Disruption of the epithelial barrier by botulinum haemagglutinin (HA) proteins – differences in cell tropism and the mechanism of action between HA proteins of types A or B, and HA proteins of type C

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Orally ingested botulinum neurotoxin (BoNT) causes food-borne botulism, but BoNT must pass through the gut lining and enter the bloodstream. We have previously found that type B haemagglutinin (HA) proteins in the toxin complex play an important role in the intestinal absorption of BoNT by disrupting the paracellular barrier of the intestinal epithelium, and therefore facilitating the transepithelial delivery of BoNT. Here, we show that type A HA proteins in the toxin complex have a similar disruptive activity and a greater potency than type B HA proteins in the human intestinal epithelial cell lines Caco-2 and T84 and in the canine kidney epithelial cell line MDCK I. In contrast, type C HA proteins in the toxin complex (up to 300 nM) have no detectable effect on the paracellular barrier in these human cell lines, but do show a barrier-disrupting activity and potent cytotoxicity in MDCK I. These findings may indicate that type A and B HA proteins contribute to the development of food-borne botulism, at least in humans, by facilitating the intestinal transepithelial delivery of BoNTs, and that the relative inability of type C HA proteins to disrupt the paracellular barrier of the human intestinal epithelium is one of the reasons for the relative absence of food-borne human botulism caused by type C BoNT.

INTRODUCTION

Clostridium botulinum produces botulinum neurotoxins (BoNT, ~150 kDa), which are the aetiological agent responsible for the disease botulism. BoNTs are classified into seven types (A–G) depending on their serological specificity (Collins & East, 1998). Each type is unique in its geographical distribution and species susceptibility. Toxin types A, B, E and F cause botulism in both humans and animals. Meanwhile, types C and D cause botulism mainly in animals, but very rarely in humans. Type G toxin-producing organisms have been experimentally isolated

from soil, but no naturally occurring outbreaks of botulism caused by type G toxin have been reported. BoNT binds highly specifically to peripheral nerve terminals, such as motor neuron nerve endings, and then enters the cytosol of the neuron. In the cytosol, the BoNT cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins involved in the fusion process of synaptic vesicles with the presynaptic membrane, thereby blocking the release of acetylcholine, causing flaccid paralysis (Schiavo *et al.*, 2000). BoNTs usually enter the body via the gastrointestinal system. In order to mediate their toxicity, BoNTs in the gastrointestinal lumen must cross the epithelial barrier to reach peripheral nerves. The detailed mechanisms by which BoNTs traverse this barrier and enter the circulation remain unclear.

When produced by the bacterium, BoNT is found in complex forms (progenitor toxins) associated with non-toxic components (non-toxic neurotoxin-associated proteins; NAPs), including haemagglutinin (HA) proteins and a non-toxic protein lacking HA activity (non-toxic non-HA; NTN), which are encoded by genes adjacent to the BoNT gene (Fig. 1). Three different forms of progenitor toxins have been identified: 12S, 16S and 19S (Oguma

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Abbreviations: BoNT, botulinum neurotoxin; HA, haemagglutinin; NAP, neurotoxin-associated protein; NTN, non-toxic non-haemagglutinin; TER, transepithelial electrical resistance.

Three supplementary figures showing the dose-dependent reduction in TER of Caco-2 cells with the apical or basolateral addition of type A or B HA-positive toxin complexes, the dose-dependent reduction in TER of MDCK I cells with apically added type A and basolaterally added type A, B and C HA-positive toxin complexes, and the effects of HA-positive complexes on rat intestinal epithelial cell lines ACL-15 and RCN-9, are available with the online version of this paper.

Fig. 1. Genes encoding HA proteins of types A, B, C and D. The figure is summarized from Minton (1995), Collins & East (1998) and Oguma *et al.* (1999).

et al., 1999; Sakaguchi *et al.*, 1984). 12S toxin is composed of a BoNT and an NTN. 16S toxin is composed of a BoNT, an NTN, and HA proteins. 19S toxin has the same components as 16S toxin, and is presumed to be a dimer of two 16S toxins linked by one of the HA subunits (Inoue *et al.*, 1996). In this paper, the 12S toxin is called the HA-negative toxin complex, and the 16S and 19S toxins are called the HA-positive toxin complex. The *C. botulinum* type A strain produces 12S, 16S and 19S toxins. Type B, C and D strains produce 12S and 16S toxins. Type E and F strains produce 12S toxin. Type G strain produces 16S toxin. The HA of types A–D consists of three different subcomponents: HA1, HA2 and HA3. All these components of the neurotoxin complex are associated with each other via non-covalent binding.

NAPs are known to markedly increase the oral toxicity of BoNT. One of the reasons for this phenomenon is the protective effect of NAPs toward BoNT in the toxin complex against the low pH and proteases in the digestive tract (Sakaguchi *et al.*, 1984). Meanwhile, the direct interaction of HA proteins with the intestinal epithelium via cell-surface glycoconjugates (Fujinaga *et al.*, 1997, 2000, 2004; Kojima *et al.*, 2005; Nakamura *et al.*, 2007) has been reported. In addition, in a human intestinal epithelial cell line (HT-29), the cell surface sialic acid-containing O-linked glycoproteins that are recognized by the type C 16S toxin have been shown to induce the internalization of the toxin into the cells (Nishikawa *et al.*, 2004; Uotsu *et al.*, 2006). Recently, it has been reported that type D HA

proteins in the toxin complex enhance permeation through a monolayer of human intestinal epithelial cells (Caco-2) (Niwa *et al.*, 2007). More recently, we have reported that type B HA proteins in the toxin complex have a novel activity, that of disrupting the paracellular barrier of the intestinal epithelium, which facilitates the transepithelial delivery of macromolecules including BoNT (Matsumura *et al.*, 2008). HA proteins of type B have a high sequence homology with those of type A, whereas they show relatively low homology with those of types C and D [these two types show ~99 % sequence identity (Fujinaga *et al.*, 1994; Nakajima *et al.*, 1998; Ohyama *et al.*, 1995; Tsuzuki *et al.*, 1990)] (Table 1). The aim of this study was to investigate the effects of HA proteins of types A and C on the paracellular barrier function of various epithelial cells in comparison with those of type B, and to elucidate the roles of HA proteins in the pathophysiology of botulism.

METHODS

Cells. Two human colon carcinoma cell lines, Caco-2 (passage 45–64) and T84 (passage 80–90), and a canine kidney epithelial cell line, MDCK I (passage 20–40), were cultured in Caco-2 medium [minimum essential medium (MEM; Gibco) supplemented with 1 mM L-glutamine and antibiotics (70 U penicillin G ml⁻¹, 70 µg streptomycin ml⁻¹)] with 20 % heat-inactivated fetal bovine serum, 17.86 mM NaHCO₃ (Wako) and 15 mM HEPES (Dojindo) at pH 7.4; T84 medium [1:1 mixture of Dulbecco's modified Eagle's medium (Gibco) and Ham's F-12 nutrient medium (Gibco) supplemented with antibiotics (70 U penicillin G ml⁻¹, 70 µg

Table 1. Comparison of percentage amino acid identities of type A NAPs and BoNTs with those of type B, type C and type D

Strains: type A=A 62A; type B=B Lamanna; type C=C Stockholm; type D=D CB-16. References: a, (Binz *et al.*, 1990); b, (East *et al.*, 1996); c, (Dineen *et al.*, 2003); d, (Lee *et al.*, 2005); e, (Kimura *et al.*, 1990); f, (Hauser *et al.*, 1990); g, (Tsuzuki *et al.*, 1990); h, (Tsuzuki *et al.*, 1992); i, (Fujinaga *et al.*, 1994); j, (Ohyama *et al.*, 1995); k, (Nakajima *et al.*, 1998); l, (Sunagawa *et al.*, 1992).

Strain	HA3	HA2	HA1	NTNH	BoNT	References
Type A	100 %	100 %	100 %	100 %	100 %	a–c
Type B	98.2 %	97.9 %	84.4 %	82.7 %	38.5 %	d
Type C	68.0 %	63.0 %	38.9 %	65.4 %	33.5 %	e–i
Type D	67.8 %	63.0 %	38.9 %	65.4 %	34.6 %	j–l

streptomycin ml^{-1})] with 10 % heat-inactivated fetal bovine serum, 14.3 mM NaHCO_3 (Wako) and 15 mM HEPES (Dojindo) at pH 7.4; and MDCK I medium [minimum essential medium (Gibco) supplemented with 1 mM L-glutamine and antibiotics (70 U penicillin G ml^{-1} , 70 μg streptomycin ml^{-1}), with 10 % heat-inactivated fetal bovine serum, 17.86 mM NaHCO_3 (Wako) and 15 mM HEPES (Dojindo) at pH 7.4, respectively, as previously described (Matsumura *et al.*, 2007, 2008). Two rat colon carcinoma cell lines, ACL-15 (passage 90–95, obtained from RIKEN Cell Bank, Japan) and RCN-9 (passage 20–25, obtained from RIKEN Cell Bank), were cultured in RPMI 1640 (Gibco) supplemented with antibiotics (100 U penicillin G ml^{-1} , 100 μg streptomycin ml^{-1}) and 10 % heat-inactivated fetal bovine serum.

Bacterial strains. *C. botulinum* type A strain 62A supplied by Dr S. Kozaki (Osaka Prefecture University), and type B strain Lamanna and type C strain Stockholm supplied by Dr K. Oguma (Okayama University), were used in this study.

Toxins

Type A toxin complexes *C. botulinum* type A strain 62A was cultured using a cellophane-tube procedure, and the culture supernatant was obtained as described previously (Inoue *et al.*, 1996). The HA-positive toxin complex (a mixture of 16S and 19S toxins) and the HA-negative toxin complex (12S toxin) were purified by a procedure described previously (Inoue *et al.*, 1999) with minor modifications. The culture supernatant was concentrated by 60 % ammonium sulfate precipitation and dialysed against 0.05 M sodium phosphate buffer (pH 6.0), before being treated with protamine (Sugii & Sakaguchi, 1975). The HA-positive toxin complex was concentrated in the precipitate that appeared during the protamine treatment (fraction 1), and the HA-negative toxin complex remained in the supernatant (fraction 2). After centrifugation (15 000 g, 30 min, 4 °C), the precipitate (fraction 1), which contained the HA-positive toxin complex, was dialysed against 0.05 M sodium acetate buffer (pH 4.2), and applied to an SP-Toyopearl 650M (Tosoh) column as described previously (Inoue *et al.*, 1996). The HA-positive toxin complex rich fraction eluted from this column was collected and dialysed against 0.01 M sodium phosphate buffer (pH 6.0). Then, this fraction was further purified by affinity chromatography using an aminophenyl beta-lactose gel (EY Laboratories) column as described previously (Arimitsu *et al.*, 2003). The HA-negative toxin complex was purified from fraction 2 as follows. Fraction 2 was dialysed against 0.05 M sodium acetate buffer (pH 4.2) and then applied to an SP-Toyopearl 650M (Tosoh) column as described previously (Inoue *et al.*, 1996). The HA-negative toxin complex rich fraction eluted from this column was collected and dialysed against 0.01 M sodium phosphate buffer (pH 6.0). Then, the HA proteins that contaminated this fraction were removed using an aminophenyl beta-lactose gel column and a rabbit anti-type A HA proteins immobilized protein G column.

Type B toxin complexes The type B HA-positive toxin complex (16S toxin) and the HA-negative toxin complex (12S toxin) were purified as described previously (Arimitsu *et al.*, 2003). The small amount of HA proteins that contaminated the preparation of the HA-negative toxin complex was removed with a rabbit anti-type B HA proteins immobilized protein G column.

Type C toxin complexes Bacteria were cultured using a cellophane-tube procedure, and the culture supernatant was obtained as described previously (Inoue *et al.*, 1999). The HA-positive toxin complex (16S toxin) and the HA-negative toxin complex (12S toxin) were purified by a procedure described previously (Inoue *et al.*, 1999) with some modifications. The culture supernatant was concentrated by 60 % ammonium sulfate precipitation and dialysed against 0.05 M sodium

phosphate buffer (pH 6.0). The HA-positive toxin complex was concentrated in the precipitate which appeared during dialysis (fraction 1), and the HA-negative toxin complex remained in the supernatant (fraction 2). After centrifugation (15 000 g, 30 min, 4 °C), the precipitate, which contained the HA-positive toxin complex, was dialysed against 0.05 M sodium acetate buffer (pH 4.2) and applied to an SP-Toyopearl 650M (Tosoh) column as described previously (Mahmut *et al.*, 2002). The HA-positive toxin complex rich fraction eluted from this column was collected and dialysed against 0.01 M sodium phosphate buffer (pH 6.0). To obtain the HA-negative toxin complex, fraction 2 was dialysed against 0.05 M sodium acetate buffer (pH 4.2), and applied to an SP-Toyopearl 650M (Tosoh) column as described previously (Mahmut *et al.*, 2002). The HA-negative toxin complex rich fraction eluted from this column was collected and dialysed against 0.01 M sodium phosphate buffer (pH 6.0). Both the HA-positive toxin complex rich fraction and HA-negative toxin complex rich fraction were applied to a hydroxyapatite (Bio-Rad) column to remove C3 enzyme as described previously (Inoue *et al.*, 1999). The HA-positive and HA-negative toxin complexes were further purified by gel filtration on a Sephacryl S-300 (Amersham) column as described previously (Fujinaga *et al.*, 1997).

All the chromatography steps were performed at room temperature, and the dialysis steps were performed at 4 °C. Purified toxin complexes were sterilized by filtration (pore size 0.2 μm ; Advantec) before being applied to cells. The concentrations of the toxin complexes in molarity were calculated from the molecular masses of 16S toxin (745 kDa) and 12S toxin (287 kDa) for the type A HA-positive and HA-negative toxin complexes; the molecular masses of 16S toxin (744 kDa) and 12S toxin (289 kDa) for the type B HA-positive and HA-negative toxin complexes; and the molecular masses of 16S toxin (749 kDa) and 12S toxin (287 kDa) for the type C HA-positive and HA-negative toxin complexes, respectively. The molecular mass of each toxin was calculated based on the stoichiometry of subunits contained in the type D toxin complexes (Hasegawa *et al.*, 2007). In the case of the type A HA-positive toxin complex (a mixture of 16S and 19S toxins), one molecule of 19S toxin was regarded as two molecules of 16S toxins, and concentrations of the toxin complex in molarity were calculated as those of 16S toxins.

Measurement of transepithelial electrical resistance (TER), and the paracellular tracer flux assay.

Caco-2, T84 and MDCK I cells were plated onto 0.4 μm pore-size filters (6.5 mm diameter) in transwell chambers (Costar), and the medium was changed every 3 days. The cells were maintained until steady-state TER was achieved. The Caco-2 cells were used 8–10 days post-plating, the T84 cells were used 11–16 days post-plating, and the MDCK I cells were used 7–10 days post-plating. To more closely simulate physiological conditions (Shiau *et al.*, 1985; Taub *et al.*, 2002), the cells were rinsed with PBS (+) (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.72 mM CaCl_2 , 0.39 mM MgCl_2) and the apical aspect was incubated in each medium supplemented with 1.27 mM NaHCO_3 and 30 mM MES (Dojindo) at pH 6.0, and the basolateral compartment was incubated in each medium supplemented with fetal bovine serum (20 % for Caco-2, 10 % for T84, 10 % for MDCK I), NaHCO_3 (17.86 mM for Caco-2 and MDCK I, 14.3 mM for T84), and 15 mM HEPES at pH 7.4. The electrical resistance was measured using a Millicell-ERS epithelial volt-ohm metre (Millipore). Inserts with no cell monolayers served as blanks to determine the baseline resistance. The TER (Ωcm^2) was calculated from the following equation: $(\text{TER}_{\text{sample}} - \text{TER}_{\text{blank}}) \times \text{surface area}$. The monolayers with baseline TER values of 600–1000 Ωcm^2 for Caco-2, 1000–2500 Ωcm^2 for T84, and 3000–5000 Ωcm^2 for MDCK I were used for the assay, and the data were expressed as a percentage of the initial resistance.

The paracellular flux of FITC-dextran was assessed as previously described (Matsumura *et al.*, 2008). The cell monolayers grown in

Fig. 2. HA proteins of types A and B and HA proteins of type C decrease TER with different cell tropisms. Monolayers were treated with vehicle (black open circles), 300 nM type A HA-positive toxin complex (red open squares), type B HA-positive toxin complex (blue open diamonds), type C HA-positive toxin complex (green open circles), type A HA-negative toxin complex (red filled squares), type B HA-negative toxin complex (blue filled diamonds), and type C HA-negative toxin complex (green filled circles). These toxins were added from the apical side (a) or the basolateral side (d) of Caco-2 cells, the apical side (b) or the basolateral side (e) of T84 cells, and the apical side (c) or the basolateral side (f) of MDCK I cells. Each point represents the mean $\pm$ SEM of triplicate wells. The experiment depicted is representative of three independent experiments.

transwells (6.5 mm diameter) were incubated with vehicle alone or toxin complexes (basolateral reservoir) in the presence of FITC-dextran (1 mg ml^{-1} , apical reservoir) with molecular masses of 4, 10 or 150 kDa (Sigma Chemical). After incubation for 24 h, basolateral media were collected, and the amount of FITC-dextran in the media was measured with a fluorometer (excitation 485 nm, emission 538 nm).

Light microscopy. The cells were cultured on round coverslips (Fisher Scientific; diameter 12 mm) in 24-well tissue-culture plates (Greiner) for 2 days. After the cells were washed with each culture medium, they were cultured in each medium (with or without 300 nM toxin complexes) for 24 h. Then, the cells were washed with PBS (–) (136.9 mM NaCl , 2.68 mM KCl , $8.1 \text{ mM Na}_2\text{HPO}_4$, $1.47 \text{ mM KH}_2\text{PO}_4$), fixed in methanol (Wako), stained with Giemsa staining solution (Merck), and observed by using phase-contrast under an Olympus BX50 microscope.

Cytotoxicity assay. The cytotoxicity of each botulinum toxin complex was assessed using cell count reagent SF (Nacalai Tesque). This assay is based on the cellular reduction of WST-8 by mitochondrial dehydrogenases, and it reflects cell viability as well as correlating with cell number. Caco-2, T84, MDCK I, ACL-15 and RCN-9 were cultured in 96-well tissue-culture plates (Greiner) at a cell density of 15 000, 25 000, 3000, 5000 and 5000 cells per well, respectively. After 1 day of culture, the cells were washed with each culture medium and cultured in 100 μl of each medium (with or without toxin complexes) for 24 h, and then

the cellular reduction of WST-8 was measured, according to the manufacturer's instructions with minor modifications. Briefly, 10 μl of cell count reagent SF was added to each well and the cells were incubated for 1.5 h in the cases of T84, MDCK I and RCN-9, 3.0 h in the case of Caco-2, or 1.0 h in the case of ACL-15. The reaction was terminated by the addition of 10 μl of 10% SDS (w/v) in water, and the absorbance was measured at 450 nm with a reference wavelength of 630 nm. For all assays, reactions containing no cells were used to determine blank values, which were subtracted from values obtained in the assays with cells. The values were reported as the percentage WST-8 reduction relative to the untreated cells.

RESULTS

HA proteins of types A or B, and HA proteins of type C decrease the TER with different cell tropisms

In the Caco-2 monolayers, which originated from human colon carcinoma, type A HA-positive toxin complexes (a mixture of 16S toxin and 19S toxin, 300 nM) and type B HA-positive toxin complex (16S toxin, 300 nM) caused a drastic decrease in TER when applied to the apical (Fig. 2a) and basolateral compartments (Fig. 2d). Neither type A HA-

negative toxin complex (12S toxin, 300 nM), nor type B HA-negative toxin complex (12S toxin, 300 nM) affected the TER, indicating the specific action of HA in these serotypes. In contrast, type C HA-positive toxin complex (16S toxin, 300 nM) as well as type C HA-negative toxin complex (12S toxin, 300 nM) did not affect TER. When added to the basolateral compartment, the type A and type B HA-positive toxin complexes (Matsumura *et al.*, 2008) induced much more pronounced activities than those obtained when these toxin complexes were added to the apical compartment (see Supplementary Fig. S1). In type A, a 50 % decrease in TER after 12 h of incubation was induced by 25–50 nM apically added HA-positive toxin complex and by <2.5 nM basolaterally added HA-positive toxin complex. In type B, a 50 % decrease in TER after 12 h of incubation was induced by >500 nM apically added type B HA-positive toxin complex and by ~2.5 nM basolaterally added type B HA-positive toxin complex. The type A HA-positive toxin complex showed more potent activity than type B, especially in the case of apical addition.

We performed the same studies, but applying toxin complexes of types A, B and C to T84 cells, another human colon originated epithelial cell line (Fig. 2b, e). In the T84 monolayers, similar results were obtained with a less potent action compared with when the complex was applied to Caco-2 cells.

We also used a canine kidney epithelial cell line, MDCK I, because MDCK I forms a polarized epithelial monolayer with a very high TER (Matsumura *et al.*, 2008) and is widely used as an *in vitro* model for the epithelial barrier (Nelson, 2003). In the MDCK I monolayers, basolaterally added type A and B HA-positive toxin complexes and, to a lesser extent, type C HA-positive toxin complex caused a loss of TER (Fig. 2f, see Supplementary Fig. S2b, c, d). A 50 % decrease in TER after 12 h of incubation was induced by ~5 nM basolaterally added type A HA-positive toxin complex, by ~10 nM basolaterally added type B HA-positive toxin complex, and by ~250 nM basolaterally added type C HA-positive toxin complex. When types A–C toxin complexes were applied from the apical side (Fig. 2c, Supplementary Fig. S2a), only type A HA-positive toxin complex caused a significant loss of TER, in a less potent manner than when the complex was applied from the basolateral side. A 50 % decrease in TER after 12 h incubation was induced by ~100 nM apically added type A HA-positive toxin complex. Also, in the MDCK I cells, neither type A HA-negative toxin complex (300 nM) and type B HA-negative toxin complex (300 nM) nor type C HA-negative toxin complex (300 nM) affected the TER, indicating the specific action of HA in these serotypes.

Types A, B and C HA-positive toxin complexes increase the paracellular permeability of macromolecules in their susceptible cells

To examine the effect of HA-positive toxin complex treatment on paracellular permeability, we used paracel-

lular tracers (FITC-dextran of various sizes). In the Caco-2 monolayers (Fig. 3a, c, e), basolaterally added type A and B HA-positive toxin complexes increased the paracellular flux of FITC-dextran of 4, 10 and 150 kDa in a size-dependent manner. After 24 h of incubation, the FITC-dextran with low molecular masses had been more efficiently transported than those with higher molecular masses. In contrast, type C HA-positive toxin complex at concentrations up to 300 nM had no detectable effect on the paracellular flux of FITC-dextran. No type of HA-negative toxin complex, up to a concentration of 300 nM, had any detectable effect on the paracellular flux of FITC-dextran (data not shown). Similar results were obtained in T84 monolayers (data not shown).

In the MDCK I monolayers, basolaterally added HA-positive toxin complexes of all types increased the paracellular flux of FITC-dextran of all sizes (Fig. 3b, d, f). However, no type of HA-negative toxin complex, up to a concentration of 300 nM, had any detectable effect on the paracellular flux of FITC-dextran (data not shown).

Type A, B and C HA-positive toxin complexes drastically alter the morphologies of their susceptible cells

The effect of HA-positive toxin complexes on the morphologies of Caco-2, T84 and MDCK I cells was assessed after 24 h of exposure. As shown in Fig. 4(a), the untreated control subconfluent Caco-2 cells formed clusters. Caco-2 cells treated with type A HA-positive toxin complex (Fig. 4d) and type B HA-positive toxin complex (Fig. 4g) showed a drastic change in morphology, with dissolution of the tight clusters and a distinct separation of the cells from each other, initially at the edges of the clusters then prevalently inside the clusters. In contrast, type C HA-positive toxin complex, at concentrations up to 300 nM, did not induce a similar, easily observable, progressive morphological change (Fig. 4j). In the T84 cells, similar results were obtained (Fig. 4b, e, h, k).

In the MDCK I cells, similar morphological changes to those found in Caco-2 and T84 cells were observed with types A and B HA-positive toxin complexes (Fig. 4c, f, i). In this cell line, type C HA-positive toxin complex caused a drastic alteration in morphology (Fig. 4l), clearly distinct from the morphological changes induced by types A and B HA-positive toxin complexes. In contrast to the loss of cell-to-cell attachment after treatment with types A and B HA toxin complexes, cells treated with type C HA-positive toxin complex appeared shrunken, and some cells were detached from the culture dishes. These results suggest distinct pathways for damaging the barrier function of MDCK I cells between type A or B HA-positive toxin complexes and type C HA-positive toxin complex. No type of HA-negative toxin complex, at a concentration up to 300 nM, disrupted the cluster morphology in Caco-2, T-84 or MDCK I cell lines (data not shown).

Fig. 3. HA-positive toxin complexes of types A, B and C increase the paracellular flux of FITC-dextran in their susceptible cells. The flux of FITC-dextran was measured in Caco-2 monolayers (a, c, e) or MDCK I monolayers (b, d, f) treated basolaterally with type A (50 nM), type B (50 nM) or type C (300 nM) HA-positive toxin complexes (type A, B or C), or vehicle (NC) for 24 h after the apical addition of 4 kDa (light-grey column), 10 kDa (mid-grey column) or 150 kDa (dark-grey column) FITC-dextran. Values are the means $\pm$ SEM of triplicate wells. * $P < 0.05$, significant differences from vehicle-treated cells; ** $P < 0.01$, significant differences from vehicle-treated cells, unpaired t test. The experiment depicted is representative of three independent experiments.

Effect of types A, B and C HA proteins on the viability of their susceptible cells

To characterize the modes of actions for types A–C HA-positive toxin complexes, the cytotoxic effects of these toxin complexes on Caco-2, T84 and MDCK I cell lines were examined by a method based on the reduction of tetrazolium dye (WST-8) that reflects cell viability as well as correlating with cell number. In Caco-2 cells (Fig. 5a), types A and B HA-positive toxin complexes did not show detectable cytotoxicity. Type C HA-positive toxin complex, however, caused a slight but reproducible cytotoxicity.

In T84 cells (Fig. 5b), types A and B HA-positive toxin complexes did not show detectable cytotoxicity, although

they increased cellular reduction of WST-8 by approximately threefold compared with the untreated control cells.

In MDCK I cells (Fig. 5c), types A and B HA-positive toxin complexes did not show cytotoxicity, whereas type C HA-positive toxin complex induced a robust cytotoxicity. Neither types A and B nor type C HA-negative toxin complexes caused detectable alteration in the WST-8 reduction of Caco-2, T84 or MDCK I cell lines.

DISCUSSION

Our results show that type A, B and C HA proteins in botulinum toxin complexes possess a potent ability to disrupt epithelial barrier function, and have distinct

Fig. 4. HA-positive toxin complexes of types A, B and C drastically alter the morphologies of their susceptible cells. Subconfluent cells of Caco-2 (a, d, g, j), T84 (b, e, h, k) or MDCK I (c, f, i, l) were untreated (NC) or treated with 300 nM of each type of HA-positive toxin complex (type A, B or C) for 24 h. Cells were fixed and stained with Giemsa staining solution. Bars, 100 μ m. The experiment depicted is representative of three independent experiments.

features in their modes of action (summarized in Table 2). These findings show that botulinum HA proteins are pathogenic factors that breach the host defence by direct interaction with the host epithelium, which is presumably linked to the intestinal transepithelial delivery of BoNTs in food-borne botulism of the susceptible species.

Type A and type B HA proteins disrupt the paracellular barrier with similar cell tropism and mechanisms

Type A and type B HA proteins (Matsumura *et al.*, 2008) induced paracellular barrier dysfunction characterized by a decrease in TER and an increase in paracellular flux of

FITC-dextran in Caco-2, T84 and MDCK I. Type A and type B HA-positive toxin complexes were more potent when applied to the basolateral side than to the apical side of these cell monolayers, suggesting that type A HA proteins, similarly to type B HA proteins (Matsumura *et al.*, 2008), act on the epithelial monolayers via the basolateral membrane only. Similarly, when type A HA-positive toxin complex was applied to the apical cell surfaces of Caco-2 monolayers containing anti-HA antisera in their basolateral reservoirs, the effect on TER was almost completely reversed (data not shown). These results imply that this is caused by the asymmetrical distribution of the molecular target(s) of HA proteins in the basolateral membrane. This site of action seems counterintuitive for an enteric pathogen; however, some pathogenic microbes

Fig. 5. Effects of HA proteins of types A, B and C on cell viability. The cellular reduction of tetrazolium dye (WST-8) of Caco-2 cells (a), T84 cells (b) and MDCK I cells (c), treated with 300 nM of each type of HA-positive toxin complex [HA (+)], or HA-negative toxin complex [HA (-)], or untreated (NC) for 24 h. Values are the means $\pm$ SEM of triplicate wells. The experiment depicted is representative of three independent experiments.

such as *Shigella* spp. are known to invade intestinal epithelial cells from their basolateral membrane after initial entry through intestinal M cells via transcytosis (Schroeder & Hilbi, 2008). Therefore, work is under way to clarify the pathway(s) taken by apically localized toxin complexes in the intestinal epithelium both *in vitro* and *in vivo*, taking into account the role of M cells.

Type A HA-positive toxin complex was significantly more potent than type B HA-positive toxin complex. In Caco-2 cells, the type A HA-positive toxin complex exhibited about a 10-fold greater potency than the type B HA-positive toxin complex when these complexes were applied to the apical compartment, and about a twofold greater

potency when these complexes were applied to the basolateral compartment. This suggests that the mode of interaction of type A HA proteins with epithelial cells is not identical to that of type B HA proteins. On the other hand, the effects of type A and type B HA proteins on the morphologies and viabilities of Caco-2, T84 and MDCK I were very similar (disruption of cell-to-cell association without a loss of attachment to dishes, and no cytotoxicity). T84 treated with type A and B HA proteins for 24 h showed an increase in the cellular reduction of WST-8 of approximately threefold compared with those observed in untreated control cells (Fig. 5b). The reason(s) for this phenomenon are unclear. This assay relies on the ability of live cells to reduce tetrazolium dye (WST-8), and therefore

Table 2. Summary of the effects of type A, B and C HA-positive toxin complexes

Barrier disruption: ++, 50% decrease in TER after 12 h incubation was induced by <5 nM toxin complex; +, 50% decrease in TER after 12 h incubation was induced by <300 nM toxin complex; $\pm$, 20% decrease in TER after 24 h incubation was induced by <300 nM toxin complex; -, decrease in TER was not induced within 24 h by 300 nM toxin complex. Cytotoxicity: +, WST-8 reduction was decreased to <50% by 300 nM toxin complex; $\pm$, WST-8 reduction was decreased to <80% by 300 nM toxin complex; -, no decrease in WST-8 reduction was induced by 300 nM toxin complex. ND, Not determined.

Strain	Cell line							
	Caco-2		T84		MDCK I		ACL-15	RCN-9
	Apical	Basal	Apical	Basal	Apical	Basal		
Barrier disruption								
Type A	+	++	±	+	+	++	ND	ND
Type B	±	++	—	+	—	++	ND	ND
Type C	—	—	—	—	—	+	ND	ND
Cytotoxicity								
Type A	—	—	—	—	—	—	+	+
Type B	—	—	—	—	—	—	—	—
Type C	±	—	—	—	+	—	+	+

indicates cellular viability and proliferation. As indicated by our experiment (Fig. 4), T84 treated with these HA proteins for 24 h does not exhibit markedly (threefold or more) enhanced proliferation compared with untreated control cells. Hence, we speculate that these HA proteins elicit a signalling event that increases the reductive capacity of each cell.

These results imply that the fundamental mechanisms of actions of type A and B HA proteins in these cell lines may have some points in common. However, the molecular target(s) for these HA proteins possibly exist in the basolateral membrane, and if so, the pathway(s) taken by apically added HA proteins to reach the basolateral side are not yet known. These are the subjects of current studies in our laboratory.

Type C HA proteins also disrupt the epithelial barrier, but their cell tropism and mode of action are different from those of type A and B HA proteins

In the Caco-2 and T84 monolayers, type C HA-positive toxin complex (up to 300 nM) did not downregulate paracellular barrier function as assessed by TER and the flux of FITC-dextran. In Caco-2 cells, type C HA proteins induced slight cytotoxicity as estimated by the tetrazolium reduction-based assay, although this cytotoxic activity caused neither a drastic alteration of morphology nor disruption of barrier function. The reduction of tetrazolium salts is dependent on cellular reductive capacity, and this reaction is sensitively influenced by the metabolic activity of cells (Vistica *et al.*, 1991). Hence, we interpreted these results as indicating that type C HA proteins slightly affect Caco-2 viability, but that this low degree of cytotoxic activity does not lead to the disruption of barrier function or a marked alteration in morphology such as shrinking or detachment from the culture dish.

However, in the MDCK I monolayers, type C HA-positive toxin complex showed a barrier-disrupting activity at a concentration about 50-fold higher than type A HA-positive toxin complex (ED_{50} at 12 h for basolateral type A ~5 nM, for basolateral type C ~250 nM), and about a 25-fold higher concentration compared with type B HA-positive toxin complex (ED_{50} at 12 h for basolateral type B ~10 nM, for basolateral type C ~250 nM). Type C HA-positive toxin complex, at concentrations up to 300 nM, caused only a slight decrease in TER when applied to the apical side of MDCK I monolayers, suggesting that type C HA proteins also act on the monolayers via the basolateral membrane only. In MDCK I cells, type C HA proteins induced a marked cytotoxicity, and elicited drastic morphological changes distinct from those induced by type A and B HA proteins. These results indicate that the disruption of barrier function by type C HA proteins can be attributed to general mechanisms of cell toxicity. There is a fundamental question that remains to be answered: does type C HA cause damage to the intestinal epithelium

of susceptible animals? The present study has shown that type C HA proteins induce marked cytotoxicity in ACL-15 and RCN-9, two different rat intestinal epithelial cell lines (see Supplementary Fig. S3). These data raise the possibility that type C HA induces damage in the intestinal epithelium of susceptible animals, which could allow the unrestricted influx of the toxin complex into the systemic circulation. However, our evidence about the epithelial barrier disruption provoked by type C HA proteins is limited to an *in vitro* cell line, MDCK I. Further *in vivo* studies will provide a better understanding of the pathological roles of this cytotoxic activity.

The correlation between epidemiology of food-borne human botulism and cell tropism of HA action

Human cases of botulism due to toxin types A, B, E and F are well documented, whereas cases of human types C and D botulism are very rare (Collins & East, 1998). The cause(s) of this phenomenon are not clear. It has been reported that neuromuscular junctions surgically excised from humans are sensitive to type C BoNT, and human syntaxin is a substrate for type C BoNT (Coffield *et al.*, 1997). It has also been reported that primates are susceptible to inhalation exposure of type C toxin (LeClaire & Pitt, 2004). On the other hand, the results obtained in this study for the action of HA toward human intestinal cell lines correlate with the epidemiology of human botulism. It has also been reported that BoNT can breach epithelial barriers by transcytosis (Maksymowych & Simpson, 1998; Couesnon *et al.*, 2008), and type C BoNT is not efficiently transcytosed compared with type A and B BoNTs in human intestinal epithelial cell lines (Caco-2 and T84) (Maksymowych & Simpson, 1998). However, comparative analyses of transepithelial transport of type B HA-positive toxin complex (16S toxin), which disrupts the paracellular barrier, and HA-negative toxin complex (12S toxin) and BoNT, which do not affect the paracellular barrier, showed that about 10-fold more HA-positive toxin complexes cross the monolayer compared with those found in cells treated with BoNT or HA-negative toxin complex (Matsumura *et al.*, 2008). Therefore, at least *in vitro*, the paracellular route appears to be the major route compared with the transcytosis route for transepithelial transport of the toxin. These observations lead us to surmise an important role for type A and B HA proteins in the pathogenesis of food-borne botulism, at least in humans. The dysfunction of the intestinal epithelial paracellular barrier induced by type A and B botulinum HA proteins greatly contributes to the development of food-borne botulism, and the relative inability of type C HA proteins to disrupt the paracellular barrier of the human intestinal epithelium is one of the reasons for the relative absence of food-borne human botulism caused by the type C toxin. Additional *in vivo* studies using species that are susceptible to oral intoxication by each serotype will be required to further investigate this intriguing possibility.

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