Oral Toxicities of *Clostridium botulinum* Type C and D Toxins of Different Molecular Sizes

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Clostridium botulinum type C progenitor toxins of different molecular sizes, C-L (16S) and C-M (12S), were purified from cultures of strains 573, Stockholm, and CB-19. C-L toxin showed some hemagglutinin activity, whereas C-M toxin did not. Neither C-L nor C-M toxin was activated upon trypsinization. Molecular dissociation of purified type C-L and C-M toxins into toxic and nontoxic components was demonstrated by sucrose density gradient ultracentrifugation and diethylaminoethyl-Sephadex chromatography at pH 8.0. The molecular construction of type C progenitor toxin appears to be analogous to that reported for botulinum toxins of other types. C-L and D-L toxins showed higher oral toxicities to mice than did C-M or D-M toxin. Such higher oral toxicities were ascribed to the higher stabilities of these toxins in gastric and intestinal juices.

Progenitor toxins of *Clostridium botulinum* types A, B, E, and F, being the complex of toxic and nontoxic components, are orally more potent to mice than are the corresponding derivative toxins, dissociated toxic components, of 5 to 7S (17, 21). Type A-L and B-L toxins of 16S possessed particularly high oral toxicities as compared with type A-M, B-M, or F toxin of 10 to 12S (17). Such higher oral toxicities shown by larger molecular-sized progenitor toxins were ascribed to their higher resistance to gastric and intestinal juices. The oral toxicity of botulinum toxin hence depends on the complexity of the molecular construction and the resulting stability in the digestive tract of the host animal (26).

Susceptibilities of different animal species to oral administration of *C. botulinum* type C and D toxins have been reported (9, 18, 24, 27-29). No attention, however, has been paid to different molecular-sized toxins in the past feeding experiments. The present investigation demonstrates that strains of *C. botulinum* type C commonly produce 12S and 16S toxins as do types A (25), B (11), and D (13), and that the oral toxicities and stabilities of type C and D toxins differ depending upon the molecular size.

MATERIALS AND METHODS

Bacterial strains and toxin production. *C. botulinum* type C strains 573, Stockholm, and CB19, and type D strains 1873, 4947, and CB16 were used. Type C strain Stockholm was supplied by J. Müller, State Veterinary Serum Laboratory, Copenhagen. Type C strain CB19 and type D strain CB16, isolated from mink feed incriminated in an outbreak occurring in Hokkaido in 1975 and from soil in Tokyo at the time of an outbreak among wild ducks in 1973, respectively, were provided by S. Sakai, Tokyo Metropolitan Re-

search Laboratory of Public Health, Tokyo. Each strain was inoculated into a medium composed of 0.5% glucose-0.5% yeast extract-2.0% peptone-0.1% cysteine-hydrochloride (pH 7.2) to which was added 0.04 volumes of a salt solution of 0.02% magnesium sulfate-0.026% calcium chloride-0.1% potassium diphosphate-0.1% potassium monophosphate-1.0% sodium bicarbonate-0.2% sodium chloride. For toxin production, an overnight culture in cooked meat medium fortified with 1% ammonium sulfate, 1% glucose, 1% yeast extract, and 0.1% cysteine-hydrochloride (pH 7.4) was inoculated into the above medium (10 ml/5,000 ml), which was incubated for 3 days at 37°C.

Preparation of culture supernatant and cell extract for ultracentrifugal analyses. Portions (0.5 ml) of *C. botulinum* type C and D strains grown overnight in the fortified cooked meat medium at 37°C were each inoculated into 10 ml of saline in dialysis casing suspended in 215 ml of the same medium in a flask. After incubation for 3 days at 37°C, the supernatant of the culture in the dialysis casing was obtained by centrifugation for 20 min at $7,800 \times g$. The cell extract was prepared by resuspending the precipitated cells in 2.0 ml of 0.2 M phosphate buffer (pH 6.0) and centrifuging for 20 min at $7,800 \times g$.

Purification of progenitor toxins of *C. botulinum* types C and D. *C. botulinum* type C and D toxins were purified by essentially the same procedures as those employed for purification of type F progenitor toxin (14, 16) except the step of acid precipitation, in which the toxin was precipitated by adjusting the culture fluid to pH 4.0 with 3 N H_2SO_4 , in the presence of added ribonucleic acid (0.4 mg/ml) as a precipitation aid (8).

Determinations of intraperitoneal and oral toxicities. The intraperitoneal mean lethal dose (i.p. LD₅₀) was determined by the time-to-death method by injecting mice intravenously with 0.1-ml doses (1, 22). Activation was studied by treating the toxin sample with trypsin at an enzyme-to-substrate ratio of 1:2 in 0.1 M acetate buffer (pH 6.0) for 30 min at 35°C.

The oral LD₅₀ was calculated by the method of Reed and Muench (19) and expressed in the equivalent number of i.p. LD₅₀. Oral administration of toxin to mice was performed as described previously (17).

Tests for stability of the toxin in gastric and intestinal juices. Gastric and intestinal juices were obtained from 280- to 350-g Wistar strain male rats by inserting polyethylene tubing into the stomach and the duodenum, respectively (26). For the stability test, 1 volume of toxin (50 µg/ml) was incubated at 35°C in 4 volumes of gastric (pH 1.4) or intestinal juice (pH 7.5). Since type D progenitor toxin is activable, the toxin to be used for the stability test was treated with trypsin at a toxin-to-trypsin ratio of 2:1 in 0.1 M acetate buffer (pH 6.0) for 30 min at 35°C, and the trypsin was removed on soybean trypsin inhibitor-coupled Sepharose 6B prepared by the method of Feinstein (4).

Other procedures. Protein was determined by the method of Lowry et al. (12). Ultracentrifugation in a 5 to 20% linear sucrose density gradient was performed by the procedure reported elsewhere (25). Disc electrophoresis at pH 4.0 in 4.5% polyacrylamide gel was carried out by the method of Reisfeld et al. (20). Neutral red was used as a marker dye to measure the relative mobility.

RESULTS

Purification and some properties of type C and D progenitor toxins. At the final step of purification of the toxins of all strains of *C. botulinum* types C and D employed, two toxin peaks were eluted from a column of Sephadex G-200 at the positions corresponding to those of B-L (16S) and B-M (12S) toxins. The first eluted peak was named L toxin, and the second one was named M toxin. In disc electrophoresis at pH 4.0, M toxin of any strain gave a single band with a relative mobility of 0.48, while L toxin gave three to four bands. The parenteral toxicities of D-L and D-M toxins increased two to six times upon trypsinization, whereas those of type C strains remained almost at the same level. All L toxins purified from type C and D cultures showed some hemagglutinin activity against the chicken erythrocytes. The toxicities and hemag-

glutinin activities of purified type C and D toxins are summarized in Table 1.

Molecular dissociation of type C and D progenitor toxins into toxic and nontoxic components. Purified toxins were each centrifuged in a 5 to 20% sucrose density gradient in 0.05 M phosphate buffer (pH 6.0), at 123,000 × g for 7 h at 6°C. All C-L and D-L toxins sedimented to the same relative position as that of B-L toxin (16S), and all M toxins sedimented to that of B-M toxin (11.6S). The presence of both L and M toxins was demonstrated in the culture supernatant and cell extract of all strains except type D strain 1873, of which the toxin peak was broad and mainly at the same position as that of B-M toxin (Fig. 1). In sucrose density gradient ultracentrifugation at pH 7.0 or above, C-L toxin was resolved into toxic and nontoxic components

TABLE 1. Toxicities and hemagglutinin activities of purified type C and D progenitor toxins

Toxin type	Strain	Toxicity (i.p. LD ₅₀ /mg of protein)		Ratio ^a	Hemagglutinin activity ^b
		Before trypsinization	After trypsinization		
C-L	573	3.2 × 10 ⁶	3.8 × 10 ⁶	1.2	2 ⁶
	Stockholm	4.6 × 10 ⁶	4.3 × 10 ⁶	0.93	2 ⁶
	CB19	1.0 × 10 ⁷	9.8 × 10 ⁶	0.98	2 ³
C-M	573	1.0 × 10 ⁷	1.2 × 10 ⁷	1.2	— ^c
	Stockholm	1.6 × 10 ⁷	1.4 × 10 ⁷	0.88	—
	CB19	1.9 × 10 ⁷	1.8 × 10 ⁷	0.95	—
D-L	4947	4.5 × 10 ⁷	2.9 × 10 ⁸	6.4	2 ⁶
	1873	5.8 × 10 ⁷	2.6 × 10 ⁸	4.5	2 ⁶
	CB16	3.0 × 10 ⁷	1.2 × 10 ⁸	4.0	2 ⁶
D-M	4947	1.5 × 10 ⁸	3.2 × 10 ⁸	2.1	—
	1873	1.8 × 10 ⁸	5.3 × 10 ⁸	2.9	—
	CB16	3.9 × 10 ⁸	1.4 × 10 ⁹	3.5	—

^a Ratio of toxicity before and after trypsinization.

^b Reciprocal of the highest dilution of toxin at 0.1 mg/ml with 0.5% chicken erythrocyte suspension at room temperature.

^c —, Negative at 1 mg/ml.

FIG. 1. (A) Ultracentrifugal analyses of the cell extract of *C. botulinum* type C strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the cell extract of type C strain 573 contained 9.4×10^4 LD₅₀, that of strain Stockholm contained 3.2×10^5 LD₅₀, and that of strain CB19 contained 3.7×10^5 LD₅₀. (a) Type C strain 573; (b) type C strain Stockholm; (c) type C strain CB19. (B) Ultracentrifugal analyses of the culture supernatant of *C. botulinum* type C strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of strain 573 contained 0.8×10^4 LD₅₀, that of strain Stockholm contained 1.2×10^4 LD₅₀, and that of strain CB19 contained 8.0×10^5 LD₅₀. (d) Type C strain 573; (e) type C strain Stockholm; (f) type C strain CB19. (C) Ultracentrifugal analyses of the cell extract of *C. botulinum* type D strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the cell extract of strain 4947 contained 8.8×10^4 LD₅₀, that of strain 1873 contained 4.6×10^6 LD₅₀, and that of strain CB16 contained 4.2×10^6 LD₅₀. (a) Strain 4947; (b) strain 1873; (c) strain CB16. (D) Ultracentrifugal analyses of the culture supernatant of type D strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the culture supernatant of strain 4947 contained 8.8×10^4 LD₅₀, that of strain 1873 contained 2.0×10^6 LD₅₀, and that of strain CB16 contained 6.0×10^5 LD₅₀. (d) Strain 4947; (e) strain 1873; (f) strain CB16.

as reported with other types (11, 13, 25); the former sedimented to the same position as that of a toxic component (7S), and the latter sedimented to that of a nontoxic component (14S) of B-L toxin. The dissociation pattern of C-L toxin is shown in Fig. 2.

C-M toxin preparations purified from *C. botulinum* type C strains 573, Stockholm, and CB19 sedimented to the same relative position as that of the toxic component of B-L toxin in ultracentrifugation at pH 8.0, but no separation into toxic and nontoxic components was ob-

FIG. 1.

served. When purified C-M toxin was chromatographed on a column of diethylaminoethyl (DEAE)-Sephadex A-50 at pH 8.0, however, two protein peaks of approximately the same areas were separated (Fig. 3); the early eluted one was toxic, and the late eluted one was nontoxic, indicating that C-M toxin is constructed with two components, toxic and nontoxic, and dissociates into the two at pH 8.0.

Oral toxicities of botulinum type C and D toxins. The oral toxicities of type C and D toxins of different molecular sizes are shown in Table 2. The oral toxicities of C-L and D-L toxins (16S) of all strains were higher than those of C-M or D-M toxin; those of C-L toxins were

FIG. 2. Ultracentrifugal analyses of the purified progenitor toxin of type C strain 573 in sucrose density gradient (5 to 20%) prepared in 0.05 M phosphate buffer, pH (a) 6.5, (b) 7.0, (c) 7.5, and (d) 8.0. A 0.2-ml sample of C-L toxin contained 85 μ g of protein and 3.5×10^5 LD₅₀. B-L, B-M, and B-S indicate the sedimentation positions of B-L (16S), B-M (12S), and B-S (7S) toxins, respectively. Symbols: ●, Protein content; ○, toxicity.

FIG. 3. Chromatography of purified C-M toxin of type C strains 573, Stockholm, and CB19 on a DEAE-Sephadex A-50 column (1.5 by 10 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0. A 2.0-mg portion of toxin was applied and eluted with a linear gradient increase in NaCl concentration from 0 to 0.5 M in 100 ml of the buffer. (a) Strain 573; (b) strain Stockholm; (c) strain CB19. Symbols: ●, Protein content; ○, toxicity; —, NaCl concentration.

TABLE 2. Oral toxicities of *C. botulinum* type C and D progenitor toxins

Toxin type	Strain	Oral toxicity (i.p. LD ₅₀)	Ratio ^a
C-L	573	6.7×10^3	2.5
	Stockholm	6.5×10^3	2.4
	CB19	2.7×10^3	1.0
C-M	573	2.7×10^5	100
	Stockholm	0.95×10^5	35
	CB19	1.2×10^5	44
D-L	4947	3.5×10^4	13
	1873	7.6×10^4	28
	CB16	7.6×10^4	28
D-M	4947	0.96×10^5	35
	1873	1.3×10^5	48
	CB16	8.8×10^5	326

^a Oral toxicity of type C-M toxin of strain 573 was taken as 100.

15 to 45 times higher than those of C-M toxins. No significant difference was found between C-M and D-M toxins in the oral toxicity expressed in equivalent mouse i.p. LD₅₀.

Stabilities of type C and D toxins in gastric and intestinal juices. Stabilities of type C and D toxins of different molecular sizes in gastric and intestinal juices were tested (Tables 3 and 4). C-L and D-L toxins of all the strains were more resistant to either gastric or intestinal juices than were C-M and D-M toxins. D-M toxin was more resistant to treatment with intestinal juices than was C-M toxin. Less than 0.02% of the original C-M toxicity remained after a 180-min exposure to intestinal juices at 35°C.

TABLE 3. *Stabilities of type C and D progenitor toxins in rat gastric juice*

Toxin type	Strain	Remaining toxicity (%) after ^a	
		40 min	120 min
C-L	573	79	16
	Stockholm	76	68
	CB19	82	68
C-M	573	3.7	0.8
	Stockholm	12	1.6
	CB19	10	1.1
D-L	4947	100	66
	1873	100	27
	CB16	79	45
D-M	4947	5.8	1.2
	1873	2.0	0.2
	CB16	2.6	0.6

^a Incubation at 35°C.

TABLE 4. *Stabilities of type C and D progenitor toxins in rat intestinal juice*

Toxin type	Strain	Remaining toxicity (%) after ^a	
		60 min	180 min
C-L	573	109	78
	Stockholm	100	79
	CB19	85	80
C-M	573	0.38	<0.02
	Stockholm	0.85	<0.02
	CB19	0.95	<0.02
D-L	4947	81	61
	1873	81	81
	CB16	91	89
D-M	4947	8.9	1.0
	1873	8.9	0.12
	CB16	7.9	0.9

^a Incubation at 35°C.

In gastric juices, however, C-M toxin was more resistant than D-M toxin.

DISCUSSION

In the present study, different molecular-sized *C. botulinum* type C toxins, C-L (16S), and C-M (12S) toxins, were detected in both the culture supernatant and the cell extract of all the strains tested. C-L and C-M toxins were purified by essentially the same procedure as that adapted for purifying type F progenitor toxin except for the step of acid precipitation, in which ribonucleic acid was added to aid precipitation of the toxin (8). It has been reported that type D strain CB16 produced both L and M toxins, whereas type D strain 1873 produced M toxin only (13). In the present study both L and M toxins were purified from cultures of type D strains 4947, 1873, and CB16, and the different molecular-sized toxins were detected in the culture supernatant and the cell extract of type D strains 4947 and CB16 by ultracentrifugation, whereas a broad toxin peak was obtained with these materials of strain 1873. This may indicate that L toxin of type D strain 1873 might have been formed artificially during the purification procedures.

In sucrose density gradient ultracentrifugation at pH 6.0, C-L and C-M toxins sedimented to the same relative position as that of B-L and B-M toxins, respectively. It was also shown that C-L toxin at pH 7.0 or above resolved into toxic and nontoxic components in ultracentrifugation and so did C-M toxin in DEAE-Sephadex chromatography at pH 8.0. The dissociated toxic component of C-L and C-M toxins sedimented to the same position as that of type D derivative toxin (7S). These results indicate that type C toxin consists of toxic and nontoxic components that have the same molecular construction as those reported for type A, B, D, E, and F progenitor toxins (10, 11, 13, 15, 25).

The oral toxicities of different molecular-sized type C and D toxins showed a similar tendency to those of type A and B toxins; the larger the molecular size, the higher the oral toxicity. In Table 5, the oral toxicities and stabilities of the toxins of types A through F of different molecular sizes are summarized. B-L toxin purified from strain Okra showed an exceptionally high toxicity to mice; the oral toxicities of both C-L and D-L toxins were also high, being close to that of B-L toxin. Such high oral toxicities always reflect the high resistance of these toxins to gastric as well as intestinal juices. The ratio of the oral toxicity to the i.p. toxicity shown in Table 5 indicates the quantity of toxin protein in micrograms equivalent to 1 oral LD₅₀ of each

TABLE 5. Oral toxicities and stabilities of *C. botulinum* toxins of types A through F

Toxin type	Toxicity		Ratio ^d	Stability ^a in	
	i.p. ^b	Oral ^c		Gastric juice ^e	Intestinal juice ^f
A-L	4.5×10^7	2.2×10^6	49		
A-M	8.5×10^7	3.6×10^6	42	<0.01	10
B-L ^g	4.5×10^7	1.5×10^3	0.03	29	88
B-M ^g	9.3×10^7	1.1×10^6	12	<0.01	6.2
C-L ^h	5.9×10^6	5.3×10^3	0.18	79	79
C-M ^h	1.5×10^7	1.6×10^5	11	8.6	<0.02
D-L ^{g,h}	2.2×10^8	6.2×10^4	0.28	93	77
D-M ^{g,h}	7.5×10^8	3.7×10^5	0.50	3.5	0.7
E ^{g,i}	1.0×10^7	3.7×10^5	37	0.7	39
F	2.1×10^7	1.1×10^6	52	<0.01	3.8

^a Remaining toxicity (percent) after inactivation.^b i.p. LD₅₀/mg of protein.^c In number of i.p. LD₅₀.^d (Oral/i.p.) $\times 10^{-3}$.^e Incubated for 40 min at 35°C.^f Incubated for 180 min at 35°C.^g Toxicity after activation with trypsin.^h Figures given for L and M toxins of types C and D are the average of three strains of each type obtained in the present studies.ⁱ G. Sakaguchi and S. Sakaguchi (21).

toxin. Since the molecular sizes of the L toxins and the M toxins are approximately the same for the different *C. botulinum* types, the ratios of the L toxin and M toxin also denote the relative mouse oral toxicity per molecule of L and M toxins. The ratios of B-L, C-L, and D-L toxins were lower than those of the others, including M toxins, showing higher oral toxicities to mice than the others. D-M toxin was the highest in the oral toxicity among M toxins of the six types. Table 5 shows also that C-M and D-M toxins (12S) were more stable in gastric juice than in intestinal juice; to the contrary A-M, B-M, E, and F toxins (10-12S) were more stable in intestinal juice. Such differences in the stability of botulinum toxins in the digestive juice may reflect different molecular configurations or conformations of these toxins.

Oral toxicities of botulinum toxins of different types to the same animal species and those of the same type to various animal species have been investigated. It was reported by Quortrup and Gorham (18) that oral administration of 5×10^7 mouse i.p. minimal lethal doses (MLD) of type C toxin did not kill foxes. Yndestad et al. (28) also reported extremely high resistance of foxes to type C toxin, 5×10^6 mouse i.p. MLD being not fatal, while other workers reported that an oral MLD of type C toxin to foxes was 10^4 mouse i.p. MLD (24). The oral lethal dose of

type C toxin to minks has been reported to be in a wide range from 10^3 to 10^5 mouse i.p. MLD; 10^3 MLD according to Wagenaar et al. (29); 5×10^3 MLD according to Tjaberg and Skulberg (27); 10^5 MLD according to Quortrup and Gorham (18); and 10^5 MLD according to Karashimada et al. (9). Since C-L toxin is 30 times more potent in oral toxicity than C-M toxin as demonstrated in the present study, such diversity in oral toxicity of type C toxin to the same animal species may have possibly been due to the difference in the ratio of C-L to C-M toxin contents of the toxin preparations used by individual authors.

C. botulinum types C and D have exclusively been involved in animal botulism; a few outbreaks of human botulism caused by type C or D toxin have been reported. It has been demonstrated that the toxicity of types C and D is governed by specific bacteriophages and that the toxigenic strain could be converted into a nontoxigenic one and vice versa (2, 3, 6, 7). However, the relationship between toxigenic bacteriophages and the host cells, *C. botulinum* types C and D, was quite unstable probably due to pseudolysogeny (2, 3). This indicates that the toxigenicity of these types in nature may easily be lost under environmental conditions. It was suggested that the alteration of environmental factors, such as temperature, pH, salt concentration, etc., might influence the probability of lysis in lysogenic bacteria and that the balance between the lysogenic and lytic cycles may be the probable cause for variation in toxin production (5). In addition, it was noticed that toxigenic strains of *C. botulinum* type C require more strictly anaerobic conditions to grow and produce the toxin than other types (23, 24). Whether C-L and D-L toxins are produced in various types of food remains to be examined; the possibility may exist that human botulism could be caused by type C and D toxins if food containing large quantities of C-L or D-L toxin is ingested.

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