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Role of PCF8775 Antigen and Its Coli Surface Subcomponents for Colonization, Disease, and Protective Immunogenicity of Enterotoxigenic *Escherichia coli* in Rabbits

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The role of the PCF8775 antigen and its antigenic subcomponents, in particular, the coli surface (CS) antigen CS6, as colonization factors and protective antigens was studied in the reversible intestinal tie adult rabbit diarrhea model. This was done by testing the abilities of different mutants which carried one or two of the CS components to colonize the intestine and to induce protective immunity against reinfection with PCF8775-positive enterotoxin-producing *Escherichia coli*. Infection with enterotoxigenic *E. coli* carrying CS4-CS6, CS5-CS6, or CS6 alone induced diarrhea in 75% or more of the rabbits, whereas the corresponding nonenterotoxigenic mutants, as well as enterotoxigenic but CS-negative strains, induced diarrhea in only a few cases. Mutants carrying CS6 alone colonized the intestine equally as well as strains carrying CS4-CS6 or CS5-CS6 did, whereas CS-negative mutants were excreted in the stool for a significantly shorter period. Rabbits previously infected with mutants carrying CS6 alone or CS6 in combination with CS4 or CS5 developed diarrhea with a significantly lower frequency after reinfection with a normally highly diarrheagenic dose of enterotoxigenic CS4-CS6-positive *E. coli* bacteria than did animals immunized with corresponding CS-negative mutants. Fecal excretion of the rechallenge strain was also of considerably shorter duration than that observed after initial infection with corresponding strains in 27 of the 30 animals (90%) immunized with strains carrying CS6 alone or in combination with CS4 or CS5. Such reduced shedding of the challenge strain was only seen in a few rabbits (3 of 12) initially infected with CS-negative bacteria. These results suggest that the CS6 component of PCF8775 is a colonization factor in rabbits and that it is also capable of inducing protective immunity.

Diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) is a major health problem in children in developing countries and the leading cause of illness in travellers to these countries (4, 16). The bacteria cause disease by the ability to colonize and multiply in the small intestine and to produce a heat-labile enterotoxin (LT) or a heat-stable enterotoxin (ST). Colonization is usually associated with adhesion of the bacteria to the intestinal mucosa; such adhesion is often mediated by antigenically specific fimbriae (11).

In ETEC strains isolated from patients with diarrheal disease, distinct types of fimbrial antigens which cause mannose-resistant hemagglutination (MRHA) of different species of erythrocytes have been described. The best characterized of these are the colonization factor antigens (CFAs) I and II (CFA/I and CFA/II) and the PCF8775 antigen (6, 9, 25, 26). The reported prevalence of these colonization factors on human ETEC strains has varied from 32 to 86% in different studies (8, 10, 27). This variation may be partly due to geographic differences but also to a loss of CFA-encoding plasmids during storage and subculturing (1, 27).

CFA/I seems to be a homogenous antigen with a fimbrial structure. CFA/II and PCF8775, on the other hand, have been shown to consist of more than one antigen component each. For CFA/II, these components are called *E. coli* surface (CS) antigens CS1, CS2, and CS3 (5, 18), and for PCF8775, these components are called CS4, CS5, and CS6 (26). PCF8775-positive *E. coli* strains of serogroup O25 usually possess CS4 and CS6, whereas strains of serogroups O6, O92, O115, and O167 often carry the combination of CS5 and CS6 (14, 26, 27). CS6-only strains of serogroups O25, O27, O148, and O159 have also been described (15). These are also spontaneous variants carrying only CS6, while strains expressing CS4 or CS5 in the absence of CS6 have not been reported. CS4 and CS5 have been found to be fimbriate and give MRHA of human group A and bovine erythrocytes, while CS5-positive strains also hemagglutinate guinea pig erythrocytes. In contrast, CS6 has a nonfimbriate structure and does not cause MRHA (26).

In recent studies, we showed that ETEC strains carrying either CFA/I or CFA/II are capable of colonizing the intestine and causing diarrhea in the reversible intestinal tie adult rabbit diarrhea (RITARD) model (19). Furthermore, infection with CFA-carrying ETEC bacteria provided strong protection against subsequent challenge with a highly diarrheagenic dose of *E. coli* bacteria of a heterologous O group but carrying the same CFA, whereas at best only partial (anti-LT) protection was induced by ETEC strains carrying the heterologous CFA (2).

In the present study, we investigated the role of the PCF8775 antigen complex and its individual components for the pathogenicity of ETEC bacteria in the RITARD model. The colonizing ability and the ability to induce diarrhea of ETEC strains which carry different combinations of the CS4, CS5, and CS6 antigens and which are enterotoxigenic or nonenterotoxigenic were tested. We also determined whether infection with these strains conferred protection

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against disease and colonization by subsequent challenge with fully virulent PCF8775 antigen-carrying enterotoxigenic organisms.

MATERIALS AND METHODS

Animals. New Zealand White rabbits of both sexes from a single breeder were used. Their weights ranged from 1.7 to 2.2 kg when the studies began.

Bacteria. ETEC strain E11881A (O25:H42 $CS4^+$ $CS6^+$ ST^+ LT^+) and different mutants of this strain which lack CS4 or both of the CS components and are toxigenic or nontoxigenic were used together with ETEC strain E17018A (O167:H5 $CS5^+$ $CS6^+$ ST^+) (see Table 1). The origin and characterization of some of these strains have been described previously (26). Stock cultures of all strains were suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% (wt/vol) glycerol and stored in multiple aliquots at −70°C. A new aliquot was used in each experiment. Strains were grown in Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract medium (9) at 37°C overnight for 6 to 7 h and then inoculated on CFA agar (7) and grown at 37°C overnight. The bacteria were harvested and washed in physiological saline, and the cultures were adjusted to the desired bacterial concentration by optical density measurements. In each experiment, the bacterial concentration was also checked by viable cell count on blood agar plates after serial dilution in saline. All cultures were also tested for MRHA, and for positive strains, the MRHA titer was determined (see below).

Agglutination tests. MRHA of human group A erythrocytes for identification of PCF8775 antigen-bearing bacteria was performed as previously described (7) by suspending bacterial cells in a 3% erythrocyte suspension containing 1% D-mannose on a glass slide. To determine the MRHA titer, serial dilutions of the bacterial cultures were mixed with an equal volume of a 1% human A erythrocyte suspension containing 1% D-mannose in microtiter plates and the MRHA titer was read after incubation at room temperature for 15 min, followed by incubation at 4°C for 90 min.

RITARD model. The RITARD model described by Spira et al. (19) was used with slight modifications (3). In short, the cecum was brought out through a midline incision and ligated permanently as close to the ileal cecum junction as possible. A 10-ml sample 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 14 days after the initial infection. Animals which did not develop fatal illnesses were sacrificed 7 days after the last infection.

Monitoring of disease. For up to 14 days after the challenge, rabbits were observed several times per day and at least twice daily for diarrhea, other symptoms of disease, or death. Rabbits were categorized as having no diarrhea, mild diarrhea (two or more loose stools), or severe diarrhea (multiple watery stools). Rectal swabs were collected daily and plated on blood agar to detect the challenge organisms. The challenge strains were identified by typical *E. coli* colony morphology, agglutination with homologous anti-O group serum, and MRHA (CS4- and CS5-positive bacteria) and, for enterotoxin-producing strains, by assessing LT and ST production in the direct culture-in-plate GM1-enzyme-linked immunosorbent assay (ELISA) described below. Animals which developed fatal illnesses were autopsied, and 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.

GM1-ELISA of LT and ST production. LT and ST production from individual colonies of fecal cultures was determined by a recent modification of the GM1-ELISA procedure (21, 24). Five individual colonies from each blood agar plate were inoculated in individual wells of GM1-coated and bovine serum albumin-blocked microtiter plates containing 200 μl of Casamino Acids-yeast extract medium per well with 45 μg of lincomycin and 2.5 mg of glucose per ml (17). After the plates were incubated with shaking (200 rpm) at 37°C overnight, the medium was saved for ST determination (see below) and the GM1-ELISA plates were washed and then developed for GM1-bound LT by using a mouse monoclonal antibody against LT in the immunodetection step, followed by anti-mouse immunoglobulin–horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) and enzyme substrate as described (24). The presence of ST type a in the medium was determined with a GM1-ELISA inhibition method, recently described, by using a ST a-specific monoclonal antibody (24).

Serological analyses. Antibodies in rabbit serum against LT were studied by using the GM1-ELISA (23) as previously described. Antibodies against lipopolysaccharides (LPSs) were determined by an ELISA with crude LPS of the homologous strain as the solid-phase antigen (20). Antibodies against whole bacteria were titrated by means of an ELISA in which whole live bacteria were attached to polylysine-treated microtiter plates with the aid of glutaraldehyde. Polystyrene ELISA plates (Nunc, Roskilde, Denmark) were treated with poly-L-lysine (10 μg/ml) at 37°C for 30 min (50 μl per well). Live washed bacteria (10^{10} organisms per ml) were then added in 50-μl volumes to each well. After centrifugation at 2,000 rpm for 5 min on a plate centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.), 50 μl of a 0.125% glutaraldehyde solution was added to each well and the plates were incubated at room temperature for 15 min. The plates were then washed twice in phosphate-buffered saline, and a 100 mM glycine–0.1% bovine serum albumin solution (200 μl per well) was incubated in the plates at room temperature for 30 min. After repeated washing in phosphate-buffered saline, 100 μl of a 0.1% bovine serum albumin–phosphate-buffered saline solution was added to each well and the plates were frozen at −30°C until they were used.

The sera were tested in fivefold serial dilutions in each assay; for the GM1-ELISA and LPS-ELISA, an anti-rabbit immunoglobulin–horseradish peroxidase preparation (Dakopatts) was used as the enzyme conjugate, and for the whole bacterial ELISA, an anti-rabbit immunoglobulin–alkaline phosphatase conjugate (Jackson Laboratory, Avondale, Pa.) was used. The endpoint titer was determined as the reciprocal value of the interpolated dilution giving an absorbance value of 0.3 above the background when the enzyme was reacted with its substrate for 20 min and read at 450 nm (for the horseradish peroxidase conjugate) or for 100 min and read at 405 nm (for the alkaline phosphatase conjugate).

RESULTS

Diarrheal response. The importance of the PCF8775 antigen components for the pathogenicity of ETEC bacteria carrying these antigens was studied in rabbits. Different ETEC strains carrying CS4 and CS6 (E11881A) or CS5 and

TABLE 1. Influence of PCF8775 CS antigens and enterotoxin production on the ability of *E. coli* to cause diarrhea and to colonize in the RITARD model

Infecting strain	Presence of E8775 antigen			Presence of enterotoxin		Diarrheal response		Bacterial excretion	
	CS4	CS5	CS6	LT	ST	No. of ill rabbits/ no. infected	Duration (days)[a]	No. of rabbits excreting >2 days/no. infected	Duration (days)
E11881A	+	−	+	+	+	5/6	2.3 ± 0.44	5/6[b]	5.4 ± 0.58
E11881E	+	−	+	−	−	1/4	0.10 ± 0.10	4/4	4.0 ± 0.35
E11881C	−	−	+	+	+	3/4	0.80 ± 0.22	4/4	6.0 ± 0
E11881F	−	−	+	−	−	1/15	<0.10	15/15	5.2 ± 0.27
E11881D	−	−	−	+	+	2/4	0.50 ± 0.25	1/4	2.3 ± 0.27
E11881B	−	−	−	−	−	2/14	0.20 ± 0.15	5/12[c]	2.8 ± 0.33
E17018A	−	+	+	−	+	6/8	1.6 ± 0.39	8/8	4.1 ± 0.37

[a] Arithmetic mean ± the standard error for the whole group of rabbits.
[b] One rabbit died after 36 h of diarrhea.
[c] Two rabbits died within 48 h.

CS6 (E17018A), as well as a series of mutants of the former strain which lack either CS4 or CS4 and CS6 and are either enterotoxigenic or nonenterotoxigenic, were tested for the ability to induce diarrhea or to colonize the intestine in rabbits infected according to the RITARD model (Table 1).

Groups of rabbits were infected with the different strains by a dose of 10^{11} bacteria per rabbit; in preliminary experiments, this dose had been shown to give diarrhea in at least 75% of rabbits infected with strain E11881A. Of the rabbits given the E11881A strain ($CS4^+$ $CS6^+$ LT^+ ST^+), five of six animals developed diarrhea with a mean duration of 2.3 days (Table 1). Of the ill rabbits, one died from diarrhea, one had severe diarrhea, and three had mild diarrhea. Rabbits given enterotoxin-producing CS4- or CS4- and CS6-deficient mutants of this strain (E11881C or E11881D) developed diarrhea (three of four animals and two of four animals, respectively). However, this diarrhea was milder and of significantly ($P < 0.05$) shorter duration than that after challenge with the original strain. Only a few rabbits (4 of 33) given non-enterotoxin-producing mutants, with or without CS4 and CS6, developed mild diarrhea of short duration (Table 1). Rabbits given the ST-only-producing $CS5^+$ $CS6^+$ strain E17018A developed diarrhea in most (six of eight) cases (Table 1). The diarrhea was comparable in both severity and duration to that induced by the fully enterotoxigenic $CS4^+$ $CS6^+$ E11881A strain (in responding animals, the mean duration was 2.2 days).

Colonizing ability. Both E11881A and those mutants of this strain carrying CS4 and CS6 or CS6 alone, as well as strain E17018A ($CS5^+$ $CS6^+$), seemed to colonize and multiply in the rabbit intestine, as judged by monitoring the fecal excretion of the infecting organisms (Table 1). Thus, the mean time of shedding of these strains in the stool after infection in the RITARD model was considerably longer than 2.1 days, which we have found to be the mean excretion time for nonenterotoxigenic, non-MRHA fecal *E. coli* bacteria isolated from human normal fecal flora (12). There was no significant difference ($P > 0.1$) in the mean time of shedding between the enterotoxin-producing and corresponding non-enterotoxin-producing mutants.

The mean time of shedding of the strains carrying CS4 plus CS6 or CS5 plus CS6 in the stool did not differ significantly from that of the mutants carrying the CS6 antigen only ($P > 0.1$). Whereas all surviving rabbits (36 of 36) excreted these challenge strains for more than 2 days (mean, 5.0 days), only 37% of the rabbits infected with mutants lacking both CS4 and CS6 antigens shed the challenge strain in feces for more than 2 days (mean, 2.7 days). Direct comparison of the excretion patterns of the different strains showed that not only bacteria carrying CS4 and CS6 or CS5 and CS6 but also the mutants carrying CS6 only were excreted for a significantly longer period than the CS-negative mutants were (CS4-CS6 versus non-CS, $P < 0.001$; CS5-CS6 versus non-CS, $P < 0.01$; and CS6 only versus non-CS, $P < 0.001$ by the Student t test).

During the initial 2 days after the infection, the challenge strain was usually the only strain detected in the fecal cultures irrespective of whether it carried CS antigens or not. Later during the infection, however, the relative proportions of the challenge bacteria in the cultures were considerably higher for the strains carrying CS6 in combination with CS4 or CS5 and for the strains expressing CS6 only than for the CS-negative mutants (results not shown).

Serological responses. In spite of the different excretion patterns in feces, the various mutants of strain E11881A induced ELISA antibody responses in serum against homologous (O25) LPS and against whole E11881A bacteria that were comparable to the antibody responses of the strain they were derived from (Table 2).

A serum antibody response against homologous whole bacteria that was similar in magnitude to that induced by strain E11881A was also seen after infection with E17018A (data not shown). The response against the CS6-shared but O and H^- serotype-heterologous E11881A bacteria was, however, considerably lower than that against homologous E17018A bacteria.

Neither the strains nor the mutants tested induced a significant anti-LT response in any of the rabbits tested.

Protective immunity. The protective effect in the RITARD model of an initial infection with strain E11881A or the various mutants of this strain against reinfection with E11881A was evaluated. Rabbits that recovered from the initial infection with 10^{11} bacteria of the respective strains were rechallenged 14 days later with 10^{11} bacteria of strain E11881A.

Rabbits initially infected with either enterotoxin-producing or nonenterotoxigenic strains carrying CS4 and CS6 were all completely protected against diarrhea from the challenge strain; i.e., none of nine animals tested developed diarrhea after the reinfection (Table 3). Also, very few of the rabbits initially infected with either of the CS6-only mutants developed diarrhea after the challenge reinfection (3 of 13 animals). Similarly, only two of eight rabbits given the $CS5^+$ $CS6^+$ strain E17018A for the initial infection developed diarrhea after challenge with the O and H^- serotype-heterologous E11881A bacteria.

TABLE 2. Antibody responses in serum after infection with enterotoxin-producing and nonenterotoxigenic *E. coli* bacteria carrying different CS antigens

Infecting strain	PCF8775 antigen	Toxin production	No. of animals tested	Antibody titers against[a]:			
				Whole bacteria (strain E11881A)		O25 LPS	
				Day 0	Day 14	Day 0	Day 14
E11881A	CS4, CS6	LT, ST	6	125	2,000	20	1,000
E11881E	CS4, CS6	None	4	200	1,550	30	2,000
E11881C	CS6	LT, ST	4	100	2,100	20	2,000
E11881F	CS6	None	15	300	1,650	30	1,500
E11881D	None	LT, ST	4	100	9,500	20	6,000
E11881B	None	None	14	250	3,000	50	2,000
E17018A	CS5, CS6	ST	8	50	200	ND[b]	ND

[a] Geometric mean of titers of all sera in the respective groups are given. Antibody titers against LT were <5 for all strains tested at days 0 and 14.
[b] ND, Not done.

In contrast, most of the rabbits (9 of 13 animals) infected with the CS-negative mutants responded with diarrhea to the challenge infection. The rate of disease to rechallenge in these animals was significantly lower than that in animals immunized with either of the complete-PCF8775-antigen strains (E11881A, $P < 0.01$; E11881E, $P < 0.01$; and E17018A, $P < 0.05$ by the Fisher exact test). Also, the difference in the diarrheal responses to the challenge after immunization with CS-negative and CS6-only mutants was statistically significant ($P < 0.03$ by the Fisher exact test).

The protective effect of an initial infection with the various mutants against subsequent colonization of the E11881A strain used for reinfection was also studied. It was found that infection with bacteria carrying CS6 in combination with CS4 gave protection against excretion of the challenge strain in all rabbits; i.e., none of nine rabbits excreted the challenge strain for more than 2 days. Also, the CS6-only mutants seemed to protect against colonization by the challenge strain in most instances (Table 3). In contrast, 9 of 12 rabbits previously infected with the CS-deficient mutants excreted the reinfection strain in the stool for more than 2 days (Table 3). This rate was significantly higher than the excretion rates in rabbits previously infected with CS6-CS4-positive or CS6-only strains ($P < 0.001$ and $P < 0.01$, respectively, by the Fisher exact test).

Irrespective of whether the rabbits were protected against diarrhea, colonization, or both, most of them responded with an antibody response against O25 LPS, as well as against whole E11881A bacteria, after the reinfection. The magnitude of this response was similar in the different groups (data not shown).

DISCUSSION

In 1982, a new putative colonization factor, PCF8775, originally designated E8775, was described (25). This factor was later shown to consist of three immunologically distinct antigens, CS4, CS5, and CS6, the latter usually being expressed together with CS4 or CS5 (26). Subsequently, strains producing CS6 only were found (14, 15). In a preliminary study, we showed that rabbits infected according to the RITARD model with a high dose of PCF8775-positive ETEC bacteria developed diarrhea (3). We extend this observation in the present study by demonstrating that enterotoxin-producing *E. coli* carrying CS6 alone or together with CS4 or CS5 were considerably more effective than corresponding CS-negative ETEC mutants in inducing diarrhea in the RITARD model. Our finding that *E. coli* bacteria carrying one or more of the CS factors were shed for a significantly longer period in the stool than CS-negative mutants of the same serotype were suggests that PCF8775 is important for colonization of *E. coli* in the intestine.

The results showing that bacteria expressing CS4 or CS5 in addition to CS6 were not significantly more effective than the CS6-only mutants in colonizing the intestine suggest that CS6 is the most important of the PCF8775 subcomponents for colonization, at least in the rabbit intestine. However, with the limitations in the number of experimental animals in the different groups, it cannot be excluded that CS4 and CS5 may also contribute to colonization since mutants expressing CS4 only or CS5 only are not available. To evaluate the role of these factors, one possible test, although less conclusive, would be to evaluate Fab fragments of anti-CS4 and anti-CS5

TABLE 3. Protection against challenge with an $CS4^+$ $CS6^+$ ST- and LT-producing strain (E11881A) by an initial infection with different *E. coli* strains which carry one or more CS antigens and are enterotoxin-producing or nonenterotoxigenic

Immunizing infection			Challenge infection		
			Diarrhea		Bacterial excretion
Strain	PCF8775 antigen	Toxin production	No. of ill rabbits/ no. tested	Duration (days)[a]	No. of rabbits excreting for >2 days/no. infected
E11881A	CS4, CS6	LT, ST	0/5	0	0/5
E11881E	CS4, CS6	None	0/4	0	0/4
E11881C	CS6	LT, ST	1/3	≥0.7	2/3
E11881F	CS6	None	2/10	0.3	0/10
E11881D	None	LT, ST	2/3	1.3	2/3
E11881B	None	None	7/10	1.2	7/9
E17018A	CS5, CS6	ST	2/8	0.3	1/8

[a] Arithmetic mean for the whole group of rabbits.

antibodies, respectively, for passive protective effect against fluid accumulation in intestinal loops induced by ETEC bacteria expressing these CS components (2, 22); we have shown that bacterial adherence factors are important for full pathogenicity of ETEC strains in this model, and monoclonal antibodies against CS4, CS5, and CS6 are now available in our laboratory (Y. Lopez-Vidal and A.-M. Svennerholm, manuscript in preparation).

In previous studies, we showed that prior colonization of the rabbit intestine with ETEC bacteria may induce protection against reinfection with homologous as well as heterologous ETEC strains if bacteria of the same adhesin or enterotoxin type or both were used for the initial and the second infection (3). We found that an initial infection with enterotoxigenic as well as nonenterotoxigenic CS6-only mutants protected against subsequent challenge with a diarrhea-causing dose of ST- or LT-producing *E. coli* that expresses CS6 together with CS4. This finding suggests that CS6 is an important protective antigen. Although these experiments were performed with bacteria of the same serotype for the initial and the second infections, the protective role of O or H antigen, as well as other surface antigens, was marginal since corresponding CS-defective mutants did not induce significant protection against the challenge despite induction of high systemic anti-LPS responses. Further, evidence for a protective role of CS6 is the result that serotype-heterologous ST-only bacteria expressing CS6 and CS5 were as effective as the serotype-homologous, CS6-only mutants in conferring protection against subsequent challenge with the CS6- and CS4-positive ETEC bacteria.

Strong evidence that CS6 may induce anticolonization immunity in the gut is our observation that initial infection with CS6-positive bacteria resulted in significantly shorter excretion of the PCF8775-positive challenge strain in the stool than was seen after immunization with CS-deficient bacteria. Thus, whereas only 3 of 30 (10%) of the rabbits excreted the challenge strain for more than 2 days after immunization with CS6-positive bacteria, 75% of those initially infected with CS-negative mutants excreted the challenge strain for more than 2 days, which has been found to be the mean excretion time for *E. coli* bacteria isolated from human normal fecal flora (12).

The colonizing ability in rabbit intestine of PCF8775-carrying bacteria seems to be comparable to that of ETEC expressing CFA/I or CFA/II. Thus, infection in the RITARD model with high doses (1×10^{11} to 5×10^{11}) of ST- or LT-producing *E. coli* carrying either of the three colonization factors resulted in diarrhea of about the same severity and duration in >80% of the animals (3, 12). Furthermore, the mean fecal excretion time (4 to 6 days) observed for the different PCF8775-positive bacteria is comparable to that previously found for CFA/I- or CFA/II-carrying *E. coli* (3, 12) and significantly longer than the excretion time for normal fecal *E. coli* (12) or the CS-deficient mutants tested in this study.

The role of antitoxic immunity for the protection observed in rabbits previously infected with LT-producing bacteria is difficult to evaluate because of the small numbers of experimental animals. In previous studies, we showed that antibodies against CFAs cooperated synergistically with anti-LT antibodies for protection against challenge with LT-producing bacteria carrying the homologous CFA in the rabbit loop model (2, 12). Using the RITARD model, we also found that strains expressing LT as well as CFA were more effective than LT-negative, CFA-positive strains or CFA-negative, LT-positive strains in protecting against subsequent infection with enterotoxigenic CFA-positive challenge organisms (3). In the present study, however, the LT-negative mutants were in all cases as effective as the corresponding LT-producing strains in conferring protection against the challenge with LT- or ST-producing bacteria. One reason for this lack of significant antitoxic immunity may be that the original PCF8775-positive strain E11881A as well as the mutants of this strain all seemed to be poor LT producers in rabbit intestine. Thus, they all failed to induce significant anti-LT antibody responses in serum after the immunizing infection. This result contrasts with the finding for CFA/I- or CFA/II-positive, LT-producing strains, which induce a substantial anti-LT response on infection in the RITARD model (12; unpublished observations).

Our finding of very similar serum antibody responses against whole bacteria as well as against homologous LPSs after infection with CS-positive and CS-negative mutants is also surprising, since we previously found that strains capable of colonization in the RITARD model are usually also quite efficient in inducing antibody formation (12, 13) and give rise to considerably higher antibody levels than noncolonizing *E. coli* bacteria do (12). We cannot exclude, however, that the antibody response in the intestine, which is of primary importance for protective immunity against noninvasive intestinal pathogens, was considerably better after infection with the colonizing bacteria than it was after infection with the noncolonizing mutants, even though the serum antibody levels did not differ between the groups.

In conclusion, the results of this study suggest that CS6 is important for the colonizing ability of ETEC strains and that this antigen is a strong protective antigen. Whether inclusion of CS6 in a future ETEC vaccine is sufficient to induce effective anticolonization immunity against strains possessing other antigen components of PCF8775 needs to be further evaluated in experimental studies with animals, as well as in clinical trials.

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