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Current Progress in Developing Subunit Vaccines against Enterotoxigenic *Escherichia coli*-Associated Diarrhea

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Diarrhea continues to be a leading cause of death in children <5 years of age, and enterotoxigenic *Escherichia coli* (ETEC) is the most common bacterial cause of children's diarrhea. Currently, there are no available vaccines against ETEC-associated diarrhea. Whole-cell vaccine candidates have been under development but require further improvements because they provide inadequate protection and produce unwanted adverse effects. Meanwhile, a newer approach using polypeptide or subunit vaccine candidates focusing on ETEC colonization factor antigens (CFAs) and enterotoxins, the major virulence determinants of ETEC diarrhea, shows substantial promise. A conservative CFA/I adhesin tip antigen and a CFA MEFA (multiepitope fusion antigen) were shown to induce cross-reactive antiadhesin antibodies that protected against adherence by multiple important CFAs. Genetic fusion of toxoids derived from ETEC heat-labile toxin (LT) and heat-stable toxin (STa) induced antibodies neutralizing both enterotoxins. Moreover, CFA-toxoid MEFA polypeptides, generated by fusing CFA MEFA to an STa-LT toxoid fusion, induced antiadhesin antibodies that broadly inhibited adherence of the seven most important ETEC CFAs associated with about 80% of the diarrhea cases caused by ETEC strains with known CFAs. This same antigen preparation also induced antitoxin antibodies that neutralized both toxins that are associated with all cases of ETEC diarrhea. Results from these studies suggest that polypeptide or subunit vaccines have the potential to effectively protect against ETEC diarrhea. In addition, novel adhesins and mucin proteases have been investigated as potential alternatives or, more likely, additional antigens for ETEC subunit vaccine development.

Diarrhea continues to be a leading cause of death among young children, especially those living in South Asia and sub-Saharan Africa, where access to clean drinking water is limited and community-wide sanitation systems are highly inadequate (1–6). Recent systematic studies conservatively estimate that >600,000 children <5 years of age die each year from diarrhea (7–9). Diarrhea is caused primarily by the ingestion of food or water contaminated with viral, bacterial, or parasitic pathogens. Among these potential pathogens, enterotoxigenic *Escherichia coli* (ETEC; strains producing heat-labile toxin [LT] and/or heat-stable type Ib toxin [STa]) is the most common bacterial cause of diarrhea in young children (1, 10, 11). ETEC strains produce fimbrial or nonfimbrial adhesins that promote the attachment of bacteria to host epithelial cells, allowing them to colonize the small intestine. They also produce enterotoxins that disrupt fluid and electrolyte homeostasis in small intestinal epithelial cells, leading to fluid hypersecretion and eventually watery diarrhea (12). Without rehydration intervention, moderate-to-severe diarrhea can lead to dehydration and death. It is estimated that ETEC strains are responsible for 280 to 400 million cases of diarrhea in children younger than 5 years, and an additional 100 million cases in children above 5 years annually (10). While the overall annual number of deaths attributable to diarrhea has dropped from nearly 5,000,000 in 1980 (13, 14) to approximately 1,000,000 in 2011 (1, 15, 16), the annual number of deaths attributable to ETEC diarrhea among children aged 0 to 59 months remains 150,000 to 300,000 (10).

It is theoretically possible to control or prevent ETEC-associated diarrhea through the installation of effective sanitation systems and country-wide access to clean drinking water (17). However, because of political and economic factors, the likelihood of accomplishing this in the coming decades is low for low-income countries in South Asia, South America, and sub-Saharan Africa. Consequently, vaccination is currently considered the most effective and practical approach to reducing the impact of ETEC diarrhea (17–19). Developing effective ETEC vaccines has become a top priority for the World Health Organization, the United Nations Children's Fund, and other public health institutions (10, 20). Unfortunately, despite these efforts, there are no currently available vaccines to effectively protect against ETEC diarrhea.

The development of an effective ETEC vaccine has provided numerous challenges. These include the heterogeneity of virulence factors among ETEC strains, which include colonization factor antigen (CFA) adhesins and enterotoxins. Investigators have identified at least 23 immunologically distinct CFA adhesins and two very distinct enterotoxins produced by various ETEC strains that cause diarrhea in humans (21–24). Since ETEC strains producing any of these CFA adhesins plus either LT or STa enterotoxin can cause diarrhea, ideally, an effective ETEC vaccine should induce protective immunity against all CFA adhesins and both enterotoxins. Unfortunately, protection against 23 immunologically distinct CFA adhesins does not appear feasible with our current technology, and simultaneous protection against two entero-

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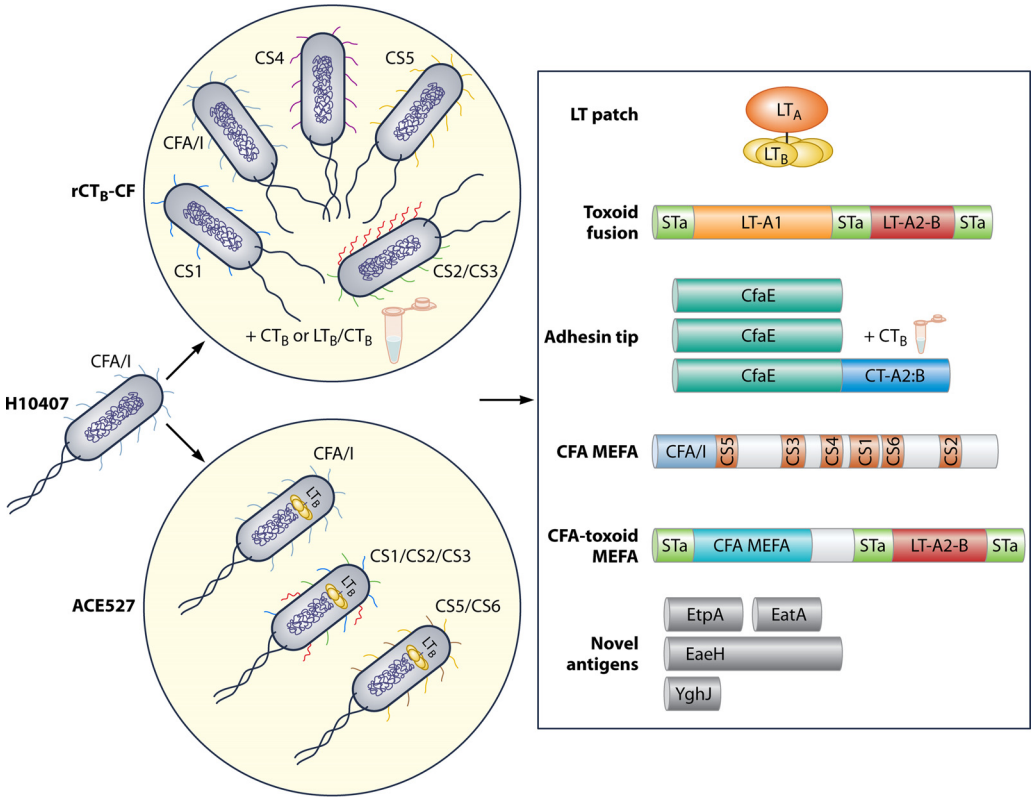

FIG 1 ETEC vaccine candidates currently under development, including the killed (rCT_B-CF) and live attenuated (ACE527) whole-cell vaccine candidates conceptually derived from the killed H10407 product and the experimental subunit vaccine candidates. Note that CS6 is a nonfimbrial outer membrane protein adhesin.

toxins has also been very challenging. Both LT and STa are potent toxins, and STa is poorly immunogenic. Thus, neither toxin can be used directly as a vaccine antigen. Detoxified LT molecules and the nontoxic B subunit of LT (LT_B) have been effectively used as immunogens to induce antibodies against LT (25, 26). Despite many early efforts, however, the development of a safe and immunogenic STa antigen as an ETEC vaccine component has not been accomplished until very recently (17).

Efforts have been made to develop a vaccine to protect against ETEC since the discovery of the role ETEC plays in children's diarrhea (17). With limited knowledge of ETEC bacterial structure and disease mechanisms, early attempts focused largely on whole-cell vaccine development (Fig. 1). The first experimental ETEC vaccine produced was colicin E2-inactivated ETEC prototype strain H10407 (O78:H11, LT^+ STa^+ CFA/I^+) (27). Adult volunteers inoculated orally with this killed whole-cell product developed IgA antibodies to CFA/I adhesin and LT and were protected against a homologous challenge (27, 28). Unfortunately, the anti-CFA/I and anti-LT antibodies produced were effective only against the homologous strain (29–31). Studies of this killed whole-cell product nevertheless led to the realization that an effective ETEC vaccine should minimally induce antibodies that protect against multiple CFA adhesins and LT (32). Consequently, products that combined multiple killed or live attenuated strains (which collectively expressed several CFA adhesins) with either the LT_B subunit protein or the homologous cholera toxin B subunit (CT_B) were generated and examined for protection against ETEC diarrhea.

The most widely studied whole-cell ETEC vaccine candidates are rCT_B-CF and ACE527 (17, 33). The rCT_B-CF vaccine consists of five formalin-inactivated strains expressing six distinct CFA adhesins (CFA/I, CS1, CS2, CS3, CS4, CS5) supplemented with recombinant CT_B subunit protein (34, 35). The ACE527 vaccine is composed of three live attenuated *E. coli* strains that collectively express five CFA adhesins (CFA/I, CS2, CS3, CS5, CS6), one CFA subunit (CS1), and the LT_B subunit (36, 37). The rCT_B-CF product induced antigen-specific antibody responses and reduced the risk of developing moderate-to-severe diarrhea by 60 to 70% for adults traveling from developed countries to countries where ETEC diarrhea is endemic (38). However, this product showed little protection against ETEC diarrhea in very young children living in areas where ETEC diarrhea is endemic, the most vulnerable group, and had some adverse effects on young children when an adult oral dose was given (39, 40). Although this product reduced the severity of disease outcomes among U.S. adults traveling to Guatemala and Mexico, it failed to reduce the overall diarrhea rate (38). The live ACE527 product also had adverse effects on volunteers (41). Oral inoculation induced antibody responses to LT_B, CFA/I, CS3, and CS6 among adults but only reduced the severity of diarrhea following a homologous challenge (36, 42).

Modifications, including refinement of the volunteer challenge model, overexpression of CFAs, and replacement of supplementary recombinant CT_B with an LT_B-CT_B hybrid, have been implemented to improve the efficacy of whole-cell vaccines (41, 43, 44). Despite these modifications, rCT_B-CF and ACE527 were deemed unlikely to provide broad protection against ETEC diarrhea be-

cause they contain only the LT_B subunit or the LT_B-CT_B hybrid as the toxin antigen. They do not contain STa or LT_A antigens and consequently fail to induce antibodies against these key toxin components. Anti-LT_B or -CT_B antibodies inhibit the binding of LT to host GM_1 receptors; they are effective only against the effects of LT and provide little protection against ETEC strains that produce STa (45, 46). Moreover, cocktail products tend to require a relatively large oral dose to simultaneously stimulate host immune responses against CFA and LT_B or CT_B. A large oral dose delivers many somatic antigens, including lipopolysaccharide, which is thought to be responsible for the adverse gastrointestinal side effects observed, especially in young children (47). Excessive somatic antigens may also mask stimulation of host antibody responses specific to the targeted CFA adhesins and LT_B or CT_B (17, 48), thus reducing protective efficacy against ETEC diarrhea.

ETEC SUBUNIT VACCINE CANDIDATES

While whole-cell vaccine products, especially live attenuated products, generally induce long-lasting host immunity, they require stringent storage conditions to maintain efficacy and pose a potential safety risk due to the possible acquisition of virulence genes after inoculation. Additionally, live attenuated vaccine products can have reduced efficacy if vaccinees have been preexposed to strains expressing virulence factors homologous to vaccine antigens and possess pre-existing antibodies, which may contribute to the rapid removal of vaccine strains from the host. In comparison, killed whole-cell products are generally safer but require multiple doses to induce long-lasting immunity. Overall, whole-cell products often contain unnecessary, unwanted, and even harmful antigens, which are likely to reduce efficacy and enhance adverse effects.

Although whole-cell products rCT_B-CF (or its modified product ETVAX) and ACE527 continue to be improved to achieve better protective efficacy, subunit vaccines have shown promise as effective alternatives for protection against ETEC diarrhea. In contrast to whole-cell products, subunit vaccines contain well-defined and highly characterized immunogens that induce immune responses specific to those immunogens. As subunit vaccines typically contain one or more well-characterized proteins or polypeptides, product safety can be more easily managed and achieved than with whole-cell vaccines. Since ETEC CFA adhesins and LT are considered among the most important virulence determinants in ETEC diarrhea, they have become the primary targets in ETEC subunit vaccine development. Recent progress in applying STa toxoids and genetic fusions of LT and STa toxoids as antigens, as well as double-mutant LT (dmLT) as an adjuvant, enhances the potential for developing a safe and broadly effective subunit vaccine against ETEC diarrhea.

LT patch. Since enterotoxins are responsible for disrupting fluid and electrolyte homeostasis in host small intestinal epithelial cells, leading to ETEC diarrhea, neutralizing the effects of these enterotoxins is considered a highly effective approach for preventing ETEC diarrhea. One early study showed that mice transcutaneously immunized with an adhesive patch embedded with purified LT developed an antibody response to LT and accumulated less fluid in their intestines after an oral challenge with LT (49). When the patch was applied to humans (placed on the upper arm), immunized adults developed strong IgG and IgA antibody responses to LT (50, 51). A subsequent placebo-controlled study, though confirming induction of anti-LT antibodies, demonstrated that immunized subjects were only marginally protected against an ETEC challenge (52). Surprisingly, a small subsequent field trial reported that transcutaneous immunization reduced the incidence of moderate-to-severe diarrhea caused by ETEC and other enteric pathogens by 75% in healthy adults traveling to Mexico or Guatemala (53). This unanticipated result, that non-ETEC diarrheal pathogens caused fewer illnesses in individuals immunized with LT, led to speculation that anti-LT immunity might provide broad-spectrum protection against a variety of enteric pathogens.

Unfortunately, a second field study failed to confirm results from the first small trial. Data from the second field study showed that the LT patch provided 60% protection against LT^+ ETEC diarrhea but provided no protection against STa^+ ETEC or any other causes of diarrhea (54). Reanalyses of data from the first field trial found that protective efficacy was overestimated in the original publication (55). Furthermore, data from a more recent field study revealed that the LT patch alone was not protective against ETEC or all-cause diarrhea (56). Consequently, on the basis of the collective results of these studies and concerns about safety discussed below, use of the LT patch alone is no longer considered a suitable approach for vaccinating against diarrhea attributable to ETEC (57). The associated transdermal antigen delivery method, however, is innovative and worthy of further exploration as a means of delivering other, more promising, immunogens with the goal of preventing ETEC diarrhea.

dmLT. Even if LT-induced antibodies were effective against ETEC diarrhea, safety concerns over the potent toxicity of native LT or the remaining toxicity of LT derivatives, such as the mutant LT (mLT) LT_{R192G}, have suppressed enthusiasm about the use of LT or mLT for ETEC subunit vaccine development. A dmLT ($LT_{R192G/L211A}$) that was shown to lack toxicity in a patent mouse assay and had greatly reduced activity in stimulating cyclic AMP levels in Caco-2 cells (58) appears to be a safer antigen for inducing anti-LT antibody responses. However, since anti-LT antibodies have not demonstrated broad efficacy in protecting against ETEC diarrhea, dmLT alone has not been considered to be an effective immunogen for ETEC subunit vaccine development. Instead, dmLT has been explored primarily as a vaccine adjuvant. Including recombinant dmLT in vaccines improved product safety (compared to mLT) and enhanced the protective efficacy of killed or live attenuated whole-cell ETEC vaccine candidates (59) and a CFA adhesin tip subunit vaccine candidate (60). We observed recently that, compared to Freund's adjuvant or ISA51 (SEPPIC), dmLT is better tolerated and equally or more effective at enhancing antitoxin or antiadhesin immune responses to an ETEC toxoid fusion ($3\times STa_{N12S}$-dmLT) or to a CFA multiepitope fusion antigen (MEFA) in intraperitoneally or subcutaneously immunized mice (unpublished data). It was also observed that when 1 μg of dmLT was used as an adjuvant with ETEC CFA MEFA as the antigen, anti-LT antibodies with *in vitro* neutralizing capacity were generated (unpublished data). This suggests that dmLT adjuvant also functions as an immunogen capable of inducing anti-LT antibodies that can enhance the overall efficacy of ETEC vaccine candidates containing this adjuvant.

LT and STa toxoid fusions. We now know that a vaccine candidate carrying LT antigen alone is not effective against ETEC diarrhea (54, 56) because anti-LT antibodies do not cross protect against diarrhea caused by STa^+ ETEC strains (45, 46). We have also learned that moderate-to-severe bacterial diarrhea in young

children is most often attributable to STa^+ ETEC strains (11). Further, more than two-thirds of the ETEC strains isolated from patients with diarrhea express STa alone or together with LT (22). Therefore, an effective (subunit or whole-cell) ETEC vaccine needs to carry STa antigens to induce protective anti-STa antibodies. However, designing a safe STa antigen that induces neutralizing anti-STa antibodies has long been a key obstacle in ETEC vaccine development. Like LT, STa is an extremely potent toxin. Unlike highly immunogenic LT, however, the small molecule STa (a peptide of 19 amino acids) is poorly immunogenic. Immunization with STa and STa derivatives or natural infection with STa^+ ETEC strains does not induce an anti-STa immune response. To effectively incorporate the STa antigen into ETEC subunit vaccine development, STa's toxicity must be greatly attenuated while enhancing its immunogenicity (61, 62).

STa's immunogenicity has been enhanced by coupling it to a strongly immunogenic carrier protein and presenting it to the host as a fusion protein or chemical conjugate (45, 61, 63, 64). Similarly, STa's toxicity has been greatly reduced or eliminated by disruption of its disulfide bonds or substitution of a single amino acid residue (65, 66). Combination of both of these results (i.e., maintaining a relevant immunogenic structure while reducing or eliminating toxicity) in a single subunit vaccine product, however, appeared to be virtually unattainable. As the three disulfide bonds play a crucial role in maintaining STa's unique structure and antigenic topology, disruption of disulfide bonds results in collapse of STa's protein structure and loss of relevant immunogenicity. Such mutant forms of STa would fail to induce protective anti-STa antibodies even if they were conjugated to a carrier protein (65). The beliefs that STa's toxicity and structure (and antigenic properties) are tightly associated and that modifications to reduce toxicity would significantly alter the protein's structure and antigenic properties were held until very recently. These concepts were invalidated by demonstrating that a nontoxic mutant form of STa fused to a carrier protein induced neutralizing anti-STa antibodies (61, 67, 68).

By substituting a noncysteine amino acid residue into a full-length porcine-type STa (heat-stable toxin type Ia), it was rendered nontoxic while its native protein structure was largely retained. Derived STa toxoids, as well as STb (another small and poorly immunogenic heat-stable toxin of ETEC associated with diarrhea in young pigs), became immunogenic after genetic fusion to a single polypeptide carrying one copy of subunit A and one copy of subunit B from LT_{R192G} (monomeric LT [mLT_{R192G}]); antibodies induced by the resultant toxoid fusions neutralized both LT and ST (61, 69). Moreover, data from challenge studies using a pig model revealed that antibodies induced by each fusion product protected against infection with an STa^+ or an STb^+ ETEC strain (61, 69). Results from these two studies encouraged further exploration of toxoid fusions, eventually leading to the breakthrough concept that a safe and immunogenic STa antigen can be produced for ETEC vaccine development.

Analogous to the findings with porcine-type STa, human-type STa with a noncysteine amino acid substitution also demonstrated greatly reduced toxicity with the native STa protein structure largely retained (68). Toxoid STa_{P13F} (in which the 13th residue of proline was replaced with phenylalanine) was genetically fused to mLT_{R192G} (with STa_{P13F} at either the C or the N terminus, between A1 and A2, or between the A and B peptides of LT_{R192G}), to generate LT_{R192G}-ST_{P13F} toxoid fusion polypeptides. Immunization with these LT_{R192G}-ST_{P13F} fusion products induced anti-LT and anti-STa antibodies that moderately neutralized STa *in vitro* (67). In order to improve anti-STa immunogenicity, three copies of a different STa toxoid (STa_{A14Q}) were fused to a triple-mLT (tmLT), $LT_{S63K/R192G/L211A}$; STa_{A14Q} was fused to the N and C termini and between the A1 and A2 peptides of the tmLT. This $3\times STa_{A14Q}$-tmLT fusion product exhibited enhanced anti-STa immunogenicity. For the first time, antibodies induced by this toxoid fusion completely neutralized purified STa in an *in vitro* antibody neutralization assay (70).

To further optimize STa toxoids for an LT-STa toxoid fusion product that induced robust anti-STa antibody responses, we selected a panel of 14 STa toxoids from a constructed STa toxoid library and genetically fused three copies of each of the 14 selected STa toxoids to a dmLT monomer to generate $3\times STa_{-toxoid}$-dmLT fusions (71). Results of mouse immunization studies that were carried out independently by two laboratories showed that STa_{N12S} was the optimal STa toxoid for generating LT-STa toxoid fusion products that induce neutralizing anti-STa antibodies. Of the 14 toxoid fusions examined, $3\times STa_{N12S}$-dmLT induced the highest anti-STa IgG titer and the third highest anti-STa IgA titer in intraperitoneally immunized mice. Moreover, antibodies induced by $3\times STa_{N12S}$-dmLT showed the strongest *in vitro* neutralization activity against STa (71). Serum pooled from mice immunized with $3\times STa_{N12S}$-dmLT neutralized not only 2 ng but also unprecedentedly 5 ng, 10 ng, and even 90% of 20 ng of purified STa in the *in vitro* assay. By utilizing 1 μg of holotoxin-structured dmLT as an adjuvant in mice immunized intraperitoneally or subcutaneously with $3\times STa_{N12S}$-dmLT, the antibody response to STa (and to LT as well) was further enhanced and the induced antibodies exhibited stronger neutralization activity against both toxins. This $3\times STa_{N12S}$-dmLT toxoid fusion is currently the most promising target for antitoxin subunit vaccine development, and human volunteer studies will be conducted to characterize antigen tolerance and safety and, more importantly, protection against STa-producing ETEC infection.

CFA adhesin tip antigen. CFA-mediated bacterial attachment to and colonization of the host's small intestine are the initial steps in gastrointestinal infection by ETEC. Consequently, blocking CFA-mediated ETEC adherence to host cell receptors becomes the first line of defense for disease prevention. The fact that ETEC strains collectively express 23 or more immunologically heterogeneous CFAs that adhere to different host receptors provides a tremendous challenge to inducing this first line of defense through immunization. To effectively block bacterial adherence, a vaccine would have to carry antigens from many CFAs, most importantly, those expressed by the most prevalent and virulent ETEC strains. One potential solution that is currently being explored at the U.S. Naval Medical Research Center is the use of a conservative CFA adhesin to induce cross-protective antibodies against different CFAs. It has been reported that some ETEC CFAs, for example, CFA/I, CS4, CS14, CS1, CS17, and CS19, have homologous antigenic domains. The major subunits of these CFAs, particularly the adhesive units or adhesins, have similar amino acid sequences (72, 73), and antibodies induced by one CFA are able to cross protect against antigenically related CFAs (72–76).

The adhesin tip from CFA/I, CfaE, has shown considerable promise for the development of a broadly protective ETEC antiadhesin vaccine (77). Mice and rabbits immunized transcutaneously with CfaE combined with LT, mLT, or dmLT (as an adju-

vant) developed a robust anti-CFA/I antibody response that inhibited the attachment of CFA/I, CS2, CS4, CS14, and CS17 ETEC strains to bovine and chicken enterocytes (60). Moreover, subsequent nonhuman primate challenge studies showed that these induced anti-CfaE antibodies conferred protection against a CFA/I ETEC challenge (77). Additionally, this CfaE tip can be coadministered with CT_B or fused to CTA2-CT_B to induce protective anti-CFA/I and anti-LT antibodies. Unfortunately, antibodies induced by CfaE and LT or CT will not protect against ETEC strains expressing STa and heterologous, non-cross-reactive CFAs. An upcoming phase 2/2b trial carried out by the U.S. Naval Medical Research Center will provide data to further assess the candidacy of this CfaE tip as a subunit vaccine against ETEC diarrhea.

CFA MEFA. As an alternative to using a conservative CFA tip antigen, we recently employed a MEFA for ETEC antiadhesin subunit vaccine development (78). The MEFA we utilized carried representative antigenic peptides or epitopes from the seven most important CFA adhesins expressed by ETEC strains. Compared to whole-organism vaccines, a single CFA adhesin or toxin protein antigen induces host antibody responses that are far more specific for the immunizing CFA or toxin. Nevertheless, much of the CFA or toxin may be nonimmunogenic or may induce nonprotective antibodies. Consequently, identifying specific peptides or epitopes within different CFAs that induce protective antibodies and combining them into a single MEFA is an efficient means of developing a vaccine for antigenically heterogeneous pathogens like ETEC. Although this process used to be extremely tedious at best, recent advances in bioinformatic tools for epitope-based vaccine development have significantly accelerated the process (78, 79).

Advantages of epitope- or peptide-based antigens include product safety, ease of production, and most importantly, precise targeting of host immune responses. A major disadvantage, however, is the generally poor immunogenicity associated with the use of a single low-molecular-weight peptide or immunogen. Using a carrier protein, a mixture of different antigen formulations, and various immunization routes can enhance the immunogenicity of epitope- or peptide-based antigens. We used the novel MEFA approach to overcome the poor immunogenicity associated with epitope- or peptide-based antigens. We used the strongly immunogenic major subunit of CFA/I (CfaB) as a backbone (or a carrier protein) and utilized epitope prediction software to retain the most important B-cell epitopes of CfaB. Then we replaced surface-exposed, but less immunogenic, epitopes of CfaB with the most immunogenic B-cell epitopes from other clinically important ETEC CFAs, including CFA/II (CS1, CS2, CS3) and CFA/IV (CS4, CS5, CS6). The end product was a CFA MEFA that induced broadly protective anti-CFA antibodies (80). Since ETEC strains expressing these seven CFAs are responsible for approximately 80% of the diarrhea cases caused by ETEC strains with known CFAs and are generally responsible for the moderate-to-severe cases (40), a vaccine protecting against these seven CFAs should be broadly effective against ETEC diarrhea. This CFA MEFA was strongly immunogenic, as it induced high titers of antibodies specific to all seven of these CFAs and probably to other CFAs homologous to CFA/I. More importantly, the antibodies induced significantly blocked the *in vitro* adherence of *E. coli* strains expressing these seven CFAs (80). Thus, this CFA MEFA represents a strong candidate for antiadhesin subunit vaccine development.

Toxoid-CFA fusions. Although the CFA MEFA induced protective antibodies against the seven most important CFAs, these antibodies would not be expected to protect against the remaining ETEC diarrhea cases caused by strains expressing different CFAs or strains with unidentified CFAs. In addition, there are geographic differences in the epidemiology of ETEC diarrhea and these seven CFAs may not always be the most prevalent globally (81). Thus, antibodies against these seven CFAs may not be sufficiently effective to protect against ETEC diarrhea in some geographic regions. On the other hand, LT and STa, alone or together, are expressed by ETEC strains causing 100% of ETEC diarrhea cases. But antibodies against LT and STa could be less effective against ETEC diarrhea without assistance from antibodies against ETEC CFAs (82). If the CFA MEFA were combined with, or further fused to, the optimal 3×STa_{N12S}-dmLT toxoid fusion that induces protective antibodies against both toxins, the derived product would be expected to induce antibodies for even broader protection against ETEC diarrhea. The induction of antitoxin antibodies by this fusion product would be expected to provide supplementary protection against ETEC strains expressing these seven CFAs and independent protection against ETEC strains expressing different CFAs.

Porcine-type adhesin-toxoid fusion antigens were demonstrated to induce antiadhesin and antitoxin antibodies that protected against ETEC diarrhea (83–85). When an STa toxoid and an LT epitope were fused to the major subunit (FaeG) of porcine ETEC fimbrial adhesin K88, derived K88-STa, K88-LT, and K88-STa-LT polypeptides induced antibodies that neutralized STa and/or LT and also inhibited the adherence of a $K88^+$ ETEC strain (83). Additionally, when peptides from the major subunit (FaeG) from the K88 adhesin and from the minor subunit (FedF) from the F18 fimbrial adhesin were fused to the A2-B peptide of mLT monomer, the tripartite adhesin-toxoid fusion peptide stimulated mouse and pig antibodies that protected against both K88 and F18 adhesins and LT. Young pigs immunized with this K88-F18-LT fusion were protected from a challenge with an ETEC strain, while control piglets developed diarrhea and dehydration after a challenge with the same ETEC strain (84, 85).

Encouraged by results from the porcine ETEC adhesin-toxin fusion studies, we further fused the human-type CFA MEFA to toxoid fusion 3×STa_{N12S}-dmLT and explored the application of CFA-toxoid MEFA in ETEC subunit vaccine development. Mice immunized intraperitoneally with CFA/I/II/IV-2×STa_{N12S}-dmLT, a single polypeptide carrying the CFA MEFA, two copies of STa_{N12S}, and the LT-A2 and LT_B peptides, developed antibodies to all seven CFAs and both toxins (86). Serum from these immunized mice inhibited the *in vitro* adherence of *E. coli* strains expressing these seven CFA adhesins and also neutralized both toxins (86). This CFA/I/II/IV-2×STa_{N12S}-dmLT MEFA has been modified to carry three copies of STa_{N12S} for further enhancement of anti-STa immunogenicity. The new CFA/I/II/IV-3×STa_{N12S}-dmLT has been shown to possess stronger anti-STa immunogenicity, and antibodies induced by this fusion product showed greater neutralizing activity against STa. Although coadministration of the CFA MEFA and toxoid fusion 3×STa_{N12S}-dmLT induces equivalently protective antibodies against seven CFAs and both toxins (86), the single CFA/I/II/IV-3×STa_{N12S}-dmLT MEFA is more cost-effective to manufacture and administer. This CFA/

I/II/IV-3×STa_{N12S}-dmLT MEFA can potentially be considered the most promising antigen for ETEC subunit vaccine development.

Additional epitopes from these seven CFAs, epitopes of other CFAs, or epitopes from newly identified antigens can be further embedded in this CFA/I/II/IV-3×STa_{N12S}-dmLT MEFA for even broader protection, specifically, for better protection against ETEC strains exhibiting different CFA profiles. The additional epitopes can be fused inside the LT-A2 or LT_B peptide of the CFA/I/II/IV-3×STa_{N12S}-dmLT. With dmLT used as the adjuvant, it is unlikely that anti-LT immunogenicity would be compromised, as this dmLT adjuvant also serves as an immunogen that stimulates the production of anti-LT antibodies.

Novel antigens. Along with the application of traditional CFA and toxin antigens for ETEC subunit vaccine development, putative pan-ETEC antigens newly identified by genome sequencing have also been explored. These novel antigens include the adhesins EtpA and EaeH and the mucin enzymes EatA and YghJ (87–90). EtpA is a 170-kDa glycoprotein secreted by a two-partner secretion system that acts as a molecular bridge between ETEC flagellin and host cell receptors (91). Antibodies induced by EtpA significantly reduced the colonization of mice by the challenge ETEC strain (H10407) (92). EaeH is another outer membrane adhesin that promotes bacterial engagement with host epithelial cell surfaces and ETEC colonization of the host's small intestine (88). EatA and YghJ are enzymes involved in mucin degradation, thereby enhancing the access of LT to intestinal epithelial cells. Antibodies against a secreted passenger domain of EatA were shown to reduce the colonization of the small intestines of mice by ETEC strain H10407 (89). Antibodies against YghJ have been suggested to provide moderate protection against extraintestinal pathogenic *E. coli* (93), but it is unknown if they protect against a challenge with ETEC strains (94).

While data are accumulating that suggest that induced antibodies against these novel antigens provide some protection against ETEC colonization, further studies are required to determine whether this translates into protection against diarrhea caused by ETEC. In addition, better characterization of the prevalence of these antigens in ETEC strains, and in commensal *E. coli* strains, is needed. In order for them to be potential vaccine candidates, they must by broadly expressed by pathogenic ETEC strains and much less prevalent in commensal *E. coli* strains; inhibition of gastrointestinal colonization by commensal *E. coli* would be an unwanted outcome of vaccination. On the basis of the contribution of these proteins to pathogenicity, namely, attachment to the host cell surface and degradation of mucin to enhance bacterial attachment and LT delivery, each of these individual antigens may not be sufficient to induce antibodies that effectively protect against ETEC diarrhea. Whether inclusion of antigenic peptides or epitopes from these proteins as supplementary antigens can improve the efficacy of other promising subunit vaccine candidates against ETEC diarrhea warrants further investigation.

CONCLUSION

Along with the continual improvement in whole-cell ETEC vaccine development, subunit or polypeptide vaccine candidates exhibit great promise. Genetic fusions of LT and STa toxoids induced antibodies that neutralized both toxins and protected young pigs against STa^+ ETEC infection. The conservative CFA/I tip antigen CfaE and CFA MEFA, which carries epitopes from the seven most important ETEC CFAs (CFA/I, CS1, CS2, CS3, CS4, CS5, CS6), induced broadly protective antiadhesin antibodies. Furthermore, CFA-toxoid MEFA, which carries representative antigens from the seven most important CFAs plus LT and STa toxoids, was found to induce strongly protective antibodies against all seven CFAs and both toxins. Additionally, the addition of newly identified novel antigens to the subunit vaccines mentioned above could potentially improve the protective efficacy of these ETEC subunit vaccines against ETEC diarrhea.

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