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Oral Toxicities of *Clostridium botulinum* Toxins in Response to Molecular Size

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Clostridium botulinum type A, B, and F toxins of different molecular sizes were fed to mice to compare the oral toxicities. The progenitor toxin, a complex of a toxic and nontoxic component, of any type was higher in oral toxicity to mice than the dissociated toxic component or the derivative toxin. The former may no doubt play a more important role in the pathogenesis of food-borne botulism. The higher oral toxicity possessed by the progenitor toxin, including the exceptionally high one found with type B-L toxin, can be explained solely by the protection afforded by the nontoxic component attached to the toxic component. The possibility of the highest oral toxicity of type B-L toxin to humans is discussed.

Clostridium botulinum toxin acts as a potent oral toxin. Human and animal botulism is caused principally by ingestion of food or feed containing preformed *C. botulinum* toxin in an amount larger than a certain threshold. Seven immunologically distinct types, types A through G, of the toxin are now known. In this laboratory, type A, B, E, and F toxins have been purified, and their molecular constructions have been studied. Three different molecular forms of type A toxin, namely, 12, 16, and 19S (designated as M, L, and LL toxins, respectively), were demonstrated (18). Crystalline type A toxin consists exclusively of the 19S material (3, 8, 18). In spent type A culture, only the 19S toxin was demonstrated by one procedure (17), whereas only the 12S toxin was shown by another method (18). Two different molecular forms of type B toxin, namely, 12 and 16S (designated as M and L toxins, respectively), were demonstrated in both spent culture and purified materials (7). The molecular sizes of type E and F toxins were found to be uniform in both culture and purified materials, the sedimentation constants being 11.6S (6) and 10.3S (10), respectively. Type E toxin in izushi, the most common vehicle for human botulism in Japan, was demonstrated to be exclusively of 12S (15). The actual molecular sizes of the toxins of other types in botulinogenic foodstuffs are not known.

All of these toxins, with molecular sizes larger than 10.3S, consist of one molecule each of the 6 to 7S toxic components and the same or larger-molecular-sized nontoxic components (13). It was proposed that the toxin in the form of a complex of the toxic and the nontoxic components, regardless of whether it is 12, 16, or 19S, should be called progenitor toxin, because it seems to be a natural state and the progenitor of the 6 to 7S toxic component or derivative toxin, which can be obtained only by laboratory manipulation at a pH above 7.2 (8).

Since the 7S toxic component, once it is separated from the nontoxic component, is much more sensitive to acid and proteolytic enzymes, it was postulated that the progenitor toxin only may act as oral toxin, whereas the derivative toxin, if any was ingested, may be destroyed rapidly in the stomach by gastric juice and pepsin (6, 11). In fact, a much larger dose of type E derivative toxin than the progenitor toxin was necessary to kill mice by oral administration (14). Sugiyama et al. (20), on the other hand, claimed that types A, B, E, and F derivative toxin had nearly the same oral toxicities to mice as the corresponding progenitor toxins.

The present investigation was undertaken to compare the oral toxicities in mice among the derivative and progenitor toxins of *C. botulinum* types A, B, and F and also among the 12, 16, and 19S progenitor toxins of types A and B.

MATERIALS AND METHODS

Toxins. Each of *C. botulinum* type A strain Hall, type B strain Okra, and type F strain Langeland were grown in a medium composed of 0.5% glucose, 0.5% yeast extract, 2.0% peptone, and 0.025% sodium thioglycolate at 30°C for 4 days. The progenitor toxins were purified by the procedures reported previously (7, 10, 18). Type A-M and B-M toxins (12S) were separated from respective L toxin (16S) by gel filtration on Sephadex G-200. Type A-L toxin used in this study was a mixture of L (16S) and LL (19S) toxins.

Type A crystalline toxin was obtained by the method described by Duff et al. (3). Its main component proved to be 19S.

The derivative toxin (7S) of each type was obtained by subjecting M toxin to chromatography on diethylaminoethyl-Sephadex A-50 at pH 8.0, as described previously (5).

Oral administration to mice. The toxin was serially diluted twofold in 0.05 M acetate buffer, pH 6.0, and 0.2-ml portions of each dilution were administered orally to five mice (strain ddY), weighing 18 to 24 g, with a slightly curved and blunt-tipped metal catheter. The mice were allowed pellet feed and drinking water ad libitum both before and after toxin administration. Oral administration of toxin was performed only in the morning between 9 and 11 a.m. Deaths in mice were observed for 5 days.

Calculation of toxicities. The intraperitoneal mean lethal dose (i.p. LD_{50}) was determined by the time-to-death method by injecting mice intravenously with 0.1-ml doses (16). Activation was accomplished by treating a toxin sample with trypsin at an enzyme-to-substrate ratio of 1:1 in 0.1 M acetate buffer, pH 6.0, for 30 min at 35°C.

The oral LD_{50} was calculated by the method of Reed and Muench (12) and expressed in an equivalent i.p. LD_{50} number.

RESULTS

The oral toxicities of *C. botulinum* type A, B, and F toxins of different molecular sizes are summarized in Table 1. The oral toxicity of the progenitor toxin of all types was significantly higher than that of the corresponding derivative toxin. This was always true, regardless of whether the derivative toxin was derived from M, L, or crystalline toxin. One oral LD_{50} of type A-M, B-M, and F progenitor toxins, whose molecular sizes were within a range of 10.3 to 12S, was of the same magnitude, being 1×10^6 to 4×10^6 i.p. LD_{50}.

Significantly higher oral toxicities were found with larger-molecular-sized toxins, such as type A crystalline toxin and type B-L toxin, than found with M toxin. The oral toxicity of type B-L toxin was particularly high; 1 oral LD_{50} of B-L toxin, corresponding to only 1,500 i.p. LD_{50}, was 700 times higher than M toxin and 16,000 times higher than the derivative toxin.

Type B-L toxin prepared from a 40-h culture instead of from a 4-day culture had an activation ratio (the ratio of the parenteral toxicity after activation to that before) of approximately 70. The oral toxicities of this preparation before and after tryptic activation were compared (Table 2). No significant difference in terms of potential i.p. LD_{50} was shown between them.

DISCUSSION

The present study has shown higher oral toxicities of the progenitor toxins than those of the derivative toxins of *C. botulinum* types A, B, and F, as was the case with the toxicities of type E (14). One oral LD_{50} was equivalent to 1×10^6 to 4×10^6 i.p. LD_{50} with respect to type A-M, B-M, and F progenitor toxins, the values being comparable to that of type E progenitor toxin

TABLE 1. *Oral toxicities of C. botulinum type A, B, and F toxins of different molecular sizes*

Type of toxin	Molecular form of toxin	Oral LD_{50} (in equivalent number of i.p. $LD_{50} \times 10^{-3}$)	Relative oral toxicity[a]
A	Crystalline	120	358
	L	2,200	19.5
	M	3,600	11.9
	Derivative	43,000	1.0
B	L	1.5	28,700
	M	1,100	39
	Derivative	24,000	1.8
F	Progenitor	1,100	39
	Derivative	>6,000	<7.2

[a] The oral toxicity of type A derivative toxin was taken as 1.0.

TABLE 2. *Oral toxicities of type B-L toxin before and after tryptic activation*

Activation	Dose in i.p. LD_{50} (potential i.p. LD_{50})[a]	No. of mice dying on day: 1	2	3	4	5	No. of dead/no. tested	Oral LD_{50} in i.p. LD_{50} (in potential i.p. LD_{50})
Before	57.1 (4,000)	0	1	2	0	1	4:5	
	28.6 (2,000)	0	1	0	0	0	1:5	40
	14.3 (1,000)	0	0	0	0	0	0:5	(2,800)
	7.1 (500)	0	0	0	0	0	0:5	
After	4,000	0	3	1	0	0	4:5	
	2,000	0	2	0	0	0	2:5	2,200
	1,000	0	0	0	1	0	1:5	
	500	0	0	0	0	0	0:5	

[a] The activation ratio (the ratio of the parenteral toxicity after tryptic activation to that before) of this material was 70.

(14). A 10- to 20-fold-larger quantity of the derivative toxin than this was necessary to kill the mice by the oral route. Since the toxic component, once separated from the progenitor toxin, becomes highly sensitive to acid and proteolytic enzymes (6, 11), binding to the nontoxic component results in the protection of the toxic component from destruction in the digestive tract. It may be only the progenitor toxin that causes food-borne botulism; the derivative toxin, even if it survived digestion by endogenous and exogenous proteolytic enzymes in food and was ingested, may hardly resist gastric juice or peptic digestion.

Among the toxins tested, type B-L showed an extraordinarily high oral toxicity to mice. Similarly, high oral toxicities for both type B progenitor toxin and derivative toxin were reported by Sugiyama et al. (20). We suspect that both of their materials contained L toxin. Dolman and Murakami (2), feeding monkeys with toxins of different types, found the highest toxicity with type B toxin. A lethal dose of type E toxin for humans was estimated to be about 500,000 LD_{50} (1), whereas that of type B toxin was as small as 3,500 LD_{50} (9). Such exceptionally high oral toxicities to humans and animals hitherto found with type B toxin can only be explained by the predominance of L toxin.

The present results may appear as though the larger the molecular size of the toxin, the higher the oral toxicity, although the reverse is the case with the parenteral toxicity. The oral toxicity of type B-L toxin (16S), however, was much higher than that of type A crystalline toxin (19S); therefore, it may depend upon not only the molecular size but also some other factor(s). The progenitor toxin may dissociate into the toxic and nontoxic components in the upper intestines, where the reaction may be slightly alkaline; therefore, the high resistance to acid and pepsin in the stomach may account for the high oral toxicity. Since the oral toxicity of the derivative toxin of M toxin is the same as that of L toxin, such exceptionally high oral toxicity found with type B-L toxin may be attributable to a specific structure of the nontoxic component attaching to the toxic component or that brought about as a result of joining the two components.

No difference in oral toxicity on a weight basis was found between unactivated and activated type B-L toxin, suggesting that the intramolecular nicking of the toxic component by trypsin (19) does not affect the oral toxicity and that activation is accomplished in the digestive tract, as is the case with type E toxin (4).

Detailed studies on the resistance of botulinum toxins of different types with different molecular sizes to acid and different proteolytic enzymes and their rates of absorption through the intestinal wall are now under way in this laboratory.

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