

AD-767 435

RAPID SOLID-PHASE RADIOIMMUNOASSAY FOR
STAPHYLOCOCCAL ENTEROTOXIN A

William S. Collins, et al

Army Medical Research Institution of Infectious
Diseases
Frederick, Maryland

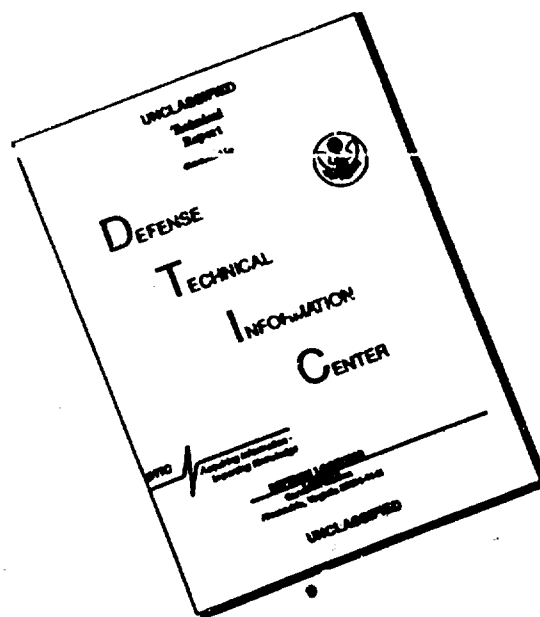
15 January 1973

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

OCT 9 1973

Rapid Solid-Phase Radioimmunoassay for Staphylococcal Enterotoxin A

WILLIAM S. COLLINS, ANNA D. JOHNSON, JOSEPH F. METZGER, AND REGINALD W. BENNETT¹
U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Received for publication 15 January 1973

A rapid solid-phase radioimmunoassay for staphylococcal enterotoxin A is described. The assay procedure requires 3 to 4 h for completion by using a competitive inhibition system in which the antibody is attached to bromoacetyl cellulose particles. It is accurate to a level of 0.01 μg of enterotoxin A/ml in a variety of media such as ham, milk products, crab meat, custard, etc. No significant interference was found with any media or food product tested.

In vitro methods for the assay and detection of staphylococcal enterotoxins were reviewed by Bergdoll (1); none seemed to be totally satisfactory for the assay of enterotoxins under a variety of conditions. Some newer more sensitive methods, principally in the field of competitive inhibition radioimmunoassay, have become available since the review by Bergdoll. Methods for the assay of staphylococcal enterotoxin B (SEB) by solid-phase radioimmunoassay were published by Johnson et al. (6) and Collins et al. (3). Solid-phase radioimmunoassay is the fastest, simplest radioimmunoassay system available and is quantitatively reliable and specific in a variety of media. There are more sensitive methods, e.g., the familiar micro-Ouchterlony procedure of Casman and Bennett (2) and the reverse passive hemagglutination which is subject to cross-reaction and not quantitatively reliable (10). However, they are cumbersome both in time and in the required processing of specimens before use.

For these reasons we developed a solid-phase radioimmunoassay for staphylococcal enterotoxin A (SEA) in culture media and in food products. This system is an outgrowth of that previously developed for staphylococcal enterotoxin B (3) using bromoacetyl-cellulose (BAC)-coupled antibody techniques, similar to those of Mann et al. (7), for measuring gamma globulins in nanogram amounts in tissue culture fluids. The system is rapid, simple, and not troubled by cross-reactions with other toxins, media constituents, and food products.

¹ Present address: Division of Microbiology, Bureau of Foods, Food and Drug Administration, Washington, D.C. 20204.

MATERIALS AND METHODS

Preparation of antibody-BAC. Antisera against SEA were prepared in goats. The toxin used was purified by the method of Schantz et al. (9) and then electrofocused in an LKB column to secure the major toxic component at its isoionic point of pH 7.4 (4 C). Initially, 1 mg of toxin in complete Freund adjuvant was injected intramuscularly, followed by biweekly 1-mg intramuscular injections of toxin over a 3-year period.

Anti-SEA serum from these goats produced a single precipitin line by Ouchterlony gel diffusion after 21 days of immunization with SEA and no cross-reactions with B, C₁, C₂, or E staphylococcal enterotoxins or the crude culture media used to prepare them. This specificity was retained during this long-term immunization.

An immunoglobulin G preparation of the SEA antiserum was prepared by using a diethylaminoethyl-Sephadex A-50 column equilibrated in phosphate buffer, 0.1 M at pH 6.5. The 0.1 M eluate contained the major portion of the immunoglobulin G as determined by electrophoresis and immunodiffusion. Previously we used tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at 0.1 M, pH 8.3, with diethylaminoethyl-Sephadex A-50 which is more efficient in separating the IgG fraction; however, it was found that the Tris buffer which remained after concentration interfered with the following coupling reaction with BAC. This 0.1 M phosphate eluate was then concentrated by ultrafiltration on a UM-2 membrane and was coupled to BAC obtained from Miles Laboratories, Elkhart, Ind., by the method described by Robbins et al. (8). Our modification of this method was previously described (3). This preparation is normally stable for 9 months or more.

Preparation of antigen. Lot P-10, one of several highly purified lots of SEA prepared in this laboratory, was used as the antigen. Electrofocused SEA was used as a test antigen and found to be no better than P-10 which has been found to be electrophoreti-

AD 767435

cally pure. P-10 has been used as an immunogen to produce monospecific anti-SEA when tested against SEA, B, C₁, C₂, E, F, and crude filtrates of culture media containing these antigens. There was an unidentified precipitin line found in micro-Ouchterlony immunodiffusions against an SED preparation which did not react in radioimmunoassay.

The P-10 lot of SEA was labeled with ¹²⁵I by the method of Greenwood and Hunter (4). Use of this method yielded a product of very high specific activity, 15 to 30 $\mu\text{Ci}/\mu\text{g}$ of SEA, but also a very unstable one. After extensive investigation and examination, it was found that Chloramine T had an adverse effect on the stability of some SEA preparations.

Another method of iodination was found to be less deleterious to the SEA molecule. Although it yielded a product of lower specific activity (1.5 $\mu\text{Ci}/\mu\text{g}$), it was in a usable range and very stable. This method as modified by Gruber and Wright (5) used microdiffusion of gaseous ¹²⁵I₂ with no contact of the protein with other reactants in the system. This method was carried out in a 50-ml Erlenmeyer flask with a section (1 by 2.5 cm) of glass tubing sealed to the bottom of the flask, forming a separate inner compartment. The SEA (250 $\mu\text{g}/\text{ml}$) to be iodinated was pipetted into the outer chamber and 0.2 ml of 0.002 M KI was pipetted into the center compartment; 2.5 mCi of ¹²⁵I (as carrier-free ¹²⁵I-sodium) was added to the center compartment, and the flask was sealed with a skirted vaccine stopper. Stock acid dichromate solution (0.27 M Na₂Cr₂O₇ in 36 N H₂SO₄) was diluted 1:20; 0.2 ml was added to the central compartment by means of a syringe with a 16-gauge needle. The flask was held at room temperature with occasional agitation for 1 h. The labeled protein solution was withdrawn with a syringe and needle and dialyzed against multiple 2-liter changes of 0.01 M phosphate-buffered saline (pH 7.1) until 95% or more of the radioactivity was precipitable with 10% trichloroacetic acid. Labeling efficiency and specific activity were essentially the same as that found for SEB by Gruber and Wright (5). The ¹²⁵I-SEA-labeled antigen could be completely removed from solution by antibody, so it was believed that there was no change in binding properties after iodination and dialysis. This preparation could be used for approximately 2 months or one half-life of the ¹²⁵I label with essentially no change in its antigenicity.

The radiolabeled SEA was stored at 4 C; before use it was diluted with 0.2 M borate buffer (pH 8.3) containing 0.7% bovine serum albumin (BSA) to give about 7,500 dpm/10 μl or 1.5 ng of ¹²⁵I-SEA in the test.

Determination of antibody-BAC concentration for inhibition tests. A titration of the antibody coupled to BAC was performed to establish optimal conditions to be used in the inhibition test. This procedure is similar to that used by Mann (7) and in the assay method for SEB (3). The antibody-BAC was diluted over a range of 1:2 to 1:10,000 in borate-BSA. A 0.5-ml amount of each dilution was added to duplicate polycarbonate centrifuge tubes (16 by 100 mm); 10 μl of ¹²⁵I-SEA antigen (approximately 6,000 counts/min) was added to each tube. These were

incubated 15 min at room temperature (25 C) and 1 h and 45 min at 4 C on a vibrator shaker. A 1.5-ml amount of borate-BSA was then added; the tubes were centrifuged at 25,000 $\times g$ for 10 min at 4 C. A 1-ml amount of the supernatant fluid buffer was counted in a well-type gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.; no. 4227) and the results were plotted (Fig. 1) for determination of the 50% end point for labeled antigen binding.

Inhibition test. A dilution of the antibody-BAC preparation was chosen from the foregoing titration which precipitated approximately 50% of the ¹²⁵I-SEA antigen. The unlabeled antigen, at a starting concentration of 1 $\mu\text{g}/\text{ml}$, was diluted over a range of 1:1 to 1:1,000. One hundred microliters of each dilution was added in duplicate to 0.5-ml portions of the diluted antibody-BAC. After adding 10 μl of the ¹²⁵I-SEA antigen, the tubes were shaken vigorously for 15 min at room temperature and 1 h and 45 min at 4 C. A 1.5-ml amount of cold borate-BSA was then added to each tube, and the tube was centrifuged at 25,000 $\times g$ for 10 min. The radioactivity in 1.0 ml of the supernatant fluid was counted to determine the amount of labeled antigen which was not bound and sedimented with the antibody-BAC.

RESULTS

The diluted ¹²⁵I-SEA antigen was reacted with serial dilutions of antibody-BAC. A curve (Fig. 1) could be plotted from this data, and from this curve a dilution was selected to bind 50% of the labeled antigen.

The inhibition assay was performed by adding known amounts of unlabeled SEA and the proper dilution of ¹²⁵I-SEA to the chosen dilution of antibody-BAC previously determined. An inhibition standard curve could then be plotted (Fig. 2). It is evident from this curve that unlabeled SEA concentrations in the range of 1 to 0.01 $\mu\text{g}/\text{ml}$ can be measured by this method.

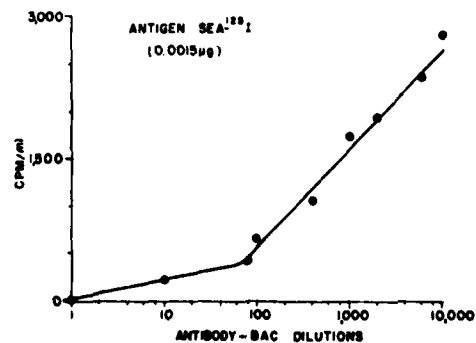


Fig. 1. Dilution curve for antibody-BAC (SEA). Vertical axis (counts per minute) reflects unbound ¹²⁵I-SEA in the supernatant fluid after reaction with dilutions of antibody-BAC plotted on horizontal axis.

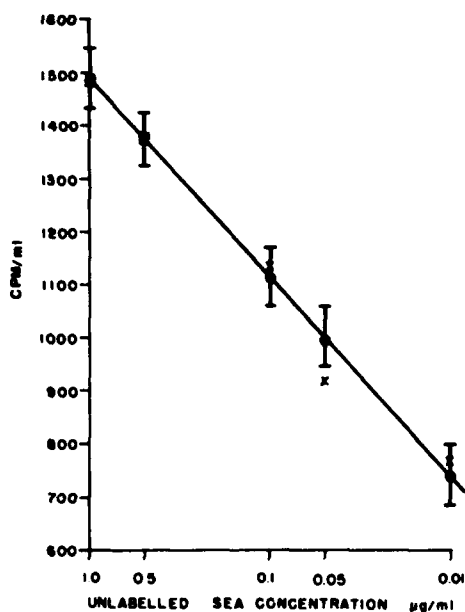


FIG. 2. Unlabelled SEA standard curve in borate-BSA showing best-fit machine curve ± 1 standard error and the raw data points (•) from which the best-fit curve (●) was derived. Y axis indicates counts per minute of unbound ^{125}I -SEA.

The log concentration and counts per minute were analyzed by least-squares fit by using a Wang 700 A programmable calculator. This program then could be used to determine the values of unknowns directly from the calculated standard curve. The program as written also gives the value of the correlation coefficient of the points on the standard curve and the standard error of these selected points. Figure 2 illustrates the plot of these points with the error term for a typical standard curve. There was an average 10% standard error in counts per minute for the assay standard curve (Fig. 2) over the range of 1 to 0.01 $\mu\text{g}/\text{ml}$ for duplicate determinations. Because of the semilog plot, this 10% error in counts per minute extrapolated to a maximum 30% error in quantitation.

Unknown preparations of SEA and SEB in food samples and culture media were provided as a blind study from a toxin survey set. These consisted of pure SEA and SEB as well as mixtures of both toxins in broth and food samples. The broth samples supplied were in Brain Heart Infusion (BHI), and negative controls were found to be inhibitory in the SEB assay system (3) when parallel studies were performed; however, this nonspecific inhibition

could be eliminated by dilution of the SEB standards in fresh BHI. None of the samples submitted interfered with the assay of SEA. The food samples consisted of a variety of preparations: salami, butter, milk, custard, crab meat, cheese, and eggs from the Food and Drug Administration, and ham prepared in this laboratory. Many of the samples supplied in the survey were at levels below the accurate quantitation level of the procedures described in this paper, as they were prepared for the evaluation of another system. However, it was found that for those within the range of assay we could accurately determine the quantity and identity of toxins; for those outside the range, the system was generally able to qualitatively identify SEA, SEB, and mixtures thereof in food samples and BHI. All samples were simply homogenized in borate-BSA buffer, and 100- μl samples of the homogenate were transferred to the assay tubes. Butter was melted at 37 C, and 100- μl samples of the water layer were used for the assay.

Of 55 samples submitted, no false-positive results for either SEA or SEB were found, and, in those specimens with too low a concentration to assay quantitatively, the correct toxins were identified qualitatively in most cases even when the other toxin was present in excess greater than 5.0 $\mu\text{g}/\text{ml}$. In assays of SEB in BHI a cutoff point of 0.05 $\mu\text{g}/\text{ml}$ was established for quantitative assay, although qualitative results were valid for all specimens submitted including those in the 0.001 $\mu\text{g}/\text{ml}$ range. The assay of SEA was quantitative to 0.01 $\mu\text{g}/\text{ml}$ when a 50% SEA ^{125}I binding end point was used; deviation greater than $\pm 10\%$ from the 50% end point resulted in a loss of sensitivity but not of specificity. As a further check on the analysis of SEA in food products, dilution of SEA in salami, milk, and custard was made at the same concentration as the standard curve in borate-BSA. A best-fit curve with limits of one standard deviation was then plotted for the borate-BSA-SEA standards, and the values found in salami, milk, and custard were then plotted on this curve. Figure 3 illustrates the plot of points for foods on a buffer standard curve of the same concentrations of SEA. Slopes were calculated individually for these values and found to be essentially the same as the buffer values. This indicated that there was no interference from the foods on the radioimmunoassay of SEA. With the ^{125}I -labeled SEA preparations available, 0.01- μg of sensitivity for quantitation was the practical level for use with 0.001 $\mu\text{g}/\text{ml}$ as a practical qualitative lower limit. Higher levels

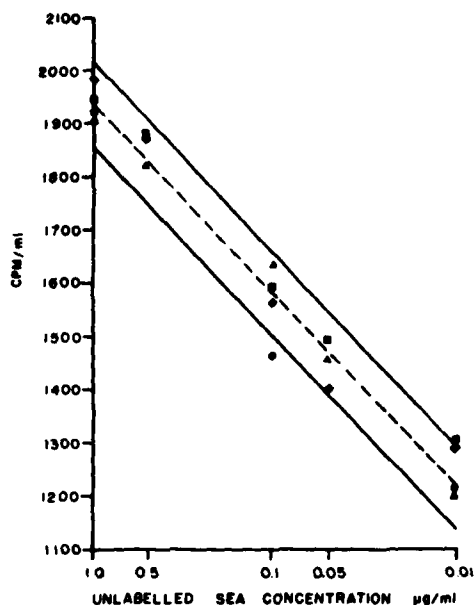


FIG. 3. Calculated best-fit standard curve ± 1 standard error for borate-BSA buffer dilutions of unlabelled SEA with actual data points for the same concentrations of unlabelled SEA as measured in borate-BSA, salami, custard, and milk. Y axis indicates counts per minute of unbound ^{125}I -SEA in 1-ml samples of supernatant fluid buffer from assay tubes. Symbols: ▲, borate; ◆, salami; ■, custard; ●, milk.

of radioactive labeling could yield more sensitivity to the system but we were not able to achieve this without degradation of some antigen preparations. Further study is in progress on this phase of the system.

DISCUSSION

The data presented here show that the solid-phase radioimmunoassay described is applicable for the sensitive and specific assay of SEA. The qualitative sensitivity is useful to 0.001 $\mu\text{g/ml}$ and quantitatively reliable to 0.01 $\mu\text{g/ml}$. A radiolabel of higher specific activity would enable greater sensitivity. The other available tests in this range (2, 10) suffer from problems of interference from nontoxic bacterial products found in culture media or foods, and from complicated, time-consuming preparation of specimens for analysis. The solid-phase radioimmunoassay reported by Johnson et al. (6) yields approximately the same sensitivity for the assay of SEB as we are reporting here for

SEA, but this system also apparently has problems with interference by nontoxic bacterial products and cultural media components. The system we are reporting is rapid, sensitive, and quantitatively accurate. It is not interfered with by bacterial products, media, or foods. Preparation of samples for assay is a simple addition of the sample to be tested, in a suitable quantity, to the test system either as an emulsion or as a borate-BSA buffer extract and then incubation, centrifugation, and counting of a sample of the supernatant fluid in a gamma counter. Results can be determined from either a graph of the standard curve or with a programmable calculator. Careful maintenance of all assay conditions must be adhered to, to secure best results. Such foods as custard which have a gelling agent must be measured with care, as a 100- μliter sample added to 0.5 ml of antibody BAC often causes the whole thing to partially gel. Centrifugation of custard at 28,000 $\times g$ yields a nongelling liquid layer which is adequate for assay. Other foodstuffs should be examined for the best method of obtaining a liquid phase; simple salt extracts are adequate but add a dilution factor which must be taken into account.

LITERATURE CITED

- Bergdoll, M. S. 1970. Enterotoxins, p. 265. In T. C. Montic S. Kadis, and S. J. Ajl (ed.), *Microbial toxins*, vol. 3. Academic Press Inc., New York.
- Casman, E. P., and R. W. Bennett. 1965. Detection of staphylococcal enterotoxin in food. *Appl. Microbiol.* 13:181-189.
- Collins, W. S., II, J. F. Metzger, and A. D. Johnson. 1972. A rapid solid phase radioimmunoassay for staphylococcal B enterotoxin. *J. Immunol.* 108:852-856.
- Greenwood, F. C., and W. M. Hunter. 1963. The preparation of ^{125}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* 69:114-123.
- Gruber, J., and G. G. Wright. 1967. Iodine-131 labeling of purified microbial and antigens by microdiffusion. *Proc. Soc. Exp. Biol. Med.* 126:282-284.
- Johnson, H. M., J. A. Hukovic, P. E. Kauffman, and J. T. Peeler. 1971. Staphylococcal enterotoxin B: solid-phase radioimmunoassay. *Appl. Microbiol.* 22:837-841.
- Mann, D., H. Granger, and J. L. Fahey. 1969. Use of insoluble antibody for quantitative determination of small amounts of immunoglobulin. *J. Immunol.* 102:618-623.
- Robbins, J. B., J. Haimovich, and M. Sela. 1967. Purification of antibodies with immunoadsorbents prepared using bromoacetyl cellulose. *Immunochemistry* 4:11.
- Schantz, E. J., W. G. Roesler, J. Wagman, E. Speck, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. *Biochemistry* 4:1011-1016.
- Silverman, S. J., A. R. Knott, and M. Howard. 1968. Rapid, sensitive assay for staphylococcal enterotoxin and a comparison of serological methods. *Appl. Microbiol.* 16:1019-1023.

RESEARCH AVAILABILITY CODES
 FIG. 3. AVAIL. AND OF SP. DIAL.
 A 20