

Enzyme-Linked Immunosorbent Assay for Detection of *Clostridium botulinum* Type E Toxin

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The enzyme-linked immunosorbent assay using the "double-sandwich" technique was utilized to determine *Clostridium botulinum* type E toxin. With this technique, about 80 mouse intraperitoneal 50% lethal doses of toxin could be detected. Cross-reaction was hardly observed with *C. botulinum* type A and B toxins. No cross-reaction was observed with culture supernatants of *C. botulinum* type C or other *Clostridium* strains. In all probability this was due to the high specificity of the antiserum prepared against the toxic component of type E toxin.

The enzyme-linked immunosorbent assay (ELISA) has proven to be a suitable technique for determining *Clostridium botulinum* type A toxin (5). Although the sensitivity for *C. botulinum* type A toxin is somewhat less than that with the hemagglutination technique (9), ELISA is more quantitative. The advantage of ELISA over the radioimmunoassay is that it is more readily usable in laboratories without special provisions being made. As shown by Notermans et al. (5), the specificity of ELISA for estimation of *C. botulinum* toxins is determined with the antiserum against the *C. botulinum* toxins used. If antiserum, prepared against the toxic component of the toxin molecule, is used, cross-reactions are almost eliminated. The same results were obtained by Sakaguchi et al. (9), who used the passive hemagglutination test for determining *C. botulinum* toxins. In the present work the use of ELISA for the quantification of *C. botulinum* type E toxin is described, as well as the results of studies on sensitivity and cross-reactivity. With this technique, rabbit serum which was prepared by immunization of rabbits with the toxoid of the toxic component (derivative toxin) of *C. botulinum* type E toxin was used.

MATERIALS AND METHODS

ELISA for the determination of type E toxin is shown schematically in Fig. 1. In this technique polystyrene tubes are coated with rabbit immunoglobulin G (IgG) against type E toxin. After incubation with the toxin, the adsorbed toxin is bound with horse serum against type E toxin. The amount of horse serum is measured with rabbit anti-horse IgG enzyme conjugate.

Type E Toxin. The medium for toxin production was composed of 2% proteose peptone, 0.5% yeast extract, 0.5% glucose, and 0.1% L-cysteine at pH 6.3.

For toxin production, a 24-h culture of type E (strain RIV1) grown in liver broth as described by Haagsma (Ph.D. thesis, Rijksuniversiteit te Utrecht, Utrecht, The Netherlands, 1973) was inoculated in the medium. Type E progenitor toxin was purified by the method of Kitamura et al. (3). The toxic component (derivative toxin) was purified from progenitor toxin by diethylaminoethyl-Sephadex chromatography at pH 8.0 as described by Kitamura et al. (3).

To obtain other toxins, liver broth was inoculated with spores of *C. botulinum* type A, B, C, and E strains. Liver broth was also inoculated with other *Clostridium* strains. For this, *C. perfringens* and *C. sporogenes* were used. The cultures were incubated anaerobically at 30°C for 4 days. The supernatant from centrifugation at 8,000 × g at 4°C for 10 min was used for the ELISA technique. Toxicity was determined after tryptic activation by the time-to-death method after intravenous injection into mice (8).

Antiserum against type E derivative toxin. Rabbit antiserum against type E derivative toxin was prepared by immunization with toxoid. Toxoid was prepared by formalinization (0.1 M phosphate buffer [pH 7.0]-0.4% Formalin for 2 days at 30°C). The formalinized toxoid was mixed with an equal volume of Freund complete adjuvant. Two 1-ml doses (0.1 mg) were injected subcutaneously into a rabbit at a 2-day interval. After 4 weeks a second injection, 1 ml of toxoid (0.2 mg/ml) without adjuvant, was injected subcutaneously. The rabbit was bled a week after the final injection. The IgG fraction was isolated by the method of Steinbuch and Audran (10). A 1-mg amount of anti-derivative toxin IgG neutralized about 60,000 intraperitoneal 50% lethal doses (i.p. LD₅₀) of type E toxin.

IgG enzyme conjugate. Horseradish peroxidase (Sigma type VI, Sigma Chemical Co., St. Louis, Mo.) was conjugated to IgG by the method described by Nakane and Kawao (4). The conjugate was used without further purification.

Determination of horseradish peroxidase activity. A substrate was prepared as described by Rui-

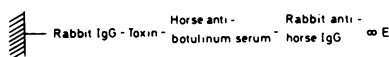


FIG. 1. Schematic presentation of the application of the ELISA technique.

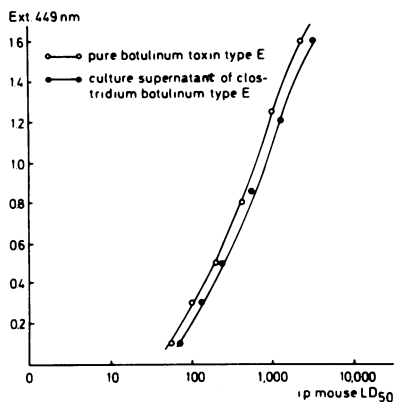


FIG. 2. Sensitivity of the ELISA technique with pure type E toxin and culture supernatant of *C. botulinum* type E.

tenberg et al. (6). An 80-mg amount of 5-aminosalicylic acid (E. Merck AG, Darmstadt, Federal Republic of Germany) was dissolved in 100 ml of hot distilled water at 70°C. Before the pH of this stock solution was adjusted to 6.0 with 1 N NaOH, a 10-ml portion of 0.05% H₂O₂ (BDH Chemicals, Poole, England) was added to 90 ml of the solution. Horseradish peroxidase activity was determined spectrophotometrically at 449 nm. The equipment used was an on-line system based on a semiautomatic apparatus for enzyme antigen analysis (Olli-System 3000, Olli Tuote, Kivenlathi, Finland), as described by Ruitenberget al. (7).

ELISA. The ELISA technique described by Engvall and Perlmann (1, 2), with some modifications, was employed. Disposable polystyrene tubes (50 by 11 mm; LKB-Produktor AB, Stockholm, Sweden) were coated with 15 µg of rabbit IgG dissolved in 1 ml of 0.07 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The tubes were incubated with rotation overnight at room temperature. After incubation, the tubes were washed twice with distilled water containing 0.05% Tween 20 (E. Merck AG). A 1-ml amount of the samples diluted with 0.05 M phosphate buffer, pH 6.5, containing 0.2% gelatin and 5 mM ethylenediaminetetraacetate was incubated with rotation at 37°C for 90 min. After washing as described above, 1 ml of a diluted commercial trivalent horse anti-*C. botulinum* serum (Wellcome Reagent Limited, Beckenham, England) was added, and this 1 ml of serum was diluted in 300 ml of 0.07 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The tubes were incubated with rotation at 37°C for 90 min. After washing, 1 ml of a dilution of rabbit anti-horse IgG enzyme conjugate was added. After incubation with rotation at 37°C for 90 min and washing, 1 ml of the substrate solution was added. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 0.1 N NaOH. The reaction product was measured spectrophotometrically at 449 nm.

RESULTS

In the first experiment the minimal detectable amount of purified type E toxin was determined by ELISA and the mouse bioassay. ELISA experiments were performed in duplicate, and the results are shown in Fig. 2. The detection of toxin from a culture filtrate of *C. botulinum* type E, strain RIV1, is also shown in Fig. 2. The results showed that *C. botulinum* toxin can be detected by ELISA. The lowest detectable dose was below 100 mouse i.p. LD₅₀. No differences

TABLE 1. Mouse i.p. LD₅₀ and ELISA extinction values of various culture filtrates of *Clostridium* strains

Bacterial strains	Toxicity (mouse i.p. LD ₅₀ /ml)	ELISA extinction value
<i>C. botulinum</i> type E, strain RIV1	4,772	1.7
<i>C. botulinum</i> type E, strain RIV1	681	0.5
<i>C. botulinum</i> type E, strain RIV1	97	0.1
<i>C. botulinum</i> type A, strain AV4	13,145	0.0
<i>C. botulinum</i> type A, strain AV4	1,878	0.0
<i>C. botulinum</i> type A, strain At2K3	4,253	0.2
<i>C. botulinum</i> type A, strain At2K3	607	0.0
<i>C. botulinum</i> type B, strain OKRA	11,449	0.2
<i>C. botulinum</i> type B, strain OKRA	1,635	0.0
<i>C. botulinum</i> type B, strain SNB77	9,744	0.2
<i>C. botulinum</i> type B, strain SNB77	1,392	0.0
<i>C. botulinum</i> type C, strain Ca	17,533	0.0
<i>C. botulinum</i> type C, strain Ca	2,504	0.0
<i>C. botulinum</i> type C, strain SNC ₂ 78	100	0.0
<i>C. perfringens</i> , strain L6539 (1:14 dilution)	ND ^a	0.0
<i>C. perfringens</i> , strain L6339 (1:14 dilution)	ND	0.0
<i>C. sporogenes</i> (1:14 dilution)	ND	0.0

^a ND, Not determined.

in extinction values were obtained between purified toxin and toxin present in the culture filtrate.

In Table 1 the mouse i.p. LD₅₀ and ELISA extinction values of various culture filtrates of *Clostridium* strains are given. Some cross-reaction could be observed with culture filtrates of *C. botulinum* types A and B. No cross-reaction occurred with other *Clostridium* strains tested.

DISCUSSION

From the results it is clear that *C. botulinum* type E toxin is measurable by ELISA. The ELISA test is simple and rapid to perform and does not require experimental animals. Tryptic activation may also be avoided since only the immunological presence of the toxin is determined. Small cross-reactions could only be observed with culture filtrates of *C. botulinum* type A and B toxins. No cross-reactions were observed with *C. botulinum* type C and other *Clostridium* species tested. Cross-reactions with *C. botulinum* type A and B toxins may be the result of the commercial trivalent antiserum used, which causes the complex of toxin and specific antibody to react nonspecifically.

With ELISA, using the "double-sandwich technique," fewer than 100 mouse i.p. LD₅₀ of toxin E could be detected. The sensitivity seems to be higher than that for *C. botulinum* type A toxin (5). In all probability this is due to the fact that type E toxin is somewhat less toxic for mice than is type A or B toxin. Based on the results obtained, ELISA may be useful for testing culture filtrates of enrichment cultures for detecting the presence of *C. botulinum* in samples. Current research is focused on this practical application of ELISA.

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