INFECTION AND IMMUNITY, Aug. 1982, p. 550–557
0019-9567/82/080550-08$02.00/0

Vol. 37, No. 2

Development of a Vaccine of Cross-Linked Heat-Stable and Heat-Labile Enterotoxins That Protects Against *Escherichia coli* Producing Either Enterotoxin

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Received 1 March 1982/Accepted 13 April 1982

A vaccine of cross-linked heat-stable (ST) and heat-labile (LT) toxins that protects against heterologous serotypes of strains of *Escherichia coli* which produce either the LT or ST enterotoxin was developed by conjugating ST to LT by the carbodiimide reaction. Three interrelated factors were found to affect the composition and properties of the final conjugate: (i) the amount of carbodiimide added to the toxins, (ii) the initial ratio of ST to LT, and (iii) the duration of the conjugation reaction. Optimal conjugation conditions were identified as a carbodiimide-to-toxin ratio of 10:1 by weight, an initial molar ratio of ST to LT of 100:1, and a conjugation reaction time of 96 h. This approach yielded a conjugate that contained 96% by moles and 36% by weight pure ST, determined with radioiodinated pure ST, and 34% by weight semipure ST, determined by the Lowry protein method. The retained antigenicities of the conjugated toxins, as determined by enzyme-linked immunosorbent assays, was ≥82%, and their toxicities, as determined by the Y1 adrenal cell assay for LT and by the suckling mouse assay for ST, were reduced to ≤0.15%. Immunization of rats with this cross-linked ST-LT vaccine provided strong protection against challenge with either the LT or the ST toxin or with viable heterologous strains which produce these toxins, either singly or together. These observations indicate that conjugation of ST to LT results in a unique new immunogen in that ST acquires immunogenicity as a function of the reaction, LT retains most of its antigenicity, and the toxic properties of each individual toxin are greatly reduced.

Acute diarrheal disease due to transient colonization of the small bowel by enterotoxigenic strains of *Escherichia coli* (ETEC) represents a major health problem of global scope. These organisms, together with rotavirus, are the principal cause of the acute diarrhea in young children which results in an estimated 10 million deaths per year among infants living in underdeveloped tropical countries (2, 24). ETEC strains are also usually responsible for the acute diarrhea that afflicts most visitors to the tropics (turista) (12, 26). They are also a frequent cause of sporadic or epidemic episodes of diarrhea among persons living in either temperate or tropical climates (25, 28) and a major cause of frequently fatal diarrhea among weanling animals, particularly lambs and piglets (20). The most practical approach for prevention of ETEC-induced diarrhea in these varied circumstances would be an immunization program that would provide protection against heterologous ETEC serotypes of strains which produce either the heat-labile (LT) or heat-stable (ST) enterotoxin.

Three different *E. coli* antigens have been shown to be capable of arousing immunological protection against ETEC strains in experimental animals. (i) Immunization with the somatic antigen protects by reducing growth of the homologous organism within the intestine, but it does not affect growth or protect against any of the multiple heterologous somatic serotypes (9, 20). (ii) Immunization with the specific fimbrial antigen responsible for adherence to the mucosal surface protects by inhibiting intestinal colonization, but this does not extend to strains possessing heterologous fimbrial antigens (6, 23), and it has recently been recognized that there are multiple antigens of this nature (7, 21). (iii) Immunization with either the LT or ST toxins evokes an antitoxin response that protects against homologous and heterologous serotypes of strains that produce the specific toxin used for immunization (15, 17, 18). Immunization with LT yields protection against strains which produce this toxin alone (LT^+/ST^-) or together with ST (LT^+/ST^+), but not against those which elaborate just ST (LT^-/ST^+) (15, 17). Long

considered to be nonantigenic, ST has now been shown to be haptenic; it becomes immunogenic when coupled to a large-molecular-weight carrier and is capable of arousing antibodies that neutralize its secretory effect in the suckling mouse model (8, 11, 14). Immunization with a semipurified preparation of ST coupled to porcine immunoglobulin G (PIG) protects against active challenge with LT^-/ST^+, but not against LT-producing strains (18).

An effective toxoid vaccine against all ETEC strains, therefore, should contain both LT and ST; the ST toxin must be rendered immunogenic by coupling it to a carrier that is suitable for administration to humans, and the component toxins should be toxoided in a manner such that their toxicities are reduced to a tolerable level and an adequate degree of antigenicity is maintained. In this report, we describe the development of such a vaccine, cross-linked ST-LT, which was made by conjugating ST to LT by the carbodiimide reaction. The influence of different conjugation conditions on the composition and properties of the final conjugates was evaluated, permitting the identification of optimal conditions which yielded cross-linking of the maximal amount of ST to LT in a reaction in which the antigenicity of the component toxins was only slightly attenuated, but their toxicities were markedly reduced. Rats immunized with this vaccine were shown to be strongly protected against challenge with either the LT or ST toxin or with viable organisms which produce either or both of these toxins.

MATERIALS AND METHODS

Enterotoxin preparations. Purified LT holotoxin was prepared by the methods described by Clements and Finkelstein (4) from *E. coli* strain 711 (F1LT), a transformed K-12 derivative bearing LT gene(s) of the Ent plasmid from porcine strain P307. Homogeneity of the LT toxin was confirmed by polyacrylamide gel electrophoresis as described previously (4).

ST toxin was purified from *E. coli* LT^-/ST^+ strain 18D (O42:H37) (kindly provided by R. Giannella) by the procedures described by Staples et al. (29), which include growth in minimal medium, followed by Amberlite XAD-2 chromatography, acetone fractionation, sequential Sephadex G-25 gel filtration, DEAE-Sephacel ion exchange chromatography, and rechromatography on Sephadex G-25. ST-containing fractions obtained during each of these procedures were identified by the suckling mouse assay (10), in which one mouse unit is defined as that amount which yields an intestinal weight/carcass weight ratio of ≥ 0.083. Thin-layer chromatography (29) of the suckling-mouse-active eluate from the final Sephadex G-25 gel filtration revealed two discrete bands: one, with an R_f of from 0.6 to 0.7, that was active in the suckling mouse assay; and another, with an R_f of from 0.3 to 0.5, that was inactive. Eluates of the active band, considered to be pure ST, contained 250 mouse units per μg. Due to large losses of toxin incurred during the purification procedures, particularly the DEAE-Sephacel ion exchange and thin-layer chromatography steps, we were unable to accrue a sufficient quantity of pure ST needed to perform all of the conjugation studies and immunize large groups of rats. For this reason, most studies employed a concentrate of the active fractions obtained from the first Sephadex G-25 gel filtration purification step. This material, which is referred to as semipure ST, contained 185 mouse units per μg of protein and 45% of the antigenicity of pure ST when assayed by enzyme-linked immunosorbent assay (ELISA) against hyperimmune antisera to pure ST (see below).

The amount of toxins used (referred to as weight) was based on protein concentrations as determined by the method of Lowry et al. (22). Molar equivalents were derived from published values of molecular weights (91,450 for LT [5] and 1,972 for ST [29]). This provided only an estimation for the heterogeneous semipure ST preparation.

Radioiodination of ST. Pure ST was radioiodinated by the chloramine-T method of Hunter (13) in a reaction mixture which contained 1 mg of ST, 0.3 mM potassium iodide, 5.5 mM chloramine-T, phosphate-buffered saline, and 1 mCi of carrier-free I^{125} (New England Nuclear Corp., Boston, Mass.). After a 10-min incubation, the reaction was terminated by the addition of sodium metabisulfite and an excess of potassium iodide. After dialysis in a 1,000 molecular weight-cutoff Spectra-Por dialysis bag (Spectrum Medical Industries, Los Angeles, Calif.) against water for 18 h at 4°C, the dialysand was applied to a column of Sephadex G-25-40 (2.5 by 80 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) and eluted with 10 mM ammonium bicarbonate in 15% isopropyl alcohol. The eluate fractions were monitored for radioactivity, optical density at 280 nm, and suckling mouse activity. Fractions in the active peak were pooled, lyophilized, and reconstituted in 2 ml of water; this material contained 500 mouse units and 8.8×10^5 cpm/ml.

Conjugation. ST was conjugated to LT by adding 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC) (Sigma Chemical Co., St. Louis, Mo.) to mixtures of ST and LT in 0.1 M phosphate buffer (pH 7.0) for 18 h at 4°C unless otherwise specified. The conjugate was then exhaustively dialyzed against water for 48 h at 4°C, using a 12,000 MWCO dialysis bag which retained all of the LT and conjugated ST but not unconjugated ST or EDAC. Repeated experiments showed that dialysis of LT alone against water resulted in a 10% loss, as measured by the Lowry protein method, due to precipitation of some of the toxin. Therefore, the amount of semipure ST conjugated to LT was determined by measuring the incremental increase in Lowry protein present in the dialysand that was in excess of 90% of the amount of LT initially added to the conjugate. The amount of radioiodinated pure ST conjugated to LT was ascertained by comparing the radioactivity of the conjugates to that added, using a PRIAS PGD auto-gamma counter (Packard Instrument Co., Downers Grove, Ill.).

Properties of conjugate. Assays conducted on uncorrected samples reflected the percentage of each specific toxin present, whereas in those made on corrected samples, the concentration of the conjugate was adjusted to represent 100% of the specific toxin tested.

In either instance, the results were expressed as the percentage of the amount of toxin in the conjugate compared with that of unconjugated LT or semipure ST that was needed to achieve a positive response in that assay.

(i) Toxicity. LT toxicity was compared by assaying serial two-fold dilutions of LT and conjugates in the Y1 adrenal cell assay (27). ST toxicity was compared in terms of mouse units per μg, which were established by determining the minimal effective dosage of serial dilutions of semipure ST or conjugates in the suckling mouse assay.

(ii) Antigenicity. Antigenicity was determined by ELISA (30). For LT, monospecific goat hyperimmune antiserum to LT (5) was used together with rabbit anti-goat antiserum conjugated to alkaline phosphatase (Miles Research Laboratories, Elkhart, Ind.). For ST, hyperimmune antiserum was raised in goats and rabbits by parenteral immunization (three occasions at monthly intervals) of a 1-mg preparation of pure ST, conjugated at a molar ratio of 100:1, to PIG (Sigma Chemical Co.), using an EDAC/total protein ratio of 45:1 by weight. To remove potential cross-reacting irrelevant antibodies, antibody to PIG was removed from the goat serum by affinity chromatography, using a Sepharose 4B column (Pharmacia Fine Chemicals) to which PIG had been covalently bound; complete removal of antibody to PIG was confirmed by ELISA of the eluate against PIG. Antibody titers to pure ST in the two antisera were ≥1:131,072 as determined by ELISA. They were used in a double-sandwich technique along with rabbit anti-goat antiserum conjugated to alkaline phosphatase (Miles Research Laboratories).

Serial two-fold dilutions, starting at 10 μg, were made of the conjugates and appropriate toxin (Fig. 1). The concentration at which these preparations yielded an adsorbance of 0.600 at 410 nm was determined, and the value for the toxin in the conjugate was expressed as a percentage of that for the unattenuated toxin.

Immunization procedures. Rats were given primary immunization intraperitoneally (i.p.), using Freund complete adjuvant followed by two peroral (p.o.) boosts 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, Puerto Rico) at a dosage of 50 mg/kg of body weight, an amount shown to be sufficient to ablate gastric secretion in rats (3).

Challenge procedures. Rats were challenged 1 week after the final boost by the instillation of test material into a single 10-cm ligated loop of distal ileum for 18 h as described previously (15, 16). Previous studies have established a correlation between significant protection in this assay system and that achieved in rats challenged by intestinal contamination of the intact intestine (19). Challenge dosages were those which evoked the maximum secretion in unimmunized animals: 0.5 ng of LT, 20 ng of semipure ST, 5 ng of pure ST (this preparation was more potent than that previously described in reference 18), and 0.1 ml of broth cultures containing 10^9 viable organisms per ml of LT^+/ST^- strain PB-258 (O15:H$^-$), LT^+/ST^+ strain H-10407 (O78:H11), and LT^-/ST^+ strain Texas 452 (O78:H12). The somatic serotype of each challenge strain was heterologous to those of the strains used to produce the toxins for immunization. Each datum point was determined with from three to five rats, and the values reported are for the mean ± standard error of the mean of the degree of reduced secretion in immunized rats as compared with unimmunized rats similarly challenged. Protection is referred to as significant when secretion in immunized rats was less than that in unimmunized rats, with a P value of <0.001 as determined by Student's t test for two independent means.

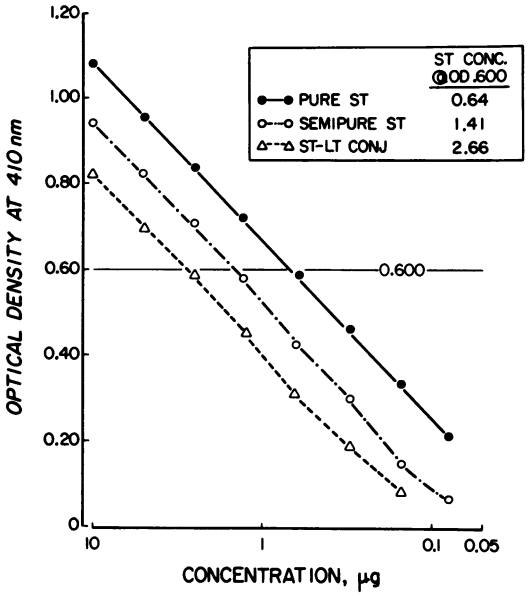

FIG. 1. Comparison of ST antigenicity (determined by ELISA), using hyperimmune antiserum to pure ST. Based on concentrations (micrograms per well) at an optical density of 0.600 nm, the antigenicity of semipure ST was 45% of pure ST and that of the LT-ST conjugate illustrated was 53% of semipure ST.

RESULTS

Effect of amount of EDAC used. Initial experiments ascertained the ratio of EDAC to the toxins needed to achieve maximal coupling of ST to LT. EDAC was added in quantities that resulted in ratios, by weight, of between 2:1 to 200:1 to the total protein contained in a 100:1 molar ratio of semipure ST to LT (Fig. 2). Progressive increases in the EDAC/total protein ratio resulted in incremental increases in the proportion of ST present in the final conjugate, with coupling of the maximum amount of ST occurring at an EDAC/total protein ratio of approximately 45:1.

Effect of ratio of ST to LT. Based on the above findings, EDAC/total protein ratios of 45:1 were used in studies concerned with the influence of the ratio of ST to LT on the degree of ST conjugation.

(i) Radioiodinated pure ST. Ten nanomoles of pure ST, together with a tracer dose of 30 μl of radioiodinated pure ST, was mixed with from 10

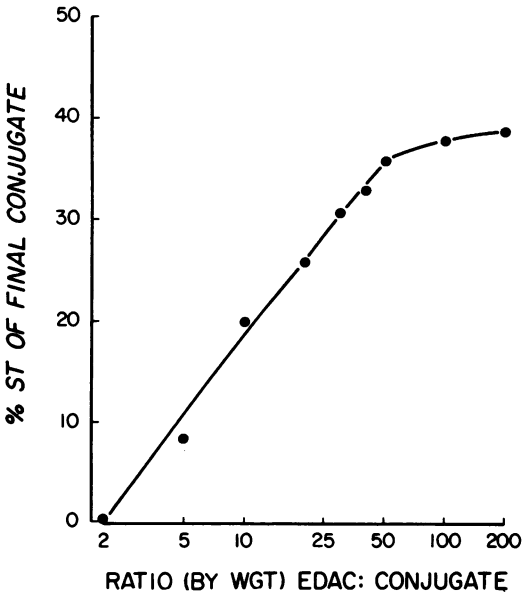

FIG. 2. Effect of the ratio of EDAC to total protein of the toxins on the degree of ST conjugation from a molar ratio of semipure ST/LT of 100:1. Values are the percentages of ST in the final conjugate based on weight (Lowry protein). Reaction time was 18 h.

to 0.1 nmol of LT, resulting in initial ST/LT molar ratios of from 1:1 to 100:1. Progressively higher initial ST/LT ratios resulted in a linear increase in the percentage of the ST added to the initial mixture that was retained in the conjugate, thus resulting in progressive increases in the proportion of ST in the final conjugate (Fig. 3). By using an initial ST/LT molar ratio of 100:1, the final conjugate contained 96% ST by moles and 36% ST by weight.

(ii) Semipure ST. A constant amount of semipure ST was added to various quantities of LT, resulting in initial ST/LT molar ratios of from 10:1 to 200:1 (Fig. 4). Progressively higher initial ST/LT ratios resulted in increasingly greater proportions of ST in the final conjugate, with an initial ST/LT molar ratio of 100:1 resulting in a final conjugate which contained 34% ST by weight.

Antigenicity of conjugated toxins. The antigenicity of the conjugates shown in Fig. 4 was tested in uncorrected samples that reflected the relative amount (percentage) of each toxin present in the conjugate. LT antigenicity fell progressively and was consistently less than the amount of toxin present in the conjugate; for example, in the conjugate derived from an initial ST/LT molar ratio of 200:1, LT comprised 62% of the final conjugate by weight, but the LT antigenicity of this material was only 12%. Although values for the antigenicity of ST rose as the proportion of this toxin in the final conjugate increased, they too were consistently less than the amount of ST in the final conjugate. Toxin antigenicity was then tested in conjugates whose concentrations had been adjusted to reflect 100% of each specific toxin; values for the antigenicity of either LT or ST did not increase by more than 15% in any instance.

These findings indicated that the antigenicity of the toxins was being reduced by the conjugation process. Although the ratio of EDAC to total protein was kept constant at 45:1 for each conjugate, the various quantities of the LT carrier in conjugates with different initial ST/LT ratios resulted in a different EDAC/specific toxin ratio for each conjugate, so that increasing the initial ST/LT ratios resulted in progressively increasing EDAC/LT ratios and decreasing EDAC/ST ratios (Fig. 4). This suggested that EDAC was responsible for the changes in antigenicity.

Effect of EDAC on toxin properties. Various quantities of EDAC were added to a semipure ST/LT molar ratio of 100:1, resulting in EDAC/total protein ratios of from 2:1 to 100:1 (Fig. 5). The properties of the toxins in the final conjugates were determined on corrected samples whose concentrations were adjusted to reflect 100% of each specific toxin. Increasing the EDAC/total protein ratio resulted in a reduction in LT toxicity (that of ST was not measured) and in the antigenicity of both toxins. LT toxicity was reduced to 50% at an EDAC/LT ratio of 6:1

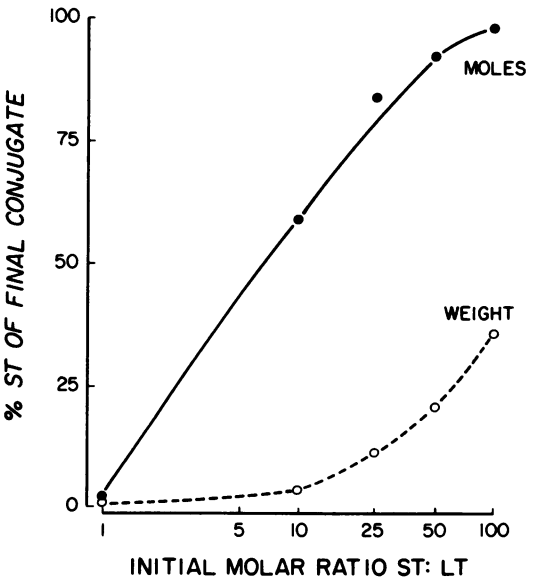

FIG. 3. Effect of the initial molar ratio of pure ST to LT on the percentage of ST present in the final conjugate as determined by using a tracer dose of radioiodinated ST. The EDAC/total protein ratio was 45:1, and the reaction time was 18 h.

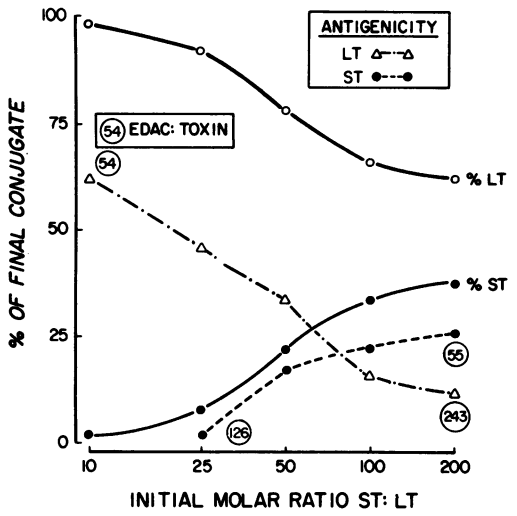

FIG. 4. Composition and properties of conjugates derived from mixing different molar ratios of semipure ST to LT, using an EDAC/total protein ratio of 45:1, with an 18-h reaction time. Values for antigenicity were derived from uncorrected samples which reflect the percentage of each toxin present. Circled numbers indicate the ratio of EDAC to specific toxin.

and to values of <1% at an EDAC/LT ratio of 32:1. EDAC/LT ratios of >32:1 caused a sharp decline in LT antigenicity (from 78 to 44%); similarly, ST antigenicity fell precipitously when EDAC/ST ratios exceeded 43:1. These observations indicated that EDAC/total protein ratios of >10:1 contained sufficient EDAC to severely compromise the antigenicity of the conjugated toxins.

Effect of duration of the conjugation reaction. Since an EDAC/total protein ratio of 10:1 yielded a submaximal degree of ST incorporation (20% by weight) in the final conjugate from the optimal ST/LT molar ratio of 100:1 after an 18 h conjugation reaction, we determined whether different conjugation times would result in a more efficient coupling of ST to LT. Increasing the conjugation reaction time between 2 and 192 h for a semipure ST/LT molar ratio of 100:1 exposed to an EDAC/total protein ratio of 10:1 resulted in a progressive increase in the amount of ST coupled to LT in the absence of significant attenuation of the antigenicity of either conjugated toxin (Fig. 6). The percentage (34%) of ST in the final conjugate after a 96-h reaction time, using an EDAC/total protein ratio of 10:1, was the same as that achieved by conjugation for 18 h, using an EDAC/total protein ratio of 45:1; antigenicity was maintained under the former conditions but was markedly compromised under the latter.

Properties of the cross-linked ST-LT vaccine. The above in vitro observations defined optimal conjugation conditions as an ST/LT molar ratio of 100:1, an EDAC/total protein ratio of 10:1, and a conjugation reaction time of 96 h. The properties of the immunogen derived in this manner are summarized in Table 1. The vaccine contained 34% ST and 66% LT by weight; the

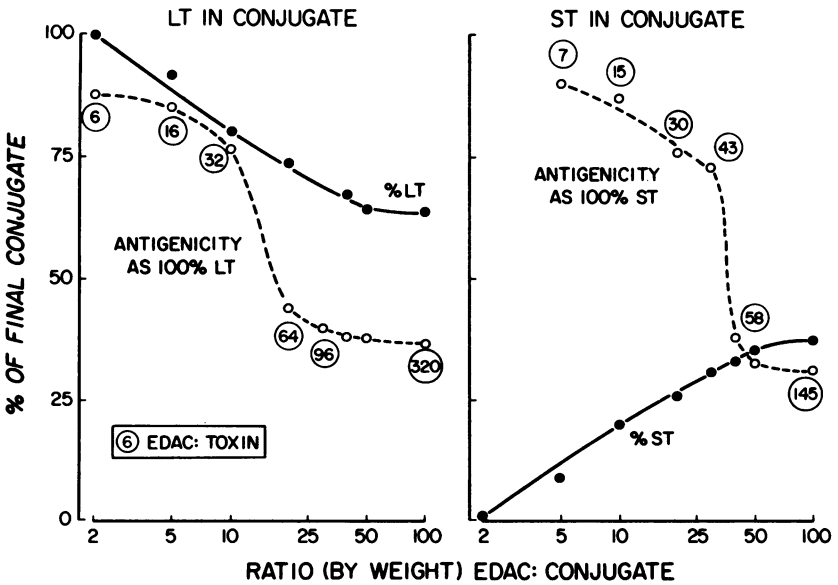

FIG. 5. Effect of varying the EDAC/total protein ratio on the antigenicity of the conjugated toxins. Semipure ST was conjugated to LT at a molar ratio of 100:1 for 18 h. Values for antigenicity were derived from corrected samples adjusted to reflect 100% of each particular toxin in each conjugate. Circled numbers indicate the ratio of EDAC to specific toxin.

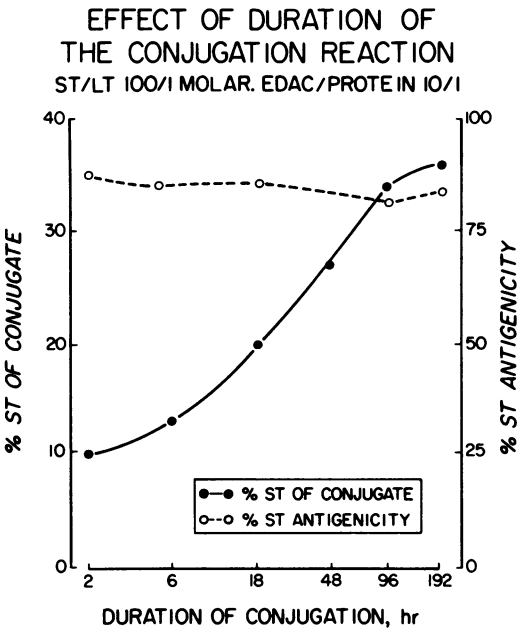

FIG. 6. Effect of the duration of the conjugation reaction on the percentage of semipure ST in the final conjugate. The ST/LT molar ratio was 100:1, and the EDAC/total protein ratio was 10:1. Values for antigenicity were derived from corrected samples adjusted to reflect 100% of the toxin.

toxins retained ≥82% of their antigenicity, but their toxicities were reduced to levels of ≤0.15% those of unconjugated toxins (Fig. 7). When expressed on a per-unit basis (i.e., the uncorrected values), the vaccine contained 29% ST and 49% LT antigenicity, with toxicities of ≤0.06%.

Results of immunization of rats. Previous studies have shown that the degree of protection in rats immunized by an i.p. prime followed by p.o. boosts is directly related to the total p.o. dosage (15, 16). Preliminary observations indicated that the amount of LT needed to achieve significant protection, using two p.o. boosts, is 1,000 antigen units (dosage in micrograms × percentage of antigenicity), whereas that of semipure ST coupled to PIG is about 1,400 antigen units. Based on this, it was calculated that a total p.o. dosage of 5,000 μg of the cross-linked ST-LT vaccine contained a sufficient quantity of both toxins: 1,392 ST antigen units determined on the basis of the adjusted sample (5,000 μg × 34% toxin × 82% antigenicity) and 1,450 antigen units determined on the basis of the per-unit values (5,000 μg × 29% antigenicity), and between 2,450 and 2,739 antigen units of LT (see Table 1).

Groups of rats were immunized with either LT, using a 100-μg i.p. prime and two 500-μg p.o. boosts, or the ST-LT vaccine, using a 1,000-μg i.p. prime and two 2,500 μg p.o. boosts (Table 2). Rats immunized with LT were significantly protected ($P < 0.001$) only against this toxin and viable LT-producing organisms, whereas those immunized with the ST-LT vaccine were significantly protected against LT, pure ST, semipure ST (95 ± 3% reduced secretion), and heterologous viable organisms which produce these toxins, either singly or together.

DISCUSSION

The development of a vaccine containing ST and LT required that each toxin be manipulated in such a way so as to attenuate its toxic properties without significantly compromising its ability to stimulate an immune response. The first consideration was to render the ST molecule immunogenic. Antibodies can be aroused to low-molecular-weight nonantigenic but haptenic compounds by conjugating them to a large-molecular-weight protein carrier; water-soluble carbodiimides have been used extensively for this purpose (1). This has already been done for ST coupled by this reaction to bovine serum albumin or to immunoglobulin (8, 11, 14). Rather than introducing an irrelevant antigen into the vaccine as the carrier for ST, we elected to use LT since, in toxoided form, it would be suitable for use in humans. The concept was to cross-link LT and ST via the available carboxyl and amino groups on the two molecules and, additionally, to intrachain link the LT molecules as a means of reducing toxicity. Our results show that conjugation of ST to LT by the carbodiimide reaction yields such an immunogenic toxoid in that the toxicities of the individual toxins were re-

TABLE 1. Properties of the cross-linked ST-LT vaccine

Toxin	%[a]	Adjusted to 100%[b]			Per unit		
		Toxicity (%)	Antigenicity[c] (%)	AU	Toxicity (%)	Antigenicity (%)	AU
ST	34	0.15	82	1,394	0.06	29	1,450
LT	66	0.06	83	2,739	0.03	49	2,450

[a] Percent by weight.
[b] Concentrations of the conjugates were adjusted to reflect 100% of each toxin.
[c] AU, The number of antigen units (amount of toxin multiplied by percentage of antigenicity) present in 5,000 μg of vaccine.

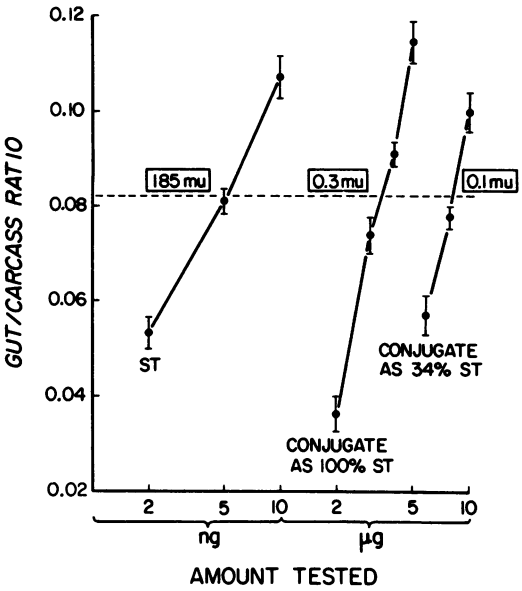

FIG. 7. Effect of conjugation on the toxicity of semipure ST. Conjugation conditions were an ST/LT molar ratio of 100:1, an EDAC/total protein ratio of 10:1, and a 96-h conjugation time. MU, Mouse units.

duced; LT retained most of its antigenicity, and ST acquired immunogenicity as a function of that reaction.

Three factors in the conjugation process modified the composition and final properties of the conjugate. The first was the initial ratio of ST to LT. Increasing this ratio resulted in a linear increase in the amount of ST coupled to the LT carrier, thus yielding conjugates which had progressively greater proportions of ST in the final conjugate. Under optimal conjugation conditions, an initial ST/LT molar ratio of 100:1 appeared to yield maximum saturation of the LT carrier, with ST comprising 96% by moles and 36% by weight of the final conjugate. The second factor was the amount of EDAC used for conjugation. Increasing the ratio of EDAC added to the toxins resulted in progressively greater proportions of ST in the final conjugate; however, it also caused a progressive decline in the antigenicity of the conjugated toxins so that an EDAC/total protein ratio of 45:1, which achieved maximum ST incorporation after an 18 h conjugation reaction, caused an unacceptable degree of reduced antigenicity. The third factor was the duration of the conjugation reaction. Extending the reaction time from 18 to 96 h resulted in maximum incorporation of ST when a lower EDAC/total protein ratio of 10:1 was used which did not markedly compromise the antigenicities of the conjugated toxins. Conjugation of a 100:1 molar ratio of ST to LT, using an EDAC/total protein ratio of 10:1 and a conjugation reaction time of 96 h, yielded an immunogen composed (by weight) of one-third ST and two-thirds LT whose antigenicities were maintained at ≥82%, but whose toxicities were decreased to levels of ≤0.15% of those of unconjugated toxins. When used as a vaccine, this immunogen provided strong protection against challenge with either toxin.

Most of the investigations conducted in this study employed a semipurified preparation of ST: it contained 74% of the suckling mouse activity of pure ST and had 45% of the antigenicity of pure ST when assayed by ELISA against hyperimmune serum to pure ST. This approach was necessitated by our inability to produce, using published methods, the relatively large quantities of homogeneous, pure ST needed to conduct all of the studies. Although the molar equivalent values used for the heterogeneous semipure ST preparation were approximations, it is noteworthy that the results achieved when the same quantities by weight of semipure and pure ST were used were similar even when assessed by two different techniques. Thus, conjugation of an initial molar ratio of pure ST to LT of 100:1 yielded a conjugate containing 36% ST by weight as determined by radioiodinated ST, whereas conjugation of semipure ST to LT at the same molar ratio yielded 34% ST in the final conjugate as determined by the incremental increase in the value for Lowry protein. Although our observations with radioiodinated pure ST established the validity of those obtained with semipure ST regarding composition of the conjugates, the applicability of our findings concerning the effect of different conjugation conditions on conjugate properties remains to be confirmed once an adequate source of this material becomes available. Although we can see no reason why the use of pure ST will significantly alter

TABLE 2. Results of challenge in rats immunized with the cross-linked ST-LT vaccine

Immunogen used	% Reduced secretion after challenge with[a]:				
	LT toxin	LT^+/ST^-	LT^+/ST^+	ST toxin[b]	LT^-/ST^+
LT	92 ± 2	69 ± 3	54 ± 1	0	3 ± 2
ST-LT	87 ± 7	70 ± 5	67 ± 3	68 ± 2	67 ± 2

[a] More than a 50% reduction in secretion represents a significant ($P < 0.001$) difference between immunized rats and unimmunized controls.

[b] Pure ST.

our basic observations, it should provide a vaccine that is more immunogenic for ST.

ACKNOWLEDGMENTS

We thank Helen Short for technical advice and Deborah Hogerman and Dianne Flint for technical assistance.

This study was supported by a grant from Johnson and Johnson Baby Products Co., Skillman, N.J.

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