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Staphylococcal Enterotoxin B: Solid-Phase Radioimmunoassay

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An immunoassay employing ^{125}I-labeled enterotoxin B and polystyrene tubes coated with specific antibody was used for assaying purified and crude enterotoxin. Antibody was adsorbed to untreated polystyrene tubes. Unlabeled enterotoxin competed with ^{125}I-labeled enterotoxin for antibody-combining sites. The uptake of ^{125}I-labeled toxin reflected the concentration of unlabeled toxin present. The test is sensitive to 1 to 5 ng of purified and crude enterotoxin B per ml, and cross-reactions with heterologous enterotoxins did not interfere with the specificity. This test possesses the combination of sensitivity and objectivity absent in current methods for assaying enterotoxin and provides a model for investigating other enterotoxin serotypes.

Of several in vitro methods available for the serological detection of staphylococcal enterotoxins (2), the most familiar and useful is the micro-Ouchterlony slide test of Casman and Bennett (3). Recently this test has been modified to increase its sensitivity (R. W. Bennett et al., Bacteriol. Proc., p. 6, 1970). Although the various techniques for enterotoxin assay have specific strong points, none appears to be totally satisfactory for the assay of enterotoxins under a variety of conditions, as Bergdoll has pointed out in a recent review (2).

For this reason, we have developed a radioimmunoassay test for assaying staphylococcal enterotoxin B in cultures and food. There are several ways of carrying out a radioimmunoassay (11); we use a so-called solid-phase system (4). In this system, antibody is adsorbed to the inside surface of untreated polystyrene tubes. After antibody treatment, the unreacted protein-adsorbing sites of the tubes are blocked by another protein unrelated to the antigen-antibody system under study. The blocking of these unreacted sites is necessary to prevent nonspecific adsorption of radioactively labeled antigen to the walls of the polystyrene tubes. Labeled antigen and unlabeled antigen compete for the antigen-binding sites of the antibody. The successful competition of unlabeled antigen for antibody sites is reflected in reduced uptake of the labeled antigen. This allows both identification and quantitation of the unlabeled antigen.

MATERIALS AND METHODS

Purified enterotoxins. Purified staphylococcal enterotoxin B (SEB) was obtained from E. J. Schantz, U.S. Army Biological Laboratory, Fort Detrick, Md. Purified staphylococcal enterotoxin A (SEA) was supplied by M. S. Bergdoll, the University of Wisconsin, Madison. The purified toxins contained less than 5% impurities (2).

Enterotoxin antisera. Antisera to enterotoxin B were produced in rabbits as previously described (6). Antisera to enterotoxin A were obtained from R. W. Bennett, Food and Drug Administration, Washington, D.C. Pools of anti-A and anti-B gave passive hemagglutination titers of 1:200,000 each with their homologous antigens (8, 9). Sephadex G-200 gel filtration indicated that the antibodies are primarily immunoglobulin G.

Staphylococcal cultures. Cultures were obtained from R. W. Bennett. Crude extracts consisted of supernatant fluids from sac cultures (6).

Iodination. All chemicals for iodination were reagent grade quality. Solutions of chloramine-T, sodium metabisulfite, and potassium iodide were prepared fresh on the day of iodination in 0.15 M phosphate-buffered saline (PBS), *p*H 7.5. Carrier-free Na^{125}I, free from preservatives or reducing agents, was obtained in 0.1 M sodium hydroxide from New England Nuclear Corp., Boston, Mass., at concentrations ranging from 5 to 250 mCi per ml. Sephadex G-25 medium was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Labeling was performed by a modification of the method of Greenwood and Hunter (5) and Hunter (7). With Eppendorf micropipettes (10 and 50 μliters), the following reagents were added in succession, with mixing after each addition, to a 3-ml glass tube: (i)

10 μliters of ^{125}I, 2 mCi in 0.1 M sodium hydroxide; (ii) 10 μliters of 0.06 M phosphoric acid; (iii) 50 μliters of potassium iodide, 0.2 to 0.4 μg; (iv) 130 μliters of PBS; (v) 50 μliters of SEB, 1 mg/ml in PBS; (vi) 50 μliters of chloramine-T, 3 to 25 μg. After 1 to 9 min, the iodination was stopped by the addition of 50 μliters of sodium metabisulfite equal to the weight of chloramine-T added. Potassium iodide (1 mg) and bovine serum albumin (BSA; 10 mg), in 0.1 ml of PBS each, were added to the reaction mixture as carriers. The sample was transferred to a Sephadex G-25 column for separation of ^{125}I-labeled SEB from unreacted ^{125}I. The column consisted of 5 g of Sephadex G-25 equilibrated with PBS and contained in a 25-ml biuret. The iodinated SEB was eluted with PBS and stored frozen in PBS containing a final concentration of 1% BSA and 0.1% sodium azide. Protein-bound ^{125}I was determined on a sample of the reaction mixture before gel filtration and on sample of the protein fraction from the column. A modification of a trichloroacetic acid precipitation test (7) was used for this determination.

Preparation of antibody-coated polystyrene tubes. The following procedure for coating polystyrene tubes with antibody is a modification of that of Askenase and Leonard (1). Polystyrene tubes (Falcon), 10 by 75 mm, were sensitized with antibody purified by sodium sulfate precipitation and Sephadex G-200 column gel filtration. A 1-ml amount of antibody in phosphate-buffered saline (PBS), *p*H 7.2 (0.07 M NaCl, 0.07 M phosphate), containing approximately 7.0 μg of protein per ml, was carefully added to the polystyrene tubes with a volumetric pipette. After 2 hr at room temperature, the antibody was removed and the tubes were washed once with 2.0 ml of 1.0% BSA in PBS to which sodium azide (0.1% final concentration) had been added as a preservative. The tubes were then filled with 1.0% BSA and left overnight at room temperature. The BSA was removed, and the tubes were washed once with PBS and stored inverted at 4 C until used.

Counting equipment. Radioactivity was measured with a sodium iodide well crystal [2 by 2 inches (5.08 by 5.08 cm)]. The detector was coupled to a Nuclear-Chicago RIDL integral counter. This system has a counting efficiency of approximately 50% for ^{125}I in a 1-ml geometry and a background count rate of 300 counts/min.

Solid-phase radioimmunoassay. Portions (1 ml) of purified or crude enterotoxins in 1.0% BSA were added to the antibody-sensitized tubes. The tubes were incubated at 37 C for 1 hr, after which 0.001 μg of ^{125}I-enterotoxin B in 0.1 ml was added. The tubes were shaken 10 times, incubated at 37 C for 4 hr, washed once with 2 ml of PBS, and counted.

Determination of 67 and 50% uptake end points. When the values are plotted on log-log paper, a linear relationship exists between the uptake of ^{125}I-labeled enterotoxin B by anti-B-coated tubes and the concentration of unlabeled enterotoxin present. This provides a simple method for approximating the concentration of unlabeled enterotoxin required for 67 and 50% uptake of labeled enterotoxin B relative to noncompetitive binding. The 67 and 50% uptake values correspond to 33 and 50% inhibition, respectively.

Alternatively, linear interpolation can be employed for estimating 67 and 50% uptake values. The formula is

$$\hat{X}_E = \log_{10}^{-1}\left\{\log_{10} X_A - \log_{10}(X_A/X_B) \cdot \left[\frac{\log_{10}(Y_A/Y_E)}{\log_{10}(Y_A/Y_B)}\right]\right\}$$

where $\hat{X}_E$ = estimated concentration of toxin resulting in 67 or 50% uptake of labeled toxin; X_A = concentration of toxin resulting in greater than 67 or 50% uptake of labeled toxin; X_B = concentration of toxin resulting in less than 67 or 50% uptake of labeled toxin. The concentration of purified toxin is expressed in micrograms per milliliter, whereas that of crude extracts is expressed as the decimal of the extract dilution. Y_E = 67 or 50% uptake; Y_A = per cent uptake of labeled enterotoxin obtained with toxin concentration X_A; Y_B = per cent uptake of labeled enterotoxin obtained with toxin concentration X_B. Log_{10}^{-1} is an operational term and indicates that the estimated concentration is to be expressed as the antilog. The detailed mathematical and statistical basis for this formula will be published elsewhere (J. T. Peeler, H. M. Johnson, and J. A. Bukovic, *in preparation*.)

RESULTS

The conditions of radioiodination consistently produced a labeled SEB in which the efficiency of labeling was in excess of 80%. The labeled enterotoxin contained an average of 1.7 atoms of iodine per molecule of toxin. Gel filtration separation of ^{125}I-labeled SEB from unreacted ^{125}I resulted in a labeled product in which more than 97% of the activity was precipitable by trichloroacetic acid.

^{125}I-labeled enterotoxin B and unlabeled toxin are compared in Fig. 1 for relative antigenic activity toward antitoxin. The migration behavior of the labeled enterotoxin was not significantly different from that of untreated toxin in the quantitative Oudin immunodiffusion test (6). This demonstrates that the antigenic activity of enterotoxin toward antitoxin to native toxin is not significantly altered by the conditions of radioiodination.

Figure 2 illustrates the specific uptake of ^{125}I-enterotoxins A and B by their respective antibody-coated tubes. Although increasing concentrations of ^{125}I-labeled enterotoxin resulted in increased counts per minute, the percentage uptake of the labeled enterotoxins decreased with increasing concentrations. For example, the addition of 0.001 μg of ^{125}I-enterotoxin B resulted in 33% up-

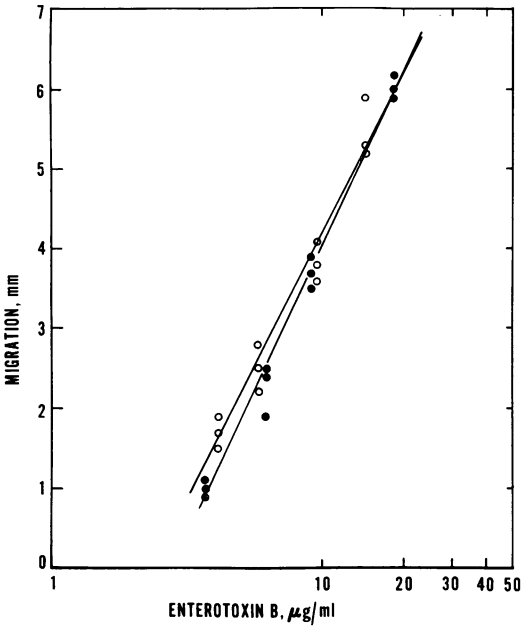

FIG. 1. *Comparative quantitative Oudin migration (6) with untreated (○) and ^{125}I-labeled (●) enterotoxin B. Labeling was performed as described with 25 μg of chloramine-T and 200 μg of enterotoxin B.*

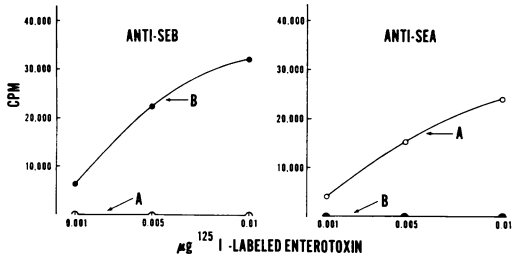

FIG. 2. *Uptake of ^{125}I-labeled enterotoxins A and B by polystyrene tubes coated with antistaphylococcal enterotoxin B (SEB) and antistaphylococcal enterotoxin A (SEA). (Left) Anti-SEB-coated tubes adsorbed only ^{125}I-labeled B. (Right) Anti-SEA-coated tubes adsorbed only ^{125}I-labeled A. CPM on the ordinate indicates counts per minute; the concentration of labeled enterotoxin added is found along the abscissa.*

take by anti-SEB tubes, whereas the addition of 0.01 μg resulted in only a 17% uptake. A similar pattern was observed with the enterotoxin A system. The concentrations of labeled enterotoxins that resulted in the higher percentage uptake by antibody-coated tubes were also the concentrations most sensitive to competitive inhibition by unlabeled enterotoxin. Nonspecific absorption of ^{125}I-labeled enterotoxins is accounted for by controls consisting of BSA-coated tubes. Although nonspecific absorption represents less than 3% of the total radioactive uptake, its value is subtracted before specific uptake values are given. Also, no difference was observed in the radioactive uptake by BSA-coated tubes and tubes coated with heterologous antitoxin or normal rabbit gamma globulin substituted for specific antibody.

Figure 3 presents a standard curve for the inhibition of uptake of 0.001 μg of ^{125}I-labeled enterotoxin B by various concentrations of unlabeled purified B toxin. Fifty per cent inhibition was obtained with approximately 0.005 μg of enterotoxin B per ml, whereas 33% inhibition (or 67% uptake) was observed with approximately 0.0025 μg of enterotoxin per ml. Both per cent uptake and concentration of enterotoxin are plotted on the log scale. Concentrations of enterotoxin A as high as 10 μg per ml had no inhibitory effects on the system.

A similar inhibition curve was obtained with a crude extract of enterotoxin B from *Staphylococcus* strain 243 (Fig. 4). The concentration of enterotoxin is expressed as the decimal of the dilution of the extract. As uptake of labeled enterotoxin B approached 100%, the curve became asymptotic, an effect not observed with purified toxin. A similar effect was observed with crude extracts of two other enterotoxin B strains. For this reason, 33 or 50% end-point calculations involving linear interpolation should not employ uptake values above 90%. The concentration of

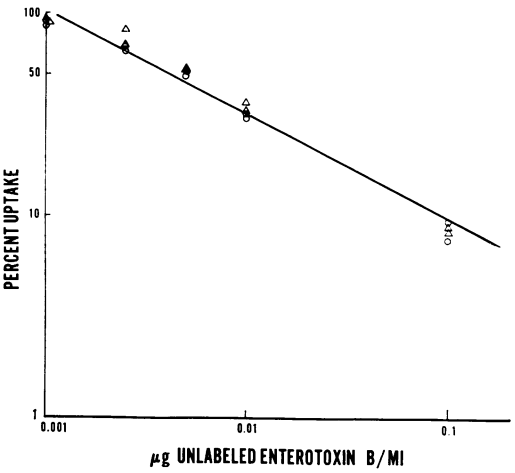

FIG. 3. *Standard curve for the solid-phase inhibition of uptake of ^{125}I-labeled enterotoxin B by unlabeled purified enterotoxin B. The polystyrene tubes were coated with antistaphylococcal enterotoxin B. Open circles and triangles represent replicate determinations performed 3 days apart.*

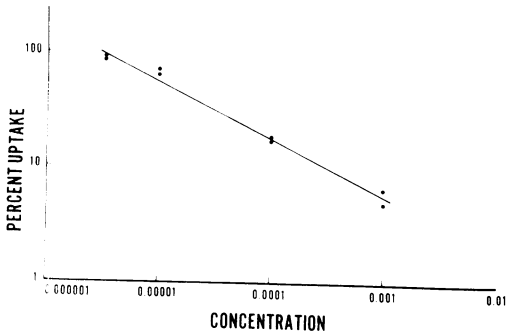

FIG. 4. *Solid-phase inhibition of uptake of ^{125}I-labeled enterotoxin B by crude B extract from Staphylococcus strain 243. The concentration of crude enterotoxin is expressed as the decimal of the extract dilution.*

toxin in crude extract was determined by the quantitative Oudin immunodiffusion method (6). The concentration of toxin resulting in 50% inhibition of uptake of ^{125}I-labeled enterotoxin B was 0.0026 μg/ml. This value is lower than that for 50% inhibition by purified toxin and may indicate that nonprecipitating enterotoxin is present in some crude extracts, whereas it is absent in purified preparations.

Table 1 illustrates the specificity of the reaction by comparing the abilities of various crude enterotoxin serotypes to inhibit uptake of ^{125}I-enterotoxin B by anti-B-coated tubes. All extracts were tested at a 1:8 dilution. Two A extracts along with an extract containing both A and D toxins inhibited the reaction by 0.5 to 6.8%. Three B extracts inhibited the reaction in excess of 99% each. Inhibitions by three C extracts tested ranged from 15.8 to 25.2%, whereas one type D inhibited to 20.6%. It is difficult to interpret the inhibitions by the heterologous toxins in terms of cross-reactivity, since nonspecific effects are indicated by the 21.9 and 9.7% inhibitions obtained with Brain Heart Infusion (BHI) broth undiluted and diluted 1:8, respectively. Also presented are the titrations of the extracts in homologous reactions by the micro-Ouchterlony test. These tests were kindly performed by R. W. Bennett and indicate that the lack of reactivity of the heterologous extracts could not result from enterotoxin not being present.

Table 2 contains data on the relative sensitivity of the solid-phase radioimmunoassay and micro-Ouchterlony tests in detecting enterotoxin B in crude extracts. The solid-phase data are expressed as the extract dilutions that resulted in 33 and 50% inhibition. Both inhibition levels are significantly outside the range of nonspecific effects or cross-reactions. If one compares the sensitivity of the solid-phase test with that of immunodiffusion at the 33% inhibition level, the radioimmunoassay test is 10 to 20 times more sensitive. At the 50% level, the sensitivity is approximately 5 to 12 times that of immunodiffusion. Preliminary data similar to those described here have been obtained with enterotoxin A, a fact which reinforces the specificity and sensitivity of the solid-phase radioimmunoassay test.

Studies are in progress on the detection of enterotoxin B in ham salad. The specificity of the system is maintained under conditions of simple salt extraction and centrifugation without further purification (3). The sensitivity of the assay is being explored, but preliminary data already sug-

TABLE 1. *Inhibition of ^{125}I-labeled enterotoxin B uptake by anti-B-labeled tubes by crude enterotoxin extracts*

Enterotoxin type	Strain	Per cent inhibition (1:8 dilution)	Ouchterlony titration
A	775	6.3	1:320
A + D	264	6.8	1:640[a]
A	16,927	0.5	
B	243	99.9	1:5,120
B	557	99.6	1:2,560
B	572	99.8	1:2,560
C	D58	20.2	1:320
C	D9	25.2	1:20,480
C	D29	15.8	1:1,280
D	4015	20.6	1:320
BHI, undiluted		21.9	
BHI, diluted 1:8		9.7	

[a] Value represents A titer.

TABLE 2. *Comparative sensitivity of solid-phase radioimmunoassay and micro-Ouchterlony immunodiffusion in detection of crude extracts of enterotoxin B*

Strain	Solid-phase inhibition dilution[a]		Ouchterlony titration end point
	33%	50%	
243	1:108,000 (0.0016)	1:63,000 (0.0026)	1:5,120
557	1:31,000 (0.002)	1:17,000 (0.0037)	1:2,560
572	1:24,000 (0.003)	1:12,000 (0.0059)	1:2,560

[a] Values in parentheses represent amount of enterotoxin B, in micrograms per milliliter, present in the 33 and 50% end points as determined by the quantitative Oudin procedure (6).

gest that the behavior of enterotoxin B in ham salad is not unlike that in crude culture extracts. Details of these studies on food will be reported later.

DISCUSSION

The data reported here show that the solid-phase radioimmunoassay procedure is applicable for the sensitive and specific detection of SEB. The sensitivity of the test is in the 1- to 5-ng range. Although this aspect has not been examined here, this sensitivity may possibly be increased by using less antibody for sensitization of the tubes and using labeled enterotoxin of higher specific activity so that amounts less than 0.001 μg may be employed in the test. Presently, reverse passive hemagglutination (10) is the only serological test comparable to solid-phase radioimmunoassay in sensitivity. Our experience with reverse passive hemagglutination indicates that factors such as staphylococcal hemolysins interfere with the test (9). Also, we believe that hemagglutination procedures, as well as others currently used, require more experience in serological procedures than does radioimmunoassay. Further, solid-phase radioimmunoassay is considerably more quantitative than these procedures. Detailed analyses of the quantitative aspects of this test will be presented in another paper.

At this time, caution should be exercised in interpreting the slight inhibition of enterotoxin B by crude extracts of heterologous enterotoxins. These inhibitions could be attributable to serological cross-reactivity, to nonspecific factors, or to a combination of both. A similar type of inhibition was obtained with BHI broth. The fact that purified SEA at a concentration of 10 μg/ml was noninhibitory is additional evidence that these reactions should be further examined to elucidate their mechanisms. In any event, the heterologous reactions do not interfere with the specific detection of enterotoxin B, since both 33 and 50% endpoint determinations are significantly out of the range of cross-reactive or nonspecific effects.

The amount of enterotoxin present in 33 and 50% inhibition end points may vary for different crude enterotoxin extracts (Table 2). Since the concentrations of enterotoxins in the stock extracts were determined by the quantitative Oudin test (6), this may indicate that radioimmunoassay and immunodiffusion do not measure exactly the same population of enterotoxin molecules in the crude extracts. For example, crude enterotoxin extracts may be heterogeneous with respect to the molecular nature of the enterotoxin; some molecules may be incomplete and thus lack the proper valence or number of antibody-binding sites for precipitation. These same molecules would, however, react in the solid-phase system because it measures the primary reaction between antigen and antibody and does not require divalency or multivalency, as does immunodiffusion, to be observed (11). Thus, purified enterotoxin may not reflect the true nature of toxin in the crude form but may represent the enterotoxin selected by the procedure of purification (2). This would explain the wide variation of sensitivity of solid-phase radioimmunoassay over micro-Ouchterlony immunodiffusion with different crude toxin extracts.

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