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Atypical Toxin Variant of *Clostridium botulinum* Type B Associated with Infant Botulism

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An atypical toxin variant of *Clostridium botulinum* (strain 657) was isolated from the feces of a 6-week-old female infant whose symptoms and clinical history were consistent with infant botulism. Toxin detected in the feces and the toxin produced by isolates from the feces and from two rectal swabs could be neutralized by type B botulinal antitoxin only at very high ratios of of antitoxin to toxin in the neutralization mixture. One international unit of type B antitoxin neutralized only about 10 lethal doses of 657 toxin as compared with approximately 10,000 lethal doses of conventional type B toxin from the Beans strain. Antitoxin prepared against 657 toxin was 10 times more effective against the conventional toxin than against the homologous toxin. Toxoid-antitoxin-binding studies indicate that both 657 toxin and type B toxin are heterogeneous and that both toxins may contain the same molecular variants, but that the proportions of the variants are different in each.

In November 1976, specimens from a 6-week-old female suspected of having infant botulism were received for testing in the Centers for Disease Control (CDC) Anaerobe Foodborne Disease Laboratory. The extract of a fecal specimen was toxic in mice, causing signs typical of botulism and death of the mice within 12 to 48 h after injection. However, the toxicity of the extract was neutralized only when the extract was diluted to contain a small amount of toxin and mixed with a relatively large amount of type B antitoxin. An organism which produced toxin with the same characteristics as those found in the extract was isolated from the fecal specimen and from two rectal swabs subsequently taken from the patient. The cultural and biochemical characteristics of the isolates were identical with those of other proteolytic strains (types A, B, and F) of *Clostridium botulinum*. The case was confirmed as infant botulism, and the causative organism was identified as a serologically atypical toxin variant of *C. botulinum* type B. The clinical details have been reported by Edmond et al. (4). Laboratory examination of the clinical specimens, characterization of the *C. botulinum* isolated from the feces of the infant, and studies of the toxin produced by the microorganism are presented in this article.

MATERIALS AND METHODS

Specimens. The specimens were obtained from a 6-week-old female (4). A serum sample, a stool specimen (specimen no. 657), and a rectal swab were obtained 7 days after the onset of the illness (6 days after admission to the hospital) and sent by air the next day to the Anaerobe Foodborne Disease Laboratory. Rectal swabs taken 11 and 25 days after the onset of the illness were also examined at CDC. The serum and the stool specimen were examined for botulinal toxin by the mouse toxicity and neutralization tests. The stool specimen and the three rectal swabs were cultured for *C. botulinum*. The methods for toxin detection and identification and for culturing are described in detail elsewhere (7).

Characterization of isolates. Pure isolates of toxigenic organisms from the stool specimen and the rectal swabs were characterized according to methods described by Dowell and Hawkins (3). Cellular morphology and spores were observed by microscopic examination of Gram-stained smears. The isolates were tested for their ability to ferment glucose, mannitol, lactose, sucrose, maltose, salicin, glycerol, xylose, arabinose, starch, mannose, rhamnose, and trehalose. They were tested for motility, oxygen tolerance, lecithinase and lipase activities, ability to digest cooked-meat particles and milk proteins, liquefaction of gelatin, production of hydrogen sulfide, urease activity, indole production, nitrate reduction, and hydrolyses of esculin and starch. Production of volatile and nonvolatile fatty acid metabolic products in peptone-yeast extract-glucose broth was determined by gas-liquid chromatography as described by Dezfulian and Dowell (2).

Preparation of toxin. Toxin was prepared from one of the *C. botulinum* isolates from the stool specimen of the patient for use in cross-neutralization tests and for the preparation of an antitoxin. This isolate was designated as strain 657, which refers to the laboratory specimen number assigned to the stool speci-

men at CDC. The organism was inoculated into two flasks, each containing 1,000 ml of Trypticase-yeast extract-glucose (TYG) medium consisting of 1.5% Trypticase (BBL Microbiology Systems), 0.75% yeast extract (BBL), and 0.5% glucose (added after autoclaving). The pH of the medium was adjusted to 7.2 with NaOH before autoclaving. After 4 days of incubation at 35°C in an anaerobic glove box, the toxin from each culture was harvested. Two alternate methods of toxin recovery were used since the precipitability and stability properties of this toxin were not known. For method 1, toxin in the first flask culture was recovered by precipitation from the whole culture by adjusting the pH to 3.9. The precipitate was allowed to settle out for 36 h, the supernatant fluid was siphoned off, and the sediment was packed by centrifugation. The sediment was suspended in 25 ml of distilled water. For method 2, toxin was recovered from the second flask culture by adding 320 g of solid $(NH_4)_2SO_4$, keeping the culture at room temperature for 36 h, and then recovering the precipitate by centrifugation. The precipitate was suspended in 25 ml of distilled water.

Preparation of antitoxins. The toxin recovered by $(NH_4)_2SO_4$ precipitation was more easily solubilized and clarified in a small volume than the acid-precipitated toxin, so it was chosen as the immunizing antigen. It was converted to toxoid by adding 0.6% Formalin after dialyzing the toxin against 0.2 M sodium succinate buffer, pH 6.0. The toxin-Formalin mixture was incubated at 35°C for 7 days, after which it was not toxic for mice. It was then stored in a refrigerator. Three rabbits were immunized with this toxoid combined with adjuvant. For two of the rabbits, the adjuvant was Holt 7/8 aluminum phosphate (9), 14 mg/ml, added to an equal volume of fluid toxoid. For the primary immunization, each rabbit received two subcutaneous injections of 1 ml each in the shoulder region. This procedure was repeated 30 days later as a booster. The third rabbit was immunized similarly with a 1:1 emulsion of toxoid in Freund complete adjuvant for the primary immunization and in Freund incomplete adjuvant for the booster. The rabbits were bled 2 weeks after the boosters, and the serum was recovered and preserved with Merthiolate at a 1:10,000 dilution and stored at 3 to 5°C.

Toxin neutralization tests. Toxin neutralization tests of fecal extract and the cultures were performed with diagnostic types A, B, E, and polyvalent (ABCDEF) botulinal antitoxins obtained from the Biologic Products Division, Bureau of Laboratories, CDC, as described elsewhere (7). Neutralization tests were performed on the acid-precipitated toxin from strain 657 toxin described above and on an acid-precipitated toxin prepared in the same manner from the Beans strain of *C. botulinum* type B. The antitoxins used in these experimental studies were the World Health Organization (WHO) type B international standard antitoxin and the rabbit antisera prepared against the 657 toxoid. The 657 toxin contained 2×10^5 50% lethal doses (LD_{50}) per 0.5 ml, and the type B (Beans strain) toxin contained 5×10^6 LD_{50}/0.5 ml. Tenfold dilutions, 10^{-1} through 10^{-8}, were made with each of the toxins, and 1.25-ml samples of each dilution were mixed with 0.25 ml of each antitoxin. After incubation for 1 h at room temperature, the mixtures were tested for toxicity by injecting 0.6 ml into each of two mice. Each of the toxins was also simultaneously tested in mice without antitoxin by injecting two mice with each dilution (0.5 ml intraperitoneally per mouse). A comparison of the titer of each toxin mixed with antitoxin and the titer of the untreated toxin showed how many lethal doses of toxin were neutralized by 0.1 ml of the antitoxin. The WHO antitoxin contained 1.0 IU/0.1 ml.

Similar neutralization tests were performed on 657 toxin by adding approximately 1.0 IU of type A, C, D, E, and F monovalent antitoxins and 0.1 ml of polyvalent ABCDEF antitoxin per 0.5 ml of each toxin dilution (CDC diagnostic antitoxins).

Antitoxin-binding tests. Antitoxin-binding tests were performed with 657 toxoid and type B toxoid against the rabbit antitoxin prepared with 657 toxoid and against the WHO type B antitoxin standard. The binding of antibodies was demonstrated by the failure of the antitoxin to neutralize 30 LD_{50} of either 657 toxin or type B toxin. The 657 toxoid was that described above, which was used for immunizing rabbits, and the type B toxoid, which contained 6.48 flocculation units per ml, was prepared according to the methods described by Wright et al. (9) by the Michigan Department of Public Health. Both toxoids contained 7 mg of $AlPO_4$ adjuvant per ml. Doubling dilutions of toxoid made in gelatin phosphate diluent (3) were tested against the antitoxins to determine the binding endpoint, at which point no residual antibodies remained for binding either 657 toxin, or type B toxin.

The test was performed by adding 0.5 ml of diluted toxoid to 0.5 ml of antitoxin, incubating the mixture for 1 h at room temperature, adding 1 ml of toxin (30 LD_{50}), incubating the mixture for another hour at room temperature, and then testing each mixture by injecting 0.5 ml into each of two mice to determine whether neutralization of the toxin had taken place. The binding endpoint was the highest dilution of toxoid which bound the antitoxin. When antitoxin is bound, the mixture causes mouse death because no neutralization of toxin takes place. The binding dose of toxoid is the volume of undiluted toxoid in the mixture at the binding endpoint.

RESULTS

Examination of specimens. No toxin was detected in the serum of the infant. The extract of the stool specimen was toxic and caused symptoms in mice suggestive of botulism, but the toxicity was not neutralized with type A, B, or E monovalent or polyvalent ABCDEF botulinal antitoxin. When diluted 1:5, the extract was still toxic, and the toxicity was neutralized with the type B and polyvalent reagents. When diluted 1:10, 0.5 ml of the untreated extract killed only some of the mice, and a 1:15 dilution of the extract was nonlethal for mice.

Enrichment cultures of the stool specimen and of the first two rectal swabs were toxic in mice.

However, like the fecal extract, the toxicity of the culture fluids was neutralized with type B antitoxin only when diluted to contain a few lethal doses in the volume treated with 1 IU of antitoxin. Enrichment cultures of the rectal swab obtained from the infant 25 days after the onset of the illness were not toxic for mice. A toxigenic organism resembling *C. botulinum* in all physiological and morphological respects was isolated from enrichment cultures of the stool specimen and of the first two rectal swabs, but not from cultures of the third rectal swab.

The toxin titers of the fecal extract and the culture flasks were relatively low. Testing 0.5 ml of serial 10-fold dilutions in mice revealed toxin titers of 1:10 for the fecal extract and the enrichment cultures and a titer of 1:100 for the pure culture isolates grown in cooked-meat–glucose–starch medium (3). Neutralization of the toxin, regardless of whether it was in the fecal extract, the enrichment cultures, or the pure cultures, required dilution of the sample to a point at which there was only about 10 LD_{50} in the injected dose which was mixed with 1 IU of type B antitoxin. One international unit of type B antitoxin is considered capable of neutralizing approximately 10,000 mouse LD_{50} of type B toxin (6).

Characteristics of the organisms. The toxigenic organisms isolated from the stool specimen and the first two rectal swabs were identical to each other with respect to toxin titer; the amount of antitoxin required for toxin neutralization; morphological, cultural, and physiological characteristics; and antibiotic susceptibility. The characteristics agreed with the findings of Dezfulian and Dowell (2) for proteolytic type B *C. botulinum* in their study of 78 type A and B (proteolytic) strains of *C. botulinum* isolated from cases of food-borne and infant botulism. The isolates from the three specimens in this case fermented glucose and maltose, but did not ferment the 11 other carbohydrates tested. All of the isolates digested milk and meat particles in chopped-meat medium, liquefied gelatin, hydrolyzed esculin, and were positive for lipase, but negative for lecithinase. Acetic, isobutyric, butyric, isovaleric, and hydrocinnamic acids were produced in peptone-yeast extract-glucose medium (3). The isolates were susceptible to penicillin, cefoxitin, tetracycline, rifampin, clindamycin, erythromycin, and metronidazole and resistant to cycloserine, sulfamethoxazole, and trimethoprim.

Concentrated 657 toxin. Each of the 1,000-ml TYG cultures had a toxicity titer of 1:1,000 when 10-fold dilutions were tested in mice, and each therefore contained 10^6 to 10^7 LD_{50} of toxin. The amount of toxin recovered by either precipitation method was similar. Each precipitate, after suspension in 25 ml of distilled water, contained about 10^7 LD_{50} (4×10^5 LD_{50}/ml). The recovery of toxin was essentially complete in each case. The toxicity was neither increased nor decreased by treatment with trypsin.

Antitoxin-toxin neutralization tests. The results of cross-neutralization tests with the anti-B and the anti-657 toxin reagents are shown in Table 1. If one considers that the highest dilution of untreated toxin which kills mice contains 1 lethal dose, then it appears that 1 IU of type B antitoxin neutralized 10,000 lethal doses of type B toxin, but only 10 lethal doses of 657 toxin and that 0.1 ml of anti-657 toxin neutralized 10,000 lethal doses of type B toxin and 1,000 lethal doses of 657 toxin. Identical results were obtained with the anti-657 toxin sera from all three rabbits.

Neutralization tests performed in the same manner with approximately 1 IU of A, C, D, E, F, and G antitoxins failed to neutralize the 657 toxin when diluted 1:100,000, at which point it contained less than 10 lethal doses. Adding type A, C, D, E, F, or G antitoxin in addition to type B antitoxin to 657 culture fluids or dilutions of the concentrated toxin gave no neutralization of toxin beyond that of the type B antitoxin alone.

Antitoxin-binding tests. The results of the studies on interaction between the toxoids and the antitoxins are shown in Table 2. These tests seem to shed some light on the nature of the toxin differences. A 125-μl amount of type B toxoid bound all of the antibodies in the type B and the 657 antitoxins; after exposure to that amount of toxoid, neither antitoxin had sufficient free antibody left for neutralizing either kind of toxin. Only 15.6 μl of type B toxoid was required for removing most of the antibodies in the 657 antitoxin effective against type B toxin. The largest amount of 657 toxoid (250 μl) did not remove all of the type B antibodies from the type B antitoxin, but only 15.6 μl was required to remove most of the antibodies effective against 657 toxin. Even less 657 toxoid was required for binding the antibodies from the 657 antitoxin: 7.8 μl bound all of the antibodies, and 2 μl bound those effective against type B toxin.

DISCUSSION

The problems encountered in neutralization of the botulinal toxin in the fecal extract and in the culture derived from specimens from this infant botulism patient made it difficult to confirm the case in the laboratory. Failure of all of the antitoxins to neutralize the toxicity of the extract suggested that the toxicity was nonspe-

TABLE 1. *Titration of toxicity of concentrated type B and 657 toxins and neutralization tests on each dilution with type B and 657 antitoxins*[a]

Toxin dilution	Type B toxin				657 Toxin			
	Lethal doses	Plain	Mixed with 1 IU of anti-B	Mixed with 0.1 ml of anti-657	Lethal doses	Plain	Mixed with 1 IU of anti-B	Mixed with 0.1 ml of anti-657
10^1	100,000	+	+	+	10,000	+	+	+
10^2	10,000	+	−	−	1,000	+	+	−
10^3	1,000	+	−	−	100	+	+	−
10^4	100	+	−	−	10	+	−	−
10^5	10	+	−	−	1	+	−	−
10^6	1	+	−	−		−	−	−
10^7		−	−	−		−	−	−
10^8		−	−	−		−	−	−

[a] Two mice were injected with 0.5 ml of plain (untreated) toxin or 0.6 ml of a toxin-antitoxin mixture. Symbols: +, the injected mice died; −, they survived.

TABLE 2. *Toxoid binding of antitoxin and serological comparison of type B and strain 657 toxins*

Dilution	Toxoid (0.5 ml) Am of toxoid (μl)	Toxoid binding[a] with following antitoxin:			
		WHO type B (0.25 IU; 0.5 ml)		657 (1:100; 0.5 ml)	
		Type B toxin (30 LD_{50})	657 toxin (30 LD_{50})	Type B toxin (30 LD_{50})	657 toxin (30 LD_{50})
Type B toxoid					
1:2	250	+	+	+	+
1:4	125	+	+	+	+
1:8	62.5	−	−	+	−
1:16	31.2	−	−	+	−
1:32	15.6	−	−	+	−
1:64	7.8	−	−	−	−
1:126	3.9	−	−	−	−
1:256	2.0	−	−	−	−
657 Toxoid					
1:2	250	−	+	+	+
1:4	125	−	+	+	+
1:8	62.5	−	+	+	+
1:16	31.2	−	+	+	+
1:32	15.6	−	+	+	+
1:64	7.8	−	−	+	+
1:128	3.9	−	−	+	−
1:256	2.0	−	−	+	−

[a] +, Toxoid bound antitoxin; mice died. −, Toxoid did not bind antitoxin; mice survived.

cific, i.e., due to something other than botulinal toxin. Tests on enrichment cultures of the stool specimen and of two of the rectal swabs also demonstrated a toxicity which was not neutralizable with the diagnostic antitoxins, but it was noted that the deaths were delayed; i.e., the mice that received culture fluids treated with type B antitoxin died on day 2 after injection, instead of on day 1. At a dilution of 1:5, the culture fluids and the extract were toxic, but the toxicity could be completely neutralized with type B (but not type A) antitoxin. At first it was assumed that the mixed cultures, as well as the extract, probably contained a mixture of botulinal toxin and a nonbotulinal toxic factor(s). Pure cultures of an organism with the cultural and physiological characteristics of proteolytic *C. botulinum* were isolated from enrichment cultures of the feces from the patient and rectal swabs. These isolates were toxigenic, and the culture fluids caused signs of botulism in mice, but the same problems experienced with neutralization of the toxin in the fecal extracts and enrichment cultures were encountered with the pure cultures. One international unit of type B antitoxin, which can neutralize approximately 10,000 LD_{50} of type B toxin, was used in each test. Isolates from three different specimens (the stool and the two rectal

swabs) all produced toxin with these same characteristics. None of the antitoxins other than type B neutralized the toxin either when used alone or in combination with type B antitoxin.

It was apparent that although the toxin produced by this strain of organism was related to type B botulinal toxin, there was clearly a difference based on the proportions of toxin and antitoxin in the neutralization reaction. One hypothesis was that the organism produces a mixture of toxic and nontoxic proteins (toxin and toxoid), both of which possess the same serologically active structure, and thus, both combine with type B antitoxin. Another hypothesis was that this strain produces more than one serological species of toxic protein and that only one corresponds to the type B specificity.

The antitoxin prepared against strain 657 toxin was more effective than the type B antitoxin against 657 toxin, but it still neutralized at least 10 times more lethal doses of type B toxin than 657 toxin (Table 1). On the basis of the results of the binding tests (Table 2), it appears that both type B toxin and 657 toxin consist of more than one molecular species and that all (or most) of the different species are common to both toxins. The number of molecular species could be limited to two, or the system may be more complex. However, the proportion of each molecular species appears to be different in each of the toxins.

Other workers have reported variations in regard to neutralization of toxins produced by various strains of *C. botulinum*. Shimizu and Kondo (8) found a variance in type B toxin specificity of toxins from the Okra strain (proteolytic) and the QC strain (nonproteolytic). Ciccarelli and Gimenez have demonstrated that in a study of 34 strains of *C. botulinum* type A, two distinct subgroups can be established on the basis of the amount of antitoxin required for neutralization of their toxins (1). One unique strain of *C. botulinum* isolated by Gimenez and Ciccarelli (strain 84) appears to produce two types of toxin (5); about 90% of the toxicity is neutralized by type A antitoxin, and the remainder is neutralized by type F antitoxin. Strain 84 obviously produces toxin of more than one molecular species. In that case, two toxin types are demonstrable by separate neutralization by two previously established antitoxins. The results presented in this report suggest that the toxin produced by any given strain of *C. botulinum* may also consist of more than a single molecular species.

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