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Non-O1 *Vibrio cholerae* Intestinal Pathology and Invasion in the Removable Intestinal Tie Adult Rabbit Diarrhea Model

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A modified removable intestinal tie adult rabbit diarrhea (RITARD) model was used to investigate the intestinal pathology, intestinal bacterial colonization, intestinal fluid volume, and onset of diarrhea caused by non-O1 *Vibrio cholerae*. Three strains of non-O1 *V. cholerae* were studied. RITARD rabbits challenged with 10^3 CFU of strain NRT36S (a strain previously shown to cause diarrhea in volunteers) developed grade 3 diarrhea at 48 to 72 h. The mean counts of non-O1 *V. cholerae* isolated were 9.3 ± 0.07 and 8.7 ± 0.7 CFU/g from the small and large intestines, respectively. Histologic examination showed necrosis of the luminal epithelium in the colon and mild inflammatory cell infiltration in the adjacent lamina propria. The severity and extent of intestinal damage by strain NRT36S was dose dependent. Higher doses of strain NRT36S caused severe necrotizing colitis and enteritis, with bacteremia and mortality at <24 h in RITARD rabbits challenged with 10^9 CFU and at <48 h in RITARD rabbits challenged with 10^4 CFU. Electron and light microscopy demonstrated invasion of NRT36S into the luminal epithelial cells of the intestine. Challenge of RITARD rabbits with non-O1 *V. cholerae* A-5 and 2076-79 (strains which did not cause diarrhea in volunteers) did not cause diarrhea or intestinal pathology. Intestinal colonization was transient: at 72 h postchallenge, animals inoculated with strain A-5 were culture negative, while only low numbers of strain 2076-79 were detectable (approximately 0.4 to 0.8 CFU/g). Our data highlight the utility of the RITARD model, when combined with appropriate pathologic and bacteriologic studies, for obtaining insights into pathophysiologic mechanisms of enteric disease by non-O1 *V. cholerae*. In agreement with volunteer studies, non-O1 *V. cholerae* NRT36S is clearly pathogenic in this model; direct cell invasion may play a role in its ability to cause illness.

The diarrheal disease of classical cholera is caused by organisms in the O1 serogroup of *Vibrio cholerae*. Epidemiologic evidence indicates that strains of *V. cholerae* in other O groups are also associated with diarrheal disease in Asia, Africa, Europe, Australia, and North and South America (1, 3, 5, 7, 8, 10, 14–16, 19, 21, 25, 28, 29, 35). Non-O1 *V. cholerae* have been isolated during epidemics of cholera, they have been implicated in food-borne disease, and they have been associated with sporadic cases of diarrheal disease.

The etiologic significance of non-O1 *V. cholerae* as a cause of diarrheal disease was demonstrated by studies in human volunteers (20). After oral administration of a serogroup 31 strain of *V. cholerae* (isolate NRT36S), six of eight volunteers receiving $>10^6$ CFU developed abdominal cramps and diarrhea. Two of the six ill volunteers had over 2 liters of diarrhea; one had over 5 liters of watery diarrhea. The clinical signs in volunteers administered this strain of non-O1 *V. cholerae* were similar to those described in non-O1 *V. cholerae*-associated food-borne disease outbreaks (1, 5). These studies of volunteers also indicated that some strains of non-O1 *V. cholerae* (A-5, 2076-79) either lack or failed to express virulence factors essential for the pathogenesis of diarrheal disease. Neither strain A (2076-79), serotype O17 (which colonized the intestinal tract for at least 5 days), or strain B (A-5), serotype 31 (which was not recovered from postchallenge duodenal and stool specimens), caused diarrheal disease in healthy volunteers.

The mechanisms of the diarrheal disease induced by non-O1 *V. cholerae* such as strain NRT36S are not known. Putative virulence factors include cholera toxin, El Tor toxin, Kanagawa hemolysin, shigalike toxin, hemagglutinins, and heat-stable toxin (2, 6, 12, 13, 15, 18, 22, 33, 36–38) and colonization ability (20, 30). The presence of these factors is variable among strains (20, 25, 30), including the three isolates compared in this study (20).

We recently reported that strain NRT36S adhered to CaCo-2 cells in large numbers and was invasive (24). The adherence and invasion assay correlated with the virulence in human volunteers, since NRT36S adhered in significantly larger numbers than the two strains, A-5 and 2076-79, that did not cause symptoms in human volunteers (20). Strains A-5 and 2076-79 were not invasive in the CaCo-2 cell assay.

To further investigate the mechanisms of diarrheal disease caused by strain NRT36S, we modified the standard removable intestinal tie adult rabbit diarrhea (RITARD) model to study the intestinal pathology of non-O1 *V. cholerae*. Our objectives were to determine the nature and location of lesions in the intestinal tract and to further study the hypothesis that strain NRT36S is invasive. We report the microbiologic, histologic, and ultrastructural features of epithelial damage in the intestine caused by non-O1 *V. cholerae* NRT36S. Our observations raise the possibility that invasion is a factor of importance in the mechanism of diarrheal disease.

MATERIALS AND METHODS

Bacterial strains. Three non-O1 *V. cholerae* strains were used in this study. A-5 is an environmental isolate cultured from frozen shrimp in Japan. This strain did not colonize or cause diarrhea in human volunteers (20). Strain 2076-79 was isolated from a patient in Mississippi who had diarrhea after

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ingestion of raw oysters. The organism colonized human volunteers after oral challenge but did not cause diarrhea (20). Strain NRT36S, isolated in Tokyo from a traveller with diarrhea, caused mild to moderate diarrhea in human volunteers (20) after doses of 10^6 to 10^9 CFU. Stock cultures were stored at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% glycerol. The characteristics of each strain have been reported previously (20, 24).

RITARD model challenge studies. For preparation of the inoculum, non-O1 *V. cholerae* A-5, 2076-79, and NRT36S were subcultured onto brain heart infusion agar (Difco Laboratories, Detroit, Mich.). After incubation for 20 h at 37°C, 20 to 30 typical *V. cholerae* colonies were suspended in 5 ml of brain heart infusion broth (Difco), vortexed, and inoculated onto modified colonization factor agar (11) consisting of 1.5 g of yeast extract per liter, 10 g of $MgSO_4$ per liter, and 0.005 g of $MnCl_2$ per liter with 2% (wt/vol) Bacto Agar, supplemented with 66 mM NaCl. After incubation for 20 h at 37°C, the bacteria were harvested from the plates with 3 ml of phosphate-buffered saline (pH 7.2). The bacterial suspension for rabbit challenge studies was diluted according to spectrophotometric standards to contain approximately 10^9 CFU of strain A-5, 2076-79, or NRT36S per ml. In addition, rabbits were challenged with doses of 10^3 and 10^4 CFU of strain NRT36S per ml. The final inocula were placed on ice until inoculated in experimental rabbits. Serial 10-fold dilutions of the inocula were plated before and after challenge for quantitative counts to confirm the challenge dose (CFU) administered.

Challenge studies were conducted in 2-kg male New Zealand White rabbits by modifications of the RITARD procedure (4, 30–32). Three RITARD rabbits were challenged for each time interval studied with each of the three strains of non-O1 *V. cholerae*. Rabbits fasted for 24 h prior to surgery and were provided water ad libitum. Anesthesia was maintained with a mixture of ketamine (50 mg/kg), xylazine (8 mg/kg), and acepromazine (1 mg/kg) administered intramuscularly. An aseptic surgical technique was used to make a 4- to 5-cm midline incision and externalize the intestine at the level of the ileocecal valve. A cecal tie was placed 1 to 2 cm from the ileocecal junction by using a 3-0 Dexon-S suture (Davis-Geck Inc., American Cyanamid Co., Manati, P.R.). The exposed intestine was kept moistened with sterile saline. A reversible slip-knot tie was placed on the ileum at a distance of 10 cm proximal to the mesoappendix, using Sialastic medical tubing (Dow-Corning Corp., Medical Products, Midland, Mich.) which was externalized through the flank by using a cutting needle. Care was taken not to disrupt the blood supply to the intestine when placing the ties. The 1-ml inoculum was injected into the lumen of the jejunum 60 cm proximal to the reversible tie, using a sterile syringe and 25-gauge needle. The bowel was returned to the abdominal cavity. The incision was closed with 2-0 chromic gut (American Cyanamid Co.) for interrupted sutures in the abdominal fascia and 2-0 Dermalon (Davis-Geck Inc.) for interrupted skin sutures. The external free end of the Sialastic tubing was secured with tape, and the abdomen was wrapped with gauze. The reversible tie was released 4 h postinoculation. After recovery from anesthesia, the rabbits were given water and food ad libitum.

Clinical observations and monitoring of infection. Rabbits were observed daily for clinical signs of diarrhea. Diarrhea was scored according to a grading system using the following characteristics: stools consisting of normal hard rabbit pellets were given a grade of 1; grade 2 diarrhea was soft mushy stools; grade 3 was catarrhal and loose diarrhea which took the form of the collection container; and grade 4 was watery, liquid diarrhea. Cultures were obtained daily by rectal swabs. Swabs were plated directly onto thiosulfate-citrate-bile-sucrose (TCBS) agar (BBL Microbiology Systems). Enrichment cultures were obtained by incubation of swabs overnight in tubes containing alkaline peptone-water (APW) broth (9). The APW broth cultures were inoculated onto TCBS agar and incubated at 37°C overnight. Colonies were selected and identified as non-O1 *V. cholerae* according to standard laboratory practices (9) and failure to agglutinate specific O1 *V. cholerae* antiserum (Difco).

Necropsy examination. Three rabbits were examined for each of the strains at each time interval postchallenge. Rabbits challenged with 10^9 CFU of A-5 and 2076-79 and 10^3 CFU of strain NRT36S were sacrificed at 24 and 72 h postchallenge by intravenous administration of sodium pentobarbital (100 mg/kg) under ketamine (50 mg/kg) anesthesia. Rabbits which died at 24 h after inoculation with 10^9 CFU of strain NRT36S were necropsied. Rabbits challenged with 10^4 CFU of strain NRT36S were sacrificed at 24 h, and two rabbits that died at 48 h were examined with the third rabbit, which was clinically depressed with diarrhea. The volume of the small and large intestinal contents was measured. The large intestinal contents were graded by using criteria similar to the diarrheal grade scoring system (see above). Specimens for bacteriologic culture were obtained for direct and enrichment culture from the small and large intestines, liver, spleen, heart blood, and mesenteric lymph node. Swabs were taken at the cecal tie and the serosa of the adjacent proximal cecum. Serial 10-fold dilutions of small intestinal and large intestinal contents were prepared in sterile 0.85% saline, plated onto TCBS agar, and incubated at 37°C.

Histology and ultrastructural examinations. The intestinal tract was prefixed in 4% paraformaldehyde–1% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). For histologic examination, cross sections of the small and large intestines were embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E).

For transmission electron microcopy, prefixed intestinal tissue was washed and postfixed in 1% OsO_4 in 0.2 M sodium cacodylate buffer (pH 7.4) for 1 h. The postfixed tissues were washed in 0.2 M sodium cacodylate buffer (pH 7.4), dehydrated with a graded series of ethanol (30 to 100%), and embedded in Epon according to standard procedures. Ultramicrotomy specimens were trimmed, sectioned, and placed on 200-mesh 0.25% Formvar-coated, carbon-coated grids. The uranyl acetate- and lead citrate-counterstained thin sections were examined in a JEOL 100B transmission electron microscope operating at an accelerating voltage of 60 kV.

For scanning electron microcopy, washed osmificated intestinal tissues were dehydrated with a graded series of ethanol (30 to 100%). They were critical point dried with liquid CO_2. The critical point-dried specimens were mounted on stubs, sputter coated with gold (approximately 4 min) in a Hummer I sputter coater, and examined on an AMR 1000 SEM apparatus at an accelerating voltage of 20 kV.

Statistical analysis. The fluid volumes and the bacterial counts of non-O1 *V. cholerae* obtained from the small and large intestines at 24 and 72 h postchallenge were analyzed by analysis of variance by comparing the data from strain 2076-79 (dose, 10^9 CFU) and strain NRT36S (doses of 10^9, 10^4, and 10^3 CFU) with those from strain A-5 (dose, 10^9 CFU) at the corresponding times. Because there were no 48-h strain A-5-challenged rabbits for comparison with the

TABLE 1. Quantitation of intestinal contents in RITARD rabbits challenged with non-O1 *V. cholerae* (three rabbits per group)

Strain	Dose (CFU)	Time postchallenge (h)	Diarrhea score	Colon contents score	Intestinal contents (ml)	
					Small intestine	Large intestine
A-5	10^9	24	1	1	9.3 ± 0.9	24.0 ± 1.0
		72	1	1	8.0 ± 2.0	22.0 ± 4.6
2076-79	10^9	24	1	1	14.0 ± 3.6	36.7 ± 12.6
		72	1	1	7.0 ± 1.7	21.5 ± 4.9
NRT36S	10^9	24	3	3	24.7 ± 6.1[a]	24.0 ± 1.4
	10^4	24	2	3+	23.3 ± 2.9[a]	47.7 ± 8.7[a]
		48	3	3+	15.2 ± 8.0	18.6 ± 1.5
	10^3	24	1–2	2–3	16.7 ± 11.5	30.0 ± 11.4
		72	3	4	3.3 ± 4.0	20.0 ± 6.5

[a] Fluid volume was significantly increased ($P < 0.05$) compared with strain A-5 at the same time interval postchallenge.

10^4 CFU NRT36S-inoculated rabbits, the intestinal counts and fluid volume data in these rabbits were compared with the 24-h results with 10^4 CFU of NRT36S by the unpaired *t* test. For the statistical analysis of intestinal bacterial counts, negative culture results were designated as zero and cultures that were negative on direct TCBS culture by serial dilution of intestinal contents but positive after enrichment were assigned an arbitrary count of 10^1 CFU. For all comparisons, differences with $P < 0.05$ were considered significant.

RESULTS

Clinical and intestinal pathologic findings. The rabbits infected with strains A-5 and 2079-76 did not develop diarrhea (Table 1). The contents in the lumen of the small and large intestines were normal (grade 1) in rabbits sacrificed at 24 and 72 h postchallenge, with the exception of one rabbit administered 2076-79 which had fluid content (grade 3) in the large intestine at 24 h postchallenge. The mucosa of the small and large intestines appeared normal on histologic examination (Fig. 1) after challenge with strains A-5 and 2076-79 in RITARD rabbits examined at 24 and 72 h postchallenge (serial sections throughout the small and large intestines were evaluated by histologic and electron microscopic examinations).

In contrast to the asymptomatic infections with strains A-5 and 2076-79, 10^9 CFU of strain NRT36S caused all rabbits to die within 24 h postchallenge. The rabbits had grade 3 (i.e., somewhat fluid, taking the form of the container) contents in the colon and grade 3 diarrhea (Table 1). The proximal colon and cecum (proximal to the permanent suture) showed mucosal edema and congestion for a distance of 3 to 4 cm distal from the ileocecal valve. The serosa was reddened. These changes were indicative of acute colitis and were confirmed by histologic examination which showed extensive and severe necrosis of the mucosa in the jejunum and ileum, proximal colon, and cecum. Large numbers of neutrophils were present in the superficial lamina propria and in the lumen. Hemorrhage was associated with the severe ulceration. The glands in the deep mucosa were intact. Bacteria were present in large numbers on the surface and within luminal epithelial cells.

Because of the mortality and the severe intestinal pathology induced by challenge with 10^9 CFU of strain NRT36S, subsequent studies were undertaken with lower challenge doses of this strain. Rabbits administered 10^4 CFU of strain NRT36S exhibited soft mucoid grade 2 diarrhea at 24 h. The contents of the proximal large intestine had a fluid consistency equivalent to a grade 3 to 4 score. Histologic examination of the intestinal tract showed focal areas of necrosis of the luminal epithelium (Fig. 2) with areas of local erosion of the mucosa in the proximal colon and cecum. Blood vessels were congested, and there was infiltration of low numbers of neutrophils. Moderate to large numbers of bacteria were adherent to the mucosa and within the superficial mucosa at the sites of mucosal damage. There was no

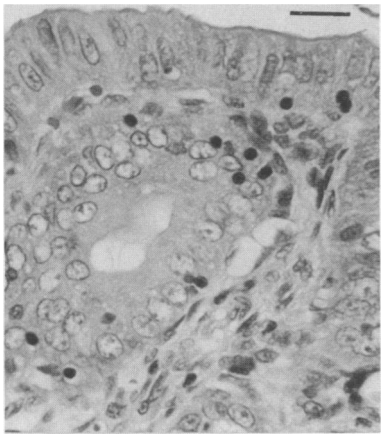

FIG. 1. Histologic appearance of normal colon in a RITARD rabbit, 72 h postchallenge with 10^9 CFU of strain A-5. H&E stain; bar = 20 μm.

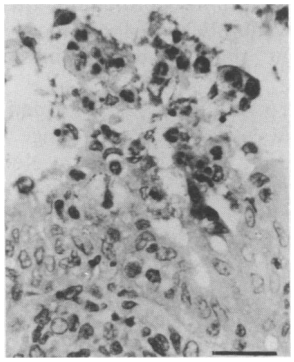

FIG. 2. Focal area of necrosis of the luminal epithelium in the colon at 24 h postchallenge with 10^4 CFU of strain NRT26S. Necrotic epithelial cells (arrows) are exfoliated into the lumen of the intestine. H&E stain; bar = 40 μm.

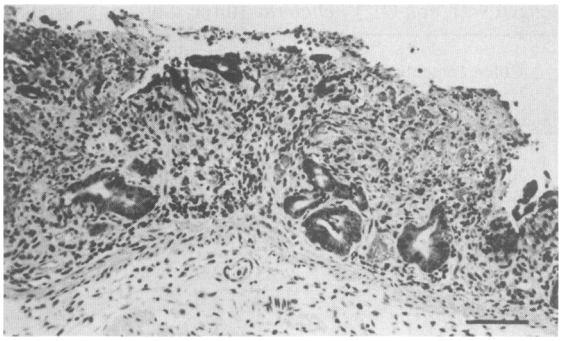

FIG. 3. Severe colitis in RITARD rabbits 48 h postchallenge with 10^4 CFU of strain NRT36S. There is destruction and loss of the epithelium and glands, with only the deeper crypts intact. H&E stain; bar = 100 μm.

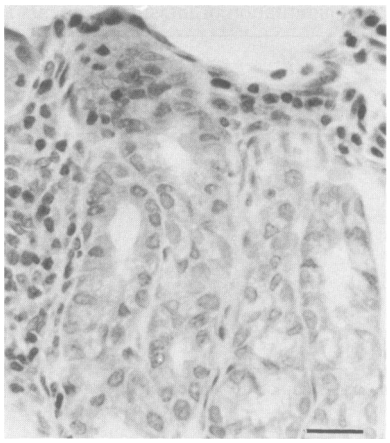

FIG. 4. Mild local damage to the colon epithelium. The luminal columnar epithelium is replaced by a flattened epithelium. There is infiltration of moderate numbers of mononuclear inflammatory cells in the lamina propria immediately beneath the luminal epithelium. H&E stain; bar = 20 μm.

histologic damage in the small intestine. The two rabbits which died and the rabbit which was sacrificed at 48 h postchallenge with 10^4 CFU of strain NRT36S showed severe and extensive ulcerative colitis with large numbers of bacteria and fibrinocatarrhal exudate on the surface. These rabbits had grade 3 diarrhea (Table 1). Blood vessels were markedly congested, and there was pronounced hemorrhage of the ulcerated mucosa (Fig. 3). The severity of the necrotizing colitis was similar to that of the RITARD rabbits which died at 24 h after challenge with 10^9 CFU of strain NRT36S.

There was no mortality in RITARD rabbits examined at 24 and 72 h postchallenge with 10^3 CFU of strain NRT36S. The rabbits exhibited normal stools to grade 2 diarrhea at 24 h, grade 2 to 3 diarrhea at 48 h, and grade 3 diarrhea in all three at 72 h postchallenge (Table 1). At 24 h postchallenge, the contents in the lumen of the large intestine were soft (grade 2) to fluid (grade 3). There was colitis of the proximal colon with mucosal edema and congestion at 72 h postchallenge, and the large intestinal contents were fluid (grade 4) in all three rabbits. Two rabbits had scanty contents (approximately 1-ml volume) in the small intestine. No enteric damage was detected in the intestines of rabbits examined histologically at 24 h postinoculation with 10^3 CFU of NRT36S. At 72 h postchallenge, the mucosal damage in the proximal colon and cecum was mild, with localized areas of necrosis of luminal epithelial cells at the crest of rugae, with replacement of the columnar cells by a flattened epithelium. In the adjacent subluminal epithelium, there was mild local infiltration of low numbers of lymphocytes (Fig. 4). There was no histologic damage to the mucosa of the small intestine.

Scanning electron microscopy confirmed the loss of villi in the small intestine at 24 h postchallenge with 10^9 CFU of strain NRT36S (Fig. 5). Transmission electron microscopy showed degenerating and necrotic luminal epithelial cells and also neutrophils in the lumen. Numerous bacteria were located extracellularly on the surface of the damaged mucosa among exfoliated cells. Ultrastructural observation confirmed histologic evidence of intracellular bacteria. Bacteria were located within membrane-bound vacuoles in the cytoplasm of epithelial cells intact on the luminal surface of the mucosa, as well as within degenerating and necrotic epithelial cells and neutrophils in the lumen of the intestine (Fig. 6).

Bacteriologic observations and intestinal fluid volumes. After challenge of rabbits with 10^9 CFU of strain A-5, the organism was recovered from the small and large intestines in low numbers at 24 h postchallenge (Fig. 7). The organism was isolated by direct culture from one rabbit (2.3×10^4 CFU/g) and only by APW enrichment from the intestines of the other two rabbits. Using an estimated count of 10^1 CFU/g for positive cultures obtained by APW enrichment, the mean small and large intestinal quantitative counts of strain A-5 at 24 h postinoculation were 1.6 ± 2.4 and $0.3 \pm$ 01 CFU/g, respectively (Fig. 7). No organisms were recovered at 72 h postchallenge either by direct culture on TCBS agar or after enrichment in APW broth (Table 2; Fig. 7). The volume of small and large intestinal contents was normal at 24 and 72 h postchallenge (Table 1).

The fluid volumes in the small intestine and large intestine were not significantly increased ($P > 0.05$) at 24 and 72 h postchallenge with 10^9 CFU of strain 2076-79 when com-

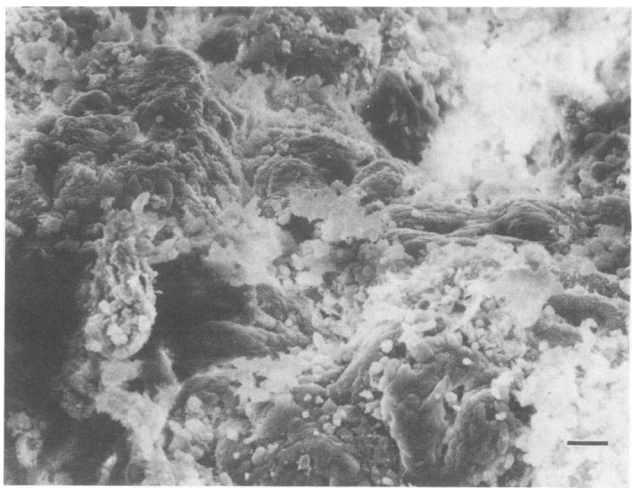

FIG. 5. Scanning electron microscopy showing loss of villi in the ileum of RITARD rabbits with severe necrotizing enteritis caused by strain NRT36S. Bacteria are adherent to the surface of the mucosa. Bar = 15 μm.

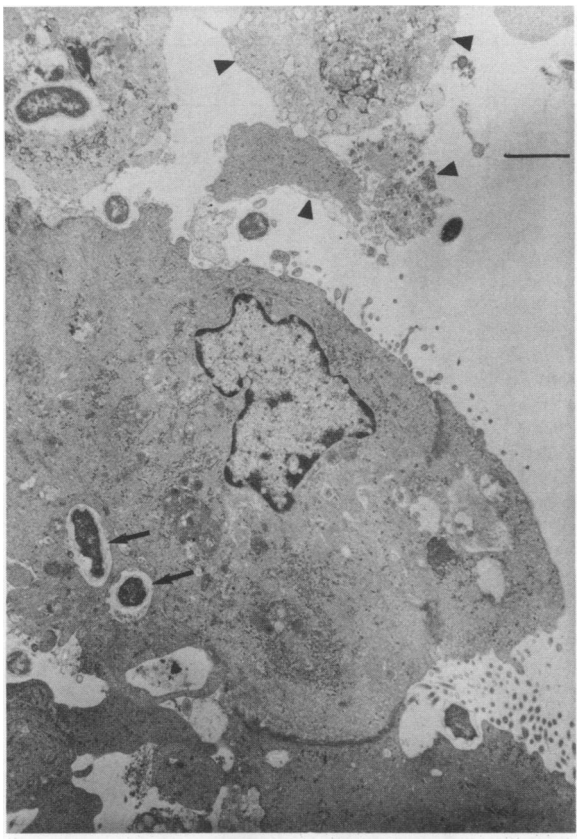

FIG. 6. Transmission electron microscopy showing intracellular bacteria in the membrane-bound vacuoles within colonic epithelium (arrows) of RITARD rabbits challenged with strain NRT36S. Bacteria are also adherent to the surface of the epithelium. Necrotic epithelial cells are exfoliated into the lumen of the colon (arrowheads), and an exfoliated cell has intracellular organisms. Bar = 2 μm.

pared to those of RITARD rabbits administered 10^9 CFU of strain A-5 (Table 1). The moderate numbers of strain 2076-79 isolated from the small intestine (Fig. 8) at 24 h were not significantly higher ($P > 0.05$) than the counts of strain A-5

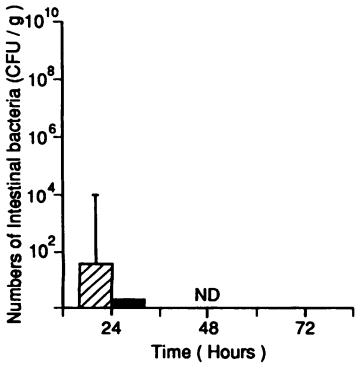

FIG. 7. Numbers of strain A-5 recovered from the small and large intestines at 24 and 72 h postchallenge with 10^9 CFU. No organisms were recovered from the large intestine by TCBS or APW enrichment culture at 72 h postchallenge. □, small intestine; ■, large intestine. ND, no RITARD rabbits were examined at 48 h postchallenge with strain A-5. Intestinal cultures at 72 h were negative.

in the small intestine at 24 h (Fig. 7). The counts of 2076-79 in the large intestine at 24 h (Fig. 8) approached significance ($P = 0.06$) in comparison with the low numbers of strain A-5 recovered from the large intestine. At 72 h, the counts of 2076-79 in the intestinal tract had declined markedly (Fig. 8) and were not significantly higher ($P > 0.05$) than the counts obtained 72 h after challenge with strain A-5 (one rabbit was positive by direct culture of the small and large intestines, and the other two rabbits were positive by APW culture of the small intestine).

All cultures of mesenteric lymph node, liver, spleen, heart blood, and peritoneum from RITARD rabbits inoculated with 10^9 CFU of strains A-5 and 2076-79 were negative (Table 2). This indicates that the infection in these RITARD rabbits was limited to transient intestinal tract colonization which had either cleared or declined to low numbers of organisms by 72 h postchallenge.

The high counts of organisms recovered from the small intestines of rabbits challenged with 10^9 CFU of strain NRT36S (Fig. 9A) were significantly elevated in comparison with strain A-5 at 24 h postchallenge ($P = 0.007$). Cultures were not obtained from the large intestine. There was a significant increase in the small intestinal fluid volume at 24 h compared with the strain A-5 rabbits at the same duration postchallenge ($P = 0.01$), but not in the large intestine (Table 1). Bacteremia was demonstrated by recovery of the organisms, usually by direct culture on TCBS agar (or APW enrichment only from some organs of some rabbits) from the mesenteric lymph node, liver, spleen, and heart blood of all three rabbits (Table 2). Cultures of the serosa over the cecum proximal to the cecal tie were positive by APW enrichment. This was attributed to invasion from the mucosa across the wall of intestine, since cultures of the peritoneum over the large intestine and at the cecal tie were consistently negative in rabbits administered A-5 and 2076-79 (Table 2).

Significantly elevated numbers of organisms (compared with rabbits challenged with 10^9 CFU of strain A-5 and sacrificed at 24 h) were recovered from the small intestine ($P = 0.04$) and large intestine ($P = 0.0001$) at 24 h postchallenge with 10^4 CFU of strain NRT36S (Fig. 9B). This was accompanied by a significant increase in the volume of fluid (Table 1) in both the small intestine ($P = 0.002$) and large intestine ($P = 0.04$). There was no systemic infection detected in RITARD rabbits at 24 h (Table 2). The numbers of strain NRT36S in the large intestine at 48 h were significantly higher by the unpaired t test ($P = 0.03$) than the counts at 24 h postchallenge with 10^4 CFU of NRT36S. The differences in bacterial counts in the small intestine were not statistically different between the two time intervals ($P > 0.05$), although the counts were higher in all three rabbits at 48 h. The fluid volumes in the small and large intestines at 48 h postchallenge were not increased (Table 1). Both of the rabbits that died were bacteremic. Positive cultures were obtained from the mesenteric lymph node, liver, spleen, and heart blood by TCBS agar. The peritoneal culture was positive from one of the two rabbits (Table 2). No bacteremia was demonstrated in the live rabbit sacrificed at 48 h postchallenge.

After challenge with 10^3 CFU of strain NRT36S the mean counts of organisms (Fig. 9C) cultured from the large intestine (6.3 ± 2.2/g) at 24 h were significantly elevated compared with counts for 10^9 CFU of strain A-5 at 24 h ($P = 0.03$). The elevated bacterial count of NRT36S in the small intestine at 24 h postchallenge was not statistically significantly different from the bacterial count of A-5 ($P > 0.05$). The counts of strain NRT36S in the small intestine and large intestine (Fig. 3) at 72 h postchallenge were significantly

TABLE 2. Culture of non-O1 *V. cholerae* from the intestinal tract and organs of challenged RITARD rabbits (three rabbits per group)[a]

Strain	Dose (CFU)	Time postchallenge (h)	Small intestine	Large intestine	Mesenteric lymph node	Liver	Spleen	Heart blood
A-5	10^9	24	+	+	−	−	−	−
		72	−	−	−	−	−	−
2076-79	10^9	24	+	+	−	−	−	−
		72	+	+	−	−	−	−
NRT36S	10^9	24	+	+	+	+	+	+
	10^4	24	+	+	−	−	−	−
		48	+	+	+[b]	+[b]	+[b]	+[b]
	10^3	24	+	+	−	−	−	−
		72	+	+	−	−	−	−

[a] +, positive culture; −, negative culture by direct and APW enrichment.
[b] Low numbers of organisms recovered; positive culture required enrichment with APW broth.

elevated (P = 0.0005 and P = 0.0001, respectively) compared with A-5. There was no significant difference in the fluid volume in either the small intestinal or large intestinal fluid at 24 or 72 h compared to strain A-5. None of the rabbits challenged with 10^3 CFU of strain NRT36S were bacteremic at 24 and 72 h postchallenge.

DISCUSSION

Diarrhea was produced experimentally in the rabbit RITARD model with strain NRT36S at doses of 10^3 CFU, whereas rabbits inoculated with 10^9 CFU of strains A-5 and 2076-79 did not develop diarrhea. These results correspond with studies in human volunteers which demonstrated that strain NRT36S caused diarrhea, whereas volunteers infected with A-5 and 2076-79 were asymptomatic. The results indicate that the RITARD rabbit model may be a suitable animal model of non-O1 *V. cholera* diarrheal disease in humans.

In addition to discriminating between the virulence of different strains, the rabbit RITARD model was utilized to obtain insights into mechanisms of the pathogenesis of enteric disease caused by strain NRT36S. The time course of fluid accumulation in the small and large intestines and the counts of non-O1 *V. cholerae* recovered from the small and large intestines; the onset, duration, and severity of diarrhea (diarrhea score); and the histologic features of the intestinal

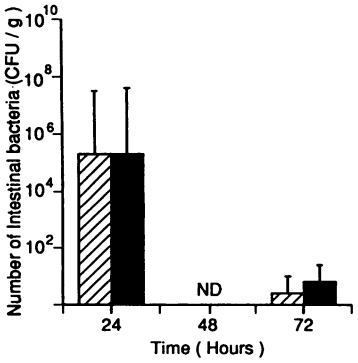

FIG. 8. Numbers of strain 2076-79 cultured from the small and large intestines of RITARD rabbits at 24 and 72 h postchallenge with 10^9 CFU. The counts of approximately 5.3 ± 2.2 CFU/g in the small and large intestines at 24 h postchallenge declined markedly to low counts of 0.4 ± 0.6 and 0.8 ± 0.7 CFU/g in the small and large intestines, respectively, at 72 h postchallenge. □, small intestine; ■, large intestine. ND, no RITARD rabbits were examined at 48 h postchallenge with strain 2076-79.

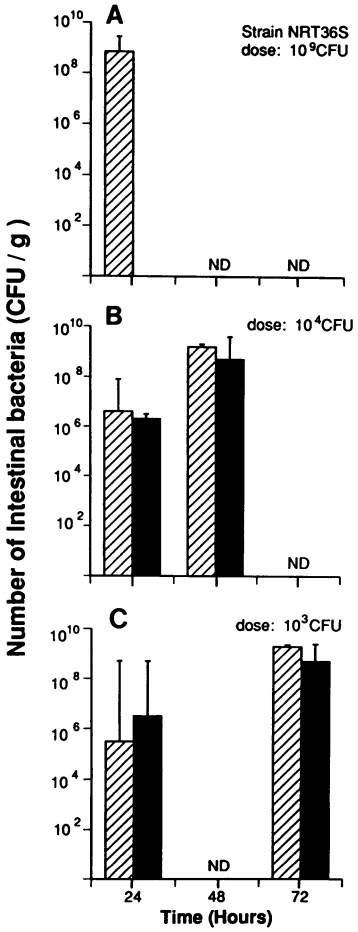

FIG. 9. Numbers of strain NRT36S recovered from the small and large intestines of RITARD rabbits. (A) High counts of NRT36S in the small intestine after challenge with 10^9 CFU. The large intestine was not cultured. All RITARD rabbits died at <24 h postchallenge. (B) High counts of NRT36S in small and large intestines at 24 and 48 h postchallenge. Two of the three RITARD rabbits died at <48 h postchallenge. No rabbits were examined at 72 h postchallenge. (C) Numbers of NRT36S in the intestines of rabbits challenged with 10^3 CFU of strain NRT36S. The counts in the small (mean, 5.5 ± 3.2 CFU/g) and large (mean, 6.3 ± 2.2 CFU/g) intestines at 24 h increased to mean counts of 9.3 ± 0.07 CFU/g in the small intestine and 8.7 ± 0.7 CFU/g in the large intestine at 72 h postchallenge. □, small intestine; ■, large intestine. ND, no rabbits survived for examination at >24 h postchallenge with 10^9 CFU or for examination at 72 h postchallenge with 10^4 CFU. No rabbits were examined at 48 h postchallenge with 10^3 CFU of strain NRT36S.

damage were analyzed to provide a better understanding of the pathophysiologic events of the intestinal and systemic disease caused by strain NRT36S in the rabbit RITARD model. We previously reported that strain 2076-79 caused diarrhea in RITARD rabbits (20). This was not confirmed in this study and may be attributed to our refinement of the model to discriminate more effectively between specific and nonspecific effects.

The histologic and scanning and transmission electron microscopic evaluations indicated that the mechanism(s) of mucosal damage and diarrhea by strain NRT36S includes bacterial invasion into the luminal epithelial cells, resulting in necrosis and exfoliation of the damaged cells. With lower doses (10^3 and 10^4 CFU), there was a propensity for the bacterial invasion and mucosal damage to be most pronounced or apparently limited to the large intestine. The initial events of focal and superficial bacterial damage were best appreciated in the rabbits inoculated with 10^4 CFU examined at 24 h postchallenge (illustrated in Fig. 2). This epithelial damage may explain the increased fluid volume in the large intestines of these rabbits and in the small and large intestines of RITARD rabbits challenged with 10^9 CFU. The severe necrotizing enterocolitis observed with higher doses of NRT36S in RITARD rabbits may be an experimental artifact induced by an overwhelming challenge dose. In a study of non-O1 *V. cholerae* isolates (from humans during an outbreak associated with raw oysters), scanning electron microscopy showed villus destruction in the ileum of infant rabbits after intraintestinal inoculation with doses of 10^5 CFU (17). While differences in inoculum size, model, and strains make direct comparisons difficult, these data confirm that some non-O1 *V. cholerae* strains have the potential for causing severe mucosal damage.

The hypothesis that invasion may be an important virulence mechanism by some strains of non-O1 *V. cholerae* is supported by in vitro adherence assays (24), which demonstrated large numbers of strain NRT36S adherent to CaCo-2 cells, whereas the avirulent strains A-5 and 2076-79 were adherent in significantly lower numbers. Strain NRT36S was also demonstrated ultrastructurally within membrane-bound vacuoles in the cytoplasm of CaCo-2 cells (24). Differences among strains in adherence to INT 407 cells has also been reported (27). Further evidence of the invasive capabilities of strain NRT36S was the development of systemic infection associated with mortality in RITARD rabbits inoculated with doses of strain NRT36S of $\geq 10^4$ CFU. No bacteremia was observed following inoculation of 10^3 CFU, indicating a dose relationship in the occurrence of bacteremia and severity of intestinal damage in the RITARD model.

The reasons for increased small intestinal fluid volume in RITARD rabbits at 24 h postchallenge with 10^4 CFU of strain NRT36S are not readily explained since histologically no epithelial necrosis was seen with this inoculum size. There may have been sufficient mucosal damage, not appreciated histologically, or another virulence mechanism(s) may have been operating. Previous studies have reported a number of bacterial toxins, including cholera toxin, El Tor toxin, Kanagawa hemolysin, shigalike toxin, cytotoxic responses in Y-1 and or CHO tissue culture cells of undetermined etiology, hemolysis of rabbit erythrocytes, and NAG-ST toxin in non-O1 *V. cholerae* organisms (2, 6, 12, 13, 15, 18, 27, 36–38). Strain NRT36S lacks cholera toxin, Kanagawa hemolysin, and shigalike toxin, although it does produce NAG-ST (20). Further studies with isogenic mutants will be needed to clarify the role of these toxins in the diarrheal response. While the severity of clinical diarrhea (diarrheal score) and intestinal histologic damage progressed with longer duration postchallenge in RITARD rabbits administered 10^4 CFU (i.e., 48 h versus 24 h postchallenge) and 10^3 CFU (72 h versus 24 h postchallenge), the fluid volumes in the small and large intestines decreased (Table 1). Again, these results cannot be explained until we have a better understanding of the pathophysiologic mechanisms underlying the diarrheal response.

Our data also show a clear difference in the colonizing ability of NRT36S compared with A-5 or 2076-79. Previous studies with non-O1 *V. cholerae* strains in the RITARD model noted that strains isolated from patients with diarrheal illness consistently colonized the rabbit intestine, while strains isolated from the environment did not (30). We observed similar results with strain A-5 (isolated from shrimp). However, data for strain 2076-79 present some ambiguities. This strain was isolated from a patient with diarrhea but did not cause illness in volunteers. It colonized volunteers at levels comparable to those of strain NRT36S, but in this study, it was not a good colonizer in RITARD rabbits. The mechanisms underlying an apparent relationship between diarrheal disease and colonization remain to be determined. It is possible that the pathogenesis of diarrheal disease involves mechanisms such as adhesion-dependent toxicity as described by Ofek et al. (23) and coregulation or coexpression of the factors responsible for illness with adherence and/or colonization factors, analogous to the relationship seen between cholera toxin and the TcpA pilus (34). As an alternative but less likely possibility, it has been proposed (based on work with rabbit models of *Escherichia coli* diarrhea) that small intestinal colonization alone is sufficient to elicit a diarrheal response (26).

In summary, the human volunteer studies, the RITARD challenge experiments reported in this study, and the CaCo-2 cell adherence studies are consistent in demonstrating the virulence of strain NRT36S compared with strains 2076-79 and A-5. These investigations further suggest that bacterial invasion plays a role in the pathogenesis of at least some non-O1 *V. cholerae* diarrheal disease. However, it is likely that gastrointestinal illness caused by non-O1 *V. cholerae* is multifactorial. Since non-O1 *V. cholerae* make up a heterogeneous collection of organisms, there may also be multiple virulence factors within and among isolates. Demonstration that invasion (and toxins and colonization) is a necessary factor causing diarrheal disease will require construction of appropriate isogenic mutants. In these and similar investigations, the RITARD model described in this study and the CaCo-2 tissue culture adherence and invasion tests reported previously provide potentially valuable assays for assessing disease mechanisms of non-O1 *V. cholerae*.

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