Identification and characterization of functional subunits of *Clostridium botulinum* type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes

Yukako Fujinaga[a], Kaoru Inoue[a], Takako Nomura[b], Junzo Sasaki[b], Jean C. Marvaud[c], Michel R. Popoff[c], Shunji Kozaki[d], Keiji Oguma[a,*]

[a] *Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[b] *Department of Anatomy, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[c] *Unite des Toxines Microbiennes, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France*
[d] *Department of Veterinary Science, College of Agriculture, University of Osake Prefecture, 1-1 Gakuen-cho, Sakai, Osaka 599-8231, Japan*

Received 20 October 1999; received in revised form 12 January 2000

Edited by Shozo Yamamoto

Abstract ***Clostridium botulinum*** **type A hemagglutinin-positive progenitor toxin consists of three distinct components: neurotoxin (NTX), hemagglutinin (HA), and non-toxic non-HA (NTNH). The HA consists of four subcomponents designated HA1, 2, 3a and 3b. By employing purified toxin and GST-fusion proteins of each HA subcomponent, we found that the HA-positive progenitor toxin, GST-HA1 and GST-HA3b bind to human erythrocytes and microvilli of guinea pig upper small intestinal sections. The HA-positive progenitor toxin and GST-HA1 bind via galactose moieties, GST-HA3b binds via sialic acid moieties. GST-HA2 and GST-HA3a showed no detectable binding.**

Key words: Toxin binding; Hemagglutinin; Microvillus; *Clostridium botulinum*

1. Introduction

Clostridium botulinum neurotoxin (NTX, $M_r \sim 150\,000$) has seven serotypes, A–G, and exists as a stable complex due to association with non-toxic proteins. The complex, designated as the progenitor toxin, is found in three forms; 12S toxin, 16S toxin and 19S toxin [1]. Type A progenitor toxin is composed of these three forms. By comparative genetic and biochemical analyses of the progenitor toxins, precise subunit structures have been elucidated [2–6]. The 12S toxin consists of a NTX and a non-toxic component having no hemagglutinin (HA) activity and is designated as non-toxic non-HA (NTNH). The 16S toxin consists of an NTX, NTNH and HA. Type A HA consist of four different subcomponents: HA3b, HA1, HA3a and HA2 that correspond to HA-52, HA-35, HA-19 ~ 20 and HA-15 [7]. The 19S toxin is a dimer of 16S toxin, and is presumed to be crosslinked through HA1 [7].

NTX is a highly potent inhibitor of the neurotransmitter release from the peripheral nerve terminus. This poisoning mechanism involves the cleavage of proteins associated with the fundamental process of exocytosis [8,9]. Food-borne botulism is caused by ingestion of material containing the progenitor toxin. Orally ingested progenitor toxin is absorbed from the upper small intestine into the lymphatic system [1], which then enters the blood stream, and reaches peripheral nerves, where neurological dysfunction is elicited. The non-toxic components play an important role in the food-borne botulism. It has been reported that the progenitor toxins possess greater potential for oral toxicity than NTX alone, since the former is more resistant to low pH and proteases in the digestive tract [1]. Recently, HA has been found to function as an adhesin allowing the type C 16S toxin to bind to the microvilli of the upper small intestine, which leads to the efficient absorption of the toxin [10].

In the toxin complex, HA-subcomponents are associated non-covalently, and it is technically difficult to isolate each viable HA-subcomponent by protein chemical procedures. Thus, the roles of each subcomponent in binding to intestinal microvilli and erythrocytes have only been vaguely defined. In this study, we produced all four type A HA subcomponents as recombinant proteins, and definitively identified the HA subcomponents that possess the ability to bind to intestinal microvilli and erythrocytes.

2. Materials and methods

2.1. Purification of progenitor toxins and their antisera

The type A HA positive progenitor toxin (a mixture of 16S and 19S toxins) and HA negative progenitor toxin (12S toxin) were purified from the culture fluid of *C. botulinum* type A strain 62A as described previously [11]. The antisera against each of these progenitor toxins were those previously prepared [11,12].

2.2. Construction of expression vectors

Purified DNA from type A strain NIH was used as a template for amplification by polymerase chain reaction as described previously [13,14]. The following primers were designed from type A HA component locus sequence [15]: Primer A1F, 5′-AACGGATCCCCGTAATCCAAAATTCATTAAAT-3′; primer A1R, 5′-ATAGTCGACTTATGGGTTACGAATATTCCA-3′; primer A2F, 5′-TGAGGATCCCCTCAGTTGAAAGAACTTTTCT-3′; primer A2R, 5′-TTTGTCGACTTATATTTTTTCAAGTTTGAACA-3′; primer A3F, 5′-TTAGGATCCCCAGTGATACTATTGATTTAG-3′; primer A3R, 5′-TGTGTCGACTTATTTAGAATTAAAAATATTGTTA-3′; primer A4F, 5′-TTAGGATCCCCGTTAGTAGCACACAACGAG-3′; primer A4R, 5′-CGTGTCGACTTAATTAGTAATATCTATATGC-3′. Sequences encoding HA1, HA2, HA3a and HA3b were obtained with the primer pairs A1F/A1R,A2F/A2R, A3F/A3R and A4F/A4R, respectively. These primer combinations introduced a novel *Bam*HI site at the 5′ end and a stop codon and *Sal*I site at the 3′ end. The products were restricted with *Bam*HI and *Sal*I and, after purification by agarose gel electrophoresis, were inserted in an expression vector

*Corresponding author. Fax: (81)-86-235 7162.
E-mail: kuma@med.okayama-u.ac.jp

PII: S0014-5793(00)01147-9

pGEX-5X-3 (Amersham Pharmacia Biotech) restricted with the same enzymes. The sequences of the constructs were verified by cycle sequencing on a 373A DNA sequencer (Perkin-Elmer).

2.3. Expression and purification of recombinant HA subcomponents

The GST-fusion proteins were expressed and affinity purified using Glutathione Sepharose 4B from lysates of recombinant *Escherichia coli* (DH5a) as recommended by the supplier (Amersham Pharmacia Biotech).

In order to isolate the HA polypeptides from the GST moiety, each fusion protein was cleaved by addition of biotin-labelled factor Xa (Restriction Protease Factor Xa Cleavage and Removal kit, Boehringer) and incubation for 5 h at 25°C (enzyme:substrate weight ratio of 1:100). After removing the biotin-labelled factor Xa by using the streptavidin gel (Restriction Protease Factor Xa Cleavage and Removal kit), the pure HA subcomponent was obtained by using a Glutathione Sepharose 4B column as described by the supplier (Amersham Pharmacia Biotech).

2.4. SDS–PAGE and immunoblot analysis

SDS–PAGE was performed by the method of Laemmli [16] by using a 4% stacking gel and a 8–16% gradient separating gel under reducing conditions. For immunoblot analysis, proteins were electrotransferred, reacted with 1:104-diluted rabbit anti-type A HA-positive progenitor toxin serum, and then reactive bands were visualized using the ECL detection system (Amersham Pharmacia Biotech) as described previously [7].

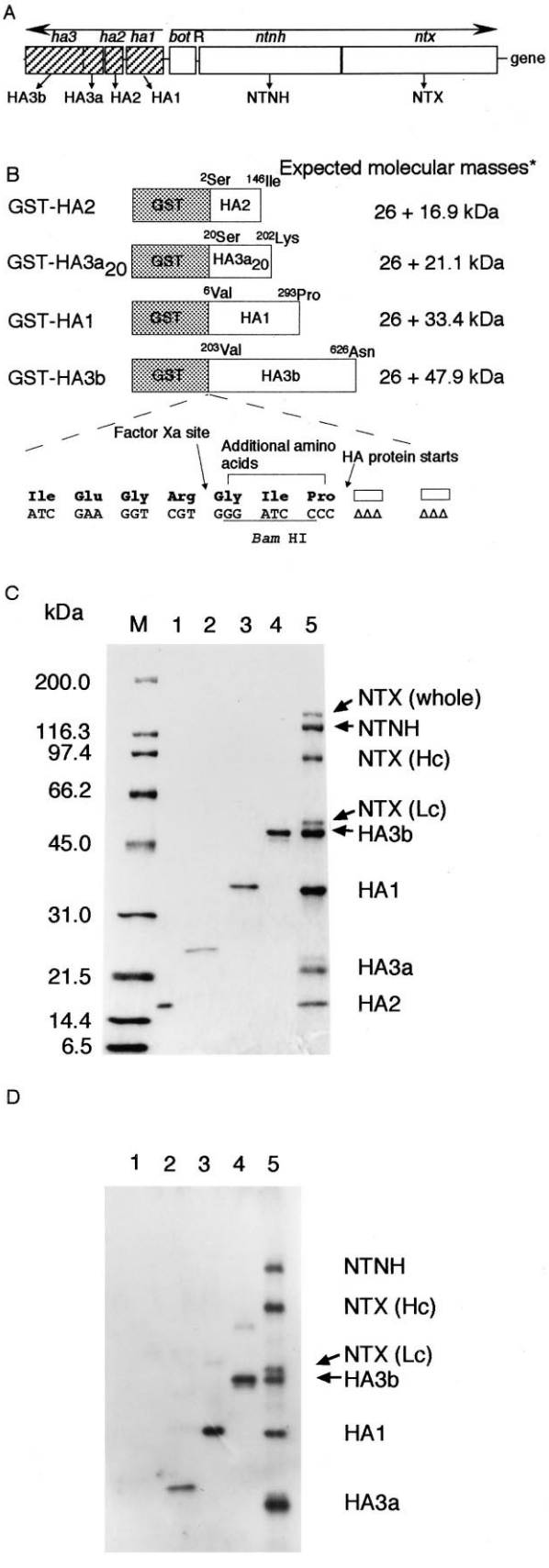
2.5. Binding assay with intestinal tissue sections

Binding experiments of the progenitor toxins and recombinant proteins to paraformaldehyde-fixed sections of guinea pig upper small intestine was performed as described previously [10]. As the first antibody, rabbit anti-type A HA-positive progenitor toxin serum (1:103 dilution), rabbit anti-type A HA-negative progenitor toxin serum (1:103 dilution), and goat anti-GST antibody (1:3×103 dilution, Amersham Pharmacia Biotech) were used for detection of type A HA-positive progenitor toxin, HA-negative progenitor toxin and GST-fusion proteins, respectively.

Sialidase treatment of the section was performed under the same condition as described previously [10], using *Arthrobacter ureafaciens* neuraminidase (highly purified preparation containing no protease, *N*-acetylneuraminic acid (NeuAc) aldolase or glycosidase; Nacalai tesque). For saccharide inhibition experiments, type A HA-positive progenitor toxin and fusion proteins were pre-incubated with saccharides (final concentration: 100 mM) for 1 h at 4°C, and then the mixture was reacted with sections.

2.6. Binding assay with erythrocytes

Binding of the progenitor toxins and recombinant proteins to erythrocytes was determined in a microassay as previously described by Hoschützky et al. [17] with minor modification. Microtiter plates (EIA/RIA Plate, Costar) were coated with the toxins or recombinant proteins (100 mg/ml, 50 ml per well) at 4°C for 16 h. The wells were



→

Fig. 1. A: Genetic organization of type A progenitor toxin genes. Data summarized from Henderson et al. [15] and Fujita et al. [13]. Hatched boxes represent the genes for HA subcomponents. Letters above boxes refer to the designation of type A progenitor toxin genes. The arrows refer to the direction of transcription of the genes. The *ha*3 gene product is a precursor of the HA3a and HA3b [2]. The N-terminal amino acid of $HA3a_{20}$ is ^{20}Ser. B: GST-HA subcomponent fusion proteins. Factor Xa cleavage sites and HA protein start sites are denoted by arrows. * Molecular masses were calculated from amino acid sequences of fusion proteins. C: Electrophoretic analysis of GST-free HA proteins and HA subcomponents from type A HA-positive progenitor toxin. Proteins were visualized by staining with Coomassie Blue R-250 (Merck). Lane M, molecular weight standards (Broad Range, Bio-Rad); lane 1, GST-free HA2; lane 2, GST-free $HA3a_{20}$; lane 3, GST-free HA1; lane 4, GST-free HA3b; lane 5, type A HA-positive progenitor toxin. Subcomponents of type A HA-positive progenitor toxin were indicated on the right side. D: Western blotting of panel C with a rabbit anti-type A HA-positive progenitor toxin serum.

washed twice with PBS, and free binding sites were blocked with 1% bovine serum albumin (BSA) in PBS at room temperature for 2 h. Then the wells were incubated with a 1% suspension of human type O erythrocytes in PBS containing 1% BSA at room temperature for 30 min. The wells were carefully washed five times with PBS, the adherent erythrocytes were lysed with H_2O (50 μl per well), and an absorbance at 405 nm of the hemoglobin was taken as a measure of the number of adherent erythrocytes. The binding inhibition test with a different amount of saccharides was also performed as described above.

2.7. Sialidase treatment of erythrocytes

Human type O erythrocytes (10% suspension in PBS) were treated with *A. ureafaciens* neuraminidase (Nacalai tesque) at a final concentration of 0.01 U/ml, at 37°C for 1 h. Thereafter, the erythrocytes were washed twice with PBS and suspended in PBS (50% suspension).

3. Results and discussion

3.1. Expression and purification of recombinant HA subcomponents

All four type A HA-subcomponents were produced as recombinant proteins fused to the GST (Fig. 1A,B). Each HA subcomponent polypeptide was designed to extend from the same N-terminal amino acid residue as the corresponding HA subcomponent of type A HA-positive progenitor toxin [7]. After elimination of the GST moiety by using factor Xa protease, the GST-free HA proteins were analyzed by SDS–PAGE (Fig. 1C). The apparent relative molecular masses of all the GST-free HA proteins except GST-free $HA3a_{20}$ were similar to those of the HA subcomponents from HA-positive progenitor toxin (native HA subcomponents). The GST-free $HA3a_{20}$ was approximately M_r 4000 (about 36 amino acid residues) larger than the native $HA3a_{20}$. As the GST-free HA-subcomponents had only three extra amino acids (Gly-Ile-Pro) derived from the vector, it is possible that the native $HA3a_{20}$ undergoes C-terminal processing by endogenous proteases. The C-terminal amino acid sequence of the native $HA3a_{20}$ will be determined to confirm this speculation.

The antigenic integrity of GST-free HA proteins was confirmed by Western blotting (Fig. 1D). All the recombinant proteins except GST-free HA2 were immuno-reactive with anti-type A HA-positive progenitor toxin serum, which was similar to results observed using the HA subcomponents from 16S and 19S toxins. The immuno-reactivity of native

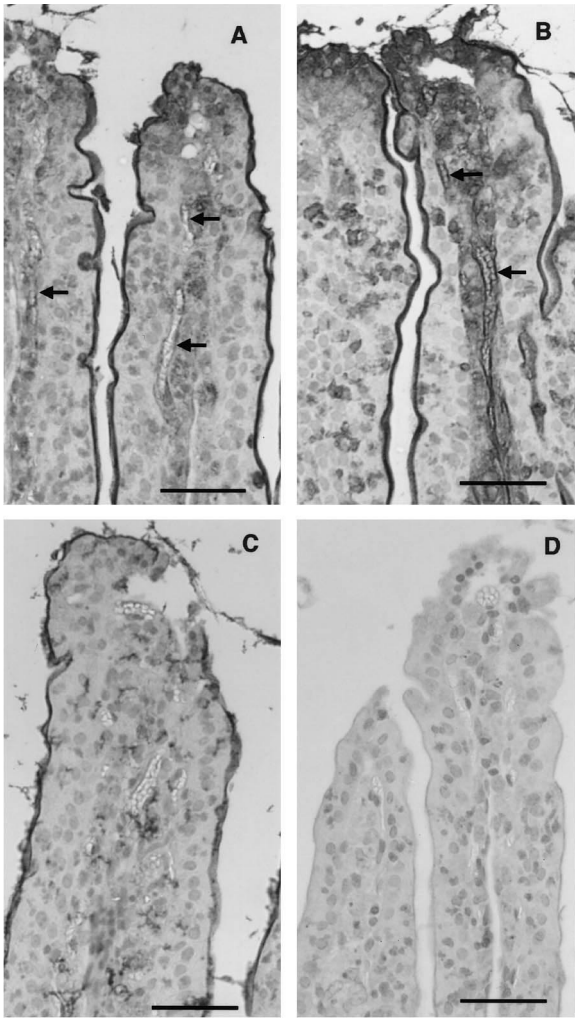


Fig. 2. Light micrograph of upper small intestine of guinea pig incubated with type A HA-positive progenitor toxin (1 μg/ml) (A), GST-HA3b (50 μg/ ml) (B), GST-HA1 (50 μg/ ml) (C), and GST alone (50 μg/ ml) (D). Adherent molecules were stained brown. As the first antibodies, rabbit anti-type A HA-positive progenitor toxin serum (A), and goat anti-GST antibody (B) (C) (D) were used. Sections were counterstained blue with hematoxylin. The same results were obtained in three repeats of this experiment, and each was done in duplicate. Arrows point to the location of stained blood capillary endothelia. Bars, 50 μm.

Table 1
Binding profiles of type A progenitor toxins and GST-HA proteins to the intestinal microvilli and erythrocytes

	HA-positive progenitor toxin	GST-HA3b	GST-HA1
Binding to intestinal microvilli			
no treatment	++	++	+
Neuroaminidase-treatment	++	−	++
In the presence of sugar (100 mM)			
Lac	∓	NT	∓
Gal	∓	NT	∓
Glc	++	NT	+
Binding to erythrocytes			
no treatment	++	++	++
Neuroaminidase-treatment	++	−	++
In the presence of sugar (100 mM)			
Lac	+	++	−
Gal	+	++	−
Glc	++	++	++

Assays were performed in three independent experiments, and gave essentially the same results. Intensity of binding: −, absent; ∓, trace; +, weak; ++, intense. For an example of ++, see Fig. 2a,b. NT, not tested.

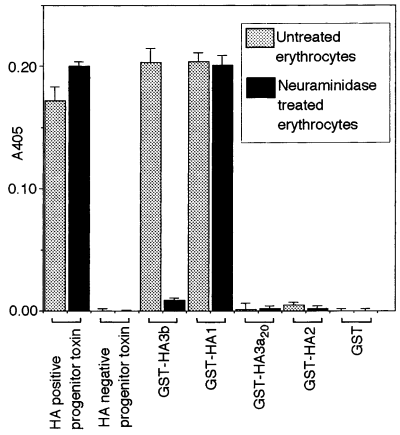


Fig. 3. Binding properties of progenitor toxins and GST-HA proteins to erythrocytes. Background binding to BSA was minimal and was subtracted from the absorbance values. Each point is the mean ± S.D. of triplicate wells. The experiment depicted is representative of the three experiments performed. A_{405}, Absorbance at 405 nm.

HA2 was very weak compared with other components as described previously [7]; likewise, GST-free HA2 was also very weak.

3.2. HA component of type A HA-positive progenitor toxin binds to the intestinal microvilli

Binding experiments were performed using intestinal tissue sections. Type A HA-positive progenitor toxin (a mixture of 16S and 19S toxins) showed intense binding to the microvilli (Fig. 2 and Table 1), whereas HA-negative progenitor toxin (12S toxin) did not show any binding, as reported for type C progenitor toxins [10]. Given that type C 16S toxin exhibits NeuAc-dependent binding activity [10], the ability of type A HA-positive progenitor toxin to adhere to sialidase-treated intestinal sections was tested. The sialidase treatment showed no change in type A HA-positive progenitor toxin binding (Fig. 2 and Table 1). In order to examine ligand(s) for type A HA-positive progenitor toxin on microvilli, binding inhibition experiments using three different saccharides were carried out (Table 1). Binding was drastically inhibited by 100 mM lactose (Lac) and 100 mM galactose (Gal), but not by 100 mM glucose (Glc). These results indicate that the HA but not NTNH or NTX, components of type A HA-positive progenitor toxin exhibit binding activity on microvilli, as observed for type C. However, unlike type C HA, the Gal moieties on the microvilli glycoconjugates mediate toxin binding.

3.3. HA1 and HA3b exhibit binding activity toward intestinal microvilli and erythrocytes

The binding of the GST-HA subcomponents to intestinal tissue sections was analyzed. Given that the immuno-reactivity of HA2 was very weak, we utilized the GST-HA fusion proteins and anti-GST antibody to detect these binding activities. As shown in Fig. 2 and Table 1, GST-HA1 and GST-HA3b showed binding to microvilli, whereas GST alone, GST-HA2 and GST-HA3a_{20} did not. In contrast to GST-HA1, GST-HA3b and HA-positive progenitor toxin also bound to blood capillary endothelia. Sialidase treatment of the sections completely abolished GST-HA3b binding to microvilli, but slightly increased GST-HA1 binding. The binding of GST-HA1 to microvilli was obviously inhibited by 100 mM Lac and 100 mM Gal, but not by 100 mM Glc (Table 1).

The binding activity of the GST-HA subcomponents to erythrocytes was also analyzed. Immobilized GST-HA proteins and progenitor toxins were used in a microassay and as shown in Fig. 3 and Table 1, GST-HA1 and GST-HA3b showed the same binding activities as type A HA-positive progenitor toxin. Type A HA-negative progenitor toxin, GST-HA2, GST- HA3a_{20} and GST alone showed no binding activity. Sialidase treatment of erythrocytes completely abolished the adhesion activity of GST-HA3b, but did not affect GST-HA1 and type A HA-positive progenitor toxin binding. The binding of GST-HA1 was drastically inhibited by 100 mM of Lac, and Gal, but not by 100 mM Glc. These inhibitions were dose-dependent and the half-maximal inhibition occurred at about 50 mM for Lac and 20 mM for Gal (Fig. 4C). The binding of type A HA-positive progenitor toxin to the erythrocytes was partially inhibited by 100 mM of Lac and Gal, but was not inhibited by 100 mM of Glc (Table 1, Fig. 4A,B). It is likely that the type A HA-positive progenitor toxin recognized the other ligand(s) in addition to Gal-based carbohydrates on erythrocyte cells. Another possibility is that type A HA-positive progenitor toxin binds with high affinity to the Gal-based carbohydrates on erythrocyte membrane and

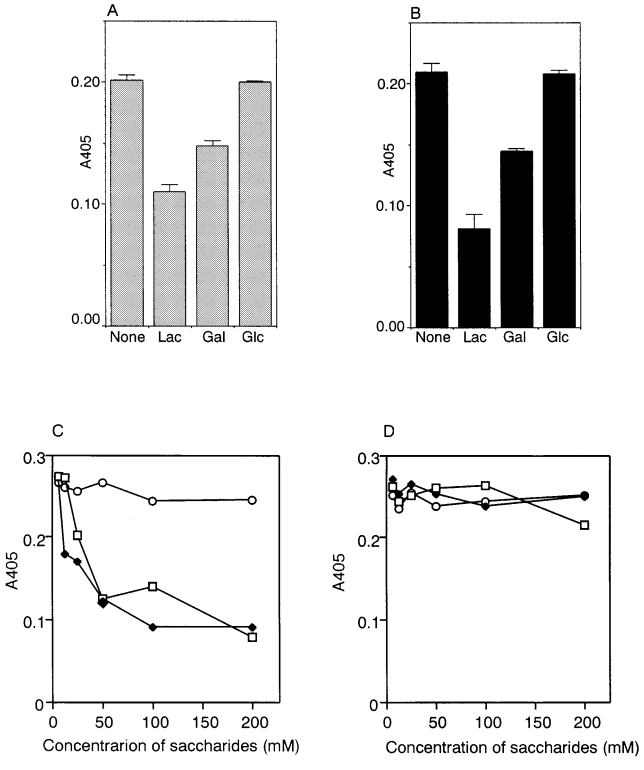


Fig. 4. Effect of saccharides on the heme-adhesion of HA-positive progenitor toxin and GST-HA1 and HA-3b. HA-positive progenitor toxin coated wells (A,B) were incubated for 1 h at 4°C with various saccharides (100 mM) prior to addition of untreated erythrocytes (A) or neuraminidase treated erythrocytes (B), and bound erythrocytes were determined. Background binding to BSA was minimal and was subtracted from the absorbance values. Each point is the mean ± S.D. of triplicate wells. Effect of different amount of saccharides (□, Gal; ◆, Lac; ○, Glc) on the binding of GST-HA1 (C) and GST-HA3b (D) to untreated erythrocytes was also observed in the same manner.

is therefore not inhibited efficiently by Gal-based mono- or disaccharides. In case of GST-HA3b, little decrease of binding to erythrocytes was observed even though 200 mM of these three saccharides were added (Fig. 4D).

From the results of binding assays using intestinal tissue sections and erythrocytes, HA1 and HA3b were identified as the adhesive subcomponents to microvilli and erythrocytes, and Gal moieties on carbohydrates side chains on microvilli and erythrocyte are important for the binding of HA1, whereas NeuAc moieties are important for the binding of HA3b.

The observation that the type A HA has two different adhesive subcomponents, HA1 and HA3b, raises the question of whether one or both of these subcomponents in the type A HA-positive progenitor toxin contribute to the binding activity of the HA-positive progenitor toxin. Our results suggest the binding of type A HA-positive progenitor toxin to sialidase-treated cells may be attributed to the HA1 molecule, as recombinant HA3b showed no binding activity on these cells. At present, there is no direct evidence indicating the contribution of the HA3b to the HA-positive progenitor toxin binding activity, however, recombinant HA3b showed more intense binding to the microvilli than recombinant HA1. In addition, the binding of type A HA-positive progenitor toxin to blood capillary endothelia on intestinal tissue sections may be attributed to the HA3b molecule, as recombinant HA1 did not bind these cells. The elucidation of the precise roles of these two subcomponents in binding and absorption of type A HA-positive progenitor toxin in the small intestine requires more detailed analysis with type A HA-positive progenitor toxin, HA1, and HA3b.

We previously reported that only HA1, out of four HA subcomponents, exists alone as a multimer in the culture supernatant of *C. botulinum* types A and D. These HA1 multimers, however, have little HA activity [7,18]. Fu et al. performed similar experiments but found that the unassembled type A HA1 possesses HA activity [19]. We are now preparing each HA subcomponent of types A–D as recombinant proteins in an attempt to clarify their binding and HA activities.

Acknowledgements: This work was supported in part by Grants 09770180 and 10670256 from the Ministry of Education, Science and Culture of Japan, and by a grant for the 'Emerging and Reemerging Infectious Disease' funded by the Ministry of Health and Welfare of Japan.

References

[1] Sakaguchi, G., Kozaki, S. and Ohishi, I. (1984) in: Bacterial Protein Toxins, pp. 435–443, Academic Press, London.
[2] Fujinaga, Y., Inoue, K., Shimazaki, S., Tomochika, K., Tsuzuki, K., Fujii, N., Watanabe, T., Ohyama, T., Takeshi, K., Inoue, K. and Oguma, K. (1994) Biochem. Biophys. Res. Commun. 205, 1291–1298.
[3] Ohyama, T., Watanabe, T., Fujinaga, Y., Inoue, K., Sunagawa, H., Fujii, N., Inoue, K. and Oguma, K. (1995) Microbiol. Immunol. 39, 457–465.
[4] Oguma, K., Fujinaga, Y. and Inoue, K. (1995) Microbiol. Immunol. 39, 161–168.
[5] Minton, N.P. (1995) Curr. Top. Microbiol. Immunol. 95, 161–194.
[6] Oguma, K., Fujinaga, Y. and Inoue, K. (1997) J. Toxicol. Toxin Rev. 16, 253–266.
[7] Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K. and Oguma, K. (1996) Infect. Immun. 64, 1589–1594.
[8] Jahn, R. and Niemann, H. (1994) Ann. N. Y. Acad. Sci. 733, 245–255.
[9] Montecucco, C. and Schiavo, G. (1994) Mol. Microbiol. 13, 1–8.
[10] Fujinaga, Y., Inoue, K., Watanabe, S., Yokota, K., Hirai, Y., Nagamachi, E. and Oguma, K. (1997) Microbiology 143, 3841–3847.
[11] Sugii, S. and Sakaguchi, G. (1975) Infect. Immun. 12, 1262–1270.
[12] Kozaki, S., Sakaguchi, S. and Sakaguchi, G. (1974) Infect. Immun. 10, 750–756.
[13] Fujita, R., Fujinaga, Y., Inoue, K., Nakajima, H., Kumon, H. and Oguma, K. (1995) FEBS Lett. 376, 41–44.
[14] Fujinaga, Y., Takeshi, K., Inoue, K., Fujita, R., Ohyama, T., Moriishi, K. and Oguma, K. (1995) Biochem. Biophys. Res. Commun. 213, 737–745.
[15] Henderson, I., Whelan, S.M., Davis, T.O. and Minton, N.P. (1996) FEMS Microbiol. Lett. 140, 151–158.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Hoschützky, H., Lottspeich, F. and Jann, K. (1989) Infect. Immun. 57, 76–81.
[18] Inoue, K., Fujinaga, Y., Honke, K., Yokota, K., Ikeda, T., Ohyama, T., Takeshi, K., Watanabe, T., Inoue, K. and Oguma, K. (1999) Microbiology 145, 2533–2542.
[19] Fu, F.N., Sharma, S.K. and Singh, B.R. (1998) J. Protein Chem. 17, 53–60.