NOTE *Bacteriology*

Involvement of Sialic Acid in Transport of Serotype C1 Botulinum Toxins through Rat Intestinal Epithelial Cells

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ABSTRACT. *Clostridium botulinum* produces a large toxin complex (L-TC) composed of neurotoxin (BoNT) and non-toxic proteins. In animal botulism, BoNT or L-TC is absorbed via the intestinal epithelium. To establish the cellular mechanisms of botulinum toxin absorption, we used cultured rat intestinal epithelial cells to test the binding and transport of serotype C1 BoNT and L-TC through the cell layers. BoNT and L-TC bound to and passed through the cell layers, with L-TC exhibiting larger binding and transport. Binding and transport of these toxins were inhibited by *N*-acetyl neuraminic acid or neuraminidase treatment of the cells. These results suggest that binding of serotype C1 BoNT and L-TC to sialic acid on the cells promoted their transport through intestinal epithelial cell layers.
KEY WORDS: botulinum neurotoxin, *Clostridium botulinum*, IEC-6, toxin absorption, toxin complex.

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Botulinum neurotoxin (BoNT) causes flaccid muscle paralysis in humans and animals by inhibiting neurotransmitter release at nerve endings. In recent years, sporadic and massive outbreaks of cattle botulism have occurred worldwide, causing large economic losses in dairy and beef cattle farming [4, 11, 15, 20]. Epidemiological studies have shown that BoNT serotypes A, B, E and F cause human botulism, whereas serotypes C1 and D appear to be causative toxins for animal and avian botulism [10, 13]. 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) [6, 7]. The first step in foodborne botulism is the trafficking of BoNT and/or L-TC from the intestinal epithelial cells to the bloodstream [19]. Recent studies have revealed that L-TC binds to sugar chains on the cell surface of intestinal epithelial cells [8, 16]. However, it is still unknown as to whether the binding of L-TC to the cells leads to its transport through the intestinal epithelia. Furthermore, the roles of sugar chains in transport of BoNT in intestinal epithelial cells are unclear. Thus, much remains to be elucidated regarding the mechanism of absorption of botulinum toxins, BoNT and L-TC, via intestinal epithelia.

Very recently, we provided the first evidence that the binding of serotype D BoNT and L-TC to sialic acid on rat intestinal epithelial cells facilitated their transport through the cell layers [17]. However, it is unknown whether both serotype C1 and D botulinum toxins that cause animal botulism are absorbed by animal intestines in a similar manner. In the present study, we examined the binding and transport properties of serotype C1 BoNT and L-TC using rat intestinal epithelial cells to determine whether absorption of serotype C1 botulinum toxins was dependent on sialic acid in a similar manner as serotype D toxins.

An anaerobic culture of *C. botulinum* serotype C strain Stockholm (C-St) and production of L-TC were performed as previously described [18]. Purification of L-TC from the culture supernatant and isolation of BoNT from L-TC were performed with sequential column chromatographic methods using an SP-Toyopearl 650 S cation exchange column (Tosoh, Tokyo, Japan) and a Mono Q anion exchange column (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.) [9, 18].

The rat intestinal cell line IEC-6 was obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. For the toxin binding tests, the cells in culture dishes were incubated with DMEM containing toxins for 1 hr at 4°C. After rinsing with phosphate buffered saline (PBS), the cells were lysed with sodium dodecyl sulfate (SDS) sample buffer without reducing agent and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Toxins bound to the cells were then detected by Western blot using a primary rabbit anti-BoNT antibody, which was made against BoNT serotype D strain 4947 (D-4947) [17], and a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). For the toxin transport tests, cells were cultured to confluence on a porous membrane of a Transwell two-chamber system (Corning, Corning, NY, U.S.A.). Toxins were added into culture medium in the upper chamber of the Transwell 2-chamber system. After incubating for 24 hr in a CO_2 incubator, the culture medium in the lower chamber was collected. The collected medium was mixed with 3 × SDS

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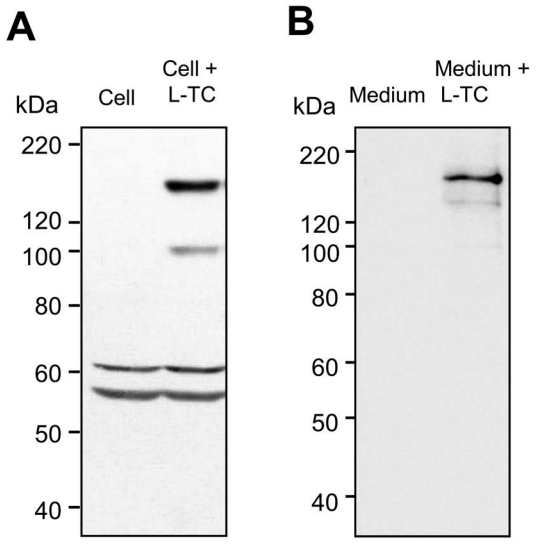

Fig. 1. Western blot analysis of the binding (A) and transport (B) of L-TC to IEC-6 cells. (A) Cells were incubated with L-TC at 100 nM for 1 hr at 4°C. BoNT in cell lysates was detected using an anti-BoNT antibody. (B) Cell monolayers were prepared in Transwell two-chamber systems. L-TC at 100 nM was added to culture medium on the apical side, and cells were incubated for 24 hr. BoNT in the culture medium on the basolateral side was detected using an anti-BoNT antibody.

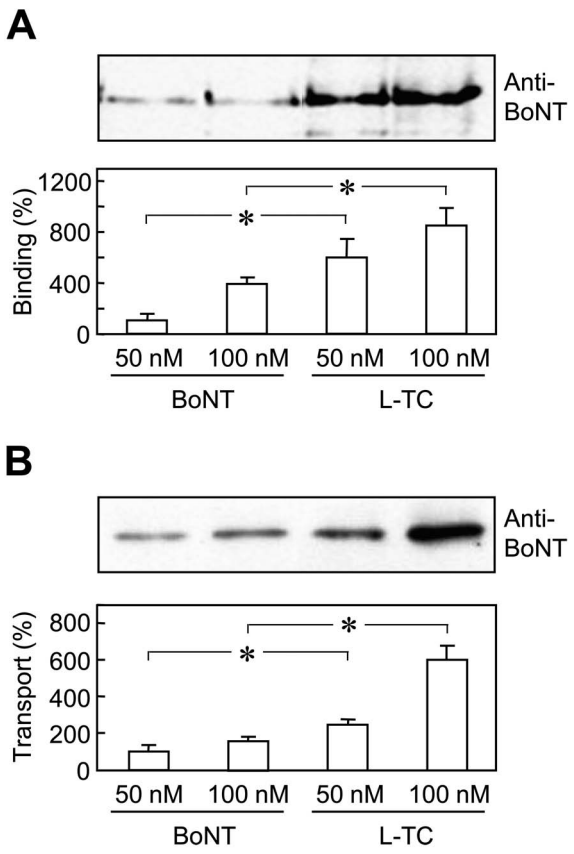

Fig. 2. Western blot analysis of the binding (A) and transport (B) of BoNT and L-TC to IEC-6 cells. Representative data (upper panel) and the calculated relative binding amount (lower plots) are shown. Band intensities were digitalized by densitometric analysis, and the amounts of binding or transport of BoNT when 50 nM BoNT was applied were set as 100%. (A) Cells were incubated with BoNT or L-TC for 1 hr at 4°C. BoNT in cell lysates was detected using an anti-BoNT antibody. (B) Cell monolayers were prepared in Transwell two-chamber systems. BoNT or L-TC was added to culture medium on the apical side, and cells were incubated for 24 hr. BoNT in the culture medium on the basolateral side was detected using an anti-BoNT antibody. Experiments were repeated 3 to 6 times, and error bars represent the SEM. Asterisks indicate significant differences ($P<0.05$; unpaired *t*-test).

sample buffer without reducing agent, and the samples were subjected to SDS-PAGE. Toxins that passed through the cell layer were detected by Western blot using a primary anti-BoNT antibody and a secondary HRP-conjugated goat anti-rabbit IgG antibody.

We first examined whether or not the anti-BoNT antibody [17] can detect C-St BoNT specifically. For this, L-TC was applied to a cell binding or transport test, and control samples (only cells or only DMEM) as well as L-TC-treated samples were subjected to SDS-PAGE and Western blotting using the anti-BoNT antibody. As shown in Fig. 1, BoNT was clearly identified as the band at 150 kDa in both the cell binding and transport tests, although several non-specific bands were observed. The band at 100 kDa may represent a heavy chain of BoNT that was dissociated from the intact BoNT molecule. Therefore, we used the band at 150 kDa as the indication of toxin binding and transport.

We examined the cell binding properties of BoNT and L-TC. As shown in Fig. 2A, both BoNT and L-TC bound to IEC-6 cell, and the binding of L-TC was 2- to 5-fold larger than that of BoNT. The properties of toxin transport through the IEC-6 cell layer were also examined. Both BoNT and L-TC passed through the IEC-6 cell monolayers, and L-TC exhibited greater levels of transport in a similar manner to that of the binding (Fig. 2B). These results were consistent with those of binding and transport of serotype D botulinum toxins to IEC-6 cells [17]. Thus, L-TC of serotypes C1 and D appeared to pass through the intestinal epithelial cell layer more effectively than BoNT.

Recently, we performed electron microscopic and X-ray crystallographic analyses for L-TC of D-4947 and reported that it has a unique ellipsoidal-shaped structure with three extended "arms" of the HA-33/HA-17 (2:1) complex [6]. We also demonstrated that two HA-33 molecules were located at the outermost region of each arm of D-4947 L-TC [6], and that the binding of L-TC to rat intestinal epithelial cells was largely dependent on its HA-33 molecules [17]. Since L-TCs of serotype C1 have similar biochemical and biophysical properties to D-4947 L-TC [14], they probably have an analogous 3D structure. Thus, the larger levels of

transport of L-TC through the intestinal cell layers, in comparison to the single BoNT molecule, may be due to the potent cell binding ability of HA-33 located at the outermost region of the L-TC. On the other hand, Matsumura *et al.* [12] showed that HA proteins of serotype A and B L-TC disrupt the barrier function of the monolayers of a human colon cancer-derived cell line, Caco-2 cells, leading to effective transport of L-TC. It still remains to be established whether HA proteins of serotype C and D have a similar disruptive effect on the intestinal barrier function of animal cells.

We next examined the role of sugar chains on the cell surface in the binding and transport of toxins. Figures. 3A and 3B shows the effect of addition of hapten sugars to the culture medium on the binding of BoNT and L-TC to the cells, respectively. Addition of galactose (Gal) and lactose (Lac) showed weak or no inhibitory effect on the binding of both BoNT and L-TC. In contrast, *N*-acetyl neuraminic acid (Neu5Ac), a major mammalian sialic acid, inhibited binding of both toxins to IEC-6 cells dose-dependently, suggesting that the sialic acid moiety of the cells contributed to BoNT and L-TC binding. To further confirm the involvement of sialic acid in toxin binding, the toxins were incubated with IEC-6 cells that were treated with neuraminidase that cleaves sialic acid. As expected, the binding of both BoNT and L-TC decreased depending on the neuraminidase concentration (Figs. 3C and 3D).

Finally, we tested whether sialic acid played an important role in the transport of toxins through cell layers. Figures. 4A and 4B show the effects of hapten sugars on BoNT and L-TC transport, respectively. Only Neu5Ac inhibited toxin transport, while Gal and Lac showed no such inhibition. Furthermore, as shown in Figs. 4C and 4D, neuraminidase-treatment of the cells dose-dependently abrogated the toxin transport. These results strongly suggest that both BoNT and L-TC were transported from the luminal to basolateral side of the intestinal epithelial cells following binding to sialic acid on the cell surface. Thus, sialic acid appears to be the primal target of serotype C1 BoNT and L-TC, facilitating their transport through the intestinal cell layers, and these results are consistent with previous studies on D-4947 BoNT and L-TC [17].

Nishikawa *et al.* [16] showed that binding of serotype C1 L-TC to human colon carcinoma HT-29 cells was decreased by neuraminidase treatment of the cells, suggesting that

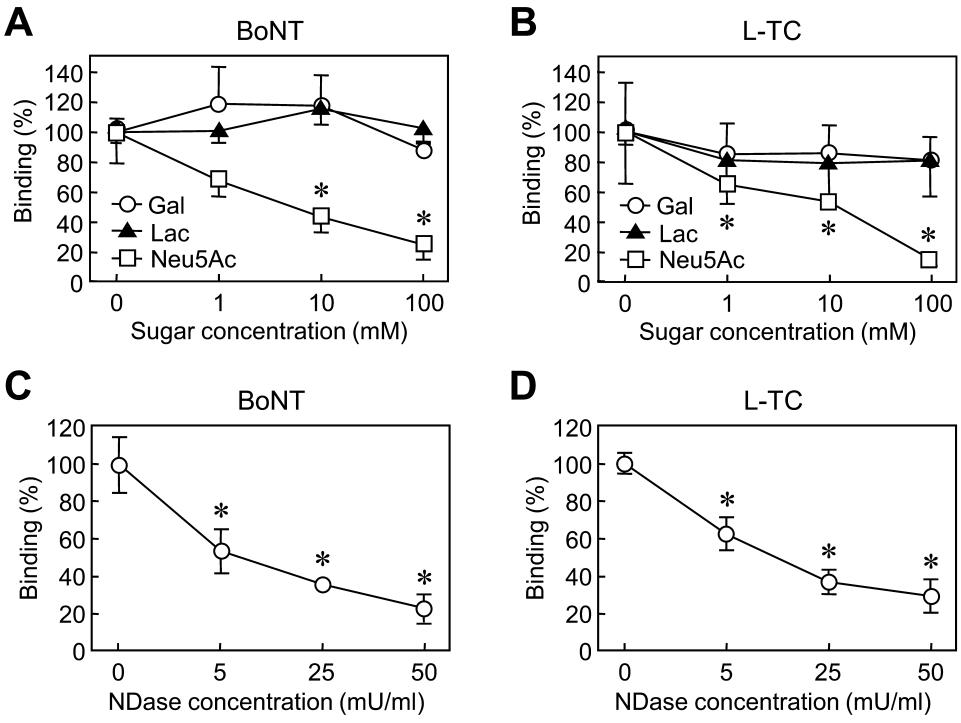

Fig. 3. Involvement of sugar chains in the binding of BoNT and L-TC to IEC-6 cells. (A and B) Effects of hapten sugars on the binding of BoNT (A) and L-TC (B) to IEC-6 cells. Cells were incubated with BoNT or L-TC at 100 nM in the presence of hapten sugars for 1 hr at 4°C. (C and D) Effects of neuraminidase (NDase) treatment on the binding of BoNT (C) and L-TC (D) to IEC-6 cells. Cells were preincubated with neuraminidase at the indicated concentrations for 18 hr in a CO_2 incubator and then incubated with BoNT or L-TC at 100 nM for 1 hr at 4°C. Band intensities obtained with Western blotting were digitalized by densitometric analysis, and the amounts of binding of toxins without hapten sugars or NDase treatment were set as 100% (control). Experiments were repeated 3 to 6 times, and error bars represent the SEM. Asterisks indicate significant differences from the control groups (P<0.05; non-repeated measures ANOVA followed by Bonferroni correction).

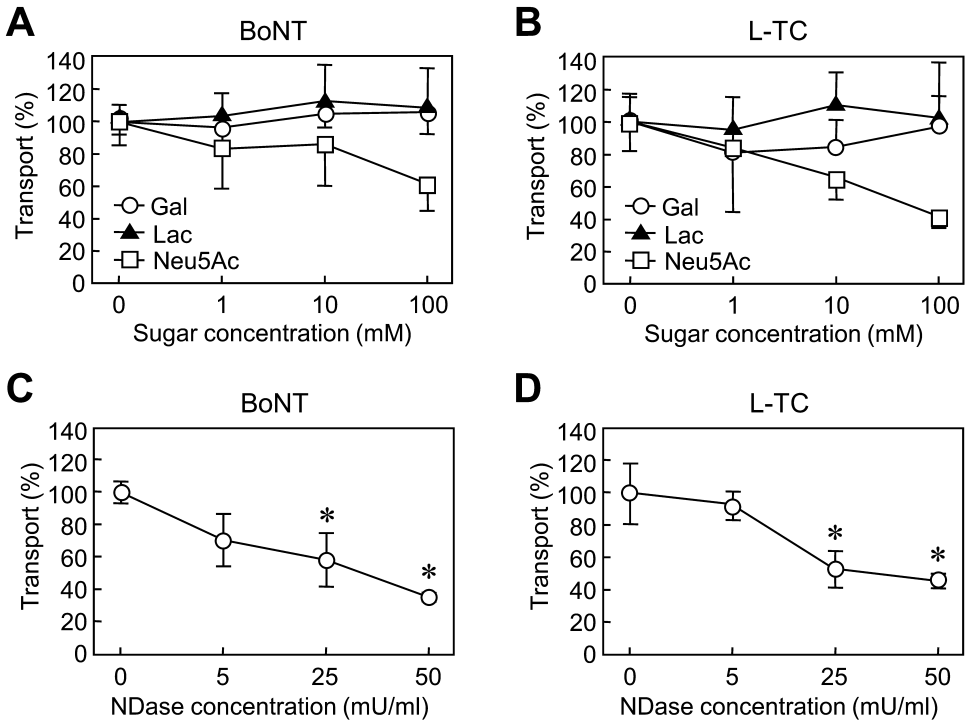

Fig. 4. Involvement of sugar chains in the transport of BoNT and L-TC through IEC-6 cells. (A and B) Effects of hapten sugars on the transport of BoNT (A) and L-TC (B) through IEC-6 cell monolayers. BoNT or L-TC at 100 nM and hapten sugars were added to culture medium in the upper chamber, and cells were incubated for 24 hr. (C and D) Effects of neuraminidase treatment on the transport of BoNT (C) and L-TC (D) through IEC-6 cell monolayers. Cells were preincubated with neuraminidase (NDase) at the indicated concentrations for 18 hr in a CO_2 incubator. BoNT or L-TC at 100 nM was added to the culture medium on the apical side, and cells were incubated for 24 hr in the presence of NDase. Band intensities obtained with Western blotting were digitalized by densitometric analysis, and the amounts of transport of toxins without hapten sugars or NDase treatment were set as 100% (control). Experiments were repeated 3 to 6 times, and error bars represent the SEM. Asterisks indicate significant differences from the control groups ($P<0.05$; non-repeated measures ANOVA followed by Bonferroni correction).

sialic acid was responsible for L-TC binding. However, they did not show whether the toxin binding to sialic acid promoted its transport through the cell layer. Additionally, human cell lines may be inappropriate for the investigation of the absorption mechanisms of serotype C1 and D botulinum toxins because humans are not a natural host for these toxins, while these toxins are toxic in animals such as cattle, rodents and birds [3, 5, 19]. Our present and previous studies [17] successfully demonstrated that binding of serotypes C1 and D botulinum toxins to sialic acid on the cell surface directly led to their transport through the intestinal epithelial cell layers of rats, a natural host of these toxins.

The recent increase in cattle botulism is now a worldwide problem that involves both economic loss as well as zoonotic potential [1]. Although the action of BoNT on nerve cells has been intensively investigated, much remains to be elucidated regarding the mechanisms of invasion of BoNT and L-TC in animals. Additionally, the effect of the toxin in cells or organs that do not fall within the nervous system is still unclear. For example, Böhnel *et al.* [2] reported that ten out of 60 tonsils of cattle that had died of botulism contained botulinum toxin, but the mechanisms by which the toxin invaded the tonsils remain unknown. The present results may provide new insight into the mechanisms involved in animal botulism. Further experiments to clarify intracellular events responsible for intestinal absorption of botulinum toxins are in progress in our laboratory.

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