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Experimental Enterotoxin-Induced *Escherichia coli* Diarrhea and Protection Induced by Previous Infection with Bacteria of the Same Adhesin or Enterotoxin Type

CHRISTINA M. ÅHRÉN AND ANN-MARI SVENNERHOLM*

Department of Medical Microbiology, University of Göteborg, S-413 46 Göteborg, Sweden

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The diarrheal response to an initial and a second infection with *Escherichia coli* expressing various enterotoxins (the heat-stable toxin [ST] alone or in combination with the heat-labile toxin [LT]) and colonization factor antigens (CFA/I, CFA/II, or E8775-type) was studied in the reversible tie adult rabbit diarrhea model. An initial infection with high doses (1×10^{10} to 5×10^{11} bacteria) of the various strains regularly induced diarrhea which was usually self-limiting (only 7 of 85 animals died). The diarrheal response to equally effective doses of different strains producing both ST and LT (ST/LT) did not differ significantly with serotype or colonization factor antigen. ST/LT-producing strains appeared to induce severe disease more regularly than ST-producing strains carrying the same adhesin. Previous infection with CFA/I-carrying, ST/LT-producing *E. coli* protected all animals reinfected with an otherwise highly diarrheogenic dose of the same strain as well as against challenge with a CFA/I-carrying, ST/LT-producing strain with different O-, K-, and H-antigens. Fecal excretion of bacteria was also significantly reduced in the protected animals, although not completely eliminated. When only one of the two antigens, CFA/I and LT, was shared by the immunizing and rechallenge strains, partial protection was evident consistent with independent antibacterial (anti-CFA) and antitoxic (anti-LT) immune mechanisms. Oral immunization with purified CFA/I significantly reduced fluid secretion in intestinal loops infected with CFA/I-carrying enterotoxigenic bacteria.

Diarrheal disease due to enterotoxigenic *Escherichia coli* (ETEC) represents a major world-wide health problem for humans as well as animals. The direct pathogenic mechanism can be attributed to the action of either or both of two entirely different enterotoxins, a heat-stable toxin (ST) and a heat-labile toxin (LT). However, colonizing ability for the small intestine involving close contact with the epithelium is also a prerequisite for the bacteria to produce disease (11). To this end ETEC possess specific adherence fimbriae (pili). Three such adhesins, the colonization factor antigens I and II (CFA/I and CFA/II [12, 16]) and E8775-type fimbrial antigen (29), have been identified on ETEC strains isolated from humans, and additional ones may emerge (7, 10, 19).

Much interest has been focused on the possibility of developing effective immunoprophylaxis against ETEC infection, but so far no human vaccine against ETEC is available. The great heterogeneity of *E. coli* strains producing enterotoxins means that such a vaccine should contain antigens which are present on ETEC of different serotypes. These could be colonization factors or enterotoxins since these antigens have been shown to induce protective immunity. Immunization of pregnant gilts or cows with purified K88 or K99 adhesins resulted in significant protection to their suckling piglets and calves against otherwise lethal challenge with ETEC strains bearing the homologous fimbrial antigen (1, 24). In addition, Evans et al. (15) have recently demonstrated protection against CFA/I-carrying *E. coli* by a sequence of parenteral and oral immunization with purified CFA/I. In rabbits, antibodies to CFA/I and CFA/II as well as anti-LT antibodies have conferred passive protection against LT-producing strains carrying the corresponding CFA (2, 16, 25, 28). Antibody responses to CFAs as well as LT have also been demonstrated in humans after both natural and artificially induced ETEC diarrhea (8, 22, 23).

In previous studies we have shown that antibodies against CFAs cooperate synergistically with enterotoxin antibodies for protection against infection with ETEC bacteria carrying the homologous CFA in the rabbit ligated ileal loop model (2, 27). In these studies, however, the mechanical defense against bacterial colonization obtained by peristalsis was not operating.

Recently, the reversible intestinal tie adult rabbit diarrhea (RITARD) model (26) was described, in which immunocompetent rabbits were susceptible to intestinal colonization with *Vibrio cholerae* or human-associated ETEC bacteria with a minimum of alteration of the intestines. The aim of this study was to evaluate the diarrheal response in the RITARD model to *E. coli* strains representing different adhesin types, enterotoxin types, and serotypes and to study the protective effect of immunization with live bacteria carrying potentially protective antigens, e.g., CFAs and LT, against diarrheogenic ETEC strains.

MATERIALS AND METHODS

Animals. New Zealand White rabbits of both sexes were used. Their weight ranged from 1.7 to 2.2 kg when studies began, unless otherwise indicated.

Bacteria. ETEC bacteria of various serotypes and enterotoxin types and carrying different adhesins were used (Table 1). All ETEC strains were isolated in Bangladesh from patients with diarrhea; strain H10407 was kindly provided by D. G. Evans (Houston, Tex.), strains E 1392-75 and C916/82 were provided by B. Rowe (London, United Kingdom), and the remaining strains originated from an epidemiological study at the ICDDR,B, Dhaka, Bangladesh (L. Gothefors, C. Åhrén, B. Stoll, D. K. Barua, F. Ørskov,

* Corresponding author.

TABLE 1. Diarrheal response in rabbits after challenge with graded doses of different ETEC strains according to the RITARD model

Strain	Serotype	Adhesin	Enterotoxin	Diarrheal response[a] to challenge dose:		
				1.0×10^{10}	10.0×10^{10}	50.0×10^{10}
H10407	O78:K80:H11	CFA/I	ST/LT	18/20[b]		
258909-3	O128:K⁻:H?	CFA/I	ST/LT	1/4	23/24	
259325-1	O8:K40:H9	CFA/II	ST/LT	0/2	2/4	7/8
E1392-75	O6:K15:H16	CFA/II	ST/LT			5/6
C916/82	O25:K?:H?	E8775-type	ST/LT			4/5
304688-2	O128:K⁻:H1	CFA/I	ST			12/17
325542-1	O78:K⁻:H12	CFA/I	ST			1/5
EF-6549	O?:K?:H?	Negative	Negative			0/8

[a] Number of animals with diarrhea or death/total number tested.
[b] A fivefold-lower dose was nondiarrheogenic.

M. A. Salek, A.-M. Svennerholm, J. Infect. Dis., in press). A nonhemagglutinating, nonenterotoxigenic, H_2S-producing *E. coli* strain isolated in feces was kindly provided by E. Falsen (Göteborg, Sweden). Stock cultures were suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% (wt/vol) glycerol and stored in working samples at −70°C. A new sample originating from the same culture of each strain was used in each experiment. Before use, strains were grown on CFA agar (14) at 37°C overnight, inoculated in Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract medium (16), and grown still at 37°C overnight. After being washed in phosphate-buffered saline (PBS), the cultures were adjusted to the desired bacterial concentration according to optical density. For control purposes, the bacterial concentration was also calculated by viable cell count on blood agar plates after serial dilution in PBS. To ascertain that the proportion of fimbriated bacteria in the cultures used for challenge was constant throughout the study, the mannose-resistant hemagglutination titer (see below) of the cell cultures was determined before use.

Antigens. Purified CFA/I was prepared from a flagellum-deficient mutant of strain H10407 (kindly provided by D. G. Evans), and purified CFA/II was prepared from strain E 1392-75 as described earlier (13). Highly purified B subunit of cholera toxin (CT-B) was obtained from Institut Mérieux, Lyon, France.

Antisera. CFA antisera were produced by giving rabbits three subcutaneous (s.c.) injections with 100 μg of purified CFA/I or CFA/II in each at 2- to 3-week intervals; the initial two immunizations were given with Freund complete adjuvant. The animals were bled by heart puncture 1 to 2 weeks after the last immunization.

Agglutination tests. Mannose-resistant hemagglutination tests of human group A and bovine erythrocytes for identification of CFA/I- and CFA/II-bearing bacteria, respectively, were performed as described previously (14) by suspending the bacterial cells in a 3% erythrocyte suspension containing 1% D-mannose on a glass slide. For quantitation of the mannose-resistant hemagglutination titer, serial dilutions of the bacterial cultures used for challenge experiments were mixed with an equal volume of the respective type of erythrocyte suspension. Agglutination of bacteria with CFA/I- or CFA/II-specific antiserum was performed on slides as previously described (27).

RITARD model. The RITARD model described by Spira et al. (26) was used, with slight modifications. Before challenge, the rabbits were starved for 24 h, but were given water ad libitum. Before surgery, animals were anesthetized with 5 mg of acepromazine (Agrivet, Uppsala, Sweden) intramuscularly followed by 20 mg of pentobarbital (ACO, Sweden) per kg intravenously. The incision site was anesthetized with 0.5% lidocaine with 0.0001% epinephrine bitartrate (Astra, Södertälje, Sweden). The cecum was brought out through a midline incision and ligated permanently as close to the ileocecal junction as possible. Ten milliliters of the bacterial inoculum was then injected into the duodenum, the ileum having first been obstructed with an umbilical tape tie about 5 cm proximal from the mesoappendix. The ileal tie was gently removed 2 h after the bacterial injection, and the animals were then returned to their cages and given food and water freely. The same technique was used for rechallenge, 7, 10, or 14 days after the initial infection. Animals not developing fatal illness were sacrificed by intravenous administration of pentobarbital within 10 days after the first infection or within 4 days after the last infection and autopsied to record the appearance of the gut.

Monitoring of disease. Rabbits were observed for diarrhea, weakness, or death every 1 to 4 h during the day up to 14 days after challenge. The time period during the night, when no observations were made, lasted for 8 to 10 h the first night after challenge and for 14 to 16 h the following nights. Rabbits were categorized as having no diarrhea, mild diarrhea (at least three loose stools), or severe diarrhea (multiple watery stools). Rectal swabs were collected one or two times daily and plated on blood agar to record the challenge organisms. The challenge strains were identified by typical *E. coli* colony morphology, mannose-resistant hemagglutination of human A or bovine erythrocytes, and by agglutination with specific anti-CFA antisera. The nontoxigenic strain was identified as an *E. coli* strain and by its ability to produce H_2S. The relative number of challenge organisms of all bacteria excreted in each specimen was scored from 0 to 3 as follows: 3, 100% of the colonies originated from the challenge strain; 2, at least 50%, but not all, of the colonies were of the challenge strain; 1, colonies of the challenge strain constituted at least 1%, but less than 50%, of all colonies; 0, less than 1% of the colonies originated from the challenge strain.

Animals developing fatal illness were autopsied, and the intraluminal fluid of the intestine was measured. A sample of the fluid was plated on blood agar to verify the presence of the infective strain.

Ten animals were sacrificed 24 to 70 h after challenge, and three 3-cm-long segments from the proximal and distal jejunum and distal ileum were removed from each rabbit. The segments were cut open and washed extensively in sterile PBS and then homogenized in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) as described previously (2). Viable counts were performed on the serially diluted

homogenates, and the number of bacteria recovered per centimeter of intestine was determined.

Rabbit ileal loop model. Protection studies by means of the rabbit ileal loop model were performed essentially as described earlier (2). Twenty-one to twenty-seven 5-cm-long intestinal loops were tied in each animal. Four graded doses of *E. coli* bacteria (strain H10407 or 258909-3, 4×10^7 to 1×10^{10} bacteria per ml) were tested in duplicates randomly positioned in each animal. Ileal loops injected with 1 ml of PBS were included in each animal as negative controls. The bacterial dose giving half-maximum fluid accumulation, the 50% effective dose (ED_{50}), was calculated for each challenge strain as the geometric mean of the ED_{50}s in four to six individual animals. The protective effect of immunization, referred to as the protection factor, was determined as the ratio between the mean ED_{50}s of the immunized and concurrently tested control animals.

Immunization with purified antigens. Groups consisting of three to six rabbits, weighing 0.9 to 1.2 kg at the onset of immunization, were used. Two groups were given two s.c. immunizations with 100 μg of CFA/I in each 2 weeks apart, and two groups obtained three weekly peroral (p.o.) immunizations with 250 μg of CFA/I in each. In addition, one group was given three weekly p.o. immunizations with 250 μg of CFA/I as well as 250 μg of CT-B in each. The oral immunizations were given in 10 ml of 0.1 M $NaHCO_3$ through a gastric tube after 24 h of starvation. Equally sized control groups of age-matched animals were given PBS s.c. or 0.1 M $NaHCO_3$ p.o. One week after the last immunization, the animals were challenged according to the RITARD or the ileal loop model.

Statistics. Statistical analyses were carried out by the Fisher exact test for comparison of attack rates of diarrhea and by the Fisher randomization test (5) for comparison of onset and termination of diarrhea between different groups of rabbits. The endpoints of the observation periods when onset or termination of diarrhea was observed were used for calculations. To evaluate duration of the diarrhea, calculations were made both for maximal and minimal duration due to the observation periods. The numerical differences in bacterial fecal score at the first and second infection in the same animals were evaluated by the Sign test. The geometric mean ED_{50}s of immunized and control animals challenged with the ileal loop technique were compared by using the Student *t* test.

RESULTS

Determination of diarrheogenic dose. Groups of rabbits were given graded doses of different *E. coli* strains, representing various enterotoxin and adhesin types, to evaluate the bacterial doses needed to induce diarrhea in the RITARD model (Table 1). The effective dose of bacteria giving rise to diarrhea in most of the animals challenged was compared for various enterotoxigenic strains. As many as 10^{10} to 10^{11} bacteria were needed of the ST/LT-producing, CFA/I-carrying strains H10407 and 258909-3 to induce diarrhea in ≥90% of the infected animals. An even higher challenge dose, 5×10^{11} bacteria, was required to induce diarrhea in ≥80% of the animals challenged with three different ST/LT-producing strains carrying CFA/II or E8775-type fimbrial antigen (Table 1). For strains producing only ST, even higher challenge doses were needed; 5×10^{11} cells of the two strains tested only gave rise to diarrhea in 20 to 70% of the inoculated animals; 5×10^{11} cells of a non-CFA-carrying nontoxigenic *E. coli* strain did not induce diarrhea in eight animals tested (Table 1).

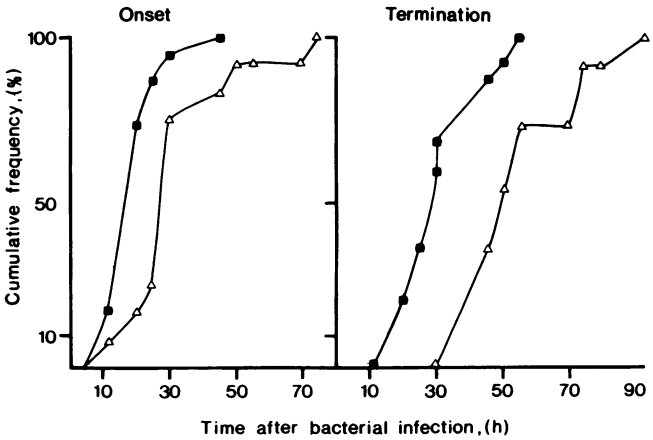

FIG. 1. Cumulative frequency of diarrheal onset and termination (when not preceded by death or sacrification) in rabbits challenged with an $ED_{\geq 80}$ of five ST/LT-producing strains (■; n = 54 [onset] and 41 [termination]) or an ED_{70} of a strain producing ST only (△; n = 12 [onset] and 11 [termination]) in the RITARD model.

Nature of diarrheal response. The nature of the diarrheal response after challenge in the RITARD model with an 80% or more effective dose ($ED_{\geq 80}$) of each strain of five different ST/LT-producing strains carrying different adhesins (Table 1) was investigated. Of the 57 animals that became ill, only 6 rabbits, all infected with the CFA/I-carrying strain H10407 or 258909-3, developed fatal disease. Three of these six rabbits died within 24 h postchallenge without passing diarrheal stools. At autopsy, their small intestines from ligamentum Treitz to the mesoappendix contained 80 to 119 ml of fluid, as compared with <30 ml in control animals given PBS only (26).

The majority (38 of 51) of the animals developing nonfatal disease passed watery stools, i.e., they developed severe diarrhea. No significant difference in time for onset (Fisher randomization test; $P > 0.21$) or termination (Fisher randomization test; $P > 0.10$) of diarrhea was seen between the groups of rabbits challenged with ST/LT-producing strains of different adhesin types or serotypes. Altogether, diarrhea had begun at 20 h postchallenge in 74% (40 of 54) and at 30 h in 94% (51 of 54) of the animals given ST/LT-producing strains. Onset of diarrhea later than 45 h postchallenge was not seen (Fig. 1). The diarrhea lasted for less than 24 h in at least 83% (34 of 41) of the animals and terminated within 55 h after infection in all except one rabbit, who finally died at 96 h.

Thirteen of 22 animals challenged with two CFA/I-carrying strains producing ST only developed symptomatic infection. This attack rate was significantly (Fisher exact test; $P < 0.003$) lower than that (41 of 44) observed after challenge with 5- to 50-fold-lower doses of ST/LT-producing bacteria carrying the same adhesin. The diarrheal response to the strains producing ST only was mild in general; eight rabbits purged loose stools, four had watery stools, and only one rabbit died from the disease. When an ED_{70} of one strain producing ST only was used, only two of the animals developed diarrhea within 20 h after challenge. Diarrhea had terminated in all animals at 93 h postchallenge (Fig. 1).

Bacterial adhesion. Ten animals developing symptomatic infection after challenge with the ST/LT-producing, CFA/I-carrying strain H10407 were sacrificed after 24 to 70 h to study adhesion of challenge bacteria in different parts of the small intestine. When bacterial counts were analyzed 24 h

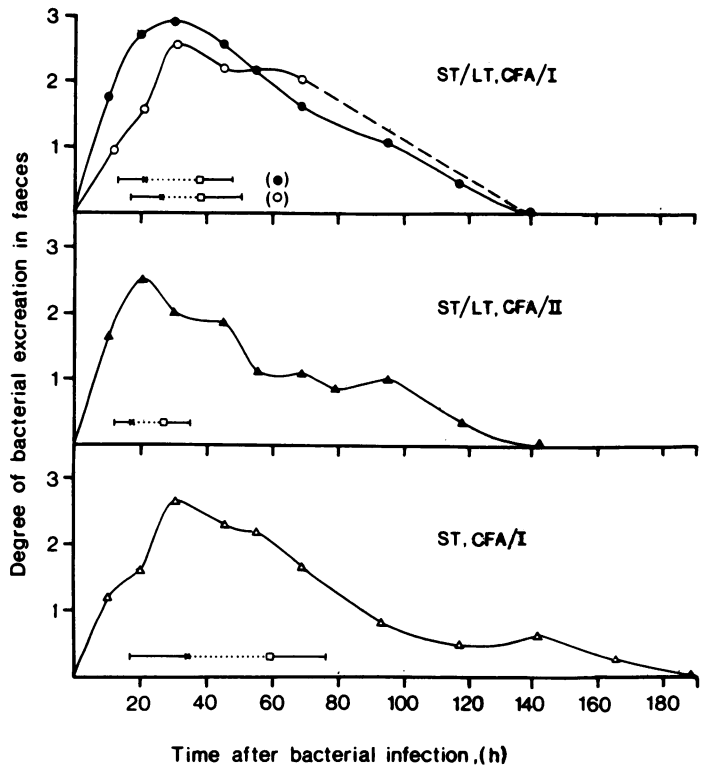

FIG. 2. Time course of bacterial excretion in feces after infection in the RITARD model with an $ED_{\geq 70}$ of ETEC strains representing different adhesin or enterotoxin types. The relative amount of challenge bacteria in each fecal specimen was scored as follows: 3, 100%; 2, ≥50% to <100%; 1, ≥1% to <50%; 0, <1% challenge bacteria. Each point represents the mean fecal score for specimens collected at a certain time from animals challenged with the same strain. Symbols: (●) 258909-3 (n = 14 to 21); (○) H10407 (n = 7 to 17); (▲) 259325-1 (n = 6 to 8); (△) 304688-2 (n = 6 to 10). The mean time (hours) ±1 standard deviation for onset (×) and termination (□) of diarrhea is shown.

after challenge from the washed and homogenized intestine from four different rabbits, only relatively few bacteria ($<10^5$ bacteria per cm of intestine) were recovered and then predominantly from the distal ileum. At 44 and 70 h postchallenge, no significant number of challenge bacteria was detected from any part of the small intestine in any of six rabbits studied.

Fecal excretion of bacteria. Fecal specimens were collected one or two times daily for 3 to 8 days after challenge to analyze shedding of the infective organisms. The different ST/LT-producing strains showed a similar excretion pattern regardless of the adhesin they carried. The infective organnisms predominated in feces during diarrhea, and as diarrhea ceased the relative amount of challenge bacteria in feces rapidly declined. No bacteria originating from the infective ST/LT-producing strains were detected in feces later than 140 h after challenge (Fig. 2).

In most of the animals challenged with the strains producing ST only, the bacterial excretion pattern was similar to that of the ST/LT-producing strains, except that the excretion usually continued for a longer period of time in correlation with the later-appearing diarrhea in these rabbits. There was no apparent difference in excretion pattern of asymptomatic rabbits and those developing diarrhea after infection with the strains producing ST only, except that excretion terminated earlier in rabbits not developing symptoms.

After challenge with 5×10^{11} bacteria of a nontoxigenic, non-CFA-carrying *E. coli* strain, bacterial excretion was seen for 2 to 3 days.

Protection induced by a previous ETEC infection. The protective effect against diarrhea or death of an initial infection with ETEC bacteria against a following reinfection with strains carrying the homologous adhesin or producing the same enterotoxins (or both) was studied in the RITARD model. At 7, 10, or 14 days after the initial infection, when shedding of the infective strain had ceased, the animals were rechallenged with highly diarrheogenic doses of the same or a heterologous strain. An initial immunizing infection with a CFA/I-carrying, ST/LT-producing strain induced solid protection against rechallenge with an equal dose of the same strain given 7 days later (zero of nine developed diarrhea) as well as against an O-, K-, and H-heterologous CFA/I, ST/LT strain (zero of five developed disease, Table 2).

To evaluate the possible protective role of antitoxic immunity, animals were given an immunizing infection with CFA/II-carrying, ST/LT-producing bacteria and challenged with O-, K-, and H-heterologous CFA/I-carrying, ST/LT-producing strains. Whereas the CFA/I-carrying, ST/LT-producing strains used for rechallenge induced diarrhea in 41 of 44 previously noninfected animals, only 3 of 7 developed a few loose stools after prior infection with the CFA/II-carrying, ST/LT-producing strains, i.e, the attack rate was significantly ($P < 0.01$) reduced (Table 2).

The possible protective effect of CFA/I against enterotoxin-induced *E. coli* diarrhea was evaluated by using a CFA/I-carrying strain not producing LT, but only the nonimmunogenic ST, for the initial infection. All but one animal (six of seven) were protected against reinfection with a dose of serotype-heterologous CFA/I-carrying, ST/LT-producing bacteria that induced disease in ≥90% of previously noninfected rabbits (Table 2). The nonprotected animal died

TABLE 2. Protection against diarrhea in the RITARD model by an initial infection with ETEC bacteria possessing the homologous adhesin (CFA) or enterotoxin or both

Immunizing infection		Challenge infection[a]		Protection P-value
Strain[b]	Attack rate[c]	Strain	Attack rate	
CFA/I, ST/LT	14/14	CFA/I, ST/LT	0/14	<0.001[d]
CFA/II, ST/LT	6/7	CFA/I, ST/LT	3/7	<0.01[e]
CFA/I, ST	11/15	CFA/I, ST/LT	1/7	<0.001[e]
CFA/I, ST	11/15	CFA/II, ST/LT	5/8	0.47[f]

[a] At 7, 10, or 14 days after the immunizing infection.
[b] CFA/I, ST/LT, strain 258909-3 or H10407; CFA/II, ST/LT, strain 259325-1 or E1392-75; CFA/I, ST, strain 304688-2.
[c] Number with diarrhea or death/total number infected.
[d] Zero of 14 at the challenge infection compared with 14 of 14 at the immunizing infection with the same strains and doses; Fisher exact test.
[e] In comparison with 41 of 44 in previously noninfected rabbits given the same strains and doses as used for the challenge infection; Fisher exact test.
[f] In comparison with 12 of 14 in previously noninfected rabbits given the same strains and doses as used for the challenge infection; Fisher exact test.

within 24 h of reinfection without developing diarrhea. However, at autopsy, no other cause of death was evident but the ETEC infection. When the CFA/I-carrying strain that produced ST only was used for the initial infection and either of two O-, K-, and H-heterologous CFA/II-carrying, ST/LT-producing strains were used for rechallenge, i.e., neither CFA/I nor LT was shared by the initial immunizing and challenge strains, five of eight animals developed diarrhea at reinfection. This attack rate was comparable to that (12 of 14) induced by the two CFA/II-carrying, ST/LT-producing strains in previously noninfected animals, indicating lack of protection at reinfection (Table 2).

Shedding of the challenge organisms was compared after an initial infection with CFA/I-bearing ETEC bacteria and after an identical reinfection in the same animals 7 days later. The relative number of CFA/I-carrying, ST/LT-producing bacteria in feces was significantly lower at 30, 45, and 69 h (Sign test; $P < 0.02$, $P = 0.02$, and $P = 0.03$, respectively) after the second infection than after the initial infection in the eight animals studied (Fig. 3).

Protective effect of immunization with purified antigens. The protective effect of active immunization with CFA/I

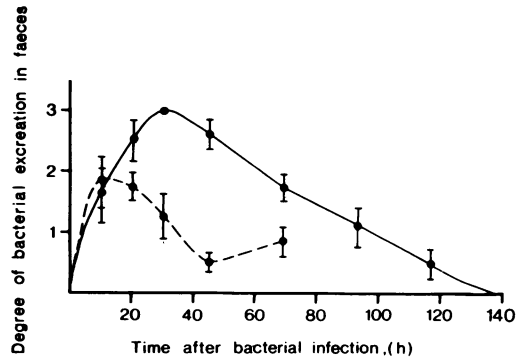

FIG. 3. Time course of bacterial excretion in feces after an initial infection (——) with a CFA/I-bearing, ST/LT-producing strain (258909-3) and after reinfection (-----) 7 days later in the same animals with the same strain and dose. The bacterial excretion was quantitated and scored as described in the legend to Fig. 2. Each point represents the mean ± standard error of the mean fecal score of eight animals at a certain time.

TABLE 3. Protection against challenge with graded doses of CFA/I-carrying ETEC bacteria in intestinal loops by p.o. and s.c. immunization with purified CFA/I

Immunization		Challenge with:			
		H10407		258909-3	
Route	Dose (no., μg)	ED_{50}[a] ($\times 10^8$)	PF[b]	ED_{50} ($\times 10^8$)	PF
s.c.	2, 100	4.7 ± 1.2	1	5.8 ± 1.1	1.6
p.o.	3, 250	24.5 ± 1.6	4.3[c]	17.8 ± 1.3	5.1[c]
Controls		5.8 ± 1.2		3.5 ± 1.3	

[a] Number of bacteria (mean ± standard error of the mean) giving half-maximal fluid accumulation in the loops.
[b] Protection factor (i.e., the ratio between mean ED_{50}s of immunized and control animals).
[c] Student t test; $P < 0.01$.

alone and in combination with enterotoxin antigen against ETEC infection was evaluated in the ileal loop or RITARD model or in both.

Rabbits given two s.c. injections with 100 μg of CFA/I in each did not respond with less fluid secretion in ileal loops to challenge with the CFA/I-bearing strains H10407 and 258909-3 than did nonimmunized control animals. On the other hand, three oral immunizations with CFA/I gave rise to significant protection (Student t test, $P < 0.01$) against both strains (Table 3). Neither the s.c. nor the p.o. immunization with CFA/I nor three p.o. immunizations with CFA/I together with cholera B subunit protected against diarrhea induced by challenge with an ED_{95} of the CFA/I-carrying, ST/LT-producing strain 258909-3 in the RITARD model.

DISCUSSION

The RITARD model was introduced by Spira et al. (26) for studying enteric infection by *V. cholerae* and ETEC. They showed that challenge with cholera vibrios as well as ST/LT-producing *E. coli* may result in watery diarrhea or death or both. In the present study we describe the diarrheal response to *E. coli* strains which produce ST/LT or ST only, which are of different serotypes, and which carry the CFA/I, CFA/II, or E8775-type fimbrial antigen. We also show that an initial ETEC infection may induce protection against reinfection with homologous and heterologous ETEC strains.

By giving as much as 1×10^{10} to 5×10^{11} bacteria of the ST/LT strains we were able to induce symptomatic disease, which was nevertheless rarely fatal, in most of the infected animals. The time for onset of diarrhea as well as the duration and severity of the disease after challenge with comparable effective doses of the various ST/LT-producing strains were similar irrespective of the adhesin or serotype of the bacteria. Although Spira et al. (26) reported symptomatic infection in somewhat lower frequencies (66%) after challenge with ST/LT-producing *E. coli*, the disease induced was usually longer lasting and more often fatal (34%) than that observed by us. This different susceptibility to ETEC observed in the two studies may be explained by genetic variation within the species (e.g., five different phenotypes have been distinguished in pigs by their different susceptibility to K88 fimbriated ETEC bacteria [3]) or by different breeding of the animals. However, it could also be due to different storage, cultivation, or preparation of the bacterial inocula, which are known to have an influence on the expression of ETEC adhesins and enterotoxin production (12, 16).

Our study shows also that *E. coli* producing ST only may

induce diarrhea in the RITARD model, although the attack rate was lower than for 5- to 50-fold-lower doses of ST/LT-producing strains carrying the same CFA. Whether ST/LT-producing *E. coli* strains more readily induce illness than ETEC producing only LT or ST in humans also is not clear, since contradictory results have been reported (4, 23).

Studies both in this and other laboratories (26) suggest that ETEC bacteria are less virulent than O group 1 cholera vibrios in the RITARD rabbit. Thus, considerably higher bacterial doses of ETEC than of *V. cholerae* are needed to induce symptomatic disease. Furthermore, the diarrhea resulting from ETEC infection is less profuse, of shorter duration, and less often fatal than after *V. cholerae* infection. This difference between the two species may reflect differences not only in toxigenicity but also in colonizing ability. Thus, *V. cholerae* has been found to colonize the rabbit intestine for several days (6; N. Lycke, A.-M. Svennerholm, and J. Holmgren, submitted for publication), whereas CFA-carrying ETEC bacteria seem to colonize the rabbit small intestine only transiently. Nevertheless, the ETEC bacteria appear to colonize when causing disease since bacterial excretion was seen for 6 to 8 days after challenge as compared with for 2 to 3 days after challenge with a nontoxigenic, non-CFA-carrying strain not causing disease. This comparatively poor colonization of CFA-carrying *E. coli* in rabbits may be explained by the fact that only ETEC strains associated with human infection were studied, since fimbrium-mediated adherence of ETEC appears to be highly species specific (17).

Prior colonization of the rabbit intestine by *V. cholerae* has been found to induce protection against reinfection with both homologous and heterologous cholera strains in the RITARD model (6). The establishment of nonfatal but diarrheogenic ETEC infection in this model made it possible to compare the diarrheal response also to an initial and a second ETEC infection. By using combinations of strains for the first and second infection that had one or more potentially protective antigens in common, the protective immunogenicity of, e.g., LT, CFAs, and maybe also other somatic antigens could be evaluated.

Our finding that an initial infection with ST/LT-producing *E. coli* carrying CFA/I induced solid protection not only against highly diarrheogenic doses of the homologous but also against a serotype-heterologous CFA/I-carrying ST/LT strain suggests that antigens other than O, K, and H, e.g., the CFAs or enterotoxins, may induce protective immunity. A protective role of ST is less likely, since this toxin appears to be nonimmunogenic unless coupled to a carrier (20). The significant protection observed when the only known antigen shared by the strains used for the initial and the second infection was CFA/I or LT also suggests that protective immunity may be induced by either or both of these antigens. This is further supported by the lack of protection when neither CFA/I nor LT was shared by the first and second challenge strains. A protective role of CFA/I is also suggested by the finding of reduced fecal excretion of CFA/I-fimbriated bacteria, probably reflecting decreased intestinal colonization, after reinfection in rabbits previously infected with CFA/I-carrying bacteria. These results of a considerably better protection induced by an homologous strain than with a strain only sharing LT and not any known CFA with the reinfective strain are consistent with findings in human volunteers (21).

Although immunization with purified CFA/I induced protection against ETEC-induced fluid secretion in ileal loops, no protection was found against challenge with CFA/I-carrying bacteria in the RITARD model. This lack of protection was probably due to the high challenge doses (ED_{95}) used in the RITARD model and the relative ineffectiveness of orally given inactivated antigens to induce a local immune response in rabbits (18). An increase in immunization dose or decrease in challenge dose or both may have resulted in protection also in the RITARD model. Thus, de la Cabada et al. (9) have reported reduced fluid accumulation in intestine of ETEC-infected rabbits after immunization with considerably higher doses of CFA/I and challenge with lower doses of bacteria than those used by us.

Although based on a small number of animals, our data suggest that effective protection against ETEC infection may be achieved if fimbrial and enterotoxin-induced immunity are operating simultaneously. Further studies are needed to evaluate whether the combined induction of antibacterial and antitoxic immunity by ETEC strains will result in considerably better immunity than that induced by toxin-deficient mutants of these strains, supporting our previous finding of a synergistic protective effect against ETEC of antibodies against the CFAs and enterotoxin antigen (2).

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