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Diarrheal Disease Caused by Enterotoxigenic *Bacteroides fragilis* in Infant Rabbits†

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Enterotoxigenic *Bacteroides fragilis* caused severe, nonhemorrhagic, watery diarrhea when 10^9 CFU of a porcine or human isolate was administered orogastrically to 3-day-old rabbits. The bacterium colonized the intestinal tract with a predilection for the large intestine (10^9 CFU/g of cecal contents). Diarrhea occurred at an average of 4.6 days postinoculation, and 84% of rabbits were dead or moribund at an average of 8.8 days postinoculation. The disease was characterized by watery diarrhea and dehydration. Severe histologic lesions including inflammation, exfoliation of epithelial cells, and crypt hyperplasia were observed throughout the colon. There was no indication of bacteremia or of bacterial adherence to or invasion of intestinal epithelial cells. Rabbits inoculated with nonenterotoxigenic *B. fragilis* were colonized with *B. fragilis* but did not develop clinical disease or intestinal lesions. While the pathogenesis of this disease is undefined, clinical signs of disease and histologic changes were consistent with a mechanism of net secretion of fluid into the small intestine and decreased absorption of fluid from the large intestine. Enteric disease caused by enterotoxigenic *B. fragilis* in infant rabbits was similar to naturally occurring enteric disease associated with the bacterium in humans and livestock. This study established that enterotoxigenic *B. fragilis* is enteropathogenic in intact infant rabbits.

Some intestinal isolates of the obligately anaerobic bacterium *Bacteroides fragilis* are enterotoxigenic, as indicated by their ability to induce fluid accumulation in the lamb or calf ligated ileal loop test (9, 12). Enterotoxigenic and nonenterotoxigenic isolates colonize the intestinal tract in diarrheic humans and livestock (calves, lambs, pigs, foals) (9–13). Enterotoxigenic isolates of *B. fragilis* caused severe enteric disease when inoculated into the ileum of the adult rabbit with ligated cecum (10, 11, 13) and when given orally to gnotobiotic pigs (J. R. Duimstra, J. E. Collins, L. L. Myers, and D. A. Benfield, Proceedings of the Conference of Research Workers in Animal Disease, abstr. no. 285, p. 50, 1986); nonenterotoxigenic isolates were avirulent in both systems.

In a preliminary study (L. L. Myers, and D. S. Shoop, Proceedings of the Conference of Research Workers in Animal Disease, abstr. no. 98, p. 17, 1987), enterotoxigenic *B. fragilis* caused enteric disease in infant rabbits. Further studies of the virulence of the bacterium in the intact infant rabbit are reported herein.

MATERIALS AND METHODS

Bacterial cultures. Two isolates of enterotoxigenic *B. fragilis* (2-078382-3 [ATCC 43858] and 86-5443-2-2) and two isolates of nonenterotoxigenic *B. fragilis* (077225-2 and 3-109-4) were used. Isolates 077225-2 and 2-078382-3 were obtained from the feces of diarrheic humans (13); the other two isolates were obtained from the feces of diarrheic pigs (10). Both toxigenic isolates were virulent when inoculated into the ilea of adult rabbits with ligated ceca (10, 13). In addition, infant rabbits in this study were inoculated with fecal isolates (17-2, 17-4, and 17-6) of enterotoxigenic *B. fragilis* from diarrheic rabbits designated 2, 4, and 6 in the litter designated 17 (inoculated with isolate 2-078382-3; Table 1). Cultures were stored aerobically (in screw-capped tubes) at room temperature for approximately 1 month on tryptose blood agar (Difco Laboratories, Detroit, Mich.) slants supplemented with 5% defibrinated bovine blood. For long-term storage, isolates were held at −70°C in heat-sealed glass culture tubes (6 by 50 mm) containing defibrinated bovine blood.

Inoculation of infant rabbits. Litters of 2-day-old New Zealand White rabbits were obtained from a local rabbitry (Janet Hanson, Livingston, Mont.). Each doe and her litter were kept together in separate cages throughout the study. Does had raised several litters prior to use in the study. Three-day-old rabbits were inoculated orogastrically with 1 ml of a 24-h brain heart infusion broth (Difco Laboratories) culture containing about 5×10^9 CFU of *B. fragilis*. A French feeding tube (size 3 1/2; Monoject, St. Louis, Mo.) was used for inoculation. A total of four rabbits were not inoculated in litters (1, 6, and 22) in which littermates were inoculated with toxigenic *B. fragilis*. Rabbits were observed for diarrhea, and those in litters 17, 18, and 20 through 22 were weighed daily. They were considered diarrheic if the perianal area, base of tail, and upper hind legs were contaminated with feces. Diarrhea was considered severe if the same areas were moist with feces.

Isolation of *B. fragilis* from rabbits. Fecal specimens were collected prior to challenge and periodically thereafter with a small rectal swab (cotton placed in the end of the disposable tip of a micropipette) moistened with brain heart infusion broth. Specimens were streaked onto a solid selective medium (PINN medium; 1), and the plates were incubated anaerobically (GasPak Anaerobe System; BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C. Individual colonies with the characteristic internal mottled appearance of *B. fragilis* were restreaked onto plates of tryptose blood agar plus 5% bovine blood and again were grown for 48 h.

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TABLE 1. Virulence of enterotoxigenic *B. fragilis* in infant rabbits

Litter no./ total no. of rabbits	Challenge isolate	No. with diarrhea	No. that died or were moribund	Avg time (days) from inoculation to death or the moribund state
1/5	2-078382-3	5	5	9.8
3/5	86-5443-2-2	4	4	5.2
6/4	86-5443-2-2	4	4	7.2
17/8	2-078382-3	8	4	9.1
20/5	17-4	5	5	12.1
21/6	17-2	6	6	6.3
22/4	17-6	4	3	11.0

Isolates were identified as *B. fragilis* by using appropriate biochemical tests (13).

At necropsy, colonization of the intestinal tract by *B. fragilis* was quantified in six rabbits. Sections (2 to 4 cm) of intestinal tissue were excised from the duodenum (2 cm from stomach), ileum (2 cm from ileocecal junction), proximal colon (immediately distal of ileocecal junction), and distal colon (5 cm from anus). Excised tissue was finely minced with scissors into 10 ml of isotonic saline solution in a 50-ml screw-cap tube, glass beads were added, and the suspension was vortexed vigorously for 20 s. Appropriate dilutions of the resulting homogenate were made in isotonic saline solution. The diluted material (0.1 ml) was spread onto each of two plates (15 by 100 mm) of PINN medium and was incubated anaerobically for 48 h at 37°C. Colonies with the characteristic mottled appearance of *B. fragilis* (usually all of the colonies) were counted and recorded as CFU of *B. fragilis* per centimeter of tissue. Approximately 0.1 g of cecal fluid was placed in a preweighed tube, the tube was again weighed, and 10 ml of isotonic saline solution was added. The mixture was vortexed rapidly for 5 s, and viable counts were determined on appropriate dilutions as described for tissue sections. Viable counts were recorded as CFU of *B. fragilis* per gram of cecal fluid. Heart blood (0.1 ml) was cultured for *B. fragilis* by direct plating on tryptose blood agar plus 5% bovine blood.

Gene fingerprinting analysis of *B. fragilis* isolates. The fingerprinting procedure was used to show that rabbits were colonized with the challenge isolates of *B. fragilis*. The two toxigenic challenge isolates (2-078382-3 and 86-5443-2-2) and four fecal isolates collected at various times after challenge from rabbits inoculated with the two toxigenic isolates were analyzed for DNA relatedness. In addition, a third isolate (porcine isolate 3-101-5) of toxigenic *B. fragilis* used for oral inoculation of gnotobiotic pigs in other studies and two fecal isolates from inoculated pigs were analyzed to further evaluate the usefulness of the test. Bacteria were grown anaerobically for 48 h on Columbia agar plates supplemented with 5% defibrinated equine blood. An inoculating loop (3 mm) of bacteria was removed from each plate and used for extraction of DNA. Extraction of plasmid and chromosomal DNA for gene fingerprinting analysis was done with a commercially available assay kit (Globex Biotechnologies Incorporated, Toronto, Ontario, Canada). Restriction enzyme digests were prepared using *Cfo*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as recommended by the manufacturer. The DNA fragments were separated by electrophoresis (30 V, 16 h) in 0.7% agarose gels (2). The nucleic acid molecular weight standard used was a 1-kilobase ladder (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.).

Histologic examination. Rabbits in two litters (14 and 15) were inoculated with *B. fragilis* isolate 86-5443-2-2 (six rabbits) or isolate 3-109-4 (three rabbits) and were euthanized (T-61 Euthanasia Solution; Taylor Pharmacal Co., Decatur, Ill.) on various days postinoculation (p.i.). In addition, selected diarrheic rabbits (from a total of eight) from litters 20, 21, and 22 (Table 1) were euthanized when moribund. Four 2-cm sections of intestinal tissue adjacent to areas described for culture of *B. fragilis* were excised, ligated at one end, partially filled with 10% neutral-buffered Formalin solution, ligated at the other end, and immersed in the Formalin fixative. Other tissues collected and fixed were heart, lung, liver, spleen, kidney, and stomach. Formalin-fixed tissues were embedded in paraffin, divided into sections 4 μm thick, and stained with hematoxylin and eosin by standard histologic techniques (17). Selected sections of colon were stained with periodic acid-Schiff reagent. Sections were examined by light microscopy.

RESULTS

A total of 31 of 37 rabbits (representing seven litters) inoculated with enterotoxigenic *B. fragilis* developed watery, nonhemorrhagic diarrhea and died or were moribund (and euthanized) by day 14 p.i. (Table 1). Three of four uninoculated rabbits also developed fatal diarrheal disease clinically similar to disease in littermates inoculated with toxigenic *B. fragilis*. Rabbits inoculated with a human or porcine isolate of the bacterium had similar clinical signs. Diarrhea occurred 2 to 12 days (average, 4.6 days) p.i., and rabbits died or were moribund on an average of 8.8 days (range, 3 to 14 days) p.i. The disease was characterized by watery diarrhea, dehydration, rough hair coat, and weak-

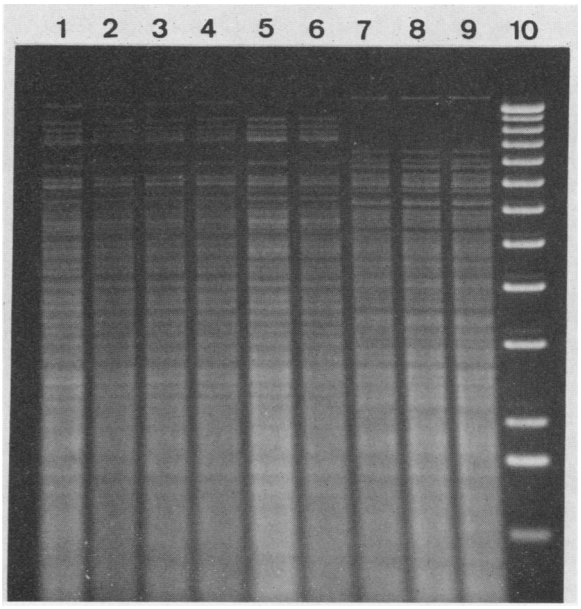

FIG. 1. Agarose gel (0.7%) electrophoresis of total cellular DNA extracted from isolates of toxigenic *B. fragilis*. The DNA was cleaved with *Cfo*I. Lanes: 1, 2-078382-3; 2 through 4, fecal isolates from rabbits inoculated with 2-078382-3; 5, 86-5443-3-3; 6, fecal isolate from a rabbit inoculated with 86-5443-2-2; 7, 3-101-5; 8 and 9, fecal isolates from pigs inoculated with 3-101-5. Lane 10 was charged with a nucleic acid molecular weight standard. The band at the top represents a 12.2-kilobase fragment, and a 1.0-kilobase fragment is at the bottom.

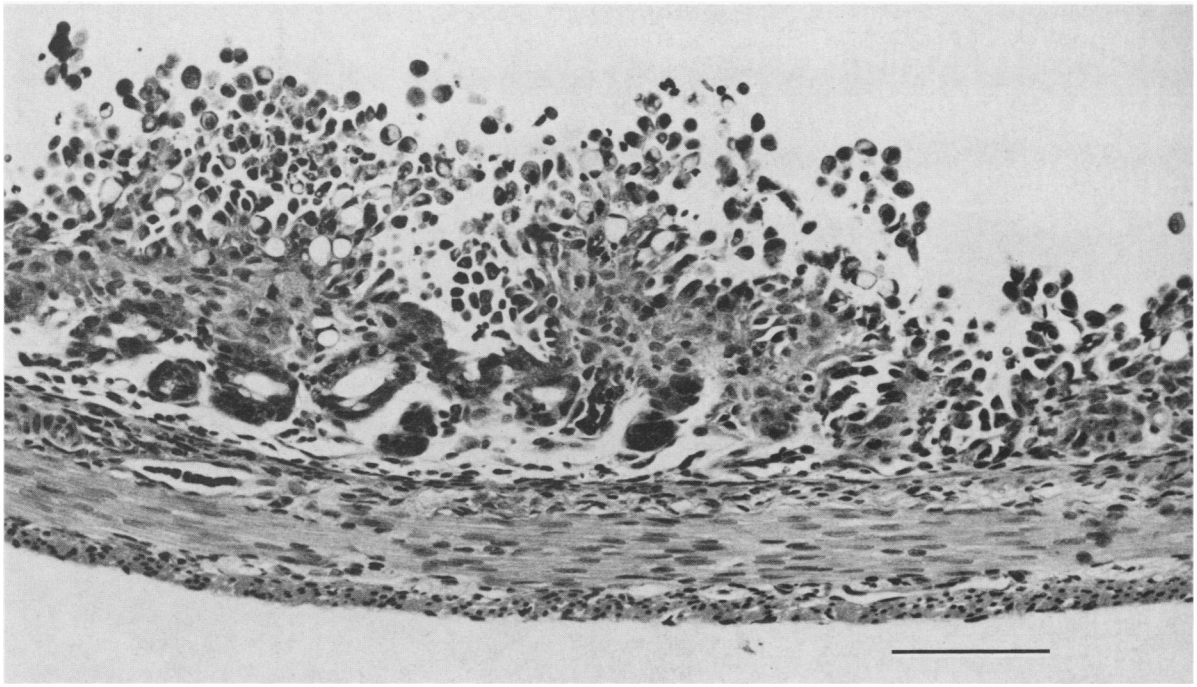

FIG. 2. Mucosa of the distal colon from a diarrheic rabbit (litter 21; rabbit inoculated with enterotoxigenic *B. fragilis*) has severe diffuse detachment of surface epithelial cells. Crypt epithelial cells are less severely affected. Cells were stained with hematoxylin and eosin. Bar, 11.0 μm.

ness. Average daily weight gain of the 18 diarrheic rabbits (litters 17, 20, 21, and 22) was 2.5 g, whereas nondiarrheic rabbits (inoculated with either toxigenic or nontoxigenic *B. fragilis*) gained an average of 10.8 g/day. Of the 18 rabbits with severe diarrhea, 13 lost weight during the 2- to 3-day period prior to death. None of 21 rabbits (from four litters) inoculated with nonenterotoxigenic *B. fragilis* developed diarrhea.

There was marked interlitter variability in the time of occurrence of diarrhea and death relative to the time of inoculation. Average time from inoculation to death or the moribund state varied from 5.2 days for rabbits in litter 3 to 12.2 days in litter 20 (Table 1). Rabbits within litters also often differed by 2 to 5 days in time from onset of diarrhea to death.

Fecal cultures were usually negative for *B. fragilis* prior to inoculation and positive on the day after inoculation. Shedding of *B. fragilis* continued throughout the p.i. observation period. There were 10^2 to 10^5 CFU of *B. fragilis* per cm of small intestine, approximately 10^7 CFU/cm of colon, and 10^9 CFU/g of cecal contents. Viable counts were similar, irrespective of the isolate used for inoculation. *B. fragilis* and other bacteria were not detected in heart blood at necropsy.

By gene fingerprinting analysis, the three isolates of toxigenic *B. fragilis* analyzed were found to be similar but distinguishable from each other electrophoretically in the 8.1- to 12.2-kilobase region (Fig. 1, lane 10, top five bands). In all cases, fecal isolates of *B. fragilis* from rabbits with diarrhea were identical with the corresponding challenge isolate, indicating that the challenge isolate did colonize the intestinal tract.

At necropsy, the cecum and proximal colon were usually at least half full of fluid with some gas, whereas the remainder of the intestinal tract was largely devoid of contents. Grossly, the mucosal surface of the small intestine appeared normal to slightly erythemic, and the large intestine was often erythemic. Mucus was observed throughout the lumen of the large intestine.

Moderate-to-severe lesions including inflammation, exfoliation of epithelial cells, and crypt hyperplasia were seen in the colon in diarrheic rabbits (Fig. 2 through 4) but not in clinically normal rabbits (Fig. 5). Exfoliating cells were seen in the proximal and distal colons in 11 of 14 and 14 of 14 diarrheic rabbits, respectively. Lesions in the two areas were qualitatively similar but usually more severe in the distal colon. Epithelial cells were detached from the surface and sides of mucosal ridges and rarely in the glandular crypts (Fig. 2). The cells were sloughed into the lumen either singly or as small clusters of two to four cells. The luminal surface of epithelial cells in the process of detachment bulged into the intestinal lumen; cells separated from adjacent cells at their lateral borders and, just before detachment, remained attached to the underlying basement membrane by thin cytoplasmic stalks. Detached epithelial cells often contained discrete cytoplasmic vacuoles, in various sizes, which were inconsistently stained by periodic acid-Schiff reagent. Other detached cells were shrunk with deeply basophilic cytoplasm and pyknotic nuclei. Infiltration of heterophils was extensive, especially in the lamina propria of the distal colon, which contained focal aggregates of heterophils and macrophages subjacent to areas of severe epithelial cell detachment. A few heterophils were evenly distributed throughout the lamina propria and were less commonly seen in glandular crypts or in the lumen of the colon. Mucus, epithelial cells, erythrocytes, and debris were seen in the lumen of the colon. Lesions in the small intestine of diarrheic rabbits were minimal. Mild villous atrophy was observed in the ileum in 2 of 14 diarrheic rabbits and was not seen in the three control rabbits. Lesions were not observed in lung, kidney, liver, spleen, stomach, or heart. There was no

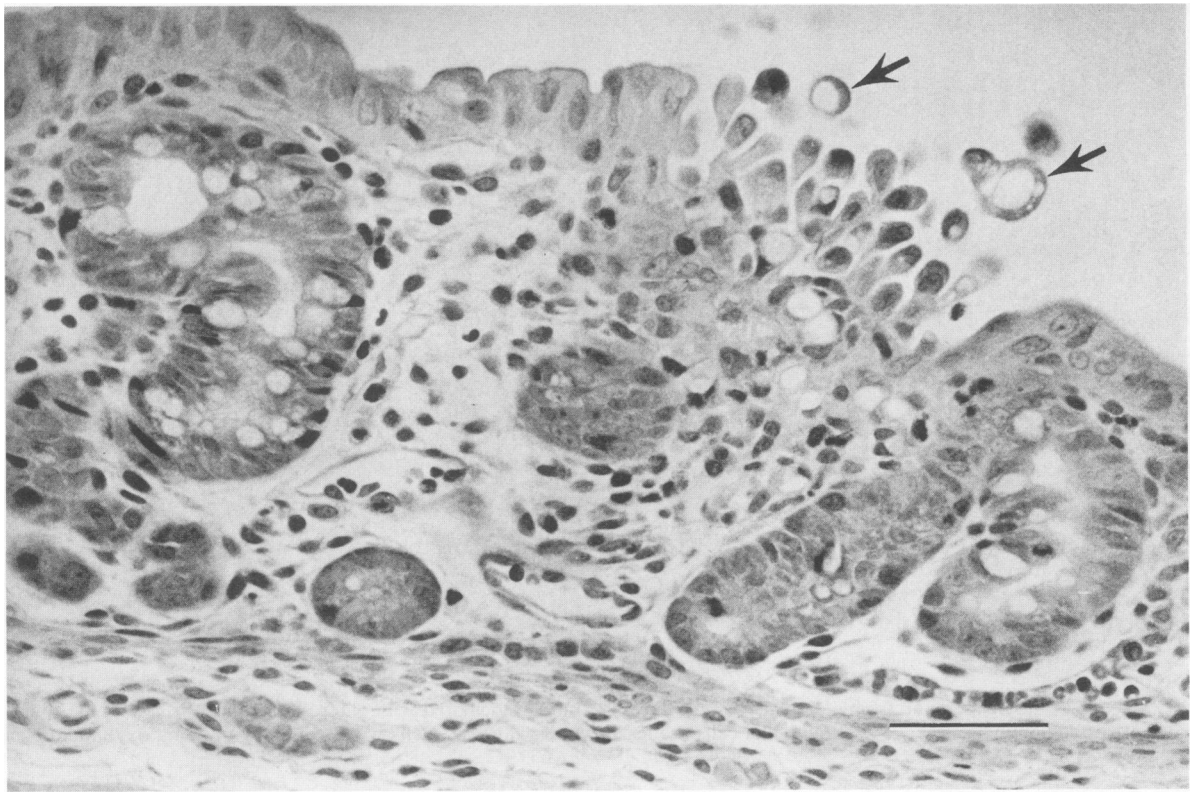

FIG. 3. Mucosa of the distal colon from a diarrheic rabbit (litter 15; rabbit inoculated with enterotoxigenic *B. fragilis*) has focal detachment of rounded, sometimes vacuolated, surface epithelial cells (arrows). Glandular crypts are lined by deeply stained epithelial cells and a few goblet cells, findings characteristic of crypt hyperplasia. Cells were stained with hematoxylin and eosin. Bar, 5.5 μm.

indication of bacterial attachment to or invasion of intestinal epithelial cells, although a few bacilli were observed in the lumen of the colon.

DISCUSSION

Enterotoxigenic *B. fragilis* caused severe, nonhemorrhagic, watery diarrhea with extensive colonic lesions apparently without adherence to or invasion of enterocytes. Nonenterotoxigenic isolates of the bacterium were avirulent. The intestinal tracts of rabbits inoculated with a toxigenic or nontoxigenic bacterial isolate were colonized with *B. fragilis* to a similar degree. The bacterium had a predilection for the large intestine. Gene fingerprinting analysis indicated that the challenge isolates of toxigenic *B. fragilis* colonized the intestinal tract. This technique has been applied successfully to epidemiologic studies of several bacterial pathogens (2–7, 15). The stability of the genotyping system has been proven reliable over time, independent of culture conditions (7). This study is the initial report of the application of gene fingerprinting analysis to the study of enterotoxigenic *B. fragilis*.

The mechanism of disease pathogenesis caused by toxigenic *B. fragilis* in the infant rabbit is unknown. We hypothesize that a bacterial enterotoxin(s) causes a net secretion of fluid into the small intestine and that a bacterial cytotoxin(s) decreases the ability of the large intestine to reabsorb that fluid; dehydration becomes progressively more severe, weakness increases, and the rabbit ceases to suckle and dies. Involvement of enterotoxin in disease pathogenesis is suggested by several factors, including the prominence of watery diarrhea in the clinical disease, the presence of enterotoxin activity (lamb ligated ileal loop test) among *B. fragilis* isolates virulent in the infant rabbit and in the adult rabbit with ligated cecum (10, 11, 13), and the absence of enterotoxin activity among isolates of *B. fragilis* avirulent in both systems. The presence of characteristic colonic lesions in toxigenic *B. fragilis*-infected diarrheic animals (including adult rabbits with ligated ceca [13], infant rabbits, gnotobiotic pigs, and young, conventional pigs with spontaneous diarrhea [J. E. Collins, L. L. Myers, North Central Conference of Veterinary Laboratory Diagnosticians, June, 1987]) also suggests the involvement of cytotoxin in disease pathogenesis.

Our intestinal isolates of toxigenic and nontoxigenic *B. fragilis* appear to have fimbria-like structures antigenically similar to the BE1 fimbrial subunit (18) (J. van Doorn, personal communication). While fimbriae may facilitate intestinal colonization, we have not observed bacterial adherence to enterocytes of diarrheic animals.

Clinical signs of disease in the infant rabbit model appeared similar to those of naturally occurring diarrheal disease associated with toxigenic *B. fragilis* in humans and livestock (9–13). The naturally occurring disease is characterized by watery, usually nonhemorrhagic diarrhea with a duration of several days to more than a week in the absence of bacteremia. Little information is available regarding intestinal lesions associated with the naturally occurring disease.

The infant rabbit model has been useful for the study of enteric disease caused by other bacteria, including *Vibrio*

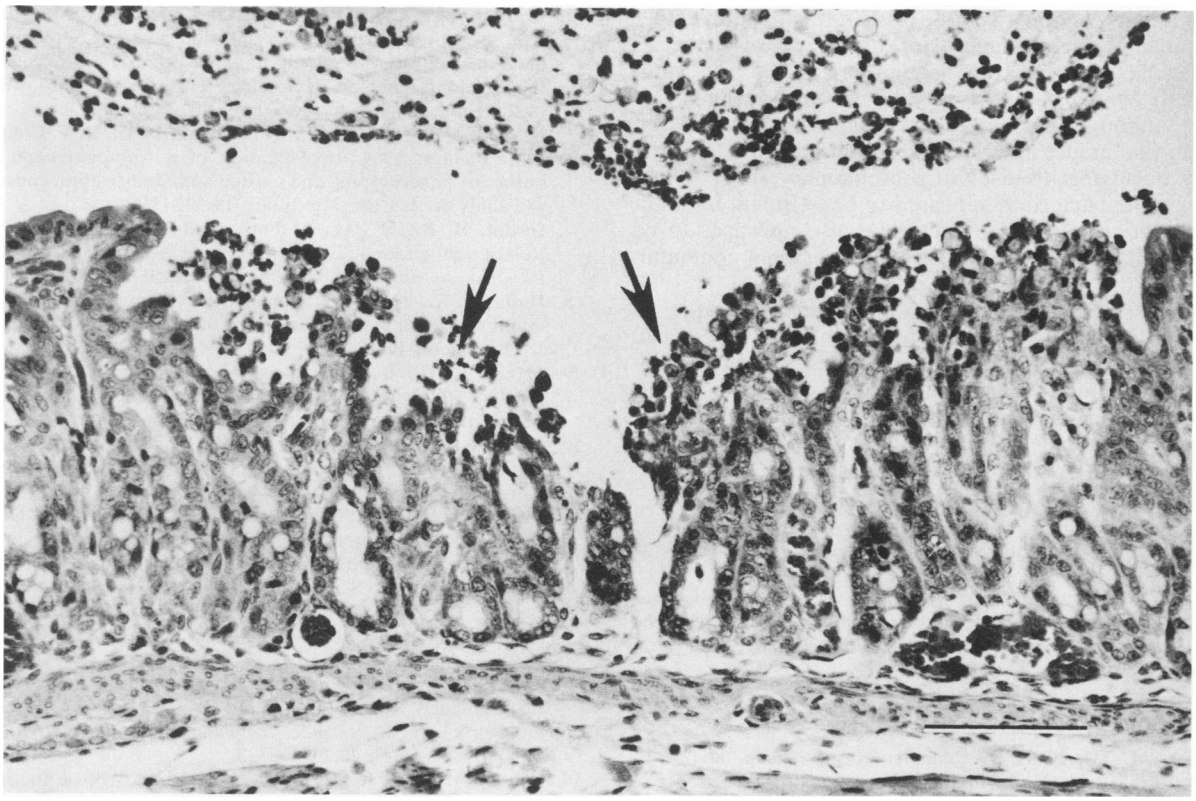

FIG. 4. Mucosa of the distal colon from an unchallenged, diarrheic rabbit (litter 22; rabbit exposed to enterotoxigenic *B. fragilis* via challenged littermates) is focally eroded (arrows) and contains elongated glandular crypts. Mucus, sloughed epithelial cells, erythrocytes, and debris are in the intestinal lumen. Cells were stained with hematoxylin and eosin. Bar, 11.0 μm.

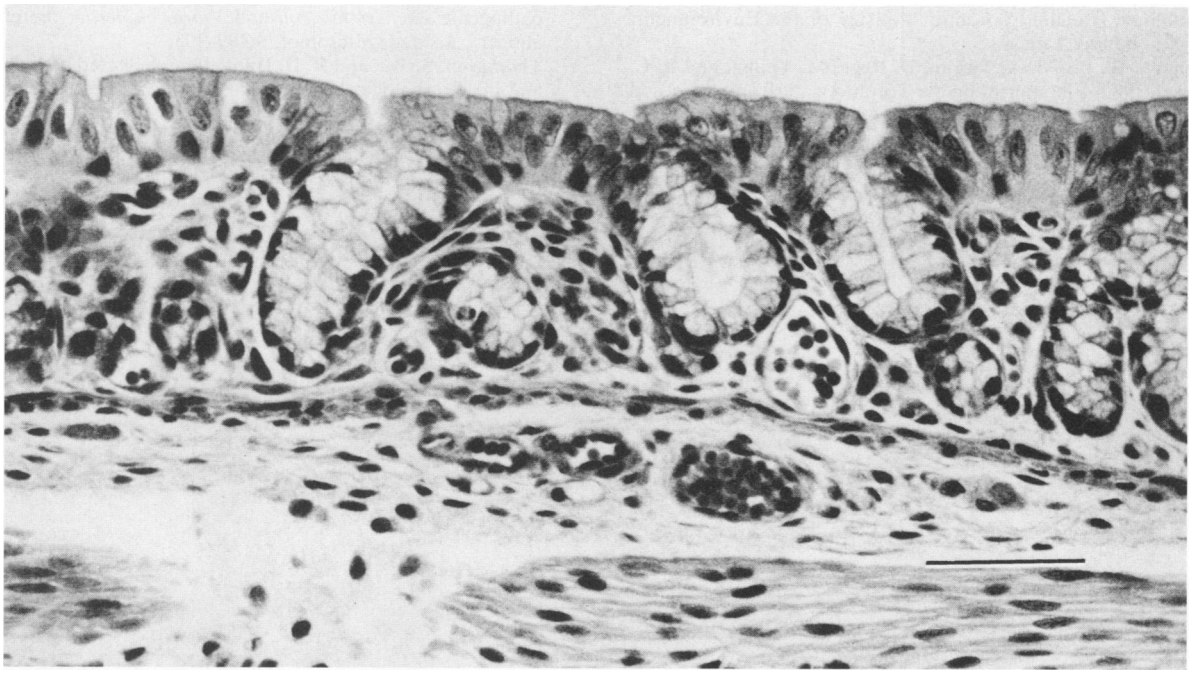

FIG. 5. Mucosa of the distal colon of a clinically normal rabbit (litter 14; rabbit inoculated with nonenterotoxigenic *B. fragilis*) is lined by tall columnar epithelial cells which are orderly in arrangement and structure. Many mucus-producing epithelial cells line glandular crypts. Cells were stained with hematoxylin and eosin. Bar, 5.5 μm.

cholerae and *Escherichia coli* (8, 14, 16). It is the only intact small animal model available for the study of diarrheal disease caused by enterotoxigenic *B. fragilis*. The adult rabbit with ligated cecum is also a useful model for this purpose, although the acute and hemorrhagic nature of disease in this model may be more pronounced than in the naturally occurring disease. In a preliminary study in our laboratory, the bacterium appeared to be avirulent in infant and young adult mice. The bacterium also appeared to be avirulent in hamsters (Connie Gebhart, personal communication).

Results of this study establish that enterotoxigenic *B. fragilis* can function as an enteropathogen. The widespread and frequent occurrence of this enterotoxigenic and enterovirulent bacterium in the intestinal tract in diarrheic humans and animals suggests an important causative role for it in the enteric disease complex.

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