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Development of Antitoxin with Each of Two Complementary Fragments of *Clostridium botulinum* Type B Derivative Toxin

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Two fragments with molecular weights of 111,000 (fragment I) and 59,000 (fragment II) were separated from each other by gel filtration of dithiothreitol and urea-treated, trypsinized derivative toxin (molecular weight, 170,000) of the proteolytic Okra strain of *Clostridium botulinum* type B on a column of Sephadex G-200 (superfine) with a buffer containing dithiothreitol and urea. Upon removal of dithiothreitol and urea by dialysis, the two fragments reassembled to reconstruct the derivative toxin molecule. Both fragments were immunogenic, and both anti-fragments neutralized type B toxin. The neutralizing activities of both anti-fragment I and anti-fragment II were, however, lower than that of the anti-derivative toxin, suggesting that the molecular integrity of derivative toxin is essential for sufficient production of the neutralizing antibody. The immunological difference found between type B toxin from a proteolytic strain and that from a nonproteolytic strain was ascribed to the antigenic difference of fragment I.

The molecule of the progenitor toxin of *Clostridium botulinum* types A, B, E, and F is composed of two components, toxic and nontoxic (4, 6-9, 12, 17). The toxic component or derivative toxin, with a molecular weight of about 150,000, is made up of two polypeptide fragments with molecular weights of about 100,000 and 50,000 (1, 3, 18). Gel filtration under reduced conditions separated trypsinized type-B derivative toxin into two fragments with molecular weights of 110,000 and 60,000, which possessed distinct antigenicities (5).

This paper reports that both of the nontoxic fragments stimulate rabbits to produce antibody capable of neutralizing the derivative toxin. The antigenic difference found between the toxin produced by a nonproteolytic type B strain and that produced by a proteolytic strain (15, 16) was ascribed to the different antigenicities found in the higher-molecular-weight fragment.

MATERIALS AND METHODS

Type B derivative toxin. *C. botulinum* type B, strains Okra (proteolytic) and QC (nonproteolytic), were used. The procedures for purifying their progenitor toxins were reported previously (6, 10). Two different-sized toxins, called L and M toxins, with molecular weights of about 500,000 and 350,000, respectively, were obtained. After treating M toxin with trypsin in 0.05 M acetate buffer, pH 6.0, containing 0.2 M NaCl (toxin-to-trypsin ratio, 20:1) for 20 min at 35°C, the product was applied to a Sephadex G-200 column (2.5 by 88 cm) equilibrated with the same buffer to remove trypsin and its autodigested materials. The toxic fractions were pooled, dialyzed against 0.01 M phosphate buffer (pH 7.5), and chromatographed on a column of diethylaminoethyl-Sephadex A-50 to separate the trypsin-activated derivative toxin from the nontoxic component, as described before (6). The derivative toxin of strain QC was obtained by the same methods.

Separation of the two fragments. The separation procedures reported previously (5) were slightly modified. The trypsin-activated derivative toxin (4 to 5 mg), adjusted to pH 7.5 with 0.05 M tris(hydroxymethyl)aminomethane, was reduced with 80 mM dithiothreitol (DTT) and treated with 1 M urea for 60 min at 25°C. This was applied to a column (2.5 by 39 cm) of Sephadex G-200, superfine, equilibrated with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, containing 5 mM ethylenediaminetetraacetate, 1 mM DTT, and 1 M urea, and eluted with the same buffer. The 280-nm absorbance of the eluted fractions was determined with a Beckman DB-GT spectrophotometer.

Anti-derivative toxin and anti-fragment sera. The derivative toxin was deotoxified by dialysis against 0.1 M phosphate buffer (pH 7.2) containing 0.4% Formalin for 5 days at 30°C. The two fragments were each dialyzed against the same buffer, containing 0.2% Formalin, for 7 days at 30°C. Each material was mixed with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Two 1-ml doses (0.1 ml of derivative toxin or fragment I [molecular weight, 111,000] and 0.05 mg of fragment II [molecular weight, 59,000]) were injected subcutaneously into each of two rabbits, weighing 2.7 to 3.5 kg, at a 2-day interval. One month after the second injection, two additional 1-ml doses (0.1 mg of derivative toxin or fragment I and 0.05 mg of fragment II), without Formalin treatment or adjuvant, were injected subcutaneously at a 2-week interval. The rabbits were exsanguinated 2 weeks after the last injection. The antiserum was titrated by the passive hemagglutina-

FIG. 1. *Polyacrylamide gel electrophoresis of trypsinized and DTT-treated derivative toxin in the presence of SDS. A sample of 0.02 mg of protein was applied to a gel column. Migration was from left to right.*

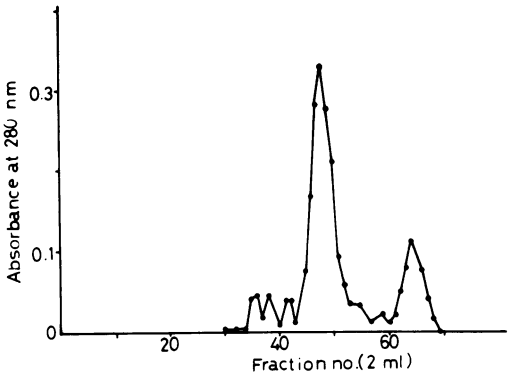

FIG. 2. *Gel filtration of trypsinized and DTT- and urea-treated derivative toxin on Sephadex G-200, superfine.*

tion (PHA) test, using the microtiter system, with sheep erythrocytes (SRBC) Formalin treated and coupled with derivative toxin or each fragment at a concentration of 0.02 mg/ml (13).

Neutralization test. Rabbit antiserum was filtered through Sephadex G-200 to obtain an immunoglobulin G (IgG) fraction. The IgG fraction was concentrated by salting out at 50% saturation of ammonium sulfate, followed by dialysis of the precipitate against 0.075 M phosphate buffer (pH 7.2) containing 0.075 M NaCl. Activated derivative toxin (400 50% lethal doses [LD_{50}]/ml) was incubated with the same volume of each serial dilution of the IgG fraction for 30 min at room temperature. Mixtures were injected intraperitoneally in 0.5-ml doses into pairs of mice; the mice were observed for death for 4 days.

Horse type B antitoxin. Horse type B antitoxin against progenitor toxin of strain Okra (Okra antitoxin) and that against strain QC (QC antitoxin) were prepared and titrated by H. Kondo, Chiba Serum Institute, Chiba, Japan.

Other methods. Toxicity was determined with the time-to-death method by intravenous injection into mice (2, 14). Protein contents were determined by the method of Lowry et al. (11). Sodium dodecyl sulfate (SDS)-gel and conventional disc electrophoreses were performed by methods previously reported (5, 6). The gel column was stained with Coomassie brilliant blue and scanned at 600 nm with a Fuji densitometer (model FD-A IV). Agar gel diffusion tests were performed by the method reported (5).

Chemicals. Diethylaminoethyl-Sephadex A-50 (medium) and Sephadex G-200 (medium and superfine) were products of Pharmacia Fine Chemicals, Uppsala, Sweden; trypsin (twice crystallized) and DTT were from Sigma Chemical Co., St. Louis, Mo.; and SDS and urea were from Wako Pure Chemical Industries, Osaka.

RESULTS

Separation of fragments and reconstruction of the toxic component. The trypsinized and reduced type B derivative toxin formed a single band in conventional disc electrophoresis. Two major bands representative of proteins of molecular weights of approximately 111,000 and 59,000 and two minor bands indicative of about 72,000 and 46,000 molecular-weights were resolved in SDS-gel electrophoresis (Fig. 1). When urea- and DTT-treated, trypsinized derivative toxin was applied to a column of Sephadex G-200 (superfine), two major protein peaks were eluted; the earlier-eluted protein was named fragment I and the late-eluted one was called fragment II (Fig. 2). Fragment I migrated in one major band and two minor bands in SDS-gel electrophoresis (Fig. 3A). The mobility rates of these bands indicated 111,000, 72,000, and 46,000 molecular weights. The minor components were demonstrated with all preparations of fragment I. Fragment II migrated in a single band representative of a protein of 59,000 molecular weight (Fig. 3B).

The two separated fragments were antigenic and contained little toxicity when tested after removing DTT and urea by dialysis (0.04 to 0.4% of the original toxicity). When mixed in an approximately equimolar ratio and dialyzed against a change of 0.025 M phosphate buffer (pH 7.2) for 48 h at 4°C without agitation, about 30% of the original toxicity was restored (Table 1). SDS-gel electrophoresis of the dialyzed sam-

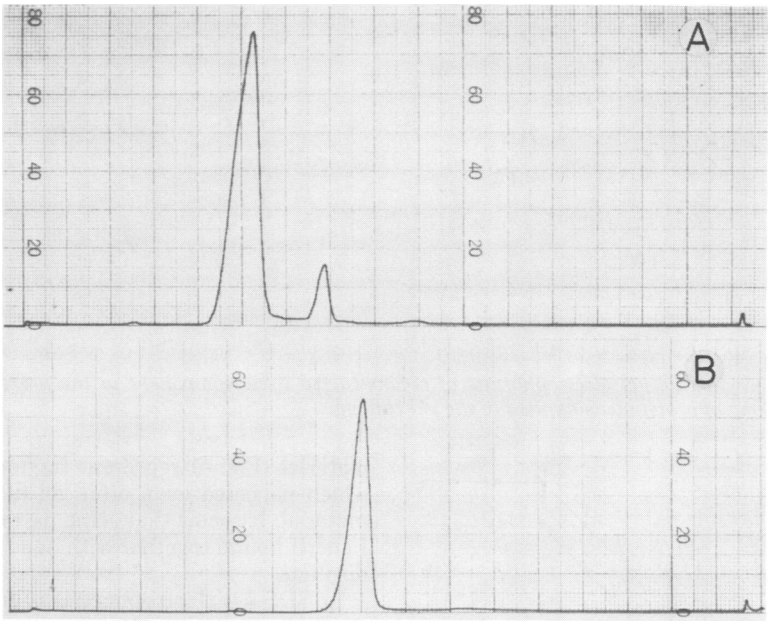

FIG. 3. *Polyacrylamide gel electrophoreses in the presence of SDS. (A) Fragment I, 0.05 mg; (B) fragment II, 0.029 mg.*

TABLE 1. *Reconstruction of type B derivative toxin with the two fragments*

Toxin	LD_{50}/mg of N (%) from:	
	Trypsin-nicked toxin	Toxin nicked endogenously in culture
Native	6.8×10^8 (100)	4.3×10^8 (100)
Fragment mixture		
Before dialysis	2.9×10^6 (0.4)	1.7×10^6 (0.4)
After dialysis	2.3×10^8 (34)	1.8×10^8 (42)

ple resulted in a single major band at the position corresponding to that of the derivative toxin (Fig. 4). In agar gel diffusion with anti-Okra derivative toxin, the reconstructed toxin formed a single precipitin line that coalesced with that of the native derivative toxin. The antigenicities of both fragments were no longer demonstrable after heating for 30 min at 60°C, but that of the derivative toxin persisted after the same treatment.

Immunogenicities of fragments I and II. The development of antibodies was followed by titrating periodically obtained serum samples by the PHA test, using SRBC that were sensitized with the derivative toxin (Fig. 5). The PHA titers of anti-derivative toxin, anti-fragment I, and anti-fragment II reached about 41,000, 10,000, and 20,000, respectively. To examine the specificity of each anti-fragment serum, agar gel diffusion tests were performed. Each anti-fragment serum formed a single precipitin line against the homologous fragment and the derivative toxin as well, but did not cross-react with the heterologous fragment (Fig. 6A and B). SRBC coupled with either fragment were not agglutinated by the heterologous anti-fragment serum.

Neutralizing activities of anti-fragment sera. The neutralizing activities of the IgG fractions prepared from the anti-derivative toxin and the anti-fragment sera are shown in Table 2. The IgG of the two anti-fragment sera neutralized the toxin. When the quantity of antiserum neutralizing 400 LD_{50} was expressed in PHA titer obtained with derivative toxin-sensitized SRBC, anti-fragment I had the same neutralizing activity as anti-derivative toxin, but anti-fragment II possessed one-fourth less activity than the other two.

Comparison of antigenicities of nonproteolytic QC toxin with those of proteolytic Okra toxin. The results of agar gel diffusion tests with various preparations, including trypsinized QC derivative toxin, are shown in Fig. 7. QC derivative toxin formed a single precipitin line with either anti-fragment. The precipitin line formed between anti-fragment I and trypsinized QC derivative toxin was not identical to that formed between anti-fragment I and fragment I (Fig. 7A); the single line formed by the reaction of anti-fragment II with fragment II joined with the line of identity formed in the reaction between anti-fragment II and trypsin-

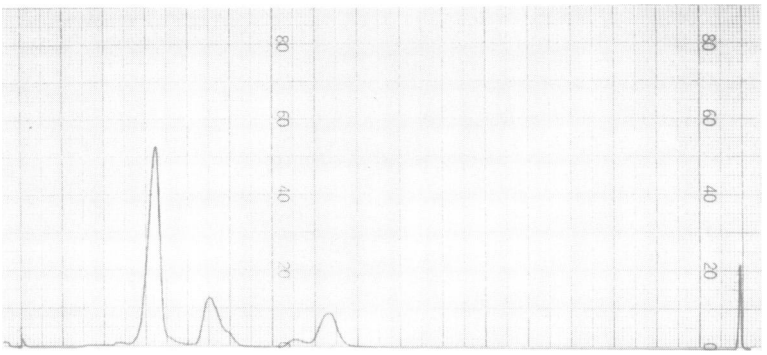

FIG. 4. *Polyacrylamide gel electrophoresis of reconstructed toxic component in the presence of SDS. A sample of 0.015 mg of protein was applied to the gel column.*

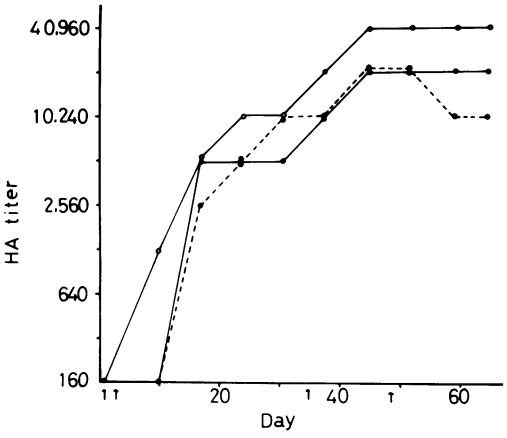

FIG. 5. *Development of anti-derivative toxin* (○——○), *anti-fragment I* (●- - -●), *and anti-fragment II* (●——●). *Arrows indicate antigen injections.*

ized QC derivative toxin (Fig. 7B). Table 3 summarizes the results of PHA tests performed on horse antitoxins against Okra and QC toxins with SRBC separately sensitized with fragment I or II. The ratio of the PHA titer of QC antitoxin to that of Okra antitoxin on the same level of antitoxic potencies was about 1 to 4 when titrated with SRBC coupled with fragment I. The PHA titers with SRBC coupled with fragment I were higher than those with SRBC coupled with fragment II with respect to Okra antitoxin.

DISCUSSION

We have reported distinct antigenicities of the two fragments of type B Okra derivative toxin (5). By treating the nicked toxin with 1 M urea, the two fragments were separated from each other without forming aggregates. Toxic molecules were successfully reconstructed with these purified fragments. The two minor bands seen in SDS-gel electrophoresis of the reconstructed material (Fig. 4) represent fragments I and II, which persisted even after gel filtration of the material. It seems that some parts of fragments I and II bound together with bond(s) other than disulfide.

In SDS-gel electrophoresis of fragment I derived from trypsinized derivative toxin, two minor bands were detected even after repeated gel filtration. These minor bands appeared to represent subfragments of fragment I formed by cleavage of an additional bond during trypsinization at pH 6 and held together with noncovalent bonds, since the sum of their molecular weights agreed with that of fragment I.

Both anti-fragments were found to neutralize type B toxin, but their potencies were lower than that of anti-derivative toxin. A mixture of equal amounts of the two anti-fragments was shown to be of no higher neutralizing activity than their sum. This may indicate that the integrity of the secondary and tertiary structures of the molecule of derivative toxin is essential for sufficient production of the neutralizing antibody. Even so, the neutralizing potency of anti-fragment I was higher than that of anti-fragment II.

In both neutralization and PHA tests, the antigenicity of type B toxin derived from a proteolytic strain was slightly different from that derived from a nonproteolytic strain (15, 16). We confirmed the difference with the purified fragments and anti-fragments by agar gel diffusion and PHA tests. Anti-fragment II formed a single common precipitin line with QC derivative toxin and fragment II. The precipitin line of QC derivative toxin against anti-fragment I, however, was not identical to that of fragment I. The toxin-neutralizing potencies of QC and Okra antitoxins for Okra toxin were directly related to the PHA titers obtained with SRBC sensitized with fragment I. The PHA titers of Okra or QC antitoxin determined with SRBC

sensitized with fragment II, however, were not related to the neutralizing potencies. It was also found that the PHA titer with SRBC sensitized with fragment II differed from one preparation to another. These findings suggest that the different antigenicities found between Okra and QC derivative toxins are due to the difference in the antigenicity of fragment I.

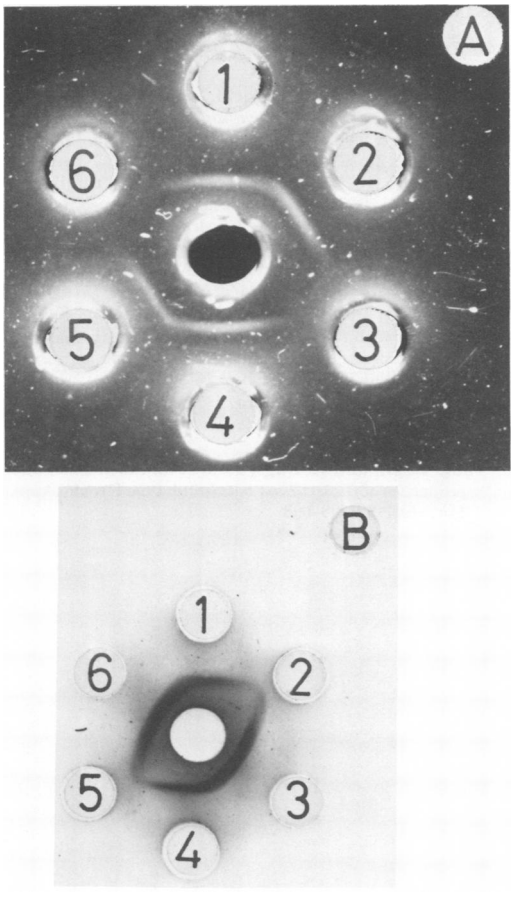

FIG. 6. *Antigenicities of Okra derivative toxin and its fragments toward each anti-fragment. (A) Center well: anti-fragment I. Lateral wells: 1 and 4, derivative toxin, 0.25 mg/ml; 2 and 5, fragment I, 0.27 mg/ml; 3 and 6, fragment II, 0.273 mg/ml. (B) Center well: anti-fragment II. Lateral wells: 1 and 4, derivative toxin, 0.345 mg/ml; 2 and 5, fragment I, 0.355 mg/ml; 3 and 6, fragment II, 0.239 mg/ml.*

TABLE 2. *Neutralizing activities of anti-derivative toxin and anti-fragment IgG*

IgG fraction	PHA titer[a]/ml	Highest dilution neutralizing 400 LD_{50}	PHA titer[a] of the dilution (ratio)
Anti-derivative toxin	40,960	800	51.2 (1)
Anti-fragment I	10,240	200	51.2 (1)
Anti-fragment II	20,480	100	204.8 (4)

[a] With derivative toxin-sensitized SRBC.

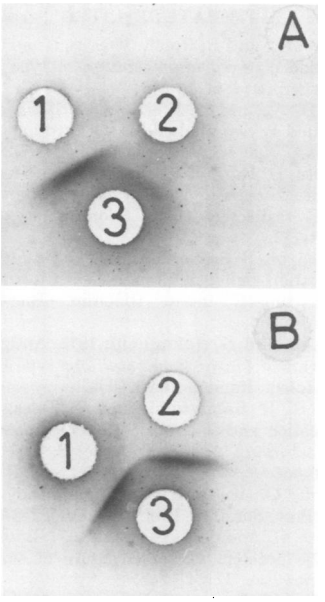

FIG. 7. *Antigenicities of QC derivative toxin toward each anti-Okra fragment. (A) 1, Fragment I, 0.257 mg/ml; 2, QC derivative toxin, 0.55 mg/ml; 3, anti-fragment I. (B) 1, Fragment II, 0.25 mg/ml; 2, QC derivative toxin, 0.55 mg/ml; 3, anti-fragment II.*

TABLE 3. *Hemagglutination titers of anti-Okra and anti-QC toxins with SRBC coupled with fragment I or II*

Antitoxin	IU/ml	SRBC coupled with:	
		Fragment I	Fragment II
Anti-Okra	800	10,240	1,280
	500	10,240	160
Anti-QC	1,050[a]	2,560	2,560
	2,200	5,120	160

[a] The antitoxin possessed a potency of 160 IU/ml when titrated with Okra toxin as the test toxin.

The slight toxicities found with fragments I (0.6 mg/ml, 4×10^4 LD_{50}/ml) and II (0.23 mg/ml, 2×10^4 LD_{50}/ml) were absorbed with the heterologous anti-fragment, without reducing the respective antigenicity. We therefore conclude that both fragments are virtually nontoxic. The toxicity of the fragment was probably due to a minor amount of undissociated toxin contained in each preparation.

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