JOURNAL OF CLINICAL MICROBIOLOGY, May 1984, p. 645–648
0095-1137/84/050645-04$02.00/0

Vol. 19, No. 5

Detection of *Clostridium botulinum* Type A Toxin by Enzyme-Linked Immunosorbent Assay with Antibodies Produced in Immunologically Tolerant Animals

MANOUCHEHR DEZFULIAN* AND JOHN G. BARTLETT

Infectious Disease Division, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 18 November 1983/Accepted 15 February 1984

Immunological tolerance is a state of unresponsiveness to foreign substances (antigens) which can develop in human and animal species as the result of continued exposure to antigens early in life. We utilized this principle for the preparation of antibodies against *Clostridium botulinum* type A toxin. By selective suppression of the immunological response of rabbits to unwanted antigens and subsequent immunization with a toxoid, we were able to produce a specific type A antitoxin without the need to purify the toxin. Despite cross-reactivity with *C. botulinum* type B, our type A antitoxin was otherwise specific since it did not react with culture filtrates of nontoxigenic variants of type B, any other *C. botulinum* type (C, D, E, F, and G), nor with 18 other *Clostridium* species, including *Clostridium sporogenes*. Using this antitoxin, we developed a sensitive enzyme-linked immunosorbent assay for detection of *C. botulinum* type A toxin.

Mouse bioassay is commonly used for the detection and typing of *Clostridium botulinum* neurotoxins. This method, however, is cumbersome and requires facilities which are not usually available to routine diagnostic microbiology laboratories. In addition, the mouse toxicity test is not suitable for examination of specimens containing other lethal substances which may cause "nonspecific" death or interfere with neutralization of botulinal toxins (9; M. Dezfulian, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C317, p. 324).

Several serological procedures including reversed passive hemagglutination (20), radioimmunoassay (1), and enzyme-linked immunosorbent assay (ELISA) (10, 12, 14–17) have been used for the detection of type-specific botulinal toxins. In all of these procedures, use of purified toxin for preparation of specific antiserum has been required.

Through induction of immunological tolerance (5) to unwanted antigens and subsequent immunization with a relatively impure toxoid, we were able to produce a type A botulinal antitoxin. A similar approach has made it possible to demonstrate tumor-specific antigens (7) and to produce antibodies specific for certain leukemic cells (6). In our study, nontoxigenic *Clostridium sporogenes* was selected as the source of antigen for induction of tolerance in newborn rabbits, and *C. botulinum* type A toxoid was selected for subsequent immunization of the animals in adult life. The only demonstrable difference between *C. sporogenes* and *C. botulinum* type A is the production of the toxin (11). Using our botulinal antitoxin, we developed a sensitive ELISA for the detection of *C. botulinum* type A toxin.

MATERIALS AND METHODS

Organisms. The organisms included 16 strains of *C. botulinum* toxigenic types A, B, C, D, E, F, and G (Table 1) and 42 strains representing 18 *Clostridium* species. These included one strain each of *C. bifermentans* (A1058), *C. innocuum* (13598), *C. paraputrificum* (14861), *C. perfringens* (A1047), *C. ramosum* (A1088), *C. septicum* (A1252), and *C. tertium* (A585); two strains each of *C. haemolyticum* (6020 and 6021), *C. histolyticum* (A1096 and A1097), *C. sordellii* (19739 and 19749), *C. sphenoides* (A1000 and A1229), and *C. novyi* A (Sm40 and 1872); three strains each of *C. butyricum* (13946, 14087, and 18851), *C. cadaveris* (A946, 19510, and 19810), *C. difficile* (A994, CPT100, and CdE2), *C. subterminale* (A753, 18041, and 18183), and *C. novyi* B (A741, 6387, and 6388); and 10 strains of *S. sporogenes* (A223, A274, A380, A742, A775, A776, A1043, A17616, A17680, and A1068). In addition, two nontoxigenic variants of proteolytic *C. botulinum* type B were employed. Since the two original toxigenic strains had been derived from single colony isolates (2), contamination with *C. sporogenes* seemed to be unlikely. All strains were from the stock collection of the Centers for Disease Control Anaerobe and Botulism Laboratories except for two strains of *C. difficile* (CPT100 and CdE_2) from our stock collection. Methods of confirming taxonomic classification have been described previously (2–4).

Preparation of culture filtrates. The filtrates of the bacterial cultures were used for the detection of toxin by mouse bioassay and the ELISA. Test strains were grown at 37°C except for two strains of *C. botulinum* type D which were grown at 30°C. A 4-day-old culture of each strain in chopped meat-glucose broth (Scott Laboratories, Inc. Fiskeville, R.I.) was centrifuged, and the supernatant was filtered through a 0.45 μm membrane filter (Millipore Corp., Bedford, Mass.). The culture filtrate of *C. sporogenes* (strains A1043 and 17616) which was used for induction of tolerance was concentrated up to 10-fold by a lyophilization process. The culture filtrates were distributed into small vials and were kept frozen at −70°C until used.

Toxoid. Alum-precipitated toxoid of *C. botulinum* type A was kindly provided by Charles Hatheway of the Centers for Disease Control, Atlanta, Ga. The toxoid was derived from an acid precipitate of botulinal toxin type A (Hall strain).

Antitoxins. Type A and polyvalent (A, B, C, D, E, and F) botulinal antitoxins were received from the Centers for

* Corresponding author.

Disease Control. The antigens to produce these antitoxins in burros are the ammonium-sulfate precipitate of spun culture fluids of *C. botulinum* types.

Induction of tolerance. Two newborn rabbits (New Zealand; Bunnyville Farm, Littlestown, Pa.) were made tolerant to the antigens present in culture filtrate of *C. sporogenes*. Beginning at birth and for a period of 14 days, infant rabbits were injected daily with a pooled culture filtrate from two strains of *C. sporogenes*. Samples of 0.2 to 0.4 ml of the concentrated filtrate were injected intraperitoneally and subcutaneously.

Immunization. Seven weeks after the last injection of *C. sporogenes* culture filtrate, the mature 9-week-old rabbits received four intramuscular injections of an alum-precipitated toxoid of type A. Before injection, the toxoid was emulsified in an equal volume of Freund adjuvant by using complete adjuvant in the first injection and incomplete adjuvant in the three subsequent injections. The sequential immunizing dose was 2, 2, 3, and then 4 ml of alum-precipitated toxoid combined with Freund adjuvant.

Serum collection. Two weeks after the last injection, each individual rabbit was bled, and the collected serum was distributed in small quantities and kept frozen at −70°C. Although in our initial experiments the two rabbit sera (antitoxins) tested appeared very similar with respect to specificity and sensitivity of the assay, detailed studies were subsequently conducted with only one antitoxin.

ELISA. The procedure described by Voller et al. (22) was adapted with some modifications. Duplicate wells of polyvinyl U-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of a 1/5,000 dilution of burro type A or polyvalent botulinal antitoxins; duplicate blank wells were coated with normal horse serum. Specificity of the test system was not affected by the type of antitoxin used, but for the sake of convenience, polyvalent antitoxin was mainly employed for coating. After overnight incubation of the coated plates, the wells were washed five times with phosphate-buffered saline–0.05% Tween 20 (PBS-T). A 100-μl sample of culture filtrates diluted in PBS-T containing 0.5% gelatin (PBS-TG) was then added to the wells (except where otherwise stated, 1:4 and 1.40 dilutions of the culture filtrates were used). Plates were incubated for 90 min at 37°C. Each well was washed five times before the addition of 100 μl of a 1:500 dilution of rabbit type A botulinal antitoxin in PBS-TG. The optimal dilutions of rabbit antitoxin and that used for coating were determined by block titration of a given antigen (culture filtrate). After a 90-min incubation at 37°C, the plates were washed as described above, and 100 μl of a 1:1,000 dilution (in PBS-TG) of alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) was added. This was incubated for 90 min at 37°C, and excess conjugate was removed by washing. A 100-μl sample of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate; Sigma Chemical Co., St. Louis, Mo.) in diethanolamine buffer (pH 9.8) was then added. Plates were incubated at room temperature for 1 h, and the absorbance was measured at 405 nm in a microplate colorimeter reader (model MR 480; Dynatech Laboratories). Net absorbance was calculated by subtracting the mean absorbance of wells coated with botulinal antitoxin from the mean absorbance of the blank wells.

Mouse bioassay. Mouse bioassay was carried out according to the procedures described previously (2, 8). Serial 10-fold dilutions of *C. botulinum* culture filtrate (in PBS–0.2% gelatin, pH 6.2) were injected intraperitoneally into each of three 20- to 25-g Swiss mice. The inoculated animals were observed daily for 4 days, and the 50% lethal dose (LD_{50}) was calculated according to the method of Reed and Muench (18). In the case of *C. botulinum* types C, D, F, and G, the culture filtrates were activated with trypsin (Flow Laboratories, McLean, Va.) before bioassay by using trypsin in a final concentration of 0.2 mg/ml. The mixture was kept at 30°C in a water bath for 30 min and then tested in mice. The toxin type of each *C. botulinum* strain was confirmed by toxin neutralization test as described previously (2, 8).

RESULTS

In the initial experiments, the minimum amount of toxin detected by mouse assay and the corresponding ELISA absorbance values were determined. Each ELISA analysis was performed in duplicate with three wells per sample. Comparison of ELISA with mouse bioassay for a culture filtrate of *C. botulinum* type A (strain 1696) is shown in Fig. 1. The lowest level of antigen detected by ELISA (0.1 ml of test sample per well) corresponds to less than 10 mouse intraperitoneal LD_{50}s. Considerable cross-reactivity was observed with culture filtrates of *C. botulinum* type B strains (Table 1). No cross-reactivity was demonstrable with the test samples of two nontoxigenic variants derived from strains of proteolytic type B (Table 1). There was also no evidence of cross-reactivity with toxigenic types C, D, E, F, and G (Table 1). Likewise, none of 10 *C. sporogenes* strains or 32 strains of other clostridial species showed reactivity in the ELISA system.

DISCUSSION

Immunological tolerance is a state of unresponsiveness to foreign substances (antigens) which can develop in humans and animals as the result of early (i.e., neonatal period) and continued exposure to antigens (5). We utilized this principle for the production of a *C. botulinum* type A antitoxin. By selective suppression of the immunological response of the animal to unwanted antigens, it was possible to produce a specific antitoxin without the need to purify the toxin. Despite cross-reactivity with *C. botulinum* type B, our type A antitoxin was otherwise specific since it did not react with culture filtrates of any other *C. botulinum* type nor with 18 other *Clostridium* species including *C. sporogenes*. The lack of reactivity with nontoxigenic variants of *C. botulinum* type B is of special interest since it indicates a link between

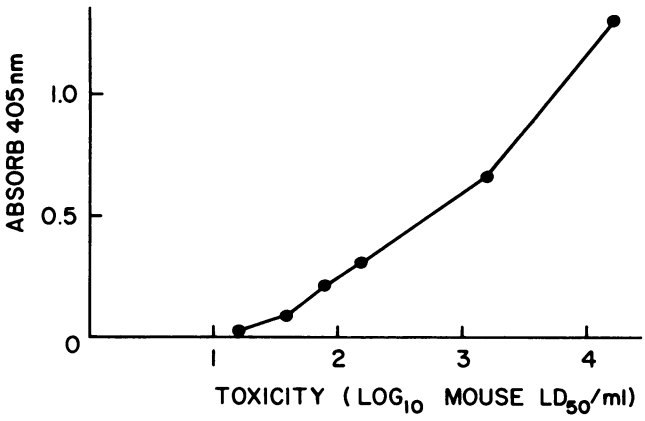

FIG. 1. Relationship between ELISA values (absorbance at 405 nm) and mouse LD_{50}.

TABLE 1. Mouse LD_{50} and ELISA values of culture filtrates of various *C. botulinum* toxigenic types

Bacterial strain	Log_{10} LD_{50}/ml	ELISA value[a] ± SD
C. botulinum type A		
207	3.9	1.52 ± .06
1839	3.9	1.52 ± .08
2294	2.9	0.58 ± 0.1
C. botulinum type B		
Proteolytic		
82	2.9	0.30 ± .09
208	2.4	0.15 ± .09
Nonproteolytic		
706	2.5	0.09 ± .01
C. botulinum type C		
64596	ND[b]	0
C. botulinum type D		
5	3.4	0
35	3.8	0
C. botulinum type E		
45	2.9	0
46	ND	0
C. botulinum type F		
Proteolytic		
RH 71-2821	ND	0
Nonproteolytic		
84-4595	1.9	0
57-KA171	0.9	0
C. botulinum type G		
2742	1.9	0
Nontoxigenic variants of *C. botulinum* type B		
B27	0	0
B30	0	0

[a] Mean net absorbance (405 nm).
[b] ND, Not done.

production of type B toxin and cross-reactivity in the ELISA.

The toxigenic differences between *C. botulinum* types A and B have recently been elucidated. Although type A produces at least three different progenitor toxins (12S, 16S, and 19S), only two (12S and 16S) are associated with type B organisms (17, 19, 20). The 16S compound, known as large toxin is dissociated into a toxic (7S neurotoxin) and a nontoxic (hemagglutinin) component (13, 17). The derivative toxin is specific for each type, but the nontoxic components of the two types are immunologically related (17, 20, 21). It appears that the antibodies directed against type A nontoxic component might indeed be responsible for the cross-reactivity of our antitoxin. As would be expected, the antitoxin neutralized type A botulinal toxin but it failed to neutralize the toxicity of the B toxin in mice (data not shown).

Cross-reactivity between types A and B progenitor toxins has previously been demonstrated by reverse passive hemagglutination test (20). Anti-derivative toxin specific for type A or type B can be produced by immunizing animals with purified derivative toxin (19, 20). Alternatively, the type-specific antitoxin can be prepared from anti-progenitor toxin, after removal of antibodies to nontoxic components, by affinity chromatography (20). Despite the use of purified derivative toxin for the production of antitoxin, some degree of cross-reactivity has often been encountered in the detection of botulinal toxin by ELISA (10, 12, 14–17).

The absorbance values measured by the ELISA correspond to less than 10 mouse intraperitoneal LD_{50}s (Fig. 1). The sensitivity of our procedure for detection of botulinal antigen is greater than the previously described ELISA for *C. botulinum* type A toxin (16). Ironically, the cross-reacting antibodies directed against antigens specific to nontoxic components of type A and type B progenitor toxins may contribute to the higher sensitivity of our system. Our ELISA procedure does not distinguish *C. botulinum* type A from type B in spite of the quantitative differences shown in Table 1. It does, however, differentiate the two toxigenic types from nontoxigenic *C. sporogenes* and other toxigenic types of *C. botulinum*. With further refinement to improve specificity, the test described may prove to be useful for detecting type A botulinal toxin in food and fecal samples and for confirming the diagnosis of type A foodborne and infant botulism.

ACKNOWLEDGMENT

This work was supported by an institutional research grant from the John J. Hopkins Fund.

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