Evidence that Botulinum C₂ Toxin Has Two Dissimilar Components

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Botulinum C₂ toxin produced by most toxigenic and nontoxigenic strains of *Clostridium botulinum* types C and D contains two distinct protein components, and these were separated by ion-exchange chromatography and gel filtration. Neither of these components manifested the original toxicity, but the original toxicity was restored when the two components were mixed together and trypsinized. This indicates that C₂ toxin consists of two dissimilar protein components and that the cooperation of the two is required to elicit toxicity.

Clostridium botulinum type C has been divided into two subtypes, C_α and C_β (5). The antitoxin serum of a type C_α strain neutralized the toxins produced by both types C_α and C_β, whereas the antitoxin serum of a type C_β strain neutralized only the toxin produced by type C_β. However, the immunological cross-reactions of toxins produced by *C. botulinum* types C and D has not been clearly elucidated. Mason and Robinson reported that type C strains produced three toxin factors (C₁ and C₂ toxins and a small amount of D toxins) and that a type D strain produced a small amount of C toxin in addition to a large amount of D toxin (13). Bulatova et al. showed that anti-C_α and -C_β sera cross-neutralized C_β and C_α toxins, respectively, and that type D strains produced a small amount of both type C_α and type C_β toxins, as well as a large quantity of type D toxin (1). Jansen reported that a type C_α strain produced C₁, C₂, and D toxins, that a type C_β strain produced only C₂ toxin, and that a type D strain produced C₁ and D toxins (7). It was later shown that some toxigenic and nontoxigenic type D strains also produced C₂ toxin (4). The C₂ toxin is produced as a protoxin in cultures of most toxigenic and nontoxigenic strains of *C. botulinum* types C and D (4, 8), and the toxicity can be enhanced by treatment with trypsin. We have reported that purified type C and D progenitor toxins are commonly constructed with toxic and nontoxic components and that no immunological cross-reactions occur between the toxic components of type C and type D progenitor toxins (14). The present work was initiated to compare the molecular structure of C₂ toxin with the structures of progenitor toxins of types A through F and also to clarify the immunological cross-reactions among the toxin factors produced by toxigenic and nontoxigenic strains of *C. botulinum* types C and D. The present paper describes resolution of C₂ toxin into two dissimilar components,

which individually exhibit a very low toxicity in mice; however, full toxicity is restored by mixing these components and treating the mixture with trypsin.

MATERIALS AND METHODS

Bacterial strains. For toxin production in various media, we used nontoxigenic strain 92-13, which produces only C₂ toxin (15). This strain, which was isolated from soil, was identified as nontoxigenic *C. botulinum* type C by agglutination tests and by its biochemical properties (20). It was provided by S. Nakamura, Kanazawa University, Kanazawa, Japan. Nontoxigenic strain C-203U28, which was derived from toxigenic *C. botulinum* type C strain 203 by curing the prophage with ultraviolet light, was provided by H. Iida, Hokkaido University, Sapporo, Japan. *C. botulinum* type C strain Stockholm and type D strain 1873 were the stock strains used in this laboratory. These strains were stored in cooked meat medium containing 1% ammonium sulfate, 1% yeast extract, 1% glucose, and 0.1% cysteine hydrochloride, pH 7.6.

Toxin production and cultural conditions. The following media were used. (i) Brain heart infusion medium consisted of 3.7% brain heart infusion (Difco Laboratories, Detroit, Mich.) and 0.1% cysteine hydrochloride, pH 7.3. (ii) Prereduced Trypticase medium consisted of 3% Trypticase (Difco), 2% yeast extract, and 1% glucose, pH 7.4. This medium was reduced with sterilized O₂-free CO₂ before autoclaving and inoculation. (iii) Peptone yeast extract glucose medium, which has been used previously for production of progenitor toxins by *C. botulinum* types A through F, was prepared according to a formula described elsewhere (6), except that the pH was adjusted to 7.6 before autoclaving. (iv) Fortified egg meat medium was prepared by the method of Segner et al. (19) except that 0.1% cysteine hydrochloride was added and the medium was adjusted to pH 7.6 before autoclaving. (v) Fortified chopped meat medium contained 100 ml of beef infusion (500 g of beef per liter), 1% peptone (Mikuni Co., Tokyo, Japan), 0.5% yeast extract, 1% glucose, 1% ammonium sulfate, 0.5% CaCO₃, 0.5% soluble starch, 0.1% cysteine hydrochloride, and 20% wet cooked beef particles. The medium was adjusted to pH 7.6 before autoclaving.

To test for toxin production, all of the media were dispensed in 10-ml quantities into test tubes (18 by 175 mm) with cotton plugs and autoclaved for 15 min at 121°C. An overnight culture of strain 92-13 in fortified chopped meat medium was heated for 15 min at 75°C, and a 0.1-ml portion was inoculated into each tube. All cultures were incubated for 2 days at 37°C. Total cell and spore numbers were counted by phase-contrast microscopy.

Determination of toxicity. Toxicity was determined primarily by the time to death method by injecting 0.1-ml doses intravenously into ddY mice weighing 18 to 25 g. A standard curve for determining the intraperitoneal (i.p.) 50% lethal dose (LD₅₀) from survival time after intravenous injection was prepared with trypsinized C₂ toxin concentrated with ammonium sulfate from a 2-day culture fluid in fortified chopped meat medium. In some experiments, mouse i.p. and intravenous LD₅₀'s were also determined by the method of Reed and Muench (18). Activation was performed with trypsin (type III; final concentration, 200 µg/ml; Sigma Chemical Co., St. Louis, Mo.) for 30 min at 35°C in 0.05 M phosphate buffer, pH 8.0.

Determination of protein. Protein contents were determined by the method of Lowry et al. (12), using bovine serum albumin as a standard.

RESULTS

Production of C₂ toxin. Table 1 shows the concentrations of C₂ toxin in brain heart infusion medium, prereduced Trypticase medium, peptone yeast extract glucose medium, and fortified egg meat medium after incubation for 2 days at 37°C and treatment with trypsin. Toxin production and spore formation in fortified egg meat medium were higher than toxin production and spore formation in the other media. However, this medium did not seem feasible for large-scale production of C₂ toxin for economic reasons. Therefore, chopped meat medium supplemented with several agents was tested, and finally fortified chopped meat medium was found

to be suitable for large-scale production of C₂ toxin. The final pH after a 2-day incubation of strain 92-13 in this medium was 5.5 to 6.0, and the potential toxicity was 1×10^3 to 3×10^3 i.p. LD₅₀ per ml. Toxicity reached a maximum after incubation for 2 days at 37°C, and maximum toxicity persisted for at least 5 days.

Activation conditions of C₂ toxin with trypsin. At first, trypsinization of C₂ toxin in the culture supernatant was performed in 0.05 M phosphate buffer (pH 6.0 or 6.5), as reported by Eklund and Poysky (4), but it was later found that maximum toxicity was obtained by treatment for 30 min at 35°C in 0.05 M phosphate buffer (pH 8.0) containing trypsin at 200 µg/ml of reaction mixture. No decrease in toxicity resulted when the concentration of trypsin was increased to 800 µg/ml to activate C₂ toxin in the culture supernatant, and the highest toxicity persisted for 180 min at 35°C.

Toxicity of C₂ toxin in mice. The intravenous and i.p. toxicities of C₂ toxin were compared in mice. C₂ toxin concentrated with ammonium sulfate was treated with trypsin and diluted with 0.05 M phosphate buffer (pH 6.0) containing 0.2% gelatin. The toxicity obtained by i.p. injection (610 i.p. LD₅₀ per ml) was lower than that obtained by intravenous injection (7,400 intravenous LD₅₀ per ml). The ratio of intravenous toxicity to i.p. toxicity was 12:1.

The relationship between the mouse i.p. LD₅₀ and survival time was examined by intravenous injections of trypsinized C₂ toxin. A linear correlation between log dose and log death time was obtained in a range of death time from 30 to 100 min (Fig. 1). In subsequent experiments, therefore, the toxicity (i.p. LD₅₀) of C₂ toxin was determined from this standard curve by inject-

TABLE 1. Production of C₂ toxin and sporulation by strain 92-13^a

Medium	Toxicity (i.p. LD ₅₀ /ml)	Total no. of cells per ml (×10 ⁷)	No. of spores per ml (×10 ⁶)
Brain heart infusion	<10	10	<1.0
Prereduced Trypticase	48	7.8	11
Peptone yeast extract glucose	<10	8.9	<1.0
Fortified egg meat	1,070	12	99

^a Strain 92-13 was inoculated into each medium and then incubated at 37°C for 2 days. Total numbers of cells and refractile spores were counted on a hemacytometer under a phase-contrast microscope. Culture supernatants were obtained by centrifugation at 12,000 × g for 20 min and were trypsinized as described in the text.

FIG. 1. Correlation between log i.p. LD₅₀ and log survival time after intravenous injection of C₂ toxin into mice.

ing specimens intravenously into mice in 0.1-ml doses.

Resolution of C_2 toxin into two components. To discover suitable procedures for the purification of C_2 toxin, we attempted chromatographic isolation of C_2 toxin on Sephadex G-100, diethylaminoethyl (DEAE)-Sephadex, and carboxymethyl (CM)-Sephadex. The supernatant of a culture of strain 92-13 incubated in fortified chopped meat medium for 2 days at 37°C was applied to a column of Sephadex G-100 (2.5 by 95 cm), which was equilibrated and eluted with 0.05 M tris(hydroxymethyl)-aminomethane hydrochloride, pH 7.5. A toxin peak emerged between elution volumes of 215 and 275 ml, but the recovery of the toxicity in this peak was only 14%. However, full toxicity was restored when the fractions (Fig. 2a, fractions I and II) which eluted around the toxin peak were pooled. When culture supernatant was applied to a DEAE-Sephadex A-50 column (0.8 by 12 cm) and eluted by a linear increase in sodium chloride concentration in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), a toxin peak emerged at 0.15 M NaCl (Fig. 2b), but only 12% of the toxicity applied was recovered in the fractions of this toxin peak (Fig. 2b, fraction II). However, when the fractions which eluted ahead of the toxin peak (Fig. 2b, fraction I) and the fractions of the toxin peak were pooled, most of the toxicity was restored. These results indicate that C_2 toxin consists of two components and that it was resolved into two fractions, which individually showed only weak toxicity in mice. More complete resolution of C_2 toxin into two fractions was attained by chromatography on a CM-Sephadex column (0.8 by 10 cm) equilibrated with 0.01 M acetate buffer, pH 6.0 (Fig. 2c). In this chromatography, the toxin peak emerged at 0.08 M NaCl when the column was eluted by a linear increase in sodium chloride concentration in 0.01 M acetate buffer (pH 6.0), but the recovery in the fractions of the toxin peak was 8% of the toxicity applied. Full toxicity was restored when the percolated fractions (Fig. 2c, fraction I) and the fractions of the toxin peak (Fig. 2c, fraction II) were mixed together. The toxicities of the fractions separated by chromatography are summarized in Table 2.

Similar results were obtained with C_2 toxin produced by toxigenic *C. botulinum* type C strain Stockholm, type D strain 1873, and non-toxigenic strain C-203U28. These strains were inoculated into fortified chopped meat medium and incubated at 37°C for 2 days. Each culture supernatant was dialyzed against 0.01 M acetate buffer (pH 6.0) and applied to a CM-Sephadex C-50 column (0.8 by 10 cm), which was equili-

FIG. 2. Chromatography on Sephadex G-100, DEAE-Sephadex, and CM-Sephadex of C_2 toxin produced by strain 92-13. A 10-ml portion of each culture supernatant was dialyzed against the buffer used for equilibration of each column and was applied to a column. The fractions (5 ml/tube) indicated by the horizontal bars were pooled. Toxicity was determined after treatment with trypsin; the reaction mixture contained 0.1 ml of each fraction, 200 μ g of trypsin, and 50 mM phosphate buffer (pH 8.0) in a total volume of 1.0 ml. (a) Sephadex G-100. (b) DEAE-Sephadex. (c) CM-Sephadex. Symbols: $\bullet$, protein contents; $\circ$, toxicity; ----, NaCl concentration.

brated with the same buffer and eluted with 0.15 M NaCl in the same buffer (Fig. 3). A very low toxicity was obtained in the eluted fractions of these strains, but full toxicity was restored when the percolated (Fig. 3a through c, fraction I) and eluted (Fig. 3a through c, fraction II) fractions were mixed together and trypsinized (Table 3).

DISCUSSION

The data presented above show that the C_2 toxin produced by both toxigenic and nontoxi-

TABLE 2. Toxicities of fractions separated by Sephadex G-100, DEAE-Sephadex, and CM-Sephadex columns

Column	Fraction	Toxicity ($\times 10^2$ i.p. LD ₅₀) ^a	% Recovery ^b
Sephadex G-100	Applied	140	100
	I	10	7
	II	10	7
DEAE-Sephadex	I + II	129	92
	Applied	145	100
	I	D ^c	
CM-Sephadex	II	18	12
	I + II	129	89
	Applied	146	100
	I	S ^d	
	II	12	8
	I + II	163	112

^a Toxicities were determined by treatment with trypsin, as described in the legend to Fig. 2.

^b The toxicity applied onto a column was defined as 100%.

^c D, Survived for 2 h, but died within 6 h.

^d S, Survived for 6 h or more.

TABLE 3. Toxicities of fractions of C₂ toxin produced by toxigenic and nontoxigenic strains of *C. botulinum* types C and D

Strain	Fraction	Toxicity (i.p. LD ₅₀) ^a
Stockholm	Applied	1,416
	I	58 ^b
	II	D ^c
1873	I + II	1,866
	Applied	1,494
	I	53 ^b
C-203U28	II	D
	I + II	1,560
	Applied	1,758
	I	D
	II	D
	I + II	1,518

^a Toxicities were determined after treatment with trypsin, as described in the legend to Fig. 2.

^b Most toxicity found in fraction I of strains Stockholm and 1873 was possibly due to the presence of type C and type D toxins, respectively; both type C and type D toxins were eluted in the percolated fraction by CM-Sephadex chromatography under the conditions employed in the present experiment. However, the toxicities in those fractions were tentatively expressed in mouse i.p. LD₅₀ of C₂ toxin, as calculated from the standard curve shown in Fig. 1.

^c D, Survived for 2 h, but died within 6 h.

genic strains of *C. botulinum* types C and D contain two distinct protein molecules and that the two can be resolved from each other by gel filtration or ion-exchange chromatography. During CM-Sephadex chromatography at pH 6.0, these two components of C₂ toxin were separated

FIG. 3. Chromatography on CM-Sephadex of C₂ toxins produced by toxigenic and nontoxigenic strains of *C. botulinum* types C and D. A 4-ml portion of each culture supernatant was applied to a CM-Sephadex C-50 column. The fractions (5 ml/tube) indicated by the horizontal bars were pooled. (a) Type C strain Stockholm. (b) Type D strain 1873. (c) Nontoxigenic strain C-203U28.

completely, whereas they were eluted close together during DEAE-Sephadex chromatography or gel filtration on Sephadex G-100 at pH 7.5. Neither of the components separated by chromatography manifested the original toxicity of the C₂ toxin which was applied to the column; however, the original toxicity was restored when two fractions were mixed together. These findings indicate that C₂ toxin produced by both toxigenic and nontoxigenic strains of *C. botulinum* types C and D contains two distinct non-toxic protein components and that cooperation of the two very dissimilar components is required to elicit lethality.

It has been shown that *C. botulinum* progenitor toxin molecules of types A through F are constructed with two components (toxic and nontoxic) and that the two components are dissociated by chromatography or ultracentrifugation at pH 7.5 or above (10, 11, 14, 16, 21).

Molecular dissociations and associations of the progenitor toxins of any type are reversible by changing the pH of the toxin solution. However, neither a decrease nor an increase in the parental toxicity has been observed (2, 9, 16). We showed in the present experiments that the elution positions of the two components of C₂ toxin during gel filtration on Sephadex G-100 were later than the position of the toxic component of any type of botulinum toxin; the molecular weights of the toxic components of the progenitor toxins produced by *C. botulinum* types A through F are approximately the same (150,000), and the elution volumes from Sephadex G-100 under the conditions described in the legend to Fig. 2a were between 165 and 195 ml. Furthermore, the optimum pH for trypsinization of botulinum type B and E toxins is 6.0 (3, 17), whereas that for C₂ toxin was found to be 8.0. These results indicate that the molecular constitution of C₂ toxin differs markedly from the molecular constitutions of botulinum progenitor toxins of types A through F.

Further experiments with purified components of C₂ toxin should be performed to substantiate the difference in molecular constitution between C₂ toxin and botulinum neurotoxin. Purification and characterization of both the components of C₂ toxin are now in progress in this laboratory.

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