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Correlation Between Oral Toxicity and In Vitro Stability of *Clostridium botulinum* Type A and B Toxins of Different Molecular Sizes

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The in vitro sensitivity to acid and pepsin differed markedly among *Clostridium botulinum* type A and B toxins of different molecular sizes. The larger the molecular size of the toxin, the higher the resistance to these agents. Type B derivative toxin was rapidly inactivated, but the progenitor toxins resisted in vitro exposure to rat intestinal juice. The molecular dissociation of the progenitor toxins did not occur in rat intestinal juice of pH 7.0, but did occur in a buffer solution of the same pH. The oral toxicity may depend mostly on the stability of toxin molecules in the stomach and, to a less extent, in the intestine. The present results seem to justify the conclusion that *C. botulinum* type A and B progenitor toxins with molecular sizes larger than 16*S* are more potent oral toxins than 12*S* progenitor toxins.

Botulism is a neuroparalytic disease generally caused by ingestion of the preformed toxin of *Clostridium botulinum* in food. Inconsistency concerning molecular size has been described for *C. botulinum* progenitor toxins: 19, 16, and 12*S* for type A, 16 and 12*S* for type B, 12*S* for type E, and 10*S* for type F (7). Each progenitor toxin is made up of a toxic component (derivative toxin) of 7*S* and a nontoxic component of the same or larger size (7).

According to Sugiyama et al. (13), there was little or no difference in the oral toxicity to mice between the progenitor and the derivative toxins. According to Sakaguchi and Sakaguchi (11), however, the oral toxicity to mice of type E derivative toxin was markedly lower than that of the progenitor toxin. They concluded, therefore, that it is not the derivative toxin but the progenitor toxin that causes food-borne type E botulism. It has been demonstrated more recently that the larger the molecular size, the more potent the oral toxicity with type A, B, and F toxins, and that type B-L toxin (16*S*) is an exceptionally potent oral toxin, its oral toxicity being 700 times higher than that of any other 12*S* progenitor toxin (10).

It has been reported that type A crystalline toxin is resistant to pepsin and papain but susceptible to trypsin, chymotrypsin, and some other proteolytic enzymes (1, 3, 8). It has also been reported that type A crystalline and type E and F progenitor toxins are significantly more stable than the corresponding derivative toxins, particularly at low pH values (5, 9).

To scrutinize the stabilities of botulinum toxins of different molecular sizes in the digestive tracts and to correlate them with the different oral toxicities, we examined the in vitro stability of type A and B toxins in gastric and intestinal juice of rats.

MATERIALS AND METHODS

Toxins. Type A (strain Hall) and type B (strain Okra) progenitor toxins were purified by the methods reported previously (6, 12). L (large-sized) toxin denotes, in this paper, a mixture of 19 and 16*S* toxins for type A and uniform 16*S* toxin for type B; M (medium-sized) toxin denotes 12*S* toxin. The derivative toxin (7*S*) was separated from each of type A-M and B-M toxins by chromatography on diethylaminoethyl-Sephadex A-50, at pH 8.0 (5). Type A crystalline toxin, consisting of mainly 19*S* molecules, was obtained according to the method of Duff et al. (2).

The specific toxicities, in mean lethal doses (LD_{50}) per milligram of N (in potential toxicities for type B toxins, which were trypsin activable, the activation ratios being 10, 10, and 4 for B-L, B-M, and B-S toxins, respectively), of the toxins were: 2.5×10^8 for A crystalline, 2.2×10^8 for A-L, 4.0×10^8 for A-M, 3.3×10^8 for A-S, 2.0×10^8 for B-L, 4.5×10^8 for B-M, and 1.2×10^8 for B-S.

Collecting gastric juice and intestinal juice from rats. Gastric juice and intestinal juice were obtained separately from 250- to 300-g Wistar male rats after abstinence for 20 to 24 h. Each rat was anesthetized with sodium pentobarbital (5 mg/100 g of body weight), and the abdomen was opened along the midline. For collecting the gastric juice, a fine glass tube was inserted through a small incision made on the upper duodenum and pushed through the pylo-

rus into the stomach. The pylorus was ligated. For collecting the intestinal juice, a segment of about 7 cm of the duodenum (from the pylorus) was tied off with two ligatures. After a few drops of penicillin (20,000 U/ml) were placed in the abdominal cavity, the incision was stitched. The surgically treated rats were kept in restraining cages for 8 h, during which period the gastric juice was collected continuously. After the rats were killed, the intestinal juice that accumulated in the lumen of the ligated duodenum was taken out. The collected gastric juice and the intestinal juice were allowed to stand for a few hours at 4°C, and the supernatant fluids were used for experiments.

Tests for stability at different pH values. Each of the progenitor and derivative toxins (5 μg/ml) was exposed to 35°C for 30 min at pH values from 1.0 to 6.0. The buffers used were 0.2 M sodium acetate-hydrochloride (pH 1, 2, 3, and 4) and 0.05 M sodium acetate-acetic acid (pH 6). One volume of toxin sample was mixed with 9 volumes of buffer, and the final pH was adjusted to the indicated value with NaOH or HCl.

Digestion with proteolytic enzymes. A portion of toxin (50 μg/ml) was incubated with pepsin (50 μg/ml) at 35°C in 0.2 M sodium acetate-hydrochloride buffer, pH 2.0. Samples, taken periodically, were diluted with 9 volumes of 0.05 M acetate buffer, pH 6.0, and injected intravenously into mice. The toxicities of these samples are given as percentages of the controls, which consisted of toxin without enzyme incubated for the same length of time.

Toxin (5 μg/ml) was incubated with pancreatin (0.8 mg/ml) in 0.1 M sodium phosphate buffer, pH 6.5, for 30 min at 35°C. The activity of 0.8 mg of pancreatin per ml was equivalent to that of 0.1 mg of trypsin per ml when assayed with casein substrate.

Tests for stability in gastric and intestinal juice. One volume of toxin (50 μg/ml) was incubated with 9 volumes of the gastric juice (pH 1.4) at 35°C. One volume of activated type B toxin (0.25 mg/ml) was incubated with 19 volumes of the intestinal juice (pH 7.0). After the indicated incubations, one volume of each sample was added to 9 volumes of 0.05 M acetate buffer, pH 6.0, to perform mouse intravenous injection. For incubation with the intestinal juice, type B toxin was activated with trypsin in 0.005 M acetate buffer, pH 6.0. The ratio of toxin to trypsin was 1:1 for the progenitor toxin and 10:1 for the derivative toxin.

Other procedures. Protein content, toxicity, and molecular size were determined by the methods described previously (12).

RESULTS

Stability at different pH values. The effect on different molecular-sized toxins of pH levels found in the stomach was studied by incubating toxin for 30 min at 35°C in buffer solutions of different pH values lower than 6. The derivative toxins of types A and B lost almost all toxicity at pH 3.0 (Fig. 1A and B). The progenitor toxins were more stable at low pH values than the derivative toxins. Type A crystalline

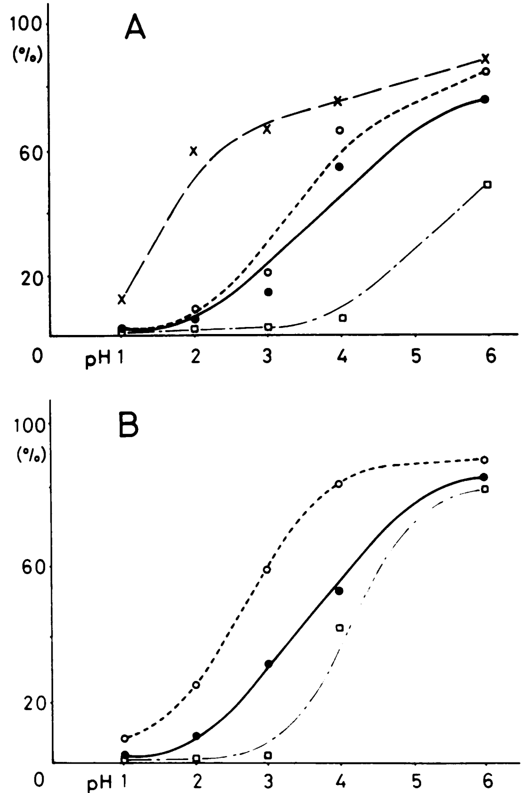

FIG. 1. *Resistance of different molecular-sized toxins to exposure to different pH values. (A) Type A toxin; (B) type B toxin. The toxicity of each toxin in 0.05 M acetate buffer, pH 6.0, was taken as 100. Symbols: ×, type A crystalline toxin; ○, L toxin; ●, M toxin; □, derivative toxin.*

toxin was considerably more stable than type A-L and A-M toxins at different pH values (Fig. 1A). Similarly, type B-L toxin was more stable than B-M toxin (Fig. 1B).

Resistance to peptic digestion. Type A and B derivative toxins lost their toxicities completely after 10 min of peptic digestion at pH 2.0 and 35°C. After incubation for 80 min, the remaining toxicity of type A crystalline and B-L toxins was about 60% or more; the remaining toxicity of type A-L, A-M, and B-M toxins was about 10% (Fig. 2).

Stability in gastric juice. Type B-L, B-M, and derivative toxins were incubated separately in gastric juice (Fig. 3). Type B-L toxin was the most stable; the derivative toxin was detoxified rapidly, becoming nontoxic within 10 min. Type B-M toxin was more stable than the derivative toxin, and about 10% of the toxicity remained after 80 min. The inactivation curves of the three toxins, respectively, resembled

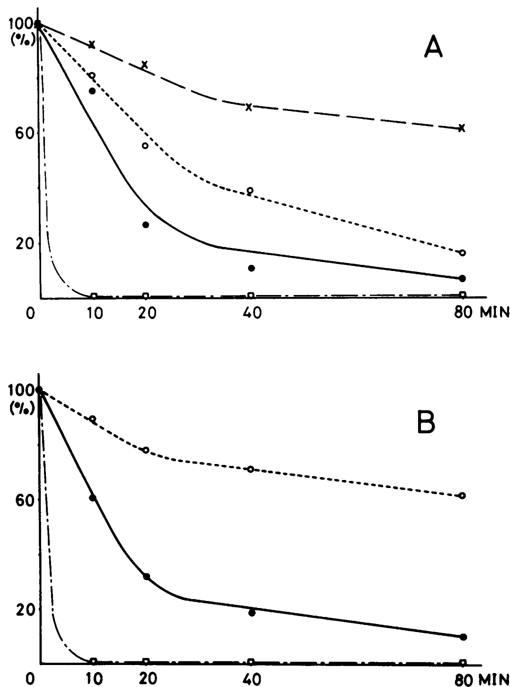

FIG. 2. *Resistance of different molecular-sized toxins to pepsin. (A) Type A toxin; (B) type B toxin. The toxicity of each toxin in 0.2 M sodium acetate-hydrochloride, pH 2.0, was taken as 100. Symbols: ×, type A crystalline toxin; ○, L toxin; ●, M toxin; □, derivative toxin.*

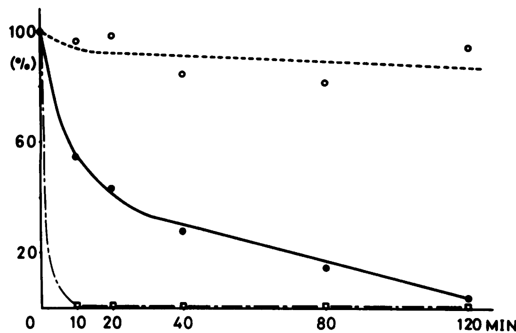

FIG. 3. *Resistance of type B toxins to in vitro exposure to gastric juice. The toxicity of each toxin in gastric juice at zero time was taken as 100. Symbols: ○, L toxin; ●, M toxin; □, derivative toxin.*

those obtained by treating them with pepsin at pH 2.0 (Fig. 2B).

Resistance to pancreatic digestion. To investigate stability of toxins in the small intestine, resistance of the toxins to pancreatin was examined at pH 6.5. Type A crystalline, A-L, and A-M toxins were found to be similarly resistant to pancreatin (Table 1). Type B-L and B-M toxins were activated with pancreatin to a similar extent. It can be seen that type A and B derivative toxins were partially inactivated with pancreatin in 30 min.

Stability in intestinal juice. When incubated in the intestinal juice of rats at 35°C, activated type B derivative toxin lost about 90% of its toxicity in 10 min and 100% in 40 min. Under the same conditions, both activated type B-L and B-M toxins retained about 60% of their toxicity after incubation for 120 min (Fig. 4). Untreated type B progenitor toxins were activated with intestinal juice.

To see whether molecular dissociation takes place in the small intestine, where the pH is about 7, type B progenitor toxins were centrifuged in a 5 to 20% sucrose density gradient at pH 6.5, 6.75, and 7.0 for 10 h at 4°C at 132,000 × *g* (Fig. 5). Type B-L toxin sedimented to the position of 16*S* at pH 6.5; type B-M toxin sedimented at 12*S*. At pH 6.75, type B-L toxin sedimented in two toxic peaks of 16*S* and 7*S*, and type B-M toxin also sedimented in two peaks of 12*S* and 7*S*. At pH 7.0, both toxins showed a single toxic peak at the position of 7*S*.

In the same sucrose density gradient pre-

TABLE 1. *Pancreatic digestion of type A and B toxins*

Toxin	Toxicity after pancreatic treatment (%)[a]
A crystalline	89
A-L	75
A-M	79
A-S[b]	56
B-L	785
B-M	785
B-S[b]	191

[a] With 0.8 mg of pancreatin per ml for 30 min at 35°C at pH 6.5.

[b] S (small-sized) denotes derivative toxin.

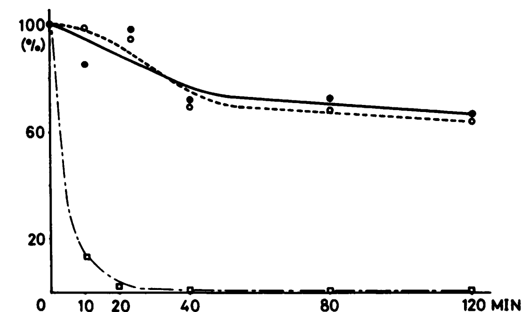

FIG. 4. *Resistance of type B toxins to in vitro exposure to intestinal juice. The toxicity of each toxin in intestinal juice at zero time was taken as 100. Symbols: ○, L toxin; ●, M toxin; □, derivative toxin.*

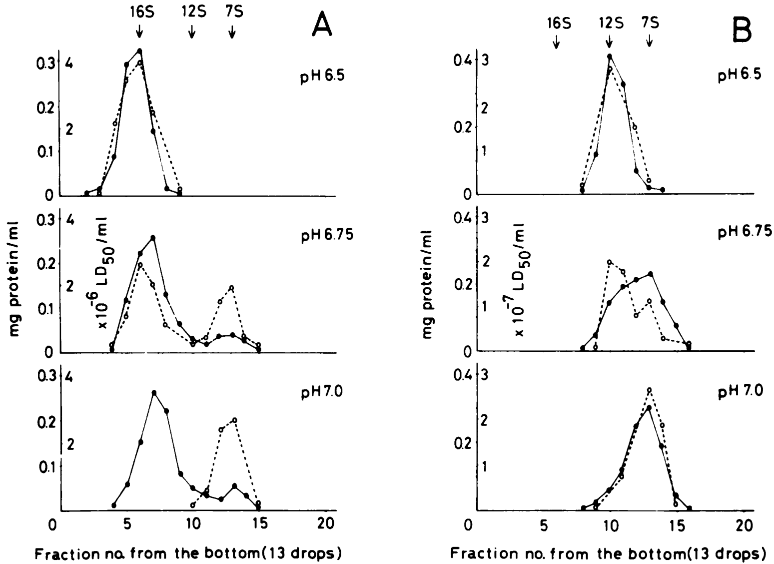

FIG. 5. *Ultracentrifugal analyses of type B progenitor toxins in a sucrose density gradient (5 to 20%) prepared in buffers at pH 6.5, 6.75, and 7.0. (A) Type B-L toxin; (B) type B-M toxin. A 0.2-ml sample of L toxin contained 0.177 mg of protein and 5 × 10^6 LD_{50} of toxicity and that of M toxin contained 0.176 mg of protein and 6.6 × 10^6 LD_{50} of toxicity. Symbols: ○, toxicity; ●, protein content.*

pared in the intestinal juice of pH 7.0, type B-L and B-M toxins sedimented to the positions of 16S and 12S, respectively (Fig. 6).

DISCUSSION

The orally ingested botulinum toxin may be attacked by various agents in the digestive tract before absorption, e.g., acid, alkali, and various enzymes such as pepsin, trypsin, chymotrypsin, etc. The oral toxicity may, therefore, largely depend on the resistance to these agents. The marked difference in in vitro stability found in type E progenitor as opposed to derivative toxins (5) may account for the different oral toxicities to mice (11). Markedly different resistance to acid and pepsin was found between the progenitor and the derivative toxins of types A and B. The different oral toxicities of the progenitor as opposed to the derivative toxins, therefore, result mostly from the differences in stability in the stomach, where the toxin is attacked by both acid and pepsin. On the basis of this as well as previous results (5, 9), there is little or no possibility for the derivative toxin to pass in its intact form through the stomach. The unknown specific structure of the complexed form of the toxic and the nontoxic components may exempt the progenitor toxin from inactivation by acid and pepsin. Thus, the oral toxicity of the progenitor toxin could not be similar to (13), but should be higher (10, 11) than, that of the corresponding derivative toxin.

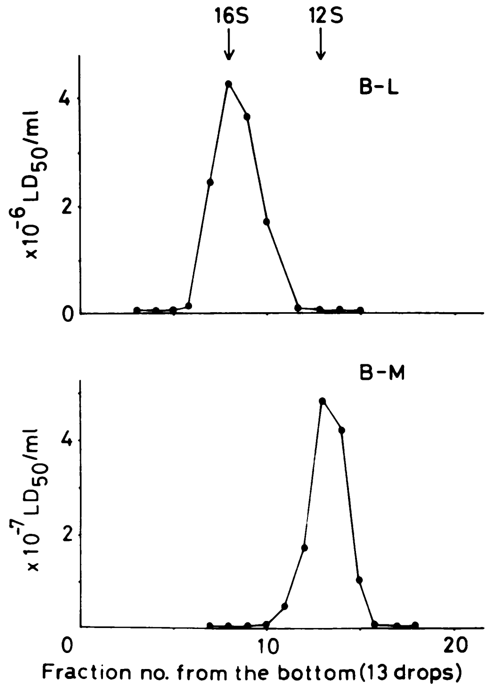

FIG. 6. *Ultracentrifugal analyses in a sucrose density gradient (5 to 20%) of type B progenitor toxins prepared in the intestinal juice, pH 7.0. A 0.2-ml portion of L toxin contained 0.245 mg of protein and 4 × 10^6 LD_{50} of toxicity, and that of M toxin had 0.150 mg of protein and 4.6 × 10^6 LD_{50} of toxicity. Symbol: ●, toxicity.*

It appeared also that the larger the molecular size of the toxin of a given type, the higher the resistance to pepsin and gastric juice. Type A crystalline toxin was previously shown to be resistant to pepsin (3, 8). It seems that this resistance is dependent not only on the molecular size but also on some other factors, since type B-L toxin was more resistant than A-L toxin (the former consisted of the 16*S* molecules only, whereas the latter was a mixture of 16*S* and 19*S* molecules). The extraordinarily high oral toxicity found with type B-L toxin (10) may have resulted at least in part from the high resistance in the stomach.

By both pancreatic digestion at pH 6.5 and exposure to intestinal juice of pH 7.0, the derivative toxin was found to be partially or completely inactivated; the progenitor toxin resisted both treatments. It has been reported that type A crystalline toxin is susceptible to trypsin and chymotrypsin (1, 3, 8); prolonged incubation with a larger amount of these enzymes would inactivate more progenitor toxins. The results indicate that the derivative toxin, if not inactivated in the stomach, would be destroyed by the proteolytic enzymes in the small intestine.

The molecular dissociation of the progenitor toxin depends upon such factors as pH and ionic strength of the medium (4, 14). Both type B-L and B-M toxins dissociated to a similar extent in a buffer solution at pH values above 6.75, whereas neither toxin dissociated in intestinal juice at pH 7.0. This fact accounts for the greater stability found with type B progenitor toxins than with the derivative toxins; also, it indicates that the progenitor toxin, after resisting destruction in the stomach, would not dissociate in the intestine and would resist the proteolytic enzymes until absorbed.

The most important factor that determines the oral toxicity of a toxin may, perhaps, be its stability in the stomach, and the next important factor may be stability in the intestine. The present results strongly support the postulate that it is not the derivative toxin but the progenitor toxin, a complex of the toxic and the nontoxic components, that acts as an oral toxin in food-borne botulism (5).

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