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Double-Antibody Radioimmunoassay for Staphylococcal Enterotoxin C_2

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A sensitive double-antibody radioimmunoassay for staphylococcal enterotoxin C_2 is described. The assay procedure employs anti-rabbit gamma globulin, prepared in goats, to precipitate the antigen-antibody complex of enterotoxin C_2 and anti-enterotoxin C_2. The test is sensitive to 100 pg of enterotoxin.

Of the several in vitro methods for the serological detection of staphylococcal enterotoxins (1), the most rapid and sensitive one is the solid-phase radioimmunoassay (2-4,8). Two systems are presently utilized in the solid-phase system: (i) anti-enterotoxin antibodies are coupled to bromoacetyl cellulose by a method described by Collins et al. (3), and (ii) polystyrene tubes are coated with antisera against the enterotoxins (4,8). Both systems were reported to be satisfactory for staphylococcal enterotoxins A and B, but the immunological reactivity of anti-enterotoxin C_2 was significantly lower by the solid-phase method. By the coated-tube method, anti-enterotoxin C_2 exhibited maximum binding capacity of 8 to 12% of total radiolabeled enterotoxins A and B bound 40 to 50% of total input (2-4).

In connection with our work on the amino acid sequence of enterotoxin C_2, we are interested in the isolation of immunologically active peptide fragments. The present radioimmunoassay method for staphylococcal enterotoxin C_2 (SEC) is inadequate to detect picogram quantities of such fragments. This paper describes a more sensitive radioimmunoassay for SEC.

MATERIALS AND METHODS

Preparation of antigen and antisera. SEC was purified in this laboratory (10). An electrophoretically pure fraction that formed one precipitin line against crude anti-SEC was used for radioiodination and for the construction of the standard curves. Antisera against SEC were prepared in rabbits as previously described (10). The antiserum that produced a single precipitin line by Ouchterlony gel diffusion was used throughout the experiments. Anti-rabbit gamma globulins (ARGG) and logit-log paper were a gift from F. Huang of Robel Research Laboratories, Ottawa, Canada.

Radioiodination of antigen. The purified fraction of SEC was radioiodinated with ^{125}I (Atomic Energy of Canada, Ottawa) to a specific activity of 80 to 120 μCi of SEC per μg by the lactoperoxidase (Calbiochem) method of Thorell and Johansson (12).

Determination of antibody titer. A titration of the anti-SEC and ARGG was performed to establish optimal conditions for the competitive binding test. Anti-SEC was diluted over a range of 1:10 to 1:5,000 in 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 2.0% normal rabbit serum. ARGG was diluted over a range of 1:2 to 1:32 with 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05 M ethylenediaminetetraacetate. Radiolabeled [^{125}I]SEC was diluted with 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1% bovine serum albumin phosphate-buffered saline [PBS] to approximately 10,000 counts/min per 100 μl. For each dilution of anti-SEC 10 glass tubes (6 by 50 mm) were used. To each of the 10 tubes was added 200 μl of PBS buffer, 100 μl of [^{125}I]SEC, and 100 μl of one of the dilutions of anti-SEC. These were counted in a Packard gamma counter and incubated overnight at room temperature (22 C). Volumes of 100 μl of each dilution of ARGG were then added to duplicate tubes and incubated overnight at room temperature. All tubes were then placed inside plastic centrifuge tubes and centrifuged in a Sorvall RC-2B centrifuge at 6,000 $\times$ g for 20 min at 4 C. The supernatant was aspirated, and after one wash with saline the tubes were counted in a well-type Packard auto gamma counter.

Competitive binding. A dilution of the antibody-SEC and ARGG preparation, which precipitated approximately 50% of [^{125}I]SEC antigen, was chosen from the foregoing titration. All tests were performed in duplicate at room temperature (22 C). The unlabeled antigen at a starting concentration of 100 ng/ml was diluted over a range of 1:10 to 1:1,000 with PBS buffer. Glass culture tubes (6 by 50 mm) or plastic tubes (12 by 75 mm) were used for the assay. The protocol of the radioimmunoassay for standards and the culture supernatant is shown in Table 1. To determine the nonspecific binding by the reaction tube and ARGG, the first two tubes in each experiment contained all the reagents except anti-SEC.

The double-antibody method was utilized for the detection of SEC in four dehydrated soups implicated in food poisoning. Each package, containing about 5 g of powder, was resuspended in 5 to 8 ml of 0.05 M phosphate buffer, pH 7.4, vortexed for 1 min, and centrifuged at 18,000 $\times$ g for 15 min at room temperature. For the radioimmunoassay 50-, 100-,

TABLE 1. *Radioimmunoassay protocol for SEC by the double-antibody method*

Tube no.	PBS buffer (μl)	SEC (ng/100 μl)	Dilution of culture supernatant (100 μl/dilution)	[^{125}I]SEC (μl)	Anti-SEC (μl)	ARGG (μl)
1–2	300	0		100	0	100
3–4	200	0		100	100	100
5–6	100	10		100	100	100
7–8	100	5		100	100	100
9–10	100	2.5		100	100	100
11–12	100	1.25		100	100	100
13–14	100	0.625		100	100	100
15–16	100	0.312		100	100	100
17–18	100	0.156		100	100	100
19–20	100	0.078		100	100	100
21–22	100		10^{-4}	100	100	100
23–24	100		5×10^{-5}	100	100	100
25–26	100		2.5×10^{-5}	100	100	100
27–28	100		10^{-5}	100	100	100
29–30	100		5×10^{-6}	100	100	100
31–32	100		2.5×10^{-6}	100	100	100
33–34	100		10^{-6}	100	100	100

200- and 300-μl aliquots of the supernatant were assayed. To maintain the final volume at 0.5 ml in the assay tube, PBS buffer was either increased or eliminated. When 300-μl aliquots were assayed, an additional 100 μl of PBS was added with the standards for a final volume of 0.6 ml.

RESULTS

Standard curves at different dilutions of anti-SEC serum were obtained by competition experiments (Fig. 1). Curves A, B, and C were obtained by the double-antibody method and curve D was obtained by the antibody-coated polystyrene tubes. Increased or decreased dilution of anti-SEC lowered the maximum binding capacity of [^{125}I]SEC by the antibody-coated tube method. The anti-SEC dilution (1:64,000) that gave the wider range of detection of SEC (curve B, Fig. 1) was used in all subsequent assays. To precipitate 50% of the [^{125}I]SEC–anti-SEC complex a 1:16 dilution of ARGG (second antibody) was necessary.

The logit transformation, the simplest method of linearization of conventionally obtained sigmoid dose-response curves of competitive binding assays (11), was applied for SEC radioimmunoassay. Figure 2 shows that plots of logit B/B_0 versus log x (11) fitted straight lines over the range of 0.1 to 100 ng for all assays and were independent of the type of tube used. Plastic differed from glass tubes in higher nonspecific binding and lower B_0 values. In the absence of anti-SEC, the average nonspecific radioactive retention was: glass tubes, 4.2%; polypropylene, 7.8%; pol-

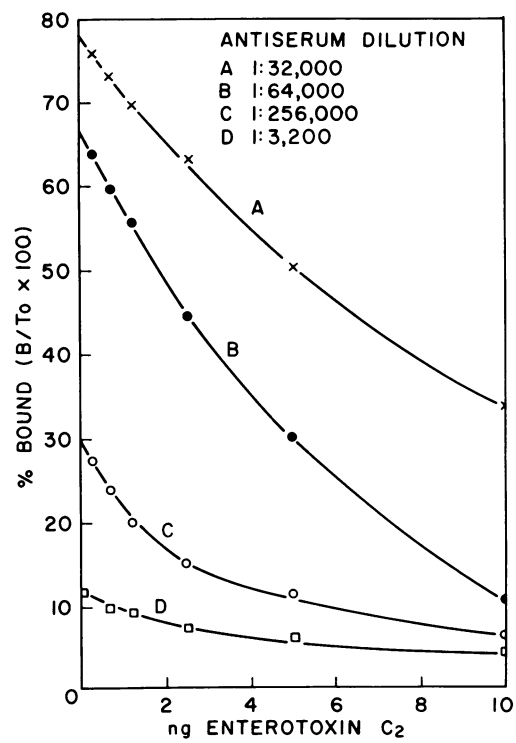

FIG. 1. *Standard curves of SEC. Curves A, B, and C were obtained from assays by the double-antibody method. Curve D was obtained by the antiserum-coated polystyrene tubes. Specific binding was calculated as the ratio of counts for the precipitate (B) to total (To) counts after subtracting the respective nonspecific binding (Table 1, tubes 1 and 2).*

ystyrene, 9.2%. The percent B_0 for SEC was: glass tubes, 66%; polypropylene, 52%; and polystyrene, 54%.

The double-antibody radioimmunoassay was utilized for the quantitation of SEC in culture supernatant, and its subsequent recovery after concentration with an Amicon ultrafiltration module. Figure 3 shows a 20% loss of SEC dur-

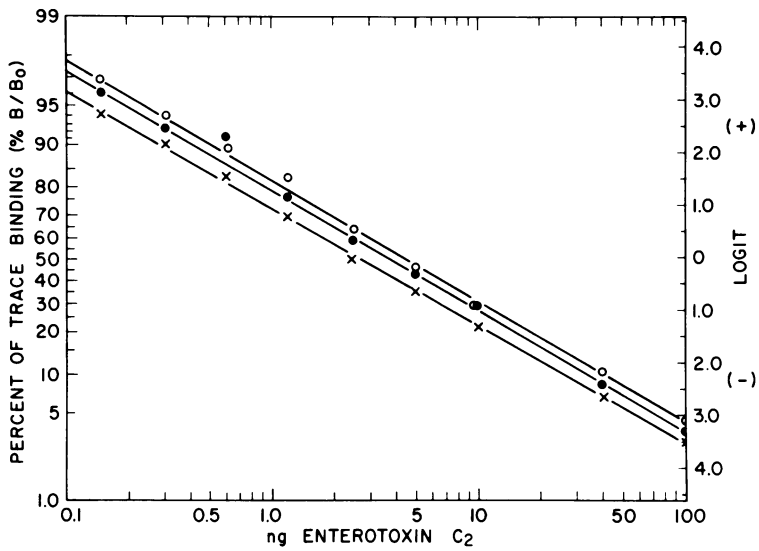

FIG. 2. *Logit-log plot for SEC from type of data obtained in Fig. 1, curve B. Symbols: X, polystyrene tubes; ●, polypropylene tubes; ○, glass tubes. The logit function is defined as: logit (Y) = ln Y/1−Y, where Y represents a measurable quantity of a response variate dependent on a dose variable × (14). Substituting B/B_0 for Y in the formula logit B/B_0 = ln $B/B_0/1 - B/B_0$ (11). B = Counts bound by the antibody for arbitrary dose, corrected for nonspecific binding. B_0 = Counts bound by the antibody for zero dose, corrected for nonspecific binding.*

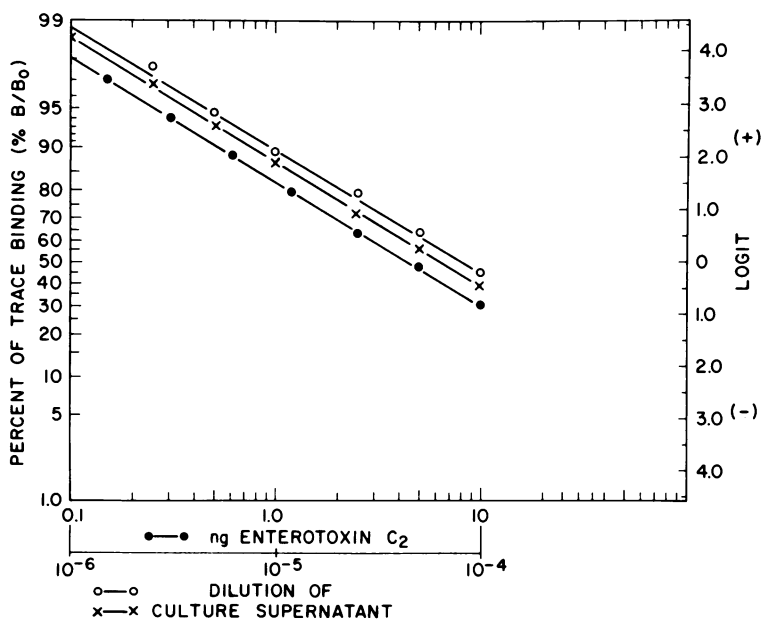

FIG. 3. *Logit-log plot for standard and culture supernatant before and after concentration with Amicon filter. Symbols: ●, standard enterotoxin C_2; ×, supernatant before concentration; ○, supernatant after concentration.*

ing the concentration step; about 68 and 54 μg of SEC per ml were detected in the supernatant before and after the concentration step, respectively.

Studies on the detection of staphylococcal enterotoxins in dehydrated soups showed the presence of SEC in the sample extract (Table 2). The curves for these samples and standard SEC were parallel (Fig. 4), indicating the presence of a protein with immunological identity.

In an effort to reduce the assay time, anti-SEC and ARGG were also added concurrently to the assay tubes and incubated overnight at room temperature. This modification reduced the percent B_0 to 40% in glass tubes, 31% in polystyrene, and 33% in polypropylene. Although this system has not been tested with unknown samples, the results indicate that the total assay time may be shortened.

DISCUSSION

The double-antibody method is based on the finding (5) that gamma globulins have separate binding sites for their antigens and antibodies. An antibody molecule may therefore form a complex with its antigen and then be complexed to a second antibody. In the radioimmunoassay, the primary antigen-antibody complex is too small to be precipitated; however, if nonimmune serum from the same animal species as the first antibody is added, followed by an antiserum specific to this antibody and raised in a second species, the whole complex can be precipitated (5). This principle was applied for the determination of SEC by radioimmunoassay. In the present work, the first antibody was raised in rabbits and the second in goats. Nonimmune rabbit serum was included as carrier (5) to form a precipitable lattice of the antigen–double-antibody complex. Complement, present in serum and dependent on calcium ions, may inhibit precipitation by the double-antibody system (6, 7). This effect was avoided by incorporation of 0.05 M ethylenediaminetetraacetate in the diluent for the second antibody.

TABLE 2. *Amount of SEC detected in dehydrated soups*

Dehydrated soup	Aliquot (μl)	SEC (ng/aliquot)	SEC (ng/ml)
Vegetable	50	0.32	6.4
	100	0.72	7.2
	200	1.5	7.5
	300	2.31	6.93
Chicken	50	0.8	16.0
	100	1.57	15.7
	200	3.4	17.0
	300	5.01	16.5
Beef-1	50	0.26	5.2
	100	0.61	6.1
	200	1.2	6.0
	300	1.93	5.7
Beef-2	50	0.21	4.2
	100	0.42	4.2
	200	0.86	4.3
	300	1.25	4.1

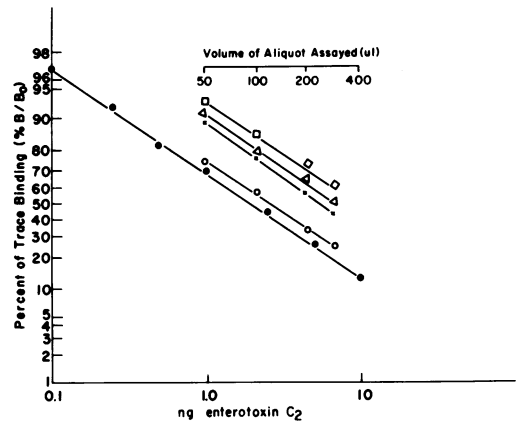

FIG. 4. *Radioimmunoassay of SEC and extracts from dehydrated soups. Symbols:* ●, *standard SEC;* ○, *chicken soup;* ×, *vegetable soup;* △, *beef soup-1;* □, *beef soup-2.*

The application of the double-antibody method was first used for the determination of growth hormone by Utiger et al. (13) and of insulin by Morgan and Lazarow (9). Our results indicate (Fig. 1) that for the determination of SEC this technique is superior to the solid-phase method. Initial binding of [^{125}I]SEC by anti-SEC was increased fivefold, the sensitivity of the assay was extended to 100 pg, and the dilution of anti-SEC was increased 20-fold.

Because of its increased sensitivity, the present method might replace the coated-tube method also for the detection of other staphylococcal enterotoxins, i.e., when these are present in extremely small quantities or when the anti-SEC adsorb poorly to plastic surfaces.

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