

Experimental Infection of Infant Rabbits with Verotoxin-Producing *Escherichia coli*

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To study the pathogenesis of diarrheal disease due to verotoxin (VT)-producing *Escherichia coli*, 3-day-old rabbits were inoculated intragastrically with live *E. coli* O157:H7 (high VT producer), *E. coli* O113:K75:H21 (low VT producer), or O157:H45 (VT negative) and were examined for clinical symptoms, bacterial colonization, presence of detectable free VT in the intestines, and histological changes. Diarrhea developed consistently with 10⁸ bacteria of *E. coli* O157:H7 but was observed only infrequently with even a higher dose of *E. coli* O113:K75:H21. VT-negative strains failed to cause diarrhea under the same experimental conditions. *E. coli* O157:H7 was recovered from the colon of infected animals in a significantly higher concentration than from the small intestine, and the clinical symptoms correlated with the presence of detectable free VT in the colon. Histological changes were seen mainly in the mid- and distal colon; these changes were characterized by a vast increase in apoptosis in the surface epithelium, increased mitotic activity in the crypts, mucin depletion, and a mild to moderate infiltration of neutrophils in the lamina propria and epithelium. Multiple foci of attached bacteria were seen on the surface epithelium of the gut-associated lymphoid tissue, cecum, and colon. Bacteria were never seen in epithelial cells or the lamina propria. These mucosal abnormalities as well as clinical symptoms were reproduced in infant rabbits by the intragastric administration of VT alone. These results are consistent with the hypothesis that VT plays a major role in the pathogenesis of diarrhea caused by *E. coli* O157:H7 and other VT-producing *E. coli*.

Production of a cytotoxin by *Escherichia coli* was reported in 1977 by Konowalchuk et al. (14) and subsequently by others (28; W. G. Wade, B. T. Thom, and N. Evans, Letter, *Lancet* ii:1235-1236, 1979). They found that culture filtrates of some strains of *E. coli* isolated from human infants and young pigs with diarrhea had a cytotoxic effect on monolayers of Vero cells that was distinctly different from the cytotoxic effect of heat-labile enterotoxin and referred to it as verotoxin (VT). However, an enteropathogenic role of VT-producing *E. coli* was not recognized until the discovery that VT was produced by *E. coli* O157:H7 strains associated with outbreaks of hemorrhagic colitis in the United States and Canada (16, 26, 34; A. D. O'Brien, T. A. Lively, T. W. Chang, and S. L. Gorbach, Letter, *Lancet* ii:573, 1983; W. M. Johnson, H. Lior, and G. S. Bezanson, Letter, *Lancet* i:76, 1983). Since then, diarrheal disease with or without grossly bloody diarrhea due to *E. coli* O157:H7 and other VT-producing *E. coli* has been reported with increasing frequency (4, 11, 22, 25).

E. coli O157:H7 strains do not produce enterotoxins (34; Johnson et al., Letter) and are noninvasive, as indicated by negative Sereny tests and tissue culture assays (34). These findings strongly suggest that VT plays an important role in pathogenesis. VT appears to be the same as a cytotoxin produced by *Shigella dysenteriae* 1 (19, 20; O'Brien et al., Letter). Association of VT-producing *E. coli* (10, 11, 22) and *Shigella* spp. (15, 24) with hemolytic uremic syndrome is also suggestive of the similarity between VT and the Shiga toxin. Furthermore, rabbits inoculated intravenously with VT developed clinical symptoms and histological lesions similar to those seen in rabbits given Shiga toxin (31). However, it is not known whether the production of VT alone would be sufficient to cause diarrhea, since mutant

strains of *Shigella* that produce the cytotoxin but are noninvasive failed to cause diarrhea in experimental infection (8, 17).

To study the pathogenesis of diarrhea due to VT-producing *E. coli*, we developed an animal model. Infant rabbits were used for this purpose, since Farmer et al. (J. J. Farmer III, M. E. Potter, L. W. Riley, T. J. Barrett, P. A. Blake, C. A. Bopp, M. L. Cohen, A. Kaufmann, G. K. Morris, R. T. Remis, B. M. Thomason, and J. G. Wells, Letter, *Lancet* i:702-703, 1983) have reported that infant rabbits are susceptible to *E. coli* O157:H7.

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MATERIALS AND METHODS

Bacteria. *E. coli* UC741 (serotype O157:H7), UC764 (O113:K75:H21), and UC761 (O157:H45), originally isolated from stools of patients with diarrhea at the Foothills Hospital, Calgary, Alberta, Canada, were used in the study. Strains UC741 and UC764 were VT positive and were the only pathogens that grew in the stool cultures of patients with bloody diarrhea. These two strains were used as representatives of high and low VT-producing *E. coli* strains. Strain UC741 produced about 30 times more VT than strain UC764 in vitro. Strain UC761 is VT negative and was isolated from a patient with diarrhea whose stool culture also grew *Salmonella* spp. All strains were stored at -70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with 30% glycerol.

Animals. New Zealand white infant rabbits (2 to 3 days old) were obtained from a commercial breeding farm (Kleefeld Rabbitry, Winnipeg, Manitoba, Canada). Each litter (6 to 10 infant rabbits) was housed as a group together with the mother. Infant rabbits (11 days old) and weaned

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rabbits (weight, 0.5 to 0.8 kg) were also used. Weaned rabbits were housed two to a cage with free access to food and water.

Preparation of inocula. Bacteria grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C on a shaker (200 rpm) were reinoculated (10% inoculum) into fresh media and grown for 4 h. Cultures were washed twice with phosphate-buffered saline by centrifugation, and bacteria were suspended in a 10% sodium bicarbonate solution to a desired density with a spectrophotometer (Spectronic 20; Bausch and Lomb Inc., Rochester, N.Y.).

Infection of rabbits. Animals were observed for 1 day to ensure that no diarrhea was present prior to infection. After rectal swabs were taken for culture, infant rabbits were inoculated with 1.0 ml of bacterial suspension through a catheter tube (17-gauge nylon; Portex, Markham, Ontario) passed into the stomach by the oral route. Handling of infant rabbits during challenge did not result in maternal rejection. Feeding tubes (size 5 French) were used for weaned rabbits after ketamine hydrochloride (40 mg/kg) was administered intramuscularly.

Challenge of infant rabbits with VT. VT was prepared from culture supernatants of *E. coli* O157:H7 by a previously described method (19) with modifications. Bacteria were grown in iron-depleted syncase broth (20) incubated for 48 h with shaking (200 rpm) at 37°C. Culture supernatants obtained by centrifugation (10,000 × *g* for 20 min) were precipitated with 60% (NH₄)₂SO₄, and the precipitates were dissolved in distilled water and dialyzed against 20 volumes of 10 mM sodium phosphate buffer (pH 7.4). The dialysate was then applied to a column containing Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 10 volumes of 10 mM sodium phosphate buffer (pH 7.4) and eluted with 0.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.4). A pool of the eluate was freeze-dried and then was dialyzed against 200 volumes of 10 mM sodium phosphate buffer (pH 7.4). Toxin activity was determined by Vero cell assay.

The VT preparation was diluted in sodium bicarbonate (final concentration 10%) and was administered to three litters of 3-day-old rabbits directly into the stomach through a catheter tube as described above; two litters (nine and seven rabbits each) were observed for clinical symptoms, and one litter (eight rabbits) was sacrificed at various times after inoculation for free VT assay and histological examination.

Experimental design. After inoculation, animals were observed daily for diarrhea. Rabbits were considered to have diarrhea when feces were stuck onto the perineum or hind legs. Diarrhea was scored as severe when the area was soiled with wet feces. At various times from the day of inoculation, animals were sacrificed with an overdose of sodium pentobarbital by intracardiac injection; and portions of the intestine were dissected for colony counts, VT assay, and histological examination.

Bacteriological examination. Sections of the intestine were weighed, carefully dissected longitudinally, and suspended in 0.5 ml of phosphate-buffered saline. The suspension was vortexed vigorously for 10 s and, after tissue was removed, was diluted serially with phosphate-buffered saline for colony counts. For *E. coli* O157:H7, which is sorbitol-negative (16, 22, 34), colony counts were performed on sorbitol-MacConkey agar in which lactose was replaced with sorbitol. After an overnight incubation at 37°C, sorbitol-negative colonies were counted. Two sorbitol-negative colonies were

picked randomly from each plate and were serotyped by slide agglutination (22). For *E. coli* O157:H45 and O113:K75:H21, which are sorbitol positive, regular MacConkey agar was used for colony counts. Colonies were identified by serotyping for *E. coli* O157:H45 and by VT assay of culture supernatants obtained from subcultures for *E. coli* O113:K75:H21. The procedures used for the preparation of anti-O157 antisera have been described previously (22).

VT assay. VT was tested by the method of Konowalchuk et al. (14) with minor modifications (22). Briefly, monolayers of Vero cells grown for 24 h in a microtiter plate in Eagle minimal essential medium (the Joklik modification; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum were inoculated with 100 µl of test material. After incubation for 24 h at 37°C in 5% CO₂, Vero cell monolayers were examined for cytopathic effect. For quantitation of VT, test materials were serially (twofold) diluted and cytotoxic dose was defined as the amount of toxin required to kill 50% of Vero cells in a microtiter well in 24 h (CD₅₀).

For VT assay of culture supernatants, bacteria were grown in Trypticase soy broth at 37°C for 24 h, and supernatant fluids were obtained by centrifugation followed by filtration (pore size, 0.22 µm). To examine the presence of free VT in the intestinal contents of test animals, sections of the intestine were obtained and treated in the same way as described above for colony counts, except that the tissue suspension, after vortexing, was centrifuged, and supernatants were obtained by filtration.

Histological examination. Sections of intestine were cut open longitudinally and stapled with the serosa surface down onto index cards. They usually adhered firmly because of surface protein. They were fixed for at least 24 h by inversion in cold fixative containing 4% formaldehyde and 1% glutaraldehyde in phosphate buffer (18). After fixation, longitudinal sections were cut and carefully embedded in paraffin wax or in glycol methacrylate to provide sections perpendicular to the mucosa. Sections were stained with hematoxylin and eosin, Brown and Brenn gram stain, and the combined periodic acid-Schiff-Alcian blue (pH 2.5) stain. All histological sections were coded for blind assessment of the histology. For selected cases portions of tissue were postfixed in osmium tetroxide and embedded in araldite for ultrathin sectioning and electron microscopy.

RESULTS

Age-dependent susceptibility of rabbits. Infant rabbits (aged 3 and 11 days) and weaned young rabbits (about 6 weeks old) were compared for their susceptibility to *E. coli* O157:H7 (Table 1). Diarrhea developed consistently in 3- and 11-day-old rabbits, although diarrhea was less severe in the latter group even with a higher inoculum dose. Weaned rabbits were significantly less susceptible to *E. coli* O157:H7 compared with 3- or 11-day-old rabbits ($P < 0.05$). Cultures

TABLE 1. Age-dependent susceptibility of rabbits to *E. coli* O157:H7

Age (days)	Inoculum dose (CFU × 10 ⁶)	No. inoculated	No. with diarrhea	No. with severe diarrhea
3	1	5	5	5
11	5	9	9	3
Weaned ^a	5	10	2	0

^a Weighted 0.5 to 0.8 kg.

of rectal swabs taken at 8 days after inoculation grew *E. coli* O157:H7 in 7 of the 10 weaned rabbits that were inoculated, but only 2 were symptomatic. We decided to use 3-day-old rabbits in the remainder of the study.

Effect of inoculum size. Three-day-old rabbits were inoculated with various doses of *E. coli* O157:H7 and were observed for diarrhea for a period of up to 14 days (Table 2). With inoculum doses of 10^8 CFU, the animals developed severe diarrhea (without blood) consistently. Severe diarrhea was observed less frequently in rabbits inoculated with 10^7 CFU. The incubation period was prolonged with smaller inoculum doses. The infectious dose causing diarrhea in 50% of the animals inoculated (ID_{50}) was about 1.4×10^6 CFU. The duration of diarrhea or mortality rate could not be determined accurately since many rabbits were sacrificed for bacteriological and histological examination before the cessation of diarrhea. In those rabbits that were kept alive for observation, diarrhea lasted for 3 to 12 days, depending on inoculum doses. Death occurred at 5 to 14 days postinoculation, except for those inoculated with 10^{10} CFU, all of which died within 3 days of inoculation. However, in no case was bloody diarrhea observed.

Other VT-producing *E. coli*. The virulence of *E. coli* O113:K75:H21 was compared with that of *E. coli* O157:H7 in 3-day-old rabbits (Table 3). The ID_{50} of *E. coli* O113:K75:H21 was 3×10^8 CFU compared with 1.4×10^6 CFU for *E. coli* O157:H7 (Table 2). Although both strains produce VT, the amount of toxin produced in culture supernatants by *E. coli* O113:K75:H21 was about 30-fold less than that produced by *E. coli* O157:H7. The difference in ID_{50} of the two strains appeared to be related to the amount of VT produced. *E. coli* O157:H45, a VT-negative strain, failed to cause diarrhea in infant rabbits.

Bacteriology. Cultures of rectal swabs obtained from infant rabbits (3 or 11 days old) at the time of oral challenge were usually negative when plated onto MacConkey agar. A few rabbits that were colonized with coliforms were excluded from the study, because the presence of a larger number of normal flora would make subsequent enumeration of challenge organisms more difficult.

Three-day-old rabbits inoculated with 10^8 bacteria became heavily colonized with each of the challenge organisms within 24 h of inoculation. Cultures of rectal swabs taken daily showed no appreciable decrease in the amount of

TABLE 2. Effect of inoculum dose on the frequency of diarrhea in 3-day-old rabbits infected with *E. coli* O157:H7

Inoculum dose (CFU)	No. inoculated	No. with diarrhea (no. with severe diarrhea)	Mortality ^a	Incubation period (days)
1×10^{10}	6	6 (6) ^b	4/4	1.0
3×10^9	2	2 (2)	1/2	1.0
1×10^9	5	5 (5)	3/3	2.0 ± 1.0^c
1×10^8	13	12 (12)	3/3	3.2 ± 2.0
1×10^7	5	5 (2)	2/3	3.4 ± 0.9
1×10^6	5	2 (1)	0/2	6.5 ± 4.9
1×10^5	3	1 (1)	0/2	3
0 ^d	7	0	0/5	

^a Number of rabbits that died per number of rabbits observed for a 2-week period.

^b No. of rabbits with severe diarrhea are shown in parenthesis.

^c Mean \pm standard deviation.

^d 10% sodium bicarbonate solution (1.0 ml) with no bacteria.

TABLE 3. Virulence of VT-producing *E. coli* in 3-day-old rabbits

<i>E. coli</i> strain	Verotoxin (CD ₅₀ /ml)	Inoculum dose (CFU)	No. with diarrhea/no. inoculated	No. with severe diarrhea	Incubation period (days)
O157:H7	1.6×10^5	10^8	12/13 ^a	12 ^b	3.2 ± 2.0
O113:K75:H21	5.1×10^3	10^9	2/4	0	4.0
		10^8	3/8 ^a	1 ^b	8.3
		10^7	0/3		
O157:H45	Negative	10^{10}	0/8		

^a $P < 0.05$ (chi-square test).

^b $P < 0.01$ (chi-square test).

growth for up to 2 weeks. To examine the degree of colonization in the different segments of the intestine, rabbits were sacrificed on days 2 through 9 of challenge; and sections (about 2 cm in length and 0.1 g in weight) of the proximal and distal small intestine and the middle colon were opened longitudinally, suspended in phosphate-buffered saline, and vortexed. After low-speed centrifugation, the supernatant fluids were diluted for viable counts (Table 4). Although all segments of the intestine were heavily colonized with *E. coli* O157:H7 on days 2 through 9 of inoculation, CFUs recovered from the mid-colon were significantly higher compared with those from the proximal and distal portions of the small intestine ($P < 0.01$). For *E. coli* O113:K75:H21, the colon was also more heavily colonized than the small intestine ($P < 0.05$). However, the degree of colonization of *E. coli* O113:K75:H21 was significantly lower than that of *E. coli* O157:H7 in each corresponding segment of the intestine ($P < 0.01$ to 0.02). The difference in the degree of colonization between these two strains might also account for the difference in virulence shown in Table 2.

The degree of colonization of *E. coli* O157:H7 was also examined in 11-day-old rabbits during 5 to 10 days of challenge with 5×10^9 bacteria. Log₁₀ CFU per g of tissue plus intestinal contents were 5.63 ± 0.04 , 7.59 ± 0.92 , and 8.45 ± 0.39 in the proximal and distal small intestine and the mid-colon, respectively. CFU recovered from the colon was significantly higher than that from the proximal small intestine ($P < 0.01$).

Correlation between clinical symptoms and presence of free

TABLE 4. Concentrations of infecting organism recovered from the intestinal lumen of 3-day-old rabbits inoculated orogastrically with VT-producing *E. coli*^a

Section of intestine	Log ₁₀ CFU ^b		<i>P</i> value ^c
	<i>E. coli</i> O157:H7	<i>E. coli</i> O113:K75:H21	
Proximal small	8.56 ± 1.34^d	6.42 ± 1.22^c	<0.01
Distal small	9.11 ± 0.71^c	7.39 ± 1.31	<0.02
Mid-colon	10.14 ± 0.31^d	8.32 ± 0.73^c	<0.01

^a Following orogastric inoculation with 10^8 organisms, one rabbit from each group was sacrificed on days 2, 3, 4, 5, 6, 7, and 9 postinoculation, and the bacterial concentration was determined by colony counts (see the text).

^b Per gram of intestinal tissue plus luminal contents; mean values \pm standard deviation of seven rabbits.

^c Difference between CFU of *E. coli* O157:H7 and *E. coli* O113:K75:H21.

^d CFU of *E. coli* O157:H7 in the mid-colon were significantly higher compared with those in the proximal or distal small intestine ($P < 0.01$; by paired-sample *t*-test, two-tail analysis, $n = 7$).

^e CFU of *E. coli* O157:H7 in the mid-colon were significantly higher compared with those in the proximal small intestine ($P < 0.05$ by paired-sample two-tail analysis, $n = 7$).

TABLE 5. Susceptibility of 3-day-old rabbits to VT

Dose (CD ₅₀ , ×10 ¹⁰) ^a	No. challenged	No. with diarrhea (no. with severe diarrhea)	No. died	Incubation period (days) ^b
7.5	9	9 (6) ^c	4	0.7 ± 0.3
2.5	7	6 (2)	1	0.8 ± 0.4

^a VT diluted with sodium bicarbonate (final concentration, 5%) was administered intragastrically.

^b Animals were examined for diarrhea twice a day.

^c One rabbit had bloody diarrhea.

VT in the intestinal lumen. Sections of the intestine from rabbits infected with VT-producing *E. coli* were obtained at various times after inoculation, as described above for the colony count experiments, and after tissues were removed by low centrifugation, the supernatant fluids were filtered through a Millipore filter (pore size, 0.22 μm) and examined for cytotoxic activity. Free VT activity was demonstrable more often in the mid-colon (17 of 27 rabbits) than in the proximal or distal small intestine (7 of 27 rabbits) ($P < 0.05$). Two control rabbits inoculated with *E. coli* O157:H45, a VT-negative strain, did not show cytotoxic activity in any part of the intestine.

Among 3-day-old rabbits inoculated with the VT-positive strains, free VT was detected in the colon more often in the rabbits that had diarrhea at the time of sacrifice than in those that did not. Of the 16 rabbits that had diarrhea, 14 were VT positive and 2 were VT negative; of the 11 rabbits that did not have diarrhea, only 3 were VT positive and 8 were VT negative ($P < 0.01$).

Susceptibility of infant rabbits to VT. VT prepared from culture filtrates of *E. coli* O157:H7 was suspended in sodium bicarbonate and administered intragastrically into 3-day-old rabbits (Table 5). Of nine infant rabbits challenged with 7.5×10^6 CD₅₀ of VT, five had diarrhea within 12 h of toxin administration. By 24 h, all animals had diarrhea, with grossly bloody diarrhea occurring in one rabbit. In contrast to infection with live bacteria, in which diarrhea lasted for 3 to 12 days, diarrhea caused by free VT lasted for only 1 to 2 days. With 2.5×10^6 CD₅₀ of VT, the results were very similar, although diarrhea was generally less severe and fewer animals died.

In another experiment, 7.5×10^6 CD₅₀ of VT was administered to eight 3-day-old rabbits, and the rabbits were sacrificed at 1 to 3 days of challenge for histological examination and free toxin assay. Free toxin was detectable in the luminal content of the proximal and distal small intestine and the mid-colon of all animals. Geometric mean titers of free toxin (the highest dilution of luminal content that causes death of 50% of Vero cells in a microtiter well within 24 h) were 6.7, 3.3, and 2.0 in the proximal and distal small intestine and the mid-colon, respectively.

Histology. From each animal, histological sections of proximal, mid-, and distal small intestine; sacculus rotundus; cecum; appendix; and proximal, mid-, and distal colon were examined blindly and independently by two investigators. In 3-day-old rabbits fed 10^8 CFU of *E. coli* O157:H7, histological changes were seen mainly in the mid- and distal colon with increased apoptosis (individual cell death [12, 13]) in the surface epithelium, increased mitotic activity in the crypts, mucin depletion, and a mild to moderate infiltrate of neutrophils in the lamina propria and epithelium (Fig. 1). These mucosal abnormalities were invariably present in the mid- and distal colon on day 2 of infection (the earliest time that

animals were sacrificed) and persisted until day 11 (the latest time that animals were sacrificed). The changes were much less severe in the proximal colon, cecum, and gut-associated lymphoid tissue (GALT; Peyer patches, sacculus rotundus, and appendix) and were minimal in the small bowel.

Another feature of histological sections prepared from *E. coli* O157:H7-infected rabbits was the presence of multiple foci of attached bacteria on the luminal surface of the colon, cecum, and GALT and only scanty foci on the villi of the mid- and distal small intestine. Attached bacteria were seen most consistently on days 2 to 6 on the colonic epithelial surface and persisted until day 11 (the latest day of experiments) on the surface of the other tissues. Electron microscopic examination demonstrated rows of adherent bacteria, and in areas of bacterial adherence, there was loss or disruption of microvilli (Fig. 2). Bacteria were never found within epithelial cells or in the lamina propria.

Histological sections prepared from rabbits infected with *E. coli* O113:K75:H21 revealed similar mucosal abnormalities and clusters of adherent bacteria as described for *E. coli* O157:H7-infected animals. However, with an inoculum size of 10^8 CFU, severe histological changes did not occur until 7 days after inoculation, whereas a similar degree of mucosal abnormalities was observed within 2 days of inoculation of *E. coli* O157:H7. In contrast, rabbits fed nontoxicogenic *E. coli* O157:H45 showed neither mucosal abnormality nor any adherent bacteria. Sections prepared from these control animals were readily identified by blind assessment of the histology.

In animals given toxin alone, there were mucosal abnormalities within 1 day of inoculation which were similar to the changes seen in the infected rabbits, with the exception that

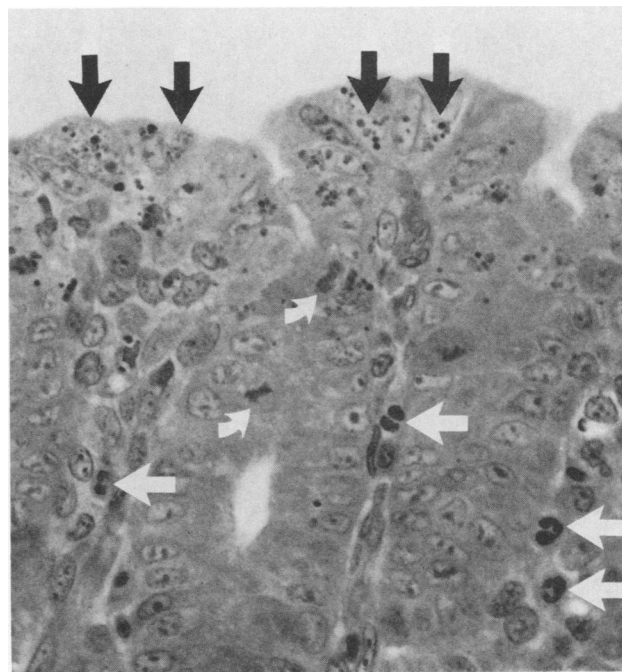


FIG. 1. Distal colon from a 3-day-old rabbit infected with *E. coli* O157:H7 and sacrificed at 4 days postinoculation. The mucosa shows many apoptotic cells (black arrows) in the surface epithelium, mitoses high in the crypts (curved white arrows), mucin depletion, and neutrophil leukocytes (straight white arrows) in the lamina propria. Stained with hematoxylin and eosin; magnification, ×650.

at no stage were adherent bacteria present. Again, the major changes were in the colon and were most severe in the distal colon. The changes consisted of numerous apoptoses in the surface epithelium and sometimes in the crypts, increased mitotic activity, mucin depletion, crypt dilatation, and heavy neutrophil infiltration with occasional crypt abscesses (Fig. 3). In one animal which had bloody diarrhea, extreme mucosal and submucosal congestion was seen in conjunction with the mucosal changes described above (Fig. 4). Histological changes were minimum to none in GALT and the small bowel.

DISCUSSION

Results of this study demonstrate that *E. coli* O157:H7, a VT-producing strain isolated from a patient with hemorrhagic colitis, is capable of causing severe diarrhea in infant rabbits in a dose-dependent fashion. VT was detectable in the luminal contents of the colon, and histological changes were seen mainly in the mid- and distal colon, which is consistent with previous observations in human infections (10, 22, 26). In patients with hemorrhagic colitis, VT was detectable regularly in diarrheal stools (10, 22), and mucosal abnormalities were demonstrated in the colon by barium enemas (22, 26). Bloody diarrhea, not seen in the experimental infection of infant rabbits, is a characteristic symptom of *E. coli* O157:H7 infection in humans (16, 22, 25, 26). However, nonbloody diarrhea was seen frequently in the household members of patients with hemorrhagic colitis who were culture positive for *E. coli* O157:H7 but did not seek medical attention (C. H. Pai, unpublished data).

The following data from the present study, together with

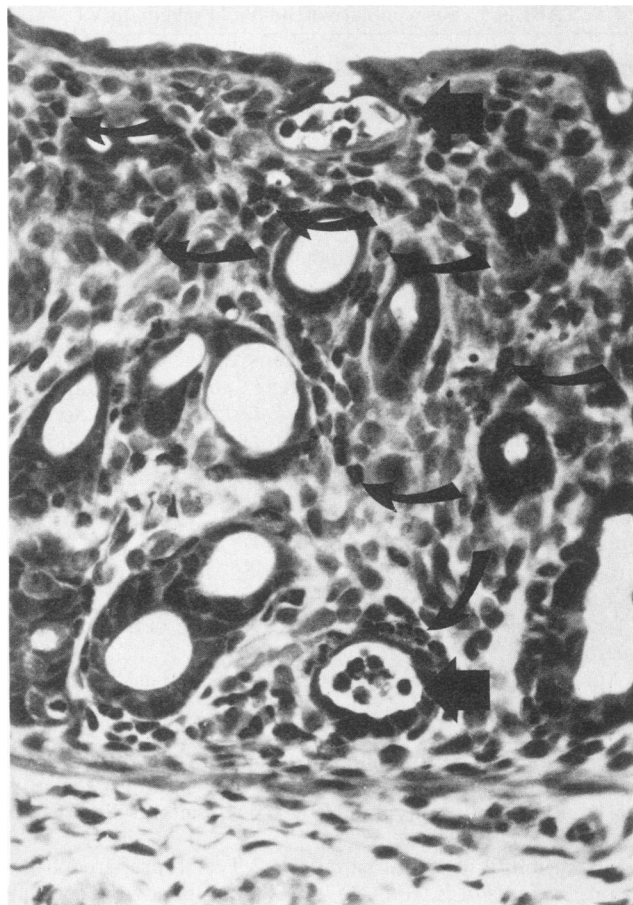


FIG. 3. The distal colonic mucosa of a 3-day-old rabbit fed VT 2 days earlier shows crypt abscesses (straight black arrows), dilated crypts, and neutrophils (curved black arrows) in the lamina propria. Stained with hematoxylin and eosin; magnification, $\times 450$.



FIG. 2. Electron micrograph of distal colon from a 3-day-old rabbit infected with *E. coli* O157:H7 and sacrificed at 2 days postinoculation, showing adherent bacteria and loss of microvilli. The cell on the right retains its microvilli. Stained with uranyl acetate and lead citrate; magnification, $\times 4,500$.

the previous findings that *E. coli* O157:H7 is neither invasive nor enterotoxigenic, support the hypothesis that VT plays an important role in the pathogenesis of diarrhea (30, 34; Johnson et al., Letter; O'Brien et al., Letter): (i) *E. coli* O113:K75:H21 produced about 30-fold less VT in vitro and was significantly less virulent than *E. coli* O157:H7 (Table 3); (ii) the presence of detectable free toxin in the mid-colon was correlated with diarrhea (see above); (iii) histological examination showed clusters of bacteria attached to the luminal surface of the colon, but bacteria were never seen within epithelial cells or in the lamina propria (Fig. 2); (iv) increased apoptosis in the surface epithelium is consistent with a cytotoxic effect of VT (Fig. 1); (v) feeding of VT alone to infant rabbits resulted in clinical symptoms and histological changes almost identical to those seen after challenge with live bacteria (Table 5 and Fig. 3 and 4). Furthermore, bloody diarrhea and extreme mucosal and submucosal congestion, the symptoms and signs characteristic of hemorrhagic colitis in humans infected with *E. coli* O157:H7, were seen following intragastric administration of VT. Crypt abscesses observed in VT-fed animals but not in those challenged with live bacteria were also seen, albeit infrequently, in rectal or colonic biopsies obtained from patients with culture-proven hemorrhagic colitis (J. K. Kelly and C. H. Pai, unpublished data). Smith et al. (31) and Cavanagh et al. (3) studied the

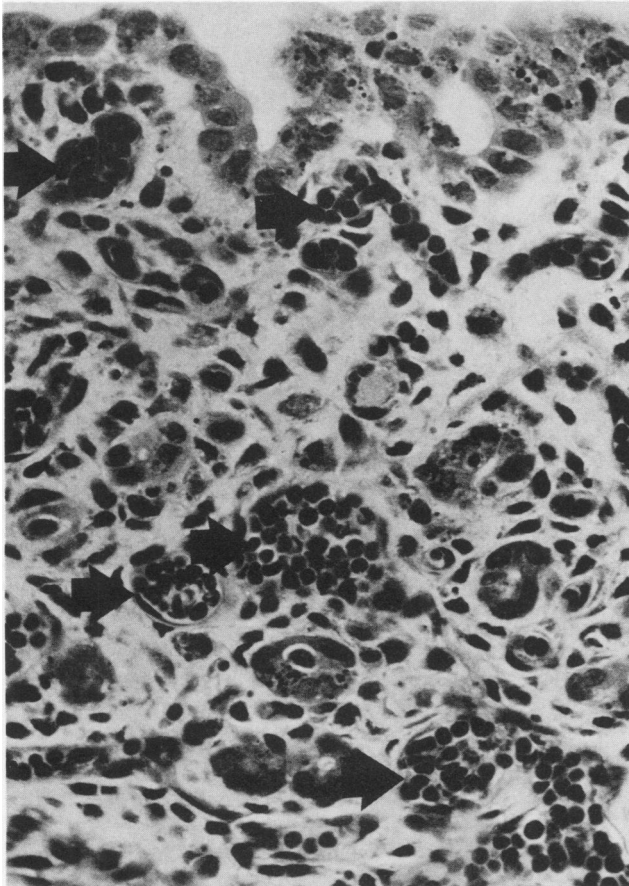


FIG. 4. The distal colonic mucosa of a 3-day-old rabbit fed VT 1 day earlier shows marked congestion of the capillaries (black arrows) in the lamina propria together with epithelial cell apoptosis. Stained with hematoxylin and eosin; magnification, $\times 450$.

toxic effect of VT and Shiga toxin in laboratory animals, including rabbits, following intravenous injection of the toxins. Histological changes were observed in many tissues, including mucosal and submucosal edemas in the cecum. To our knowledge, this is the first report of the effect of orally administered VT in a laboratory animal. Contrary to the effect of intravenously administered VT, the histological lesions were seen mainly in the mid- and distal colon in our study. The fact that the small intestinal epithelium was spared the changes observed in the colonic epithelium, in spite of higher concentrations of VT in the small intestine than in the colon, suggests that the colonic epithelial cells may be specifically susceptible to VT.

The histopathological changes in animals infected with *E. coli* O157:H7 or *E. coli* O113:K75:H21 were mainly in the mid- and distal colon and consisted of bacterial adhesion, increased apoptosis, increased mitosis, mucin depletion, and neutrophil leukocytic infiltration. The full spectrum of changes apart from adherent bacteria was produced by feeding to rabbits VT alone, suggesting that those changes are due to VT. Apoptosis is the process of individual cell death with preservation of tissue architecture (12, 13). The cell cytoplasm undergoes condensation, the nucleus becomes pyknotic or karyorrhectic, and the cell rapidly disintegrates and is phagocytosed by macrophages or adjacent epithelial cells. Apoptosis occurs as a physiological balance

to mitosis in many tissues and as a pathological process following cell injury due to cell-mediated immunity, radiation, and cytotoxic drugs (12, 23, 29). In this study, scanty apoptoses were found in the surface epithelium of the cecum, appendix, and colon of the control animals, indicating physiological cell death under normal conditions. In infected animals or VT-fed animals the quantity of apoptoses was enormously increased with a concomitant increase in mitotic activity in the crypts, suggesting increased epithelial turnover. Mucin depletion may also reflect rapid epithelial turnover. The neutrophil leukocytic infiltration may also be related to increased cell death, since proteinase released by a variety of cells can activate complement and induce chemotaxis (32). Thus, the full spectrum of histological changes are consistent with a direct cytotoxic effect of VT and its sequelae.

The ability to adhere to intestinal epithelial cells is an important virulence factor of noninvasive enteropathogens, such as enterotoxigenic and enteropathogenic *E. coli* (1, 5, 7, 9, 27, 30, 33), but little information is available on the adhesive properties of *E. coli* O157:H7 strains. Wells et al. (34) have shown that isolates of *E. coli* O157:H7 from outbreaks of hemorrhagic colitis were negative for colonization factor antigen (CFA)/I or CFA/II by mannose-resistant hemagglutination, and one strain tested did not attach to human ileal brush border membranes. In a recent study by Beery et al. (2), the attachment of an *E. coli* O157:H7 strain to the cecal epithelium was demonstrated by immunoperoxidase techniques following peroral inoculation of 1-day-old chicks. Diarrhea was not observed, however; and no bacteria were detected on the surface of the colonic epithelium. Although the organism was also observed penetrating into the subepithelial lamina propria of the cecae 14 to 28 days postinoculation, the penetration appeared to be a result of epithelial cell death and sloughing. In the present study, histological examination demonstrated rows of adherent bacteria on the luminal surface of the cecum, GALT, and colon of infant rabbits infected with *E. coli* O157:H7 and *E. coli* O113:K75:H21. In areas of bacterial adherence, there was loss or disruption of microvilli. Although attempts were not made to identify those bacteria attached to the epithelial surface, the absence of attached bacteria in control animals and recovery of the challenge organism in significantly greater numbers from the colon than from the small intestine strongly suggests that the attached bacteria are those of the challenge strains. This may be the first evidence of the importance of adherence to the colonic epithelial cells in the pathogenesis of VT-producing *E. coli* diarrhea. O'Brien has reported that VT is produced not only by *E. coli* strains isolated from patients with hemorrhagic colitis but also by fecal isolates from healthy individuals and a laboratory strain of *E. coli* K-12 (unpublished data). Apparently, not all VT-producing *E. coli* strains are pathogenic, probably because some produce only a low level of toxin, but more importantly, some strains do not possess the specific attachment factors that enable them to colonize the gut.

Although the toxin produced by shigellae is the same as VT of *E. coli* (19, 20; O'Brien et al., Letter), the role of Shiga toxin in the pathogenesis of shigellosis is unclear. The current understanding is that Shiga toxin is responsible for the diarrheal phase of shigellosis that often precedes the dysenteric phase by a few days, and the site of action of Shiga toxin is the small intestine (6, 8, 17). However, *E. coli* O157:H7, which, unlike shigellae, is probably noninvasive, produces lesions primarily in the large intestine, as evidenced by sigmoidoscopy, colonic biopsies, and barium

enemas of patients with hemorrhagic colitis (22, 26) and also by data presented in this study in experimental animals. These lesions produced by *E. coli* O157:H7 are presumed to be caused by VT. Further studies are warranted to define the role of Shiga toxin and VT in the pathogenesis of diarrhea. Recently, Scotland et al. (S. M. Scotland, H. R. Smith, G. A. Willshaw, and B. Rowe, Letter, Lancet ii:216, 1983), Smith et al. (31), and O'Brien et al. (21) have shown that VT production in *E. coli* is determined by genes carried on bacteriophage. In vivo studies with a nontoxigenic, isogenic strain of *E. coli* in which the VT-converting phage has been cured may help to define the role of VT in *E. coli* diarrhea.

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