

Monoclonal Antibody-Based Immunoassay for Type A *Clostridium botulinum* Toxin Is Comparable to the Mouse Bioassay

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Received 18 January 1985/Accepted 9 April 1985

A monoclonal antibody (BA11) has been produced against *Clostridium botulinum* type A neurotoxin by the fusion of myeloma cells (P3 NS1/1-Ag4-1) with spleen cells from BALB/c mice immunized with botulinum type A neurotoxin. The antibody bound specifically to botulinum type A neurotoxin, showing no cross-reactivity with types B and E botulinum toxins or with any of several other bacterial toxins tested. The monoclonal antibody did not bind to botulinum type A neurotoxin which had been denatured with sodium dodecyl sulfate and bound only weakly to each of the separated heavy and light subunits of the neurotoxin, suggesting a conformational requirement for the antigenic determinant of the antibody. A sensitive immunoassay for *C. botulinum* type A toxin with monoclonal antibody BA11 in conjunction with an enzyme amplification system has been developed which allows detection of 5 to 10 mouse 50% lethal doses ml⁻¹ of purified neurotoxin. The assay was equally sensitive when applied to the detection of crude toxin in food stuffs; the average value for the minimum level of detectable toxin in extracts of tinned salmon or corned beef was 9 ± 3.1 mouse 50% lethal doses ml⁻¹.

The neurotoxins produced by the eight types of *Clostridium botulinum* act primarily at the neuromuscular junction and cause the syndrome botulism, which is frequently a fatal condition in humans and animals (13, 14). Much effort has been imparted by the food industry to ensure the adequacy of food treatment processes to prevent the growth and toxin production of *C. botulinum*, and there is considerable need for rapid, sensitive assays for these toxins that do not require the use of laboratory animals.

Acute toxicity tests in mice (12) presently provide the only assay for botulinum toxins of sufficient sensitivity to allow the detection of low levels (<5 mouse 50% lethal doses [MLD_{50s}] ml⁻¹) in food stuffs and biological samples. Assays involving intraperitoneal injection of toxin take up to 4 days to complete, and although direct injection into a tail vein can shorten the assay time to a few hours, the latter procedure requires considerable operator skill and is tedious to perform and monitor. Furthermore, mouse toxicity results are not in themselves specific; specificity is only imparted by carrying out parallel toxin neutralization tests with homologous antisera. Enzyme-linked immunosorbent assays (ELISAs) developed for botulinum toxins (1, 7, 9-11), although they provide relatively rapid and specific assays for these toxins, are at present insufficiently sensitive to replace the mouse bioassay.

Hybridoma cell lines which secrete antibody specific to a toxin provide a potentially inexhaustible supply of antibody which may be used in ELISAs. In this report, we describe the properties of a monoclonal antibody produced against *C. botulinum* neurotoxin and its use in an amplified ELISA for the toxin with a lower limit of detectable toxin approaching that of the mouse bioassay.

MATERIALS AND METHODS

Sources of toxins. *C. botulinum* type A (NCTC 2916) type B (Okra) and type E (Eye Alaska) were used. Bacteria were

grown in 20-liter cultures for 48 h, after which toxin was precipitated by addition of 1.5 M H₂SO₄ to spent cultures to give a final pH of 3 to 3.5 by a modification (5) of previously described methods (2).

Diphtheria toxoid was kindly provided by K. Redhead, National Institute for Biological Standards and Control, Hampstead, London, *Staphylococcus aureus* enterotoxins and *Clostridium perfringens* enterotoxin A were kindly donated by H. S. Tranter of Public Health Laboratory Service, Center for Applied Microbiology and Research, Porton Down, England, and *Clostridium difficile* toxins were a kind gift from N. Sullivan of the Virginia Polytechnic Institute and State University, Blacksburg, Va. Tetanus toxin was a gift from P. Walker, Wellcome Laboratories, Dartford, England.

Purification of *C. botulinum* neurotoxins. *C. botulinum* type A neurotoxin (specific toxicity, 2×10^8 MLD_{50s} mg of protein⁻¹) was purified by previously described methods (5) including affinity chromatography on *p*-aminophenyl- β -D-thiogalactopyranoside-Sepharose-4B (8) followed by chromatography on DEAE-Sepharcel at pH 7.9.

The purification of *C. botulinum* type B neurotoxin was identical to that described for type A neurotoxin, except for the final purification. Affinity-purified type B neurotoxin (8×10^9 MLD_{50s}) was dialyzed against Tris-hydrochloride buffer (0.1 M, pH 7.9) and loaded onto a column of DEAE-Sepharcel (10 by 0.6 cm) equilibrated in the same buffer. Type B neurotoxin (specific toxicity, 1.1×10^8 MLD_{50s} mg of protein⁻¹) was eluted from the column with 0.15 M Tris-hydrochloride (pH 7.9) containing 18 mM NaCl.

Impure *C. botulinum* type E neurotoxin was obtained by the method described for type A neurotoxin (5). To purify the neurotoxin component, impure toxin (7×10^8 MLD_{50s}) was dialyzed against 0.15 M Tris-hydrochloride buffer (pH 7.9) and loaded onto a column of DEAE-Sepharcel equilibrated on the same buffer. After washing with a further 20 ml of the Tris-hydrochloride buffer, elution was continued with a gradient of NaCl (100 ml of 0 to 0.5 M NaCl in 0.15 M

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Tris-hydrochloride [pH 7.9]). Pure type E neurotoxin (2×10^8 MLD_{50s} mg of protein⁻¹) was obtained by selecting the fractions of highest specific toxicity from the first protein peak to elute after application of the salt gradient.

Purification of neurotoxin subunits. *C. botulinum* type A neurotoxin (5 mg, 1.5 mg ml^{-1}) was dialyzed against Tris-hydrochloride buffer (0.1 M, pH 7.9) containing 2.5 M urea and 1 M NaCl. Dithiothreitol was then added (final concentration, 100 mM) to the toxin solution, which was incubated for 16 h at 20°C; the resulting precipitate of light chain was collected by centrifugation ($10,000 \times g$, 10 min), and the supernatant fluid containing the heavy chain was retained. The precipitated light subunit was washed, by resuspension with a ground glass homogenizer, in 1 ml of Tris-hydrochloride buffer (0.1 M, pH 7.9) containing 2.5 M urea, 1 M NaCl, and 10 mM dithiothreitol and further washed twice with 1-ml portions of Tris-hydrochloride buffer (0.1 M, pH 7.9) containing 0.5 M NaCl and 10 mM dithiothreitol.

The solution of the heavy subunit was dialyzed overnight at 4°C against Tris-hydrochloride buffer (0.1 M, pH 7.9) containing 0.2 M NaCl and 10 mM dithiothreitol and centrifuged at $10,000 \times g$ for 10 min. Both subunit preparations were stored at 4°C.

Production of hybridoma cell lines. Purified botulinum type A neurotoxin (0.5 mg ml^{-1}) was dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) and inactivated with 0.6% neutralized formaldehyde (30°C, 14 days); subsequently, excess formaldehyde was removed by dialysis against 0.1 M sodium phosphate buffer (pH 7.0).

BALB/c mice were immunized by intraperitoneal injection of 0.5 ml of neurotoxin (50 µg) in Freund complete adjuvant. Further doses (50 µg each) in Freund incomplete adjuvant were given 2 and 6 weeks later, and a final dose (without adjuvant) was given at 8 to 9 weeks by both intraperitoneal (50 µg, 0.5 ml) and intravenous injection (50 µg, 0.3 ml). Mice were sacrificed 4 days after the final dose, and their spleens were removed.

Spleen cells were fused with myeloma cells (P3 NSI/1-Ag4-1) with polyethylene glycol, and the resulting hybridomas were cloned twice by limiting dilution by methods previously described (6).

To obtain antibody at high concentration, approximately 10^7 hybridoma cells were inoculated into the peritoneal cavity of BALB/c mice previously primed with pristane; ascitic fluid was collected 10 to 20 days later. Antibody was purified from ascites fluids by affinity chromatography on protein A-Sepharose (4).

Analytical techniques. Ouchterlony double immunodiffusion with a range of antimouse immunoglobulin subclass sera (immunoglobulin M [IgM], IgA, IgG1, IgG2a, IgG2b, IgG3; Miles Laboratories, Inc.) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis on polyacrylamide slab gels (4 to 30% acrylamide; PAA/4/30; Pharmacia Fine Chemicals) were carried out as described previously (15).

Isoelectric focusing was performed on precast thin-layer polyacrylamide gels (Ampholine Pagplate, pH 3.5 to 9.5; LKB Bromma) by using a LKB Multiphor apparatus with 1 M H₃PO₄ as the anode electrode solution and 1 M NaOH at the cathode electrode. After electrophoresis, gels were fixed in 0.7 M trichloroacetic acid–0.14 M 5-sulphosalicylic acid for 1 h, stained with 0.1% Coomassie blue R250 in 25% (vol/vol) ethanol–8% (vol/vol) acetic acid, and destained in 25% (vol/vol) methanol–8% (vol/vol) acetic acid.

Toxin neutralization test. Antibody ascites fluids (0.1 ml, containing 5 to 10 mg of antibody ml⁻¹) were mixed with dilutions of *C. botulinum* type A neurotoxin (0.25 ml, 5 to

5,000 mouse MLD_{50s} ml⁻¹) in 0.07 M sodium phosphate buffer (pH 6.5) containing 0.2% gelatin, incubated for 1 h at 30°C, and diluted to 2.5 ml with the same buffer; 0.5-ml volumes were injected intraperitoneally into Porton mice in groups of four. Deaths were recorded over a period of 4 days.

ELISA for monoclonal antibodies. Hybridomas were screened, and the cross-reactivity of monoclonal antibodies was assessed by an ELISA with neurotoxin-coated microtiter plates. Neurotoxin solution (20 µg ml^{-1} in 50 mM sodium phosphate buffer [pH 8.0]; 0.1 ml well⁻¹) was used to coat polystyrene microtiter plates (Dynatech M129A) and left overnight at 4°C; the toxin solution was then removed, and the plates were washed three times with phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST) and incubated for 1 h at 37°C with 0.07 M sodium phosphate buffer (pH 6.5) containing 0.2% gelatin and 1% bovine serum albumin (GPBSA). After a further three washes with PBST, hybridoma supernatants (50 µl) were added to the wells, which were then incubated for 90 min at room temperature with shaking. The plates were then washed twice with PBST, rabbit antimouse peroxidase conjugate (100 µl per well, Dakopatt diluted 1 in 200 with GPBSA) was added, and the plates were incubated with shaking for 90 min at room temperature. After three washes with PBST, 100 µl of substrate solution (5-aminosalicylic acid [1 mg ml⁻¹] [3], 0.05 M sodium phosphate buffer [pH 6.0], 0.06% hydrogen peroxide) was added, and the reactions were allowed to proceed for 20 min; absorbances were measured at 450 nm with a Dynatech MR580 plate reader.

Preparation of alkaline phosphatase-conjugated guinea-pig antitoxin type A toxin. Guinea pigs were immunized by intramuscular injection of (1 ml, 100 µg) neurotoxin in Freund complete adjuvant followed by further doses (100 µg) in Freund incomplete adjuvant at 2, 6, 10, and 14 weeks. Immunoglobulins were precipitated from the guinea pig serum by slow addition of an equal volume of 80% saturated ammonium sulfate solution (pH 7.0). After centrifugation at $20,000 \times g$ for 15 min, the precipitate was dissolved in phosphate buffer (0.1 M, pH 6.8; 5 ml ml of original serum⁻¹), and the precipitation process was repeated. The precipitate was then resuspended in phosphate buffer (0.075 M, pH 6.8; 0.5 ml ml of original serum⁻¹), dialyzed against 4 liters of the same buffer at 4°C, and mixed with DEAE Sephacel (0.2 g ml of original serum⁻¹) equilibrated in the phosphate buffer. After stirring for 20 min, the supernatant was obtained by centrifugation at $15,000 \times g$ for 10 min, aliquoted, and stored frozen at –20°C.

Conjugation of 2-ml aliquots of guinea pig IgG fraction (1 ml of protein neutralized 95,000 MLD_{50s} of botulinum type A toxin) to calf intestinal alkaline phosphatase was performed by IQ Bio Ltd. by using succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (details presently commercial in confidence). The conjugate was diluted 600-fold with a solution containing 0.5 M NaCl, 0.2 M ammonium sulfate, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.1% (vol/vol) Triton X-100, 4% (wt/vol) bovine serum albumin, and 0.1 mM triethanolamine at pH 7.5 before use.

Amplified ELISA for *C. botulinum* type A neurotoxin. Microtiter plates (Nunc) were coated (100 µl well^{-1}) with 15 µg of purified BA11 monoclonal antibody ml⁻¹ in 20 mM citrate buffer (pH 5.0) and incubated overnight at 37°C. The plates were washed three times with a solution containing 5% (wt/vol) lactose, 0.5% (wt/vol) bovine serum albumin, 0.1% (vol/vol) Triton X-100, and 0.01% (wt/vol) Thiomersal at pH 7.5, allowing the final wash to incubate for 30 min

before tapping dry. Coated plates were then freeze dried and stored desiccated at 4°C until use.

Toxin solutions, diluted with 100 mM Tris-hydrochloride buffer (pH 7.5) containing 0.2% gelatin, were pipetted into plate wells (100 μ l well⁻¹) followed by 20 μ l of alkaline phosphate-conjugated guinea pig antitoxin type A toxin solution, and the mixture was incubated for 4 h with shaking, after which plates were washed six times with 100 mM Tris-hydrochloride buffer (pH 8.0) containing 150 mM NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, and 0.005% (vol/vol) Triton X-100. Freshly prepared NADP⁺ substrate solution (0.2 mM in 50 mM ethanolamine buffer [pH 9.0]) was added (80 μ l well⁻¹), and the reaction was allowed to proceed for 30 min at 20°C with shaking. Amplifier solution containing 0.2 mg of alcohol dehydrogenase ml⁻¹, 0.2 mg of diaphorase ml⁻¹, 3% (vol/vol) ethanol, and 1 mM iodinitrotetrazolium violet in 25 mM phosphate buffer (pH 7.0) was then added (220 μ l well⁻¹), and the color was allowed to develop for 10 min at 20°C before the reaction was stopped by the addition of 50 μ l of 0.2 M H₂SO₄.

Absorbances were measured at 490 nm, and the resulting data were analyzed with a Dynatech MR580 ELISA reader interfaced with a BBC microcomputer. Toxin in extracts was determined (in duplicate) by the mouse bioassay, and the concentration of toxin giving an absorbance of 0.3 U above the blank value in the ELISA was determined.

RESULTS AND DISCUSSION

Hybridoma cell line BA11 produces antibody with a high binding activity for *C. botulinum* type A neurotoxin in a solid-phase immunoassay. Isotype analysis of the hybridoma product revealed the antibody to be of the IgG1 class, and subsequent examination of the purified antibody by sodium dodecyl sulfate gel electrophoresis under reducing conditions showed the molecular weights of the heavy and light chains to be consistent with this observation. Isoelectric focusing of the purified antibody revealed several strong protein bands in a limited region with no indication that more than one antibody was present in the hybridoma product.

The binding of monoclonal antibody BA11 to a variety of bacterial toxins was assessed by an ELISA with solid-phase toxin (Table 1). Antibody BA11 bound specifically to *C. botulinum* type A neurotoxin and showed no cross-reactivity with type B and E neurotoxins or with any other toxin tested. Similar high specificity was also observed with homologous polyclonal antisera used in this study and elsewhere (10).

Using similar assays, the binding of monoclonal antibody BA11 to the separated heavy (100,000-dalton) and light (50,000-dalton) subunits of botulinum type A neurotoxin was assessed (Fig. 1a). There was a strong reaction between the antibody and the native toxin, whereas both the separated neurotoxin subunits gave only a weak response in the ELISA with signals that plateaued at absorbance values below 0.1 in each case. In a control experiment, native toxin and both subunits each reacted strongly with homologous mouse polyclonal antisera (Fig. 1b).

The comparatively weak binding of the antibody to the individual toxin subunits implies that the structure of the antigenic site on the native toxin is largely destroyed during the separation of the subunits. This suggests that the antigenic determinant involved is of the discontinuous type, i.e., one in which the epitope comprises amino acid sequences that are separated in the primary sequence of the neurotoxin but are brought together as a consequence of the tertiary

TABLE 1. Binding of monoclonal antibody BA11 to various toxin types measured by ELISA

Solid-phase toxin	<i>A</i> ₄₅₀ of the following dilution of BA11 monoclonal antibody culture supernatant ^a	
	1×	10×
Blank	0	0
<i>Clostridium botulinum</i>		
Neurotoxin type A	1.37	1.31
Neurotoxin type B	0	0
Neurotoxin type E	0	0
Neurotoxin type A denatured with SDS	0.03	0
<i>Clostridium tetani</i> neurotoxin	0.01	0
<i>Clostridium perfringens</i> enterotoxin A	0	0
<i>Clostridium difficile</i>		
Toxin type A	0.01	0
Toxin type B	0	0
<i>Corynebacterium diphtheria</i> toxoid	0	0
<i>Staphylococcus aureus</i>		
Enterotoxin type A	0	0
Enterotoxin type B	0	0
<i>Vibrio cholerae</i> toxin	0.02	0

^a All data are average absorbance values of six ELISA wells (standard deviation, <15% in each case).

structure of the protein. Consistent with this possibility is the fact that denaturation of the neurotoxin with sodium dodecyl sulfate and 2-mercaptoethanol abolishes the anti-toxin reaction (Table 1).

Ascites fluid (containing ca. 5 mg of antibody ml⁻¹) derived from BA11 hybridoma cell line failed to precipitate botulinum type A neurotoxin in double immunodiffusion tests but did display weak toxin-neutralizing activity; 0.1 ml of BA11 ascites neutralized approximately 50 MLD_{50s} of botulinum type A neurotoxin. This weak neutralizing activity may be an indication that the antibody-binding site(s) is not on or close to a biologically active region of the molecule. The inability of BA11 to precipitate the toxin may reflect the presence of a single binding site for the antibody on the neurotoxin molecule but could equally be due to BA11 being monovalent or unable to form the necessary lattice structures because of steric hindrance.

An amplified ELISA developed for the detection of botulinum type A toxin is depicted in Fig. 2. Each molecule of NAD⁺ generated by the antibody-alkaline phosphatase conjugate initiates the formation of several hundred colored formazan molecules thus providing an amplification factor.

With this amplified ELISA, three separate determinations of purified *C. botulinum* type A neurotoxin (specific toxicity, 1.5 × 10⁸ MLD_{50s} mg of protein⁻¹), gave values for the lowest detectable level of toxin of 5, 7, and 10 MLD_{50s} ml⁻¹. Determination of toxin by using a conventional single-sandwich assay similar to that depicted in Fig. 2 but with guinea pig anti-botulinum peroxidase conjugate (5-amino salicylic acid as substrate) in place of the alkaline phosphatase system gave a lower limit of detectable toxin of between 300 and 1,000 MLD_{50s} ml⁻¹ (C. Shone, unpublished data). Thus, the enzyme amplification system afforded almost a

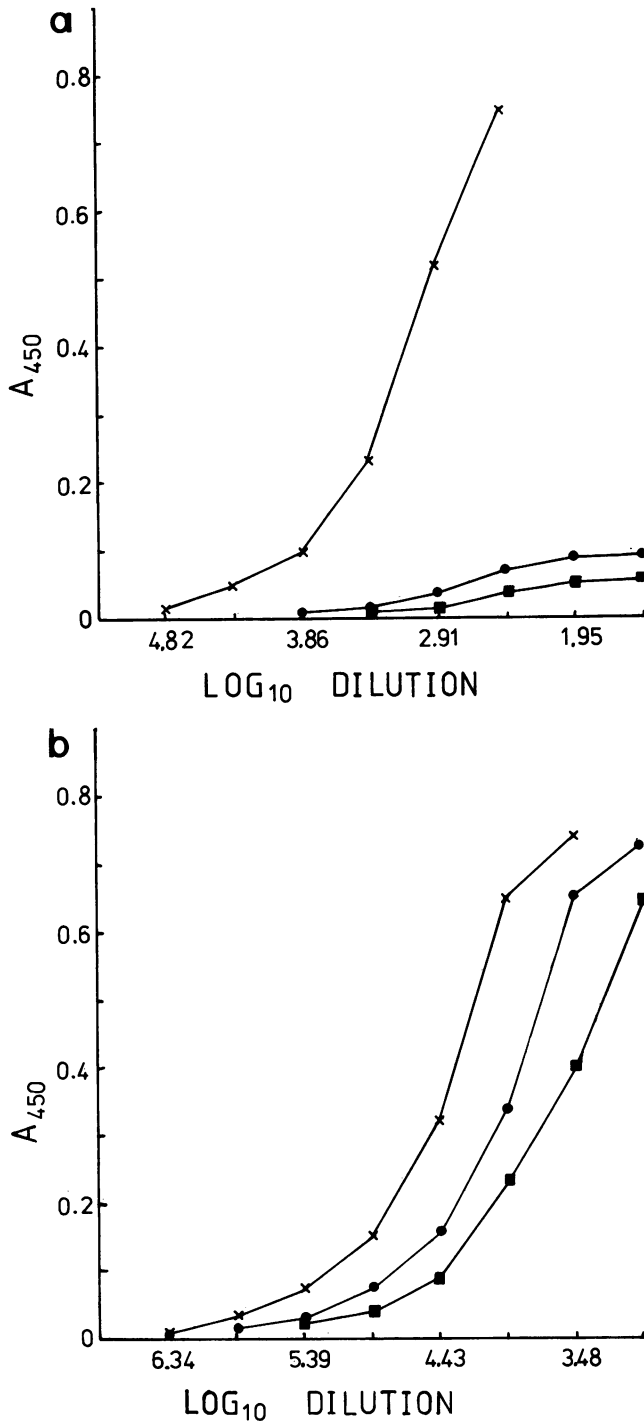


FIG. 1. Binding of monoclonal antibody BA11 (a) and polyclonal mouse antbotulinum type A neurotoxin (b) in ELISAs with solid-phase toxin components: type A neurotoxin (x), heavy subunit (●), and light subunit (■). Subunit preparations of the neurotoxin were coated onto microtiter plates at 20 μg ml⁻¹ in 0.1 M sodium phosphate buffer (pH 8.0) containing 20 mM 2-mercaptoethanol. The partially soluble light subunit was dispersed with a glass homogenizer before coating.

100-fold increase in the sensitivity of the ELISA compared with the unamplified system.

To investigate the potential of the amplified ELISA for the assay of botulinum toxin in foodstuffs, cans of salmon and

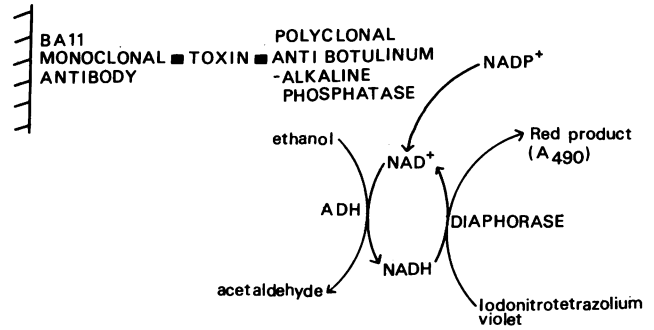


FIG. 2. Amplified ELISA system for *C. botulinum* type A toxin.

corned beef were artificially contaminated with either clostridial spores or portions of culture supernatant; toxin present in food extracts was determined with the mouse bioassay and the immunoassay. Figure 3 shows the results of a typical assay for botulinum toxin in salmon extract in which approximately 10 MLD₅₀ ml⁻¹ could be detected by the ELISA. Nine determinations of botulinum toxin food extracts (Table 2) gave an average lower limit of detectable toxin an arbitrary end point of 0.3 absorbance units above background levels) equivalent to approximately 9 (standard deviation, 3.1) MLD₅₀ ml⁻¹. Careful analysis of precisely measured absorbance values may allow a lower endpoint value to be used, which would result in a more sensitive

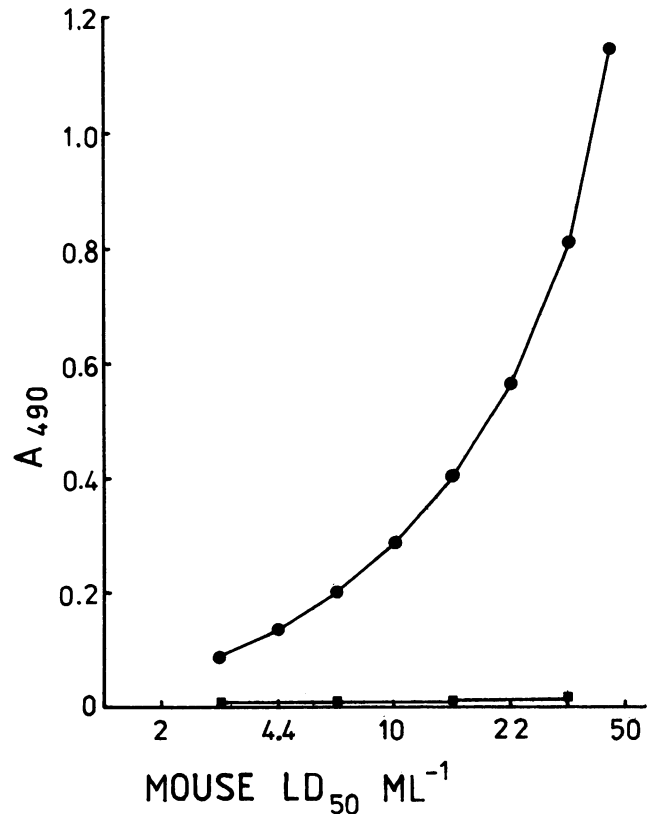


FIG. 3. Determination, with an amplified ELISA, of toxin levels in an extract of tinned salmon contaminated with *C. botulinum* type A toxin (●) and *C. botulinum* type B culture supernatant (■) (MLD₅₀ for type B toxin were 10 times those indicated).

TABLE 2. Detection of botulinum toxin A in food extracts by ELISA

Sample (MLD ₅₀ s ml ⁻¹ in extract)	Reciprocal of dilution at ELISA endpoint	Minimum toxin concentration detected (MLD ₅₀ s ml ⁻¹)
Culture supernatant added to salmon		
1.5 × 10 ³	250	6
1.5 × 10 ³	214	7
3.0 × 10 ³	300	10
3.0 × 10 ³	215	14
<i>C. botulinum</i> grown in salmon		
10 ⁴	985	10
10 ⁴	2,578	4
7.0 × 10 ³	1,150	6
Culture supernatant added to corned beef		
2.0 × 10 ³	235	8.5
6.0 × 10 ³	496	12

assay. The sensitivity of the immunoassay was unaffected by the constituents of the salmon extract, since the level of detection was unchanged even when uncontaminated salmon extract was used as a diluent for the toxin instead of Tris-hydrochloride-gelatin buffer.

Previously developed *in vitro* assays for *C. botulinum* type A toxin (1, 10) have proved insufficiently sensitive to replace the mouse bioassay for the diagnosis of type A food-borne or infant botulism: the sensitivities of these assays are approximately 50-fold lower than the currently used mouse bioassay. The immunoassay developed by Dezfulian and Bartlett (1) has the additional drawback that nontoxic proteins of type A *C. botulinum* also appear to be detected, which may increase the possibility of false-positive reactions occurring in the ELISA.

The amplified immunoassay described in this report provides a convenient test for *C. botulinum* type A toxin with a sensitivity approximately 10-fold greater than that of the previously reported *in vitro* assays. At the arbitrary endpoint value of 0.3 absorbance units above background levels, an endpoint easily discernible by eye, approximately 9 MLD₅₀s ml⁻¹ could be detected in the foodstuffs tested, which is equivalent to detection of less than 1 MLD₅₀ in the 0.1-ml portion used for the immunoassay. Compared with the sensitivity of the mouse bioassay (2 MLD₅₀s ml⁻¹, assuming 0.5 ml of toxin injected per mouse) the sensitivity of the amplified ELISA is only fivefold lower and as such is sufficiently sensitive to replace the mouse assay in a majority of food industrial and laboratory applications. This could greatly reduce the usage of mice in the detection and quantitation of type A toxin. The ELISA has the additional advantage in that a result can be obtained in less than 6 h compared with the 4 days necessary for the intraperitoneal mouse lethality test which is so widely used at present.

In conclusion, monoclonal antibody BA11 appears to be a useful reagent for the detection of botulinum type A toxin in foods. The antibody appears specific to *C. botulinum* type A neurotoxin and, when used in conjunction with the ELISA

amplification system developed by IQ Bio Ltd., provides an immunoassay for the toxin with a sensitivity approaching that of the mouse bioassay.

Monoclonal antibodies are presently being produced in this laboratory against *C. botulinum* type B, E, and F neurotoxins, which will allow the development of equally sensitive immunoassays for these toxins.

ACKNOWLEDGMENTS

We thank Nigel Bailey, Brian Capel, Roger Rhind-Tutt, and Sarah Bennett for their excellent technical assistance and Janet Peat for typing the manuscript.

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