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Cooperative Role of Antibodies against Heat-Labile Toxin and the EtpA Adhesin in Preventing Toxin Delivery and Intestinal Colonization by Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrheal disease in developing countries, where it is responsible for hundreds of thousands of deaths each year. Vaccine development for ETEC has been hindered by the heterogeneity of known molecular targets and the lack of broad-based sustained protection afforded by existing vaccine strategies. In an effort to explore the potential role of novel antigens in ETEC vaccines, we examined the ability of antibodies directed against the ETEC heat-labile toxin (LT) and the recently described EtpA adhesin to prevent intestinal colonization *in vivo* and toxin delivery to epithelial cells *in vitro*. We demonstrate that EtpA is required for the optimal delivery of LT and that antibodies against this adhesin play at least an additive role in preventing delivery of LT to target intestinal cells when combined with antibodies against either the A or B subunits of the toxin. Moreover, vaccination with a combination of LT and EtpA significantly impaired intestinal colonization. Together, these results suggest that the incorporation of recently identified molecules such as EtpA could be used to enhance current approaches to ETEC vaccine development.

Enterotoxigenic *Escherichia coli* (ETEC) strains comprise a genetically and phenotypically diverse group of organisms that share the ability to produce and effectively deliver heat-labile (LT) and/or heat-stable (ST) toxins to effector sites in the small intestine (14). Effective delivery of these toxins results in increases of intracellular concentrations of cyclic nucleotides, cyclic AMP (cAMP) and cGMP, respectively. Both toxins stimulate cellular kinases responsible for the phosphorylation of the cystic fibrosis transmembrane regulatory channel (CFTR) in the cell membrane (7, 17). Activation of the CFTR in turn leads to efflux of chloride into intestinal lumen with commensurate salt and water losses responsible for the watery diarrhea associated with these pathogens.

ETEC strains are a leading cause of diarrheal illness in developing countries, where they are responsible for hundreds of thousands of deaths, largely among young children. Although ETEC strains were discovered more than 40 years ago, the development of a broadly protective vaccine has been hampered by a number of factors (5, 35), including (i) the lack of complete sustained protection afforded by anti-LT immunity, (ii) the poor inherent immunogenicity of ST molecules, typically short peptides, and (iii) the antigenic heterogeneity of plasmid-encoded fimbrial colonization factors (20), one of the principle targets of ETEC vaccines to date.

Despite these challenges, the development of immunity following either a naturally occurring (4, 34) or an experimental (22) infection with ETEC has suggested that the development of an ETEC vaccine is technically feasible. In addition, recent molecular (30, 31) and immunoproteomic (26) studies have demonstrated that the pathogenesis of these organisms is considerably more complex than previously appreciated, and consequently additional antigens have emerged that might be targeted in development of a broadly protective vaccine.

In essence, ETEC vaccines must prevent the delivery of toxins to their cognate epithelial cell receptors. Theoretically, this might be accomplished by direct toxin neutralization or by interrupting steps that facilitate effective delivery of these molecules, an approach similar to that used in acellular subunit vaccines for pertussis (11, 18), which stimulate antibodies against both adhesins of *Bordetella pertussis* and pertussis toxin (36).

Interestingly, pertussis toxin, which, like LT, possesses ADP-ribosylating activity, plays a clear role in promoting mucosal infection (2, 6). Similarly, previous studies have suggested that in addition to stimulating fluid efflux into the lumen of the small intestine, LT likely plays a complex role in ETEC pathogenesis since it facilitates adherence to intestinal epithelial cells *in vitro* (21) and promotes small-intestinal colonization *in vivo* (1, 3).

Also similar to *B. pertussis*, ETEC produces multiple potential adhesins. The recently identified EtpA adhesin is a large extracellular protein belonging to the two-partner secretion family of molecules (16), for which filamentous hemagglutinin from *B. pertussis* is the prototype. Although vaccination with EtpA has been shown to induce significant protection against intestinal colonization (27, 28), further studies are needed to investigate the utility of EtpA as a component of subunit vaccines for ETEC. We demonstrate here that vaccination with LT and EtpA provides robust protection against intestinal colonization in a murine model, that EtpA is required for optimal delivery of LT to epithelial cells, and

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
H10407	Wild-type ETEC strain O78:H11; CFA/1; LT$^+$/ST$^+$; EtpA$^+$	12, 13
jf876	Δ*lacZYA*::Km^r derivative of H10407	10
jf1668	Isogenic *etpA* mutant of H10407; *etpA*::Cm^r	27
jf1697	jf1668 complemented with pJY019 expression plasmid	30
jf1700	jf1668 transformed with pBAD/myc-HisA vector control plasmid	30
jf1696	Top10(pJL017; pJL030): EtpA-myc-His expression strain	15, 30
Plasmids		
pJY019	pBAD/myc-HisA-based etpBAC locus expression plasmid	16
pBAD/myc-HisA	Arabinose-inducible expression plasmid	Invitrogen

[a] Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

likewise that antibodies against EtpA enhance LT neutralization *in vitro* compared to antitoxin alone.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in these studies are outlined in Table 1. Strains were grown in Luria broth with antibiotics as appropriate. Strain jf1668 containing a chloramphenicol resistance cassette interrupting the *etpA* gene (EtpA::Cm^r) was grown in chloramphenicol (15 μg/ml). Strain jf876 containing a kanamycin resistance cassette in the *lacZYA* locus (LacZYA::Km^r) was grown in kanamycin, (25 μg/ml). Strains containing recombinant plasmids were grown in the presence of ampicillin (100 μg/ml). EtpA expression from plasmid pJY019 was induced at a final concentration of 0.0002% arabinose as previously described (15).

Antibody purification. Polyclonal rabbit antisera against the A and B subunits of LT were provided by John Clements of Tulane University. Polyclonal rabbit anti-EtpA antibodies were produced against a recombinant 110-kDa fragment (16) as previously described. Antibodies were purified from nonimmune sera (control) and from immune polyclonal rabbit antisera using agarose-immobilized protein A-beads (Protein A Plus Agarose; Pierce). Briefly, 100 μl of 50% agarose slurry was added to 100 μl of sera, and the suspension was incubated at 4°C for 1 h. Antibodies were eluted from beads in 500 μl of 1 M Tris (pH 8.8), dialyzed with phosphate-buffered saline (PBS; pH 7.2), and concentrated to a final volume of ~100 μl, resulting in antibody concentrations of 200 to 400 μg/ml.

***In vitro* assessment of toxin delivery.** Caco-2 epithelial cell monolayers were infected with ETEC H10407, *etpA* mutant, or complemented mutants at multiplicity of infection of ~100 (bacteria/cell). Briefly, cultures of bacteria grown overnight in Luria broth from frozen glycerol stocks were diluted 1:100 and grown for 1 h. Then, 5 μl of each culture with or without antibodies as indicated was then added to confluent Caco-2 monolayers seeded into 96-well plates. For mutants complemented with expression plasmids, the medium was supplemented with 0.0002% arabinose. Two hours after inoculation, the monolayers were washed three times with tissue culture medium, and the medium was replaced with 100 μl of fresh medium/well and returned to the incubator (37°C, 5% CO$_2$) for 1 h. cAMP competitive enzyme-linked immunosorbent assay (ELISA; Amersham/GE Healthcare) was then used to examine the efficiency of toxin delivery as previously described (10).

LT and production of recombinant EtpA. The heat-labile toxin used in these studies was kindly provided by John Clements. Recombinant EtpA-myc-His was prepared as previously described from Top10(pJL017; pJL030) (15). Briefly, Top10(pJL017; pJL030) was grown overnight in ampicillin (100 μg/ml), diluted 1:100 into fresh medium, and grown to an optical density at 600 nm of ~0.5, and then the culture was induced with 0.0002% arabinose for an additional 2.5 h. The supernatant was recovered and sterile-filtered through a 0.22-μm-pore-size low-protein-binding fil-ter, concentrated by ultrafiltration using a 100K molecular-weight-cutoff filter, and equilibrated with metal affinity chromatography equilibration buffer (50 mM sodium phosphate, 300 mM NaCl [pH 7.0]) before binding to cobalt metal affinity resin (Talon; Clontech). Protein was eluted in 150 mM imidazole buffer as previously described and dialyzed against PBS in the presence of protease inhibitors (Sigma).

Mouse immunization with LT and LT-EtpA. In LT dose-ranging studies, groups of 10 mice (CD-1; Charles River) each were immunized intranasally as previously described (28) with either 2.5 or 1 μg of LT or an equal volume of PBS (controls) on days 0, 7, and 21. To evaluate EtpA combined with LT, mice were immunized intranasally on days 0, 7, and 21 with 1 μg of LT with or without 20 μg of EtpA-myc-His. The Institutional Animal Care and Use Committees of the University of Tennessee Health Sciences Center and the VA Medical Center approved the studies described here. All procedures involving mice complied with Public Health Service guidelines and the *Guide for the Care and Use of Laboratory Animals*.

Assessment of immune responses to LT and EtpA vaccination. Immune responses to LT and EtpA were determined by kinetic ELISA (37) as previously described (19). Briefly, ELISA wells were incubated at 4°C overnight with proteins at a final concentration of 4 μg/ml in 0.1 M NaHCO3 buffer (pH 8.6), washed the following day with Tris-buffered
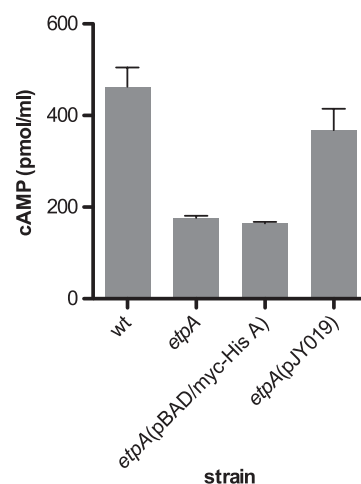

FIG 1 EtpA is required for optimal delivery of heat-labile toxin to intestinal epithelial cells. The strains used in these experiments were as follows: wt, H10407; *etpA*, jf1668 isogenic deletion mutant; *etpA*(pBAD/myc-HisA), transformed with cloning vector alone; *etpA*(pJY019), *etpA* mutant complemented with *etpA* locus expression plasmid. The graph depicts the mean cAMP values (*n* = 3) ± the standard errors of the mean.
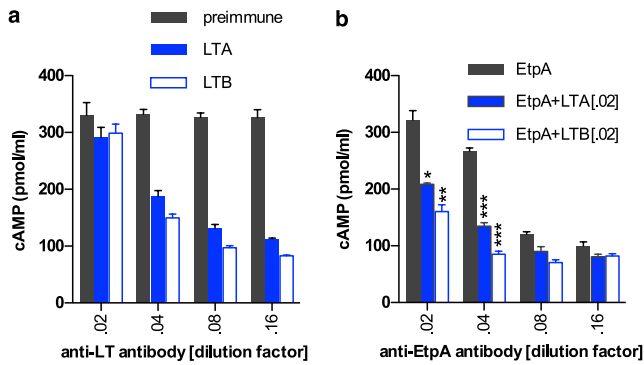
FIG 2 Additive role of anti-EtpA and anti-LT antibody in preventing toxin delivery by ETEC *in vitro*. (a) cAMP activation in Caco-2 cells infected with ETEC H10407 in the presence of antibodies against the heat-labile toxin A (LTA) and B (LTB) subunits. (b) cAMP activation in target Caco-2 cells after infection with H10407 in the presence of anti-EtpA antibody with or without subinhibitory concentrations (0.02 dilution factor) of anti-LTA or anti-LTB antibody. Comparisons between groups using unpaired (two-tailed, Welch's correction) *t* testing are indicated: *, $P \leq 0.02$; **, $P \leq 0.004$; and ***, $P \leq 0.001$.

saline containing 0.005% Tween 20 (TBS-T), and blocked with 1% bovine serum albumin (BSA) in TBS-T for 1 h at 37°C prior to the addition of the samples. Sera were diluted 1:500 in TBS-T with 1% BSA, and 100 μl was added to each ELISA well, followed by incubation at 37°C for 1 h. After three washes with TBS-T, horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgA, IgM, and IgG) was added at a final dilution of 1:5,000, followed by incubation for an additional hour before washing and development with TMB (3,3′,5,5′-tetramethylbenzidine)-peroxidase substrate (KPL).

Intestinal colonization studies in mice. The murine intestinal colonization model of ETEC infection (1) was used to examine the potential utility of LT and EtpA as immunogens. Briefly, mice were treated with streptomycin (5 g/liter of drinking water) to eliminate competing flora. At 12 h prior to challenge with bacteria, food was withheld, and streptomycin solution was replaced with sterile water. To reduce gastric acidity, mice received famotidine at 50 mg/kg via intraperitoneal injection 2 h prior to administration of bacteria by gavage. The mice then received 10^4 to 10^5 CFU of strain jf876 containing a Km^r marker in the *lacZYA* locus by gavage. At 24 h after challenge, the mice were sacrificed, and the small intestines were harvested. Ileal segments were treated with 5% saponin solution for 10 min, followed by plating of serial dilutions in PBS onto Luria agar plates

containing 25 μg of kanamycin/ml. Each experiment used 10 mice in each experimental group, and challenge studies were performed on day 42 after the first immunization.

RESULTS

EtpA is required for optimal delivery of LT to epithelial cells. Although the cellular effects of LT and the structurally similar cholera toxin have been carefully detailed, less is known about the mechanism by which LT is delivered efficiently to host epithelial cells. Studies to date have demonstrated that *etpA* is essential for the efficient adherence of EtpA-producing ETEC strains *in vitro* and for intestinal colonization in a murine model of infection (16, 28, 30). Although both bacterial adhesion and intestinal colonization are felt to be important prerequisites for efficient toxin delivery (14), the precise role of EtpA in toxin delivery was previously unknown. However, as shown in Fig. 1, *etpA* deletion mutants were less efficient at stimulating cAMP responses in target intestinal epithelial cells relative to wild-type ETEC (H10407) or the complemented mutant. These data support earlier assumptions regarding the importance of bacterial adhesion and provide further evidence that toxin delivery is a complex process involving multiple virulence genes (10, 31).

Antibodies against LT and EtpA impair toxin delivery by ETEC. The involvement of EtpA in toxin delivery by ETEC suggests that generating antibodies against this adhesin could complement existing approaches, including the direct neutralization of toxin. Interestingly, there is scant existing data regarding whether anti-LT antibodies can neutralize toxin as delivered by the bacteria, and earlier published reports had suggested that ETEC can deliver toxin in a way that circumvents neutralization (23, 39). Therefore, we conducted additional *in vitro* experiments to examine the utility of both anti-EtpA and anti-LT antibodies in mitigating toxin delivery to target intestinal epithelial cells. As shown in Fig. 2a, antibodies against either subunit of the heat-labile toxin inhibited the LT-mediated activation of cAMP in target epithelial cells in a dose-dependent fashion, up to a dilution of 1:50 (dilution factor of 0.02). Interestingly, anti-EtpA antibody exhibited a similar dose-dependent inhibition of toxin delivery that was significantly enhanced by the addition of subinhibitory concentrations of either anti-LTA or anti-LTB antibodies (Fig. 2b).

Immunization with purified heat-labile toxin impairs intestinal colonization. In earlier studies, we demonstrated that vaccination with outer membrane vesicles or commercial preparations
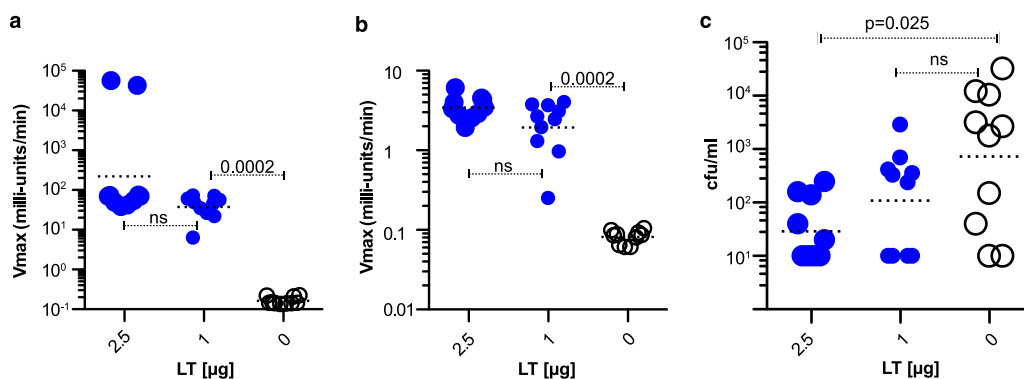

FIG 3 Immunization with heat-labile toxin impairs colonization in a murine model of ETEC infection. (a) Determination of total (IgG, IgM, and IgA) anti-LT serum antibody after intranasal administration of heat-labile toxin by kinetic ELISA expressed in mU/min. Dashed horizontal lines represent geometric mean values. (b) Anti-LT fecal IgA antibody after vaccination. (c) Reduction in intestinal colonization with ETEC (jf876) after immunization with either 2.5 or 1 μg of LT per dose (statistical comparisons by Mann-Whitney two-tailed nonparametric testing for $n = 10$ mice in all groups).
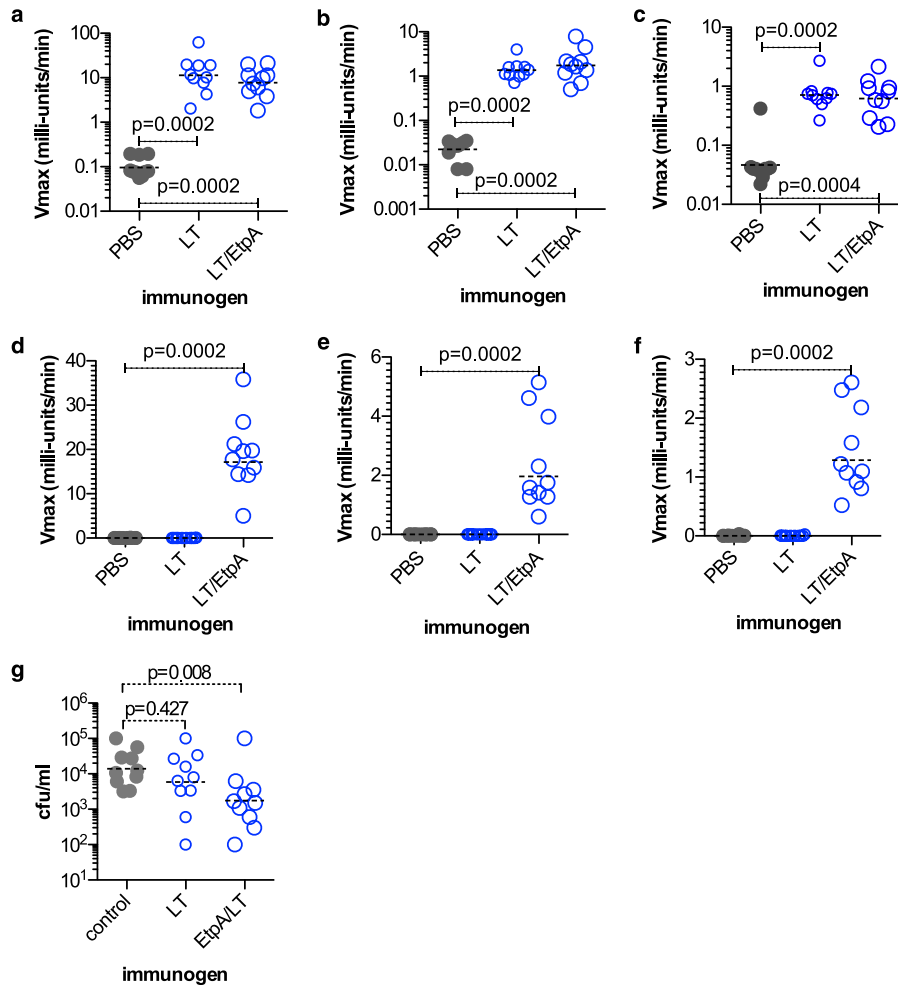
FIG 4 Vaccination with EtpA adjuvanted by LT stimulates mucosal immune responses to both proteins and protects against intestinal colonization with ETEC. (a to f) Antibody responses determined by kinetic ELISA after vaccination with either LT alone (1 μg/dose), a combination of LT (1 μg) and EtpA (20 μg/dose), or PBS controls. Horizontal lines indicate geometric means. (a) Total anti-LT serum antibody (IgG, IgM, and IgA). (b) Total fecal antibody responses to LT. (c) Anti-LT fecal IgA. (d) Serum anti-EtpA (IgG, IgM, and IgA). (e) Anti-EtpA total fecal antibody. (f) Anti-EtpA fecal IgA. (g) Intestinal colonization with ETEC strain jf876 after immunization with LT or EtpA/LT. Statistical comparisons by Mann-Whitney two-tailed nonparametric testing for $n = 10$ mice/group are indicated.

of LT delivered intranasally at doses of 5 or 2.5 μg significantly impaired intestinal colonization with ETEC (29). To examine the ability of LT to inhibit intestinal colonization at lower doses and to investigate its potential use as an adjuvant for other antigens, we conducted additional dose ranging studies with highly purified LT, followed by challenge with ETEC to examine the effect of immunization on intestinal colonization. As shown in Fig. 3, immunization with either 2.5- or 1-μg doses of purified LT resulted in both serum and fecal antibodies directed against the heat-labile toxin (Fig. 3a and b), with the higher dose of LT engendering correspondingly higher anti-LT fecal IgA (geometric mean kinetic ELISA values of 3.43 mU/min) compared to the 1-μg dose (1.94 mU/min). Similarly, the degree of intestinal colonization in these studies paralleled the mucosal antibody responses to LT in each group (Fig. 3c), with the higher dose affording significant protection against colonization relative to controls.

Vaccination with LT-adjuvanted EtpA prevents ETEC intestinal colonization. One potential approach to the development of ETEC vaccines is the identification of candidate immunogens that

could be combined in a recombinant subunit approach. The *in vitro* studies included here would suggest that combining an EtpA-based anti-adhesin strategy with LT-antitoxin could prove beneficial. To investigate this possibility, we vaccinated mice with either LT alone at the 1-μg dose or this dose of LT combined with EtpA. As shown in Fig. 4, vaccination of mice with either LT or the combination of antigens stimulated significant serum (Fig. 4a), as well as mucosal (Fig. 4b and c), antibody responses to the toxin. Likewise, vaccination with LT-adjuvanted EtpA stimulated significant serum (Fig. 4d) and fecal (Fig. 4e and f) antibody responses to the adhesin. Finally, vaccination with LT-adjuvanted EtpA yielded significant protection against ETEC infection (Fig. 4g) relative to unimmunized controls.

DISCUSSION

Now more than 40 years after their discovery (32), the enterotoxigenic *E. coli* remain a leading cause of death due to diarrheal illness in developing countries, and an essential target for vaccine development (38). Although most vaccinology efforts for these patho-

gens have to date focused on a relatively small number of antigens, namely, colonization factors and heat-labile toxin (5, 38), technological advances, including proteomics (26) and genomics (33), have permitted the identification of additional antigens for consideration in ETEC vaccines.

Given the underlying plasticity of *E. coli* genomes in general, it is likely that success in formulating a broadly protective ETEC vaccine will rely on the inclusion of multiple antigens to achieve broad representation. Similar to other *E. coli* pathotypes and other pathogens (25), the genome of ETEC is considered to be "open" (24), in that sequencing new ETEC genomes will theoretically add an unlimited number of genes to the ETEC pangenome. Nevertheless, of the ETEC sequences available to date, some genes appear to be represented in a diverse population of ETEC (33).

Among the genes that appear to be relatively conserved in the ETEC pathovar, based on currently available sequence data, are those encoding the EtpA two-partner secretion system, which appears in six of eight (75%) ETEC genomes sequenced to date (8, 24, 33). Likewise, EtpA was recently shown to be present in a diverse population of ETEC obtained in Chile, suggesting that EtpA is both conserved and geographically dispersed among these pathogens (9). Importantly, EtpA appears to be significantly immunogenic since it was detected both in immunoproteomic studies using convalescent-phase sera from patients with ETEC (26) and after experimental infection in mice. In addition, data have demonstrated that EtpA affords protection in a murine model of ETEC infection and is required for efficient intestinal colonization (27, 28).

The studies here further demonstrate that effective pathogen-host interactions are paramount in directing toxin delivery. Adhesion of bacteria to the target epithelium is felt to be an essential prerequisite for toxin delivery by ETEC (39) and, indeed, earlier *in vitro* studies demonstrated that both flagellar motility and the intimate interaction of ETEC with host cells were strict requirements for LT-mediated activation of cAMP (10). The present experiments demonstrate that EtpA, thought to mediate adhesion by acting as a molecular bridge between ETEC flagella and host cells (30), likewise plays an important role in the delivery of heat-labile toxin. Interestingly, EtpA antibodies exhibited at least an additive inhibitory effect on cAMP activation in intestinal epithelial cells when combined with antibodies directed against either the A or the B subunit of the toxin, suggesting that a multivalent strategy designed to interdict delivery of toxin at multiple levels could be used in the rational design of ETEC vaccines.

Likewise, the animal infection studies reported here appear to support this concept. Because earlier data suggested that LT plays a significant role in bacterial adhesion (21), as well as in intestinal colonization (1, 3), both important steps in ETEC pathogenesis, we sought to determine whether LT could be combined with EtpA to act both as a mucosal adjuvant and an immunogen. Together, the data from the present study suggest that LT-based vaccine strategies could serve as a platform for adopting additional novel antigens for inclusion in a multivalent approach that yields broad-based protection against ETEC.

ACKNOWLEDGMENTS

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