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PURIFICATION AND CHARACTERIZATION OF HEMAGGLUTININ OF *CLOSTRIDIUM BOTULINUM* TYPE C STRAIN STOCKHOLM

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Clostridium botulinum type C hemagglutinin (HA) was purified and characterized for clarifying the function of HA in botulinum intoxication.

The purified HA showed a single band on disc electrophoresis and a single precipitin line on agar gel double diffusion test. The specific activity of the purified HA was 1.6×10^4 units/mg protein for rat erythrocytes. The molecular weight of HA was 230,000 by ultracentrifuge analysis. HA bound not only to erythrocytes but also to intestinal cells. The HA activity was inhibited by the addition of gangliosides (GM₃, GM₄, GD_{1a}, GD_{1b}, and GT_{1b}) and fetuin, and was not shown to erythrocytes treated previously by neuraminidase. These results suggest that HA binds to sialic acid on cell surface. In botulinum intoxication, HA may play as a carrier for the internalization of toxin through small intestine cell.

Key words: *Clostridium botulinum*, hemagglutinin purification, hemagglutinin characterization

INTRODUCTION

Botulinum toxin, which is the most poisonous toxin produced by *Clostridium botulinum*, causes botulisms in human and animals. The usual cause of death in botulism is respiratory failure by neuromuscular paralysis. The mechanisms of the paralysis have been extensively studied.¹⁶⁾ The toxin peroral is taken up into the small intestine cell, enters the blood stream with lymph through the thoracic duct, and reaches the motor nerve endplate, where toxin blocks acetylcholine release from the presynaptic membrane.^{3,4)} However, the mechanisms of the entrance of toxin through the small intestine cell, which is the first step of intoxication, is still obscure.

Botulinum toxin in the culture medium exists as a complex with hemagglutinin.¹¹⁾

Although it has been reported that the toxin contained hemagglutinin showed higher oral toxicity than the purified toxin,^{8,9,15)} the role of HA is not clarified. In this paper,

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a procedure for the purification of hemagglutinin is described and some properties and binding characteristics of the purified HA are discussed.

MATERIALS AND METHODS

Materials: GM₃(NeuAc), GM₃(NeuGc), GM₂(NeuAc), GM₁(NeuAc) and GD_{1a}(NeuAc) were kindly supplied by Dr. M. Naiki (Department of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University). GM₄(NeuAc) was kindly supplied by Prof. R. Yu. (Department of Neurology, School of Medicine, Yale University).

GD_{1b}(NeuAc) and GT_{1b}(NeuAc) were purchased from Seromed (West Germany) and from Biosynth AG (Switzerland), respectively. Fetuin and neuraminidase (EC 3. 2. 1. 18) from *Clostridium perfringens* were purchased from Sigma Chemical Co.

Organism and HA production: *Clostridium botulinum* type C strain Stockholm was used.¹⁸⁾ HA was produced by the dialyzing cultivation method reported previously.¹⁹⁾

Toxin preparation: Type C toxin was purified by the method reported previously.²⁰⁾

HA activity assay: HA activity was assayed by previous method¹⁸⁾ with slight modifications. Two fold serial dilutions of each sample were made in 0.25 ml volumes with 0.15 M sodium phosphate buffer-saline at pH 7.0 (PBS) by using glass tubes. An equal volume of 0.3% fresh rat blood cell suspension was added to sample solution, and the mixture was incubated for 2 h at room temperature. One hemagglutinating unit is defined as the least amount of the sample causing the positive agglutination reaction.

Hemagglutination inhibition (HI) test: The inhibition of HA activity by gangliosides and fetuin in PBS at pH 7.0 were determined by using microtiter U plates with 4 units of HA. HA solution pretreated with inhibitors and 1% horse erythrocyte suspension were equally mixed, and allowed to stand for 2 h at room temperature. The HI titer was obtained from the lowest concentration of inhibitor required to block the hemagglutination reaction.

Column chromatography: DEAE-cellulose (Brown) in 0.5 N NaOH was packed into column (3.5×55cm) under slight pressure and then equilibrated with 27.5 mM borax-45 mM sodium dihydrogen phosphate buffer at pH 8.0 (Buffer A). Sephacryl S-400 Superfine (Pharmacia Fine Chemicals Inc.) was packed into a column (1.6×135cm) after removing fine particles by decantation. Column chromatography was performed at room temperature. The eluted proteins were monitored with an ultraviolet absorptiometer (LKB UV cord II) at 280 nm.

Electrophoresis on polyacrylamide gel: Disc electrophoresis was carried out by the method of Gabriel⁷⁾ using 5% polyacrylamide separation gels with Tris-glycine buffer at pH 8.0 as a running buffer. The marker bromophenol blue was allowed to migrate equally to 10 cm from the top of the gel at 2.5 mA per gel. Electrophoresis in the presence of sodium dodecylsulfate (SDS) was carried out by the method of Weber et

al.²¹⁾ with 8% polyacrylamide gels at 8 mA per gel. All gels were stained overnight with 0.05% Coomassie Brilliant Blue R-250 in 50% methanol-10% acetic acid solution, and destained in 10% acetic acid and 10% methanol solution.

Immunodiffusion test: Ouchterlony double diffusion test was performed in 0.02M Tris-glycine buffer at pH 8.0-0.5 M NaCl containing 1% special agar B (Wako).

Molecular weight determination: Molecular weight of HA was determined by sedimentation equilibrium method¹⁴⁾ using a HITACHI DA-7 ultracentrifuge processor (Hitachi Koki Co. Ltd.) in Buffer A.

Protein determination: Protein concentration was determined by the method of Lowry et al.¹¹⁾ with bovine serum albumin as standard. For electrophoretic separation of the molecular components, protein was estimated densitometrically by the method of Kahn and Rubin.¹²⁾

Anti HA: Anti-HA was prepared as reported previously.¹⁸⁾ The crude HA containing toxin (fraction II of Table 1) was detoxified with 0.5% formaldehyde for 7 days at 37 C, and injected to rabbits.

RESULTS

Purification of hemagglutinin

Table 1 shows the result of purification procedure of HA of *Clostridium botulinum*

TABLE 1 Purification of hemagglutinin of *Clostridium botulinum* type C strain Stockholm

FRACTION	VOLUME (ml)	PROTEIN CONC. (mg/ml)	TOTAL HA ACTIVITY (units × 10 ⁵)	YIELD (%)	SPECIFIC ACTIVITY (U/mg)	PURIFICATION (fold)
I. Culture supernatant	800	52	3.4	100	8.2	1
II. (NH ₄) ₂ SO ₄ precipitation	50	67	2.7	79	8.1 × 10 ²	94
III. DEAE-cellulose column chromatography	74	0.84	1.9	56	3.1 × 10 ³	378
IV. Sephacryl S-400 gel filtr.	16	0.83	1.5	44	1.1 × 10 ⁴	1341
V. Rechromatography of Sephacryl S-400 gel filtr.	20	0.34	1.1	32	1.6 × 10 ⁴	1951

Hemagglutination activity was estimated as described in Materials and Methods.

type C strain. The culture supernatant was mixed with a half volume of the saturated ammonium sulfate solution and allowed to stand for 2 days at 4°C. The resultant precipitate was centrifuged and dialyzed against Buffer A for 2 days at 4°C. After centrifuged the dialyzate to remove the insoluble materials, it was applied on a column of DEAE-cellulose. By this procedure, HA was absorbed on DEAE-cellulose but not toxin. Then the column was washed with 0.02M Tris-glycine buffer (pH 8.0), followed by elution with increasing NaCl concentration of eluate by a linear gradient method. After applying the gradient elution, 71% of HA activity of the applied sample was contained in the first peak (fig. 1). This peak fraction was concentrated, and chromatographed on the Sephacryl S-400 column. HA activity was eluted at the second peak (Fig. 2). Fractions from 50 to 53 were pooled and concentrated by ultra-

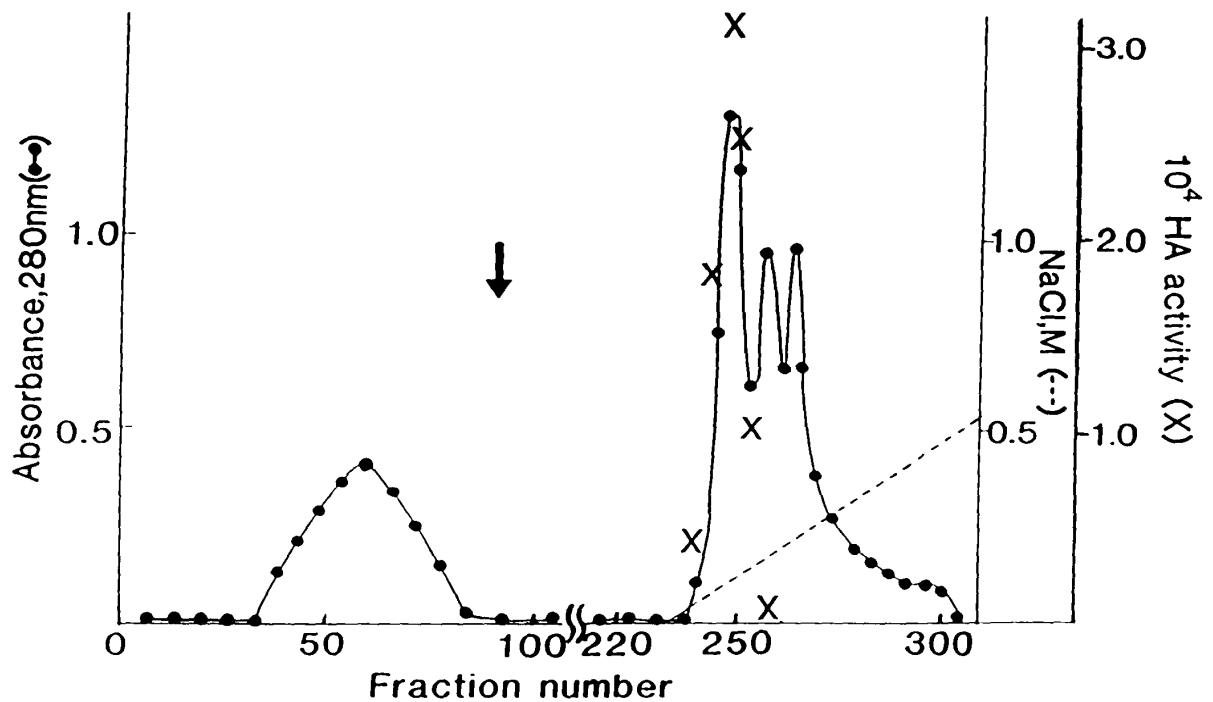


Fig. 1 DEAE-column chromatography. The material (335 mg) dialyzed against Buffer A was applied to the column (3.5 x 55 cm) at 50 ml/h. After toxin was passed through (fractions 31-85), the column was washed with 2.5 column volumes of 0.02 M Tris-glycine buffer at pH 8.0 (indicated by arrow). Then the linear gradient was performed with 1 liter of the buffer containing 1.5 M NaCl at a flow rate of 35 ml/h. HA activity of each fraction was assayed with rat erythrocytes. Each fraction contained 9 ml.

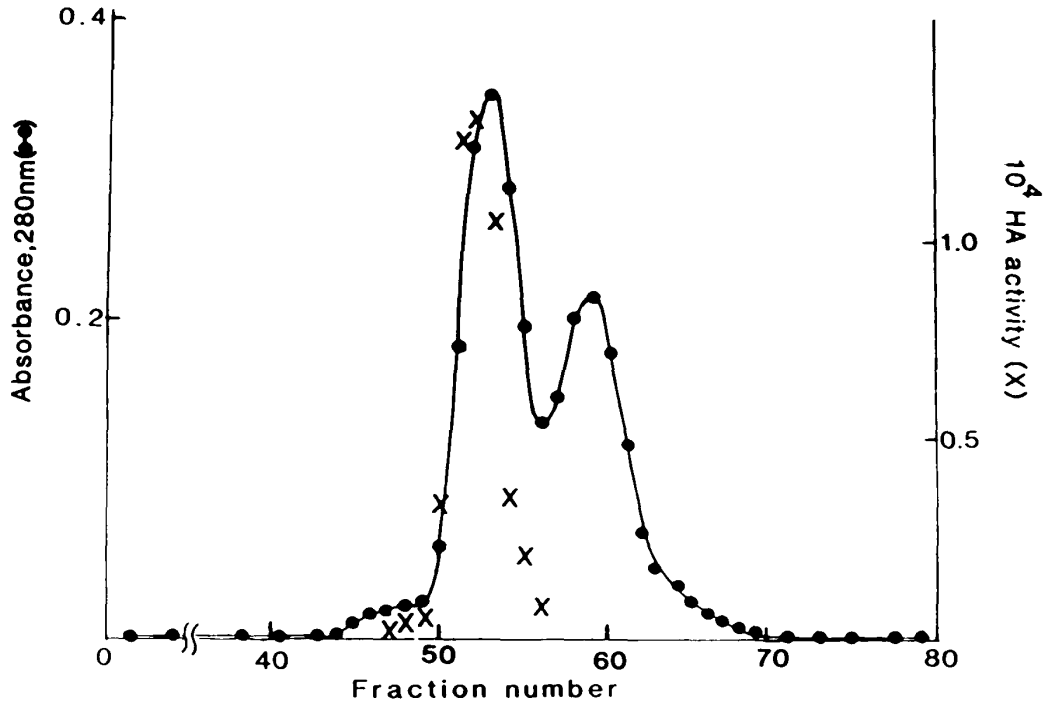


Fig. 2 Sephacryl S-400 column chromatography. A concentrated HA fraction (11.8 mg) from DEAE-cellulose column was applied on the Sephacryl S-400 column (1.6 x 135 cm) at 6.5 ml/h. The elution buffer was 0.02 M Tris-glycin buffer at pH 8.0 containing 0.5 M NaCl. Each fraction contained 4 ml.

filtration, and then rechromatographed on the Sephacryl S-400 column. The specific HA activity was constant from fraction 49 to 53 (fig. 3), and an overall yield of this fraction HA was 32% (tab. 1). The purified HA showed a single zone in disc electrophoresis (fig. 4) and gave only a single precipitin line in the immunodiffusion test (fig. 5).

Molecular weights and molecular components

The molecular weight of purified material was calculated from the result of the ultracentrifuge analysis with following equation:

$$M = \frac{2RT}{(1 - V\rho)\omega^2} \times \frac{d \ln C}{dr^2}$$

where R is the gas constant, T the absolute temperature, ω the angular velocity, r the distance from the axis of rotation, and C the protein concentration (O. D. 280 nm).

V (specific volume, 0.72 ml/mg) was calculated as buoyancy factors. ρ (density of solution) was 0.999 (gm/ml). The molecular weight given was 230,000. Components of the purified material were analysed by SDS-PAGE (fig. 6). HA was dissociated

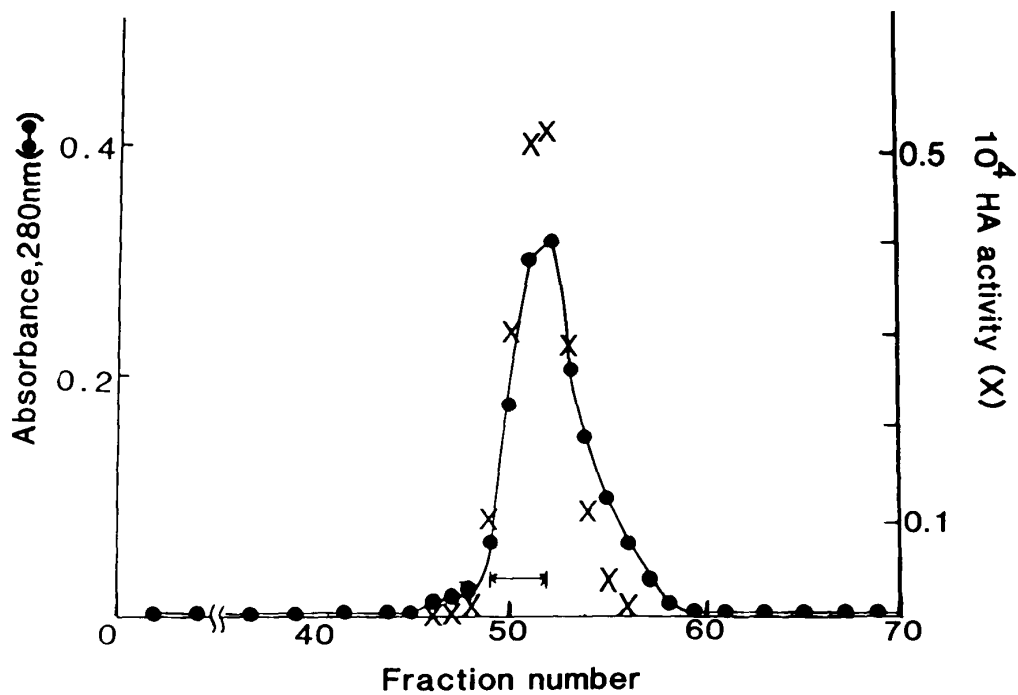


Fig. 3 Rechromatography on Sephacryl S-400. The concentrated HA (1.5 mg, fractions 50-53 in Fig. 2) was applied on the same column described in Fig. 2 at a flow rate of 2 ml/h. Each fractions contained 4 ml.

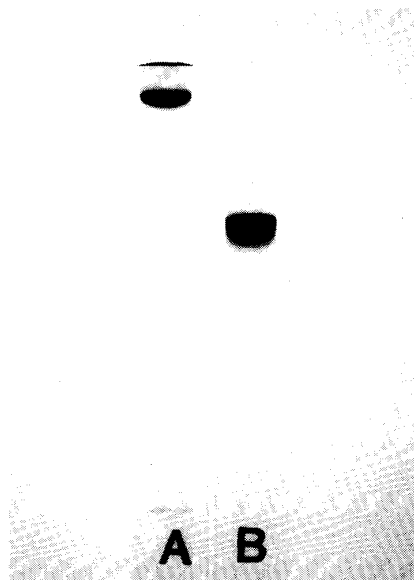


Fig. 4 Disc electrophoresis of HA and toxin. Fifty μ g sample were applied on disc electrophoresis, which was carried out by the method of Gabriel et al. (7) with 5.0% polyacrylamide gel at pH 8.9. A, type C HA; B, type C toxin.

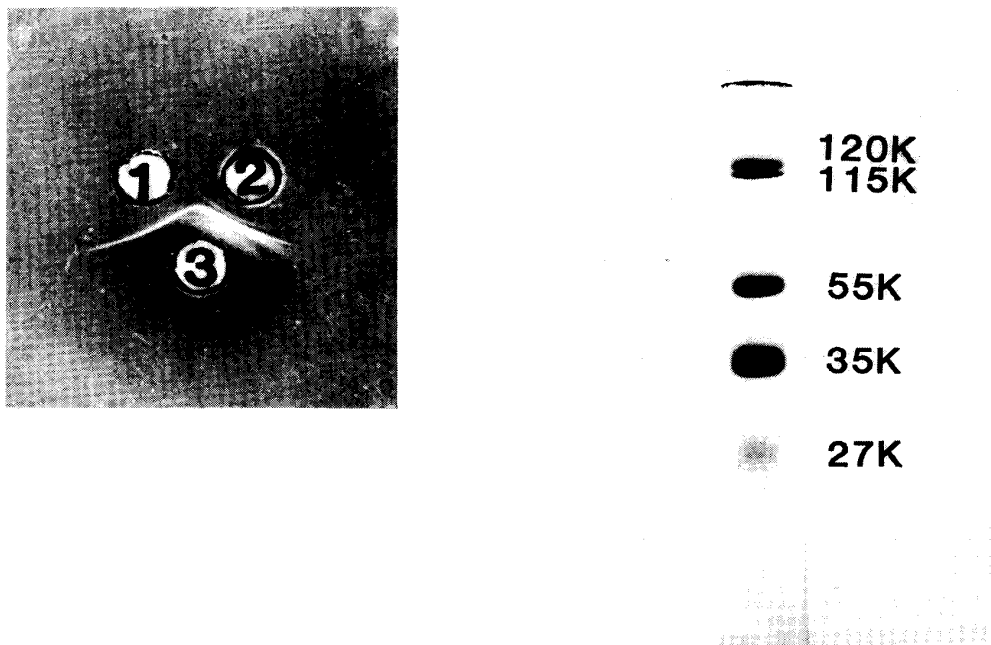


Fig. 5 Immunodiffusion test of HA fractions of culture supernatant and the purified HA. Gel contained 1% agarose in 0.02 M Tris-glycine-0.5 M NaCl buffer at pH 8.0, and 50 μ l of each fraction were applied to well. (1), purified HA ; (2), culture supernatant ; (3), rabbit anti-crude HA (including HA, type C toxin and other protein in supernatant).

Fig. 6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified HA. Protein (50 μ g) was applied on a gel, and the electrophoresis was carried out by the method of Weber et al. (21) with 8% polyacrylamide gel at a constant current of 8 mA per gel.

into five components approximately 120,000, 115,000, 55,000, 35,000 and 27,000. Molar ratio of these subunits was estimated as 1 : 1 : 2 : 3 : 1 by densitometry.

Agglutination of erythrocytes and binding to intestinal epithelial cells

Susceptibility of animal erythrocytes to HA is shown in Table 2. HA showed the highest hemagglutination reactivity to human, horse and cat erythrocytes, which are eight times higher than that of rabbit. When the erythrocytes were previously treated with neuraminidase, their susceptibility to HA were lost. HA was also absorbed with the small intestinal epithelial cells.

Inhibition of hemagglutination

Hemagglutination activity of HA to erythrocyte was inhibited in the presence of gangliosides, GM₄, GM₃, GD_{1a}, GD_{1b}, GT_{1b} and fetuin, but not D-galactose, α -Methyl-D-mannoside, D-glucose and fetuin treated with neuraminidase (tab. 3).

TABLE 2 *Hemagglutination activity to erythrocytes*

	($\mu\text{g/ml}$) ^a
Human	60
Horse	60
Cat	60
Rat	120
Mouse	244
Dog	244
Chicken	240
Sheep	320
Rabbit	480

^aMinimum HA protein concentration giving hemagglutination.

TABLE 3 *Hemagglutination inhibition test with gangliosides and fetuin using horse erythrocytes*

SUBSTANCES ^a	CONCENTRATION ^b	EQUIMOLAR PER ONE
	μmol	MOL OF SIALIC ACID μmol
GM ₄	1.8	1.8
GM ₃ (NeuAc)	6.5	6.5
GM ₃ (NeuGc)	13.0	13.0
GM ₂ (NeuAc)	10.8	10.8
GM ₁ (NeuAc)	83.0	83.0
GD _{1a} (NeuAc)	16.0	8.0
GD _{1b} (NeuAc)	34.0	17.0
GT _{1b} (NeuAc)	3.6	1.2
Fetuin ^c	10.3	0.76
Fetuin treated by neuraminidase	100	ND ^d

^aTerminology for gangliosides is that proposed by Svennerholm (17). Molecular weights of GM₃(NeuGc) and GM₃(NeuAc) were calculated as C₂₄(lignoceric acid). The others molecular weights of gangliosides were calculated as C₁₈(stearic acid). Molecular weight of fetuin and fetuin treated with neuraminidase were calculated as 48,000.

^bMinimum substances concentration giving hemagglutination inhibition.

^cSialic acid contents of fetuin were calculated as 0.7%.

^dNot determined.

DISCUSSION

Clostridium botulinum type C hemagglutinin was purified approximately 2,000 fold from the culture supernatant. The purified HA was homogenous in the purity assays. In the presence of sodium dodecyl sulfate with or without the reducing agent, HA was dissociated into five components, 120,000, 115,000, 55,000, 35,000 and 27,000, whose molecular ratio was 1 : 1 : 2 : 3 : 1-2. Since the molecular weight of the purified HA has been estimated 240,000 by analytical ultracentrifugation, two molecular types of HA may exist, one of them consists of four components of 120,000, 55,000, 35,000 and 27,000 with the molecular ratio of 1 : 1 : 1 : 1, and the other consists of three components of 115,000, 55,000 and 35,000 with the molecular ratio of 1 : 1 : 2. Similar subunit structure was observed on types A and B HAs purified by affinity chromatography.⁶⁾ DasGupta et al.⁵⁾ reported that three sizes of type A HA could be separated from crystalline toxin by gel filtration. Botulinum HA may have several aggregated forms. From the results of the hemagglutination and hemagglutination inhibition tests, type C HA is considered to bind to sialic acids in the surface of erythrocytes. This observation is in agreement with Balding's report.²⁾ Probably HA binds the similar binding sites in the intestinal cells.

HA binds to toxin and forms HA-toxin complex at low pH, however, HA-toxin complex dissociates to HA and toxin above pH 7.2 and partially around pH 7.0.¹⁶⁾ Since the pH in the animal small intestine was around 7, botulinum toxin may bind through HA on intestinal epithelial cells as the first step of botulinum intoxication. HA may play a role as a kind of a carrier for the toxin passing the intestinal barrier.

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