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Sialic acid-dependent binding and transcytosis of serotype D botulinum neurotoxin and toxin complex in rat intestinal epithelial cells

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ABSTRACT

A large toxin complex (L-TC) produced by *Clostridium botulinum* is composed of neurotoxin (BoNT), non-toxic non-hemagglutinin (NTNHA) and hemagglutinin subcomponents (HA-70, -33 and -17). In animal botulism, BoNT or L-TC is internalized by intestinal epithelial cells. Previous studies showed that L-TC binds to intestinal cells via sugar chains on the cell surface, but the role of toxin binding to sugar chains in the toxin absorption from intestine is unclear. To clarify whether the toxin binding to sugar chains on intestinal cell surface leads to its transcytosis across the cells, we examined binding and permeation of BoNT and L-TC of *C. botulinum* serotype D strain 4947 to the rat intestinal epithelial cell line IEC-6 in semi-permeable filters in Transwell systems. Both BoNT and L-TC bound to and permeated the cell monolayers, with L-TC showing greater binding and permeation. In addition, both binding and permeation of toxins were potently inhibited by *N*-acetyl neuraminic acid in the cell culture medium or by treatment of the cells with neuraminidase. However, neither galactose, lactose nor *N*-acetyl galactosamine inhibited binding or permeation of toxins. These results support the idea that permeation of both BoNT and L-TC through the intestinal cell layer depends on prior binding to sialic acid on the cell surface. This is the first report demonstrating that the binding of botulinum toxins to cell surface sialic acid leads to their transcytosis through intestinal epithelial cells.

1. Introduction

Neurotoxin produced by *Clostridium botulinum* (BoNT) causes flaccid muscle paralysis in human and animals by preventing neurotransmitter release at nerve endings. Epidemiological studies have shown that BoNTs serotypes A, B, E, and F cause human botulism whereas serotypes C and D appear to be causative toxins for animal and avian botulism (Montecucco and Schiavo, 1994; Li and Singh, 1999). In naturally contaminated foods, BoNT (150 kDa) associates with non-toxic non-hemagglutinin (NTNHA; 130 kDa) and three types of hemagglutinin: HA-70, HA-33 and HA-17 (70, 33 and 17 kDa, respectively), forming a large toxin complex (L-TC; 750 kDa) (Inoue et al., 1996; Hasegawa et al., 2007). The first event in food-borne botulism is toxin absorption in the upper small intestine (Sugii et al., 1977; Sakaguchi, 1983; Maksymowych et al., 1999). Recent studies showed that L-TC permeates

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intestinal epithelial cell layers more effectively than BoNT alone, although BoNT alone can be internalized via the intestines and exert toxicity by oral inoculation (Niwa et al., 2007; Maksymowych and Simpson, 1998, 2004).

It has been shown that L-TC binds to sugar chains on the surface of intestinal epithelial cells and is subsequently internalized by the cells (Fujinaga et al., 2000; Nishikawa et al., 2004; Kojima et al., 2005). Thus it seems that the binding of L-TC to sugar chains is the first step for the absorption of L-TC. However, no study has demonstrated that the binding of L-TC to sugar chains leads to its permeation across the cells. Furthermore, the roles of sugar chains have never been examined in the context of the endocytosis and transcytosis of BoNT in intestinal epithelial cells. Accordingly, we investigated the relationship between the binding of serotype D toxins to sugar chains and their permeation through intestinal epithelia using rat intestinal epithelial IEC-6 cells.

2. Materials and methods

2.1. Production and purification of the botulinum toxins

C. botulinum serotype D strain 4947 (D-4947) was cultured anaerobically for 5 days using the dialysis tube method previously described (Hasegawa et al., 2004). The culture supernatant was fractionated with ammonium sulfate at 60% saturation and the resulting precipitate was dialyzed against 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl. The sample solution was applied to an SP-Toyopearl 650S (Tosoh, Tokyo, Japan) cation-exchange column (1.6 cm × 40 cm) equilibrated with dialysis buffer and was eluted with a linear gradient of NaCl (0.2–0.8 M). The fraction containing L-TC was pooled and precipitated with ammonium sulfate at 80% saturation. The precipitated L-TC was dissolved in 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and then applied to a HiLoad 16/60 Superdex 200 pg (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) gel-filtration column (1.6 cm × 60 cm) equilibrated with the same buffer. The L-TC fraction was further applied to a Mono S HR5/5 (GE Healthcare Bio-Sciences) cation-exchange column equilibrated with 50 mM acetate buffer (pH 5.0).

The separation of BoNT from L-TC was performed as previously described (Hasegawa et al., 2004) using a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with 20 mM Tris–HCl buffer (pH 8.8) containing 0.4 M NaCl. The BoNT fraction was applied to a Mono Q HR5/5 anion-exchange column (GE Healthcare Bio-Sciences) equilibrated with 20 mM Tris–HCl buffer (pH 7.8) and eluted with a linear gradient of NaCl (0–1.0 M).

For the isolation of the HA-33/HA-17 complex from L-TC, the concentrated L-TC fraction was mixed with 20 mM Tris–HCl buffer (pH 7.8) containing 4 M guanidine hydrochloride (Gdn buffer) for 4 h at 21 °C. The mixtures were applied to a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with the Gdn buffer, and the fraction corresponding to HA-33/HA-17 complex was dialyzed against 20 mM Tris–HCl buffer (pH 7.8).

2.2. Cell culture

The rat small intestine epithelial cell line IEC-6 was obtained from RIKEN BioResource Center (Tsukuba, Japan). Cells were grown in Dulbecco's modified Eagle medium (DMEM) 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 °C. Culture medium was renewed every 2–3 days.

2.3. Assay for toxin binding and permeability

For toxin binding assay, IEC-6 cells were prepared in 24-well dishes (Corning, NY, USA) 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 phosphate buffered saline (PBS), and lysed with 200 μl of SDS buffer. Proteins bound to cells were separated on SDS-PAGE and detected by Western blot.

For toxin permeability assay, cells were grown 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 volumes of culture medium in the inside and outside of the culture insert were 200 and 900 μl, respectively. Cells were cultured for 6 days to allow formation of tight connections. Toxins were suspended in 200 μl of DMEM containing 5% FBS and added to the inner chamber. Outer chamber was filled with 900 μl of DMEM containing 5% FBS. Cells were incubated in a CO_2 incubator for 1, 6, or 24 h. The culture medium (200 μl) in the outside of the inserts was collected and treated with 100 μl of 3× SDS sample buffer. Samples were then subjected to SDS-PAGE and Western blot.

2.4. Inhibition assay

To investigate the role of sugar chains on the toxin binding or permeation to the cells, cells were incubated with BoNT or L-TC and hapten sugars (β-D-lactose [Lac], β-D-galactose [Gal], *N*-acetyl galactosamine [GalNAc], or *N*-acetyl neuraminic acid [Neu5Ac]) during the binding or permeability assay.

Where indicated, cells were incubated with *Clostridium perfringens* neuraminidase (Sigma, St. Louis, MO, USA) at a final concentration of 16.7 mU/ml for 1, 2, and 18 h in a CO_2 incubator prior to the binding or permeation assay. The neuraminidase-treated cells were then washed three times with PBS and used for the assay. For toxin permeation assay, neuraminidase was concomitantly added to culture medium with toxins throughout the incubation time (24 h).

When the effects of antibodies on the binding or permeation of L-TC were examined, L-TC was mixed with antibodies in DMEM and incubated for 1 h at 37 °C prior to the binding or permeation assay.