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Immunological Properties of *Escherichia coli* Heat-Stable Enterotoxins: Development of a Radioimmunoassay Specific for Heat-Stable Enterotoxins with Suckling Mouse Activity

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Antiserum was raised against the purified heat-stable enterotoxin (ST) produced by enterotoxigenic *Escherichia coli* strain 431, a class II porcine enteropathogen. The antiserum was used to examine the antigenic determinants of STs produced by enterotoxigenic strains of different host origins and develop a sensitive radioimmunoassay specific for ST having biological activity in suckling mice and piglets (ST_A). The antiserum neutralized one effective dose of toxin at a dilution of 1:5,000 and neutralized approximately 40 μg of toxin per ml of serum. In the radioimmunoassay, protein A-bearing staphylococci was used as the primary solid-phase adsorbent. The purified STs produced by a class I enteropathogen (strain 667) and by a bovine enterotoxigenic strain (B-41) exhibited patterns of competitive inhibition identical to those of homologous unlabeled strain 431 ST in the radioimmunoassay when specific antibody to strain 431 ST was used. The levels of ST in culture supernatants determined by the suckling mouse assay correlated with the concentrations of toxin measured by the radioimmunoassay. The antiserum was specific for ST_A produced by enterotoxigenic *E. coli* of porcine, bovine, and human origins and did not react with heat-labile enterotoxin or with ST that had biological activity in piglets but not in suckling mice (ST_B). These results suggest that ST_A molecules having different host origins share at least one antigenic determinant.

The enterotoxigenic (ENT^+) *Escherichia coli* strains implicated in diarrheal diseases of humans and neonatal animals have been shown to produce at least two different types of enterotoxins (9, 13, 20). The heat-labile enterotoxin consists of two kinds of subunits (4, 14), is antigenic, and cross-reacts immunologically (4) with cholera toxin. Antisera prepared against highly purified cholera toxin have been used to develop immunoassays (7, 27) and a G_{M1} ganglioside enzyme-linked assay for heat-labile enterotoxin (24).

Several workers have described the purification and chemical characterization of heat-stable enterotoxins (STs) having molecular weights ranging from 1,980 to 5,100 (1, 11, 15, 23, 25). The chemical properties common to *E. coli* STs produced by porcine, bovine, and human ENT^+ strains include (i) unique heat stability, (ii) stability to acidic but not alkaline pH, (iii) high content of half-cystine, and (iv) absence of several amino acids (e.g., basic residues) common to proteins.

Most workers depend on the suckling mouse bioassay to detect and quantitate STs (6); however, this assay is impractical for screening large numbers of clinical isolates for ST production, is fairly expensive, and does not detect all forms of ST (2, 10, 17, 19). An immunological assay would facilitate clinical diagnoses of diarrheal disease and be useful for detecting and quantitating STs in partially purified preparations; however, development of such an assay has been hindered by difficulties in obtaining antisera to ST. In contrast to the antigenicity of cholera toxin and *E. coli* heat-labile enterotoxin, attempts to raise antisera to ST by injecting ST either as a crude extract or in a highly purified form have been unsuccessful (1, 20). In this paper we describe the preparation of high-titer antisera to a porcine ST which reacts with STs produced by other strains of ENT^+ *E. coli* and the development of a sensitive radioimmunoassay (RIA). The RIA was used to examine the immunological relatedness of different ST_A molecules.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The ENT^+ *E. coli* strains used were kindly supplied by Harley Moon, National Animal Disease Center, Ames,

Iowa, and R. Bradley Sack, Johns Hopkins University School of Medicine, Baltimore, Md. Stock cultures were maintained on Trypticase soy agar plates or as lyophilized ampoules, as described previously (1). The preparation of media and the growth conditions were as described previously (1).

Toxin bioassay and neutralization test. The suckling mouse assay for ST was performed essentially as described previously (1), except for an incubation time of 2 h, which improved sensitivity at low doses of purified toxin. Mice 2 to 3 days old were selected on the basis of a visible absence of milk in their stomachs and by weight (average weight, 1.95 to 2.05 g). Three mice were used per group to estimate each experimental point. Neutralization activity was assayed by incubating dilutions of antiserum or phosphate-buffered saline with 40 or 10 mouse units of ST at 37°C for 3 h in a final volume of 100 μl. The contents of each tube were diluted with phosphate-buffered saline to 1.0 ml before the bioassay. Neutralization of ST was calculated from the following expression: [dose (nanograms of protein) × dilution which neutralized one effective dose]/[0.1 ml (volume injected)].

Purification of ST. The STs produced by ENT$^+$ *E. coli* strains 431, 667, and 213C$_2$ were purified essentially as described by Alderete and Robertson (1). A total of 80 liters of culture supernatant was subjected to chromatography with Amberlite XAD-2 (50 to 100 mesh) resin (23); this step was substituted for the original UM-2 ultrafiltration step. A gel filtration step with a Bio-Gel P-6 column (2.4 by 90 cm) preceded preparative gel electrophoresis, ion-exchange chromatography with diethylaminoethyl-agarose, and gel filtration with a Bio-Gel P-4 column (1 by 100 cm) instead of a P-10 column. The gel filtration steps were run in the presence of 200 mM NaCl to prevent aggregation of ST. The purified toxins had molecular weights which varied from 4,200 to 4,400 and from 3,400 to 3,600 as determined by gel electrophoresis and amino acid analysis, respectively (L.A. Dreyfus, J.C. Frantz, and D.C. Robertson, manuscript in preparation). Gel filtration in the presence of 200 mM NaCl yielded a more highly purified form of ST than previously described (1). A basic polypeptide containing of 12 or 13 amino acids copurified with our original preparations; however, we observed no differences in biological activity. The purified toxins had effective doses of 0.4 ng per suckling mouse.

Radiolabeling of toxin. In preliminary experiments, toxin was labeled to a low specific activity by using chloroamine-T; biological activity was lost rapidly under a variety of storage conditions when ST was radiolabeled to a high specific activity. Subsequently, we found that ST could be radiolabeled to a high specific activity with a minimal loss of biological activity by using lactoperoxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, Calif.). Suckling mouse activity was not required for the binding of ^{125}I-labeled ST to antibody; however, we used biologically active radioligand whenever possible. Toxin was radioiodinated with chloroamine-T (8) in a reaction mixture (100 μl) which contained 20 μg of ST, 0.5 M sodium phosphate buffer (pH 7.5), 0.9 mM chloroamine-T, and 1.0 mCi of carrier-free Na^{125}I (Amersham Corp., Arlington Heights, Ill.). After 10 min of incubation at room temperature, the reaction mixture was applied to a column (0.8 by 6 cm) containing 2.0 g of Amberlite XAD-2 (50 to 100 mesh) resin to separate free iodine from the labeled toxin. The column was washed with 30 ml of distilled water, and the toxin was eluted with 10 ml of 99% methanol–1% acetic acid. The eluted material was flash evaporated to dryness and dissolved in phosphate-buffered saline. More than 95% of the toxin (specific activity, 100 to 200 Ci/mmol) was recovered, and there was no detectable loss of biological activity when the toxin was assayed within 24 h.

Preparation of conjugates. Toxin was coupled to a bovine serum albumin (BSA) or hemocyanin (Sigma Chemical Co., St. Louis, Mo.) carrier in a reaction mixture which contained a twofold molar excess of ST (0.5 mg), 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3), 4.0 mg of BSA or hemocyanin, 0.2 ml of 0.5 M sodium phosphate (pH 5.5), and trace levels of ^{125}I-labeled strain 431 ST as a recovery marker. This reaction mixture was incubated in the dark for 18 h at room temperature. After extensive dialysis to remove free ST, the amount of toxin coupled to the carrier ranged from 35 to 40%.

Immunization procedure. Each ST conjugate (equivalent to 100 μg of free ST per ml) was mixed with an equal amount of Freund complete adjuvant. New Zealand white rabbits (12 months old) were injected intradermally at multiple sites along the back with 1.0 ml (equivalent to 50 μg of free ST) of either a BSA-ST conjugate or a hemocyanin-ST conjugate. These rabbits received intramuscular booster doses (equivalent to 50 μg of ST each) suspended in Freund incomplete adjuvant at 4- to 5-week intervals for 3 months. The fourth booster dose was increased to the equivalent of 350 μg of ST and was injected subcutaneously in the upper back. Sera were collected before immunization and on a weekly basis starting 3 weeks after the first immunization and were stored at −20°C until they were assayed for anti-ST activity.

RIA procedure. Protein A-bearing staphylococci were used as the primary immunoadsorbent (18) and were obtained commercially (Calbiochem-Behring Corp., La Jolla, Calif.) or were prepared as described by Kessler (12), using *Staphylococcus aureus* Cowan serotype 1 ATCC 12598 (American Type Culture Collection, Rockville, Md.). Routinely, 0.5 ml of a 10% suspension of Formalin-treated *S. aureus* was incubated for 30 min at room temperature with 0.1 ml of serum. The antibody-coated cells were washed and stored at 4°C as a 10% suspension. Further manipulations were carried out at 4°C. To standardize the RIA, the anti-ST immunoglobulin-coated staphylococci were diluted with a 1% suspension of non-immunoglobulin-coated staphylococci (1:8) so that approximately 30% of the radioligand was bound in the absence of unlabeled ligand. The components of the reaction mixture (100 μl) were added as follows: 60 μl of buffer (0.1 M NaCl, 1.0 mM ethylenediaminetetraacetate, 0.1% Triton X-100, 10 mM sodium phosphate, pH 7.5); unlabeled ST; 0.181 pmol of ^{125}I-labeled strain 431 ST; and 20 μl of a 1% suspension of anti-ST-coated staphylococci. After 18 h of incubation at 4°C, 1.0 ml of ice-cold buffer was added to each tube. The tubes

were centrifuged at 3,000 × g for 10 min at 4°C, the supernatant fractions were aspirated, and the pellets were counted with a gamma scintillation counter. The average net counts per minute of each unlabeled ST standard or sample was normalized to a percentage of the net counts per minute of the standard which contained no unlabeled ST. Nonspecific association of ^{125}I-labeled ST to staphylococci which were either uncoated or coated with nonimmune serum was less than 0.1% of the total amount of toxin added.

The toxin-binding activity present in an immune serum was estimated by incubating a 10% solution of staphylococci with the immune serum. The immunoglobulin-coated bacteria were washed, diluted to a 1% suspension, and incubated with increasing amounts of ^{125}I-labeled strain 431 ST. After 18 h of incubation, the immune complexes adsorbed to the staphylococci were pelleted by centrifugation and washed with buffer; the pellets were counted, and the data were analyzed by using Scatchard plots (21). Separate tubes containing ^{125}I-labeled immunoglobulin G were used to quantitate the percentage of the total immunoglobulin adsorbed. The amount of ST bound per milliliter of serum was calculated from the following formula: amount of serum bound = [(nanograms of ST bound at saturation) × (1/serum dilution)]/(percentage of immunoglobulin G adsorbed).

RESULTS

Immune response to ST. Our objectives in this study were to prepare high-titer antisera to *E. coli* ST in order to examine the structural similarities between the STs produced by ENT$^+$ strains having different host origins and to develop a sensitive diagnostic test for ST. In preliminary experiments, the route of immunization and dosage were varied, and different coupling agents and protein carriers were tested. Since ST not coupled to a carrier was a poor antigen (1), toxin was coupled to cyanogen bromide-activated Sepharose 4B and to either BSA or hemocyanin by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. A limited but detectable humoral response was observed with ST coupled to BSA or hemocyanin, whereas no response was detected with ST coupled to Sepharose 4B. In subsequent experiments, BSA was used as a carrier because BSA conjugates were more soluble and had more uniform consistencies than conjugates prepared with hemocyanin. A total of 14 rabbits were immunized by various routes and with different doses. All of these animals responded to the ST-BSA complex and yielded high-titer antisera acceptable for biochemical and immunological studies. Data on the nature and kinetics of the immune response to ST will be described elsewhere.

Antisera obtained at weekly bleedings were incubated with protein A-bearing staphylococci, and their ST-binding capacities was determined. The humoral response rose slightly after each booster dose (equivalent to 50 μg of ST). At 16 weeks, 1 ml of antiserum bound 100 ng of ST. The fourth booster (equivalent to 350 μg of ST) caused the titer to increase significantly, reaching a maximum 2 weeks postinjection; 1 ml of 19-week antiserum bound 36.4 μg of ST (data not shown), and this serum source was used for all of the experiments described below.

Neutralization of ST. The neutralization activity of an antiserum was determined by the suckling mouse assay after incubation of twofold dilutions of antiserum with strain 431 ST (Fig. 1). The data showed that a dilution between 1:5,000 and 1:10,000 completely neutralized 1 effective dose of strain 431 ST and that significant neutralization of 4 mouse units occurred at a 1:1,000 dilution. Based on an effective dose of 0.4 ng (that is, the amount of ST which elicited a ratio of gut weight to body weight of 0.09), 1 ml of antiserum neutralized more than 16 but less than 40 μg of toxin, which was in good agreement with the binding data. Nonimmune rabbit serum at a 1:10 dilution did not show neutralizing activity.

Development of an RIA for ST. In the RIA developed for ST, protein A-bearing staphylococci were used as the primary solid-phase immunoadsorbent (18). Figure 2 shows the dose responses with increasing amounts of unlabeled strain 431 ST. The binding of ^{125}I-labeled ST was inhibited 50% by a 1.6-fold excess of unlabeled ST. Since ENT$^+$ *E. coli* strain 431 is a class II enteropathogen (16, 26), it was of interest to determine whether the STs purified from a class I enteropathogen (strain 667) and a bovine ENT$^+$ strain (strain B-41) were immunologically

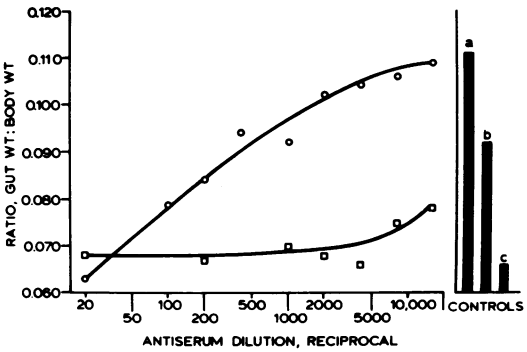

FIG. 1. *Neutralizing activity of anti-strain 431 ST serum. Symbols: ○, neutralization of 4 mouse units per mouse; □, neutralization of 1 mouse unit per mouse. Bars a, b, and c show the ratios of gut weight to body weight for 4 mouse units not treated with antiserum, for 1 mouse unit not treated with antiserum, and for phosphate-buffered saline, respectively.*

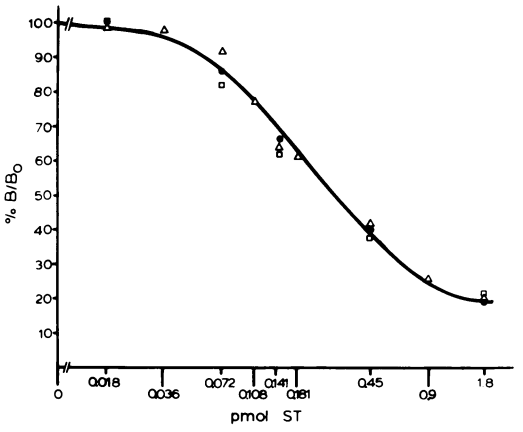

FIG. 2. *RIA for ST. Symbols: △, strain 431 ST; □, strain B-41 ST; ●, strain 667 ST. % B/B_0, ——.*

similar to strain 431 ST. The results indicated that the two heterologous STs were as effective as homologous unlabeled toxin in competition for ^{125}I-labeled strain 431 ST. The data suggested that all three ST preparations were serologically cross-reactive, which was consistent with similar amino acid compositions and molecular weights (Dreyfus et al., manuscript in preparation).

Culture supernatant fractions were assayed for ST to determine (i) whether the toxin could be detected by the RIA as it was in purified preparations and (ii) whether the levels of ST determined by the RIA correlated with the levels detected by the suckling mouse assay. Table 1 shows that there was a good correlation between the two assays. The effective dose (0.4 ng) of each pure toxin was used to calculate the expected yield of the ST in culture supernatants, and the resulting values were compared with those determined by the RIA. All of the strains positive by the suckling mouse assay were also positive by the RIA. We have no evidence to suggest production of multiple species of ST by strain $213C_2$; thus, the difference shown in Table 1 probably reflects variability in the suckling mouse assay. Culture supernatants containing heat-labile enterotoxin or ST_B were negative, which suggested that the antisera were specific for ST_A. The RIA was reproducible, with day-to-day variations of less than ±5%.

DISCUSSION

The nonantigenic nature of *E. coli* ST has been noted by several workers (1, 20); however, the data described here indicate that ST is antigenic when it is coupled to a protein carrier. Several booster doses were required to produce high-titer antisera in rabbits.

Protein A-bearing staphylococci were used as the solid-phase immunoadsorbent, which rapidly immobilized and facilitated purification of immunoglobulin G from whole sera. By estimating the amount of immunoglobulin G adsorbed, the RIA could be used to quantitate the amount of ST-specific antibody present in an immune serum. The low level of nonspecific binding of ^{125}I-labeled ST to non-immunoglobulin-coated staphylococci (less than 0.1%) allowed the use of immunoglobulin-coated cells in the development of the RIA for ST_A. Furthermore, immunoglobulin-coated cells stored at 4°C were stable for at least 1 month. The time of the reaction between toxin and immunoglobulin-coated staphylococci could be decreased to 4 h, so that assays could be completed in 1 day. Most preparations were incubated overnight at 4°C for convenience. Day-to-day variations were usually less than ±5%.

The antisera described here were raised against ST produced by porcine ENT^+ *E. coli* strain 431, but they reacted with purified and crude STs produced by other ENT^+ strains of porcine, bovine, and human origins. These antisera were specific for ST_A and did not exhibit any apparent cross-reactivity with either heat-labile enterotoxin or ST_B. Heterologous ST_A molecules purified from ENT^+ strains 667 and B-41 exhibited patterns of competitive inhibition identical to the pattern of homologous unlabeled

TABLE 1. *Detection of ST by the suckling mouse assay and the RIA*

Strain	Host	Toxin	Culture supernatant		ST concn (μg/ml)	
			Mouse units/ml[a]	Effective dose (ng)[b]	Mouse assay	RIA
431	Porcine (class II)	ST_A	2,400	0.4	0.96	1.05
667	Porcine (class I)	ST_A	800	0.4	0.32	0.42
1657	Porcine	ST_B	0	NR[c]	0.00	0.00
B-41	Bovine	ST_A	4,800	0.4	1.92	2.33
$213C_2$	Human	ST_A	800	0.4	0.32	0.51
$286C_2$	Human	LT[d]	0	NR	0.00	0.00

[a] The number of mouse units per ml was determined by the suckling mouse assay.

[b] The effective dose was the amount of purified ST required to produce a ratio of gut weight to body weight of 0.90. The amount of ST in each culture supernatant fraction was estimated in the RIA by using purified strain 431 ST as the standard. The protein content of each purified ST was measured by the method of Ehresmann et al. (5), using crystalline BSA as a standard.

[c] NR, Not reactive.

[d] LT, Heat-labile enterotoxin.

strain 431 ST, which suggests that ST_A molecules of different origins share a similar antigenic determinant. We found a high degree of correlation between the amount of toxin measured by the suckling mouse assay and the amount detected by the RIA. The RIA described here was somewhat more sensitive than the suckling mouse assay, and the sensitivity of the RIA could be enhanced by increasing the specific activity of the radiolabeled ST and decreasing the amount of antibody-coated staphylococci. More important, ST could be detected and quantitated in culture supernatants or purified toxin fractions by the RIA.

In contrast to the immunological homogeneity observed in this work, ST preparations have been shown to vary with respect to molecular weight (1, 11, 23), biological activity in different animal models (10, 16, 17, 19, 26), heat stability (2), and methanol solubility (2). Five different STs produced by class I and II porcine enteropathogens and ENT^+ strains of bovine and human origins have been purified in our laboratory (Dreyfus et al., manuscript in preparation). Amino acid analyses have revealed that each of these STs contains six half-cystine residues, no basic amino acid residues, and 11 of the 18 amino acids usually present in proteins. The amino acid compositions of the different STs differed by one or two residues; thus, small differences in chemical composition may be partially responsible for the observed differences in the species and age responses to STs. Since most STs contain six half-cystine residues (1, 23), the half-cystine-rich regions proximal to the carboxy termini of STs (22) may form a common immunodeterminant.

It appears that crude ST preparations have been unacceptable for raising antisera because of small amounts of toxin. In our opinion, only preparations that are more than 90% pure can be used for coupling to protein carriers. Once antisera are raised in other animal species, enzyme-linked immunosorbent assays which do not require radioactivity can be developed. Then it should be possible to undertake rapid screening of large numbers of clinical isolates of enteropathogenic *E. coli*, and this should facilitate epidemiological studies on the prevalence and importance of STs in diarrheal disease.

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