Heat-Stable Enterotoxin of Enterotoxigenic Escherichia coli as a Vaccine Target

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Enterotoxigenic Escherichia coli (ETEC) is responsible for 280 million to 400 million episodes of diarrhea and about 380,000 deaths annually. Epidemiological data suggest that ETEC strains which secrete heat-stable toxin (ST), alone or in combination with heat-labile toxin (LT), induce the most severe disease among children in developing countries. This makes ST an attractive target for inclusion in an ETEC vaccine. ST is released upon colonization of the small intestine and activates the guanylate cyclase C receptor, causing profuse diarrhea. To generate a successful toxoid, ST must be made immunogenic and nontoxic. Due to its small size, ST is nonimmunogenic in its natural form but becomes immunogenic when coupled to an appropriate large-molecular-weight carrier. This has been successfully achieved with several carriers, using either chemical conjugation or recombinant fusion techniques. Coupling of ST to a carrier may reduce toxicity, but further reduction by mutagenesis is desired to obtain a safe vaccine. More than 30 ST mutants with effects on toxicity have been reported. Some of these mutants, however, have lost the ability to elicit neutralizing immune responses to the native toxin. Due to the small size of ST, separating toxicity from antigenicity is a particular challenge that must be met. Another obstacle to vaccine development is possible cross-reactivity between anti-ST antibodies and the endogenous ligands guanylin and uroguanylin, caused by structural similarity to ST. Here we review the molecular and biological properties of ST and discuss strategies for developing an ETEC vaccine that incorporates immunogenic and nontoxic derivatives of the ST toxin.

According to the World Health Organization, enterotoxigenic Escherichia coli (ETEC) is responsible for 280 million to 400 million episodes of diarrhea, many of which lead to malnutrition, and about 380,000 deaths annually. Most of the victims are children less than 5 years of age living in developing countries. This makes ETEC one of the most important enteropathogens in impoverished children. ETEC is also considered the most common cause of travelers’ diarrhea (63).

ETEC is transmitted by the fecal-oral route, whereupon it colonizes the small intestine. Adhesion to the intestinal epithelium is mediated by colonization factor antigens (CFs), which are fimbriae or fibriils, filamentous protein structures on the bacterial surface (11). To date, 25 distinct CFs have been identified (61). The essential ETEC secretogenic virulence factors are the heat-stable (ST) and heat-labile (LT) enterotoxins. Both elicit net secretion of ions and water, resulting in watery diarrhea, in the most serious cases producing a profuse cholera-like condition. ETEC can produce two distinct heat-stable toxins, STa/STI and STb/STII, which are unrelated structurally, functionalaly, and immunologically (18). Only the former, hereinafter referred to as “ST,” is thought to play an important role in human disease and will be discussed in this context. LT is a large (84,000 Da), immunogenic oligo-toxin related to cholera toxin in sequence, structure, and mechanism (48). In contrast, ST is a small polypeptide (2,000 Da) which is nonimmunogenic in its natural form. Epidemiological data suggest that strains secreting ST, with or without LT, induce the most severe disease among children of developing countries (61, 62). Two ST variants are known: the 19-aminoc acid STh (synonyms, STb and STII), found in human ETEC strains, and the 18-aminoc acid STp (synonyms, STIa and STIb), isolated from human and porcine strains. STh and STp are very similar, with 14 identical residues, including the cysteines of the 3 disulfide pairs (34, 72).

A variety of strategies have been pursued in attempts to develop a vaccine against ETEC. The most promising vaccine candidate to date is a killed whole-cell vaccine comprising 5 different ETEC strains which express the most prevalent CFs, coadministered with recombinant cholera toxin (CT) B subunit (CT-B). This approach exploits the immunological cross-reactivity between CT and LT (8, 48). Although efficacious against more-serious diarrhea among American travelers to Guatemala, this vaccine was not protective in Egyptian children (45, 61).

An epidemiological study in Guinea-Bissau found that antigens other than the CFs may contribute substantially to the acquisition of natural anticolonizing immunity (58). This interpretation suggests that other surface antigens should be considered for inclusion in an ETEC vaccine. In addition, ST is present in approximately 75% of all clinical ETEC isolates and seems to be associated with more-serious illness than LT, which makes it a very attractive candidate to include as a component of an ETEC vaccine (61).

The main challenges for making a vaccine component from ST are to engineer the molecule so as to render it immuno-
genic while abolishing toxicity without losing protective epitopes. Such a mutant variant is often referred to as an ST toxoid. Here we review the molecular and biological properties of ST and discuss strategies for developing an ETEC vaccine that incorporates immunogenic and nontoxic derivatives of the toxin.

**ST BIOLOGY**

**Heat-stable toxin biogenesis.** The heat-stable toxins of ETEC are encoded on transmissible plasmids (47, 55) and are expressed as 72-amino-acid prepropeptides (33). The presequence (amino acids 1 to 19) is a signal peptide that directs translocation of the prepropolypeptide across the inner membrane, mediated by the Sec machinery (36). Outer membrane (OM) translocation is accompanied by cleavage of the propeptide (STh amino acids 20 to 53; STp, 20 to 54), generating the mature ST (STh, 54 to 72; STp, 55 to 72); OM translocation is believed to require the TolC channel (67, 68), though this has not been directly demonstrated. The periplasmic thiol/disulfide interchange protein DsbA is required for the formation of the three disulfide bridges found in the mature ST (66).

**ST toxicity in the gut.** ETEC colonization of the small intestine is facilitated by CF-mediated mucosal adhesion. Thereupon, the colonizing strain expresses its enterotoxins, though expression levels do not seem to be influenced by colonization (46). In the intestine, ST binds to and activates the intestinal brush border guanylate-cyclase-C (GC-C) receptor, which is the receptor of the endogenous ligands guanylin and uroguanylin (15, 43, 56, 57). ST activates GC-C when present in submicromolar concentrations and is known to bind to the receptor at nanomolar concentrations (10). The immediate result of ST-mediated GC-C receptor activation is an increase in intracellular messenger cyclic GMP (cGMP). cGMP in turn mediates decreased absorption of sodium and chloride ions and increased secretion of bicarbonate and chloride ions, ultimately resulting in watery diarrhea (56). In normal physiology, the GC-C receptor plays an important role in fluid homeostasis, pH control, and electrolyte balance, regulated by the endogenous ligands (15). Mutant mice that lack the GC-C receptor are viable and resistant to ST, and they are protected against ETEC diarrhea, underscoring the pivotal role of GC-C in ST-induced diarrhea (44).

**Structure and function of ST.** The ETEC heat-stable enterotoxins closely resemble guanylin and uroguanylin both in sequence and in three-dimensional structure (Fig. 1). The endogenous GC-C ligands have two disulfide bridges (Cys7-Cys15 and Cys10-Cys19, shown as yellow sticks), and ST has an additional one (Cys6-Cys11). Note that Cys6 in ST is replaced with 5-beta-mercaptopropionate (shown as blue/yellow stick). The proposed GC-C receptor-interacting residues of ST (STp, Asn11-Pro12-Ala13) are shown as sticks. (B) Sequence alignment of the human GC-C ligands (top) and bacterial GC-C ligands (bottom). Disulfide bonds are marked with lines. Residue numbering is according to STh. The species abbreviations are as follows: Hs, Homo sapiens; Ec, Escherichia coli; Yk, Yersinia kristensenii; Ye, Yersinia enterocolitica; Vc, Vibrio cholerae.

![FIG. 1. Structures and sequence alignment of guanylate cyclase C receptor ligands. (A) Structures of the A form of human uroguanylin (left, PDB:1UYA) and the toxic domain (residues 6 to 18) of STp (right, PDB:1ETN). The N and C termini are marked. The two structures have a similar fold, and the part of the uroguanylin structure that corresponds to the ST structure is shown in cyan. The structures share two disulfide bridges (Cys7-Cys15 and Cys10-Cys19, shown as yellow sticks), and ST has an additional one (Cys6-Cys11). Note that Cys6 in ST is replaced with 5-beta-mercaptopropionate (shown as blue/yellow stick). The proposed GC-C receptor-interacting residues of ST (STp, Asn11-Pro12-Ala13) are shown as sticks. (B) Sequence alignment of the human GC-C ligands (top) and bacterial GC-C ligands (bottom). Disulfide bonds are marked with lines. Residue numbering is according to STh. The species abbreviations are as follows: Hs, Homo sapiens; Ec, Escherichia coli; Yk, Yersinia kristensenii; Ye, Yersinia enterocolitica; Vc, Vibrio cholerae.](image-url)
Interestingly, the third cystine found in the ST peptides seems to lock the conformation in the active form. The amino acid sequence from the first to last cysteine of ST forms the so-called toxic domain and has been reported to contain all the properties necessary for full biological activity (1, 72). However, an STh variant lacking the four N-terminal residues was reported to have a 10-fold reduction of potency compared to full-length ST, suggesting that the residues outside the toxic domain may also be required for full potency (60).

The structure of a synthetic, fully toxic analog of the effector domain of STp, where the first cysteine was replaced with \(-\text{mercapto} \)propionic acid, has been solved by X-ray crystallography (34) (Fig. 1A). This analysis revealed that the backbone forms a right-handed spiral comprising three \(-\text{turns} \) in a U-shaped configuration, tightly connected by the three disulfide linkages. Interestingly, the core of the structure does not seem to be stabilized by a prominent hydrophobic core. The structure of a synthetic, fully toxic analog of the effector domain of STP, where the first cysteine was replaced with \(-\text{mercapto} \)propionic acid, has been solved by X-ray crystallography (34) (Fig. 1A). This analysis revealed that the backbone forms a right-handed spiral comprising three \(-\text{turns} \) in a U-shaped configuration, tightly connected by the three disulfide linkages. Interestingly, the core of the structure does not seem to be stabilized by a prominent hydrophobic core. The importance of the conserved cystines for ST function had previously been elucidated by mutagenesis: disruption of the 2-5 and 3-6 cystines (Cys6Ala and Cys17Ala STp mutants), which are shared with the endogenous ligands, resulted in a complete loss of toxicity (31, 51). In contrast, toxicity was diminished but not abolished by breaking the ST-specific 1-4 cystine (Cys5Ala STp mutant). The functional effects of the cystine-breaking mutants probably reflect their important structural role.

Mutagenesis studies suggest that the region consisting of the three residues of the second \(-\text{turn} \) (STp, Asn11-Pro12-Ala13) (Fig. 1B), which is completely conserved among the bacterial GC-C ligands, is important for interaction with the GC-C receptor (34). As would be predicted, mutation of these three residues (32, 60, 70, 71, 73) more or less diminishes toxin activity (Fig. 1 and 2). Furthermore, structural analysis revealed that the three residues protrude as a patch, thus creating a possible site of interaction with the GC-C receptor (34). One weakly toxic (STp, Ala13Gly) mutant and one nontoxic (STp, Ala13Leu) mutant showed only minor structural differences compared to the fully toxic analog (42). This observation further corroborates the functional importance of this conserved patch.

**ST Vaccinology**

ST made immunogenic through chemical conjugation to carriers. Due to its small size, ST is nonimmunogenic in its natural form, and hence, the first step toward the generation of an ST toxoid is to couple it to an appropriate carrier. The first report of a successful attempt to make ST immunogenic was chemical conjugation of ST to porcine immunoglobulin G (21).
Rats immunized with this ST-IgG conjugate were protected against ST-only-producing ETEC when tested using the ligated ileal loop assay (19). In the next step toward developing an ST vaccine, synthetic ST was conjugated to the B subunit of the heat-labile enterotoxin (LT-B) and shown to be immunogenic in both rats and rabbits. These immunized animals were protected in challenge experiments with ST and ST-producing ETEC (22, 23, 27). Interestingly, the chemical coupling of ST to LT-B reduced the toxicity more than 600-fold (22). LT-B is an attractive carrier since it provides immunization against LT as well as having the ability to target the conjugate to the GM1 ganglioside (30). After showing protection in animals, the ST-LT-B conjugate was also tested by peroral immunization of human volunteers, and Klipstein and coworkers were able to show a strong antitoxin response in both serum (IgG) and jejunal aspirates (IgA) (25). A completely synthetically produced peptide vaccine consisting of ST and 26 amino acids of LT-B yielded similar results in human volunteers (24). In both cases, the jejunal aspirates neutralized ST in the suckling mouse assay (12), indicating that it is also possible to mount an immunogenic response to ST in humans. It is worth noting that the synthetic ST toxin used by Klipstein and colleagues was based on the STp sequence published by Chan and Giannella (5), which was later discovered to have two interchanged residues (STp. Asn11Tyr and Tyr18Asn) (54). However, this mutant ST peptide apparently had the same biological and immunological properties as native ST (16, 27). In spite of the apparent successful generation of both a chemical conjugate and a synthetic ST vaccine candidate, these promising studies have not been further pursued.

One advantage of chemical conjugation is that it provides the opportunity to attain high hapten-to-carrier ratios, which may be important for achieving sufficiently high titers of anti-ST antibodies for effective neutralization in vivo. The results obtained with ST-LT-B chemical conjugates are included in Table 1. In addition to porcine IgG and LT-B, ST has been chemically conjugated to bovine serum albumin (17) and cholera toxin B (CT-B) (52).

**Genetic fusion of ST to carriers.** Recombinant techniques have the advantage of a simple and cheap mode of toxoid production, providing precisely defined and homogeneous proteins that can be delivered by live bacterial vectors (9). The first carrier to which ST was genetically fused was the A subunit of the heat-labile enterotoxin (LT-A), and the LT-A-ST fusion protein was shown to react with an ST monoclonal antibody (41). In later studies, ST has been fused to a number of different carriers in various ways, including LT-B (4, 9, 14), pLT(Arg192Gly) (73), CT-B (39, 40), the outer membrane protein OmpC (38), the ZZ moiety derived from *Staphylococcus aureus* protein A (28), the major subunit ClpG of *Escherichia coli* CS31A fimbriae (2), *Salmonella* flagellin (35), and green fluorescent protein (GFP) (65). The most successful genetic fusions of ST to suitable carriers, from a human vaccine perspective, are summarized in Table 1.

The first successful attempt at rendering ST immunogenic by genetic fusion was the coupling of a fragment of STh (positions 6 to 15), carrying a Cys7Ala mutation, to the N terminus of CT-B (40). This study was followed by experiments where various ST-related fragments, including the full sequence of STh, were fused to the N and C termini of CT-B,
resulting in several fusion proteins that elicited ST-specific immune responses in rabbits (39). The obtained level of anti-ST antibodies only partially neutralized ST in challenge experiments with suckling mice. It is also worth noting that all fusion proteins were recognized by one of two neutralizing monoclonal antibodies (MAb) against ST, whereas a free ST C terminus was required for recognition by the second neutralizing MAb. However, the fusions retained some toxicity (A.-M. Svennerholm, personal communication). The CT-B component of the currently licensed cholera vaccine contributes to its protection against LT-ETEC diarrhea (6–8). A CT-B-ST vaccine could accordingly give protection against both ETEC enterotoxins.

Due to the success of using LT-B in chemical conjugation constructs, it was also chosen as a carrier for genetic fusions to ST (4, 9). When ST was fused directly to the C terminus of LT-B, no anti-ST antibodies were elicited in immunized mice (9). The insertion of a seven-amino-acid linker, however, rendered ST immunogenic. Furthermore, the resulting antibodies, when mixed with ST, were able to neutralize ST toxicity in suckling mice (9). In a follow-up study, a Salmonella enterica serovar Dublin strain that expressed a differently shaped LT-B-ST fusion construct with an eight-amino-acid linker was used to immunize mice orally (4). The results were rather puzzling, since neither sera nor mucosal secretions exhibited reactivity to native ST in an enzyme-linked immunosorbent assay (ELISA), but they were both able to neutralize native ST in suckling mice. Interestingly, the genetic fusions of ST to LT-B also rendered ST nontoxic when assessed in the suckling mouse assay (9).

More recently, the genetic fusions of the mutants STp (Pro12Phe) and STp (Ala13Gln) to porcine LT (Arg192Gly) were reported to elicit neutralizing antibodies in rabbits after intramuscular immunization (73). ST has also been genetically coupled to the S. aureus protein A ZZ fragment (28) and Salmonella flagellin (35). The ZZ-ST fusion peptide, where ST was fused to the N terminus of ZZ, elicited an immune response in rabbits with serum levels that were able to neutralize native ST in suckling mice. The toxicity of ZZ-ST was lower than that of native ST, and a polymeric form of ZZ-ST was about 100 times less toxic than the monomeric fusion protein (28). When ST was inserted into the hypervariable region of the Salmonella flagellin, a good antigenic response was observed only after two of the disulfide bonds were disrupted by substitution of cysteine residues (StH, Cys7Ala Cys18Ala) (35). The flagellin-ST fusion protein was delivered using an attenuated Salmonella strain. These genetic fusion experiments show that the mode of presenting ST epitopes is important for proper antigenic recognition and that minor changes can have profound effects on immunogenicity.

The ability to attain a high hapten-to-carrier ratio by genetic fusion, as opposed to chemical conjugation, is limited. However, recombinant constructs of ST-CT-B and ST-LT-B have been reported to pentamerize, thereby creating a 5:1 hapten-to-carrier ratio (37, 40).

Enhancing ST antigenicity. One very interesting result obtained with ST chemically conjugated to LT-B suggests that it is possible not only to render ST immunogenic but also to increase its immunogenicity through proper manipulation of the polypeptide (26). By manipulating the conditions used to promote disulfide bond formation in synthetically produced ST, Klipstein et al. were able to isolate hyperantigenic ST variants.

Detoxifying ST. Detoxification of ST can be achieved, at least in part, by genetic fusion or chemical conjugation, but it has often been combined with mutagenesis (2, 35, 39). Several mutants with effects on toxicity have been reported and are summarized in Fig. 2.

The previously mentioned reduction or complete loss of toxicity resulting from mutations of the conserved cysteines emphasizes the important structural role of the disulfide bridges (31, 51). The next residue that was targeted for mutagenesis was Asn11 of STp. Six different amino acids were substituted for Asn11 (Fig. 2), and all six ST mutants were recognized by anti-ST antibodies, suggesting that the variants have structural conformations similar to that of native ST. Two of the variants, Asn11Lys and Asn11Arg, were apparently nontoxic, and the other four showed reduced toxicity (32). One substitution in the same position, namely, Asn11Val, was later reported not to affect toxicity (70).

Mutations of the two conserved amino residues immediately following Asn11 in the sequence, Pro12 and Ala13, showed results similar to those for the Asn11 mutants (60, 71, 73). All published substitutions in these two positions have reduced toxicity (Fig. 2).

One challenge in the development of an ST vaccine is to separate toxicity from antigenicity (2). Due to the small size of ST, it is likely that single-amino-acid substitutions which reduce toxicity may also impinge on the resulting toxoid’s ability to induce neutralizing antibodies. The literature reports substitutions that do not effect antigenicity (32), as well as others that clearly disrupt the ability to elicit neutralizing immune responses to the native toxin (3). The discovery of a neutralizing MAb that is specific for the N terminus of the molecule which does not include the toxic domain is encouraging in this respect (53). ST variants with greatly reduced toxicity, resulting from the mutation of two cysteines (STp, Cys6Ala and Cys17Ala), are also promising, since they are still recognized by a neutralizing antibody (51). Finally, the recently published STp (Pro12Phe) and STp (Ala13Gln) vaccine candidates also retained antigenicity while showing greatly reduced toxicity in GC-C receptor cell-based and porcine ligated loop assays (73).

ST vaccine delivery. Most published studies use antibody responses in serum as a measure of immunogenicity and antigen response. There is good evidence, however, that a strong mucosal antibody response with production of secretory IgA is needed for prevention of ETEC disease (50). Oral delivery of the vaccine is therefore a natural first approach, presenting antigens directly to the gut-associated lymphoid tissue. An oral vaccine is therefore a natural first approach, presenting antigens directly to the gut-associated lymphoid tissue. An oral vaccine is also desirable, because it can be administered easily to children without the need for injections. Attempts at expressing ST in attenuated bacteria intended for oral delivery include use of attenuated Salmonella, Shigella, and Lactobacillus reuteri (4, 65, 74). Alternative routes to elicit mucosal responses could include transgenic foods or nasal or rectal administration. Some evidence suggests that transcutaneous immunization can induce secretory IgA, as can parenteral priming followed by mucosal boost (13). Most published animal experiments have used oral or intraperitoneal administra-
tion, with a few studies administering the vaccine candidate by subcutaneous or intramuscular injections.

Vaccine challenges and safety issues. A successful ST vaccine must be able to elicit a strong immune response to ST in the gut. Provided this can be achieved, there are still concerns as to whether an ST-based vaccine can be efficacious (49). The molar ratio of anti-ST antibody needed for neutralization of the toxin in the suckling mouse assay seems to be approximately 1:1 (53). As far as we are aware, there are no estimates of the amount of ST toxin produced during an ETEC infection in humans, and a concern is that the gut might be unable to produce the amounts of anti-ST antibodies required to effectively neutralize ST. However, even if it may be impossible to achieve a complete neutralization of ST, an ST vaccine may give partial protection against the ST toxin and hence effectively reduce the ETEC disease burden.

A particular safety issue that must be addressed prior to clinical evaluation of a vaccine candidate is the possibility of cross-reactivity between anti-ST antibodies and endogenous peptides of the guanylin family. Since the endogenous GC-C ligands and ST have similar structures and activate the same receptor, cross-reactivity due to epitope mimicry may occur. The ideal ST vaccine should elicit neutralizing antibodies that bind specifically to ST epitopes not shared by the guanylin peptides. It should be noted, however, that cross-reactivity shown in vitro does not necessarily imply that a vaccine will induce clinical autoimmune disease (64). Conversely, lack of demonstrable cross-reactivity in vitro does not exclude autoimmune reactions. We are not aware of any publications that have addressed these important ST vaccine safety aspects.

CONCLUSIONS

The most promising ETEC vaccine to date is a killed whole-cell vaccine comprising five different ETEC strains, which express the most prevalent CFs, coadministered with recombinant cholera toxin B subunit (8, 48). The information reviewed here suggests that a multivalent ETEC vaccine can be improved by adding an ST toxoid component. Since ST is present in approximately three-quarters of symptomatic ETEC infections (61), an ST toxoid-containing vaccine has the potential of covering a broad range of ETEC infections. Even if an ST vaccine does not induce full protection against the ST toxin, it could still substantially reduce the severity of ETEC infections, as has been demonstrated for cholera vaccines (50, 59).

To summarize, a successful ST vaccine should consist of a nontoxic ST mutant coupled, either by chemical conjugation or by recombinant fusion, to a carrier, in order to evoke an adequate immune response. Despite the fact that only a few residues have been subject to extensive substitution experiments and the exact mechanism of interaction between ST and the GC-C receptor remains unknown, several promising toxoid candidates have been developed. These results encourage further research on ST as a vaccine target.

ACKNOWLEDGMENTS

We thank Hans Steinisland for providing valuable comments on the manuscript and Ann-Mari Svennerholm for providing supplementary information on her experiments with ST mutants. This work was supported by the Research Council of Norway, GLOBVAC program, grant number 185872/S50.

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Editor: A. T. Maurelli