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Production and Evaluation of Antibody to the Heat-Stable Enterotoxin from a Human Strain of Enterotoxigenic *Escherichia coli*

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Escherichia coli heat-stable enterotoxin was coupled to bovine serum albumin by a carbodiimide reagent. Antibody to the conjugate was produced by immunization of rabbits. Data from radioimmunoassay and infant mouse tests indicate the presence of antibody to the enterotoxin. The antisera can be used in a radioimmunoassay to measure enterotoxin in various fluids.

The most common test for detection of the heat-stable enterotoxin (ST) of *Escherichia coli* is the infant mouse assay described by Dean et al. (3). This method depends upon the accumulation of fluid in the mouse gut after intragastric injection of ST. Although this method has gained widespread use, it has certain drawbacks, including lack of quantitation, research nature, and failure to detect ST in some epidemic *E. coli* strains, have led to a search for other methods.

Immunochemical methods which are highly sensitive, specific, and easy to perform have not been developed because of the scarcity of purified, well characterized ST. Recently, several investigators (1, 2, 4, 6-9), using *E. coli* from various animal and human sources, have described methods for the purification of ST. The characterizations of the toxin by these researchers indicated the existence of multiple molecular sizes and different compositions of amino acids. The antigenic nature of ST has not been resolved; it appears to be weakly antigenic even when coupled to a protein carrier.

Recently, the Food and Drug Administration obtained a quantity of ST prepared in accordance with the method of Staples et al. (8). This ST, derived from a strain of *E. coli* pathogenic for humans, has a molecular weight of approximately 2,000. This paper describes the preparation of a conjugate of ST and bovine serum albumin, the immunization of rabbits, and the evaluation of the antisera by radioimmunoassay and by the infant mouse tests.

MATERIALS AND METHODS

Reagents. *E. coli* ST was obtained through Food and Drug Administration contract 223-77-2115, in accordance with the method of Staples et al. (8). The toxin, produced by strain 18D serotype 042:k86:H37, was isolated from a case of infant diarrhea and produced only ST. Test cultures of enterotoxigenic *E. coli* were obtained from Food and Drug Administration, Division of Microbiology stocks. Cultures were grown in Casamino Acids-yeast extract broth, and sterile filtrates were used in the tests. Other reagents were obtained as follows. Bovine serum albumin (BSA) fraction V powder was from Calbiochem; rabbit serum albumin (RSA) fraction V powder was from Nutritional Biochemicals Corp.; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (ECDI) was from Pierce Chemical Co.; 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluene sulfate (CMC) was from Aldrich Chemical Co.; ^{125}I (100 mCi/ml) was from Amersham Corp.; and Pansorbin (10% [wt/vol] suspension of *Staphylococcus aureus* Cowan 1 cells) was from Calbiochem. Reagent-grade chemicals were used to prepare phosphate-buffered saline (PBS; 0.07 M phosphate and 0.07 M sodium chloride, pH 7.2). RSA/PBS was prepared with 0.5% RSA plus 0.1% sodium azide (NaN_3) as a preservative.

Preparation of ST-BSA and ST-RSA conjugates. A mixture of 20 mg of BSA and 10 mg of purified ST was dissolved in 2 ml of distilled water, and the pH was adjusted to 5.5. Dry ECDI (13 mg) was added, the pH was readjusted to 5.5, and the mixture was stirred gently overnight at room temperature. The reaction mixture was dialyzed against PBS for 48 h at 4°C. The ultraviolet spectrum was obtained on a diluted portion of the reaction mixture and compared with the spectrum of a reaction mixture from which ST was omitted. The ST-BSA conjugate was stored at −20°C.

A 20-mg portion of RSA and 10 mg of purified ST were combined with 30 mg of CMC under the same reaction conditions as those chosen for the ST-BSA conjugate.

Antisera. Antisera to the ST-BSA conjugate were produced by immunization of three New Zealand albino rabbits. The rabbits were injected intramuscularly with 1-mg amounts of conjugate in Freund complete adjuvant. Additional injections were made at days 14 and 53, and the animals were bled on day 67. A later conjugate injection was made on day 209, and

the animals were bled on day 223.

Iodination of ST-RSA. ST-RSA (50 μg) was labeled with ^{125}I by a modification of the chloramine-T method (5).

Antibody-binding determination. Appropriate dilutions of rabbit anti-ST-BSA in 0.1 ml of RSA-PBS, 0.1 ml of ^{125}I-labeled ST-RSA (ca. 40,000 dpm), and 0.5 ml of RSA/PBS were incubated at 37°C for 4 or 15 h. A portion of Pansorbin reagent (0.1 ml) was added to each tube, and the contents were mixed and incubated at room temperature for 30 min. A 2.5-ml portion of saline-Tween 20 (9 g of NaCl and 0.5 g of Tween 20 in 1 liter of distilled water) was added to each tube. The samples were centrifuged for 6 min at 3,000 × *g*, and the supernatant was discarded. The ^{125}I activity associated with the Pansorbin reagent was measured in a gamma spectrometer. Control tubes containing diluted preimmune rabbit serum and RSA/PBS alone were included with each determination.

Biological activity. The infant mouse test was used to detect ST activity in culture filtrates from *E. coli*. An outline of the test as it was performed in this laboratory has been presented (P. L. Spaulding and J. Lovett, Abstr. Annu. Meet. Am. Assoc. Lab. Anim. Sci. 1977, p. 59–60). For neutralization experiments, antisera from various bleedings were pooled and diluted 1:2 and 1:10 with sterile saline. A mixture of 1.0 ml of sterile culture filtrate and 1.0 ml of the appropriate dilution of antisera was incubated at 4°C overnight and then for 95 min at 37°C. The samples were analyzed for ST immediately after incubation. A positive control consisting of an ST-positive culture plus two dilutions of pooled preimmune rabbit serum was incubated and included in the mouse test.

RESULTS

The formation of conjugates between ST and BSA or RSA was inferred from the ultraviolet spectra (data not shown). The conjugate spectrum showed increased absorption in the 260- to 280-nm region, compared with native BSA or RSA spectrum. In the absence of ST, the reaction of BSA and ECDI had no effect on the spectrum, compared with the reaction of native BSA. No estimates were made of the molar ratios of the reactants in the conjugates.

Figure 1 shows the results of the binding of ^{125}I-ST-RSA to various dilutions of antisera. This figure represents binding data for pooled sera from the two bleedings of the three rabbits. The maximum radioactivity bound at the highest serum dilution (1:10) represented 13,031 of 20,837 cpm or 63% of the added ^{125}I-ST-RSA. Even at a serum dilution of 1:20480, 9% of the added radioactivity bound. The preimmune serum at 1:10 and the binding assay buffer bound 4 and 5% of ^{125}I-ST-RSA, respectively. The binding curves for each bleeding and each rabbit were generally similar to the one presented in Fig. 1. Rabbits 1 and 3 may have had greater responses to the booster injection, as illustrated by a percentage of ^{125}I-ST-RSA binding activity higher than that for the first bleeding.

Data on the inhibition of ^{125}I-ST-RSA binding to serum by purified ST are presented in Table 1. Decreased binding (B/B_0, <100) of the ^{125}I-ST-RSA reflected this inhibition. As little as 2 ng of ST per ml in RSA/PBS inhibited the binding by about 43% at a serum dilution of 1:2,000, whereas at a dilution of 1:640, the same concentration inhibited binding at 9%. As the serum dilution increased, the sensitivity of the inhibition increased.

The serum neutralization of the biological activity of ST in the culture filtrate, as determined by the infant mouse test, is presented in Table 2. At a serum dilution of 1:10, culture B7A was the only positive one of the eight ST samples tested. The preimmune serum did not neutralize the activity, as shown by the results for culture 694, which was incubated with two different dilutions of the serum. The cultures were isolated from porcine and human strains. The purified ST used for antibody production was obtained from a human source. The data suggest the existence of common antigenic and biological activity sites. This observation is supported by the data obtained for culture fluids examined in

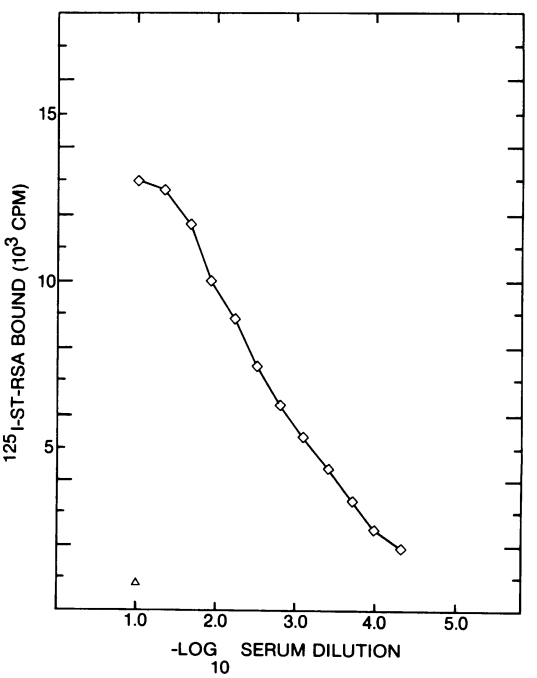

FIG. 1. *Binding of ^{125}I-ST-RSA to dilutions of pooled immune sera (◇) and a dilution of pooled preimmune sera (△). Sera and labeled reagent were incubated overnight at room temperature.*

the ^{125}I-ST-RSA-binding determination. The data, presented in Table 3, show a decreased binding (B/B_0, <100) which reflects this inhibition. The heat-labile toxin (LT) cultures did not significantly inhibit binding, whereas all the ST-positive cultures caused more than 50% inhibition of binding.

DISCUSSION

Carbodiimides can activate functional groups on proteins, including carboxylic acids, sulfhydryls, and tyrosines. Based on this property, *E. coli* ST was conjugated to BSA and RSA. Sera from rabbits immunized with ST-BSA were evaluated with ^{125}I-ST-RSA by radioimmunoassay binding procedure. A different protein and carbodiimide were used for the preparation of ST-RSA conjugate to avoid any possible complications from antibodies to BSA-carbodiimide by-products.

The data presented here demonstrate the formation of antibody against ST-BSA conjugate by immunological and biological methods. Immunological activity was evident from the increased binding of ^{125}I-ST-RSA to antisera when compared to the binding to preimmune sera (Fig. 1) and the inhibition of binding of ^{125}I-ST-RSA to antisera by ST in the buffer (Table 1) and culture filtrates (Table 3). The neutralization of biological activity of ST in culture filtrates by antisera was observed in the infant mouse assay (Table 2).

Iodination of ST-RSA conjugate produced a useful reagent which could be incorporated into the development of a sensitive radioimmunoassay. Direct iodination of ST would have presented technical difficulties, owing to its size and its sensitivity to oxidizing and reducing agents. Preliminary investigations indicated that ST dissolved in buffer at 1 ng/ml could be detected by radioimmunoassay.

Various studies (1, 2, 4, 6–9) show that the molecular size of *E. coli* ST varies. However, in two of the studies (1, 8) amino acid analysis showed a similarity by the presence of six half-cystine residues but a difference in N-terminal residues. The data obtained for neutralization and inhibition of ST in culture filtrates by the antisera suggest that there is a similarity in the active regions of STs derived from animal and human strains.

This study indicates that an immunoassay test can be used to measure ST in various fluids. An enzyme immunoassay which makes use of the described antibody is currently under investigation.

TABLE 1. *Inhibition by ST of ^{125}I-ST-RSA binding to antisera*

ST concn (ng/ml)	(B/B_0[a]) at an antiserum dilution of:	
	1:640	1:2,000
100	44.5	30.4
10	56.1	36.1
2	91.2	57.5
1	96.9	71.0
0	100	100

[a] Ratio of bound cpm at given ST concentration to bound cpm at zero ST concentration multiplied by 100.

TABLE 2. *Antisera neutralization of ST activity in the infant mouse test*

Culture	Infant mouse test result[a]		
	Without antisera	With antisera dilution of:	
		1:2	1:10
263 (LT)	−	−	−
1408 (LT)	−	−	−
1362 (LT, ST)	+	−	−
B7A (LT, ST)	+	−	+
694 (ST)[b]	+	−	−
987 (ST)	+	−	−
E-38 (negative)	−	−	−

[a] Suckling mice (1 to 4 days old) were inoculated with 0.1 ml of sample containing 2% Chicago Blue dye. Five mice were used for each assay. After 4 h at room temperature, the mice were killed, and the intestinal tracts were removed. A ratio of intestinal weight to the remainder of body weight greater than 0.08 represented a positive response to ST.

[b] With preimmune sera dilutions of 1:2 and 1:10, culture 694 (ST) was positive.

TABLE 3. *Culture filtrate inhibition of ^{125}I-ST-RSA binding to antisera*

Culture	B/B_0[a]
263 (LT)	93.2
1408 (LT)	95.3
1362 (LT, ST)	23.8
B7A (LT, ST)	21.2
694 (ST)	22.1
987 (ST)	46.8
E-38 (negative)	100

[a] Ratio of bound cpm for the particular culture to bound cpm of the negative culture multiplied by 100.

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