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Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of *Clostridium botulinum* Type F Toxin

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Clostridium botulinum Type F *Toxin*의 면역학적 효소방 법에 의한 검출에 관한 연구

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Abstract

The enzyme-linked immunosorbent assay using the so-called "double-sandwich" technique was applied to determine *Clostridium botulinum* type F toxin. Polystyrene tubes were coated with horse anti-type F toxin serum and then toxin sample was added. The tubes were subsequently treated with rabbit anti-type F toxin IgG, and sheep anti-rabbit serum IgG-horseradish peroxidase conjugate. By this technique, about 10 mouse intraperitoneal 50% lethal doses (ip LD_{50}) of type F toxin could be detected. Low back-ground reading was achieved with the use of phosphate-buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin as diluents of rabbit IgG and conjugate. Addition of EDTA in the diluent of toxin increased ELISA extinction value significantly. No cross-reaction was observed with botulinum type A and B toxin, but type E toxin gave slight corss-reaction.

INTRODUCTION

Several immunological methods have been developed to detect *C. botulinum* toxins quantitatively. Of them the enzyme-linked immunosorbent assay (ELISA) has been given attention due to its advantages—speed, convenience, sensitivity, independence of radioactivity—and applied to determine botulinum toxin types A, B, and E respectively [1, 2, 3] The results indicate that ELISA is sensitive as other immunological methods and suitable for detection of botulinum toxins. In the present work, the application of ELISA to determine *C. botulinum* type F toxin in culture filtrate is described as well as the results of studies on back-ground reading and cross-reactivity.

Materials and Methods

Toxins

C. botulinum type F, strain Langeland grown in cooked meat medium was inoculated into the toxin production medium which was composed of 1% enzymatic digest of casein (N-Z-amine, type B), 2% proteose pep-

tone, 1% yeast extract, 1% glucose, and 0.05% sodium thioglycolate at pH 7.4. The culture was incubated for 5 days at 30°C, and then solid ammonium sulfate was added to whole cultures to final 60% saturation (390g/). After holding 2 days at 4°C, the clear supernatant fluid was siphoned off and the remaining precipitate was collected by centrifuging at 10,000 x g for 30 min at 4°C. The toxic precipitate was extracted with 0.07 M phosphate buffer, pH 6.0 and dialyzed against the same buffer to remove ammonium sulfate. This concentrated toxin was used as the sample of ELISA.

For cross reactivity test, toxin production media used for type F toxin were innoculated with spores of *C. botulinum* type A, B, and E strains. The cultures were incubated at 37°C for type A, B and at 30°C for type E for 5 days. After centrifugation at 10,000 x g at 4°C for 30 min, the supernatants were used for the ELISA technique. Toxicity was determined intraperitoneally by Reed and Muench method[4] and intravenously by the time-to-death method[5] with mice.

Antiserum

For preparation of rabit anti-type F serum, type F toxin was further purified according to the procedure of Yang and Sugiyama[6]. The flow sheet for purification of type F toxin is shown in Fig. 1. Two different antisera were prepared: antiserum against partially purified toxin which was the toxic fraction of CM-cellulose chromatography and antiserum against purified toxin were obtained by immunizing albino rabbits. Toxic sample was toxoided by adding formalin to final 0.3% and incubating at 37°C for two weeks. A volume of toxoid containing about 1 mg of protein was homogenized with an equal volume of complete Freund's adjuvant and injected sc at several sites. One month later 1 mg of toxoid was injected sc with Freund's incomplete adjuvant. The rabbit was bled out 10 days after the final injection. Serum was collected, and heated at 56°C for 30 min. Antiserum titer was determined according to the Center for Disease Control (CDC) titration method[7] using CDC type F antiserum as a standard. The immunoglobin G (IgG) fraction was isolated by the procedures of ammonium sulfate precipitation[8] and DEAE-cellulose chromatography[9].

Horse anti-type F serum was obtained from CDC and sheep anti-rabbit serum IgG, provided by Dr. H. Sugiyama, Univ. of Wisconsin was used.

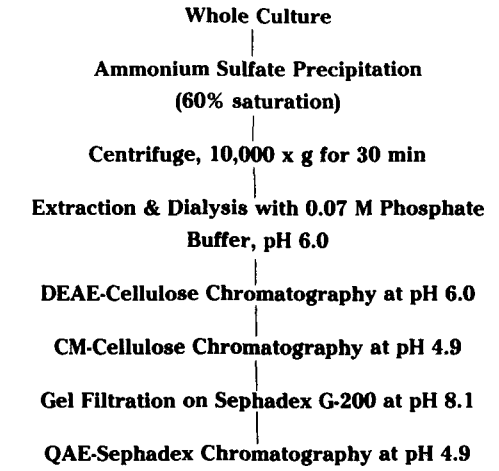

Fig. 1. Flow Sheet for Purification of *C. botulinum* Type F Toxin.

ELISA

The ELISA technique described by Engvall and Perlmann[10, 11] with some modification was employed. Disposable polystyrene tubes (75 by 10 mm, Falcon Plastics, Cockeysville, MD) were coated with 15 μg of horse anti-F serum dissolved in 1 ml of 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The tubes were incubated with shaking overnight at room temperature. After incubation, the tubes were washed three times with distilled water containing 0.05% Tween 20 (Sigma). One milliliter of toxin sample, diluted with 0.07 M phosphate buffer, pH 6.0, was added to tubes and incubated with shaking at 37°C for 90 min. After washing as described above, 1 ml of rabbit anti-F toxin IgG was added (30 μg/ml diluted in 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.05% Tween 20 and 1% bovine serum albumin). The tubes were incubated with shaking at 37°C for 90 min. After washing, 1 ml of sheep anti-rabbit serum IgG and horseradish peroxidase conjugate, prepared according to the method of Nakane and Kawaoi[12], was added. After incubation with shaking at 37°C for 90 min and washing, 1 ml of substrate solution, prepared as described by Ruitenberg *et al.*[13] was added. After incubation at 37°C for 30 min, the reaction was stopped by adding 0.1 ml of 0.1 N NaOH. The reaction product was measured spectrophotometrically at 449 nm. The schematic presentation of ELISA is shown in Fig. 2.

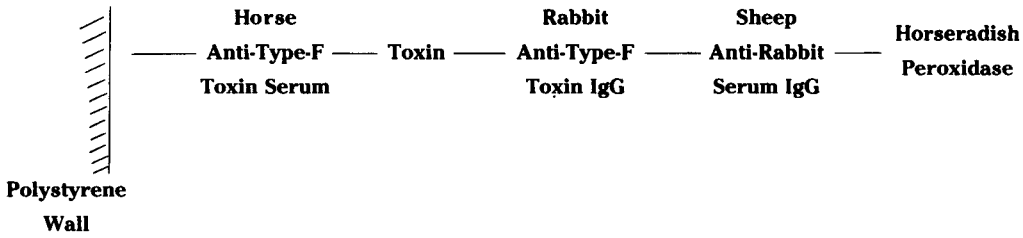

Fig. 2. Schematic Presentation of the Application of the ELISA Technique.

Results and Discussion

Our initial attempts to apply reported ELISA procedures for type A, B and E toxin to type F toxin determination have been failed due to unusually high back-ground reading. This high back-ground reading might be due to the non-specific attachment of rabbit IgG and sheep IgG-enzyme conjugate to the coated wall of polystyrene tube. Since Tween 20 or BSA, or combination of both have been used as protective blocking agents in ELISA[14, 15], attempts were made to use those agents to reduce background reading. As shown in Table 1, addition of Tween 20 (0.05%, v/v) and BSA (1%, w/v) to the diluent of rabbit IgG and sheep IgG-enzyme conjugate significantly reduced the background reading of ELISA.

Table 1. Effects of Tween 20 and Bovine Serum Albumin (BSA) in the Diluent on ELISA Value[a]

Sample	ELISA value (O.D. at 449 nm)	
	With Tween 20 and BSA	Without Tween 20 and BSA
Saline	0.03	0.41
Toxin 1 x 10^2 LD_{50}/ml	0.60	0.86
0.5 x 10^2 LD_{50}/ml	0.42	0.74

a Tween 20 (0.05%, v/v) and BSA (1%, w/v) were added to the diluent of rabbit IgG and sheep IgG-enzyme conjugate.

Fig. 3 shows ELISA values of type F toxin using rabbit anti-type F IgG against partially purified toxin. The lowest detectable concentration was about 10 mouse ip LD_{50}. When 5 mM of EDTA was added to the diluent of toxin, the extinction values were increased significantly compared to that of EDTA-free sample of same toxicity. Kozaki *et al.*[1] suggested that addition of EDTA in toxic sample prevents negative influence on extinction value of ELISA possibly by inhibiting proteolytic enzyme activity in samples.

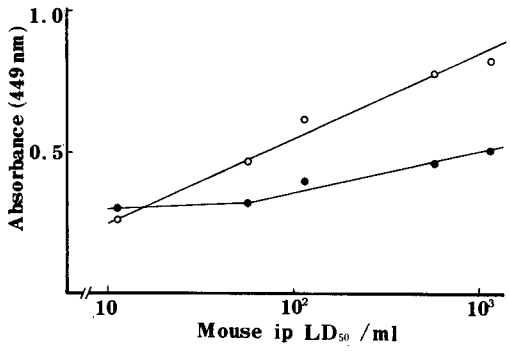

Fig. 3. ELISA Sensitivity for the Detection of C. *botulinum* Type F Toxin using Rabbit Anti-type F Toxin Serum against Partially Purified Toxin.
—○— Toxin sample with 5mM EDTA; —●— Toxin sample without EDTA.

In Fig. 4 ELISA values of type F toxin using rabbit anti-F toxin IgG against purified toxin were shown. When rabbit IgG against purified toxin was used ELISA exinction values were significantly increased over that of antiserum against partially purified toxin (Fig. 3), even though the lowest detectable concentration still lies around about 10 mouse ip LD_{50}. The relationship between optical density and toxicity was linear over a toxicity range of 10 to 10^3 mouse ip LD_{50}/ml.

In Table 2 the mouse ip LD_{50} and ELISA extinction values of different types of botulinum toxin were given. Some cross-reaction was observed with type E toxin. But

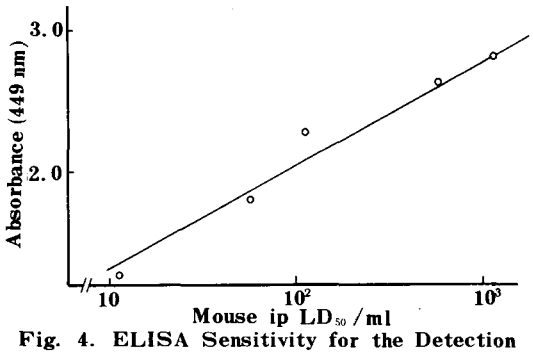

Fig. 4. ELISA Sensitivity for the Detection of *C. botulinum* Type F Toxin using Rabbit Anti-type F Toxin Serum against Purified Toxin.

no cross-reaction occurred with types A and B toxin. The slight cross-reaction between type F and type E toxin is not surprising since a slight but measurable neutralization of type E toxicity by F antitoxin has been reported[6, 16, 17]

Table 2. Mouse ip LD_{50} and ELISA Extinction Values of Culture Supernatants of *Clostridium butulinum* Types.

Types	Toxicity (Mouse ip LD_{50}/ml)	ELISA extinction values[a]
C. botulinum Type F	1.1×10^3	2.80
C. botulinum Type A	2.0×10^4	0.04
C. botulinum Type B	6.3×10^5	0.03
C. botulinum Type E	8.0×10^3	0.85

a O.D. at 449 nm.

The overall significance of our study is that it provided a rapid means for detecting miniscule amounts of *C. botulinum* type F toxin using ELISA technique. Sensitivity of the procedure is greater than the ELISA procedures previously described for *C. botulinum* type A, B and E which are in the range of 50 to 100 mouse LD_{50}[1, 2, 3]. It must be pointed out that serological titrations of botulinum toxin in toxicity units should be interpret cautiously since nontoxic but still antigenic derivatives of toxin react with antitoxic antibodies. Unless fully toxic antigen is used, the LD_{50} points of the standard toxin curve could understate significantly the actual amounts of antigen in the standards. In this regards, the ELISA for detection of botulinum toxin will be useful as a rapid screening test if the positive reaction is then identified with the conventional test with mice.

요 약

Clostridium botulinum type F toxin을 면역학적 효소방법으로 간단히 검출할 수 있는 방법을 연구하였다. 폴리스티렌 튜부 벽에 horse anti-type F toxin serum을 부착시킨 후 toxin sample을 가하였고, 여기에 rabbit anti-type F toxin IgG를 반응시킨 다음 sheep anti-rabbit serum IgG와 horseradish peroxidase를 conjugation시킨 것을 최종적으로 반응시켜 효소의 양을 측정 함으로써 toxin의 양을 간접적으로 정량하였다. 본 방법에 의해 측정할 수 있는 toxin의 최소량은 10 mouse ip LD_{50} 정도이었으며 type A와 B의 toxin과는 cross-reaction이 없었고 type E toxin과는 약간의 cross-reaction이 있었다. Back-ground reading을 줄이기 위해 0.05%의 Tween 20과 1%의 bovine serum albumin을 희석액에 첨가 하였고 EDTA를 toxin sample에 첨가하여 extinction value를 증가시켰다.

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