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Intestinal Absorption of Botulinum Toxins of Different Molecular Sizes in Rats

SHUNJI SUGII, IWAO OHISHI, AND GENJI SAKAGUCHI*

College of Agriculture, University of Osaka Prefecture, Sakai-shi, Osaka 591, Japan

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During a period of 10 to 12 h after injection of type B 16S (L) toxin into the ligated duodenum of rats, 0.01 to 0.1% of the total toxicity administered was found in the lymph drawn by cannulation of the thoracic duct. The recovery was 50 to 100 times higher than that of the rat given type B 12S (M) or 7S (S) toxin. During the same period, 0.6 to 1.5% of the specific antigens were recovered, regardless of the molecular size of the toxin that had been administered. In lymph of the B-L or B-M toxin recipient, the toxic and nontoxic components were detected in comparable quantities, indicating that the undissociated progenitor toxin molecule is absorbed through the intestinal wall. Although the toxic component had lost its toxic activity, the two components of B-M toxin appearing in lymph reassembled to reconstruct the 12S molecule, whereas those of B-L toxin did not, although the toxic component was still active. Type B-L, B-M, and B-S toxins showed similar stabilities to in vitro exposure to rat lymph (pH 8.2), but B-L toxin showed a considerably higher stability to intestinal juice (pH 7.0) than did B-M toxin. Thus, the toxicity of lymph of rats administered botulinum toxin intraduodenally depends not upon the rate of absorption, but largely upon the stability in the intestine.

Of the seven immunologically distinct types, A through G, of botulinum toxin, human disease has been caused more frequently by one or more of types A, B, and E and less frequently by type F toxins. The progenitor toxins of these four types have been purified, and their gross molecular constructions have been clarified. They are heterogeneous in molecular size: 19, 16, and 12S for type A (24); 16 and 12S for type B (12); 12S for type E (10); and 10S for type F (17). The progenitor toxin of any size is a complex of a 6-7S toxic component and a 6-7S, or larger-molecular-size, nontoxic component (13).

We have reported that it is not the derivative toxin but the progenitor toxin that causes foodborne botulism (18, 21). Type A crystalline (19S) and type B-L (16S) toxins showed higher oral toxicities than other progenitor toxins of types A, B, and F, and type B-L toxin showed an exceptionally high oral toxicity (18). Such different oral toxicities were ascribed to the different stabilities, principally in the stomach (23).

The orally ingested progenitor toxin, surviving attacks by gastric juice and pepsin, is absorbed from the upper intestine into the lymphatic system (7, 9, 15). The toxin appearing in lymph and blood of experimental animals fed orally with a type A crystalline or type E progenitor toxin was found to possess a molecular dimension of about 7S (9, 11, 19). It is not known, however, whether both the toxic and nontoxic components of the progenitor toxin, in the intact or dissociated form, are absorbed, or whether the dissociated toxic component only is absorbed through the intestinal barriers.

We undertook the present investigation to answer these questions, using rats and purified preparations of three type B toxins of different molecular sizes. To locate the site of absorption, the stomach and each portion of the intestine were tied off. Such ligation may have obstructed physiological peristalsis of the intestine, but accelerated absorption of the toxin and facilitated location of the portion where absorption of the largest quantity of the toxin occurred. The results demonstrated the absorption of both components of type B toxins of different molecular sizes from the rat small intestine. Attempts were also made to explain oral toxicities so markedly different from one progenitor toxin to another regardless of similar absorption rates.

MATERIALS AND METHODS

Toxins. The progenitor toxins of types A (strain Hall), B (strain Okra), E (strain German sprats), and F (strain Langeland) were purified by the methods reported previously (10, 12, 17, 24). Two type A and B progenitor toxins of different molecular sizes were

eluted from Sephadex G-200: one was 12S (medium-sized or M toxin), and the other was 16S for type B and a mixture of 19 and 16S toxins for type A (large-sized or L toxin) (12, 24). Type A crystalline toxin (mainly 19S) was obtained according to Duff et al. (8). The derivative toxin (7S) (small-sized or S toxin) was separated from type B-M toxin by chromatography on diethylaminoethyl-Sephadex A-50 at pH 8.0 (11). The activation ratios of type B and E progenitor toxins were 5 and 200, respectively. Untrypsinized type B or E toxin was administered to rats, but its toxicity is expressed as potential toxicity (the toxicity attainable by trypsinization at pH 6.0 for 30 min at 30°C).

Chemicals. Twice-crystallized trypsin was the product of Sigma Chemical Co., St. Louis, Mo.; potassium penicillin G was from Meiji Seika, Tokyo; heparin (sodium salt) was from Wako Pure Chemical Industries, Osaka; and sodium pentobarbital was from Abbott Laboratories, Chicago, Ill. Sephadex G-200 (particle size, 40 to 120 μm), diethylaminoethyl-Sephadex A-50 (medium), and cyanogen bromide-activated Sepharose 4B were the products of Pharmacia Fine Chemicals, Uppsala, Sweden.

Collecting intestinal juice and lymph from rats. Lymph and intestinal juice (actually a mixture of bile and pancreatic and intestinal juices) were obtained from 250- to 300-g Wistar male rats by the methods described elsewhere (23). The animals were anesthetized by intraperitoneal (i.p.) injection with sodium pentobarbital (5 mg/100 g of body weight), and the abdomen was opened along the midline.

To collect intestinal juice, a segment of about 7 cm was made on the duodenum from the pylorus with two ligatures. The rat was killed in 8 h, and the intestinal juice accumulated in the segment was taken out.

Lymph was collected continuously by cannulation of the thoracic duct with fine polyethylene tubing (Intramedic polyethylene tubing, Clay Adams, Parsippany, N.J.; inner diameter, 0.58 mm; outer diameter, 1.0 mm) according to Bollman et al. (1). Lymph was drawn into test tubes (13 by 75 mm), the inside of which had been coated with a heparin solution in saline (100 U/ml). The lymph flow was 0.25 to 0.5 ml/h.

After the cannulation, a 0.1-ml dose of toxin was injected through the curvature into the stomach with a 0.5-ml syringe attached to a fine hypodermic needle. To inject the toxin into the desired part of the intestine, an approximately 5-cm segment was isolated with two ligatures. A 0.5-ml dose of toxin was injected into the segment through the pylorus or the proximal end.

Care was taken not to spill the toxin into the abdominal cavity by using a piece of absorbent cotton wet in 70% ethanol. Drops of penicillin (20,000 U/ml) were applied to the abdominal cavity, and the incision was sutured. The rats were allowed free access to drinking water and kept in restraining cages during collection of lymph.

To draw blood periodically from the portal vein, the abdominal incision was not sutured, and rats were kept under light anesthesia throughout the experimental period.

RPHA. Rabbits were immunized with the 12S toxin of each type according to Sakaguchi and Sakaguchi (20). Anti-toxic and anti-nontoxic component immunoglobulins were purified from the anti-12S toxin serum by affinity chromatography on cyanogen bromide-activated Sepharose 4B coupled with each of the toxic and nontoxic components (22). Sheep erythrocytes Formalin treated and coupled with each of the purified anti-toxic and anti-nontoxic component immunoglobulins were used for reversed passive hemagglutination (RPHA) to titrate lymph for each of the toxic and nontoxic components (22). With the microtiter technique, the minimal quantity of each component detectable was 3.9 to 15.6 ng/ml.

Stabilities of type B toxins in intestinal juice and lymph. Each toxin was trypsinized at pH 6.0 by the method reported (23). One volume of the activated toxin (0.25 mg/ml) was incubated with 19 volumes of intestinal juice (pH 7.0) or lymph (pH 8.2) at 35°C. After incubation, each sample was diluted 10-fold in 0.05 M acetate buffer, pH 6.0, to inject mice.

Toxicity assay and other determinations. Toxicities of lymph and blood serum were determined by the time-to-death method by injection into mice. From a standard dose-response curve prepared with the 12S toxin of each type, the i.p. mean lethal dose (LD_{50}) per milliliter was estimated (10, 12, 17, 24).

Protein contents and molecular sizes were determined by methods reported previously (24).

RESULTS

Toxicities of lymph of rats administered progenitor toxins of different types intragastrically. A 0.1-ml dose each of type A, B, E, and F progenitor toxins, containing 1.5×10^6 to 8.2×10^8 i.p. LD_{50}, equivalent to about 1 oral LD_{50} of M toxin, was injected into the stomach. A 0.5-ml portion of lymph drawn during every 4-h period was injected i.p. into a mouse. Lymph of the type A crystalline or B-L toxin recipient only was lethal to mice throughout the 24- or 28-h period, during which period none of the surgically treated rats died (Table 1).

Site of absorption of the toxin in the alimentary canal. To find the site of absorption of the toxin, 0.5-ml doses of type B-L toxin, each containing 4×10^7 i.p. LD_{50}, were introduced into ligated segments of the duodenum, jejunum, ileum, cecum, colon, and rectum made

TABLE 1. *Toxicity of lymph after intragastric administration of toxin*

Toxin	Dose (i.p. LD_{50})	Toxicity[a] at time (h) after administration					
		4	8	16	20	24	28
A-crystalline	37×10^5	D	D	D	D	D	
A-L	15×10^5	S	S	S	S	S	S
A-M	33×10^5	S	S	S	S	S	S
B-L	32×10^{5b}	D	D	D	D	D	D
B-M	57×10^{5b}	S	S	S	S	S	S
E	41×10^{3b}	S	S	S	S	S	S
F	72×10^5	S	S	S	S	S	S

[a] D, 0.5 ml of lymph was lethal to mice; S, nonlethal.
[b] Apparent toxicity.

in separate animals. The lymph of the rat given the toxin intraduodenally was the most toxic, reaching about 10^4 LD_{50}/ml in about 6 h; the specimens of lymph of other rats showed toxicities lower than 10^3 LD_{50}/ml. The blood drawn from the rat administered the toxin intraduodenally contained a toxicity lower than 10 LD_{50}/ml. No toxic lymph was obtained from the rat given the toxin into the stomach that had been tied off. Nearly 30% of the toxicity administered was recovered by washing the stomach with saline in 10 h.

Toxicities of lymph of rats administered B-L, B-M, or B-S toxin intraduodenally. The rates of absorption from the duodenum of type B toxins of different molecular sizes were compared. A 0.5-ml dose each of B-L, B-M, and B-S toxins contained 8×10^7 i.p. LD_{50} and 1.5, 1.0, and 0.5 mg of protein, respectively. Each was injected into the ligated duodenum. The lymph of the type B-L toxin recipient reached a toxicity of 2.5×10^4 LD_{50}/ml in 6 h; that of the type B-M toxin recipient reached a toxicity of 2×10^2 LD_{50}/ml (Fig. 1). The toxicity recovered in lymph of the B-L toxin recipient was about 0.01 to 0.1% of that administered; that of the B-M toxin recipient was only 1 to 2% that of the B-L toxin recipient. Similarly, low toxicities (lower than 100 LD_{50}/ml) of lymph were detected after intraduodenal administration with 1.0 mg of type A-L, A-M, or E or F progenitor toxin (Table 2). Within a range of 10^7 to 10×10^7 LD_{50}/injection, the toxicity appearing in lymph increased by 8 to 10 times as the dose was doubled. When kept enclosed in the ligated duodenum for 10 to 12 h, both type B and E progenitor toxins became no longer activable with trypsin. During the enclosure, the toxicity of either toxin decreased to about 10 to 15%.

RPHA tests with lymph of rats administered B-L, B-M, or B-S toxin intraduodenally. RPHA tests detected both the toxic and nontoxic components to the same titers in lymph of rats administered any toxin (Fig. 1). The rates of appearance of the constituent components of each toxin in lymph were nearly the same, reaching the highest titer in about 2 h, in contrast to the 6 h needed to reach the highest toxicity. RPHA tests detected about 0.6 to 1.5% of the toxic and the same percentage of the nontoxic components in lymph of either the B-L or B-M toxin recipient.

In vitro stabilities of type B toxins in intestinal juice and lymph. Stabilities of B-L and B-M toxins in intestinal juice (pH 7.0) and lymph (pH 8.2) were examined to explain the different toxicities, but the same RPHA titers, of lymph determined after intraduodenal administration. The remaining toxicities of the

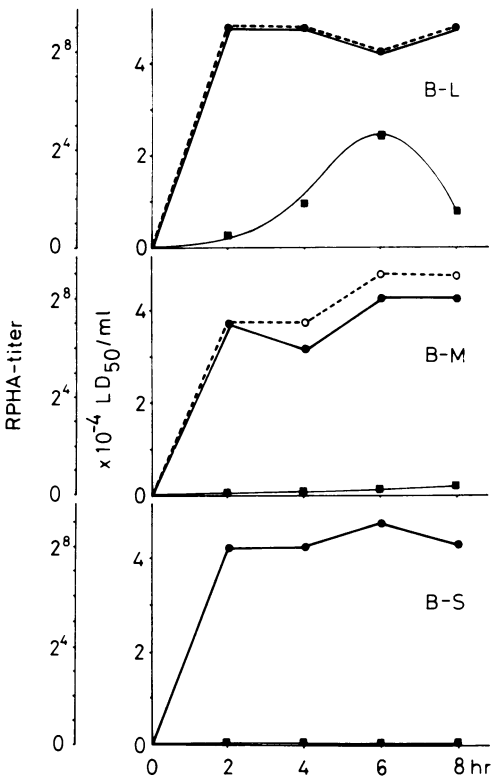

FIG. 1. *Absorption of B-L, B-M, and B-S toxins from the ligated duodenum. A 0.5-ml dose of each toxin containing 8×10^7 i.p. LD_{50} was injected into a 5-cm segment of the duodenum of rats. Symbols: ■, toxicity; ●, RPHA titer of the toxic component; ○, RPHA titer of the nontoxic component. The plots of the latter two coincided at each determination.*

two toxins in intestinal juice of pH 7.0 did not significantly differ from each other within 2 h. Upon further incubation, however, B-M toxin lost about 60% of the toxicity in 3 h and 100% in 6 h, whereas type B-L toxin retained about 15% of the toxicity even after 12 h (Fig. 2). When exposed to rat lymph of pH 8.2, little difference was found in stabilities among the three toxins (Fig. 3).

Molecular sizes of the antigenic proteins in lymph. Each of the three type B toxins was introduced into the ligated duodenum; the molecular sizes of the toxic and nontoxic components appearing in lymph were determined. A 0.5-ml dose contained a toxicity of 7×10^7 i.p. LD_{50} and 2.0 mg of protein for B-L toxin and 3.5×10^8 i.p. LD_{50} and 3.5 mg of protein for B-M toxin. A 0.2-ml portion of lymph taken 4 to 6 h after administration, containing 6×10^3 i.p. LD_{50}, was centrifuged in a 5 to 20% sucrose density gradient in 0.05 M phosphate buffer, pH 6.0, at 132,000 × *g* for 6 h at 4°C. Both toxic and

TABLE 2. *Toxicity of lymph after intraduodenal administration of 1.0 mg of toxin*

Toxin	Dose (i.p. LD_{50})	Toxicity[a] at time (h) after administration				
		2	4	6	8	10
A-crystalline	4×10^7	D	D	D	D	D
A-L	3×10^7	D	D	D	D	D
A-M	6×10^7	D	D	D	D	D
B-L	4×10^{7b}	5,100[c]	6,000	6,400	1,900	1,500
B-M	8×10^{7b}	D	D	D	D	D
E	15×10^{6b}	D	D	D	D	D
F	12×10^6	D	D	D	D	D

[a] D, 10^2 i.p. LD_{50}/ml or lower.
[b] Potential toxicity.
[c] i.p. LD_{50} per milliliter.

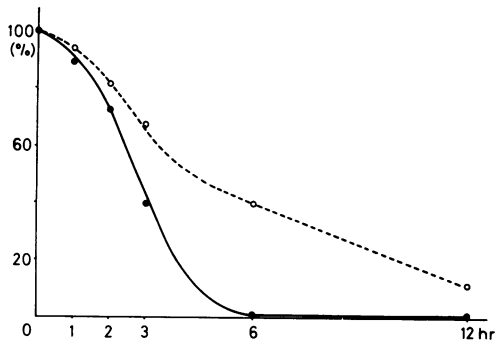

FIG. 2. *In vitro stabilities of B-L and B-M toxins in rat intestinal juice (pH 7.0). The toxicity of each toxin in intestinal juice at zero time was taken as 100. Symbols: ○, B-L toxin; ●, B-M toxin.*

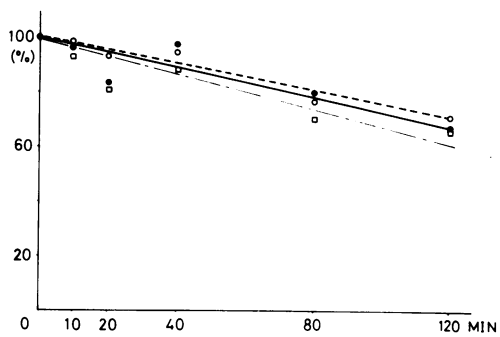

FIG. 3. *In vitro stabilities of B-L, B-M, and B-S toxins in rat lymph (pH 8.2). The toxicity of each toxin in lymph at zero time was taken as 100. Symbols: ○, B-L toxin; ●, B-M toxin; □, B-S toxin.*

nontoxic components in lymph of the B-M toxin recipient showed the highest RPHA titers at the position of 12S (Fig. 4). Similar results were obtained in lymph of A-M, E, and F progenitor toxin recipients. On the other hand, the toxic component of the B-L toxin recipient showed the highest RPHA titer at the position of 7S, whereas the nontoxic component showed it at the position of about 14S (Fig. 4).

DISCUSSION

The orally ingested botulinum toxin is absorbed mostly from the upper intestine (3, 7, 9, 15). The absorbed toxin is transported mostly into the lymphatic and only slightly into the blood stream (9, 15). The present results confirmed these findings. Some absorption of botulinum toxin from the stomach, the colon, and the ileum has been observed in dogs and monkeys (3, 5, 6). We also demonstrated some absorption of the toxin from any part of the intestine, but the rate of absorption from the duodenum was by far the highest. In the present investigation, each portion of the intestine was ligated, unlike in prior published work (9, 15), but the present findings may also hold true for normal rats. No appreciable absorption from the stomach was demonstrated.

The lymph drawn from the B-L toxin recipient was 50 to 100 times more toxic than that from the B-M toxin recipient and recovered 0.01 to 0.1% of the toxicity administered. Nevertheless, the RPHA titers of lymph of the B-L, B-M, and B-S toxin recipients were on the same level. The antigenicity recovered in lymph was 0.6 to 1.5% of that administered, and the recovery in antigenicity was 15 to 60 times higher than that in toxicity. Furthermore, RPHA tests demonstrated both toxic and nontoxic components in comparable quantities in lymph of the progenitor toxin recipients. It seems that toxins of different molecular sizes are absorbed at similar rates from the intestine, but the smaller the molecular size, the larger the portion detoxified before appearing in lymph. Such detoxification does not accompany appreciable impairment of the antigenicity of either component.

Such a high oral toxicity found with B-L toxin, being 700 times as high as that of B-M toxin (18), may have been due partly to the high resistance to acid and pepsin in the stomach

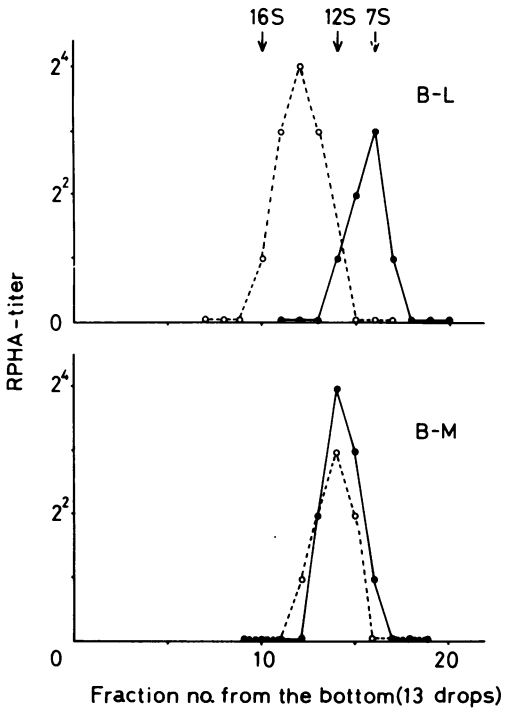

FIG. 4. *Ultracentrifugation in 5 to 20% sucrose density gradient in 0.05 M phosphate buffer, pH 6.0, of lymph of rats given B-L or B-M toxin intraduodenally. A 0.2-ml sample of each toxin contained 6 × 10^3 LD_{50}. Symbols: ●, RPHA titer of the toxic component; ○, RPHA titer of the nontoxic component. For sedimentation markers, B-L (16S), B-M (12S), and B-S (7S) toxins were used.*

(23) and partly to the high stability in the intestine.

The recovery of the lethal activity in lymph after intraduodenal administration of B-L toxin was 0.01 to 0.1%, which is comparable to that after administration of type A crystalline toxin (9, 14, 15). A recovery of 1.5% or more of the lethal activity in the blood of rabbits administered type E toxin orally was reported (19). Trypsinization of type B or E progenitor toxin before administration may not significantly affect the toxicity of lymph, as tryptic activation takes place in the small intestine (18, 23). The discrepancy may have been due to either the animal species or the preparation of the toxin.

A marked difference in the stability between B-L and B-M toxins was found by prolonged exposure to rat intestinal juice of pH 7.0. The difference within 2 h was too small to be recognized by the mouse test (23). On the other hand, the stabilities of the three different type B toxins in rat lymph of pH 8.2 were similar. The different toxicities of lymph between B-L and B-M toxin recipients may, therefore, have resulted from the different susceptibilities to the proteolytic enzymes in the duodenum.

In sucrose density gradient centrifugation, both toxic and nontoxic components in lymph of the B-M toxin recipient sedimented in a single band to the position of 12S, whereas in lymph of the B-L toxin recipient the toxic component sedimented to the position of 7S and the nontoxic component sedimented to that of about 14S. When administered orally with type A crystalline (19S) or type E progenitor toxin (12S), the toxin appearing in lymph or blood has a dimension of 7S (9, 11, 19). Neither B-L nor B-M toxin is dissociated in rat intestinal juice of pH 7.0 (23). The same rates of destruction shown with B-L, B-M, and B-S toxins in lymph of pH 8.2 (Fig. 3) indicate that both B-L and B-M toxins are dissociated into B-S toxin; otherwise they should have shown distinct destruction curves. From these findings, it seems justified to conclude that the undissociated molecule of the progenitor toxin, no matter whether it is fully or partially toxic or has been detoxified, is absorbed through the upper intestine into the lymphatics, where molecular dissociation occurs.

Since the sucrose density gradient was made in a buffer of pH 6.0, the dissociated 7S toxic and 7S nontoxic components in lymph reassembled to reconstitute the 12S molecule (16). The 7S toxic and 14S nontoxic components failed to reassemble under the same conditions, probably because the structure of the 14S nontoxic component had been modified in rat lymph. In fact, the two components of B-L toxin dissociated in vitro at pH 8.0 reassembled only partially, although those of B-M toxin reassembled completely, in a sucrose density gradient at pH 6.0, indicating that the 14S nontoxic component undergoes modification more easily.

The present studies demonstrated for the first time the nontoxic component of B-L or B-M toxin in rat lymph. The RPHA test with sheep erythrocytes coupled with the anti-nontoxic component immunoglobulin is sensitive enough to detect minute quantities of the nontoxic component in lymph.

The nontoxic component of type B-L toxin is made up of two distinct antigenic subcomponents (12). We found that the two subcomponents remained in the complex form in lymph.

The exact mechanism of absorption of botulinum toxin from the intestine is not known. We demonstrated that type B-L, B-M, and B-S toxins are absorbed at similar rates from the ligated duodenum. This indicates that endocytosis is the most likely mechanism of absorption

of the toxin molecule. It is known that lactoglobulin (7S) in colostrum is absorbed through the neonatal small intestine by pinocytosis (2, 4). If pinocytosis or endocytosis is the case, it seems possible that the toxin molecules of 16S or 19S pass through the intestinal barriers at the same rates.

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