***Clostridium botulinum* type A haemagglutinin-positive progenitor toxin (HA⁺-PTX) binds to oligosaccharides containing Gal β 1-4GlcNAc through one subcomponent of haemagglutinin (HA1)**

Kaoru Inoue,¹ Yukako Fujinaga,¹ Koichi Honke,² Hideyuki Arimitsu,¹ Nazira Mahmut,¹ Yoshihiko Sakaguchi,¹ Tohru Ohya,³ Toshihiro Watanabe,³ Katsuhiko Inoue³ and Keiji Oguma¹

Author for correspondence: Keiji Oguma. Tel: +81 86 235 7162. Fax: +81 86 235 7162.
e-mail: kuma@med.okayama-u.ac.jp

¹ Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

² Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

³ Department of Food Science, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2422, Japan

Haemagglutinin (HA) activity of *Clostridium botulinum* type A 19S and 16S toxins (HA-positive progenitor toxin; HA⁺-PTX) was characterized. HA titres against human erythrocytes of HA⁺-PTX were inhibited by the addition of lactose, D-galactose, N-acetyl-D-galactosamine and D-fucose to the reaction mixtures. A direct glycolipid binding test demonstrated that type A HA⁺-PTX strongly bound to paragloboside and some neutral glycolipids, but did not bind to gangliosides. Type A HA⁺-PTX also bound to asialoglycoproteins (asialofetuin, neuraminidase-treated transferrin), but not to sialoglycoproteins (fetuin, transferrin). Although glycopeptidase F treatment of asialofetuin abolished the binding of HA⁺-PTX, endo- α -N-acetylgalactosaminidase treatment did not. Thus these results can be interpreted as indicating that type A HA⁺-PTX detects and binds to Gal β 1-4GlcNAc in paragloboside and the N-linked oligosaccharides of glycoproteins. Regardless of neuraminidase treatment, type A HA⁺-PTX bound to glycophorin A which is a major sialoglycoprotein on the surface of erythrocytes. Both native glycophorin A and neuraminidase-treated glycophorin A inhibited the binding of erythrocytes to type A HA⁺-PTX. Since the N-linked oligosaccharide of glycophorin A is di-branched and more than 50% of this sugar chain is monosialylated, type A HA⁺-PTX probably bound to the unsialylated branch of the N-linked oligosaccharide of glycophorin A and agglutinated erythrocytes. One subcomponent of HA, designated HA1, did not agglutinate native erythrocytes, although it did bind to erythrocytes, paragloboside and asialoglycoproteins in a manner quite similar to that of HA⁺-PTX. These results indicate that type A HA⁺-PTX binds to oligosaccharides through HA1.

Keywords: binding, sugar chain, glycolipid, glycoprotein

INTRODUCTION

Clostridium botulinum strains produce immunologically distinct neurotoxins (type A to G) which inhibit the release of acetylcholine at neuromuscular junctions and synapses. The molecular masses of all types of these neurotoxins are approximately 150 kDa. The neuro-

toxins associate with non-toxic components in culture fluids to form larger complexes which are designated progenitor toxins. In the type A strain, three different sized progenitor toxins with molecular masses of 900 kDa (19S), 500 kDa (16S) and 300 kDa (12S) are produced. Type B, C and D strains produce both the 16S and 12S toxins, whereas the type E and F strains produce only the 12S toxin and type G strain produces only the 16S toxin (Sakaguchi *et al.*, 1984). In all these strains, the non-toxic components of the 19S and 16S toxins display

Abbreviations: CBB, Coomassie brilliant blue; HA, haemagglutinin; HA⁺-PTX, haemagglutinin-positive progenitor toxin; PAS, periodic acid-Schiff.

haemagglutinin (HA) activity but that of the 12S toxin does not. The 12S toxin is formed by association of a neurotoxin with a non-toxic component having no HA activity (designated here as non-toxic non-HA), while 19S and 16S toxins are formed by conjugation of the 12S toxin with HA. The non-toxic components (HA and non-toxic non-HA) are considered to be very important to the development of food poisoning because the non-toxic components protect the neurotoxin from the barrier of gastric juice when the progenitor toxins pass through the stomach (Sugii *et al.*, 1977). Therefore, the larger the molecular size of the progenitor toxin of the same type, the higher the oral toxicity (Ohishi & Sakaguchi, 1980; Ohishi *et al.*, 1977).

We purified different sized progenitor toxins from *Clostridium botulinum* type A and C culture fluids, and demonstrated that the HAs consist of subcomponents that have molecular masses of 52–53, 33–35, 19–23 and 15–17 kDa, which are designated here HA3b, HA1, HA3a and HA2, respectively (Fujinaga *et al.*, 1994; Inoue *et al.*, 1996). In a previous study, we purified types C and D 16S toxins, and characterized their HA activity and binding to glycolipids and glycoproteins (Inoue *et al.*, 1999). Types C and D 16S toxins agglutinated human erythrocytes and both HA activities were reduced by employing erythrocytes that had been treated with neuraminidase. Types C and D 16S toxins bound to sialylglycolipids and sialoglycoproteins but did not bind to neutral glycolipids or asialoglycoproteins. In the present study, we performed haemagglutination and haemagglutination-inhibition tests, and direct binding tests to glycolipids and glycoproteins, by employing purified type A HA⁺-PTX and HA1 to determine how they bind to erythrocytes.

METHODS

Bacteria and toxin. *C. botulinum* type A strain A-NIH was used. The mixture of type A 19S and 16S toxins, designated here as type A HA⁺-PTX, was purified from culture fluid (Inoue *et al.*, 1996). Partially purified type A HA1 was further purified with a hydroxyapatite (Seikagaku) column (0.7 × 5 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) and eluted with 10 mM phosphate buffer containing 1 M NaCl (Fig. 1). We previously reported that *C. botulinum* type A culture fluid contained HA⁺-PTX (19S and 16S toxins), HA⁺-PTX (12S toxin), free HA and free HA1 (Inoue *et al.*, 1996). The HA or HA⁺-PTX contained in the HA1 sample was eluted with 0.2 M phosphate buffer (pH 6.0) and possessed HA activity.

Glycolipids and glycoproteins. Glycosphingolipids were purified from the following sources as described previously by Gasa *et al.* (1983): GM4 (NeuAc α 2-3Gal β 1-Cer), GM1 [Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-Cer], GD1a [NeuAc α 2-3Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-Cer], GD1b [Gal β 1-3GalNAc β 1-4(NeuAc α 28NeuAc α 2-3)Gal β 1-4Glc β 1-Cer] and SM4 (HSO $_3$ -3Gal β 1-Cer) from bovine brain; GM2 [GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-Cer] from a Tay-Sachs brain; ceramide monohexoside (a mixture of Gal β 1-Cer and Glc β 1-Cer) and LacCer (Gal β 1-4Glc β 1-Cer) from horse erythrocytes; Gb3Cer (Gal α 1-4Gal β 1-4Glc β 1-Cer) and SM3 (HSO $_3$ -3Gal β 1-4Glc β 1-Cer) from human kidney; Gb4Cer (GalNAc β 1-3Gal α 1-

Fig. 1. Hydroxyapatite column chromatography. The sample was applied to a hydroxyapatite column equilibrated with 10 mM phosphate buffer (pH 6.0). After washing the column with 1.5 column vols equilibrating buffer, the elution was performed successively with 10 mM phosphate buffer (pH 6.0) containing 1 M NaCl and 200 mM phosphate buffer (pH 6.0). Fractions of 1 ml were collected. The fractions indicated by a solid bar were pooled. ○, A₂₈₀; ▲, HA titre.

4Gal β 1-4Glc β 1-Cer) and SPG (NeuAc α 2-3Gal β 1-4GlcNAc β -3Gal β 1-4Glc β 1-Cer) from human erythrocytes; and GM3 (NeuAc α 2-3Gal β 1-4Glc β 1-Cer) and GD3 (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc β 1-Cer) from rat liver. Asialo GM1 (bovine brain) was purchased from Wako Pure Chemical Industries. Paragloboside was obtained by treatment of SPG with neuraminidase. Fetuin was purchased from Wako Pure Chemical Industries. Asialofetuin and glycophorin (from blood type MN, predominantly glycophorin A) were purchased from Sigma.

SDS-PAGE and electroblotting. SDS-PAGE was performed by the method of Laemmli (1970) using 12.5% acrylamide linear gels. Protein bands were stained with Coomassie brilliant blue (CBB) R-250. The molecular mass markers used were myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The bands separated by SDS-PAGE were electroblotted to PVDF membranes (Immobilon; Millipore) with a semidry blotting apparatus (Nippon Eido) according to the methods described by Hirano & Watanabe (1990).

Preparation of antisera and immunoblotting. Antiserum against type A HA prepared previously in rabbit was employed (Inoue *et al.*, 1996). Antiserum against type A HA1 was also prepared in rabbit by the method described previously (Inoue *et al.*, 1996). Immunoblotting was performed as described previously (Inoue *et al.*, 1999).

Enzyme treatment of human erythrocytes. Ten millilitre aliquots of 10% (v/v) washed human erythrocytes were incubated with 0.1 U *Arthrobacter ureafaciens* neuraminidase (Nacalai tesque), at 37 °C for 1 h. After treatment, the erythrocytes were washed three times in PBS (pH 6.0).

Determination of HA titre and inhibition tests. The HA titre was obtained by microtitration methods using multiwell plates as described previously (Inoue *et al.*, 1999). Inhibition of HA activity of the type A HA⁺-PTX with several different saccharides was examined as follows. Each diluted preparation (20 µl) was mixed with 20 µl of an appropriate concentration of saccharide solution and incubated at 37 °C for 1 h. Thereafter, 40 µl 1% (v/v) erythrocyte suspension was added to the mixtures. Following incubation at room temperature for 2 h, haemagglutination was assessed. All tests were performed in duplicate and repeated twice.

TLC immunostaining. Binding of type A HA⁺-PTX and HA1 to the glycolipids was analysed by TLC immunostaining as described previously (Inoue *et al.*, 1999) with some modifications. The glycolipids were developed on high-performance thin-layer chromatography (HPTLC) aluminium sheet silica gel 60 plates (Merck) with chloroform/methanol/water (65:35:8, by vol.). The developed plates were dipped in 0.2% (w/v) polyisobutyl-methacrylate in n-hexane for 1 min and dried. The plates were incubated in PBS (pH 6.0) containing 3% (w/v) BSA (blocking buffer 1) for 1 h, followed by incubation with 10 µg type A HA⁺-PTX ml⁻¹ or 100 µg HA1 ml⁻¹ (in blocking buffer 1) for 1 h. The plates were then washed three times with PBS (pH 6.0) containing 0.05% (v/v) Tween 20 (PBS/Tween) and reacted for 1 h with antiserum against type A HA or antiserum against type A HA1 diluted 1:1000 with blocking buffer 1. After washing three times with PBS/Tween, the plates were incubated with a peroxidase-labelled anti-rabbit IgG antibody (DAKO A/S) diluted 1:1000 with blocking buffer 1 for 1 h. The immunoreactive bands were detected by the enhanced chemiluminescence Western blotting (immunoblotting) system (ECL; Amersham). All of the procedures were performed at room temperature.

Enzyme treatment of glycoproteins. Enzyme treatment of glycoproteins was performed as follows. Transferrin (25 µg) or glycophorin (25 µg) was incubated with neuraminidase (2.5 mU) in the reaction mixture (25 µl) containing 10 mM phosphate buffer (pH 7.4) and 0.15 M NaCl at 37 °C overnight. Transferrin and asialofetuin (25 µg each) were incubated with glycopeptidase F (1 mU, Takara Shuzo) in the reaction mixture (25 µl) according to the instruction manual at 37 °C overnight. Asialofetuin (25 µg) was incubated with endo- α -N-acetylgalactosaminidase (2.5 mU, Seikagaku) in a reaction mixture containing 20 mM citrate buffer (pH 4.5) at 37 °C for 20 min.

Binding to ghost membrane proteins or glycoproteins. We analysed the binding of type A HA⁺-PTX to ghost membrane proteins and glycoproteins. The PVDF membranes blotted with proteins were immersed overnight in PBS (pH 6.0) containing 10% (w/v) BSA (blocking buffer 2) at 4 °C. The membranes were incubated in 10 µg type A HA⁺-PTX ml⁻¹ or 100 µg HA1 ml⁻¹ in blocking buffer 2 for 2 h and then washed three times with PBS/Tween. Bound type A HA⁺-PTX and HA1 to glycoproteins were detected by antiserum against type A HA and antiserum against type A HA1, respectively, and the immunoreactive bands were detected by ECL (Amersham).

Binding of erythrocytes to toxins. The binding of type A HA⁺-PTX and HA1 to erythrocytes was analysed using 96-well microtitration plates as previously described by Hoschutzky *et al.* (1989) with minor modification. Fifty microlitre aliquots of HA⁺-PTX or HA1 (10 µg ml⁻¹) were incubated in microtitre plates and left to stand overnight at 4 °C. The plates were washed three times with 200 µl PBS (pH 6.0), then 100 µl PBS (pH 6.0) containing 1% BSA was added to each well and incubated for 2 h at room temperature. After washing the

wells three times with 200 µl PBS (pH 6.0), 100 µl PBS (pH 6.0) containing glycoprotein was added to each well. After 1 h incubation at room temperature, 10 µl 10% (v/v) native or neuraminidase-treated erythrocytes in PBS (pH 6.0) was added. Plates were incubated for 30 min at room temperature and washed six or seven times with 200 µl PBS (pH 6.0). Bound erythrocytes were lysed by adding 50 µl distilled water and the absorbance at 405 nm was analysed. All tests were performed in duplicate and repeated three times.

RESULTS AND DISCUSSION

SDS-PAGE and immunoblot analysis of HA⁺-PTX and HA1

Type A HA⁺-PTX and HA1 were subjected to SDS-PAGE. HA⁺-PTX showed seven major bands (Fig. 2a, lane 4). On the basis of our previous report, each protein band was assigned as non-toxic non-HA, heavy chain of neurotoxin, HA3b, light chain of neurotoxin, HA1, HA3a and HA2, respectively (Inoue *et al.*, 1996). Type A HA1 demonstrated a single band with a molecular mass of 35 kDa (Fig. 2a, lane 3). The mobility of this protein band was the same as that of HA1 of HA⁺-PTX. When HA1 was subjected to SDS-PAGE without heat treatment, a single protein band with molecular mass of 60 kDa was evident by CBB staining (Fig. 2a, lane 2). The antiserum against type A HA reacted with HA3b, HA1 and HA3a of HA⁺-PTX, and free HA1 as reported previously (Fig. 2c, lanes 3 and 4; Inoue *et al.*, 1996). In the preparation of non-heated free HA1, two bands of 60 and 30 kDa appeared with either antiserum against type A HA or HA1. Thus, free HA1 may exist as a dimer in culture fluid and the native HA1 monomer may have more a compact form than that denatured with SDS.

Haemagglutination and haemagglutination-inhibition tests

The minimum concentration of type A HA⁺-PTX to give haemagglutination was determined using neuraminidase-treated and non-treated (native) human erythrocytes. Non-treated erythrocytes were agglutinated by 0.098 µg type A HA⁺-PTX ml⁻¹. Using the erythrocytes treated with neuraminidase (at 37 °C for 1 h), 0.195 µg type A HA⁺-PTX ml⁻¹ were needed to cause haemagglutination. Previously, Balding *et al.* (1973) reported that the HA activity of type A HA was activated by the treatment of erythrocytes with neuraminidase. This discrepancy might be ascribed to differences in the concentration of neuraminidase used, difference of neuraminidase and/or difference in the strains.

To elucidate the carbohydrate binding specificity of type A HA⁺-PTX, haemagglutination-inhibition tests were performed. Previously, Dasgupta & Sugiyama (1977) reported that D-galactose and some of its derivatives were inhibitors of type A HA. Balding *et al.* (1973) proposed that type A HA is inhibited by D-galactose. In this study, seven kinds of saccharide were subjected to

Fig. 2. SDS-PAGE (a) and immunoblots (b, c) of purified type A HA⁺-PTX and HA1. The samples were mixed with sample buffer containing β -mercaptoethanol and incubated at room temperature (lane 2) or heated at 100 °C for 7 min (lanes 1, 3 and 4). Electrophoresis was performed on a 12.5% acrylamide gel. The gel was stained with CBB R-250 (a). Samples were blotted onto a PVDF membrane and successively reacted with the anti-type A HA1 serum (b) and anti-type A HA serum (c). Lane 1, protein standards; lanes 2 and 3, type A HA1; lane 4, type A HA⁺-PTX.

Fig. 3. Binding of type A HA⁺-PTX to glycolipids on TLC plates. TLC plates were developed in a solvent system with chloroform/methanol/water (65:35:8, by vol.). The plates were incubated with type A HA⁺-PTX, stained by TLC-immunostaining as described in Methods (b) and then glycolipids were visualized by orcinol/H₂SO₄ reagent (a). Glycolipids used are as follows: lane 1, GM1; lane 2, asialoGM1; lane 3, SPG; lane 4, paragloboside; lane 5, LacCer; lane 6, Gb3Cer; lane 7, Gb4Cer.

the inhibition tests. The HA activity of type A HA⁺-PTX was inhibited by lactose, D-galactose, N-acetyl-D-galactosamine and D-fucose. To inhibit 50% of the HA activity 27.5 mM D-fucose, 15 mM N-acetyl-D-galactosamine, 15 mM D-galactose and 5 mM lactose were required, whereas no inhibition was observed with treatment by 100 mM D-glucose, N-acetyl-D-glucosamine and D-mannose (data not shown).

Binding of type A HA⁺-PTX to glycolipids

The results from the haemagglutination tests and corresponding inhibition tests suggest that polysaccharides on the surface of human erythrocytes play an important role in the binding of type A HA⁺-PTX. To explore this hypothesis further, we analysed the direct binding of type A HA⁺-PTX to glycolipids by TLC-immunostaining.

As shown in Fig. 3, type A HA⁺-PTX strongly bound to

Table 1. Binding of type A HA⁺-PTX to glycolipids

Glycolipid	Structure	Binding
GalCer	Gal β 1-Cer	–
GlcCer	Glc β 1-Cer	–
LacCer	Gal β 1-4Glc β 1-Cer	+
Gb3Cer	Gal α 1-4Gal β 1-4Glc β 1-Cer	+
Gb4Cer	GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-Cer	+
AsialoGM1	Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-Cer	++
Paragloboside	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-Cer	+++
Gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD3, SPG)		–
Sulfated glycolipids (SM3, SM4)		–

paragloboside and asialoGM1 (Fig. 3b, lanes 2 and 4). Only weak binding was observed to Gb3Cer, Gb4Cer and LacCer (Fig. 3b, lanes 5–7). No binding was observed to GM1 and SPG (Fig. 3b, lanes 1 and 3). The results of direct binding tests of HA⁺-PTX to purified glycolipids are summarized in Table 1. Type A HA⁺-PTX scarcely bond to gangliosides, GalCer, GlcCer or to sulfated glycosphingolipids (SM3, SM4). These results suggest that N-acetylneuraminic acid or sulfate at the terminus of the carbohydrate structure disturbed the binding of type A HA⁺-PTX to glycolipids.

Binding of type A HA⁺-PTX to glycoproteins

To investigate the binding property of type A HA⁺-PTX to oligosaccharides, a binding test to enzyme-treated and non-treated glycoproteins was performed. Although

Fig. 4. Binding of type A HA⁺-PTX to glycoproteins and membrane proteins from human erythrocytes. Glycoproteins (10 µg each) and membrane proteins from human erythrocytes (300 µg each) were electrophoresed and stained with CBB R-250 (a, c) or PAS (d) or electroblotted onto PVDF membranes for toxin binding analysis as described in Methods (b, e). Lane 1 contains protein standards. Glycoproteins used are as follows: lane 2, transferrin; lane 3, neuraminidase-treated transferrin; lane 4, glycopeptidase F-treated transferrin; lane 5, fetuin; lane 6, asialofetuin; lane 7, glycopeptidase F-treated asialofetuin; lane 8, endo- α -N-acetylgalactosaminidase-treated asialofetuin; lane 9, membrane proteins from human erythrocytes; lane 10, membrane proteins from neuraminidase-treated human erythrocytes; lane 11, human glycophorin A; lane 12, neuraminidase-treated glycophorin A.

type A HA⁺-PTX did not bind to transferrin or fetuin (Fig. 4b, lanes 2 and 5), it bound to neuraminidase-treated transferrin and asialofetuin (Fig. 4b, lanes 3 and

6). As shown in Fig. 5a, transferrin contains two N-linked di-branched sugar chains which are fully sialylated (complex type; Yamashita *et al.*, 1993). Fetuin contains six carbohydrate moieties/molecules, at least three O-linked oligosaccharides and three N-linked oligosaccharides (Carr *et al.*, 1993). The N-linked oligosaccharides are di- or tri-branched (complex type) and about 80% of these oligosaccharides are fully sialylated (Green *et al.*, 1988). Therefore, our results indicate that type A HA⁺-PTX bound to unsialylated oligosaccharides. HA⁺-PTX also bound to endo- α -N-acetylgalactosaminidase-treated asialofetuin (Fig. 4b, lane 8) but no binding was observed to glycopeptidase F-treated transferrin and asialofetuin (Fig. 4b, lanes 4 and 7). Thus type A HA⁺-PTX binds to unsialylated N-linked sugar chains (complex type) and not to three unsialylated O-linked sugar chains contained in fetuin. Since paragloboside and unsialylated N-linked sugar chains (complex type) which were bound by the toxin have a common structure, containing the Gal β 1-4GlcNAc at the terminus of their oligosaccharides (Table 1, Fig. 5b), type A HA⁺-PTX should detect and bind to this structure of oligosaccharide. The Gal β 1-3GalNAc structure contained in asialoGM1 is also found in the desialylated O-linked sugar chain of glycoproteins. AsialoGM1 was detected by type A HA⁺-PTX, but desialylated O-linked sugar chains of fetuin were not. The structure of the sugar chain of asialoGM1 is Gal β 1-3GalNAc1-4Gal1-4Glc, but that of desialylated O-linked sugar chain is only Gal β 1-3GalNAc. Thus, not only Gal β 1-3GalNAc but also the whole sugar chain of asialoGM1 may be important for the binding of toxin. Alternatively, O-linked sugar chains of fetuin may not be exposed to the surface of the molecule, unlike asialoGM1.

Binding of type A HA⁺-PTX to glycoproteins of erythrocytes and binding of erythrocytes to type A HA⁺-PTX

To confirm which glycoproteins of erythrocytes were detected by type A HA⁺-PTX, membrane proteins were prepared from erythrocytes and the binding of toxin was analysed. The membrane proteins prepared from non-treated and neuraminidase-treated erythrocytes exhibited multiple protein bands on SDS-PAGE by CBB staining (Fig. 4c, lanes 9 and 10). The results show that mainly a single band was bound by type A HA⁺-PTX in each preparation (Fig. 4e, lanes 9 and 10). The mobilities of these bands were identical to those detected by periodic acid-Schiff (PAS) staining (Fig. 4d). The PAS-positive band indicated by the arrow in Fig. 4d is considered to be a dimeric form of glycophorin A (PAS1) from a previous report (Furthmayr *et al.*, 1975). Commercial glycophorin A and neuraminidase-treated glycophorin A were also bound by type A HA⁺-PTX (Fig. 4e, lanes 11 and 12). These protein bands were not detected by CBB staining, but the mobilities of protein bands bound by type A HA⁺-PTX were identical to those detected by PAS staining. In the other experiment, the binding of erythrocytes to type A HA⁺-PTX-coated 96-

Fig. 5. The proposed structures of native and enzyme-treated glycoproteins and binding of type A HA⁺-PTX (a) and the typical structures of N-linked (complex type) and O-linked sugar chains (b). The structures of HA⁺-PTX (19S and 16S toxins) are cited from our previous report (Oguma *et al.*, 1999).

well microtitre plates was analysed (Fig. 6a, b). Non-treated and neuraminidase-treated erythrocytes both bound to type A HA⁺-PTX, and the binding was not affected by the presence of fetuin. Contrary to this, dose-dependent inhibition was observed by the addition of asialofetuin, glycophorin A and neuraminidase-treated glycophorin A to the reaction mixture. However, the concentration of glycoproteins required for inhibition of

neuraminidase-treated erythrocyte binding seemed to be higher than that required for non-treated erythrocyte binding inhibition.

Glycophorin A is a major glycoprotein of the membrane of human erythrocytes (Marchesi *et al.*, 1972) and the molecule contains approximately 15 O-linked sugar chains and one N-linked sugar chain (Tomita *et al.*,

Fig. 6. Binding of erythrocytes to type A HA⁺-PTX and HA1 on microtitre plates. Type A HA-PTX (a, b) and HA1 (c, d) were coated on plates, and incubated in the presence of fetuin (○), asialofetuin (⊕), glycoporphin A (△) or asialoglycophorin A (▲). Native erythrocytes (a, c) or neuraminidase-treated erythrocytes (b, d) bound to type A HA⁺-PTX or HA1 were lysed and the amount of bound erythrocytes was determined spectrophotometrically at 405 nm as described in Methods. The amount of bound erythrocytes in the absence of glycoprotein was determined as 100%.

1975). The structure of the *N*-linked oligosaccharide is a dibranched complex type sugar chain having *N*-acetylglucosamine linked at the mannosyl residue of the core portion and fucose linked at the proximal *N*-acetylglucosamine residue. More than 50% of this sugar chain is monosialylated at the C-6 position of one of two terminal galactoses, and the other terminal galactose is not sialylated. The remaining *N*-linked sugar chains are disialylated (Yoshida *et al.*, 1980). Therefore, HA⁺-PTX can agglutinate erythrocytes by binding to one unsialylated branch of an *N*-linked sugar chain in glycoporphin A in the case of non-treated erythrocytes and by binding to two desialylated branches of the protein in the case of neuraminidase-treated erythrocytes.

Characterization of HA1

Haemagglutination tests, and binding tests to glycolipids, glycoproteins and erythrocytes were also performed using purified type A HA1. Purified HA1 did not agglutinate native erythrocytes even though 400 µg ml⁻¹ was used. However, neuraminidase-treated erythrocytes were agglutinated by 100 µg HA1 ml⁻¹. Type A HA1 bound to paragloboside and asialoGM1 similar to HA⁺-PTX. However, binding to the other glycolipids was not observed (Fig. 7b). HA1 did not bind to native transferrin and fetuin, but bound to neuraminidase-treated transferrin and asialofetuin (Fig. 7c). HA1 also bound to endo- α -*N*-acetylgalactosaminidase-treated asialofetuin, but did not bind to glycopeptidase F-treated transferrin

and asialofetuin (Fig. 7c) as observed in HA⁺-PTX. Native erythrocytes bound to purified type A HA1 the same as HA⁺-PTX and the binding was not affected by fetuin. The binding of erythrocytes to HA1 was reduced by asialofetuin, glycoporphin A and neuraminidase-treated glycoporphin A (Fig. 6c). The effect of glycoporphin A seemed to be smaller than that of asialofetuin and neuraminidase-treated glycoporphin A. Using erythrocytes treated with neuraminidase, inhibition by glycoporphin A was not observed (Fig. 6d). The similar binding specificities of HA1 and HA⁺-PTX indicates that type A HA⁺-PTX binds to glycolipids, glycoproteins and erythrocytes through HA1. We previously reported that HA⁺-PTX and HA1 bind via galactose moieties using recombinant HA subcomponents (GST-fusion proteins) expressed in *Escherichia coli* (Fujinaga *et al.*, 2000). Fujinaga *et al.* (1998) also reported that polyclonal antibodies against HA1 inhibit the haemagglutination caused by HA⁺-PTX and that the toxin agglutinates erythrocytes through HA1. These results support the conclusions obtained in this study.

Orally ingested botulinum progenitor toxins are supposed to be absorbed from the upper intestine into the lymphatic system (Sugii *et al.*, 1977). Recently, we proposed that type C HA plays an important role in the absorption of the progenitor toxins from the small intestine of guinea pigs (Fujinaga *et al.*, 1997). We also reported that type A HA⁺-PTX and GST-HA1 bound to epithelial cells of the guinea pig small intestine and that their binding was reduced by galactose and lactose

Fig. 7. Binding of HA1 to glycolipids and glycoproteins. The TLC plates were developed in a solvent system with chloroform/methanol/water (65:35:8, by vol.) The plates were incubated with type A HA1, stained by TLC-immunostaining as described in Methods (b) and then glycolipids were visualized by orcinol/H₂SO₄ reagent (a). Glycolipids used are as follows: lane 1, Gb3Cer; lane 2, Gb4Cer; lane 3, GM1; lane 4, asialoGM1; lane 5, SPG; lane 6, paragloboside. (c) Glycoproteins were electrophoresed, electroblotted onto a PVDF membrane and binding of HA1 was analysed. Glycoproteins (10 µg each) used are as follows: lane 1, transferrin; lane 2, neuraminidase-treated transferrin; lane 3, glycopeptidase F-treated transferrin; lane 4, fetuin; lane 5, asialofetuin; lane 6, glycopeptidase F-treated asialofetuin; lane 7, endo- α -N-acetylgalactosaminidase-treated asialofetuin.

(Fujinaga *et al.*, 2000). While Maksymowych & Simpson (1998) found that type A neurotoxin itself might be absorbed, by performing *in vitro* experiments with human carcinoma cells, they also reported that the neurotoxin as well as HA⁺-PTX was absorbed from the stomach and small intestine using an *in vivo* mouse model (Maksymowych *et al.*, 1999). In this study, we have demonstrated that the type A HA⁺-PTX binds to some glycolipids and glycoproteins, but does not bind to gangliosides. However, it has been reported previously that type A neurotoxin binds to gangliosides but does not bind to some neutral glycolipids (Kamata *et al.*, 1997). Therefore, it is postulated that unsialylated oligosaccharides on the surface of the small intestine are

the receptors at least for type A HA⁺-PTX (not neurotoxin). Neurotoxin and/or 12S toxin may have a different receptor and/or different adsorptive pathway from those of HA⁺-PTX. The mechanisms of how HA-positive and -negative toxins (including neurotoxin alone) are absorbed and enter the lymphatic vessel through the epithelial cells is a topic for further analysis.

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