in tests for mucinase activity.

The important contribution of the adhesive properties of K88 antigen to virulence is clearly demonstrated in the present study. The K88-positive strain and the selected K88-negative strain were equally virulent in gnotobiotic piglets. In contrast, the K88-negative strain was virtually nonvirulent in conventionally reared suckling piglets. Recent results with early weaned piglets support this conclusion (42), and also demonstrate that the ability to synthesize enterotoxins contributes to enteropathogenicity. Failure to attach and thus reach high numbers in the anterior small intestine presumably accounts for the low virulence of our K88-negative strain in conventionally reared piglets. Successful colonization of the lumen of the anterior small intestine of gnotobiotic piglets by the K88-negative strain, as judged by the FA technique, may be attributable to reduced motility of the germ-free gut (1) or to colonization of the stomach with constant spillage into the small intestine. The death of these piglets indicates that attachment to the gut epithelium is not a prerequisite for the manifestation of enterotoxin activity. Enterotoxin may have been produced by bacterial growth in the lumen of the anterior small intestine. Alternatively, enterotoxin may have been synthesized by proliferation of bacteria in the stomach and caused a fluid loss while passing through the intestine.

The pattern of colonization of the intestine by K88-positive bacteria is poorly understood. Although previous results (2, 26) indicate that colonization proceeds from initial establishment in the posterior intestine, it is easier to visualize

adhesion of ingested organisms to the intestinal wall as they pass down the gut. Rutter and Anderson (*unpublished results*) found that K88-positive (W1) bacteria given to conventionally reared piglets by stomach tube are detected by viable counts 3 hr after infection only in the terminal ileum, and 6 hr after infection throughout the small intestine. One interpretation of these results is that K88 antigen was present on the surface of ingested bacteria, and immediate adhesion of a small proportion of organisms to the intestinal wall occurred during passage of the inoculum through the small intestine. If subsequent growth occurs as proliferation of microcolonies over the mucosal surface, these will eventually coalesce to form dense sheets of bacteria.

Loss of K88 antigen after ethidium bromide treatment indicates that the gene determining K88 antigen synthesis is extrachromosomal in strain W1 as in other strains (30). The temperature dependence of K88 antigen synthesis is consistent with the proposal that phenotypical modifications may result from an episomal gene alternating between two states (23). The consequences of this proposal in the life cycle of pathogenic bacteria are particularly interesting. Environmental pressures may have selected for retention of the K88 gene in a flexible form that allows enteropathogenic K88-positive strains of *E. coli* to survive not only in the intestinal tract by producing K88 antigen, but also outside the host by repressing antigen synthesis to maintain cell economy. It would now be of interest to determine whether other virulence factors have similar adaptive properties.

There are now many reports of the adhesion of bacteria to intestinal tissue, and recently it has been found that *Clostridium welchii* type C attaches to the epithelial surface of the small intestine in diseased piglets (4). Thus, bacteria that are responsible for enteric disease may colonize the small intestine in a manner similar to K88-positive strains. If mechanisms analogous to the K88 antigen exist, they should be characteristic of a particular enteropathogenic group. Therefore, it is of considerable interest that a common antigen has recently been detected in strains of *E. coli* enteropathogenic for either calves (43) or human infants (7). A better understanding of the associations of enteropathogenic bacteria with mucous membranes is fundamental to our knowledge of the pathogenesis and control of enteric disease. Inhibition of in vitro attachment by antisera suggests that specific antibodies may neutralize adhesion of K88 antigen to receptors in the pig gut. If similar mechanisms occur in other enteric diseases, this could lead to the development of effective vaccines.

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