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Rapid Solid-Phase Radioimmunoassay for Staphylococcal Enterotoxin A

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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 bromacetyl 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.

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 isoinonic 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 Ouchterlonv gel diffusion after 21 days of immunization with SEA and no cross-reactions with B, C, or E staphylococcal enterotoxins. 

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 electrophoresi-
cally pure. P-10 has been used as an immunogen to produce monospecific anti-SEA when tested against SEA, B. C., C., 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 μCi/μg 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 μCi/μg), it was in a usable range and very stable. This method as modified by Gruber and Wright (5) used microdilution of gaseous 125I, with no contact of the protein with other reagents 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 μg/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 ml of 0.15 M Na2CrO4 in 36 N H2SO4 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 dis/min/10 μg 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 × 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 μg/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 × 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 μg/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.](image-url)
per minute of unbound performed; assay system (3) controls were found to be inhibitory in the Brain Heart samples. The broth samples supplied were in the foods on the radioimmunoassay of consisted of pure as a blind study from a toxin survey set. These individually for these value-, and found to be food samples and culture media were provided concentrations of to a maximum this determination. Because of the semilog plot. the range of average term for a typical standard curve. There was an salami, milk, and custard was made at the same gives the value of the correlation coefficient of greater than this

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 SEB. 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-μliter samples of the homogenate were transferred to the assay tubes. Butter was melted at 37°C, and 100-μliter 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 μg/ml. In assays of SEB in BHI a cutoff point of 0.05 μg/ml was established for quantitative assay, although qualitative results were valid for all specimens submitted including those in the 0.001 μg/ml range. The assay of SEA was quantitative to 0.01 μg/ml when a 50% SEA binding end point was used; deviation greater than ±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 121I-labeled SEA preparations available, 0.01-μg of sensitivity for quantitation was the practical level for use with 0.001 μg/ml as a practical qualitative lower limit. Higher levels

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Unlabeled SEA standard curve in borate-BSA showing best-fit machine curve ±1 standard error and the raw data points (x) from which the best-fit curve (o) was derived. Y axis indicates counts per minute of unbound 121I-SEA.
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 µg/ml and quantitatively reliable to 0.01 µg/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 × 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**


