Mechanisms of intestinal absorption of botulinum neurotoxin complex

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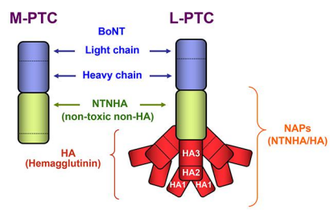
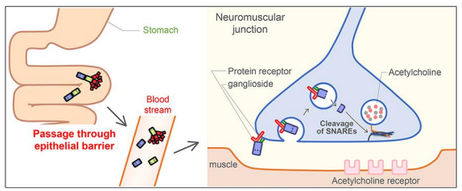
1 Abstract

Introduction: Botulinum neurotoxins (BoNTs) are produced along with one or more neurotoxin-associated proteins (NAPs) that non-covalently associate with BoNT to form progenitor toxin complexes (PTCs). The NAPs include non-toxic non-hemagglutinin (NTNHA) and hemagglutinin (HA), and these are known to markedly increase the oral toxicity of BoNT. To cause food-borne botulism, BoNT in the gastrointestinal lumen must traverse the intestinal epithelial barrier. The invasion site(s) and mechanism of BoNT *in vivo* were largely unknown. We have uncovered the site(s) and mechanism of intestinal translocation of the type A1 BoNT (BoNT/A1) complex *in vivo*.
Methods: Fluorescently labeled toxins, NTNHA/HA, HA subcomponents, reconstituted HA and PTCs were injected into ligated mouse intestinal loops containing Peyer's Patches (PPs), or orally administrated. After incubation for several hours, PPs were excised from intestines. Whole-mount specimens were stained and observed by confocal microscopy.
Results: L-PTC (a complex of neurotoxin, NTNHA and HA), which makes the predominant contribution to causing illness because of it's highest oral toxicity, binds to microfold (M) cells in the follicle-associated epithelium (FAE) of mouse PPs, and is transported to their basolateral sides via the interaction of HA in L-PTC with glycoprotein 2 (GP2) on the M-cell surface, which do not have thick mucus layers. Susceptibility to orally administered L-PTC was dramatically reduced in M cell–depleted mice and GP2-deficient (*Gp2*$^{-/-}$) mice.
Conclusions: Type A1 L-PTC invades the host through intestinal M cells by using GP2 on the apical surface of M cells as a transcytotic receptor, and this process is mediated by a non-toxic protein, HA. (**Matsumura T. *et al.*, *Nat Commun.* 2015**)

2 *Clostridium botulinum* toxins and food-borne botulism

Botulinum neurotoxin (BoNT) complex





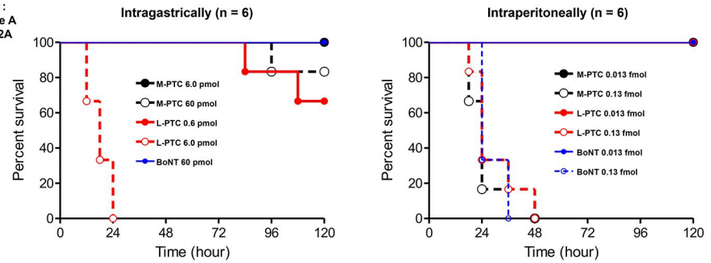
The site(s) and mechanism by which ingested BoNT invades the host remain unclear.

How dose botulinum neurotoxin complex cross the epithelial barrier?

BoNTs are produced by *Clostridium botulinum* and related species, along with one or more neurotoxin-associated proteins (NAPs) that non-covalently associate with BoNT to form toxin complexes (TCs, also called progenitor toxins). The NAPs include non-toxic non-hemagglutinin (NTNHA) and hemagglutinin (HA). HA is composed of three different subcomponents: HA1, HA2, and HA3. *C. botulinum* type A1 strains produce HA-negative TC (M-PTC) and HA-positive TC (L-PTC) simultaneously. M-PTC contains BoNT and NTNHA, whereas L-PTC consists of BoNT, NTNHA, and HA.

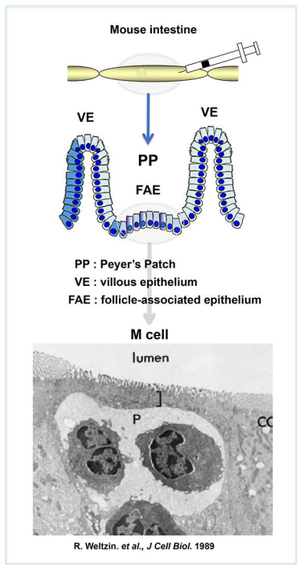
3 Type A1 L-PTC exhibits highly oral toxicity than M-PTC

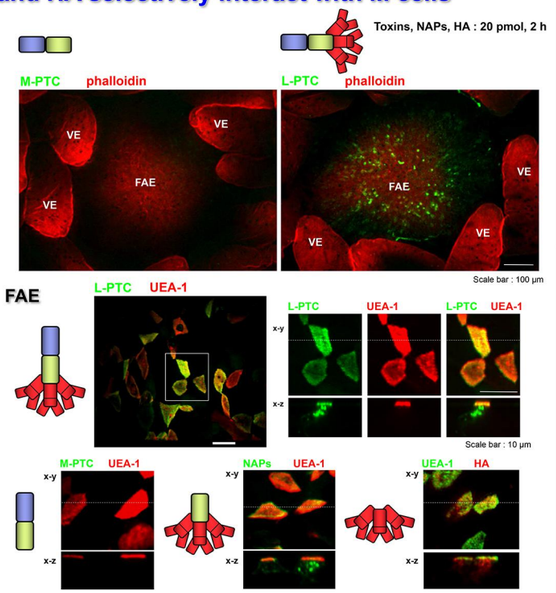
Toxins : from type A strain 62A

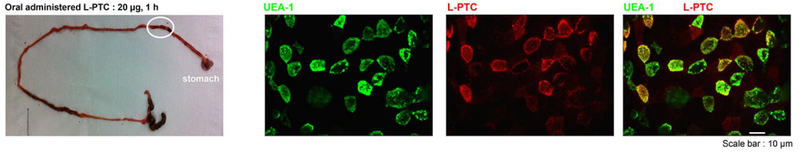


To compare the toxicities of type A1 M-PTC and L-PTC, we intragastrically (M-PTC 6.0 pmol: 1.72 μg, 60 pmol: 17.2 μg, L-PTC 0.6 pmol: 0.45 μg, 6 pmol: 4.5 μg, BoNT 60 pmol: 9.0 μg) or intraperitoneally (M-PTC 0.013 fmol: 3.85 pg, 0.13 fmol: 38.5 pg, L-PTC 0.013 fmol: 10 pg, 0.13 fmol: 100 pg, BoNT 0.013 fmol: 2.01 pg, 0.13 fmol: 20.1 pg) administered each toxin to mice. When intragastrically administered, the toxicity of L-PTC was highest, approximately two orders of magnitude greater than that of M-PTC, and M-PTC was more toxic than BoNT alone. By contrast, there was no significant difference among the toxicities of the intraperitoneally administered toxins. These results indicate that L-PTC makes the predominant contribution to the onset of food-borne botulism.

4 L-PTC, NAPs and HA selectively interact with M cells

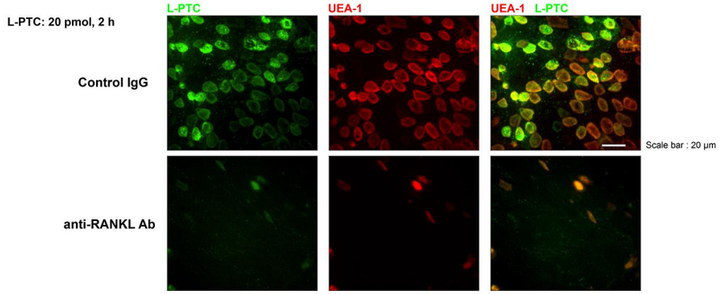


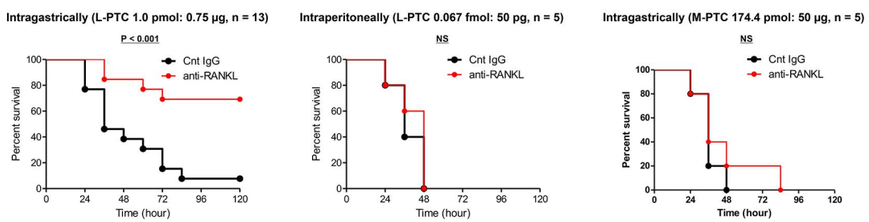




To determine the site(s) responsible for intestinal translocation of the toxin, we assessed localization of TCs by *in situ* intestinal loop assays in mouse. Alexa 488 or 568-labeled L-PTC, M-PTC, NAPs, or HA (20 pmol) were injected into mouse ligated loop, and whole-mount intestinal FAE region were stained with Rhodamine-labeled phalloidin or FITC- or Rhodamine-labeled UEA-1 (M cell marker). As the results, L-PTC was selectively localized at the follicle associated epithelium (FAE) covering Peyer's patches (PPs), whereas M-PTC exhibited no such clear localization to any sites in the intestinal tissue. These data imply that L-PTC binds to, and is internalized by, specific cells present in the FAE.
L-PTC, its NAPs (a complex of NTNHA/HA), and HA bound to the UEA-1+ M cells and were then transported to their basolateral sides. By contrast, the M-PTC exhibited minimal interaction with M cells. Thus, HA is the critical factor in the interaction with M cells.
On the other hand, L-PTC were taken up by M cells in duodenum ~ jejunum region 1 h after oral administration.

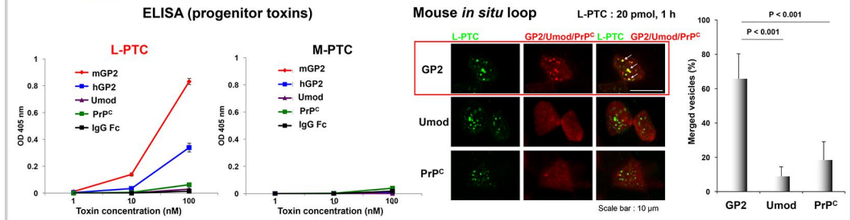
5 M cells are the major sites at which L-PTC breaches the intestinal epithelial barrier

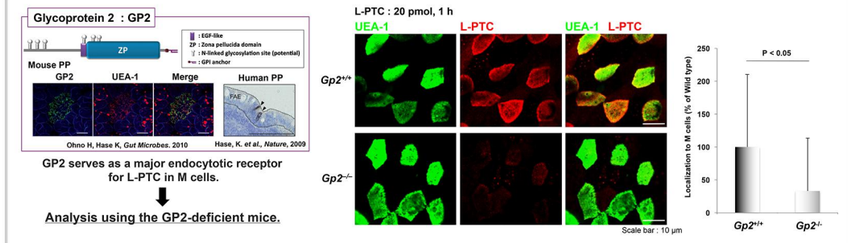


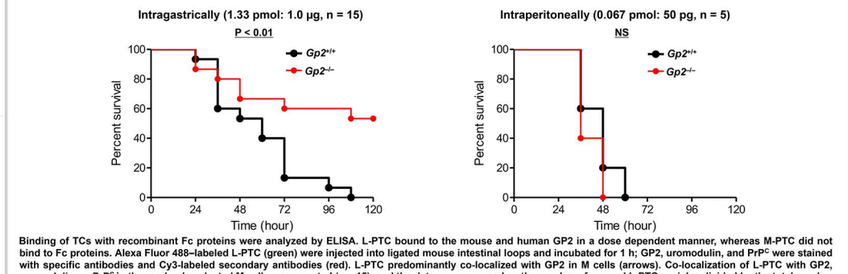


Signaling via the TNF superfamily member receptor activator of NF-kB ligand (RANKL) and receptor activator of NF-kB (RANK) plays a key role in the differentiation of M cells, which can be transiently depleted by RANKL neutralization. BALB/c mice were treated i.p. with 250 μg of anti-RANKL antibody or an isotype control rat IgG on days 0, 2, 4, and 6. On day 8, L-PTC were administrated to each mice, and mice were observed for morbidity and mortality. Statistical analyses were performed with the log-rank test. NS, not significant. Furthermore, the localization of L-PTC was analyzed in anti-RANKL Ab treated mouse. In mice treated with anti-RANKL Ab, the number of M cells was drastically reduced relative to control mice, and this reduction was accompanied by loss of localization of L-PTC at FAE. Correspondingly, susceptibility to orally administered L-PTC was significantly reduced in the mice treated with anti-RANKL Ab. By contrast, M-cell depletion did not influence lethality upon systemic challenge with L-PTC and oral administration of M-PTC. These results provide evidence that M cells make a significant contribution as a major portal for L-PTC.

6 L-PTC is taken up by M cells via the GP2-HA interaction



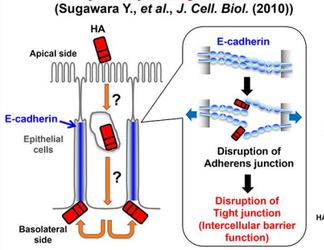


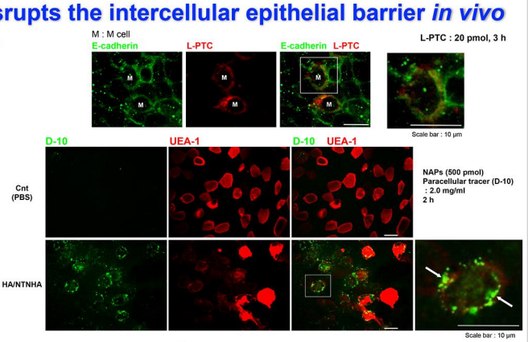


Binding of TCs with recombinant Fc proteins were analyzed by ELISA. L-PTC bound to the mouse and human GP2 in a dose dependent manner, whereas M-PTC did not bind to Fc proteins. Alexa Fluor 488–labeled L-PTC (green) were injected into ligated mouse intestinal loops and incubated for 1 h; GP2, uromodulin, and PrP^C were stained with specific antibodies and Cy3-labeled secondary antibodies (red). L-PTC predominantly co-localized with GP2 in M cells (arrows). Co-localization of L-PTC with GP2, uromodulin, or PrP^C in the randomly selected M cells was counted (n = 15), and the data are expressed as the number of merged L-PTC vesicles divided by the total number of L-PTC vesicles. Error bars indicate s.d. Statistical analyses were performed with the Student's t-test.
Alexa Fluor 568-labeled L-PTC (red) were injected into ligated mouse intestinal loops and incubated for 1 h. PPs removed from *Gp2*$^{+/+}$ or *Gp2*$^{-/-}$ mice were incubated with FITC-labeled UEA-1 (green). Images from FAE regions were analyzed quantitatively using the MetaMorph software. The data are expressed as percentages of the level in *Gp2*$^{+/+}$ mice. Error bars indicate s.d. For each group, six different PPs obtained from three different mice were examined (*Gp2*$^{+/+}$, 127 cells; *Gp2*$^{-/-}$, 161 cells). Statistical analyses were performed with the Mann–Whitney U-test. *Gp2*$^{-/-}$ mice exhibited significantly lower susceptibility to orally administered L-PTC than wild-type (*Gp2*$^{+/+}$) mice. *Gp2*$^{+/+}$ or *Gp2*$^{-/-}$ mice were inoculated intragastrically (1.33 pmol: 1.0 μg) or intraperitoneally (0.067 fmol: 50 pg) with L-PTC (intragastrically, n = 15 per group; intraperitoneally, n = 5 per group). Statistical analyses were performed with the log-rank test. NS, not significant. Consistent with this, uptake of L-PTC by M cells was significantly reduced in *Gp2*$^{-/-}$ mice. By contrast, there was no significant difference between *Gp2*$^{+/+}$ and *Gp2*$^{-/-}$ mice in susceptibility to intraperitoneally administered L-PTC. Taken together, these results indicate that L-PTC is taken up by M cells via the GP2–HA interaction.

7 NAPs (HA/NTNHA) disrupts the intercellular epithelial barrier *in vivo*

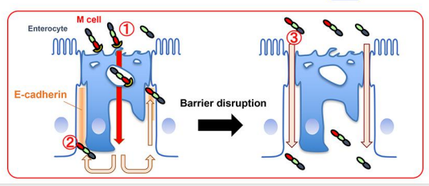
HA disrupts the intercellular epithelial barrier by directly binding E-cadherin.
(Sugawara Y., *et al., J. Cell. Biol.* (2010))





Alexa Fluor 568-labeled L-PTC (red) were injected into ligated mouse intestinal loops and incubated for 3 h, and whole-mount intestinal FAE region were stained with E-cadherin. Right panel shows a higher-magnification image of the boxed region. L-PTC were co-localized with E-cadherin at basolateral side around M cells.
NAPs (NTNHA/HA) and paracellular tracer (fixable FITC–dextran 10K; D-10, green) or vehicle control (PBS) and the tracer were injected into ligated mouse intestinal loops and incubated for 2 h, and FAE regions were stained with rhodamine-labeled UEA-1 (red). Right panel shows a higher-magnification image of the boxed region. Paracellular tracer penetrated between M cells and enterocytes (arrows).

8 Conclusion



★ We propose a three-step mechanism by which the type A BoNT complex breaches the intestinal epithelial barrier.
1, Apically located L-PTC (HA) translocates via M cells without disrupting the paracellular barrier.
2, Once located in the basolateral side, the HA of L-PTC disrupts the paracellular barrier.
3, A large amount of toxins accumulates in the basolateral area via paracellular movement.

Matsumura T., *et al.*, *Nat Commun.* (2015)
Sugawara Y., *et al., PLOS ONE* (2014)
Sugawara Y., *et al., J. Cell. Biol.* (2010)
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