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Development of a Monoclonal Antibody Capable of Interacting with Five Serotypes of *Staphylococcus aureus* Enterotoxin

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A monoclonal antibody capable of binding to determinants shared in common by staphylococcal enterotoxin serotypes A, B, C_1, D, and E was developed. To accomplish this, BALB/c mice were immunized by alternating injections of serotypes A and D to enrich for spleen lymphocytes programmed to produce antibody to possible common determinants. These cells, fused with mutant myeloma cells (P3-X-63Ag8.653) produced hybrids that formed monoclonal antibodies to either serotype A only or to both serotypes A and D. A cloned hybridoma from the latter group produced an antibody (subclass immunoglobulin G1) which interacted with five serotypes. Highest affinity was to B and C_1. An immunomatrix consisting of this antibody cross-linked to protein A-Sepharose CL-4B with dimethyl pimelimidate was capable of binding enterotoxin. Bound toxin was eluted with diethylamine. Because of its ability to interact with all five serotypes, the monoclonal antibody should prove useful in the development of a rapid method for screening foods for the presence of staphylococcal enterotoxin.

Staphylococcal enterotoxins are a group of at least five structurally related emetic proteins produced by *Staphylococcus aureus*. Although the actual number of cases varies from year to year, in the United States about one-fourth of all reported bacterial food poisonings are caused by ingestion of staphylococcal enterotoxin (5).

Although *S. aureus* can easily be detected in foods, its presence does not necessarily imply that enterotoxin was produced. Many strains are not toxigenic (18). Similarly, the absence of viable staphylococci in food does not mean that toxin is also absent. The organisms die rapidly after the stationary phase and may be succeeded by harmless saprophytic bacteria. Furthermore, the toxin is more heat stable than the cells and can be present in heated food (19). As a result, there is a need for rapid and sensitive methods for toxin detection.

There are no biological or chemical methods which can be employed on a practical basis for routine detection of enterotoxin. The enterotoxins are antigenic, and various immunological methods with antibody raised in rabbits have been described (4, 9, 15, 16, 20). However, there are difficulties in preparing high yields of specific antibody by conventional immunological procedures, and the supply of good antisera is limited (1). The amount of antibody produced is dependent on individual characteristics of the rabbit, toxin serotype, and the immunization protocol used. The antigenic diversity of the enterotoxins complicates their detection because each one must be assayed separately. There is a continuing search for improved methods of antibody production (2).

We have been investigating the use of monoclonal antibodies to detect enterotoxin. The aim of this research was to obtain a monoclonal antibody capable of binding to antigenic regions shared in common by all toxin serotypes. Such an antibody would be extremely useful from a regulatory viewpoint in developing immunological methods for screening food samples for the toxins.

Hybridomas able to produce a common antibody were isolated. The monoclonal antibody was partially characterized, and preliminary results on development of an affinity purification procedure for enterotoxin are described.

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MATERIALS AND METHODS

Toxins and antisera. Purified enterotoxins A through E were provided in lyophilized form by Gopal Murthy (Microbial Biochemistry Branch, Food and Drug Administration, Cincinnati, Ohio) who purified them from cell-free culture broth by the method of Oda (17). The purity of the enterotoxins was 99% or better (G. Murthy, personal communication). Antisera to the enterotoxins were raised in rabbits as previously described (3) and lyophilized. Toxins and antisera were stored at 4°C, rehydrated in sterile distilled water, and diluted in phosphate-buffered saline (PBS) for use. Toxins were detected qualitatively in column effluents by production of precipitin lines with antisera by the microslide gel double-diffusion method of Casman et al. (4). The reported sensitivity is 0.1 μg/ml. Goat anti-mouse immunoglobulin G (IgG) (γ) labeled with horseradish peroxidase for use in enzyme-linked immunosorbent assays (ELISA) was obtained from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md. and stored at 4°C.

Cells and media. Myeloma cells (P3-X63-Ag8.653, repository no. GM 3570) were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.J. These cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10% NCTC-109, 1% nonessential amino acids, penicillin (50 U/ml), and streptomycin (50 μg/ml) (all from Microbiological Associates, Bethesda, Md.). Myeloma cells were grown in the presence of 8-azaguanine (M. A. Bioproducts) 2 weeks before fusion experiments. Rat hepatocytes obtained from Ellen Fishberg, Albert Einstein College of Medicine, Bronx, N.Y., were grown in DMEM supplemented as above but without NCTC-109. Littlefield HAT medium (14) was prepared by the addition of hypoxanthine, thymidine (both from M. A. Bioproducts), and aminopterin (Sigma Chemical Co., St. Louis, Mo.) to the myeloma

medium described above except the fetal bovine serum was increased to 20%.

Chemicals and reagents. Polyethylene glycol 1500 was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Bovine serum albumin and substrate for horseradish peroxidase [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)] were from Kirkegaard and Perry Laboratories, Inc.

Immunization. A total of seven mice were immunized by intraperitonal (i.p.) injection of 50 to 200 μg of enterotoxin A (SEA) or enterotoxin D (SED). A varied pattern of injections was employed, and time intervals between injections 1, 2, and 3 ranged from 1 to 4 weeks. Mice injected first with one toxin were injected next with the other toxin. The purpose of alternating injections was to enrich for spleen lymphocytes having the capability of producing antibody to determinants shared in common by both toxin serotypes. Serum antibody was detected by the ELISA described below.

Fusion. Spleen lymphocytes used for the fusion experiment were from a mouse which had first been injected (i.p.) with 175 μg of SED in complete Freund adjuvant (1:1), followed by injection (i.p.) with 100 μg of SEA in incomplete Freund adjuvant (1:1) on day 31, and a final injection with 200 μg of SEA (0.1 ml i.p. and 0.1 ml intravenously) on day 137. After 2 days, the spleen was removed from the mouse, and the cells were flushed out by injecting 5 ml of DMEM into the spleen capsule. The cells were washed twice with DMEM and collected by centrifugation at 400 × *g* for 10 min at room temperature.

The procedure outlined by Galfré and Milstein (7) was modified and used for fusion. In brief, myeloma cells were harvested from a suspension culture in the logarithmic phase of growth, pelleted by centrifugation at 400 × *g* for 10 min at room temperature, and washed three times with DMEM. Suspensions containing about 10^7 myeloma cells and 10^8 spleen cells were mixed together at a ratio of eight spleen cells per myeloma cell and pelleted by centrifugation. After the supernatant liquid was removed, the pellet was loosened by gentle tapping, and 0.8 ml of 50% polyethylene glycol 1500 prewarmed at 40°C was added to the cells dropwise over a period of 1 min with gentle stirring. The cell suspension was diluted by the slow addition of DMEM over a period of about 10 min. The cells were pelleted, washed with DMEM, and resuspended in 49 ml of DMEM containing 20% fetal bovine serum. This suspension was distributed in three 96-well tissue culture plates (Linbro model no. 76-003-05; Flow Laboratories, Inc., McLean, Va.), containing 10^5 feeder cells (spleen cells) per well. Hybrids were observed after about 14 days of incubation at 37°C under 10% CO_2 and high humidity. The next day and at intervals thereafter, HAT medium was added to each of the fusion wells.

ELISA. Hybrids capable of producing antibody were detected by an ELISA developed as described by Voller et al. (23). Wells in polystyrene plates (Immulon 2, U bottom; Dynatech Laboratories, Inc., Alexandria, Va.) were sensitized by the addition of 100 μl of enterotoxin (2 μg/ml) in PBS, pH 8.0. After standing overnight at 4°C, the liquid was removed, and the wells were filled with 3% bovine serum albumin to block residual binding sites. After 1 h at 37°C, the blocking reagent was removed, the wells were washed (PBS containing 0.02% Tween 20), and 50 to 100 μl of test antibody preparation was added to each well. After 2 h, wells were aspirated and washed three times. Enzyme-labeled goat anti-mouse IgG (50 μl) was added to each well and incubated for 1 h at 37°C. Wells were washed 10 times, and 50 μl of enzyme substrate was added and allowed to react for 30 min at room temperature. Absorbance at 410 nm was measured in an ELISA reader (Dynatech MR 590).

Cloning in soft agarose. The hybridoma cells were cloned by the method of Coffino et al. (6). Hybrids were plated over a layer of rat hepatocytes as feeder cells. After about 10 days, the clones were picked with a micropipette and placed in supplemented DMEM in 96-well culture plates. After 3 to 4 days, the culture fluid was tested for antibody.

Production of antibody in ascites fluid. BALB/c mice were injected i.p. either two or three times with 0.5 ml of pristane at weekly intervals. After a rest period (2 to 10 days), ca. 10^6 log-phase hybrid cells were injected i.p. into each mouse. Ascites fluid was collected when mouse abdomens became distended. The frequency and amounts of each collection varied with individual mice and pristane treatment.

Pooled centrifuged ascites fluid was precipitated twice with 50% saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 0.05 M potassium phosphate buffer (pH 7.0), and dialyzed for 48 h against the same buffer. The dialyzed preparation was centrifuged to remove a slight precipitate and frozen in 3-ml volumes.

Immunoaffinity purification of enterotoxin. The immunomatrix described by Schneider et al. (21) was investigated as a possible means for affinity purification of enterotoxin. The matrix was constructed by first binding antibody to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) and then cross-linking the entire complex by treatment with dimethyl pimelimidate. According to the authors, this procedure allows optimal spatial orientation of antibodies and, therefore, maximum binding efficiencies for antigens.

Protein A-Sepharose CL-4B (0.75 g) was equilibrated in 0.1 M sodium borate buffer (pH 8.2) and added to 2.8 ml (33.7 mg of protein) of monoclonal antibody preparation [$(NH_4)_2SO_4$-fractionated ascites fluid] previously dialyzed against the same buffer. After mixing for 30 min with gentle shaking at room temperature, the slurry was collected on a sintered glass filter and washed with 10 ml of borate buffer followed by 10 ml of 0.2 M triethanolamine, pH 8.2. The antibody-protein A-Sepharose CL-4B complex was suspended in 60 ml of 0.02 M dimethyl pimelimidate dihydrochloride (Sigma) in 0.2 M triethanolamine, pH 8.2. After gentle agitation for 45 min at room temperature, the mixture was collected by centrifugation (1 min at 500 × *g*), resuspended in 60 ml of 0.02 M triethanolamine, and washed three times with borate buffer. Antibody cross-linked to the matrix by the above procedure was used to prepare a column (diameter, 0.7 mm) for immunoaffinity purification of enterotoxin.

Samples of enterotoxin in 0.1 M borate buffer (pH 8.2) were applied to the top of the column which was then washed in sequence with (i) 4 ml of 0.5 M NaCl in 0.05 M Tris-hydrochloride (pH 8.2)–0.5% Nonidet P-40 (Particle Data Laboratories, Elmhurst, Ill.); (ii) 4 ml of 0.15 M NaCl in 0.05 M Tris-hydrochloride (pH 8.2) containing 0.5% Nonidet P-40 and 0.1% lauryl sulfate; and (iii) 8 ml of 0.15 M NaCl in 0.5% sodium deoxycholate. Bound enterotoxin was eluted from the immunomatrix with 8 ml of 0.05 M diethylamine (pH 11.5) in 0.5% sodium deoxycholate. After elution, fractions were adjusted to pH 7.4 with 0.5 M NaH_2PO_4.

RESULTS

All seven immunized mice produced circulating antibodies to SEA or SED or both. Since the goal was to produce monoclonal antibody to antigenic determinants held in common by two or more enterotoxin serotypes, a mouse with a high titer to both SEA and SED was selected for the fusion

experiment. In this experiment, 65 of 149 wells produced hybridomas that were visible macroscopically. Culture medium from positive wells was screened in separate ELISA plates for IgG to SEA and SED. A total of 30 wells contained IgG to SEA, and 11 were positive to both SEA and SED. None was positive only to SED. Selected hybridomas were cloned and recloned on soft agar, and isolated hybrids were found to produce an antibody which interacted with both toxin serotypes. For example, of 45 clones obtained from one original hybridoma, 30 cultured and monitored for antibody production over a 4-month period consistently produced antibody to both SEA and SED. A stabilized clone capable of producing this antibody was used to produce ascites fluid which was fractionated by 50% ammonium sulfate and dialyzed.

Specificity of monoclonal antibody. Antibody in dialyzed ascites fluid interacted with all five serotypes tested (Table 1). Interestingly, the highest titers were against serotypes B and C_1. Controls consisting of the specific enterotoxin without added monoclonal antibody were all negative, indicating that the enzyme-labeled anti-mouse IgG did not bind nonspecifically to the enterotoxin or to other sites in the wells. The test was repeated on several occasions, always with the same results. The monoclonal antienterotoxin did not bind with any of a number of other antigens tested. These antigens were randomly selected and included *Staphylococcus* cells, *Rhizobium* cells, infectious bovine rhinotracheitis virus, and purified fungal glucose oxidase.

Inhibition of ELISA. An experiment was performed to determine whether preincubation of enterotoxin with monoclonal antienterotoxin inhibited binding of the antibody to wells in ELISA plates. Antienterotoxin (0.2 ml) from a preparation having a known titer (i.e., highest 10-fold serial dilution producing an ELISA value above a background value of 10^{-4} against SEA and 10^{-5} against enterotoxin B [SEB]) was added to 0.2 ml of PBS containing 100 μg of purified SEA or SEB.

The mixtures were incubated for 30 min at 37°C and then tested by the usual ELISA procedure for interaction with SEA or SEB. A control containing 0.2 ml of PBS instead of enterotoxin was also incubated and tested. The results are shown in Table 2.

ELISA values represented by the controls were considerably decreased by pretreatment of antienterotoxin with SEB. Almost complete inhibition probably occurred since ELISA values of 0.26 and 0.32 were only slightly above the background value (0.2) and the preparation tested by ELISA represented a 1:2 dilution of the antibody preparation which had original titers of $1:10^4$ (SEA) and $1:10^5$ (SEB). SEA also blocked binding of antibody to SEA but to a lesser extent. The ELISA value of 0.6 for SEA versus 0.26 for SEB suggests about a 10-fold difference in titer based on values in the comparable range shown in Table 1.

TABLE 1. Interaction of monoclonal antienterotoxin with various toxin serotypes

Dilution (log_{10})	ELISA values at 410 nm[a] for enterotoxin serotypes:				
	A	B	C	D	E
0	1.9	>2	>2	1.9	1.9
1	0.9	>2	>2	1.0	0.8
2	0.8	>2	>2	0.7	0.5
3	0.3	1.7	1.9	0.3	0.2
4	0.1	0.7	0.8	0.1	0.1
5	0	0.1	0.1	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0	0	0	0	0

[a] After subtracting a background value of 0.2. Background was measured in wells containing all reagents except antibody.

TABLE 2. Inhibition of monoclonal antienterotoxin with SEA and SEB antigens

Antigen in ELISA wells	ELISA values at 410 nm for monoclonal antibody adsorbed with:		
	SEA	SEB	Control
SEA	0.6	0.26	>2
SEB	>2	0.32	>2

Decrease in titer to SEB antigen is not determinable from the data in Table 2 because only a 1:2 dilution was tested and the amount of residual antibody activity as measured by ELISA exceeded 2, the highest value measurable by the ELISA reader. These results agree with our earlier observation that affinity of monoclonal antienterotoxin is higher for SEB than for SEA.

Determination of antibody subclass and type. The monoclonal antibody was characterized as to its mouse immunoglobulin subclass and light-chain type. Double immunodiffusion was performed in agar gel coated on glass microslides. Wells (3 mm) were cut in the agar, charged with goat anti-mouse immunoglobulin subclass-specific antisera (Litton Bionetics, Kensington, Md.), and run against ammonium sulfate-fractionated culture fluid. The monoclonal antienterotoxin was determined to belong to the IgG1 subclass with kappa light chain.

Development of an immunoaffinity matrix. To construct an immunomatrix as described by Schneider et al. (21), it was first necessary to determine the amount of antibody which binds to protein A-Sepharose CL-4B. Ammonium sulfate-fractionated ascites fluid (3 ml) containing 36 mg of protein (optical density at 280 nm, 0.8) was applied to the top of a column (5-ml height; 1.5 g [dry weight]; 0.7-mm diameter) of protein A-Sepharose CL-4B previously equilibrated in 0.05 M potassium phosphate buffer, pH 8.0. The column was washed with about 30 ml of the same buffer, and 3-ml fractions were collected. Adsorbed IgG was eluted with 30 ml of 0.1 M glycine buffer (pH 3.0) and each fraction was immediately passed through a column (1.8 by 10 cm) of Sephadex G-25 (Pharmacia) to desalt it and raise the pH. About 6 mg of protein was adsorbed and eluted from the protein A column as compared with 31 mg which passed through in the wash fractions (Fig. 1). Fractions representing the small elution peak were assayed on separate ELISA plates for ability to interact with SEA and SED. Both sets of values correlated with those for protein.

Although not all fractions were assayed, a considerable amount of antibody was detected in the wash, indicating that the amount applied exceeded the capacity of the column. For example, a 10^{-2} dilution of fraction no. 3 gave an ELISA value (optical density at 410 nm) of greater than 2. A rather low affinity for protein A is typical for mouse subclass IgG1. From these experiments we estimated that, at most, 3 mg of monoclonal antienterotoxin could be expected to have been cross-linked with 0.75 g (dry weight) of protein A-Sepharose CL-4B by the pimelimidate treatment described above. To test whether this immunomatrix could bind enterotoxin, two small (2.5-ml) columns were prepared. The second column contained protein A-Sepharose CL-4B without cross-linked antibody as a control. Both columns were equilibrated with 0.1 M borate buffer, pH 8.2. Equal volumes (4 ml) of a crude

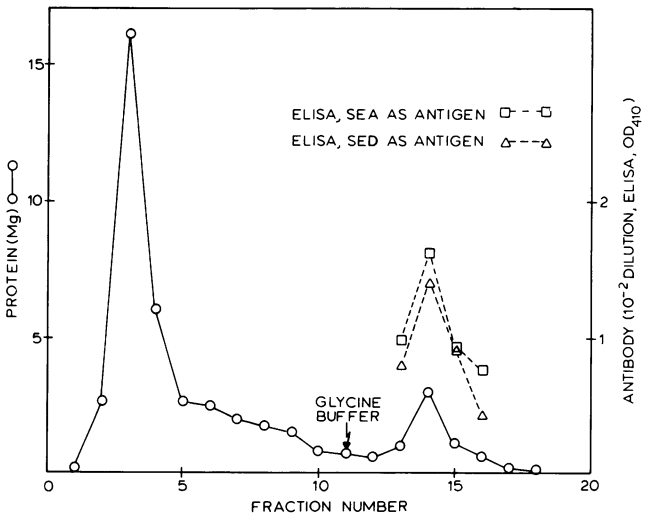

FIG. 1. Elution profile for monoclonal antienterotoxin on a column of protein A-Sepharose CL-4B. Antibody was eluted after application of glycine buffer beginning at fraction no. 11. OD_{410}, Optical density at 410 nm.

preparation containing an unknown amount of SED were applied to both columns which were washed and eluted as described above. Because materials in the wash and eluting buffers had high extinction values at 280 nm, it was impossible to follow the protein elution profile spectrophotometrically. All fractions were analyzed qualitatively for SED by the microslide double-diffusion procedure. The first fraction collected was the only one from the control column that was found to contain SED. The first fraction from the affinity column was free of SED, as were fractions collected during the subsequent three wash steps, indicating that the toxin was bound to the immunomatrix. After the wash, detectable toxin was eluted from the matrix with 0.05 M diethylamine.

In a second experiment, 0.25 mg of purified SEA in 0.1 M borate buffer (pH 8.2) was applied to each column. An additional 10 ml of borate buffer was passed through each column before initiation of the wash and elution steps employed previously. The protein profile as measured spectrophotometrically at 280 nm on the first 12 ml of column effluent is shown in Fig. 2. However, protein absorbance values for subsequent fractions were impossible to obtain because of their high extinction values at 280 nm.

By difference in absorbance, we estimate that about 75% of the protein applied to the affinity column was bound to the immunomatrix. Although toxin was detected by the microslide procedure in the first 12 ml of buffer passed through the control column, none was detected in the same fractions from the affinity column. After being washed with the series of three buffers, toxin bound to the immunomatrix was eluted with diethylamine and detected by the microslide method.

DISCUSSION

In the fusion experiment, over one-third of the hybridoma culture wells that were positive for IgG contained antibody to both SEA and SED. Apparently, injecting mice alternately with SEA and SED was an effective immunization strategy to enrich for spleen lymphocytes programmed to produce antibody to a common site on the two antigens. Bergdoll and co-workers (1982 Annual Report, Food Research Institute, University of Wisconsin-Madison, p. 339–348) isolated hybrid cell lines capable of producing monoclonal antibodies reactive with SEA, SEB, and enterotoxins C_1, C_2, and C_3. Some of these antibodies were specific for only one serotype, others interacted with more than one serotype, but none was able to bind with the five serotypes as reported here.

Although we had hoped that the monoclonal antibody would bind to other enterotoxin serotypes, it was surprising that the titer was about 10-fold higher for B and C_1 than for those used to immunize the mouse (A and D). Spero et al. (22) found that a polyclonal antibody preparation had cross-reactivity for SEB and enterotoxin C_1 but not SEA and proposed that one of the major antigenic determinants of these two enterotoxins possesses a significant similarity. Lee and co-workers (13) from the Bergdoll group reported that rabbits immunized with SEB produced antibody that precipitated with B, C_1, and C_2 in gel diffusion plates. This group also isolated common antibody to A and E (12). Although reported (8, 11), cross-reactivity of polyclonal antibody to SEA and SEB has not been confirmed (12). These investigations and the work reported here for monoclonal antienterotoxin suggest that there are two groups of toxins with common antigenic sites, B and C_1 in one group and A, D, and E in the other. Monoclonal antienterotoxin has lower affinity for A, D, and E, suggesting that it only partially fits the site on these antigens and that the common epitope is not the same stoichiometric structure in both groups.

The toxic action of the enterotoxins is speculated to be due to a common molecular structure (10). Possibly, monoclonal antienterotoxin binds to a site on or near that structure.

The immunomatrix studied here does not contain a large amount of antibody because IgG1 has low affinity for protein A. Schneider et al. (21) developed more efficient matrices for such antibodies by indirectly binding them to protein A

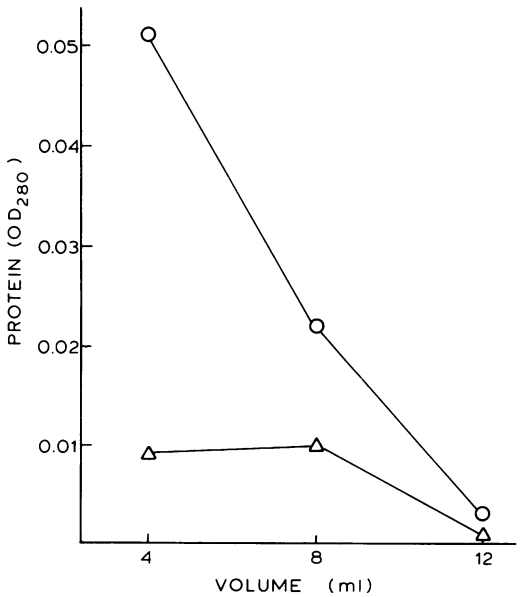

FIG. 2. The amount of enterotoxin (protein) in the initial effluent from a column containing monoclonal antibody cross-linked to protein A-Sepharose CL-4B (△) as compared with that in effluent from a similar column without cross-linked antibody (○). OD_{280}, Optical density at 280 nm.

through intermediate coupling to anti-mouse IgG, followed by cross-linking the entire complex with pimelimidate. Alternatively, cyanogen bromide-activated Sepharose could be used to form an immunomatrix but, according to these authors, multisite attachment resulting from this procedure does not spatially orient immunoglobulins for efficient antibody-antigen interaction.

We propose to use the monoclonal antibody described here to develop a method for detecting enterotoxin in food extracts. Antienterotoxin could be used as the first antibody in a double-sandwich procedure to screen samples for the presence of any of the five serotypes. The second antibody could be from a battery of type-specific monoclones, provided attachment sites are not blocked by the first one.

Monoclonal antibodies are potentially useful for the development of rapid, sensitive, and specific methods for detection of enterotoxin. Application of these methods at both the food processing and regulatory levels should facilitate protection of individuals from the hazard of staphylococcal food poisoning.

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