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Oral Immunization of Rabbits with Enterotoxigenic *Escherichia coli* Protects against Intraintestinal Challenge

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The development of a successful oral vaccine against enterotoxigenic *Escherichia coli* depends upon the identification of appropriate protective antigens which can be delivered effectively to intestinal mucosa. We have determined in a modified RITARD model the relative protection against intraintestinal challenge afforded by oral immunization with live enterotoxigenic *E. coli* carrying different candidate antigens. Studies were done with both wild-type strains and genetically manipulated strains of enterotoxigenic *E. coli* (parent strain E1392/75 2A) which carried plasmids containing intact heat-labile toxin (LT) gene sequences or various mutations of the LT genes. Immunizations were done by orogastric tube inoculation on days 0, 7, and 14; challenges were done on day 33. Protection against diarrhea with a homologous challenge was found to be 84 to 100% ($P < 0.01$). Protection against diarrhea with challenges in which specific antigens could be tested included the following: (i) O and H antigens (O6:H16), 87 to 100% protection with different *E. coli* strains with identical O and H antigens ($P < 0.01$) but no protection against a heterologous challenge; (ii) LT or the B subunit of LT only, approximately 50% protection ($P < 0.02$). These findings suggest that O antigens are highly protective in this model but afford only serotype-specific protection and that the B subunit (with or without the A subunit) affords less protection but confers cross-protection against heterologous strains producing LT. This model should be useful in further defining appropriate protective antigens for candidate enterotoxigenic *E. coli* vaccine strains.

Prospects for the control of acute infectious diarrheal disease through the use of live attenuated oral vaccines seem promising. Experimental studies have suggested that the secretory immune system of the gut can be stimulated most effectively by microbes actively colonizing the mucosal surfaces of the intestinal tract and releasing appropriate protective antigens. Live attenuated rotavirus vaccines of animal origin have been shown in the field to be protective against clinically important rotavirus diarrheas in children (17). Live oral attenuated *Vibrio cholerae* strains have been found protective in volunteers (Editorial, Lancet ii:722–723, 1986), and nonliving *V. cholerae* oral vaccines have been found to be protective in an endemic cholera area (3). Live attenuated oral vaccines against shigellosis are being developed and tested in animals and volunteers (2). In all of these situations, of concern are the safety of the vaccine and the degree of protection it provides. For species containing antigenically diverse strains, it will be more difficult to produce broad-spectrum protection. Success is more likely to be achieved in protection against *V. cholerae* infections, where only two cross-reacting serotypes and a single antigenic enterotoxin are involved.

Enterotoxigenic *E. coli* (ETEC) are known to be antigenically diverse, and therefore the problems of vaccine design are many (12). ETEC are found in at least 10 or more relatively common O serogroups (many more have been described with less frequency); they possess one of at least four known colonization factor antigens (CFAs), and more are being continually described (9). Furthermore, one of the enterotoxins they produce, the heat-stable toxin (ST), is not antigenic in its natural state. Fortunately, the single heat-labile enterotoxin (LT) is immunologically identical (or nearly so) in all ETEC which produce it.

Candidate ETEC vaccines will thus have to include strains which carry the most common cell wall antigens and CFAs as well as an inactive form of LT. It seems highly unlikely that even the most common ETEC antigens can be packaged in a single strain. Therefore it is important to be able to determine the degree of protection offered by known candidate antigens and to consider how these may be combined optimally in a protective vaccine. At present, this is being done in human volunteer studies. However, because of the complexity of the issue, an efficient animal model would greatly facilitate this work. We describe here a modification of our original RITARD model for study of ETEC-induced acute diarrheal disease and a series of oral immunization and RITARD challenge studies which further define the protective role of the cell wall antigens and the LT of ETEC.

MATERIALS AND METHODS

Animals. Outbred New Zealand White rabbits of both sexes were obtained from a single supplier and weighed between 1.6 and 2.5 kg. They were acclimated in the animal care facilities for 1 week.

Bacterial strains. *Escherichia coli* strains were obtained from the culture collections of the authors. The naturally occurring strains (Table 1) were human isolates from diarrhea patients; many have been used and reported previously. A series of genetically manipulated strains (kindly supplied by James Kaper) (Table 2) were prepared from a single parent strain, E1392/75 2A (O6:H16), which had spontaneously lost sequences encoding LT and ST but which retained sequences expressing CFA/II (CS1 and CS3) (10). A further derivative of this strain, E1392/75 7A, had lost the plasmid encoding CFA/II. Into these strains were transformed plas-

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TABLE 1. Sources and descriptions of naturally occurring *E. coli* strains used in this study[a]

Strain	Serotype	Colonization factor	Enterotoxin	Source
408-3	O78:H12	Neg	LT-ST	Diarrhea patient, Calcutta, 1968
408-4	O78:H12	NT	Neg	Same patient as 408-3
B_2C	O6:H16	CFA/II	LT-ST	Diarrhea patient, Vietnam, 1970
H10407	O78:H11	CFA/I	LT-ST	Diarrhea patient, Dhaka, 1969
M408-C_1	O6:H16	CFA/II	LT-ST	Travelers' diarrhea patient, Morocco, 1977
04404-C_3	O159:H44	Neg	LT only	Diarrhea patient, Arizona, 1981
03526-C_1	O25:H^-	Neg	LT only	Diarrhea patient, Arizona, 1981
21720-C_5	O27:H20	NT	ST only	Diarrhea patient, Arizona, 1981
03780-C_5	O166:H15	NT	ST only	Diarrhea patient, Arizona, 1981
M130-C_3	O6:H16	NT	Neg	Control patient, Morocco, 1977
Rab-C_4	Negative	NT	Neg	Normal rabbit isolate, 1978

[a] Neg, Negative; NT, not tested.

mids containing intact LT gene sequences or various mutations of the LT genes. These plasmids were prepared by Walter S. Dallas and are described in detail elsewhere (5). Briefly, pWD600 contains human LT genes from *E. coli* H74-114 cloned on a 5.4-kilobase *Pst*I fragment in pBR322. pWD601 is a derivative of pWD600 in which the DNA sequences from the *Xba*I site in the *eltA* cistron in the *Sma*I site in the *eltB* cistron have been deleted. *E. coli* containing pWD601 are negative in both Y1 adrenal cells and GM1 enzyme-linked immunosorbent assay and are thus LT-A^- and LT-B^-. The *elt* sequences remaining on the plasmid could theoretically encode in frame a hybrid peptide composed of amino acids 1 through 10 of the A subunit and 54 through 103 of the B subunit, but this has not been confirmed by peptide analysis. pWD605 is a derivative of pWD600 in which the *Sst*I site in *eltB* has been destroyed by treatment with the Klenow fragment of DNA polymerase I. The result of this mutation is a frameshift at sequences encoding the first residue of the mature B subunit. *E. coli* containing pWD605 are negative in both Y1 adrenal cells and GM1 enzyme-linked immunosorbent assay and are thus LT-A^+ and LT-B^-. The final plasmid, pWD615, is a derivative of pWD600 in which the *Xba*I site of *eltA* was filled in with Klenow fragment, thus inserting a stop codon at sequences encoding residue 12 of the A subunit. *E. coli* containing this plasmid are negative in Y1 adrenal cells but positive in GM1 enzyme-linked immunosorbent-assay and are thus LT-A^- and LT-B^+.

These genetically manipulated strains have also been tested for the presence of shigalike toxin and found to have only low levels (Alison O'Brien, personal communication), similar to other nontoxigenic *E. coli* strains.

Bacterial inoculum. Brain heart infusion broth was inoculated from frozen stock cultures and incubated at 37°C for 15 h on a roller drum at 50 rpm. This culture was used to inoculate (1%) a 2-liter flask containing 400 ml of casein-yeast extract medium, which was then placed in a shaking water bath at 37°C at 100 rpm for 4 to 5 h. The culture was centrifuged at 6,000 × *g* for 10 min, and the pellet was suspended in 10 ml of sterile phosphate-buffered saline (PBS). The bacterial concentration was estimated in a spectrophotometer at 540 nm and diluted in PBS for use as the inoculum. Viable plate counts were made on MacConkey agar.

RITARD model procedure. At the time this study began, the RITARD procedure originally developed for *V. cholerae* (14) was used. Briefly, this consisted of (i) ligating the cecum, (ii) making a temporary tie of the distal ileum, (iii) inoculating the bacterial suspension into the proximal jejunum, and (iv) removing the temporary ileal tie at 2 h.

Since the procedure did not result in comparable high attack rates of lethal watery diarrhea when ETEC were used for challenge, the procedure was modified as follows. The animals were given only water for 24 h preceding the challenge. Just before surgery, each animal was placed on a surgical board and given 0.1 ml of fentanyl citrate-droperidol in the thigh muscle and 2 ml of lidocaine infiltrated intradermally along the abdominal midline. A midline incision 4 to 5 in. (ca. 10 to 25 cm) long was made into the peritoneal cavity under the local anesthesia, and the cecum was brought out through the incision and ligated with no. 11 umbilical tape as close to the ileum-cecum junction as possible without compromising either the continuity of the intestinal tract or the blood supply. The upper small intestine was identified, and 10 ml of sterile PBS was injected at this point into the jejunum and used to wash the small intestine by allowing gravity and gentle finger manipulation to drain the fluid distally into the large intestine. A temporary tie (slip knot) with umbilical tape was then placed around the distal ileum near the mesoappendix. The bacterial suspension (10 ml) was then injected into the upper jejunum at the same area where the previous PBS had been injected. The contents of the peritoneal cavity were then returned, and the incision was partially closed, being sure that the loose ends of the slip knot were brought out through the incision and clipped to the skin with metal skin clips. The animals were kept on the surgical boards and 90 min later were given 2 ml of tincture of opium intraperitoneally through a small sterile infant feeding tube attached to a syringe that was easily introduced into the peritoneal cavity through the partially closed inci-

TABLE 2. Genetically manipulated *E. coli* strains used in this study[a]

Strain	Colonization factor	Enterotoxin[b]
E1392/75 2A(pWD600)	CFA/II	A^+ B^+
E1392/75 2A(pWD601)	CFA/II	A^- B^-
E1392/75 2A(pWD605)	CFA/II	A^+ B^-
E1392/75 2A(pWD615)	CFA/II	A^- B^+
E1392/75 7A(pWD600)	Neg	A^+ B^+
E1392/75 7A(pWD601)	Neg	A^- B^-
E1392/75 7A(pWD605)	Neg	A^+ B^-
E1392/75 7A(pWD615)	Neg	A^- B^+
E1392/75[c]	Neg	Neg

[a] All are derived from the same parent strain; all are serotype O6:H16 and ST negative. See the text for further details. Neg, negative.

[b] Designates the complete subunits of LT produced by the strain; strains listed as negative for the subunit still contain some residual amino acid sequences (see the text).

[c] Strain contains no plasmids.

sion. Thirty minutes later (2 h after the initial surgery) the slip knot was removed and the abdominal incision was sutured completely. The rabbit was then returned to its cage and given food and water immediately.

The rabbits were observed for 5 days for signs of weakness, diarrhea, and death. Each dead animal was autopsied, and the amount of fluid collected in the intestines was estimated to establish that diarrhea was the cause of death.

Colonization assays. Animals were sacrificed with intravenous Somlethal; immediately the abdominal cavity was opened, and 10-cm sections of both the jejunum (taken about 5 cm distal to the most proximal portion which could be identified) and the ileum (taken about 5 cm above the mesoappendix) were isolated with double ties. The individual sections were cut and removed intact; fluid in the intestinal loops was drained. The sections were then cut longitudinally and placed into 50-ml beakers containing 20 ml of sterile PBS and washed 10 times. The sections were then weighed and placed into a Potter-Elrehjem homogenizer containing 5 ml of cold PBS. The tissue was homogenized for 1 to 3 min at full speed and serially diluted in PBS. Colony counts were done on MacConkey agar. After incubation at 37°C for 24 h, colony counts were done and expressed as CFU per gram of tissue.

Oral inoculation procedure. The oral inoculum (4) was given through a stomach tube in the following manner. At time zero, the rabbits were given 50 mg of cimetidine into an ear vein. At 15 min, a stomach tube was placed per os, and 15 ml of a 5% solution of sodium bicarbonate was given. At 30 min, the stomach tube was again placed, and another 15 ml of 5% sodium bicarbonate solution was given, followed immediately by the bacterial inoculum suspended in 10 ml of PBS. At 60 min, 2 ml of tincture of opium was given intraperitoneally, and the rabbits were returned to their cages. No detectable diarrheal illness developed after this procedure in any rabbit.

Oral immunization and challenge procedure. The rabbits were orally inoculated as described above on days 0, 7, and 14. On day 33 (19 days after the last oral inoculation) the animals were challenged by the modified RITARD procedure described above. A similar number of control unimmunized rabbits were challenged on the same day as the immunized rabbits.

Statistical analysis. Bacterial mean counts were compared by using the Student *t* test; attack rates after challenge were compared by using the Fisher exact test. Protection was calculated by dividing the attack rate in immunized rabbits by the attack rate in controls and subtracting from 100.

RESULTS

Modification of the RITARD challenge procedure. With the original RITARD model as described for *V. cholerae*, challenge strains of ETEC produced recognizable diarrheal disease in greater than 75 to 80% of challenged rabbits; the lethal diarrhea rate, however, was not greater than about 50% at the largest doses used, 10^{11} (14). Of 12 rabbits challenged with 10^{10} cells of strain 408-3, 6 developed severe, lethal diarrhea; at a challenge of 10^{11} cells the attack rate of lethal diarrhea was the same (3 of 6). With strains B_2C, 10407, and M408-C_1, the lethal attack rates were between 17 and 33% (total of 4 of 17 rabbits) with these different strains.

We sought therefore to modify the RITARD model in hopes of achieving a higher rate of lethal diarrhea. We made two modifications to the model: (i) a PBS wash of the small bowel was made to remove any remaining intestinal contents that might have interfered with intestinal colonization, and (ii) tincture of opium was given 30 min before the removal of the temporary tie to inhibit intestinal motility and retard further the normal peristaltic mechanism for removal of organisms.

With these modifications, the attack rates for two of these strains (408-3 and B_2C) markedly increased; at challenges of 10^{11} organisms, the attack rate for any recognizable diarrhea was greater than 90%, and that of lethal diarrhea was greater then 80% (9 of 18 versus 48 of 56; $P = 0.004$, Fisher exact test) (Table 3). However, the attack rates of the other two strains of ETEC, 10407 and M408C1, did not change with the modified technique. Because of the increased attack rates of lethal diarrhea achieved with the modified RITARD in these two ETEC strains, this procedure was adopted for testing all of the remaining ETEC strains. The results from challenges with naturally occurring strains are shown in Table 3. With the exception of the two highly virulent strains (408-3 and B_2C) the attack rates of lethal diarrhea were comparable among the different ETEC, regardless of whether they produced both or only one of the two enterotoxins (between 11 and 40%). Challenge with nonenterotoxigenic *E. coli* or PBS resulted in detectable diarrhea in 6 of 34 challenges; three rabbits died after challenge, but none had a typical acute watery diarrheal illness.

Challenges with the genetically manipulated strains by the modified RITARD technique are shown in Table 4. None of these strains produced ST; they produced either whole LT ($A^+ B^+$) or only one of the complete LT subunits, and some produced, in addition, CFA/II. The strains producing complete LT were highly virulent, comparable to strains 408-3 and B_2C, with attack rates of lethal diarrhea of 85 to 100%. The presence or absence of the CFA/II antigen did not affect

TABLE 3. Modified RITARD challenge of *E. coli* strains from natural sources

Strain or challenge	No. of rabbits challenged	No. (%) with any diarrhea	No. (%) with lethal diarrhea[a]
Strains producing LT and ST			
408-3	56	54 (96)	48 (86)
H10407	6	4 (66)	2 (33)
B_2C	25	23 (92)	21 (84)
M408-C_1	6	4 (66)	1 (17)
Strains producing LT only			
04404-C_3	8	1 (11)	1 (11)
03526-C_1	5	2 (33)	1 (20)
Strains producing ST only			
21720-C_5	5	3 (60)	2 (40)
03780-C_5	4	3 (75)	1 (25)
Nonenterotoxigenic strains[b]			
408-4	12	3 (25)	2[c] (17)
M130-C_3	6	1[d] (17)	0
RabC_4	10	1 (10)	0
PBS only	6	1 (17)	0

[a] These rabbits are included also in those developing any diarrhea.

[b] All strains were negative in rabbit ileal loops, the adrenal cell assay, and the infant mouse assay.

[c] Two rabbits died on day 3 after challenge; only one rabbit had any fluid in the small bowel.

[d] One rabbit died in 24 h with distended cecum but no fluid in the small bowel.

TABLE 4. Modified RITARD challenge of genetically manipulated *E. coli* strains[a]

pWD series[b]	Strains producing: LT	CFA/II	No. of rabbits challenged	No. (%) with any diarrhea	No. (%) with lethal diarrhea
600	$A^+ B^+$	+	26	24 (92)	22 (85)
		−	12	12 (100)	12 (100)
605	$A^+ B^-$	+	6	3 (50)	2 (33)
		−	6	5 (83)	4 (67)
615	$A^- B^+$	+	10	1 (10)	0
		−	9	4 (44)	1 (11)
601	$A^- B^-$	+	16	8 (50)	6[c] (38)
		−	5	1 (20)	0[c]
	Negative[d]	−	6	0	0

[a] All strains are O6:H16, ST-negative derivatives of E1392/75.
[b] See Table 2 regarding further designation of strains.
[c] $P = 0.001$, comparing these two $A^- B^-$ strains.
[d] Strain has no plasmids.

the virulence in these strains. Strains that produced complete A subunit but not B subunit ($A^+ B^-$) also produced lethal diarrhea but at a decreased rate (33 to 67%), suggesting that the intact B subunit was not necessary to effect severe secretory diarrhea in this model. Strains that produced no A subunit but did produce the complete B subunit ($A^- B^+$) produced only low rates of mild diarrhea but essentially no lethal diarrhea. One strain, E1392/75 7A(pWD601), which produced neither complete A nor B subunit and was CFA/II negative, was avirulent; its matched strain, however, which did produce CFA/II but was otherwise identical, did unexpectedly produce a significant rate (38%) of lethal diarrhea. The strain that contained no virulence plasmids was also avirulent.

Dose responses and colonization studies. Dose responses were determined for the four naturally occurring strains (Table 5). Only one of the strains, 408-3, produced any lethal diarrhea below a challenge of 10^7 organisms. In all strains, a very large challenge, 10^9 organisms and above, was required for the production of high rates of both diarrhea and lethal diarrhea.

TABLE 5. Dose response to modified RITARD challenge with selected ETEC strains from natural sources

Strain	Challenge dose (CFU/rabbit)	No. of rabbits challenged	No. (%) with any diarrhea	No. (%) with lethal diarrhea
408-3	10^3	4	2 (50)	0
	10^5	5	4 (80)	2 (40)
	10^7	4	3 (75)	2 (50)
	10^9	5	3 (60)	2 (40)
	10^{11}	56	54 (96)	48 (86)
B_2C	10^7	6	2 (33)	0
	10^9	5	3 (60)	1 (20)
	10^{11}	25	23 (92)	21 (84)
H10407	10^7	6	0	0
	10^9	5	3 (60)	2 (40)
	10^{11}	6	4 (66)	2 (33)
M408-C_1	10^7	6	2 (33)	0
	10^9	6	3 (50)	0
	10^{11}	6	4 (66)	1 (17)

Colonization studies of the jejunum and ileum were done to determine whether the early ability to colonize the small bowel correlated with the virulence of the organisms. Counts of colonized ETEC at 6 h after the modified RITARD challenge are summarized in Table 6. In the jejunum, counts were essentially equal regardless of the virulence of the strain. In the ileum, however, two of the three poorly virulent strains had significantly reduced colony counts at this time.

Colonization of the small bowel was also determined after the oral immunization procedure (Table 7). These studies were done 18 h after the oral inoculation and are compared with similar data obtained after an inoculation by the original RITARD procedure. (This was done because of the small numbers of survivors found at 18 h when some of the strains were inoculated by the modified RITARD procedure.) Colonization after oral inoculation was clearly less, as compared with that with the RITARD procedure, in both the jejunum and the ileum at this time. However, each ETEC strain did colonize the ileum, which suggests that the oral route of administration might be sufficient to induce a local immune response.

Oral immunization and challenge studies. As the first step in developing an oral immunization schedule, we determined whether a single oral immunization would have any measurable effect on subsequent colonization, as had been shown with *V. cholerae* studies done in a similar way (4). In preliminary experiments in which two ETEC strains were used, first for a single oral immunization and then as an homologous challenge, no decrease in small intestinal colonization was seen (data not shown), which suggested that it was unlikely that protection would have been induced. Therefore, to maximize the possibility for protection, the oral immunization procedure was modified to include three oral inoculations at 10^{11} organisms each given 1 week apart, followed by a bacterial challenge of 10^{11} organisms given 19 days after the last oral immunization. All of the subsequent oral immunization RITARD challenges were done with this standardized time schedule. All challenges were done using the modified RITARD technique with strains known to be the most virulent in this model.

Homologous challenges. Homologous challenge studies (Table 8) were done with one naturally occurring strain, 408-3, and one genetically-manipulated strain, E1392/75 2A(pWD600), which produced both complete LT and CFA/II. In both of these experiments a high degree of

TABLE 6. Colonization of small intestine at 6 h after modified RITARD challenge with 10^{11} CFU per animal[a]

Strain	CFA antigen	Colonization[b] Jejunum	Ileum
408-3	I, II Neg	7.3 ± 0.2	8.2 ± 0.4
H10407	I	7.1 ± 0.1	7.4 ± 0.2
408-C_1	II	8.2 ± 0.3	7.1 ± 0.9
E1392/75 2A (pWD615)[c]	II	8.3 ± 0.7	5.5 ± 0.3[d]
E1392/75 7A (pWD601)[c]	Neg	7.8 ± 0.7	4.5 ± 0.6[d]
E1392/75 7A (pWD615)[c]	Neg	8.3 ± 0.2	7.2 ± 0.5

[a] Three rabbits were used for each challenge. Neg, Negative.
[b] Geometric mean $\log_{10}$ ± standard error (CFU per gram of tissue).
[c] Avirulent strains (see Table 4).
[d] $P = <0.05$, as compared with E1392/75 7A(pWD615).

TABLE 7. Colonization of small intestine at 18 h after either oral or RITARD challenge[a] with 10^{10} CFU per animal

Strain	Oral challenge			RITARD challenge		
	No. of rabbits	Colonization[b]		No. of rabbits	Colonization[b]	
		Jejunum	Ileum		Jejunum	Ileum
H10407	3	0.5 ± 0.9	4.30 ± 1.11	3	6.3 ± 0.9	6.6 ± 1.8
B_2C	3	0	1.32 ± 1.11	3	4.6 ± 1.9	5.9 ± 2.6
408-3	6	0.9 ± 1.0	4.55 ± 1.25	3	5.4 ± 0.3	6.5 ± 1.3
M408-C_1	3	2.7 ± 1.7	4.40 ± 1.12	3	6.6 ± 0.5	7.3 ± 1.1
E1392/75 2A(pWD600)	6	3.8 ± 0.7	4.41 ± 1.02	6	3.9 ± 0.8	5.2 ± 1.0

[a] Original RITARD method was used in these experiments.
[b] Geometric mean $\log_{10}$ ± standard error (CFU per gram of tissue).

protection (84 to 100%) was shown against the homologous challenge.

Heterologous challenges. Strains for immunization and challenge were chosen to determine the protection afforded by each of the candidate antigens: O and H and LT subunits. In all instances the challenge strains were highly virulent, reproducibly giving attack rates of lethal diarrhea of 80 to 100% (Table 9).

(i) O and H antigens. The immunizing strain was a genetically manipulated strain that contained no genes for LT, ST, or CFA. The challenge strains were fully virulent, naturally occurring ETEC; one was of the same serotype as the immunizing strain, and the other was of a heterologous serotype. A high degree of protection was seen with the homologous serotype challenge (Table 9, experiment 1); no protection was seen after the heterologous serotype challenge (Table 9, experiment 2).

(ii) LT subunits. In the LT subunit experiments, the immunizing strains antigenically shared only the LT subunit structure with the challenge strains; the immunizing strains were of a different serotype and were CFA negative. In these two sets of experiments, a significant protection (43 to 50%) was found, but this was considerably less than the 87% protection afforded by the serotype antigens (Table 9, experiment 1). The presence of the A subunit in one of the immunizing strains (Table 9, experiment 4) did not change the degree of protection from that produced by the B subunit alone (Table 9, experiment 3).

(iii) O and H antigens plus partial B subunit. In the experiments with O and H antigens and partial B subunit (experiments 5 and 6), the immunizing strain was of the homologous or heterologous serotype as the challenge strains (as in experiments 1 and 2). This immunizing strain, however, also carried pWD601, which contains a 49-amino-acid fragment of the B subunit. Complete protection was seen with the homologous challenge (Table 9, experiment 5); partial protection was seen in the heterologous challenge, similar to that seen with the intact B subunit (Table 9, experiments 3 and 4).

DISCUSSION

Virulence of ETEC and small bowel colonization. The newly described modified RITARD model allows the study of ETEC-induced acute lethal diarrhea in rabbits in a way more nearly analogous to that of *V. cholerae*. With the original RITARD adult rabbits were found to develop severe diarrhea at a considerably lower rate after ETEC challenge as compared with that after *V. cholerae* challenge (14). At a maximum challenge of 10^{11} organisms, only 30 to 50% of rabbits developed severe, lethal diarrhea after ETEC challenge, whereas nearly 100% developed similar diarrhea in response to a lesser dose (10^8 organisms) of *V. cholerae*. The explanation for this is not known, although it is possibly due to prior exposure of the animals to related *E. coli* antigens, thus rendering them partially immune. Another possibility was that colonization was less efficient with ETEC, perhaps based on nonimmune grounds, such as poorly defined species specificity. With the modifications introduced into the model, predictable and reproducible attack rates of approximately 85% or greater can now be produced with some ETEC strains. Presumably, this is due to more efficient colonization of the small bowel mucosa, possibly due to the initial washing of the small intestine which may remove nonspecific inhibitory substances (15), or the opium treatment, which further retards intestinal motility and thus would allow the organisms to be in contact with the mucosa for longer periods of time.

Because a high lethal diarrhea rate can now be produced, this model should be of use in testing possible immunologic

TABLE 8. Response to modified RITARD challenge in rabbits orally immunized with homologous strains[a]

ETEC strain[b]	Controls			Immunized			% Protection (*P*)	
	No. of rabbits	No. (%) with any diarrhea	No. (%) with lethal diarrhea	No. of rabbits	No. (%) with any diarrhea	No. (%) with lethal diarrhea	Against any diarrhea	Against lethal diarrhea
408-3	10	8 (80)	3 (30)	10	0	0	100 (<0.001)[c]	100 (0.105)[c,d]
E1392/75 2A(pWD600)	10	8 (80)	6 (60)	8	2 (25)	1 (13)	69 (0.031)[c,e]	78 (0.057)[c,f]

[a] All immunized rabbits were given 10^{11} *E. coli* orally on days 0, 7, 14 and challenged on day 33 with 10^{11} *E. coli*.
[b] See Tables 1 and 2 for descriptions of strains.
[c] Fisher's exact test with only concurrent controls.
[d] With historical controls also, 48 of 56 had lethal diarrhea ($P < 0.0001$).
[e] With historical controls also, 24 of 26 had diarrhea ($P < 0.0001$).
[f] With historical controls also, 22 of 26 had lethal diarrhea ($P < 0.001$).

TABLE 9. Response to modified RITARD challenge in rabbits orally immunized with heterologous strains of ETEC[a]

Oral immunizing strain (expt no.)	Virulence antigens in immunizing strain	Challenge strain	Virulence antigens in challenge strain	Known common antigens
Strains sharing ± O and H antigens				
E1392/75 7A (expt 1)	O6:H16[b] (no plasmids)	B_2C	O6:H16, LT-ST, CFA/II	O, H
E1392/75 7A (expt 2)	O6:H16[b] (no plasmids)	408-3	O78:H12, LT-ST, CFA negative[d]	None
Strains sharing only subunits of LT				
E1392/75 7A(pWD615) (expt 3)	O6:H16, A^- B^+	408-3	O78:H12, LT-ST, CFA negative[d]	B^+
408-3 (expt 4)	O78:H12, LT-ST, CFA negative	E1392/75 2A(pWD 600)	O6:H16, LT, CFA/II	A^+ B^+
Strains sharing B subunit fragment ± O and H antigens				
E1392/75 7A(pWD 601) (expt 5)	O6:H16[f]	B_2C	O6:H16, LT-ST, CFA/II	O, H, B subunit fragment
E1392/75 7A(pWD 601) (expt 6)	O6:H16[f]	408-3	O78:H12, LT/ST, CFA negative	Only B subunit fragment

[a] All immunized rabbits were given 10^{11} *E. coli* orally on days 0, 7, and 14 and challenged on day 33 with 10^{11} *E. coli*.
[b] All strains carry no plasmids and are A^- B^- and CFA/II negative.
[c] All *P* values determined by the Fisher exact test with concurrent controls.
[d] Strain 408-3 is negative for CFA/I and CFA/II.
[e] NS, Not significant.
[f] All strains carry plasmid which codes for 49 amino acids of the B subunit.

means of preventing diarrheal illness without using large numbers of animals.

One of the drawbacks of this model is the inability to predict, at present, which ETEC strains will be virulent in this model. A number of ETEC strains with similar known virulence properties (LT, ST, CFA, and O antigens) were tested, but not all were of the same virulence. Of four naturally occurring LT- and ST-producing strains, only two (408-3, B_2C) were highly virulent. Of two naturally occurring strains producing LT only, neither was highly virulent; however, two genetically derived strains, which also produced only LT, were highly virulent, equivalent to the strains producing LT and ST. Of two strains producing ST only, neither was highly virulent, although diarrhea was produced in half of the rabbits. A smaller number of nonenterotoxigenic strains have been examined. Although these appear to be benign in this model, one strain (408-4) did produce lethal diarrhea in 2 of 12 rabbits. This occurred during one experimental challenge only, and the explanation is not clear; this strain is clearly negative for LT and ST in all standard assays, including the rabbit ligated intestinal loop model, adrenal cell assay, and infant mouse assay. Some rabbits also developed detectable diarrhea after the surgical procedure alone with only PBS challenge, but this was mild and would not be confused with the severe watery diarrhea produced by ETEC infection. Thus the severe diarrheal disease was specific to challenge with certain enterotoxigenic organisms.

Of particular interest is the observation that the genetically manipulated *E. coli* strains that made intact A subunit were virulent in this model. This suggests that B subunit is not necessary for the A subunit to enter intact intestinal mucosal cells; as others have suggested, A can be introduced into the cell after nonphysiologic binding (6). To our knowledge these is the first data that suggest that this can occur in an intact model, although such strains have not been described in nature.

The highly lethal strains have retained their virulence in this model for as long as we have studied them, which at this point is about 3 years.

Studies were done to determine whether there were differences in colonization of the small bowel between virulent and avirulent ETEC strains and between strains with and without known colonization factors. These studies, unfortunately, were not conclusive. It appeared that ETEC strains colonized the rabbits well at 6 h after inoculation, regardless of their ability to subsequently produce lethal diarrhea. Colonization studies beyond this time were difficult to do because of the high death rate by 18 h in the highly virulent strains. Also there seemed to be no clear advantage for an ETEC to possess the CFA/I or CFA/II antigens as far as its ability to either colonize the small bowel or to produce diarrheal disease. Of the genetically manipulated strains, avirulent strains colonized to the same degree, whether or not they possessed the CFA/II antigen, and virulent strains produced high attack rates of lethal diarrhea, whether or not they produced CFA/II. The only exception which suggested that CFA/II might play a role in diarrhea production was in the pWD601 strains which were A^- or B^- but carried a plasmid which coded only for a partial B fragment. In these paired strains, the one that also produced CFA/II had a significantly higher attack rate of diarrheal disease, including lethal diarrhea. Previous studies have also suggested that CFA alone may result in mild diarrheal disease (13); lethal diarrhea, however, seems to be beyond this spectrum. A recent study, in which younger adult rabbits were used, also suggested that the CFA alone could result in mild diarrheal illness (18). Since this finding was not observed with similar paired strains (pWD615) which produce an intact B subunit, this finding is presently unexplained.

TABLE 9—*Continued*

Control			Immunized rabbits			% Protection (*P*)	
No.	No. (%) with diarrhea	No. (%) with lethal diarrhea	No.	No. (%) with diarrhea	No. (%) with lethal diarrhea	Against any diarrhea	Against lethal diarrhea
9	9 (100)	9 (100)	8	1 (13)	1 (13)	87 (0.0004)[c]	87 (0.0004)
10	10 (100)	10 (100)	10	10 (100)	7 (70)	0 (NS[e])	30 (NS)
19	16 (84)	14 (74)	19	9 (47)	8 (42)	44 (0.019)	43 (0.05)
10	10 (100)	10 (100)	10	5 (50)	5 (50)	50 (0.016)	50 (0.016)
10	9 (90)	9 (90)	9	0	0	100 (0.0001)	100 (0.0001)
18	18 (100)	18 (100)	18	8 (44)	7 (39)	56 (0.00017)	61 (0.00005)

Immunization and protection studies. The oral immunization procedure was similar to that used successfully in stimulating protective immunity against *V. cholerae* (4). With *V. cholerae*, however, a single oral immunization prevented intestinal colonization on a subsequent challenge; in the present study, a single oral immunization did not prevent subsequent colonization, and therefore a series of three doses was used in an attempt to maximally stimulate a protective response. Colonization studies with ETEC showed that oral inoculation was relatively inefficient at establishing small bowel colonization, and this may explain the differences seen with *V. cholerae*. The oral immunization procedure did not produce symptoms of diarrhea in the rabbits, in spite of the fact that large numbers of organisms (10^{11}) were inoculated; had these been given by the modified RITARD method, lethal diarrhea would have resulted.

The challenge studies successfully defined the protective nature of some of the virulence antigens of ETEC. (i) Protection afforded by specific cell wall antigens was high (85 to 100%) but serogroup specific. Since in these studies both O and H antigens were identical, it was not possible to be sure which of these two was more important. From previous studies cell wall antigens are known to stimulate strong protective immunity (16), and it is thus presumed that most of the protection came from the lipopolysaccharide cell wall antigen. (ii) The B subunit of LT also afforded significant protection, but of lesser degree (~50%). This protection, however, was broad spectrum and occurred with strains of different serotypes. The addition of A subunit to the immunizing strains which also produced B subunit did not add to the degree of protection seen. This corroborates other evidence (7) that the A subunit is only weakly antigenic.

Of considerable interest is the observation that protection was afforded by an *E. coli* strain which carried the pWD601 plasmid coding only for amino acids 54 through 103 of the B subunit. Although this strain was GM1 enzyme-linked immunosorbent assay negative and therefore designated B^-, it clearly resulted in protection comparable to those strains carrying the complete B subunit. This corroborates the data of Klipstein and co-workers, in which a synthetic peptide of LT-B consisting of amino acids 58 through 83 was antigenic when given orally to volunteers (8).

The ST antigen was not specifically examined as an immunogen in these studies; previous work has demonstrated that this small-molecular-weight peptide is not antigenic unless it is attached to a larger-molecular-weight carrier protein. One might expect, however, that when the challenge ETEC produced ST, diarrhea may have been possible in these otherwise immunized animals. This was clearly not the case, since nearly complete protection was provided even against strains that produced ST and LT as well as only LT. This also strongly suggests that colonization or elaboration of ST was being prevented in these protected animals.

Similar results have been recently reported with the RITARD model used to study the protective antigens of ETEC (1). In these studies, both the initial immunization and the challenge were done by the original RITARD method. Therefore, only survivors of the first immunizing challenge could be used for the second challenge, and most of these immunized animals were thus also convalescent from diarrheal disease. This technique, although similar to the present one, is limited in a practical way because only one immunizing challenge can be used because of the necessity for doing two surgical procedures. Furthermore, in these studies, lethal diarrhea was infrequently seen, and protection was measured by the prevention of any diarrhea rather than by the prevention of lethal diarrhea. In these studies (1), however, similar findings of homologous protection and cross-protection with LT were found; in addition, protection with CFA/I was demonstrated. Those studies further support the value of this model in defining suitable antigens and immunizing strains which may ultimately be useful in the development of suitable oral ETEC vaccines for humans.

It is still not clear how directly comparable these studies in rabbits will be to the human illness induced by ETEC. In the one human volunteer challenge study, in which persons who had recovered from an initial ETEC challenge were rechallenged with an ETEC of a different serotype but which shared LT antigens with the original strains, no protection

was seen (11). This suggests that LT was not a protective immunogen as measured under the conditions of the study. In other studies in which *V. cholerae* O antigens and enterotoxin have been used to study protective immunity, the protection afforded by the enterotoxin has been significant (3).

Since protection can clearly be defined in this model, additional parameters need to be more clearly defined: measurement of serum and secretory small intestinal antibodies, the optimum numbers of bacterial organisms and doses required for protection, and the length of protection. These will be the subject of additional work with this model. One class of important virulence antigens, the CFAs, have not been studied yet in this model for two reasons: a highly virulent strain of ETEC which produces CFA/II and is of a serogroup other than O6 is still being sought, and similar genetically manipulated *E. coli* strains which carry the other known CFA antigens (CFA/I and E8775) are not yet available.

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