A Comparison of Histopathological Changes in Calves Associated with K99$^-$ and K99$^+$ Strains of Enterotoxigenic *Escherichia coli*

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ABSTRACT

Enterotoxigenic colibacillosis was experimentally produced in four colostrum-deprived calves given 10^{10} *Escherichia coli* strain 210 (serotype 09+:K30+:K99$^-$:F41$^-$:H$^-$) orally and the histopathological changes compared to those seen in colostrum-fed calves infected in an earlier study with strain B44 (serotype 09+:K30+:K99+:F41+:H$^-$). *Escherichia coli* strain 210 caused diarrhea, atrophic villi with cuboidal epithelium, and focal accumulations of a few neutrophils in the dome villi above Peyer's patches but neither the clinical nor the histopathological changes were as pronounced as with strain B44. The extent and distribution of adherence to the mucosal surface differed between the two strains. Strain B44 adhered as a continuous layer over most of the absorptive epithelial surface of both the jejunum and ileum. Adherence of strain 210 was restricted to the ileum and the bacteria often adhered focally in "clumps" rather than as a continuous layer, especially on the distal half of the villous surface.

Key Words: Enterotoxigenic *E. coli*, histopathology, neonatal calves, bacterial adherence.

RÉSUMÉ

Cette expérience visait à produire une colibacillose entérotoxinogène, chez quatre veaux privés de colostrum; on leur administra à cette fin, par la voie buccale, 10^{10} de la souche 210 d'*Escherichia coli*, laquelle correspond au sérotype 09$^+$: K30$^+$: K99$^-$: F41$^-$: H$^-$. On compara ensuite les lésions microscopiques ainsi provoquées à celles qu'on avait déjà enregistrées chez des veaux également privés de colostrum et qu'on avait infectés, au cours d'une expérience antérieure, avec la souche B44 qui correspond au sérotype 09$^+$: K30$^+$: K99$^+$: F41$^+$: H$^-$. La souche 210 causa de la diarrhée, de l'atrophie des villosités, une métaplasie cubique de leur épithélium et l'accumulation de quelques neutrophiles, au sommet de certaines des villosités qui surplombaient les plaques de Peyer; ni les signes cliniques, ni les lésions microscopiques, n'égalèrent cependant ceux que provoqua la souche B44. L'étendue et la distribution de l'adhésion à la muqueuse, propre à chacune des deux souches, affichèrent des différences. La souche B44 adhéra sous la forme d'une couche continue, au-dessus de la plupart des cellules absorbantes du jéjunum et de l'iléon. L'adhésion de la souche 210 se limita à l'iléon et les colibacilles adhéraient en paquets, seulement ici et là, plutôt que sous la forme d'une couche continue, surtout sur la moitié distale de la surface des villosités.

Mots clés: *Escherichia coli* entérotoxinogènes, histopathologie, veaux nouveau-nés, adhésion bactérienne.

INTRODUCTION

Enterotoxigenic colibacillosis is a common enteric problem in calves under five days of age. The pathogenesis of this disease involves colonization of the small intestine by strains of enterotoxigenic *Escherichia coli* (ETEC) resulting in acute, profuse, watery diarrhea which is often fatal. An initial step in the colonization process appears to be attachment of the ETEC to the intestinal epithelium followed by the formation of microcolonies which cover the villi (1). Enterotoxigenic *E. coli* isolated from a variety of animal species, as well as humans, have been shown to possess pili which are the mechanism of attachment. The first pilus identified on bovine strains of ETEC was the K99 antigen (2).

Recently, other attachment factors have been found on bovine ETEC. Morris *et al* demonstrated F41 pili, which often occurs together with K99 pili on ETEC which also have 09 or 0101 somatic antigens (3, 4). This pilus has recently been biochemically purified (5). In addition, Girardeau and co-workers identified two other pili, called (F)Y and F31a on bovine isolates and suggested that they may be involved in coloniza-

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tion (6, Girardeau, J.P., H.C. Dubourguier and M. Contrepois. 1979. Attachment des *E. coli* entéropathogènes à la muqueuse intestinale. In gastro-entérites néonatales du veau, Société Française de Buiatrie. pp. 53-66). Other workers have compared the ability of strains of ETEC which possess different combinations of somatic, capsular and pilus antigens to colonize the small intestine (7, 8, 9). While the strains used in these studies were not examined for the presence of the recently discovered pili, maximum colonization in calves was seen with K99+, encapsulated strains, and was reduced when K99- or acapsular mutants were used. We recently reported the histopathological changes in the small intestine during natural and experimental infections of calves with ETEC strain B44 (09:K30+:K99+:F41+:H-). These included: 1) stunted jejunal and ileal villi covered by cuboidal epithelium, 2) layers of Gram negative bacteria adherent to the infected mucosa and 3) focal accumulations of neutrophils above the dome areas of Peyer's patches (10). The present study was done to determine the histopathological changes seen in colostrum deprived calves infected with an encapsulated strain of ETEC which lacks K99 and F41 pili, and to compare the lesions to those seen in colostrum-fed calves infected with strain B44.

MATERIALS AND METHODS

STRAIN OF *E. COLI*

A strain of *E. coli* with the serotype 09+:K30+:K99$^-$: F41$^-$:H$^-$ (VIDO strain 210), which produced heat stable enterotoxin as detected by the infant mouse assay, was supplied by Dr. H.W. Smith, Houghton Poultry Research Station. Strain 210 is a laboratory derivative of an 09:K30:K99 calf-enteropathogenic strain, believed to be reference strain B44, previously designated as the 0+K+99$^-$ variant by Smith, and obtained by the methods previously outlined (7). When grown on Minca agar containing 1% isovitalex (Minca-IS, BBL, Cockeysville, Maryland) this strain did not agglutinate in standard K99, K88 or 987P antiserum but did agglutinate in K30 antiserum. In addition, this strain did not possess F41 pili as determined by immunofluorescent staining of cell cultures (personal communication, J.A. Morris). Other pili, as yet unidentified, were detected on strain 210 by electron microscopy (11).

PREPARATION OF ANTISERA

The K99 antiserum was prepared by inoculating an adult New Zealand white rabbit with purified K99 antigen (supplied by Dr. R.E. Isaacson, Department of Epidemiology, University of Michigan, Ann Arbor, Michigan) and had an agglutinating titer of 1/2048 when tested against K99 reference strain B41 (0101:K99). The K30 antiserum was prepared as described previously (1) and had an agglutinating titer of 1/64 against strain 210 grown on blood agar. Antiserum against 987P was also provided by Dr. R.E. Isaacson, and that against K88 was obtained from the Diagnostic Laboratory, Department of Veterinary Microbiology, Western College of Veterinary Medicine.

INOCULATION OF CALVES

Three newborn Holstein bull calves were removed from their dams immediately after birth and prior to nursing. They were placed in individual isolation rooms and were challenge exposed orally by inoculating a trypticase soy broth culture of *E. coli* strain 210 into the back of the mouth using a 20 mL syringe as outlined in Table I. Immediately following challenge inoculation, each calf was allowed to suck 1-2 L of homogenized milk from a nipple bottle. A fourth calf (calf 80-52), also deprived of colostrum, was challenge inoculated in a similar manner with 2.0×10^{10} viable *E. coli* when three hours old. Immediately after inoculation the calf was anesthetized using halothane (Fluothane, Ayerst Laboratories, Montreal), a laparotomy was performed in the left paralumbar fossa using sterile surgical technique, and a biopsy (approximately 0.5×1.0 cm in size) of the ileal wall was taken from a location approximately 30 cm anterior to the ileocecal junction. For additional samples, taken at 2, 4, 6, 8, 10, 12 and 18 h after challenge inoculation, the location of each biopsy was 10 to 15 cm anterior to the previous site. During the time between biopsies the surgical incision was closed and the calf allowed to regain consciousness. The calf died 18 h after inoculation at the time the last biopsy was taken.

To ensure that the broth cultures used for challenge were K99-, they were tested for agglutination with standard K99 antiserum and were also streaked onto Minca-IS immediately before inoculating the calves. Following incubation at 37°C for 16 h ten isolated colonies were picked from the Minca-IS plates and tested for agglutination with K99 antiserum.

COLLECTION OF SAMPLES

Each calf, except 80-52, was fed 2 L of homogenized milk twice a day and was examined at regular intervals following challenge inoculation for the presence of diarrhea. Following the onset of diarrhea, calves were euthanatized by an intravenous injection of sodium pentobarbitol (Euthanyl, MTC Pharmaceuticals, Hamilton, Ontario) and tissue sections were taken from five equally spaced sites in the small intestine and preserved in 10% neutral buffered formalin. The first site was located approximately 90 cm distal to the pylorus and the last site (section 5) was the same distance anterior to the ileocecal junction. The fixed tissues were dehydrated, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H & E). Tissue sections from the same locations were also stored at -70°C for fluorescent antibody staining with K99, K88 and 987P antiserum by the method described previously (11). Fecal samples and ileal contents were also collected at the time of euthanasia from calves 80-40 and 80-42

and serial tenfold dilutions of each sample were made in phosphate buffered saline (0.1 M, pH 7.2) and plated onto MacConkey's agar to determine the number of viable *E. coli*, and onto Minca-IS for detection of K99 antigen. Ten colonies from each sample were picked from MacConkey's agar or Minca-IS and tested for agglutination with K30 or K99 antiserum respectively. Ileal contents were also examined for the presence of bovine rotavirus using an enzyme-linked immunosorbent assay (ELISA).

RESULTS

CLINICAL AND NECROPSY FINDINGS

None of the broth cultures used to challenge the calves, nor any of the ten colonies grown from each challenge culture on Minca-IS reacted with K99 antiserum.

Calves 80-40, 80-42 and 80-44 all became diarrheic within 25 h following challenge (Table I); feces were watery, yellow and copious and although diarrhea continued until the time of euthanasia, none of the calves became clinically depressed, dehydrated or anorectic. At necropsy the small intestine appeared similar to those found in colostrum-fed calves challenged with K99+ *E. coli*. Yellow, watery contents were present at all levels of the small intestine and in the cecum and spiral colon, but no other gross abnormalities were noted. The number of viable *E. coli* in the ileal contents ranged from 7.3×10^8 to 1.0×10^9 and were slightly higher in feces (Table I). None of the 20 colonies grown on Minca-IS from ileal contents or feces taken from calves 80-40 or 80-42 agglutinated with K99 antiserum; however, all 20 colonies from each calf reacted with K30 antiserum. Feces and ileal contents of all three calves were negative for the presence of rotavirus.

HISTOPATHOLOGICAL OBSERVATIONS

The morphological changes observed in the small intestine of calves infected with *E. coli* strain 210 are summarized in Table II. Pathological changes were restricted primarily to the ileum with only a few changes noted in the lower jejunum of one calf. Lesions were absent in the duodenum. In general, the intestinal pathological changes seen with

TABLE I. Age at Onset of Diarrhea and occurrence of *E. coli* in Ileal Contents and Feces of Calves Following Oral Inoculation with *E. Coli* Strain 210 (09:K30+:K99⁻:F41⁻:H⁻)

Calf Number	Challenge dose (CFU)[a]	Age at (h)		*E. coli* per mL (CFU)[a]		Number of Colonies Agglutinated[b] K30		K99	
		Challenge	Onset diarrhea	Ileum	Feces	Ileum	Feces	Ileum	Feces
80-40	1.0×10^{10}	3	28	1.0×10^9	2.8×10^9	10	10	0	0
80-42	3.8×10^{10}	3	20	7.3×10^8	2.5×10^9	10	10	0	0
80-44	6.0×10^9	14	31	ND	ND	ND	ND	ND	ND

[a]CFU — colony forming units
[b]Number of colonies out of a total of ten, which agglutinated with K30 or K99 antiserum when grown on MacConkey's agar or Minca-IS respectively

TABLE II. Intestinal Pathological Changes in Calves Inoculated with *E. coli* Strain 210 (09:K30⁺:K99⁻:F41⁻:H⁻)

Calf Number	Age (h) at Necropsy	Villus Atrophy			Cuboidal Absorptive Epithelium			Bacterial Adherence to Mucosa			Focal Epithelial Cell Loss			Focal Neutrophil Emigration		
		D[a]	J	I	D	J	I	D	J	I	D	J	I	D	J	I
80-40	42	-	+	+	-	+	+	-	+	+	-	-	-	-	-	+
80-42	24	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+
80-44	30	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+
80-52	18	ND[b]	-	-	ND	-	-	ND	-	+	ND	-	+	-	-	-

[a]D, J and I represent duodenum, jejunum and ileum respectively
[b]ND represents not done

TABLE III. Comparison of the Frequency of Intestinal Pathological Changes Between Calves Infected With a K99⁺ *E. coli* (Strain B44) and Those Infected With A K99⁻ Strain (210)

Serotype of *E. Coli*	Villus Atrophy			Cuboidal Absorptive Epithelium			Bacterial Adherence to Mucosa			Focal Epithelial Cell Loss			Focal Neutrophil Emigration		
	D[a]	J	I	D	J	I	D	J	I	D	J	I	D	J	I
09⁺:K30⁺:K99⁺:F41⁺:H⁻	0/8	7/8	8/8	0/8	7/8	4/8	0/8	6/8	8/8	0/8	6/8	6/8	0/8	6/8	8/8
09⁺:K30⁺:K99⁻:F41⁻:H⁻	0/4	1/4	3/4	0/4	1/4	3/4	0/4	1/4	3/4	0/4	0/4	1/4	0/4	0/4	3/4

[a]D, J and I represent duodenum, jejunum and ileum respectively

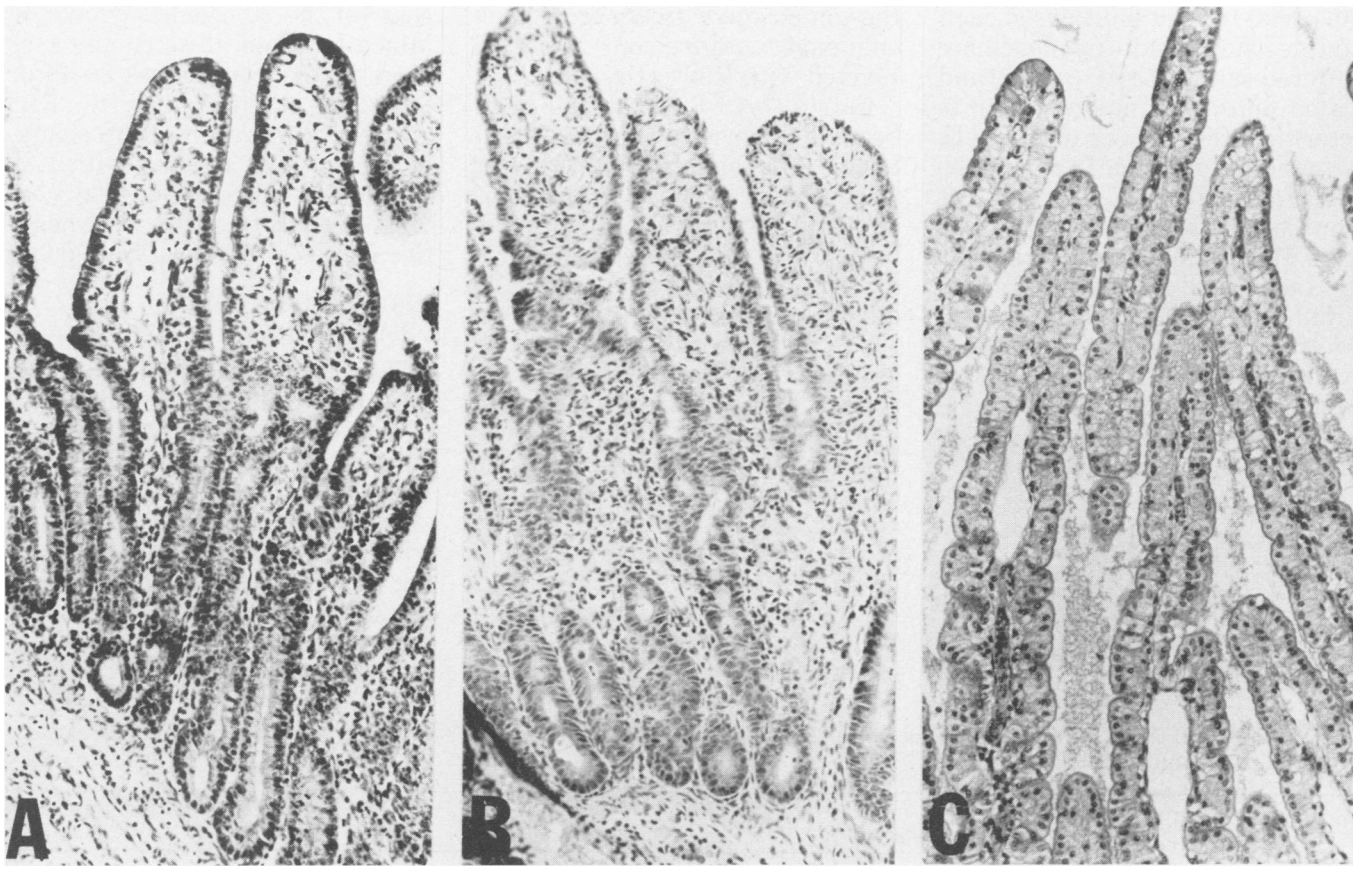

Fig. 1. Ileum of calves infected with a K99$^-$ *E. coli* strain 210 (A), a K99+ *E. coli* strain B44 (B) and a control calf (C). The villi of both infected calves show a similar degree of atrophy compared to the control. With strain 210 the stunted ileal villi were covered by an intact layer of cuboidal epithelium with basal nuclei (A) compared to the control with columnar epithelium and apically situated nuclei (C). With strain B44 (B) cuboidal epithelium was evident in both the ileum and jejunum and focal areas of epithelial loss were observed on some villus tips (H & E stain).

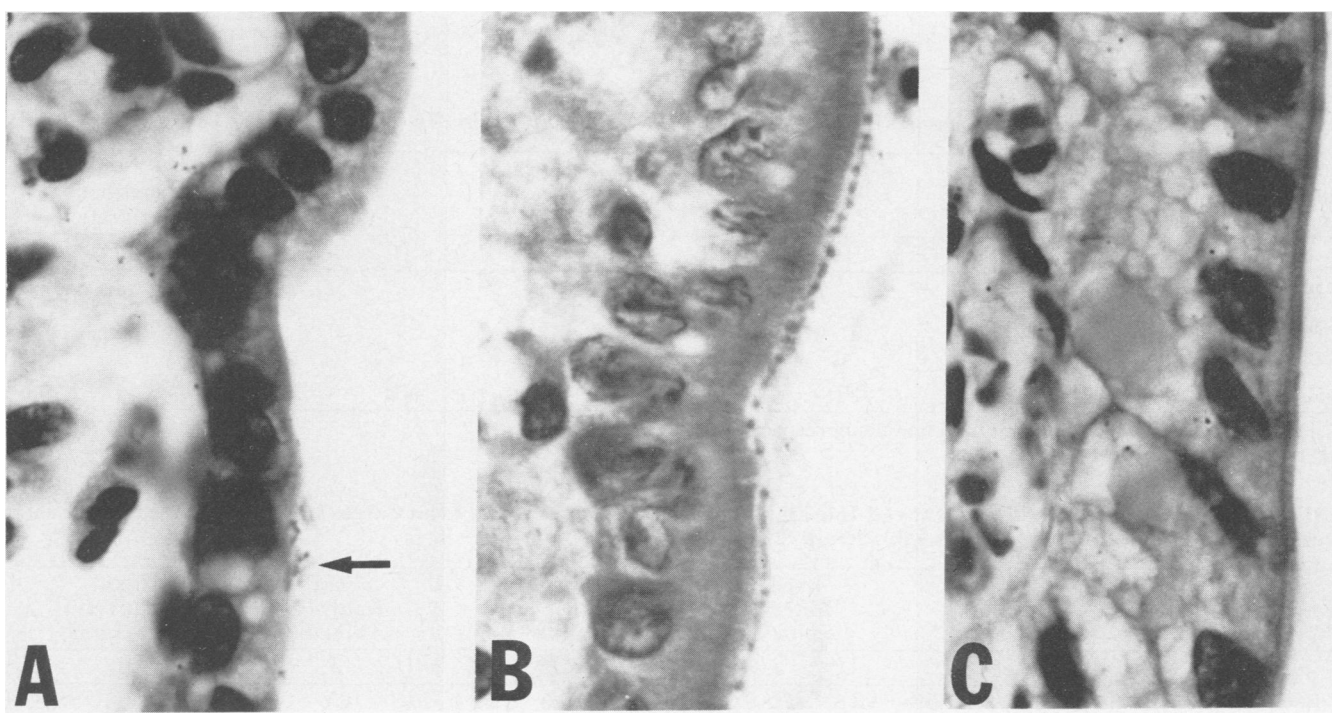

Fig. 2. Ileum of calves infected with *E. coli* strain 210 (A), *E. coli* strain B44 (B) and a control calf (C). Bacterial adherence was often focal (arrow) with strain 210 (A) compared to a continuous layer of adherent bacteria with strain B44 (B) (H & E stain).

strain 210 were less extensive, and observed less frequently than were the changes seen in colostrum-fed calves infected with strain B44 (10) (Table III). Villous atrophy occurred to a similar degree with both strains (Fig. 1) but strain B44 affected a larger segment of the small intestine. The epithelium covering the stunted villi was cuboidal rather than columnar with both strains, but the focal degeneration and exfoliation of absorptive epithelial cells at the tips of jejunal and ileal villi in calves infected with strain B44 was not observed in the group infected with strain 210. Ileal epithelial nuclei were invariably situated basally in infected calves in contrast to control calves of the same age in which the nuclei often occupied an apical position in the cell.

Both strains of *E. coli* adhered to the epithelial surface of the small intestine but the extent and distribution of adherence differed between the two (Fig. 2). Strain B44 adhered as a continuous layer, often several cells thick, over much of the absorptive epithelial surface of both the jejunum and ileum. Adherence of strain 210 was limited mainly to the ileum and the bacteria usually adhered in focal areas as "clumps" of bacteria rather than as a continuous layer. This focal bacterial adherence was especially prominent on the distal half of the villous surface (Fig. 2A) whereas on the proximal portion of villous epithelium, near the crypt openings, the bacteria often adhered as one continuous layer (Fig. 3). When tissue sections from the ileum of calves infected with strain 210 were stained with anti-K30 antiserum a sparse layer of fluorescent bacteria was seen adhering to the villous surface (Fig. 4A). This was in contrast to the continuous layers of bacteria several cells thick which were seen in sections from calves infected with strain B44 and stained with K99 antiserum (Fig. 4B). No fluorescence was seen in sections from

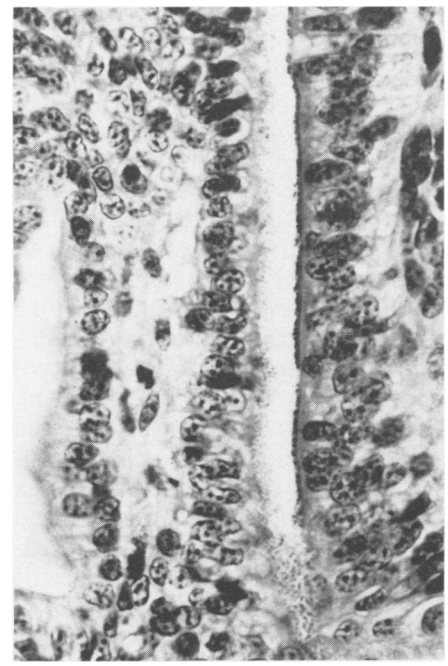

Fig. 3. Ileum of a calf infected with *E. coli* strain 210 showing the continuous layer of bacteria adherent to the proximal villus epithelial surface near crypt openings compared to the focal adherence seen on the villus tips of the same calf (Fig. 2A) (H & E stain).

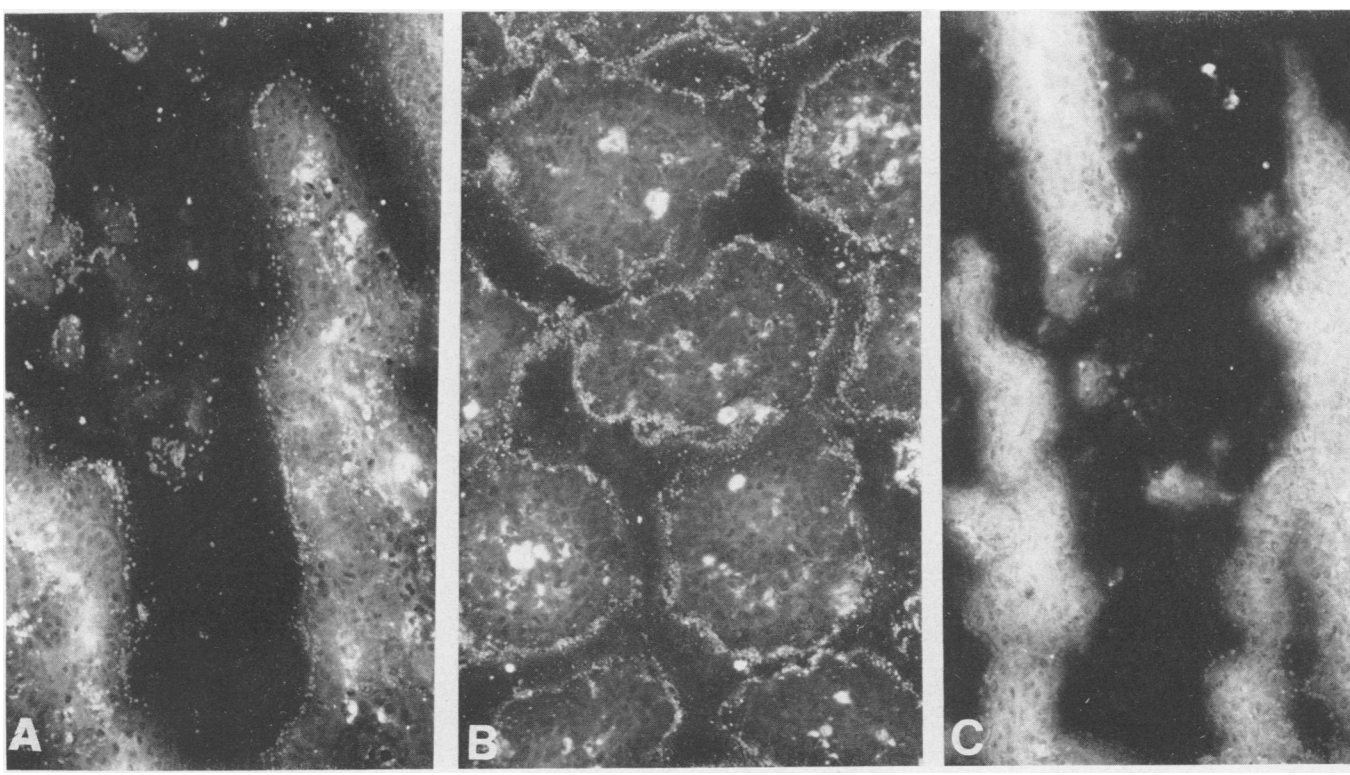

Fig. 4. Ileum of calves infected with either *E. coli* strain 210 or strain B44 and stained with fluorescent antibody. With homologous OK antiserum, *E. coli* strain 210 were seen as an incomplete layer covering the villi (A). Bacterial adherence was much more intense in the ileum of a colostrum fed calf infected with strain B44 and stained with K99 antiserum (B) Continuous layers of bacteria several cells thick were seen on the villi. No fluorescence was seen when ileal sections from calves infected with strain 210 were stained with K99 (C), K88 or 987P antisera (not shown).

calves infected with strain 210 and stained with K99 (Fig. 4C), K88 or 987P antisera (not shown).

Increased neutrophil emigration through the "dome" villi above Peyer's patches occurred in the calves infected with strain 210 (Fig. 5A) but it was not nearly as prominent as in calves infected with strain B44 (Fig. 5B).

DISCUSSION

The results indicated that *E. coli* strain 210, which lacks the known attachment factors but which possesses another unidentified pilus, can cause diarrhea and pathological changes in colostrum-deprived calves. Attachment of cells of strain 210 to the epithelial surface was focal on the distal villous surface and limited mainly to the ileum. This distribution of attachment differed from that seen with strain B44 which adhered as a more continuous layer throughout the jejunum and ileum (8, 9, 10). Previous studies by other workers suggested that the maximum degree of colonization of the small intestine in calves is achieved by strains of ETEC which possess both K99 antigen and capsule (7, 8, 9). However the strains used in these earlier studies were not examined for the presence of F41 antigen or other potential attachment pili and hence the relative contribution of the pili and the capsule to the colonization process remains unclear. The mechanism by which strain 210 attaches is unknown and could involve the unidentified pilus or the K30 antigen; however, further studies will be required to determine this.

It is tempting to try to relate the difference in the amount of adherence between strains 210 and B44 to the differences observed in the clinical disease and the intestinal pathological changes caused by the respective strains. Calves infected with the extensively adherent strain B44 developed severe diarrhea in six to eight hours and soon became severely dehydrated and weak. Calves infected with the focally adherent strain 210 developed diarrhea later after inoculation and did not become severely dehydrated or depressed even though they were deprived of colostrum. Similarly, although both strains elicited similar pathological changes including villus atrophy, cuboidal absorptive epithelium in the distal small intestine, and focal neutrophil emigration through dome villi above Peyer's patches, the changes were quantitatively much more extensive with strain B44. Although this correlation between degree of bacterial adherence and mucosal pathological changes seems real it is difficult to give an adequate explanation for the differences.

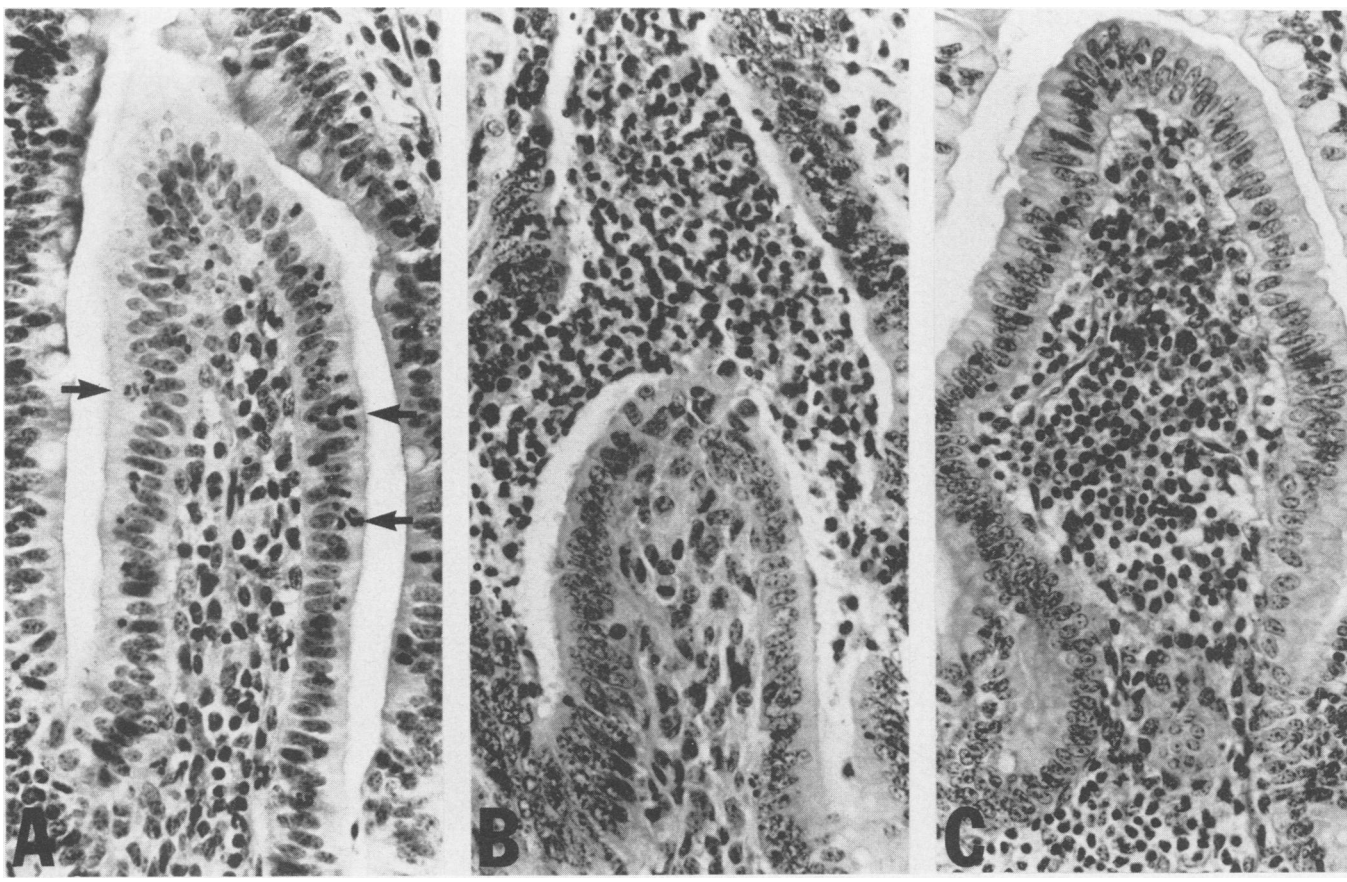

Fig. 5. Ileum of calves infected with *E. coli* strain 210 (A), *E. coli* strain B44 (B) and a control calf (C). With B44 large numbers of neutrophils emigrated through the dome villi above Peyer's patches into the lumen (B) whereas with strain 210 only a few focal areas of neutrophils (arrows) were seen in dome villi (A) and uninfected calves had no neutrophils (C) (H & E stain).

The colostrum-deprived calves infected with strain 210 were compared to colostrum-fed calves infected in an earlier study with strain B44 (10). The conclusions therefore are based on the assumption that the time difference and the colostrum had no appreciable effect on the different enteric histopathological changes found in the two groups. Attempts to infect colostrum-fed calves with strain 210 were unsuccessful. Although neutrophil emigration into the intestinal lumen of pigs can be influenced by their immune status (12) and the presence of colostrum in the calves infected with strain B44 may have, in a similar way, had some effect on the large numbers of neutrophils seen in that group, this seems unlikely because the colostrum fed to the calves had no detectable agglutinating activity for the B44 strain (10).

Focal epithelial cell loss from the tips of villi observed with strain B44 (10) was not a prominent feature of the intestine infected with strain 210. The debate about whether focal epithelial cell loss from the villous tip in calves infected with strains of ETEC is a "lesion" (10) or an "artefact" (8, 13, 14) is worthy of comment. Pearson and Logan showed that intestinal samples collected from an anaesthetized calf infected with *E. coli* and rotavirus had an intact epithelial surface whereas samples from the same calf collected five to seven minutes after death had loss of epithelium from some villus tips (14). Similarly, Hadad and Gyles found that intestinal samples collected from an anaesthetized calf infected with a K99$^+$ *E. coli* had an intact epithelial surface whereas samples collected 15 minutes after death showed loss of epithelium from the tips of villi (8). Pearson and Logan, however, also showed that uninfected control calves did not show the loss of epithelial cells from villus tips if samples were taken five to seven minutes after death (14). Similarly, we found that the epithelial loss seen in calves infected with K99$^+$ *E. coli* did not occur in control calves in which samples were collected from the same sites and at the same time intervals after death (10). What is important in this set of observations is not whether the epithelial cell loss is referred to as an "artefact" or a "lesion" but rather that there are differences between the infected and uninfected animals that could relate to the pathogenesis of the disease. The fact that this change occurs in infected animals at five minutes postmortem and not in controls strongly implies that the bacteria and/or their products are exerting some deleterious effect on epithelial cells. Although there may not be denuded villi *in vivo* there has been some change in the epithelium of infected calves that results in the very rapid exfoliation that is not present in uninfected animals. Certainly, the other changes noted in infected calves, including villus atrophy, cuboidal absorptive epithelium, replacement of vacuolated epithelial cells with apical nuclei by nonvacuolated epithelial cells with basal nuclei, all, in the absence of obvious crypt injury, imply an increased rate of epithelial exfoliation in infected calves and lend further support to the notion that the bacteria have a degenerative effect on the absorptive epithelial surface.

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