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Acta Med. Okayama, 2012
Vol. 66, No. 3, pp. 253-261
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Acta Medica Okayama
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Original Article

Clostridium botulinum Type E Toxins Bind to Caco-2 Cells by a Different Mechanism from That of Type A Toxins

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Cultured *Clostridium botulinum* strains produce progenitor toxins designated as 12S, 16S, and 19S toxins. The 12S toxin consists of a neurotoxin (NTX, 7S) and a non-toxic non-hemagglutinin (NTNH). The 16S and 19S toxins are formed by conjugation of the 12S toxin with hemagglutinin (HA), and the 19S toxin is a dimer of the 16S toxin. Type A cultures produce all 3 of these progenitor toxins, while type E produces only the 12S toxin. The 7S toxin is cleaved into heavy (H) and light (L) chains by a protease(s) in some strains, and the H chain has 2 domains, the N-terminus (Hn) and C-terminus (Hc). It has been reported that type A toxins bind to the intestinal cells or cultured cells via either HA or Hc. In this study, we investigated the binding of type A and E toxins to Caco-2 cells using Western blot analysis. Both the type E 7S and 12S toxins bound to the cells, with the 7S toxin binding more strongly, whereas, in the type A strain, only the 16S/19S toxins showed obvious binding. Pre-incubation of the type E 7S toxin with IgG against recombinant type E Hc significantly inhibited the 7S toxin binding, indicating that Hc might be a main binding domain of the type E toxin.

Key words: *Clostridum botulinum*, neurotoxins, Caco-2, binding, Hc

Clostridium botulinum produces poisonous neurotoxins (NTX, 7S), and the cultures can be classified into 7 types, A to G, based on the antigenicity of the 7S toxin. The 7S toxin is produced as a single chain protein (single chain form, approximately 150 kDa). In the type A strain, the cells produce a protease(s) which can cleave the 7S toxin to a light (L) chain (50 kDa) and a heavy (H) chain (100 kDa) linked by a disulfide bridge (di-chain form). In the type E strain, the cells produce no such protease, and therefore, the 7S toxin remains in a single chain form (Fig. 1A) [1]. Recently, it has become clear that the L chain is a catalytic domain showing protease activity, whereas, the H chain has 2 domains: the N-terminus (Hn) is a channel-forming or transmembrane domain, and the C-terminus (Hc) is a binding domain to the target neuronal cells [2, 3].

In culture liquid and foods, the 7S toxin exists as various sizes of progenitor toxins that are composed of a 7S toxin and non-toxic components, including a hemagglutinin (HA) and a non-toxic non-HA (NTNH). There are 3 kinds of progenitor toxins, 12S (300 kDa), 16S (500 kDa), and 19S (900 kDa) toxins (Fig. 1B). The type A strain produces 12S, 16S, and 19S toxins, the type B, C, and D strains produce 12S and 16S toxins, while the type E and F strains produce only the 12S toxin. The progenitor toxins dissociate into a 7S toxin and non-toxic components under an alkaline condition. The non-toxic components protect the 7S toxin against low pH and

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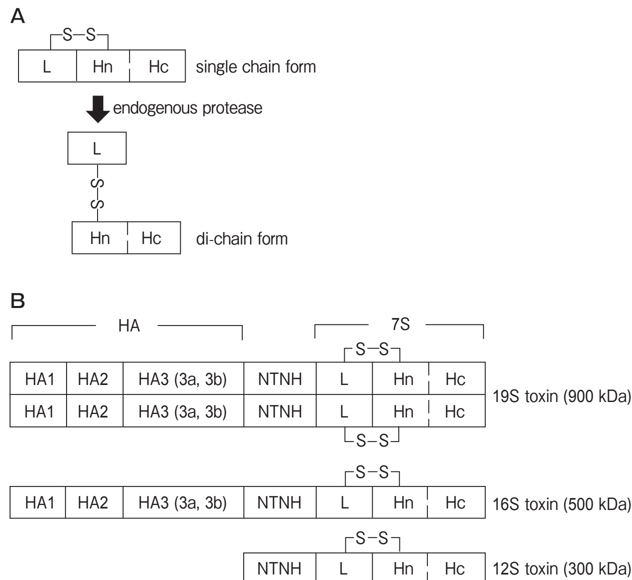

Fig. 1 Schematic models of the structures of *C. botulinum* 7S toxin and progenitor toxins. (**A**) The 7S toxin is synthesized as a single chain form protein, and cleaved into the di-chain form by endogenous protease in some strains. The di-chain form toxin is composed of a light (L) chain and a heavy (H) chain, and the two chains are linked by a disulfide bond. (**B**) The progenitor toxins consist of neurotoxin and nontoxic components. The 12S toxin is formed by association of a neurotoxin with a nontoxic component having no HA activity (nontoxic non-HA, NTNH); the 16S toxin is formed by conjugation of the 12S toxin with HA; the 19S toxin is a dimer of the 16S toxin. L, L chain; Hc, half C-terminal of H chain; Hn, half N-terminal of H chain.

protease digestion across the gastrointestinal tract, and foster the process of botulism [4].

In food-borne botulism, orally ingested toxins must traverse the gastrointestinal tract before arriving and affecting the nerve endings. Oguma *et al.* found that 1) the 19S toxin is a dimer of the 16S toxin; 2) HA consists of 3 to 4 subcomponents designated HA-1, -2, and -3 (3a and 3b); 3) HA3b and HA1 bind to sialic acid and galactose, respectively, existing on the surface of intestinal cells, cultured cells, and erythrocytes; 4) type A, B, C, and D HA-positive progenitor toxins bind to the cells much more strongly than the 7S toxin via either HA1 or HA3b; 5) the binding occurs at both 4℃ and 37℃, but internalization into the cells appears only at 37℃ [5-11]. Ohyama *et al.* reported that both the 7S and 16S toxins of types C and D were able to bind to IEC-6 cells (a rat intestinal epithelial cell line) and Caco-2 cells (a human colonic carcinoma cell line) at 4℃, but the 16S toxin bound more strongly than the 7S toxin because the 16S toxin has HA; HA was important for higher binding of the 16S toxin [12-14]. However, Popoff *et al.* reported that both the type A 7S toxin and complex toxin (progenitor toxin) bound to m-IC_{cl2} cells (a mouse intestinal epithelial cell line) and Caco-2 cells at a similar level via Hc [15, 16].

The type E strain produces only the 12S toxin, and its binding domain(s) to the intestinal cells has not yet been clarified. Caco-2 cells can differentiate into polarized cells, and are connected by tight junction, as similar to human intestinal barriers [17]. Therefore, in this study, we compared the binding properties of type A and E toxins to the Caco-2 cells, in an attempt to determine the binding domain(s) of the type E toxin.

Materials and Methods

Production and purification of botulinum toxins of type A and type E. Production and purification of type A toxins from *C. botulinum* type A strain 62A were performed as described previously [18]. The spores were germinated at 35℃ overnight in 50 ml of cooked meat medium (pH 7.2; Becton, Dickinson and Company, Sparks, MD, USA), and then transferred to toxin production medium. The medium was composed of 2% peptone, 0.5% yeast extract, 0.5% glucose and 0.025% sodium thioglycolate. After incubation at 30℃ for 5 days, the toxins were collected by acid (3 N H_2SO_4) precipitation. The precipitates were suspended in distilled water, centrifuged (8,700 × g for 20 min), and re-suspended into 0.2 M phosphate buffer (pH 6.0). This preparation was then treated with protamine to remove RNAs. Thereafter, the toxins were precipitated with a 60% saturation of ammonium sulfate, and dissolved in 50 mM acetate buffer (pH 4.2). The solution was then applied to an SP-Toyopearl 650S (Tosoh, Tokyo, Japan) column. The 16S/19S and 12S toxins were eluted with a linear gradient of NaCl (0-500 mM). After dialysis against 10 mM phosphate buffer (pH 6.0), the toxin fractions were applied to a lactose gel (EY Laboratories Inc., San Mateo, CA, USA) column. The 12S toxin passed through the column, whereas the 16S/19S toxins bound to the column were eluted with the same buffer containing 0.2 M lactose. To purify 7S toxin, the 16S/19S toxins were dia-

lyzed against 10 mM phosphate buffer (pH 8.0) to dissociate them to 7S toxin and non-toxic components, and re-applied to the same lactose column. The 7S toxin was collected in the path-through fraction.

Type E 7S and 12S toxins were purified from *C. botulinum* type E strain Iwanai according to the methods previously reported with slight modifications [19, 20]. The spores were germinated under anaerobic conditions at 30℃ for 2 days in 50 ml of cooked meat medium (pH 7.2) supplemented with 1% (w/v) proteose peptone, 1% (w/v) polypeptone, 1% (w/v) lactalbumin hydrolysate, 1% (w/v) yeast extract, 0.1% (w/v) L-cysteine hydrochloride monohydrate and 1% (w/v) glucose. Then, the culture was transferred to large-scale culture medium (pH 6.3) consisting of 2% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) glucose and 0.025% (w/v) sodium thioglycolate. After incubation at 30℃ for 3 days, the bacterial cells were collected by centrifugation at 10,000 × g for 30 min, and washed with 50 mM acetate buffer (pH 5.0). The toxins were extracted with 200 mM phosphate buffer (KH_2PO_4/Na_2HPO_4, pH 6.0) at 37℃ for 2 h and then at 4℃ overnight. The cell extract was precipitated with ammonium sulfate at 60% saturation, and collected by centrifugation at 15,000 × g for 15 min. The precipitate was dissolved in 20 mM acetate buffer (pH 6.0) and dialyzed against the same buffer.

Cell extract was loaded onto a CM-Sepharose™ Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column previously equilibrated with 20 mM acetate buffer (pH 6.0). The pass-through fractions were treated with Ribonuclease A from bovine pancreas (7.5 μg/ml final concentration) for 5 h at 30℃, and then centrifuged at 1,000 × g for 10 min to remove RNAs. The resultant supernatant was loaded onto a CM-Sepharose Fast Flow column equilibrated with 20 mM acetate buffer (pH 6.0). After washing with the same buffer, the proteins were eluted with an exponential gradient of NaCl (30–120 mM) in equilibration buffer. The toxic fractions were pooled, concentrated and loaded onto a Sephacryl™ S-200 High Resolution (GE Healthcare Bio-Sciences AB) gel filtration column pre-equilibrated with 20 mM acetate buffer (pH 6.0). 12S toxin was eluted in the first peak. Next, the toxin was dialyzed against 10 mM phosphate buffer (pH 8.0) to dissociate 12S toxin to 7S toxin and NTNH, and loaded onto a Toyopearl DEAE-650S (Tosoh, Tokyo, Japan) column equilibrated with the same buffer. 7S toxin and NTNH were eluted with a linear gradient of NaCl (0–200 mM). All the centrifugation and dialysis steps were performed at 4℃. All purification steps were carried out at room temperature.

Preparation of recombinant proteins. The recombinant type E NTNH (rE-NTNH) was produced by using a pET-32 Ek/LIC vector (Novagen, Darmstadt, Germany). The gene encoding whole NTNH was amplified from *C. botulinum* type E strain Iwanai by a polymerase chain reaction (PCR) using the following primers: for NTNH-F, 5'-GACGACGACAAGATG AAATAAATGGTAATTTAAATTG-3'; and for NTNH-R, 5'-GAGGAGAAGCCCGGTTTATATATT TTTTAATATCCATAGATA-3'. According to the manufacturer's protocol, the PCR product was cloned into a pET-32 Ek/LIC vector. After verifying the DNA sequence by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), the constructed plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS-competent cells. The His-tagged proteins were expressed by addition of IPTG at 25℃, purified by Ni-NTA Agarose (QIAGEN GmbH, Hilden, Germany) chromatography, and then cleaved with recombinant Enterokinase (rEK), which was subsequently eliminated by an Enterokinase Cleavage Capture Kit (Novagen, Darmstadt, Germany). Finally, rE-NTNH proteins were obtained in pass-through fractions after applying the cleavage-protein solution to the Ni-NTA Agarose column.

Recombinant type E Hc (rE-Hc) was prepared in order to study the role of Hc and in particular its ability to interact with membranes. The following primers were designed for amplification of the gene fragment encoding Hc of *C. botulinum* type E strain Iwanai: for Hc-F, 5'-CGCGGATCCGCGTCATATT TTAATAAATTCTTTAAGAGAATTA-3'; and for Hc-R, 5'-CCGCTCGAGCGGTTATTTTTCTTGCC ATCCATGTTCTT-3'. The PCR product was digested by *Bam*HI and *Xho*I and cloned into a pGEX-6P-3 vector (GE Healthcare Bio-Sciences AB) that was already restricted with the same enzymes. After introducing pGEX-6P-3-*rE-Hc* into *E. coli* 21 (DE3) pLysS-competent cells, the expression and purification of rE-Hc protein were performed according to the procedure described previously [21].

Preparation of antisera. Antisera against rE-NTNH, rE-Hc and A-16S/19S toxins were generated by immunizing rabbits (New Zealand white

rabbits; 2.5 kg, female) as described previously [22]. The rE-NTNH and rE-Hc were utilized with no pretreatment, whereas, the A-16S/19S toxins were made with 0.4% Formalin treatment. Initially, 200 μg of each antigen was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously. Two weeks later, the rabbit was given a second subcutaneous immunization of 200 μg of antigen with Freund's incomplete adjuvant. After 2 weeks, a final booster injection of 100 μg of antigen was administered intravenously without adjuvant. One week later, the blood was collected from the rabbit, and the antiserum was collected by centrifugation at 3,000 g for 20 min. To partially purify IgG, proteins in the serum were precipitated by 50% saturated ammonium sulfate, and dissolved in 17.5 mM phosphate buffer (pH 6.3). After dialysis against the same buffer, proteins were added to a Toyopearl DEAE-650S column. The pass-through fractions which contained IgG were collected and stored at −80℃ until use. All animal experiments were carried out in accordance with the animal experiment guidelines of Okayama University (OKU-2009318). Efforts were made to minimize the suffering of the animals.

Cell culture. Caco-2 cells (RIKEN Bio Resource Center) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco™ Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were maintained in a humidified environment of 5% CO_2 at 37℃.

Toxin-binding assay. Caco-2 cells (2×10^5/well) were seeded in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown to 100% confluence. Toxins or proteins were suspended in 300 μl of cold phosphate-buffered saline (PBS; pH 6.5) to the indicated concentrations. Cells were then incubated with toxins for 1 h at 4℃, washed three times with cold PBS, and lysed with 150 μl of SDS sample buffer. Proteins from 10 μl of cell lysate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting. The intensity of detected bands was measured with Image Gauge Ver 4.0 (Fujifilm, Tokyo, Japan). The Western blotting with different amounts of type A 16S/19S and type E 12S toxins was first performed, and the standard linear lines were prepared by assay the intensity of H chain and 7S toxin in type A and E, respectively. Thereafter, the levels of cell-bound type A and E toxins in the different cell lysates (10 μl) were estimated by using this standard lines after the Western blotting. The quantity of toxins in 10 μl of cell lysates thus obtained was multiplied by 15 to estimate the amounts of toxins in 150 μl of whole cell lysates.

Results

Purification of type A and E toxins. As shown in Fig. 2A, we have successfully purified type A 7S, 12S, and 16S/19S toxins. As described in the Introduction, the type A strain of *C. botulinum* produces endogenous protease(s) that can cleave the 7S toxin into a di-chain form. Therefore, the 7S toxin showed 2 bands of 50 kDa and 100 kDa corresponding to the L chain and H chain, respectively, on SDS-PAGE in the presence of 2-mercaptoethanol (2-ME). The 16S/19S toxins demonstrated seven bands: the L chain and H chain of 7S toxin, intact NTNH and four subcomponents of HA (HA1, HA2, HA3a, and HA3b). The SDS-PAGE pattern of the type A 12S toxin showed the disappearance of a 120-kDa intact NTNH accompanied by the appearance of a 106-kDa band, indicating that the NTNH of 12S toxin was cleaved at the N-side of Phe-145 by protease, as reported previously [23]. The type A 12S toxin is comprised of the 7S toxin and cleaved NTNH. Unlike the type A strain, the type E strain does not produce protease, and the 7S toxin exists as a single chain toxin. Therefore, the type E 7S toxin demonstrated a 150-kDa band on SDS-PAGE in the presence of 2-ME (Fig. 2B). The type E 12S toxin was comprised of a 7S toxin (~150 kDa) and an NTNH (~130 kDa).

Binding of type A toxins to Caco-2 cells. As described above, the functions of nontoxic components during passage of type A progenitor toxins through the intestinal barrier remain controversial. To further investigate the binding properties of type A toxins, a toxin-binding assay was conducted by using the Western blotting method, as described under the Materials and Methods. The 7S and 12S toxins showed no binding to Caco-2 cells, whereas the 16S/19S toxins exhibited significantly high binding (Fig. 3A). These results indicate that the binding of type A toxins to Caco-2 cells is mediated by HA. This lends support to our previous report that HA plays

crucial roles in the binding of type A toxins to intestinal microvilli and erythrocytes [8]. The intensity of the H chain band of 7S toxin was measured, and the amounts of bound toxins were calculated by the standard linear line (Fig. 3B). The binding amounts of 16S/19S toxins at the final concentrations of 50, 100, and 200 nM were 1.74, 2.55, and 4.38 pmol, respectively.

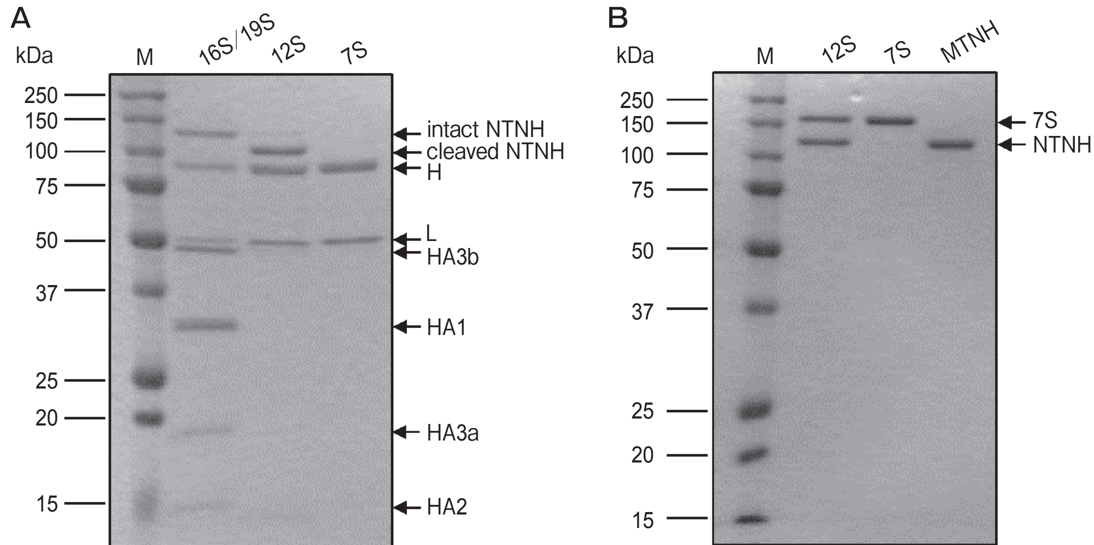

Fig. 2 SDS-PAGE patterns of the purified toxins and nontoxic components. Proteins were separated by SDS-PAGE using 12% polyacrylamide gel in the presence of 2-ME, and stained by Coomassie brilliant blue R-250. (**A**) Type A 7S, 12S, and 16S/19S toxins. (**B**) Type E 7S, 12S, and NTNH. M, standard molecular mass markers.

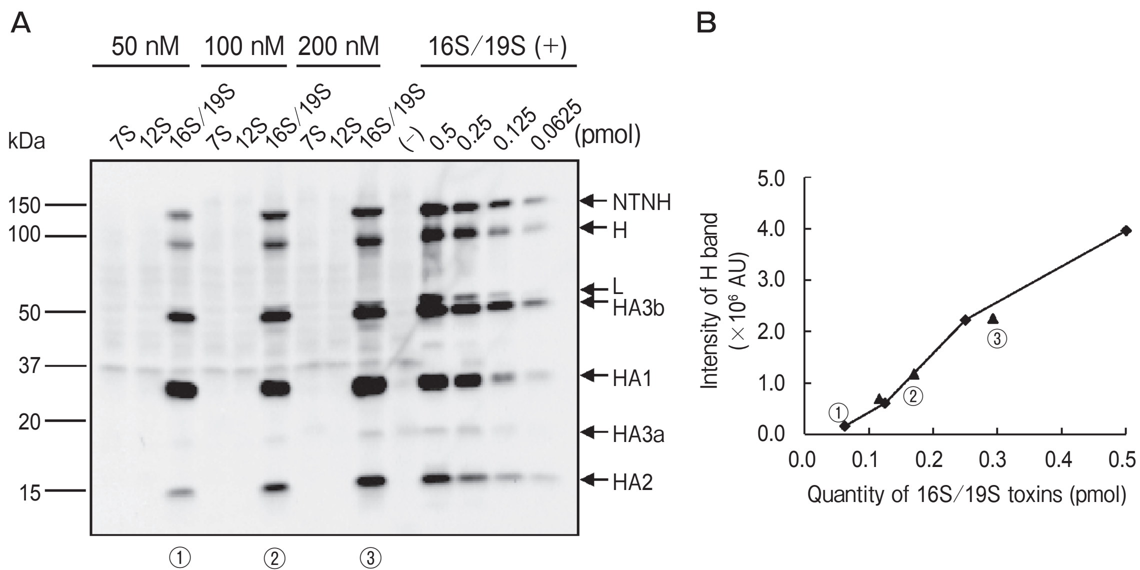

Fig. 3 Binding of type A toxins to Caco-2 cells. (**A**) Caco-2 cells were incubated with type A 7S, 12S, and 16S/19S toxins at the final concentration of 50, 100, and 200 nM for 1 h at 4℃. Toxins bound to cells were detected by Western blotting using anti-A-16S/19S IgG. (**B**) The standard linear line was first prepared by assay the intensity of H chain after doing the Western blotting with different amounts of 16S/19S toxins. Then, the levels of cell-bound toxins in preparations of ①, ②, and ③ of (**A**) were estimated by using this standard line. (−), only cell lysate; 16S/19S (+), only 16S/19S toxins.

Binding of type E toxins to Caco-2 cells. We next investigated the binding properties of the type E 7S and 12S toxins. The effects of temperature and time on the binding and the absorption of type E toxins were first examined. The type E 7S (Fig. 4A) and 12S (Fig. 4B) toxins were found to bind to Caco-2 cells in a time-dependent manner at 4℃ and 37℃. The level of binding activity was significantly higher at 37℃ than that at 4℃, suggesting that some toxins may be internalized into the cells at 37℃ after binding.

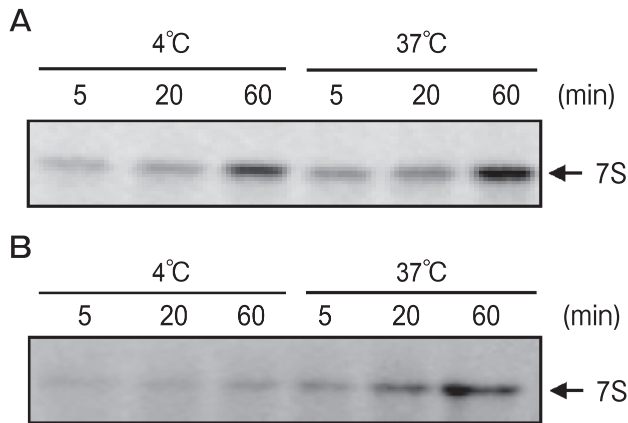

Fig. 4 Temperature dependence and time course of the binding of type E toxins to Caco-2 cells. Cells were incubated with 7S toxin (**A**) or 12S toxin (**B**) at 100nM for 5, 20, and 60min at 4℃ or 37℃, respectively. Toxins bound to the cells were detected using anti-rE-Hc IgG.

The toxin-binding activities at various concentrations of type E 7S and 12S toxins were also examined. The amount of binding of the type E 7S and 12S toxins increased as the concentrations of the toxins increased (Fig. 5A). By quantifying the intensity of the 7S toxin band, the level of 7S binding activity at the final concentrations of 50, 100, and 200nM was estimated to be 0.27, 0.41, and 1.01 pmol, respectively. On the other hand, the level of binding activity for the 50 and 100nM 12S toxin dilutions was unfortunately too low for calculation, and the level of binding activity of the 12S toxin at the final concentration of 200nM was calculated to be 0.2 pmol (Fig. 5B). Thus, the level of binding activity of the 7S toxin was approximately 5-fold greater than that of the 12S toxin. We also found that neither NTNH nor rNTNH exhibited any detectable binding to Caco-2 cells (data not shown). Therefore, the 7S toxin, but not the NTNH, might play a key role in the binding of type E toxins to Caco-2 cells.

Inhibitory effect of antibody on the binding of type E toxins. The above results indicate that the mechanism of binding of type E toxins to Caco-2 cells is different from the mechanism of binding of type A toxins to Caco-2 cells, and the binding domain(s) of the type E toxins may exist in the 7S toxin. The 7S toxin has been reported to bind to neuronal cells via Hc [24, 25]. To assess whether the Hc domain is involved in the interaction with the receptor on Caco-2 cells, an inhibition assay was performed using anti-

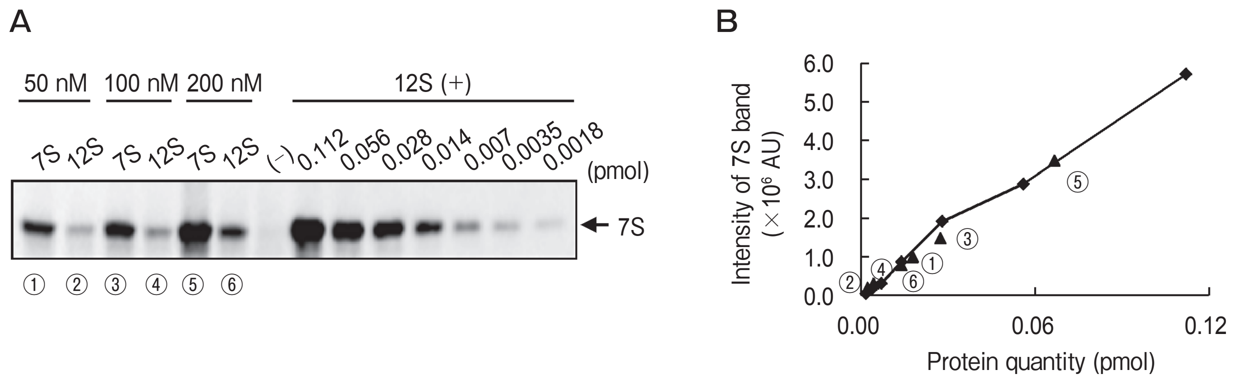

Fig. 5 Effect of the concentrations of type E toxins on their binding to Caco-2 cells. (**A**) Caco-2 cells were incubated with the type E 7S and 12S toxins (final concentrations of 50, 100, and 200nM) for 1h at 4℃. Toxins in 10μl of the cell lysates were detected by Western blotting using anti-rE-Hc IgG. (**B**) The standard linear line was first prepared by assay the intensity of 7S toxin after doing the Western blotting with different amounts of 12S toxins. Then, the levels of cell-bound toxins in the preparations of ①, ②, ③, ④, ⑤, and ⑥ of (**A**) were estimated by using this standard line. (−), only cell lysate; 12S (+), only 12S toxin.

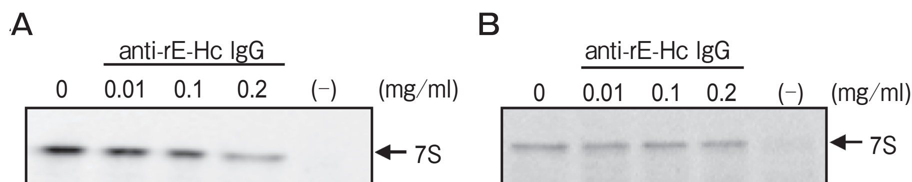

Fig. 6 Effect of antibody on the binding of type E 7S (**A**) and 12S (**B**) toxins. Prior to incubation with Caco-2 cells, the 7S and 12S toxins (100 nM final concentration) were incubated with anti-rE-Hc IgG (final concentrations of 0, 0.01, 0.1, and 0.2 mg/ml) for 1 h at 37℃. Toxins bound to the cells were detected by Western blotting using anti-rE-Hc IgG.

rE-Hc IgG. Toxins were incubated with specified concentrations of IgG for 1 h at 37℃ prior to the toxin-binding assay. Pre-incubation of the 7S toxin with anti-rE-Hc IgG resulted in an obvious decrease of 7S toxin binding. The addition of 0.2 mg/ml of anti-rE-Hc IgG led to an approximately 50% reduction in the toxin binding (Fig. 6A). On the other hand, pre-incubation of the 12S toxin with anti-rE-Hc IgG did not inhibit the toxin binding to Caco-2 cells (Fig. 6B). These data indicate that the type E 7S toxin may bind to Caco-2 cells mainly via Hc.

Discussion

Binding of type A and E toxins to Caco-2 cells was observed. Recent research has shown that botulinum toxins bind to the cell surface at 4℃, but are not internalized [13]. Therefore, in this study, the toxin-binding experiments were done at 4℃. The binding of type A 7S, 12S, and 16S/19S toxins to Caco-2 cells was first investigated. As we expected, only HA-positive 16S/19S toxins bound to Caco-2 cells, which corresponds to our previous research. Thus, HA is considered to be essential for the binding of type A 16S/19S toxins. However, Popoff *et al.* documented that Hc is important for the binding and absorption of type A 7S and progenitor toxins [15, 16]. What led to these different conclusions is not clear. The conflict might be related to the use of different detection systems or different final concentrations of toxins between the 2 experiments.

We also investigated the binding of type E 7S and 12S toxins (containing no HA) to Caco-2 cells. In contrast to type A toxins, the 7S toxin exhibited a higher level of binding than the 12S toxin, and no binding of native NTNH or rNTNH to Caco-2 cells was observed. Hence, it was considered that the binding domain may exist in the 7S toxin. In order to confirm this, inhibition assays of antibody were conducted. Pre-incubation of the 7S toxin with anti-rE-Hc IgG led to significant, but not complete inhibition, even though a large amount of IgG was employed. This indicates that Hc is the main binding domain, but other domain(s) such as the Hn or L chain might also act in binding. In contrast, no obvious inhibition by anti-rE-Hc IgG on 12S toxin binding was observed. As to the reason for the low binding activity of the 12S toxin, we suppose that, in the 12S toxin, the Hc domain might be covered by NTNH or the structure of the Hc domain might be changed after 7S toxin associates with NTNH. This might also explain why anti-rE-Hc IgG did not inhibit the 12S toxin binding.

In this work, type A 16S/19S toxins showed slightly higher binding than type E toxins. We speculate that there are more type A HA receptors than type E toxin-binding receptors on the cell surface. The HA of type A, B, C, and D progenitor toxins has been reported to bind to galactose and/or sialic acid on the cells [5-14], while the intestinal receptors of type E toxins remain unclear. Regarding the receptors, synaptic vesicle glycoprotein 2 (SV2) proteins have been reported to act as receptors for type A and type E 7S toxins on neuronal cells [26, 27]. Popoff *et al.* reported that the type A 7S toxin binds to the SV2C on the intestinal cells via Hc [15]. Therefore, in a future study we will investigate whether SV2 proteins of intestinal cells act as receptors for type E 7S toxin.

In order to induce food-borne botulism, the toxins must be absorbed from the small intestine. Our previous work showed that the type C 16S toxin was internalized into HT-29 cells (a human colon carcinoma grade II cell line) within at least 4 min after administration at 37℃ [9]. In the present study, we investigated the binding of type E toxins to Caco-2 cells at 4℃ and 37℃. Both 7S and 12S toxins showed higher

binding activity at 37℃ than 4℃. We consider that some amounts of toxins may have been internalized into the cells after binding at 37℃. Since (1) no studies have been conducted into the internalization of type E toxins through intestinal cells, and (2) Caco-2 monolayers incubated on membrane filters are generally used to investigate the permeability of drugs and chemicals [17], we are currently planning to investigate the transcytosis of type E toxins by using a Caco-2 cell monolayer system.

Previously, it was reported that the progenitor toxins dissociate into a 7S toxin and non-toxic components after absorption from the small intestine, in the lacteal vessels [28]. If type A and E 7S toxins are absorbed from the small intestine, the progenitor toxins must dissociate into 7S toxin and nontoxic components before reaching the small intestine. We are also planning to clarify this point by subjecting the progenitor toxins to different pH levels and to different amounts of the gastrointestinal juice, in order to mimic conditions in the gastrointestinal tract.

Acknowledgments. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 22500427 to Y.Y. and No. 23659897 to K.O. and Y.Y.). This work also was supported by China Scholarship Council.

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