Determination of *Clostridium botulinum* Toxin by Reversed Passive Latex Agglutination

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(Received 20 February 1984/Accepted 16 April 1984)

ABSTRACT. The agglutination test with latex beads sensitized with antitoxic-component immunoglobulin was evaluated in detection of botulinum type A, B and E toxins. The test detects 4–8 ng/ml of toxin and no cross-reaction was observed among type A, B and E toxins. The minimum detectable amount by reversed passive latex agglutination (RPLA) of crude toxin in culture did not differ from that of purified toxin. The toxin produced in beef was also detectable by RPLA. Beef extract did not interfere with specific agglutination of latex beads. The RPLA test may be useful for routine laboratory tests for botulinum toxin.—*Key words:* agglutination, *Clostridium botulinum*, polystyrene latex, toxin.

Jpn. J. Vet. Sci. 46(4): 487–491, 1984

INTRODUCTION

The mouse injection method is usually used for detection and titration of botulinum toxin. The mouse test, however, is of little value if the sample contains other toxic substance superseding botulinum toxin. Several immunological methods such as hemagglutination, enzyme-linked immunosorbent assay, radioimmunoassay, have been developed [1, 3, 7, 10, 11, 13]. Radioimmunoassay is not practicable in all laboratories and it is not so simple to prepare antibody-enzyme conjugates for enzyme linked immunosorbent assay. Reversed passive hemagglutination (RPHA) is often used; however, it may often be affected by some nonspecific hemagglutinin possessed by various foodstuffs.

Since the first application of latex agglutination for detection of staphylococcal enterotoxin B [14], many attempts have been made to apply it for other bacterial toxins. Detection of botulinum toxin with latex particles coated with specific antitoxin, however, has not been reported.

This report deals with reversed passive

latex agglutination (RPLA) applied for detecting botulinum type A, B and E toxins, both purified and crude ones in culture and in meat.

MATERIALS AND METHODS

Type A, B and E derivative toxins: Type A, B, and E progenitor toxins were purified by the methods reported previously [5, 8, 15]. The toxic component or derivative toxin was purified by DEAE-Sephadex chromatography of the progenitor toxin (M or 12S toxin) at pH 7.5 [8, 15] or 8.0 [6].

Anti-toxic component immunoglobulin (AT): Rabbit antiserum against derivative toxin was prepared by immunization with toxoid with an equal volume of Freund's complete adjuvant and then with toxin. One milliliter of the mixture containing 0.1 mg of toxoid was injected subcutaneously into a rabbit. Four weeks later, the same quantity of derivative toxin without adjuvant was injected also subcutaneously. The rabbits were bled a week after the final injection. Rabbit AT was purified by affinity chromatography [13]. The anti-toxic-component titers of type

Fig. 1. Grading of latex agglutination

A, B and E ATs (1 mg/ml) determined by passive hemagglutination were 25,600, 12,800 and 25,600, respectively.

Freeze-dried sheep ATs, supplied by Dr. Serve Notermans, National Institute of Public Health, the Netherlands, were reconstituted in 0.2 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and purified by affinity chromatography. Type A, B and E ATs (1 mg/ml) had 12,800, 25,600 and 25,600 PHA titers, respectively.

AT-sensitized latex particles: Polystyrene latex particles (48GE, 0.75 μ m, Takeda Chemical Industry, Osaka) were suspended to 0.25% in 0.075 M phosphate buffer, pH 7.2, containing 0.075 M NaCl (PBS), centrifuged and washed twice with PBS, and resuspended in PBS at 0.25%. The latex suspension was mixed with an equal volume of rabbit or sheep AT at various concentrations. The mixture was incubated at 37°C for 1 hr with occasional shaking. After washing twice with PBS to remove uncoupled AT, the latex was resuspended to 0.125% in PBS containing bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO.) at 0.5% and polyvinylpyrrolidone (for cosmetics, Wako Pure Chemical Industries, Osaka) at 0.005%. The suspensions were stored at 4°C.

RPLA: A toxin sample was diluted two-fold serially in a U-type microtiter plate (Tomy Seiko, Tokyo). To each well containing 0.025 ml of a dilution, 0.025 ml of the sensitized latex was added. After mixing, the plates were incubated at room temperature for 14 to 18 hr. The agglutination was scored as shown in Fig. 1.

Toxic meat: Beef was macerated with a stomacher (Seward, London) in two volumes of 1.0% peptone and 0.08% disodium phosphate (and 0.5% glucose for type E strain). The macerate was dispensed into test tubes each with 10 ml. Graded numbers of spores were inoculated into the beef macerate. The tubes were heated at 60°C for 15 min and incubated anaerobically for 7 days at 30°C. The supernatant was titrated by the time-to-death method and RPLA.

RPHA: Formalinized sheep erythrocytes coupled with each of type A, B and E ATs were prepared by the procedures reported [4, 13]. RPHA was performed in wells in microtiter plates with 0.05-ml portions of twofold serial dilutions of toxin in PBS containing BSA at 0.25% and 0.025-ml portions of a 0.5% suspension of AT-coupled FSRBC. Agglutination was read in 4 hr of incubation at room temperature.

Protein contents: Protein contents of toxin and antitoxin preparations were determined by the method of Lowry *et al.* [9] and expressed in BSA equivalent.

Toxin titration: The time-to-death method [2, 12] was used. A standard curve was prepared with each of type A and B progenitor toxins and type E activated progenitor toxin. The toxin potency was expressed in ip LD₅₀/ml.

RESULTS

To sensitize polystyrene latex particles, 15 μ g/ml of rabbit and 30 μ g/ml of sheep ATs were found to give the best results. By RPLA with rabbit AT-and sheep AT-sensitized

Table 1. Sensitivities and specificities of latex coupled with rabbit IgG

Toxin type	Agglutination titer (ng toxin/ml) with latex coupled with		
	AT-A	AT-B	AT-E
A	×256 (3.9) ^{a)}	—	—
B	—	×128 (7.8)	—
E	—	—	×128 (7.8)

a) The lowest detectable concentration of toxin.

Table 2. Sensitivities and specificities of latex coupled with sheep IgG

Toxin type	Agglutination titer (ng toxin/ml) with latex coupled with		
	AT-A	AT-B	AT-E
A	×128 (7.8) ^{a)}	—	—
B	—	×128 (7.8)	—
E	—	—	×128 (7.8)

a) The lowest detectable concentration of toxin.

Table 3. Sensitivities of RPLA with culture supernatants

Toxin type	LD ₅₀ /ml	Aggl. titer with latex coupled with	
		Rabbit IgG	Sheep IgG
A	3.4 × 10 ⁶	2,560	1,280
B	1.1 × 10 ⁶	1,280	1,280
E	6.5 × 10 ⁴	1,280	1,280

Table 4. RPLA titers of beef inoculated with type A, B and E spores

Spores inoculated/g	Type A		Type B		Type E	
	Titer ^{a)}	LD ₅₀ /ml ^{b)}	Titer	LD ₅₀ /ml	Titer	LD ₅₀ /ml
1,000	64	41,000	32	<6,300	64	62,000
100	64	94,000	8	<6,300	512	100,000
10	64	73,000	32	<6,300	256	29,000
1	16	4,300	16	<6,300	64	29,000

a) The reciprocal of the highest dilution of the beef extract giving positive agglutination.

b) Determined by the time-to-death method.

ed latex, the lowest detectable concentrations of A-M, B-M and E toxins were almost the same, being 4–8 ng/ml (Tables 1 and 2). There was no cross-reaction among type A, B and E toxins by use of either AT for coupling latex.

The results with latex beads coupled with rabbit AT and those with sheep AT (types A, B and E) and with type A, B and E crude toxins in culture supernatant are shown in Table 3. With rabbit AT-coupled latex, the lowest detectable levels of type A, B, and E

toxins in culture were 1,300 LD₅₀/ml or 13 ng/ml, 900 LD₅₀/ml or 9 ng/ml, 50 LD₅₀/ml or 6 ng/ml, respectively. Nearly the same quantities of the toxins were detected with the latex coupled with sheep AT. With rabbit AT-coupled and sheep AT-coupled latex, no cross-reaction was found among type A, B and E culture supernatants.

The results of RPLA with latex coupled with rabbit ATs and the extracts of beef inoculated with type A, B or E spores are summarized in Table 4. RPLA with type A

AT-coupled latex showed titers of 16–64 with beef extract containing 4,300–94,000 ip LD₅₀/ml. Type B AT-sensitized latex agglutinated with the beef extract containing type B toxin less than 6,300 ip LD₅₀/ml. Type E AT-sensitized latex gave titers of 64–512 with the beef extract containing 29,000–100,000 ip LD₅₀/ml. None of the sensitized latex agglutinated with extract of uninoculated beef.

The same purified toxin solution was titrated by RPLA and RPHA to compare their sensitivities. RPLA and RPHA gave the same titers with type A and type E toxins.

DISCUSSION

We found it simpler to couple antibody to latex particles than to sheep erythrocytes, since the former required neither formalinization nor any coupling reagent. Benzidine for preparing bisdiazotized benzidine is no longer commercially available for its carcinogenicity and the properties of tannic acid as a coupling reagent differ from one preparation to another.

It took 14–18 hr to read RPLA as compared with 2–4 hr to read RPHA, but the period for latex beads to settle can be shortened by centrifugation of the microtiter plate in a plate holder, which did not affect the RPLA titer.

RPLA has an advantage over the mouse test and RIA, since trypsinization of activable toxins, killing experimental animals, or use of isotopes is not necessary.

The sensitivity of RPLA was found to be the same as that of RPHA in detecting botulinum toxin. The lowest detectable quantity of type A toxin (4–8 ng/ml) by RPLA was equivalent to that by RIA [1]. RPLA tests can detect about 13 ng/ml of type A, 9 ng/ml of type B, and 6 ng/ml of type E toxins in culture supernatant.

No cross-reaction was observed among type A, B and E toxins in RPLA, as anti-derivative toxin instead of anti-progenitor toxin was used for sensitization of latex beads

[13].

The minimum detectable quantity of purified toxin and that of crude toxin in culture were nearly the same, indicating that the constituents of the culture medium, direct hemagglutinin possessed by type A (LL and L toxins) and B (L toxin) toxins, or any other metabolite would not interfere with agglutination of latex particles nor cause false positive reaction. It is known that SRBC sometimes agglutinate with serum or meat extract. Type A, B and E toxins produced in beef were detectable by RPLA, although its titer did not necessarily parallel to the mouse lethal toxicity. Beef extract did not cause nonspecific agglutination of latex beads.

From the present results, it may be concluded that the RPLA test is simple to perform and specific and sensitive enough for detection, typing, and quantitation of botulinum toxin in culture and in food.

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要 約

逆受身ラテックス凝集反応によるボツリヌス菌毒素の検出: 堀口安彦・小崎俊司・阪口玄二 (大阪府立大学農学部獣医公衆衛生学教室)——ラテックス凝集反応 (RPLA) による A, B, E 型ボツリヌス菌毒素の検出を試みたところ, 4–8 ng/ml の精製毒素を検出でき, A, B, E 型毒素間に交差反応はみとめられなかった。菌培養上清中の毒素の RPLA による検出感度は精製毒素の検出感度と同程度であった。牛肉中で産生された毒素も RPLA によって検出が可能で, 牛肉抽出液による特異性および感度への影響はなかった。以上の成績から, RPLA はボツリヌス菌毒素の検出に使用できると考えられた。