Properties of Cross-Linked Toxoid Vaccines Made with Hyperantigenic Forms of Synthetic Escherichia coli Heat-Stable Toxin

FREDERICK A. KLIPSTEIN,1* RICHARD F. ENGERT,1 AND RICHARD A. HOUGHTEN2

Department of Medicine, University of Rochester Medical Center, Rochester, New York 14642,1 and Molecular Genetics Group, Scripps Clinic and Research Foundation, La Jolla, California 920372

Received 17 October 1983/Accepted 1 February 1984

The ability of hyperantigenic preparations of synthetically produced Escherichia coli heat-stable toxin (ST) to provide an immunogenically more potent vaccine when cross-linked by the glutaraldehyde reaction to the heat-labile toxin B subunit was assessed. Three synthetic ST preparations were evaluated: ST(S) had the same antigenicity and toxicity (secretory potency in the suckling mouse assay) as native ST, ST 1056 had 3.5-fold more antigenicity and 1% toxicity, and ST(C) had 15-fold greater antigenicity and 31% toxicity. Vaccines that contained equal antigenic proportions of ST and B subunit, as determined by enzyme-linked immunosorbent assays, consisted by weight of 52% ST(S), 25% ST 1056, and 9% ST(C). The initially lower toxicity and smaller proportions by weight of hyperantigenic ST preparations yielded vaccines that had nearly 10-fold less residual ST toxicity than the ST(S) vaccine. Immunization of rats with graded dosages of vaccines containing 9% ST(C) and 51% ST(S) by weight, but equal amounts of ST(S) antigenicity, raised to the same degree dose-dependent increases in mucosal immunoglobulin A antitoxin titers to ST(S) which correlated with the amount of protection against challenge with a viable LT+/ST− strain. These observations indicate that hyperantigenic synthetic ST preparations provide immunologically more potent vaccines than those obtained with the previously used synthetic ST(S) preparation, which has the same biological properties as native ST.

One approach currently being considered for its potential to provide immunological protection against diarrheal disease caused by enterotoxigenic strains of Escherichia coli (ETEC) is immunization with cross-linked toxoid forms of the heat-stable (ST) and heat-labile (LT) toxins that are produced by such strains. In contrast to the multiplicity, antigenic diversity, and high prevalence of unidentifiable forms of specific pilus antigens responsible for mucosal adherence among human ETEC strains (9, 28, 38), both enterotoxins have been purified to homogeneity, and their properties have been well characterized. Although some degree of structural and immunological variation exists between toxins produced by human versus animal ETEC strains (1, 6, 11, 14), both LT (or its nontoxic B subunit) and ST from either source have sufficient immunological homology so that immunization of experimental animals with them provides uniform active or passive protection against challenge with toxins produced by heterologous strains or with viable ETEC strains regardless of their source, somatic serotype, or pilus antigens (17, 21, 33). LT and ST are antigenically distinct, however, so that immunization with either toxin form provides protection only against those ETEC strains which produce the homologous toxin, either singly or together or with the heterologous toxin (16, 18, 21). This has been resolved by cross-linking the haptenic ST toxin to LT, thereby creating a vaccine that is immunogenic for both toxin forms (19).

We have recently described the properties of a vaccine made by cross-linking ST to the B subunit of LT toxin (20). The synthetically produced ST preparation used, ST(S), consists of the same amino acid sequence and has the same secretory potency and antigenic properties as native ST (22). Based on our finding that the immunogenicities of ST(S) and the LT B subunit are the same when tested by immunization with graded dosages of each in experimental animals, the cross-linked vaccine is prepared to contain equal antigenic proportions of each toxin component (21). The B subunit is nontoxic, the residual toxicity of the ST component is markedly reduced by the cross-linking reaction, and the vaccine has proven to be innocuous when given parenterally and/or perorally (p.o.) to various animal species, including dogs, rabbits, rats, guinea pigs, and mice (23; F. A. Klipstein, and R. F. Engert, unpublished data). When given by p.o. immunization to rats or rabbits, the vaccine raises dose-dependent increases in specific immunoglobulin A (IgA) antitoxin titers to each toxin component in intestinal mucosal washings which correlate with the degree of protection against active challenge in ligated ileal loops with either the ST or LT toxin or with viable bacteria which produce them, either singly or together (19, 21, 23).

In the present study, we determined whether a vaccine that is immunogenically more potent but has even less residual ST toxicity could be obtained by substituting other synthetically produced ST preparations whose stereochemistry was modified to yield toxins that have greater antigenicity but less toxicity (i.e., secretory activity) than ST(S). The composition, antigenicity, and residual ST toxicity of vaccines made by cross-linking two such hyperantigenic synthetic ST preparations to human LT B subunit were compared with the one previously tested which used ST(S). The increased immunogenicity of one of the hyperantigenic ST preparations was confirmed by determining the mucosal IgA antitoxin response and degree of protection in rats that were given p.o. immunization with graded dosages of vaccines containing either ST(S) or the hyperantigenic ST preparation.
MATERIALS AND METHODS

Enterotoxin production. The complete procedure for the solid-phase synthesis and purification of synthetic ST based on the structural formula of human ST reported by Chan and Giannella (4) has been described in detail previously (22; R. A. Houghten, J. M. Ostrech, and F. A. Klipstein, submitted for publication). Briefly, the peptide was synthesized on a Beckman model 990 B peptide synthesizer using benzhydramine resin and appropriately protected amino acids. The crude peptide was extracted from the resin with acetic acid, desalted on a Sephadex G-10 column (Pharmacia Fine Chemicals, Piscataway, N.J.), and incubated with dithiothreitol to maintain it in the reduced state. It was purified by passage through a DEAE-cellulose column and a Beckman high-performance liquid chromatography (HPLC) system. The HPLC eluate was shown to be homogeneous by amino acid analysis, thin-layer chromatography, paper electrophoresis, and analytical HPLC.

The HPLC-purified linear sequence of ST was oxidized by exposing it at a concentration of 1.0 mg/ml (500 mM) in the presence of 0.5 M NaHCO₃ to air at room temperature for 18 h; this material, which was not further processed, is designated ST(C). Another portion of the linear ST was oxidized under the same conditions, except that the concentration used was 0.1 mg/ml (50 mM). After lyophilization, this material was passed through a Sephadex G-50 column equilibrated with 0.2 M NaHCO₃. This yielded two peaks: the initial peak was determined to be multimeric and was designated ST 1056. The second peak was monomeric; this material was repassaged through preparative HPLC, and that eluate which had the identical secretory potency and antigenicity as pure native ST was designated ST(S) (22). The details of these procedures concerned with altering the stereochemistry of the disulfide bridges of synthetic ST will be presented in a subsequent communication.

The LT toxin B subunit was obtained from cultures of E. coli pDF87, a transformed K-12 derivative bearing the B subunit plasmid of human E. coli H10407 (5); it was purified by published chromatographic techniques (7). The amount of toxins used was based on their protein concentrations determined by the method of Lowry et al. (29); their molar equivalents were derived from published values (13, 25).

Conjugation conditions. The three synthetic ST preparations were cross-linked to B subunit by mixing various initial molar ratios of these toxins in the presence of Sigma grade I glutaraldehyde (GA) (Sigma Chemical Co., St. Louis, Mo.). The ratio of GA to total protein of the toxin mixtures was varied in each reaction so that the GA to B subunit molar ratio was kept constant at 700:1. This concentration of GA was used because it was found to provide effective cross-linking without attenuation of B subunit antigenicity (F. A. Klipstein and R. F. Engert, unpublished observations). After a 2-h reaction at room temperature, the conjugates were rapidly chilled, exhaustively dialyzed for 48 h against TEAN buffer (Tris, EDTA, sodium azide, NaCl) at 4°C, and then processed as described previously (20).

The antigenities of the toxin materials, either alone or in conjugated form, were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (20, 22). The antigenicity of each of the three synthetic ST preparations was measured by a double-sandwich technique using goat and rabbit hyperimmune antisera to ST(S); that of the B subunit was determined using goat hyperimmune antiserum to human B subunit. The antigenicity of each toxin component of the conjugates was expressed as a percentage of that of the concomitantly assayed respective unattenuated toxin; the percent antigenicity of each toxin component multiplied by 1,000 yielded the number of antigen units (AU) per milligram of vaccine (10, 21). The antigenicity of each of the three synthetic ST preparations was expressed in terms of that of ST(S) and in each instance is referred to as ST AU. One ST AU is the equivalent of the antigenicity of 1 μg of unattenuated native ST.

The residual ST toxicity of the vaccines was determined by testing graded dosages in the suckling mouse assay, in which 1 mouse unit is defined as that amount which yields an intestinal/carcass weight of >0.083 (19). Values for each of the synthetic ST preparations in unattenuated and conjugated forms were compared, and the results were expressed either as the fold reduction in secretory potency for that particular preparation or as the equivalent amount of unattenuated ST (in terms of secretory potency) present per unit of vaccine.

Immobilization procedures. Sprague-Dawley rats (150 to 175 g) were given primary immunization intraperitoneally using Freund complete adjuvant, followed by two boosters given p.o. at 4-day intervals. Immunization p.o. was given via an intragastric tube 2 h after the p.o. administration of cimetidine (Tagamet; Smith Kline & French Laboratories, Carolina, P.R.) at a dosage of 50 mg/kg of body weight to ablate gastric secretion.

Challenge procedures. The immunized rats were challenged 4 to 6 days after the final booster by the instillation into a single 10-cm ligated loop of distal ileum for 18 h of 0.1 ml of a broth culture containing 10⁸ viable organisms of E. coli LT/ST strain TX 452 (O78:H12) per ml as described previously (18, 21). Each datum point was determined in four to six immunized rats, and the results reported are for the mean ± standard error of the mean percentage of reduced secretion in immunized rats as compared with the value in five uninunmunized control rats similarly challenged. Reduced secretion of >50% represented a significant (P < 0.001) difference, as determined by Student’s t test for two independent means, between values in immunized and control animals.

Antitoxin response. Mucosal IgA antitoxin titers to ST(S) and the B subunit were determined by ELISA as described previously (19, 21). Antitoxin titers to ST in animals immunized with vaccines containing either ST(S) or ST(C) were determined by a double-sandwich technique in which goat hyperimmune antiserum to ST(S) was used as the solid phase and ST(S) was the antigen; in each instance the titers are expressed in reference to ST(S). The values reported are the mean fold increases, rounded to the nearest integer value, in the titers of immunized rats over those in unimmunized control rats. Antitoxin titers in the controls were 1:2 against both toxin components; thus, a titer of 1:64 in immunized animals represented a sixfold increase.

RESULTS

Properties of the synthetic ST preparation. The antigenicity of ST 1056 was 3.5-fold greater and that of ST(C) was 15-fold greater than the antigenicity of ST(S) as determined by ELISA using hyperimmune antisera to ST(S) (Fig. 1). Values for 1 mouse unit in the suckling mouse assay were 5.7 ng for ST(S), 445 ng for ST 1056, and 18.2 ng for ST(C); thus, ST 1056 had 13% and ST(C) had 31% of the secretory potency of ST(S).

Conjugation with the B subunit. Each of the three synthetic ST preparations was cross-linked to B subunit using various initial molar ratios of ST to B subunit (Fig. 2). Conjugates
containing equal proportions of ST and B subunit by weight were derived from an ST(S) to B subunit ratio of 45:1 and an ST 1056 to B subunit ratio of 40:1; ratios of ST(C) to B subunit sufficient to yield such a conjugate were not used. The antigenicity of the toxin components was not reduced by the cross-linking reaction. Conjugates with approximately equal antigenic proportions for each toxin component contained 52% ST(S), 25% ST 1056, and 9% ST(C) by weight. Since the proportion by weight of B subunit was highest in conjugates with the least ST, conjugates with the hyperantigenic ST preparations contained more B subunit AU. In conjugates with approximately equal AU proportions of each toxin component, the ST(S) conjugate had 500 AU, the ST 1056 conjugate had 725 AU, and the ST(C) conjugate had 900 AU of each toxin component per mg (Table 1).

The residual ST toxicity present in conjugates which contained 1,000 AU of each component was the equivalent of approximately 100 ng of unattenuated ST in those made from each of the hyperantigenic ST preparations; this amount was nearly 10-fold less than that of the ST(S) conjugate. The difference was due principally to the fact that conjugates made from the hyperantigenic preparations contained less ST by weight. Although the hyperantigenic ST preparations initially had a lower secretory potency than ST(S), this property was counterbalanced by the fact that their toxicities were less strongly attenuated than that of ST(S) by the cross-linking reaction. Thus, when the secretory potency of each ST preparation in vaccine form was compared with its original value, that of ST(S) was reduced 727-fold, that of ST(C) was reduced 256-fold, and that of ST 1056 was reduced only 44-fold.

**Immunization of rats with ST-B subunit vaccines.** Groups of rats were immunized with two vaccines. In one, ST(S) was cross-linked at an initial molar ratio of 70:1 to B subunit by the carbodiimide reaction as described previously (21); this vaccine contained 51% ST(S) and 49% B subunit by weight and had 450 AU of each toxin component per mg. In the second, ST(C) was cross-linked at an initial molar ratio of 10:1 to B subunit by the GA reaction; this conjugate contained 9% ST(C) and 91% B subunit by weight and had 1,300 ST AU and 917 B subunit AU per mg. The dosages used were based on the amount of ST AU per milligram in each vaccine; 1,000 ST AU were contained in 2.2 mg of the ST(S) vaccine and in 769 µg of the ST(C) vaccine.

All rats received intraperitoneal primary immunization with 200 ST AU, followed by various dosages of the two p.o. booster immunizations such that total p.o. dosages ranged between 500 and 3,000 ST AU (Fig. 3). When the total p.o. immunization dosage was expressed in ST AU, responses to the ST(S) and ST(C) vaccines were identical: each vaccine raised the same dose-dependent increase in the mucosal IgA ST antitoxin response and in the degree of protection against challenge with a viable LT⁻/ST⁺ strain. However, when the p.o. dosages were expressed on the basis of the amount of ST given by weight, ST(C) was markedly more effective: the total p.o. dosage of ST(C) necessary to yield 50% reduced secretion in challenged rats was 70 µg, or 15 times less than the 1,050 µg of ST(S) required to achieve this.

![FIG. 1. Antigenicity of different synthetic ST preparations as determined by the double-sandwich ELISA technique using hyperimmune antiserum to ST(S). Values for antigenicity were derived by comparing the number of micrograms of ST required to yield an optical density of 0.600 in ELISA; those for toxicity (secretory potency) are from data in suckling mouse assays presented in the text. In each instance, the value for ST(S) was considered to be 100%.](image-url)

**FIG. 2.** Composition and antigenicity of conjugates obtained by cross-linking the synthetic ST preparations at various initial molar ratios to the B subunit. Values for the percentage of each toxin component are based on Lowry protein (29). Values for AU were determined by ELISA and are expressed per milligram of vaccine. All ST AU values are expressed in terms of ST(S).

**TABLE 1.** Properties of cross-linked vaccines made from different synthetic ST preparations.

<table>
<thead>
<tr>
<th>Conjugation</th>
<th>Vaccine</th>
<th>% (wt)</th>
<th>AU/mg</th>
<th>1,000-AU vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST(B)</td>
<td>ST</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>ST(S)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST 1056</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST(C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>ST (mg)</th>
<th>ST (µg)</th>
<th>ST (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST(S) (45/1)</td>
<td>52</td>
<td>48</td>
<td>500</td>
</tr>
<tr>
<td>ST 1056 (20/1)</td>
<td>25</td>
<td>75</td>
<td>849</td>
</tr>
<tr>
<td>ST(C) (10/1)</td>
<td>9</td>
<td>91</td>
<td>1,300</td>
</tr>
</tbody>
</table>

a Initial molar ratio of ST to B subunit.
b Amount of vaccine needed to provide 1,000 AU of each toxin component and amount of ST by weight contained in this vaccine.
c Equivalent amount of unattenuated ST secretory potency, as determined by suckling mouse assay, in this amount of vaccine.
Additional studies were carried out to determine whether an ST(C) vaccine containing a higher proportion of ST by weight and AU is equally immunogenic and whether this vaccine is effective when given to rats exclusively by the p.o. route. The vaccine was derived from an initial molar ratio of ST(C) to B subunit of 30:1; it contained 30% ST(C) and 70% B subunit by weight and had 4,000 ST AU and 690 B subunit AU per mg. Rats were given four p.o. immunizations at weekly intervals with 250 µg of vaccine containing 1,000 ST AU. This raised a sixfold increase in mucosal IgA ST antitoxin titers and provided strong protection, with reduced secretion of 86 ± 8% (mean ± standard error of the mean), against challenge with the viable LT−/ST+ strain.

DISCUSSION

ST has been purified from human (35, 37), porcine (3, 24), and bovine (34) ETEC strains, and the amino acid sequence of the human (1, 4, 32, 36) and porcine (24, 25) toxins has been determined. The toxins consist of 18 or 19 amino acids, although the active site is confined to the C-terminal 14 amino acid residues. One-third of the molecule consists of six cysteine residues which are joined together by disulfide bridges. Although some variation has been noted among different preparations, principally in the presence of tyrosine versus asparagine at the C-terminal 4 and 8 positions, the basic structure of human and porcine ST is remarkably similar. ST preparations based on this structure have now been produced synthetically by several laboratories. The ST(S) preparation which we have used previously (20, 21, 23) is based on the 18-amino acid sequence described by Chen and Giannella for human ST (4); it is identical in terms of biological and antigenic properties to native ST (22). Ikekura et al have used solution methods to produce a 19-amino acid peptide, containing tyrosine rather than aspara-
ST(C) was 15 times more immunogenic than ST(S) in immunized rats, a value that correlated completely with the determination by ELISA that ST(C) is 15 times more antigenic than ST(S).

Two other modifications were incorporated into the present study which facilitated the production of a more efficiently produced vaccine. The first was that the B subunit was obtained directly from an *E. coli K-12* strain that had been modified by recombinant techniques to produce only human B subunit (5), rather than by dissociation procedures from the LT holotoxin as we have done in the past. The second was that GA was substituted for carbodiimide as the cross-linking reagent. GA has a number of advantages. (i) At the proper concentration, it provides effective cross-linking of ST to the B subunit without affecting their antigenicities. (ii) It yields more efficient coupling of ST to B subunit, so that vaccines containing equal antigenic proportions of ST and B subunit can be derived from lower initial molar ratios of ST to B subunit, thereby significantly reducing the amount of ST needed to make the vaccine (F. A. Klipstein and R. F. Engert, unpublished observations). (iii) Unlike carbodiimide, there is a considerable body of experience that attests to the safety of administering GA conjugates to humans (8, 26, 31). GA toxoids lost favor a decade ago when immunization with GA-treated cholera toxoid provided disappointing results in humans (27, 30); however, methods such as ELISA were not then available to determine the actual antigenicity of these toxoids, so that immunization dosages were unknown in terms of the number of cholera AU given. When expressed in this manner in the present study, the dose-dependent responses to vaccines cross-linked using either GA or carbodiimide were identical.

ACKNOWLEDGMENT

This work was supported by a grant from Johnson and Johnson, New Brunswick, N.J.

LITERATURE CITED


