RESEARCH ARTICLE

HA-33 facilitates transport of the serotype D botulinum toxin across a rat intestinal epithelial cell monolayer

Hiroaki Ito[1], Yoshimasa Sagane[2], Keita Miyata[1], Ken Inui[1], Tomohito Matsuo[1], Ryohta Horiuchi[1], Toshihiko Ikeda[3], Tomonori Suzuki[4], Kimiko Hasegawa[5], Hirokazu Kouguchi[6], Keiji Oguma[4], Koichi Niwa[1], Tohru Ohyama[1] & Toshihiro Watanabe[1]

[1]Department of Food and Cosmetic Science, Faculty of Bioindustry, Tokyo University of Agriculture, Abashiri, Japan; [2]Sars International Centre for Marine Molecular Biology, Bergen, Norway; [3]Yokohama College of Pharmacy, Yokohama, Japan; [4]Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [5]Rigaku Corporation, Akishima, Japan; and [6]Hokkaido Institute of Public Health, Sapporo, Japan

Correspondence: Toshihiro Watanabe, Department of Food and Cosmetic Science, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493, Japan. Tel./fax: +81 152 48 3843; e-mail: t-watana@bioindustry.nodai.ac.jp

Received 9 August 2010; revised 21 December 2010; accepted 22 December 2010. Final version published online 1 February 2011.

DOI:10.1111/j.1574-695X.2011.00779.x

Editor: Nicholas Carbonetti

Keywords

Clostridium botulinum; neurotoxin; toxin complex; haemaggulutinin; intestinal epithelium; IEC-6.

Abstract

A large size botulinum toxin complex (L-TC) is composed of a single neurotoxin (BoNT), a single nontoxic nonhaemagglutinin (NTNHA) and a haemagglutinin (HA) complex. The HA complex is comprised of three HA-70 molecules and three arm structures of HA-33/HA-17 that consist of two HA-33 and a single HA-17. In addition to the mature L-TC, smaller TCs are present in cultures: M-TC (BoNT/NTNHA), M-TC/HA-70 and immature L-TCs with fewer HA-33/HA-17 arms than mature L-TC. Because L-TC displays higher oral toxicity than pure BoNT, it was presumed that nontoxic proteins are critical for food poisoning. In this study, the absorption of TCs across intestinal epithelial cells was assessed by examining the cell binding and monolayer transport of serotype D toxins in the rat intestinal epithelial cell line IEC-6. All TCs, including pure BoNT, displayed binding and transport, with mature L-TC showing the greatest potency. Inhibition experiments using antibodies revealed that BoNT, HA-70 and HA-33 could be responsible for the binding and transport. The findings here indicate that all TCs can transport across the cell layer via a sialic acid-dependent process. Nonetheless, binding and transport markedly increased with number of HA-33/HA-17 arms in the TC. We therefore conclude that the HA-33/HA-17 arm is not necessarily required for, but facilitates, transport of botulinum toxin complexes.

Introduction

Botulinum neurotoxin (BoNT; 150 kDa), produced by *Clostridium botulinum*, is immunologically classified into seven distinct serotypes A–G. Serotypes A, B, E and F are the causative agents of human botulism, whereas serotype C and D toxins are involved in animal and avian botulism (Montecucco & Schiavo, 1994; Li & Singh, 1999). Orally ingested BoNT reaches the neuromuscular junction and enters nerve cells via receptor-mediated endocytosis. Once in the nerve cell, the zinc protease activity of the toxin cleaves specific sites on target proteins that are involved in the release of the neurotransmitter. Botulism occurs via a series of processes that cause muscular paralysis in humans and animals.

In culture fluids and naturally contaminated foods, BoNT forms a toxin complex (TC) in conjunction with auxiliary nontoxic proteins, including the 130-kDa nontoxic nonhaemagglutinin (NTNHA) subunit and/or three haemagglutinin (HA) subcomponents with molecular masses of 70, 33 and 17 kDa (HA-70, HA-33 and HA-17). *Clostridium botulinum* produces both haemagglutination-positive and -negative TCs with different molecular masses. The M-TC is comprised of BoNT and NTNHA, the L-TC is formed by the association of the M-TC with HAs, and the LL-TC is presumed to be a dimer of the L-TC. The haemagglutination-positive TC is a 750-kDa L-TC (serotype B–D and G) or a mixture of the L-TC and the LL-TC (serotype A), whereas the haemagglutination-negative TC is a 280-kDa M-TC (serotype A-F) (Inoue *et al.*, 1996; Hasegawa *et al.*, 2007). The proposed assembly pathway for mature L-TC formation from individual subcomponents is as follows (Kouguchi *et al.*, 2002; Mutoh *et al.*, 2003): (1) the

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

association of BoNT and NTNHA yields the 280-kDa M-TC, (2) assembly of the M-TC and HA-70 forms the intermediate 490-kDa M-TC/HA-70 and (3) further conjugation with the HA-33/HA-17 complex leads to the formation of mature 750-kDa L-TC. In addition to these TCs, two haemagglutination-negative L-TCs (610 and 680 kDa) in the culture fluid of serotype C and D strains correspond to intermediate products in the pathway leading from the 490-kDa M-TC/HA-70 to the mature 750-kDa L-TC, which has a smaller number of HA-33/HA-17 complexes than mature L-TC (Mutoh *et al.*, 2003, 2005). Transmission electron microscopy of negatively stained TCs (M-TC, M-TC/HA-70, 610-kDa L-TC, 680-kDa L-TC and mature 750-kDa L-TC) and crystal structure analysis of the HA-33/HA-17 complex revealed the three-dimensional structure of a 14-meric mature L-TC (three-arm L-TC; Fig. 1a): the ellipsoid shape of a single BoNT; a single NTNHA; and three HA-70s, each attached to an arm structure (two HA-33 molecules and a single HA-17) (Hasegawa *et al.*, 2007). The 610-kDa L-TC contained one HA-33/HA-17 arm (one-arm L-TC; Fig. 1a) while the 680-kDa L-TC contained two (two-arm L-TC; Fig. 1a). More recently, Nakamura *et al.* (2009) solved the crystal structure of serotype C HA3 (which corresponds to HA-70). In the crystal packing, three HA-70 molecules assemble to form a three-leaved propeller-like structure, which coincides with our 14-meric model of the L-TC.

The TCs (M-TC, L-TC and LL-TC) display greater oral toxicity than pure BoNT (Ohishi *et al.*, 1977; Sakaguchi *et al.*, 1984). Pure BoNT without auxiliary nontoxic components is susceptible to the proteolytic and acidic conditions in the digestive tract (Halliwell, 1954; Ohishi *et al.*, 1977; Sugii *et al.*, 1977a, b; Bonventre, 1979; Ohishi & Sakaguchi, 1980; Ohishi, 1984; Chen *et al.*, 1998; Niwa *et al.*, 2007), indicating that the nontoxic components serve to protect BoNT against the conditions of the gastrointestinal tract. There is evidence that the L-TC transports across the intestinal epithelial cell monolayer more effectively than pure BoNT (Fujinaga *et al.*, 1997, 2004; Niwa *et al.*, 2007,

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

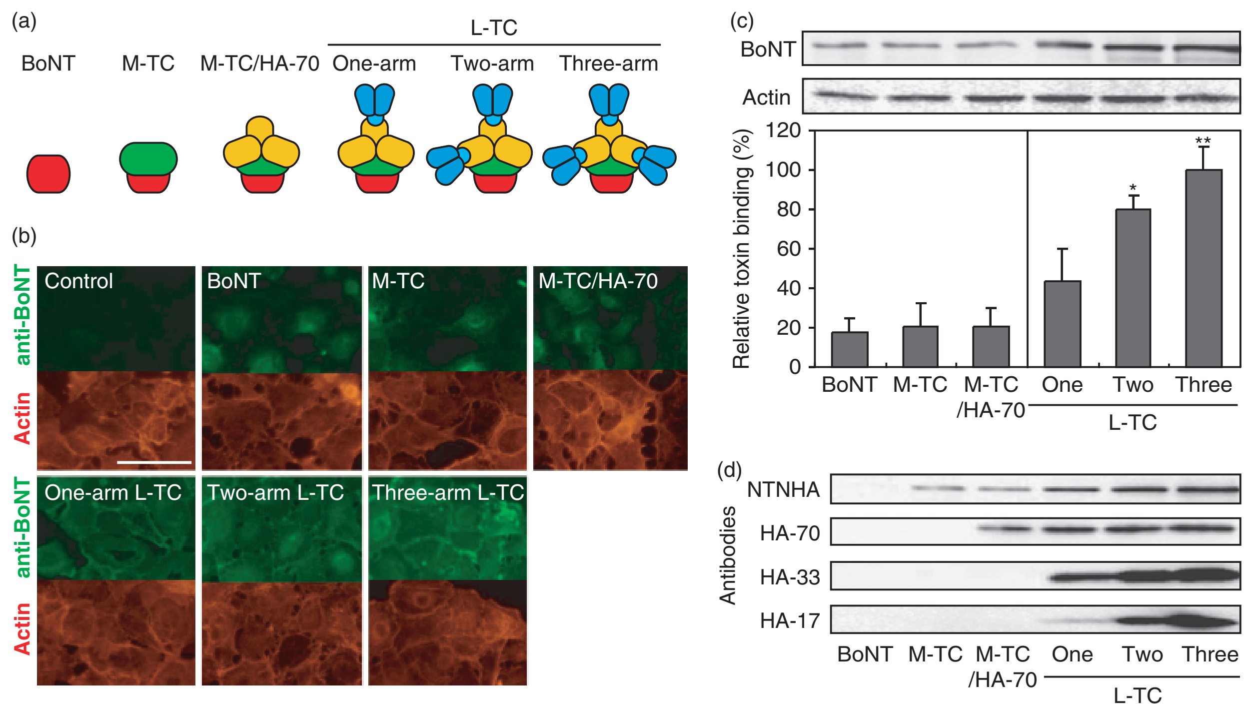

Fig. 1. Toxin complexes (TCs) produced by *Clostridium botulinum* type D strain 4947 and the binding of TCs to IEC-6 cells. (a) Schematic structures of the botulinum TCs based on the report by Hasegawa *et al*. (2007). Red, BoNT; green, NTNHA; yellow, HA-70; light blue, HA-17; and blue, HA-33. (b) Cells were incubated with BoNT, M-TC, M-TC/HA-70, and one-, two- and three-arm L-TCs at 100 nM for 1 h at 4 °C. Toxins bound to the cells and cytoskeletal actin were visualized by immunostaining using BoNT antibody followed by Alexa Fluor 488-conjugated secondary antibody (green) and Alexa Fluor 546-conjugated phalloidin (red). Cells without toxin treatment were used as a control. Scale bar indicating 50 μm at upper left applies to all images. (c) Western blot analysis of the binding of TCs to IEC-6 cells. Cells were incubated with 20 nM TC. BoNT in the cell lysates was detected using anti-BoNT antibody. Representative data (upper panels) and calculated relative amounts (lower panels) are shown. The relative binding was calculated based on band intensities. Experiments were repeated in triplicate, and error bars represent the SEM. Binding of the two-arm L-TC ($P < 0.05$) and three-arm L-TC ($P < 0.01$) were significantly increased over BoNT. Statistical analyses were performed using nonrepeated measures ANOVA with a Bonferroni's correction. (d) The cell-binding assay was performed in the same manner for panel (c). TC components were detected using anti-NTNHA, anti-HA-70 and anti-HA-33/HA-17.

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

2010; Inui *et al*., 2010). Therefore, HA components appear to play a role in the transport of TCs across the intestinal epithelium in addition to protection from BoNT digestion. However, there are conflicting data that the absorption of botulinum toxin across the intestinal epithelium is independent of the presence of HA components (Maksymowych & Simpson, 1998, 2004; Maksymowych *et al*., 1999). In this study, we used pure BoNT and the TCs from serotype D strain 4947 to assess cell binding and cell monolayer transport in the rat intestinal epithelium cell line IEC-6. The results of these assays suggest that the HA-33/HA-17 arm facilitates transport of the toxin across the intestinal epithelium cell monolayer. While the ease of movement depends on the number of arms, all TCs, including pure BoNT, can transport across the cell monolayer.

Materials and methods

Antibodies

Rabbit polyclonal antibodies were raised against BoNT, NTNHA, HA-70, HA-33 and the HA-33/HA-17 complex (Niwa *et al*., 2007).

Production and purification of botulinum toxins

Clostridium botulinum serotype D strain 4947 was cultured anaerobically for 5 days using dialysis (Hasegawa *et al*., 2004). Crude TC was precipitated with 60% (w/v) saturated ammonium sulphate, dialysed against 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl and applied to a Toyopearl SP-650S (Tosoh) cation-exchange column (1.6 cm × 40 cm) equilibrated with dialysis buffer. TC species bound to the resin were eluted with a linear gradient of NaCl (0.2–0.8 M). Each fraction was pooled separately, concentrated and further purified with a HiLoad 16/60 Superdex 200 pg (GE Healthcare, UK) gel-filtration column (1.6 cm × 60 cm) equilibrated with 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. The TC fraction was then applied to a Mono S HR5/5 (GE Healthcare) cation-exchange column (0.5 cm × 5 cm) equilibrated with 50 mM acetate buffer (pH 5.0) and eluted by a linear gradient of NaCl (0–0.5 M).

Separation of BoNT from the three-arm L-TC was achieved using a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with 20 mM Tris-HCl (pH 8.8) containing 0.4 M NaCl (Hasegawa *et al*., 2004). Each separated sample was applied to a Mono Q HR5/5 (GE Healthcare) anion-exchange column (0.5 cm × 5 cm) equilibrated with 20 mM Tris-HCl (pH 7.8) and eluted by a linear gradient of NaCl (0–1.0 M).

Cell culture

The rat small intestine epithelial cell line IEC-6 was obtained from the RIKEN BioResource Center. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). Cells were maintained in a humidified environment of 5% CO_2 at 37 °C. The culture medium was renewed every 2–3 days.

Fluorescent microscopy

IEC-6 cells were seeded on a glass chamber-slide (Nunc). The cells were incubated with each toxin diluted to 100 nM in DMEM for 1 h at 4 °C, rinsed three times with cold phosphate-buffered saline (PBS) and fixed with 3.7% (v/v) formaldehyde for 15 min. After three rinses with PBS and quenching with 50 mM NH_4Cl for 15 min, the cells were incubated in PBS containing 2% (w/v) bovine serum albumin (BSA) for 1 h and further incubated with anti-BoNT diluted 1 : 300 in PBS containing 2% (w/v) BSA for 2 h. The cells were rinsed with PBS and incubated with 2% (w/v) BSA–PBS containing Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG) antibody (1 : 500; Invitrogen) and Alexa Fluor 546-conjugated phalloidin (5 U mL^{-1}; Invitrogen) for 1 h. Cells were then observed under a fluorescent microscope (Axiovert 40 CFL, Carl Zeiss) with standard fluorescein isothiocyanate (FITC) excitation/emission filters for Alexa Fluor 488 and rhodamine filters for Alexa Fluor 546.

Assay for toxin binding and transport

Toxin binding to IEC-6 cells and transport through the cell monolayer were assayed as described previously (Niwa *et al*., 2010). IEC-6 cells were prepared in 24-well dishes (Corning) and grown to confluence. Each toxin was suspended in 300 μL of DMEM without FBS at the indicated concentrations and added to culture dishes at 4 °C. Cells were then incubated with toxins for 1 h at 4 °C, rinsed three times with cold PBS and lysed with 200 μL of sodium dodecyl sulphate (SDS) buffer. Proteins bound to cells in 20 μL of the treated sample were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and detected by Western blot analysis.

Toxin transport through an IEC-6 cell layer was assessed in Transwell culture inserts comprised of a two-compartment culture system separated by a polycarbonate membrane (Corning). Cells were seeded at a confluent density (5.0×10^5 cells cm^{-2}) on the bottom membrane of the culture insert. The cells were cultured for 5 days to allow formation of tight connections. Integrity of the connection was confirmed by the passage of FITC-dextran (MW 20 kDa; Sigma-Aldrich) through the cell layer (Supporting Information, Fig. S1). Toxins were suspended in 200 μL of DMEM containing 5% (v/v) FBS and 0.2 mg mL^{-1} FITC-dextran (MW 500 kDa; Sigma-Aldrich), and added to the inner chamber. The outer chamber was filled with 900 μL of DMEM containing 5% (v/v) FBS. Cells were incubated in a

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

CO_2 incubator for 1, 6, 24 or 48 h at 37 °C. Medium from the outer chamber (200 μL) was collected and treated with 100 μL of 3× SDS sample buffer. A 20-μL aliquot of the treated sample was then used for SDS-PAGE and Western blot analysis. Concomitant passage of FITC-dextran through the cell layer was evaluated by measuring fluorescent intensity of the outer medium using a fluorometer (excitation, 495 nm; emission, 510 nm). Outer chamber medium (100 μL) was diluted with 700 μL PBS before measuring. Binding and transport tests were repeated three to four times with different batches of purified toxin.

Inhibition test

The effect of antibodies on the binding and transport of TCs was assessed by mixing toxins (40 nM) with antibodies diluted 1 : 10 in DMEM and incubating for 1 h at 37 °C before the binding and transport tests.

The role of sialic acid in cell binding or monolayer transport of the TCs was investigated by incubating cells with toxins (20 nM) and *N*-acetylneuraminic acid (Neu5Ac; 100 mM) during the binding and transport tests.

The effect of neuraminidase on binding and transport was evaluated with a preliminary incubation of cells with *Clostridium perfringens* neuraminidase (Sigma-Aldrich) at a final concentration of 16.7 $mU\,mL^{-1}$ for 18 h in a CO_2 incubator. The neuraminidase-treated cells were then washed three times with PBS and used in binding and transport tests with TCs. During the transport test, neuraminidase was concomitantly added to culture medium with toxins throughout the incubation time. All inhibitory tests were repeated three times with different batches of purified toxin.

Western blot analysis

Samples from the toxin-binding or transport assay were electrophoresed on 10% (w/v) SDS gels. The separated proteins were blotted onto a nitrocellulose membrane (GE Healthcare) and incubated with antibodies diluted 1 : 300 in TTBS [20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20] with 3% (w/v) skim milk at 4 °C overnight. After rinsing three times with TTBS, the membranes were incubated with anti-rabbit IgG–horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology) diluted 1 : 1000 in TTBS containing 3% (w/v) skim milk at 4 °C overnight. After rinsing, proteins were visualized by chemiluminescence detection. Goat anti-actin antibody and bovine anti-goat IgG antibody conjugated with HRP (Santa Cruz Biotechnology) were used for actin detection. The intensity of detected bands was analysed with IMAGE J 1.38 v software (http://rsb.info.nih.gov/ij/). In the transport assay, a series of pure BoNT dilutions (0.2, 0.4, 1.0, 2.0, 4.0 and 8.0 nM) was loaded onto every SDS gel to calibrate the staining intensity of the BoNT band from each toxin. The calibration curve generated from the plot of BoNT concentration vs. staining intensity was used to estimate toxin concentration.

Results

Cell binding and cell monolayer transport of TCs

BoNT, M-TC (BoNT/NTNHA), M-TC/HA-70 and three types of L-TCs (designated here as one-, two- and three-arm L-TC) with different numbers of the HA-33/HA-17 arms attached were purified from cultures of *C. botulinum* serotype D strain 4947 (schematic structures of the toxins are shown in Fig. 1a). The toxins were incubated with IEC-6 cells at 4 °C for 1 h. Toxins bound to the cells and cytoskeletal actins were visualized by immunohistochemistry using anti-BoNT and phalloidin (Fig. 1b). Fluorescence from the BoNT-, M-TC- and M-TC/HA-70-treated cells displayed similar intensities, whereas higher intensities were observed after cells were treated with one-, two- or three-arm L-TCs. To quantitatively assess binding of the TCs, toxins bound to the cells were extracted into the sample buffer, applied to SDS-PAGE and subjected to Western blot analysis using anti-BoNT. Staining intensities were compared to determine the relative amount of the toxins bound to the cells (Fig. 1c). BoNT, M-TC and M-TC/HA-70 displayed similar cell-binding potencies. In contrast, the one-, two- and three-arm L-TCs exhibited two, four and five times greater binding than pure BoNT.

Toxin transport through the IEC-6 cell monolayer was examined using the Transwell two-chamber system (Niwa *et al.*, 2010). Toxins suspended in the medium were placed in the inner chamber for apical application to the cell monolayer. Following a 1–48-h incubation at 37 °C, toxins transported to the basal side of the layer were collected from the outer chamber and used for Western blots, which indicated that all of the TCs were transported across the cell monolayer (Fig. 2a). L-TCs displayed greater transport potency than the smaller TCs, whereas pure BoNT, M-TC and M-TC/HA-70 exhibited similar transport efficiencies at all time points (Fig. 2b). After a 24-h incubation, the one-, two- and three-arm L-TCs showed 2, 2.5 and 3 times greater cell monolayer transport, respectively, than pure BoNT. During the 48-h incubation, the L-TCs displayed an asymptotic flattening of the transport velocity, probably because of equilibration of the concentrations of the media in the inner and outer chambers, whereas the velocity of the transport of pure BoNT, M-TC and M-TC/HA-70 displayed an almost linear curve. Passage of FITC-dextran through the cell layer also significantly increased in the presence of the L-TCs. However, dextran passage was not facilitated by the presence of pure BoNT, M-TC and M-TC/HA-70 (Fig. 2c).

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

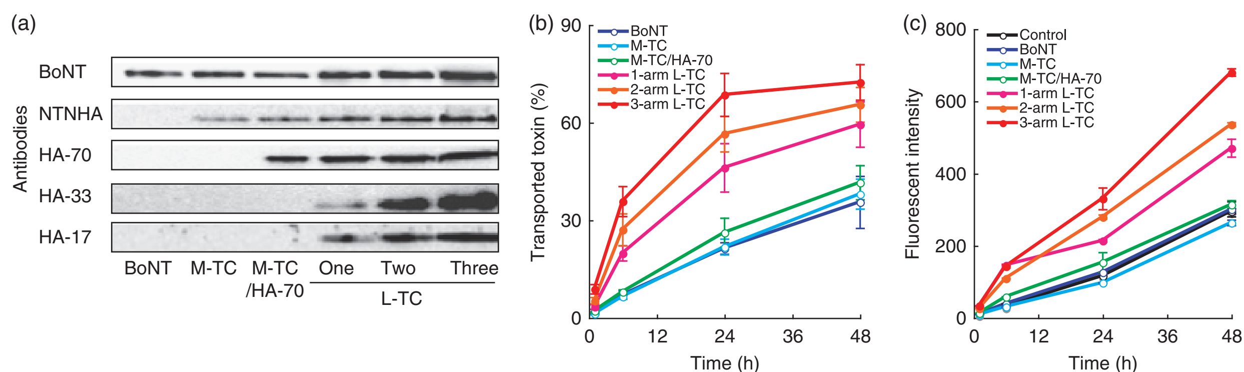

Fig. 2. Transport of TCs through the IEC-6 cell monolayer and concomitant passage of FITC-dextran in toxin transport. (a) Western blot analysis of the transport of TCs through the cell monolayer. Each 20 nM toxin suspension containing 0.2 mg mL^{-1} FITC-dextran (MW 500 kDa) was poured into the inner chamber of the Transwell two-chamber system separated by a membrane covered with an IEC-6 cell layer. Following a 24-h incubation, each component of the TC in the outer chamber medium was detected by Western blots with anti-BoNT, anti-NTNHA, anti-HA-70 and anti-HA-33/HA-17. (b) Time course of toxin transport through the cell layer. Toxin concentration in the outer chamber medium was estimated from the band intensities of BoNT on the Western blot as described in the Materials and methods. Amounts of the transported toxins are indicated as percentages of the original toxin amounts applied to the cell layer. (c) Time course of concomitant FITC-dextran transport. Passage was evaluated by measuring the fluorescent intensity of the outer chamber medium. Experiments were repeated four times, and error bars represent SEMs. Statistical analyses were performed using nonrepeated measures ANOVA with a Bonferroni's correction. Transport of the L-TCs was significantly higher than transport of BoNT at all time points ($P < 0.01$) with the exception of one-arm L-TC after 1 h of incubation. Passage of FITC-dextran in the presence of the L-TCs was significantly higher ($P < 0.01$) than in the control cell layer (without toxins) at all time points.

Antibodies detected each of the nontoxic proteins in the cell-binding and cell-monolayer-transport assays, indicating that all of the nontoxic proteins transported through the cell monolayer (Figs 1d and 2a).

TC components responsible for cell binding and monolayer transport

Of all the TCs examined, the three-arm L-TC demonstrated the greatest binding to IEC-6 cells and showed the greatest transport through the cell monolayer. Additionally, binding and transport of the toxins were significantly elevated with an increase in the number of HA-33/HA-17 arms. These findings suggested that the HA-33/HA-17 arm in L-TCs is responsible for cell surface binding and subsequent transport through the cell monolayer. However, smaller TCs without an HA-33/HA-17 arm exhibited low but certain cell binding and cell monolayer transport. To identify the component responsible for the binding and transport of each TC, pure BoNT, M-TC, M-TC/HA-70 and three-arm L-TC were preincubated with one of the antibodies against their constituents before the binding or transport assay (Fig. 3). The binding and transport facilitated by pure BoNT were significantly inhibited by preincubation with anti-BoNT. Binding and transport by M-TC was significantly inhibited by preincubation with anti-BoNT, and slightly by anti-NTNHA treatment. As for the M-TC/HA-70, anti-BoNT and anti-HA-70 significantly reduced both the cell binding and the cell monolayer transport of the toxin, while anti-NTNHA had very little effect. Of the antibodies used for three-arm L-TC, the anti-HA-33 significantly interfered with both the cell binding and the monolayer transport. Because of the failure to isolate the HA-17 component under a mild denaturant condition (Kouguchi *et al.*, 2002), a polyclonal antibody could not be raised against HA-17. Therefore, the effect of anti-HA-17 on the binding and transport of L-TCs could not be examined in this study. However, the effect of anti-HA-33 and anti-HA-33/HA-17 on the assays was not significantly different.

Further examination of anti-HA-33 on cell binding and monolayer transport of immature L-TCs showed that anti-HA-33 was a significant inhibitor of the immature L-TC (one- and two-arm L-TC), comparable to the antibody inhibition of the three-arm L-TC (data not shown).

Sialic acid-dependent cell binding and monolayer transport of M-TC, M-TC/HA-70 and L-TCs

As demonstrated in a previous study, the cell binding and monolayer transport of the pure BoNT and three-arm L-TC produced by the serotype D strain are mediated in a sialic acid-dependent manner (Niwa *et al.*, 2010). Therefore, sialic acid-dependent binding and transport by other toxins (M-TC, M-TC/HA-70 and one-arm and two-arm L-TCs) were examined. Incubation with sialic acid (Neu5Ac) significantly reduced the cell binding and transport of toxins (Table 1). Treatment of IEC-6 cells with neuraminidase, which cleaves sialic acid, produced a significant decline in binding and transport.

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

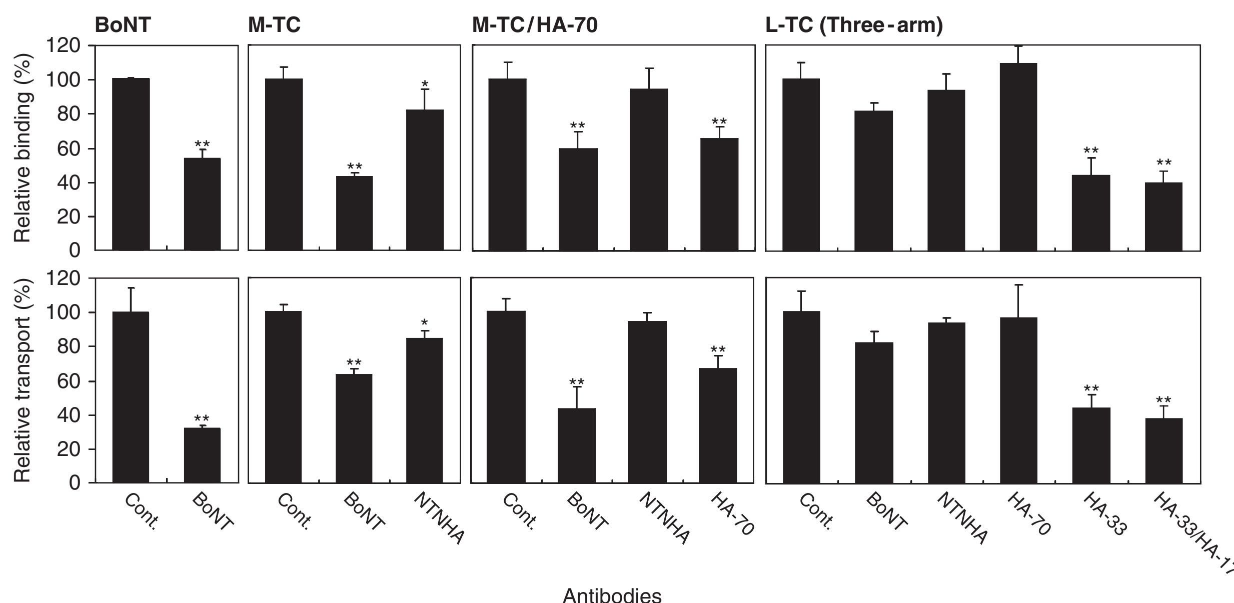

Fig. 3. Effect of antibodies against each TC component on the cell binding and monolayer transport of BoNT, M-TC, M-TC/HA-70 and three-arm L-TC. The toxins (40 nM) were preincubated with antibodies (diluted to 1 : 10 in incubation buffer) against BoNT, NTNHA, HA-70, HA-33 and HA-33/HA-17 for 1 h at 37 °C before cell binding (upper panels) and cell monolayer transport (lower panels) assays. Relative binding and transport were calculated (see Fig. 2) and compared with the control without antibody treatment. Experiments were repeated in triplicate, and the error bars represent SEMs. Columns with a single asterisk or double asterisk denote a significant decrease in binding or transport ($^{*}P < 0.05$; $^{**}P < 0.01$) compared with the control (Cont.) that was not preincubated with antibody. Statistical analyses were performed using the unpaired *t*-test for BoNT and nonrepeated measures ANOVA with a Bonferroni's correction for other toxins.

Table 1. Effects of *N*-acetylneuraminic acid and neuraminidase treatment on cell binding and cell transport of M-TC, M-TC/HA-70 and L-TCs

	M-TC		M-TC/HA-70		One-arm L-TC		Two-arm L-TC		Three-arm L-TC	
Treatments	Binding (%†)	Transport (%)	Binding (%)	Transport (%)	Binding (%)	Transport (%)	Binding (%)	Transport (%)	Binding (%)	Transport (%)
Control	100 ± 12.1	100 ± 6.6	100 ± 14.1	100 ± 4.4	100 ± 12.6	100 ± 18.3	100 ± 3.8	100 ± 8.3	100 ± 11.0	100 ± 16.3
Neu5Ac	68.0 ± 9.6*	55.6 ± 5.0**	63.4 ± 4.4*	65.7 ± 1.5**	39.8 ± 5.3**	51.5 ± 13.0*	52.8 ± 8.9**	59.8 ± 9.9**	55.1 ± 5.4**	61.0 ± 12.5*
NDase	46.8 ± 4.9**	66.3 ± 5.2**	53.1 ± 9.3**	51.9 ± 3.4**	34.3 ± 4.9**	40.9 ± 6.9**	66.6 ± 7.5**	48.2 ± 8.2**	41.0 ± 3.7**	61.7 ± 1.8*

†Percentages represent the ratios of cell binding and transport of TCs coincubated with *N*-acetylneuraminic acid (Neu5Ac) or neuraminidase (NDase)-pretreated cells compared with the control.

Experiments were repeated in triplicate. Values are the means ± SE of the percentages. The asterisk indicates a value that is significantly different ($^{*}P < 0.05$; $^{**}P < 0.01$) from the control.

Statistical analyses were performed using nonrepeated measures ANOVA with a Bonferroni's correction.

Discussion

Clostridium botulinum produces TCs of different sizes that consist of BoNT and auxiliary serotype-specific nontoxic proteins. Because the large TCs exhibit significantly higher oral toxicity than pure BoNT, the auxiliary nontoxic proteins have been considered essential for food poisoning (Sakaguchi *et al.*, 1984). Although their most accepted function is as a safeguard against the harsh conditions in the digestive tract (Halliwell, 1954; Ohishi *et al.*, 1977; Sugii *et al.*, 1977a, b; Bonventre, 1979; Ohishi & Sakaguchi, 1980; Ohishi, 1984; Chen *et al.*, 1998; Niwa *et al.*, 2007), the role of the nontoxic proteins is still under debate. There is some evidence that the HA components of auxiliary nontoxic proteins play a role in absorption across the intestinal epithelium (Fujinaga *et al.*, 1997, 2004; Nishikawa *et al.*, 2004; Uotsu *et al.*, 2006; Niwa *et al.*, 2007, 2010). However, some serotype A, E and F strains do not possess the genes that encode the HA components (Sakaguchi *et al.*, 1990; East *et al.*, 1996; Johnson & Bradshaw, 2001), implying that absorption of the botulinum toxin into intestinal epithelial cells does not depend on the presence of HA components.

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

Inhibition experiments using specific antibodies revealed the components responsible for cell binding and monolayer transport in an intestinal epithelial cell line. The binding and transport of pure BoNT, M-TC and M-TC/HA-70 were significantly inhibited by preincubation with anti-BoNT, implying that the BoNT in these toxins is predominantly responsible for cell binding and transport across the intestinal epithelia. At the neuromuscular junction, BoNT internalizes into nerve cells via a receptor-mediated process where glycosphingolipids (Montecucco & Schiavo, 1994; Li & Singh, 1999) and proteins such as synaptotagmin (Rossetto & Montecucco, 2007) serve as BoNT receptors. However, recent data indicate that serotype A BoNT transports across the intestinal epithelial cell monolayer via a process in which ganglyosides and the synaptic vesicle protein SV2 serve as dual receptors (Couesnon *et al.*, 2008). Although the receptor in the intestinal epithelial cells for serotype D BoNT is unknown at present, the transport of BoNT across the IEC-6 cell monolayer was inhibited by sialic acid (Niwa *et al.*, 2010), implying that serotype D BoNT might be transported across the intestinal epithelial cell monolayer through mediation of at least one sialic acid-dependent receptor.

Both anti-BoNT and anti-HA-70 significantly interfered with the cell binding and cell monolayer transport of M-TC/HA-70, implying the involvement of both BoNT and HA-70 components in binding and transport. The binding and transport of M-TC/HA-70 is also inhibited by the pretreatment of cells with sialic acid and neuraminidase. Because the binding and transport of BoNT is also inhibited by sialic acid and neuraminidase treatment (Niwa *et al.*, 2010), it was not possible to determine whether these inhibitions affected only BoNT mediation, only HA-70 or both. However, the HA-70 component in the serotype C toxin can interact with the terminal sialic acid moieties of a sugar chain (Fujinaga *et al.*, 1997, 2004). Additionally, Nakamura *et al.* (2009) demonstrated that serotype C HA-70 binds to sialic acid on mucin with high affinity. HA-70 produced by serotype D-4947 shares a high sequence similarity with serotype C HA-70; thus, the D-4947 HA-70 might interact with sialic acid-dependent receptor(s) on the cell surface.

In the three-arm L-TC, anti-BoNT and anti-HA-70 demonstrated no or weak interference with cell binding and transport. Transmission electron microscopy showed that the HA-33/HA-17 arms in L-TCs are exposed on the outermost surface of the complex (Hasegawa *et al.*, 2007). Therefore, HA-33/HA-17 arms might overlay the receptor recognition sites in BoNT and HA-70, which results in a decreased cell-binding ability. The HA-33 component in this complex could play a key role in cell binding and subsequent transport across the cell layer in a sialic acid-dependent manner, as demonstrated recently (Niwa *et al.*, 2010).

These results led to the conclusion that all serotype D TCs, including pure BoNT, can interact with putative sialic acid-dependent receptor(s) on intestinal epithelial cells through the BoNT, the HA-70 or the HA-33 component for transport across the cell monolayer. This idea is consistent with evidence showing that BoNT transport is independent of the presence of HA components (Sakaguchi *et al.*, 1990; East *et al.*, 1996; Maksymowych & Simpson, 1998, 2004; Maksymowych *et al.*, 1999; Johnson & Bradshaw, 2001). L-TCs with HA-33/HA-17 arms exhibited a greater binding and transport than smaller TCs (BoNT, M-TC and M-TC/HA-70) without any HA-33/HA-17 arm attached. The binding and transport of L-TCs are also markedly increased depending on the number of attached arms. These findings imply that the HA-33/HA-17 arm can facilitate the cell binding and the monolayer transport activities of the TCs. This idea is consistent with evidence that HA components play a role in the transport of botulinum toxin across the intestinal epithelium (Fujinaga *et al.*, 1997, 2004; Nishikawa *et al.*, 2004; Uotsu *et al.*, 2006; Niwa *et al.*, 2007, 2010). It appears that the HA components are not necessarily required for transport of the toxin through the intestinal epithelium, but the HA-33/HA-17 arm can facilitate passage of the toxin, with higher levels of translocation as the number of arms increase.

Passage of FITC-dextran (500 kDa) was also facilitated during transport of the L-TCs (one-, two- and three-arm L-TC) across the cell monolayer, but not during transport of pure BoNT, M-TC and M-TC/HA-70. This finding indicates that the presence of HA-33/HA-17 arms can facilitate the passage of large size molecules, other than toxins, through the cell layer. The HA component of serotype A and B complexes (L-TC, LL-TC and NTNHA/HA-70/HA-33/HA-17), with the exception of M-TC and pure BoNT, aids toxin transport by disrupting the paracellular barrier of human intestinal cell lines (Matsumura *et al.*, 2008; Jin *et al.*, 2009). In addition, the serotype C L-TC disrupts the cell barrier of the nonhuman epithelial cell lines MDCK-I, ACL-15 and RCN-9 (Jin *et al.*, 2009). After cell barrier disruption, all types of TCs can easily transport across the intestinal cell layer. However, in actual food-poisoning processes, L-TCs initially transport across the intestinal epithelium from the luminal to basolateral side without the HA mechanism due to the disrupting action of basolateral polarity (Matsumura *et al.*, 2008; Jin *et al.*, 2009). Sugawara *et al.* (2010) identified epithelial cadherin (E-cadherin) as a target molecule of serotype B HA. The HA basolaterally binds E-cadherin and disrupts E-cadherin-mediated cell–cell adhesion. In the human cell line Caco-2, both apically and basolaterally applied HA disrupts the cell barrier. Apically applied HA migrated to the basal surface through transcytosis and localized to the lateral cell membrane with E-cadherin (Sugawara *et al.*, 2010). In contrast, the canine cell line MDCK-I was

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

insensitive to apically applied HA but sensitive to basolaterally applied HA, implying that the presumptive apical receptor involved in initial transcytosis of the toxins before disruption of the basolateral barrier might differ based on the susceptibility of animal species to different serotypes of botulinum toxin (Sugawara *et al.*, 2010). HA-33/HA-17 arms facilitated cell binding in addition to transport through the cell layer. Zhou *et al.* (2005) isolated synaptotagmin II using a pull-down assay from the rat brain synaptosomal protein extract as a putative protein receptor for HA-33 on nerve cells. In future studies, pull-down assays using isolated HA-33 protein and intestinal epithelial cell extract could help identify the presumptive apical receptor involved in transport of the botulinum toxins. The ability of serotype D HAs to disrupt the basolateral cell barrier remains unclear and should be examined. Although the number of reports on the ability of botulinum HAs to disrupt the cell barrier is limited, the mode of action of serotype C HA, and its susceptible cells, are different from those of serotypes A and B (Jin *et al.*, 2009). Therefore, investigation of the morphological alterations and the mortality of various epithelial cell lines during the toxin translocation process of serotype D botulinum toxins is necessary.

Acknowledgements

The authors thank Michiru Kamiyoshihara, Natsuki Takahashi, Satoshi Kano, Masashi Muramatsu, Shin-Ichiro Miyashita and Yuhma Yoshida for technical assistance. This work was supported by grants-in-aid from the Japan Society for the Promotion of Science (No. 21580383 and No. 22590405).

References

Bonventre PF (1979) Absorption of botulinal toxin from the gastrointestinal tract. *Rev Infect Dis* **1**: 663–667.

Chen F, Kuziemko GM & Stevens RC (1998) Biophysical characterization of the stability of the 150-kilodalton botulinum toxin, the nontoxic component, and the 900-kilodalton botulinum toxin complex species. *Infect Immun* **66**: 2420–2425.

Couesnon A, Pereira Y & Popoff MR (2008) Receptor-mediated transcytosis of botulinum neurotoxin A through intestinal cell monolayers. *Cell Microbiol* **10**: 375–387.

East AK, Bhandari M, Stacey JM, Campbell KD & Collins MD (1996) Organization and phylogenetic interrelationships of genes encoding components of the botulinum toxin complex in proteolytic *Clostridium botulinum* types A, B, and F: evidence of chimeric sequences in the gene encoding the nontoxic nonhemagglutinin component. *Int J Syst Bacteriol* **46**: 1105–1112.

Fujinaga Y, Inoue K, Watanabe S, Yokota K, Hirai Y, Nagamachi E & Oguma K (1997) The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin. *Microbiology* **143**: 3841–3847.

Fujinaga Y, Inoue K, Watarai S *et al.* (2004) Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. *Microbiology* **150**: 1529–1538.

Halliwell G (1954) The action of proteolytic enzymes on *Clostridium botulinum* type A toxin. *Biochem J* **58**: 4–8.

Hasegawa K, Watanabe T, Sato H *et al.* (2004) Characterization of toxin complex produced by a unique strain of *Clostridium botulinum* serotype D 4947. *Protein J* **23**: 371–378.

Hasegawa K, Watanabe T, Suzuki T *et al.* (2007) A novel subunit structure of *Clostridium botulinum* serotype D toxin complex with three extended arms. *J Biol Chem* **282**: 24777–24783.

Inoue K, Fujinaga Y, Watanabe T, Ohyama T, Takeshi K, Moriishi K, Nakajima H, Inoue K & Oguma K (1996) Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect Immun* **64**: 1589–1594.

Inui K, Ito H, Miyata K, Matsuo T, Horiuchi R, Ikeda T, Watanabe T, Ohyama T & Niwa K (2010) Involvement of sialic acid in transport of serotype C1 botulinum toxins through rat intestinal epithelial cells. *J Vet Med Sci* **72**: 1251–1255.

Jin Y, Takegahara Y, Sugawara Y, Matsumura T & Fujinaga Y (2009) 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. *Microbiology* **155**: 35–45.

Johnson EA & Bradshaw M (2001) *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. *Toxicon* **39**: 1703–1722.

Kouguchi H, Watanabe T, Sagane Y, Sunagawa H & Ohyama T (2002) *In vitro* reconstitution of the *Clostridium botulinum* type D progenitor toxin. *J Biol Chem* **277**: 2650–2656.

Li L & Singh BR (1999) Structure–function relationship of clostridial neurotoxins. *J Toxicol Toxin Rev* **18**: 95–112.

Maksymowych AB & Simpson LL (1998) Binding and transcytosis of botulinum neurotoxin by polarized human colon carcinoma cells. *J Biol Chem* **273**: 21950–21957.

Maksymowych AB & Simpson LL (2004) Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. *J Pharmacol Exp Ther* **310**: 633–641.

Maksymowych AB, Reinhard M, Malizio CJ, Goodnough MC, Johnson EA & Simpson LL (1999) Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. *Infect Immun* **67**: 4708–4712.

Matsumura T, Jin Y, Kabumoto Y, Takegahara Y, Oguma K, Lencer WI & Fujinaga Y (2008) The HA proteins of botulinum toxin disrupt intestinal epithelial intercellular junctions to increase toxin absorption. *Cell Microbiol* **10**: 355–364.

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Montecucco C & Schiavo G (1994) Mechanism of action of tetanus and botulinum neurotoxins. *Mol Microbiol* **13**: 1–8.

Mutoh S, Kouguchi H, Sagane Y, Suzuki T, Hasegawa K, Watanabe T & Ohyama T (2003) Complete subunit structure of the *Clostridium botulinum* type D toxin complex via intermediate assembly with nontoxic components. *Biochemistry* **42**: 10991–10997.

Mutoh S, Suzuki T, Hasegawa K, Nakazawa Y, Kouguchi H, Sagane Y, Niwa K, Watanabe T & Ohyama T (2005) Four molecules of the 33 kDa haemagglutinin component of the *Clostridium botulinum* serotype C and D toxin complexes are required to aggregate erythrocytes. *Microbiology* **151**: 3847–3858.

Nakamura T, Kotani M, Tonozuka T, Ide A, Oguma K & Nishikawa A (2009) Crystal structure of the HA3 subcomponent of *Clostridium botulinum* type C progenitor toxin. *J Mol Biol* **385**: 1193–1206.

Nishikawa A, Uotsu N, Arimitsu H *et al*. (2004) The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells. *Biochem Bioph Res Co* **319**: 327–333.

Niwa K, Koyama K, Inoue S, Suzuki T, Hasegawa K, Watanabe T, Ikeda T & Ohyama T (2007) Role of nontoxic components of serotype D botulinum toxin complex in permeation through a Caco-2 cell monolayer, a model for intestinal epithelium. *FEMS Immunol Med Mic* **49**: 346–352.

Niwa K, Yoneyama T, Ito H *et al*. (2010) Sialic acid-dependent binding and transcytosis of serotype D botulinum neurotoxin and toxin complex in rat intestinal epithelial cells. *Vet Microbiol* **141**: 312–320.

Ohishi I (1984) Oral toxicities of *Clostridium botulinum* type A and B toxins from different strains. *Infect Immun* **43**: 487–490.

Ohishi I & Sakaguchi G (1980) Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. *Infect Immun* **28**: 303–309.

Ohishi I, Sugii S & Sakaguchi G (1977) Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. *Infect Immun* **16**: 107–109.

Rossetto O & Montecucco C (2007) Peculiar binding of botulinum neurotoxins. *ACS Chem Biol* **2**: 96–98.

Sakaguchi G, Kozaki S & Ohishi I (1984) Structure and function of *Clostridium botulinum* toxins. *Bacterial Protein Toxins* (Alouf JE, Fehrenbach FJ, Freer JH & Jeljaszewicz J, eds), pp. 435–443. Academic Press, London.

Sakaguchi G, Sakaguchi S, Kamata Y, Tabita K, Asao T & Kozaki S (1990) Distinct characters of *Clostridium botulinum* type A strains and their toxin associated with infant botulism in Japan. *Int J Food Microbiol* **11**: 231–241.

Sugawara Y, Matsumura T, Takegahara Y, Jin Y, Tsukasaki Y, Takeichi M & Fujinaga Y (2010) Botulinum hemagglutinin disrupts the intercellular epithelial barrier by directly binding E-cadherin. *J Cell Biol* **189**: 691–700.

Sugii S, Ohishi I & Sakaguchi G (1977a) Correlation between oral toxicity and *in vitro* stability of *Clostridium botulinum* type A and B toxins of different molecular sizes. *Infect Immun* **16**: 910–914.

Sugii S, Ohishi I & Sakaguchi G (1977b) Intestinal absorption of botulinum toxins of different molecular sizes in rats. *Infect Immun* **17**: 491–496.

Uotsu N, Nishikawa A, Watanabe T, Ohyama T, Tonozuka T, Sakano Y & Oguma K (2006) Cell internalization and traffic pathway of *Clostridium botulinum* type C neurotoxin in HT-29 cells. *Biochem Biophys Acta* **1763**: 120–128.

Zhou Y, Foss S, Lindo P, Sarkar H & Singh BR (2005) Hemagglutinin-33 of type A botulinum neurotoxin complex binds with synaptotagmin II. *FEBS J* **272**: 2717–2726.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Paracellular flux of FITC-dextran through the developing IEC-6 cell monolayer.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Downloaded from https://academic.oup.com/femspd/article/61/3/323/552641 by guest on 25 April 2024

© 2011 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved