The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin

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Binding of the purified type C 7S (neurotoxin), 12S and 16S botulinum toxins to epithelial cells of ligated small intestine or colon of the guinea pig (*in vivo* test) and to pre-fixed gastrointestinal tissue sections (*in vitro* test) was analysed. The 16S toxin bound intensely to the microvilli of epithelial cells of the small intestine in both *in vivo* and *in vitro* tests, but did not bind to cells of the stomach or colon. The neurotoxin and 12S toxin did not bind to epithelial cells of the small intestine or to cells of the stomach or colon. Absorption of the toxins was assessed by determining the toxin titre in the sera of guinea pigs 6–8 h after the intra-intestinal administration of the toxins. When the 16S toxin [1×10^5 minimum lethal dose (MLD)] was injected, 200–660 MLD ml⁻¹ was detected in the sera, whereas when the 12S toxin (2×10^5 MLD) or 7S toxin (2×10^5 MLD) was injected, little toxin activity was detected in the sera. Therefore, the haemagglutinin of type C 16S toxin is apparently very important in the binding and absorption of botulinum toxin in the small intestine.

Keywords: haemagglutinin, *Clostridium botulinum*, toxin binding, absorption

INTRODUCTION

Botulinum neurotoxin, a protein of approximately 150 kDa, is produced in serologically distinct forms (types A–G) by the Gram-positive spore-forming bacterium *Clostridium botulinum*. The neurotoxin produced by the bacterium is in the form of a stable complex due to its association with non-toxic proteins. The complex, designated progenitor toxin, is found in three forms: 12S toxin (molecular mass ~ 300 kDa), 16S toxin (~ 500 kDa) and 19S toxin (~ 900 kDa) (Sugii *et al.*, 1977a, b; Ohishi & Sakaguchi, 1980). Type A progenitor toxin is composed of all three forms; type B, C and D toxins are composed of two forms, namely 12S and 16S; type E and F toxins are composed of a single

form, 12S; and type G toxin is composed of a single form, 16S. Comparative genetic and biochemical analyses of these progenitor toxins have been facilitated by cloning and sequencing the representative operons encoding them and by purifying the corresponding toxins (Kimura *et al.*, 1990; Tsuzuki *et al.*, 1990, 1992; Fujinaga *et al.*, 1994; Hauser *et al.*, 1994; Fujita *et al.*, 1995; Minton, 1995; Oguma *et al.*, 1995; Ohyama *et al.*, 1995; Henderson *et al.*, 1996; Inoue *et al.*, 1996). From these studies, the following conclusions were drawn. The 12S toxin consists of a neurotoxin and a non-toxic component having no haemagglutinin (HA) activity, designated non-toxic non-HA. The 16S toxin consists of a neurotoxin, a non-toxic non-HA, and a HA, associated in the ratio 1:1:2. The molecular mass of the non-toxic non-HA is 120–140 kDa, but the non-toxic non-HA of the 12S toxin is dissociated into 105–130 kDa and 10–15 kDa components on SDS-PAGE with or without 2-mercaptoethanol. The HA consists of subcomponents having molecular mass values of approximately 53, 33,

Abbreviations: DAB, 3,3'-diaminobenzidine tetrahydrochloride; HA, haemagglutinin; MLD, minimum lethal dose; PLP, periodate/lysine/paraformaldehyde.

22–23 and 17 kDa in type C (Fujinaga *et al.*, 1994) and D (Ohyama *et al.*, 1995) toxins, and 52, 35, 19–20 and 15 kDa in type A toxin (Fujita *et al.*, 1995; Inoue *et al.*, 1996). The type A 19S toxin consists of the same protein components as the 16S toxin; it is presumed to be a dimer of the 16S toxin conjugated through HA-35 molecules (Inoue *et al.*, 1996).

Human and animal food-borne botulism is caused by ingestion of food or feed containing the progenitor toxin. The orally ingested progenitor toxin is absorbed from the upper small intestine into the lymphatic system (Sugii *et al.*, 1977b); it then enters the bloodstream and reaches peripheral nerves, where neurological dysfunction is elicited. The non-toxic components are considered to be very important in the development of food poisoning because they protect the neurotoxin from the acidity and proteases in the digestive tract (Sugii *et al.*, 1977a, b; Ohishi & Sakaguchi, 1980). It has also been reported that the dissociation of the type B 16S toxin into neurotoxin and non-toxic components occurs in a buffer solution of pH >7.0, but does not occur in rat intestinal juice of pH 7.0 (Sugii *et al.*, 1977a).

Despite increased understanding of the biochemistry of the progenitor toxin, the role of the non-toxic components in oral toxicity has not been fully clarified. The current study reports that the HA of the type C 16S toxin binds to epithelial cells of the upper small intestine of guinea pigs, and that only the 16S toxin is efficiently absorbed there.

METHODS

Toxins. The type C 16S and 12S toxins, and the 7S (neurotoxin) toxin were highly purified from the culture fluid of *C. botulinum* type C strain Stockholm (C-ST) according to the procedure previously employed for purifying type A toxins (Inoue *et al.*, 1996). The toxins were precipitated by 50% saturation with ammonium sulphate. The harvested precipitates were dialysed against 50 mM sodium acetate buffer (pH 4.2), and then applied on to an SP-Toyopearl 650M column. The 16S and 12S toxins separated by this column chromatographic procedure were further purified by gel filtration on a Sephacryl S-300 column, and by density-gradient centrifugation on a 5–20% sucrose gradient at pH 6.0. The 7S toxin was isolated from the 16S toxin thus obtained by density-gradient centrifugation on a 5–20% sucrose gradient at pH 8.8. Toxins were dissolved in toxin dilution buffer (20 mM sodium phosphate buffer, pH 6.0, containing 0.2%, w/v, gelatin) at a concentration of about 1 mg ml⁻¹ and stored frozen at -80 °C until used.

Antisera. Two previously described antisera against the purified type C neurotoxin and the non-toxic components containing both HA and non-toxic non-HA (Oguma *et al.*, 1980) were used in this study. An antiserum against the purified type C 16S toxin was produced in a rabbit by the same procedure described previously by Oguma *et al.* (1980). For the binding inhibition experiments, the immunoglobulin fractions obtained by DEAE-cellulose chromatography from these antisera were employed (Tsuzuki *et al.*, 1988).

Animals. Female Hartley strain guinea pigs (Shimizu Laboratory Supplies) were used at between 8 and 12 weeks of age

(400–600 g). The animals were not fed for 24 h prior to use, with water given freely. The experimental protocols were approved by the Animal Experiment Control Committee of Okayama University Medical School, and were in accordance with its guidelines. The minimum number of guinea pigs necessary to obtain valid results was used.

Toxin binding to ligated intestinal loops (*in vivo* test).

Guinea pigs were anaesthetized by intraperitoneal injection of sodium pentobarbital, and the abdomen was opened along the midline. One ligated intestinal loop (3–4 cm in length) was made in the upper small intestine (3–8 cm from the pylorus), lower small intestine (2–7 cm from the caecum), proximal colon (2–7 cm from the caecum) and distal colon (3–8 cm from the anus). Care was taken to leave the blood supply intact. Each loop was inoculated with 0.4–0.8 ml toxin solution (20 nM) by a 27 gauge needle, and a second tie was made to isolate the site of injection. Care was taken to maximize the distribution of the toxin solution throughout the loop. Loops were incubated for 1 h in the abdominal cavity, while the guinea pigs were kept warm and under anaesthesia. In the case of the stomach, 1 ml of the toxin solution (20 nM) was orally inoculated by a feeding needle, and reacted for 1 h.

Thereafter, the loops and stomach were excised from the guinea pig, opened, briefly washed in two changes of ice-cold PBS (0.149 M NaCl; 0.01 M sodium phosphate; pH 6.0), and fixed in periodate/lysine/paraformaldehyde (PLP) fixative for 5 h at 4 °C (Yoshimura *et al.*, 1980). The tissues were dehydrated and paraffin-embedded before cutting 5-µm-thick sections. The sections were deparaffinized, hydrated, rinsed in PBS, and incubated with 0.3% H₂O₂ in methanol for 30 min at room temperature to inactivate the endogenous peroxidase. After washing with PBS/0.1% Triton X-100, they were incubated with 1 M glycine in PBS/0.1% Triton X-100 for 1 h at room temperature to block free aldehyde groups generated by treatment with the fixative. After washing with PBS/0.1% Triton X-100, the sections were first incubated with 3% normal goat serum for 1 h at room temperature to block the non-specific reaction, and then with the properly diluted rabbit anti-type C 16S toxin in a humidity chamber at 4 °C overnight. Finally, they were treated with biotinylated goat anti-rabbit antibody (Vectastain Elite ABC kit; Vector Laboratories) at room temperature for 1 h, and developed by using avidin-biotin peroxidase complexes (Vectastain Elite ABC kit; Vector Laboratories) and DAB solution (0.25 mg 3,3'-diaminobenzidine tetrahydrochloride ml⁻¹ and 0.002% H₂O₂ in 25 mM Tris/HCl buffer, pH 7.2) according to the manufacturer's instructions. Sections were then counterstained with haematoxylin and examined using a light microscope.

Toxin binding to pre-fixed sections of intestine (*in vitro* test).

Samples of the stomach, the upper and lower small intestine, and the proximal and distal portions of the colon were removed from the anaesthetized guinea pigs. All samples were fixed with a solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 4 °C, dehydrated and embedded in paraffin, and 5-µm-thick sections were cut. The sections were deparaffinized, hydrated, rinsed in PBS (pH 7.2) and incubated with 1 M glycine in PBS (pH 7.2) for 1 h at room temperature. After washing with PBS (pH 6.0), they were incubated with the toxin solutions (2 nM) in a humidity chamber for 1 h at room temperature. To diminish the non-specific binding of the toxin, the sections were washed in six changes of PBS (pH 6.0)/0.1% Triton X-100 for 10 min. They were then incubated with 0.3% H₂O₂ in methanol for 30 min at room temperature. After washing with PBS (pH 6.0)/0.1% Triton X-100, the sections were incubated

with 3% normal goat serum for 1 h at room temperature, and then stained with the diluted rabbit anti-type C 16S toxin, followed by biotinylated goat anti-rabbit antibody, avidin-biotin peroxidase complexes and DAB substrate. Sections were counterstained with haematoxylin and examined with a light microscope.

Inhibition assays

(i) **Antibody inhibition.** The 16S toxin (final concentration $1 \mu\text{g ml}^{-1}$) was incubated for 1 h at 25°C with purified anti-16S (final concentration $30 \mu\text{g ml}^{-1}$), anti-non-toxic components (final concentration $30 \mu\text{g ml}^{-1}$) or anti-neurotoxin immunoglobulins (final concentration $30 \mu\text{g ml}^{-1}$), and then the mixtures were reacted with the pre-fixed sections. The binding assay was then performed as described above.

(ii) **Neuraminidase-treated sections.** Tissue sections were treated with *Arthrobacter ureafaciens* neuraminidase (highly purified preparation containing no protease, N-acetylneuraminic acid aldolase or glycosidase; Nacalai tesque) at 3 U ml^{-1} according to the procedure of Caldero *et al.* (1988). Briefly, the sections were incubated with neuraminidase at 37°C in a humidified environment for 12 h. Following this, the sections were incubated with 1 M glycine in PBS/0.1% Triton X-100 and then the toxin-binding test was performed.

Immunoelectron microscopy. Toxin (20 nM) or toxin dilution buffer was injected in the ligated intestinal loops and reacted in the same way as described for the *in vivo* test. The ligated areas were resected out and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for more than 6 h at 4°C . After washing with PBS, the intestine was cut into 20- μm -thick cross-sections with a vibratome (Microslicer DTK-1000; Dousaka). The resultant sections were pre-incubated with 0.3% H_2O_2 in methanol for 30 min, with 1 M glycine and 3% normal goat serum each for 1 h at room temperature, and then reacted with rabbit anti-type C 16S toxin at 4°C overnight. Thereafter, the sections were successively reacted with biotinylated goat anti-rabbit antibody, avidin-biotin peroxidase complexes, and DAB solution same as the staining method for the light microscopy, and then reacted with 1% OsO_4 for 1 h. After dehydration with an acetone series, the samples were embedded in epoxy resin. Ultrathin sections were cut with an ultramicrotome (Sorval MT-5000) and observed under a transmission electron microscope (JEM 100-CX) operated at 80 kV.

Intra-intestinal administration of toxins to guinea pigs. Guinea pigs were anaesthetized with Sevoflurane (Maruishi Seiyaku) and the intestine was approached via a small midline incision. A 0.4 ml dose of toxin was injected into the upper small intestine (3–4 cm from the pylorus) with a 27 gauge needle. Care was taken not to spill the toxin into the abdominal cavity. After the incision was sutured, the guinea pigs were kept in restraining cages. The guinea pigs were killed after 6–8 h, and blood was collected to analyse the amount of toxins absorbed.

Tests for stability of the toxin in digestive juice. Digestive juice (actually a mixture of gastric, bile, pancreatic and intestinal juices; pH of about 8.0) was obtained from two guinea pigs separately as follows. A guinea pig was anaesthetized with Sevoflurane. The intestine was approached via a small midline incision and then the upper small intestine (about 3 cm below the major duodenal papilla) was ligated. After the incision was stitched, the guinea pig was kept in a restraining cage for 8 h. After the guinea pig was killed, the digestive juice that had accumulated in the duodenum and stomach was taken out. The collected intestinal juice was centrifuged at 3000 g for 10 min and the supernatant fluid was

used for experiments. Twenty microlitres of the 16S, 12S or 7S toxin, having 10^4 times the minimum lethal dose (MLD) for mice, was mixed with 380 μl digestive juice and incubated for up to 8 h at 35°C . The toxicity of the samples was then determined in mice.

Other procedures. Toxicity (MLD) of the preparations was determined by injecting the diluted samples into mice intraperitoneally as reported previously by Inoue *et al.* (1996). Protein concentration was determined by the method of Bradford as described previously by Inoue *et al.* (1996).

RESULTS

Reactivities of the anti-type C 16S toxin serum with 16S, 12S and 7S toxins differed

Since binding of the toxins to the epithelial cells was studied by using anti-type C 16S toxin antiserum as a primary antibody, the reactivity of this antiserum with the 16S, 12S or 7S toxin was first evaluated. Each toxin (2 μl 5 nM solution) was dot-blotted on a nitrocellulose membrane and then the serial tenfold- and twofold-diluted anti-16S toxin serum was reacted, followed by the secondary antibody and the substrate. The reactivities of this antiserum with the 16S, 12S and 7S toxins were slightly different. Therefore, the following binding experiments *in vivo* and *in vitro* were performed using 0.25×10^{-3} -, 0.5×10^{-3} - or 1×10^{-3} -diluted sera to detect the 16S, 12S or 7S toxins, respectively.

16S toxin binds selectively to epithelial cells of the upper small intestine (*in vivo* test)

The intestinal loops of guinea pigs were ligated and then reacted with 16S, 12S or 7S toxin. Binding of the toxins to cells was determined by immunostaining. Fig. 1(a) illustrates the specific staining of oxidized 3,3'-diaminobenzidine corresponding to the 16S toxin at the epithelial surface of the upper small intestine. The 16S toxin bound weakly to the lower small intestine as compared to the upper region (Table 1). In other tissues, including the stomach (Fig. 1c; Table 1) and colon (Fig. 1d; Table 1), little of the 16S toxin attached to the cells. None of the loops inoculated with the toxin dilution buffer (negative control) were stained (Fig. 1b, upper small intestine). The same binding profile of the 16S toxin was obtained by using anti-non-toxic components antiserum instead of anti-16S toxin serum. Binding of the toxin was also detected by using anti-neurotoxin antiserum. When the tissues were fixed in 4% paraformaldehyde, the intensity of the 16S toxin binding was considerably diminished. In the cases of the 7S and 12S toxins, no binding was observed to any portions of the digestive tract (Table 1). These data reveal that the 16S toxin can bind strongly to the epithelial surface of only the upper small intestine.

16S but not 12S or 7S toxin binds to pre-fixed sections of intestine (*in vitro* test)

To determine the progenitor toxin component(s) required for binding to the epithelial surface of the upper small intestine, the binding test *in vitro* was undertaken.

Fig. 1. Localization of type C 16S toxin *in vivo*. (a) Guinea pig upper small intestine incubated with type C 16S toxin ($10 \mu\text{g ml}^{-1}$, 20 nM). Arrows point to the location of the toxin. (b) Upper small intestine incubated with toxin dilution buffer (not exposed to toxin, but otherwise treated identically). (c) Stomach incubated with type C 16S toxin ($10 \mu\text{g ml}^{-1}$). (d) Colon incubated with type C 16S toxin ($10 \mu\text{g ml}^{-1}$). The same results were obtained in three repeats of this experiment using three guinea pigs, and each was done in duplicate. Bars, 100 μm .

Table 1. Binding of type C toxins to epithelial cells of the gastrointestinal tract

Assays were performed in at least three independent experiments (using three guinea pigs), and each was done in duplicate, giving the same results each time. Intensity of staining: —, absent; $\pm$, traces; +, weak; ++, intense. For an example of ++ staining, see Fig. 1(a).

	Toxin		Binding				
	Type	Concn	Stomach*	Small intestine		Colon	
				Upper	Lower	Proximal	Distal
<i>In vivo</i> (ligated intestine)	16S	$10 \mu\text{g ml}^{-1}$ (20 nM)	—	++	+	$\pm$	$\pm$
	12S	$6 \mu\text{g ml}^{-1}$ (20 nM)	—	—	—	—	—
	7S	$3 \mu\text{g ml}^{-1}$ (20 nM)	—	—	—	—	—
<i>In vitro</i> (section)	16S	$1 \mu\text{g ml}^{-1}$ (2 nM)	$\pm$	++	++	$\pm$	$\pm$
	12S	$0.6 \mu\text{g ml}^{-1}$ (2 nM)	ND	—	—	—	—
	7S	$0.3 \mu\text{g ml}^{-1}$ (2 nM)	ND	—	—	—	—

* Toxin preparations (1 ml) were orally administered.

In this assay, each segment of the digestive tract of healthy guinea pigs was fixed in 4% paraformaldehyde instead of the PLP fixative, because the PLP fixative modifies the carbohydrate chains in the tissue, which might have an adverse influence on the binding of toxin. In the pre-fixed sections of the upper and lower small intestine, the 16S toxin showed intense binding to brush border and goblet cells, whereas 12S and 7S toxins did not show any binding to any sites (Table 1). In sections of the stomach and colon, all these toxins could not obviously bind to any sites (Table 1). The same binding

property of the 16S toxin was observed by using anti-non-toxic components serum instead of the anti-16S toxin serum.

Antibodies or neuraminidase treatment inhibit binding of the 16S toxin

The effects of pre-incubation of the toxins with anti-toxin antibodies and of pre-treatment of the tissue sections with neuraminidase were examined. Pre-incubation of the 16S toxin with the purified anti-16S

Fig. 2. Electron micrographs comparing type C 16S-treated intestinal epithelium and normal epithelium. (a) Epithelial cells from a ligated intestinal loop subjected to 16S toxin ($10 \mu\text{g ml}^{-1}$, 20 nM) for 1 h. (b) Epithelial cells in an adjacent intestinal loop injected with toxin dilution buffer. The same results were obtained in two repeats of this experiment using different guinea pigs. Bars, 1 μm .

toxin immunoglobulins or anti-non-toxic components immunoglobulins resulted in an apparent inhibition of the binding to brush border and goblet cells, whereas pre-incubation of the 16S toxin with the purified anti-7S immunoglobulin did not influence the toxin-binding properties. Neuraminidase treatment of the tissue sections prior to incubation with the toxin completely abolished binding of the 16S toxin to the luminal surface of intestinal epithelial cells. These inhibition assays were repeated three times using tissue sections from three different guinea pigs; these gave the same results each time.

Ultrastructural localization of the 16S toxin to the microvilli of the epithelial surface

With the light microscope, the 16S toxin was detected at the epithelial surface of the upper small intestine as mentioned above. To more precisely determine the toxin-binding site on the intestinal epithelial surface, an immunoenzymic labelling technique was used in conjunction with electron microscopy. The microvilli membranes were stained with electron-opaque reaction product (Fig. 2a), whereas no staining was observed in the control (Fig. 2b).

16S but not 12S or 7S type C toxin is absorbed in the intestine of guinea pigs

Absorption of the toxins was assessed by determining the toxin titres in the sera of guinea pigs 6–8 h after the intra-intestinal administration of the toxins (Table 2). When the 16S toxin (1×10^5 MLD) was injected, 200–660 MLD ml^{-1} was detected in the serum, whereas when the 12S toxin (2×10^5 MLD) or 7S toxin (2×10^5 MLD) was injected, little toxin activity was detected, i.e. 0.5 ml of each threefold-diluted serum did not kill mice. The guinea pigs which received the 12S or 7S toxin intra-intestinally did not show signs of botulism during

Table 2. Absorption of type C progenitor toxins from the small intestine

The toxin titre in the blood was determined by intraperitoneal injection into two mice. Assays for absorption of each type of the toxin were performed in three independent experiments (using three guinea pigs).

Type	Toxin (intra-intestinal administration)	Toxin titre in blood (MLD ml^{-1})
	Amount ($\text{MLD in } 0.4 \text{ ml}$)	
16S	1×10^5	2×10^2 , 2×10^2 , 6.6×10^2
12S	2×10^5	<6, <6, <6
7S	2×10^5	<6, <6, <6

this period, but those which received the 16S toxin manifested severe botulism about 6 h after inoculation of the toxin.

When each of the 16S, 12S and 7S toxins was injected in the ligated upper small intestine, all guinea pigs receiving these three types of type C toxin manifested severe botulism about 2 h after the inoculation, and more than 200 MLD ml^{-1} was detected in the sera. It seemed that all forms of the type C toxin spilled artificially through the damaged tissue caused by ligation of the intestine. Therefore, we consider that the ligated intestine system is not appropriate for toxin absorption experiments.

12S and 7S toxins are slightly less stable than 16S toxin in digestive juice

The stabilities of the different forms of type C toxin in the digestive juice of guinea pigs were tested (Table 3). The 16S toxin was resistant to the digestive juice and

Table 3. Stabilities of type C toxins in digestive juice

The experiment was repeated twice using the digestive juices from different guinea pigs and gave essentially the same results. After incubation with digestive juice, each sample was diluted as indicated, then the toxicity was determined using two mice. D, Died; M, manifested a symptom of botulism; S, survived.

Toxin	Sample dilution	Toxicity at time (h) after incubation with digestive juice		
		0 (control)	2	8
16S	1×10^{-3}	D, D	D, D	D, D
	1×10^{-4}	M, M	M, S	M, M
	0.3×10^{-4}	S, S	S, S	S, S
12S	1×10^{-3}	D, D	D, D	D, D
	1×10^{-4}	D, D	D, M	D, D
	0.3×10^{-4}	D, M	D, M	M, M
7S	1×10^{-3}	D, D	D, D	D, D
	1×10^{-4}	D, D	D, M	D, D
	0.3×10^{-4}	M, M	S, S	S, S

maintained its original toxicity after incubation for 8 h. The 12S and 7S toxins were partially inactivated, but they still possessed more than 33% of their original toxicity after 8 h exposure to the digestive juice.

DISCUSSION

Binding and absorption of the purified 16S (HA-positive), 12S (HA-negative) and 7S (neurotoxin) forms of type C botulinum toxin in the small intestine of the guinea pig were studied. Binding of toxin was observed in two different systems, namely *in vivo* and *in vitro* tests. We used the *in vivo* test to investigate the binding of toxin in as native a state as possible, and the *in vitro* test to confirm the detailed binding properties by employing inhibition tests with anti-toxin antibodies and neuraminidase treatment of cells. The results (Table 1) indicated that neither the neurotoxin (7S) nor the non-toxic non-HA exhibited specific binding to the small intestine, whereas the HA of the 16S toxin did. In the *in vivo* test, binding of the 16S toxin could also be detected by using anti-neurotoxin antiserum. This result implies that the neurotoxin is not dissociated from the 16S toxin in the small intestine in the presence of intestinal juice, just as observed in the rat (Sugii *et al.*, 1977a), and that the HA moiety anchors the neurotoxin on the epithelial cells of the small intestine. The optimum concentration of the 16S toxin was tenfold lower in the *in vitro* test (2 nM) compared with the *in vivo* test (20 nM). This finding is reasonable because in the *in vivo* test, dilution of the toxin by the intestinal juice may occur and also the mucus layer may interfere in the binding of toxin to the microvilli. Binding of the 16S toxin was inhibited by polyclonal antibodies against both the 16S toxin and the non-toxic components, but not by the anti-neurotoxin antibody. Binding was also

inhibited by treating the intestinal tissue sections with neuraminidase before addition of the 16S toxin. From these results, we conclude that the HA of 16S toxin is involved in the binding, and that terminal sialic acid residues of the membrane-bound glycoconjugates (glycolipids or glycoproteins or both) are important for binding.

We employed PLP fixative for the *in vivo* binding test because intense binding of the 16S toxin was observed in the case of PLP fixative, but there was weak binding in the case of the paraformaldehyde fixative. PLP fixes both carbohydrate and protein, whereas paraformaldehyde fixes only the protein moiety. Although the reason for the above-mentioned phenomenon may not be answered until the toxin receptor structure is identified, it seems that the 16S toxin, which bound to the carbohydrate chains of glycolipids in the membrane, is detached from the cells during dehydration with ethanol in the case of the paraformaldehyde fixative. In contrast, in the *in vitro* binding test, the degree of toxin binding was not so different between the PLP-fixed and paraformaldehyde-fixed preparations. The reason for this was not clear either, but we employed paraformaldehyde because we wanted to observe the binding of toxin to the carbohydrate chain in a chemically non-modified form.

In the *in vivo* experiment, the 16S toxin bound more strongly to the epithelial surface of the upper region than to that of the lower region of the small intestine. This is consistent with the previous findings, namely that the orally ingested botulinum toxin is absorbed mostly from the upper small intestine (Sugii *et al.*, 1977b). However, in the *in vitro* experiments with paraformaldehyde-fixed normal tissue sections, the 16S toxin bound uniformly to the lower small intestine as well as to the upper region. This difference might be caused by the mucus layer overlying the intestinal epithelium. The nature of the mucus layer of the upper and lower small intestine may be somewhat different. It is possible that the mucus layer of the lower small intestine may inhibit the binding of 16S toxin to the brush border only in the *in vivo* experiment, and that this mucus layer is washed out during the preparation of the pre-fixed sections for the *in vitro* experiment. Further experiments will be needed to define the role of the mucus layer in binding of the 16S toxin to the brush border of the upper and lower small intestine.

To clarify the role of the non-toxic components in the intestinal absorption of the toxin, we injected the 16S, 12S and 7S toxin preparations directly into the small intestine without ligation. The absorption rate of the 16S toxin, estimated by the toxicity of the sera from guinea pigs 8 h after toxin administration, was at least 100-fold higher than those of the 12S and 7S toxins. However, the toxicities of the 7S and 12S toxins were not considerably decreased by incubating them with guinea pig digestive juice when compared with that of the 16S toxin, the 7S and 12S toxins retaining at least 33% of their toxicity. These findings indicate that the

reason for little binding and absorption of the 12S and 7S toxins is not that they are destroyed by the digestive juice but that they lack the HA. The HA may have a critical role in the binding and then the efficient absorption of the toxin. The HA moiety consists of four different subunits, namely HA-53, HA-33, HA-22-23 and HA-17, non-covalently associated in the ratio 1:2:1:1 (Fujinaga *et al.*, 1994; Inoue *et al.*, 1996). Further research to determine how the 16S toxin is translocated from the epithelial surface to the circulation and which subcomponent(s) of the HA is(are) required for binding and absorption is now being undertaken.

It has been reported that the type C 12S toxin also has oral toxicity, although very low compared with that of the 16S toxin (Ohishi & Sakaguchi, 1980). We think that the low oral toxicity of the 12S toxin may be caused by its low binding affinity to the epithelial cells of the small intestine rather than its lower resistance to digestive juice in the case of the guinea pig. In our experiment, binding of the 12S toxin to the cells was hardly observed, and the guinea pigs showed no symptoms of botulism 8 h after injection of the 12S toxin. Observations of duration longer than 8 h and autoradiography of the animals injected with isotope-labelled toxins may be needed to clarify the oral toxicity of the 12S toxin. In type E and F botulinum toxins, only HA-negative 12S toxins are produced. The oral toxicity of E and F toxins is also low (similar level to type C 12S toxin) compared with type C 16S toxin (Ohishi & Sakaguchi, 1980); the binding of type E and F toxins to the epithelial cells of the small intestine may also be low. The detailed absorption mechanism of these 12S toxins, including types E and F, should be studied.

ACKNOWLEDGEMENTS

This work was supported in part by grants 08457088 and 08770192 from the Ministry of Education of Japan.

REFERENCES

- Caldero, J., Campo, E., Calomarde, X. & Torra, M. (1988). Distribution and changes in glycoconjugates in rat colonic mucosa during development. *Histochemistry* **90**, 261–270.
- Fujinaga, Y., Inoue, K., Shimazaki, S., Tomochika, K., Tsuzuki, K., Fujii, N., Watanabe, T., Ohyama, T., Takeshi, K., Inoue, K. & Oguma, K. (1994). Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. *Biochem Biophys Res Commun* **205**, 1291–1298.
- Fujita, R., Fujinaga, Y., Inoue, K., Nakajima, H., Kumon, H. & Oguma, K. (1995). Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. *FEBS Lett* **376**, 41–44.
- Hauser, D., Eklund, M. W., Boquet, P. & Popoff, M. R. (1994). Organisation of the botulinum neurotoxin C1 gene and its associated non-toxic protein genes in *Clostridium botulinum* C468. *Mol Gen Genet* **243**, 631–640.
- Henderson, I., Whelan, S. M., Davis, T. O. & Minton, N. P. (1996). Genetic characterisation of the botulinum toxin complex of *Clostridium botulinum* strain NCTC 2916. *FEMS Microbiol Lett* **140**, 151–158.
- Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K. & Oguma, K. (1996). Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect Immun* **64**, 1589–1594.
- Kimura, K., Fujii, N., Tsuzuki, K., Murakami, T., Indoh, T., Yokosawa, N., Takeshi, K., Syuto, B. & Oguma, K. (1990). The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. *Biochem Biophys Res Commun* **171**, 1304–1311.
- Minton, N. P. (1995). Molecular genetics of clostridial neurotoxins. *Curr Top Microbiol Immunol* **95**, 161–194.
- Oguma, K., Syuto, B., Iida, H. & Kubo, S. (1980). Antigenic similarity of toxins produced by *Clostridium botulinum* type C and D strains. *Infect Immun* **30**, 656–660.
- Oguma, K., Fujinaga, Y. & Inoue, K. (1995). Structure and function of *Clostridium botulinum* toxins. *Microbiol Immunol* **39**, 161–168.
- Ohishi, I. & Sakaguchi, G. (1980). Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. *Infect Immun* **28**, 303–309.
- Ohyama, T., Watanabe, T., Fujinaga, Y., Inoue, K., Sunagawa, H., Fujii, N., Inoue, K. & Oguma, K. (1995). Characterization of nontoxic-nonhemagglutinin component of two types of progenitor toxin (M and L) produced by *Clostridium botulinum* type D CB-16. *Microbiol Immunol* **39**, 457–465.
- Sugii, S., Ohishi, I. & Sakaguchi, G. (1977a). Correlation between oral toxicity and *in vitro* stability of *Clostridium botulinum* type A and B toxins of different molecular sizes. *Infect Immun* **16**, 910–914.
- Sugii, S., Ohishi, I. & Sakaguchi, G. (1977b). Intestinal absorption of botulinum toxins of different molecular sizes in rats. *Infect Immun* **17**, 491–496.
- Tsuzuki, K., Yokosawa, N., Syuto, B., Ohishi, I., Fujii, N., Kimura, K. & Oguma, K. (1988). Establishment of a monoclonal antibody recognizing an antigenic site common to *Clostridium botulinum* type B, C1, D, and E toxins and tetanus toxin. *Infect Immun* **56**, 868–902.
- Tsuzuki, K., Kimura, K., Fujii, N., Yokosawa, N., Indoh, T., Murakami, T. & Oguma, K. (1990). Cloning and complete nucleotide sequence of the gene for the main component of hemagglutinin produced by *Clostridium botulinum* type C. *Infect Immun* **58**, 3173–3177.
- Tsuzuki, K., Kimura, K., Fujii, N., Yokosawa, N. & Oguma, K. (1992). The complete nucleotide sequence of the gene coding for the nontoxic-nonhemagglutinin component of *Clostridium botulinum* type C progenitor toxin. *Biochem Biophys Res Commun* **183**, 1273–1279.
- Yoshimura, S., Komatsu, N. & Watanabe, K. (1980). Purification and immunohistochemical localization of rat liver glutathione peroxidase. *Biochim Biophys Acta* **621**, 130–137.

Received 12 March 1997; revised 23 July 1997; accepted 28 August 1997.