Botulinum Toxin Accessory Proteins: Are They Just an Accessory?

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BACKGROUND Botulinum neurotoxins produced by *Clostridium botulinum* consist of a complex of a core neurotoxin protein and one or more nontoxin accessory proteins. The accessory proteins are generally thought to protect the neurotoxin from the gastric environment in botulism poisoning, dissociating away upon absorption. Other than their questionable immunogenicity, they are rarely mentioned in botulinum toxin therapy.

OBJECTIVE To review evidence that accessory proteins potentially play a role in neurotoxin activity.

RESULTS Evidence suggests that the accessory proteins do not dissociate from the neurotoxin complex and enhance neurotoxin activity. Complexed type A botulinum toxin has dramatically higher endopeptidase activity than noncomplexed neurotoxin. A primary accessory protein, hemagglutinin-33, exhibits this same effect on both type A and type E core neurotoxin proteins, the latter not natively having this accessory protein. A clinical study using an objective computer assessment assay has shown a correlation between type A complex size and glabellar strain reduction, which reflects increasing clinical efficacy. Finally, a systematic review found no correlation between type A complex size and neutralizing antibody formation.

CONCLUSION Accessory proteins may play a role in the efficacy of botulinum toxin and could remain complexed to the neurotoxin for longer than previously reported.

Botulinum Toxin Structure

Botulinum neurotoxin (BoNT) is synthesized by *Clostridium botulinum* bacteria. The core neurotoxin component is a ~150 kDa protein. The bacteria also synthesize nontoxin accessory proteins (NAPs) that form a complex with the core neurotoxin protein (Table 1). All type A *C. botulinum* strains produce NAPs of varying sizes, resulting in overall complexes ranging from ~300 to ~900 kDa in size (Figure 1).[1]

In the United States (US), there are currently 5 BoNT type A (BoNT/A) formulations approved for use that have varying molecular weights. The complexes of prabotulinumtoxinA-xvfs (PraA) and onabotulinumtoxinA (OnaA) are ~900 kDa due to their full complement of NAPs. IncobulinumtoxinA (IncA) and daxibotulinumtoxinA (DaxA) are at the other end of the spectrum, consisting of only the 150 kDa neurotoxin protein. AbobotulinumtoxinA (AboA) is believed to contain an intermediate number of accessory proteins; its complex size is often quoted as ~500 kDa, but its actual molecular weight has not been disclosed by the manufacturer.[1,2]

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The details regarding manufacturing are not in the public domain. All commercially available BoNTs are synthesized by *C. botulinum* strains that make a complexed neurotoxin.[1] PrabotulinumtoxinA-xvfs and OnaA have the full 900 kDa structure that is preserved and in the final product.[3,4] AbobotulinumtoxinA does not disclose the full size of the molecule that their strain produces, whereas both DaxA and IncA remove the accessory proteins as part of their manufacturing process.[1,2,5–7]

Botulinum Toxin Mechanism of Action

Discussions around the mechanism of action of neurotoxins tend to revolve around the 150 kDa structure, which is composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC). The role of the HC is to facilitate entry of the toxin into neuronal cell through endocytosis and the LC is a Zn^{+2}-dependent endopeptidase that cleaves synaptosomal-associated protein of 25 kDa (SNAP25) after its release into the cell cytosol.[8] Once SNAP25 is cleaved, acetylcholine vesicles in the presynaptic neuron are unable to dock and release neurotransmitter into the neuromuscular junction, resulting in the inhibition of muscular contraction and relaxation of muscle. The reduction of the disulfide bond between the HC and LC in the cytosol is essential to the activation of the endopeptidase activity, perhaps leading to secondary protein unfolding and exposure of the proteolytic site.[9]

Systemic botulism most commonly occurs by the ingestion of food contaminated with *C. botulinum*. It is generally accepted that the NAPs shield the 150 kDa neurotoxin protein from the hostile gastric environment, in which the neurotoxin protein is susceptible to digestive

TABLE 1. Molecular Weights of Commercial Botulinum Toxin Products in the United States

Generic Name	Brand Name	Molecular Weight (kD)
IncobotulinumtoxinA	Xeomin	150
DaxibotulinumtoxinA	Daxxify	150
AbotulinumtoxinA	Dysport	<500*
OnabotulinumtoxinA	Botox	900
PrabotulinumtoxinA	Jeuveau	900

* Estimated based on various reports as the molecular weight is not reported by the manufacturer.[1]
AboA, abobotulinumtoxinA; DaxA, daxibotulinumtoxinA; IncA, incobotulinumtoxinA; OnaA, onabotulinumtoxinA; PraA, prabotulinumtoxinA.

proteases and a pH < 3. Once in the intestines, the NAPs may also interact with intestinal epithelial cells to assist in transporting the neurotoxin protein into the circulation, resulting in botulism.[8]

Dissociation of Accessory Proteins

Whether the NAPs dissociate from the neurotoxin protein is a controversial topic, particularly in the context of injection into a muscle or subcutaneous tissue for treatment.[1] Some report that BoNT complexes are most stable at a pH of less than 6.25 and that NAPs dissociate at neutral pH levels or higher.[10,11] Studies in laboratory preparations of BoNT/A (900 kDa complex) have shown dissociation of the NAPs in less than 1 minute at a pH over 7.0, suggesting that the complex dissociates after reconstitution and possibly before it is even injected into tissue.[1] However, other studies have found the exact opposite, suggesting that the complex remains intact at physiologic pH levels.[1,9] It is important to note that studies are performed in artificial environments using unformulated BoNTs that were purified with varying methods and without any of the excipients/stabilizers that are found in the final commercialized product formulations.

Support for the stability of the complex across a wide pH range can be found in the manufacturing of PraA. The PraA 900 kDa complex is exposed in solution to a wide range of pH levels, ranging from 3.2 to 6.8, as it undergoes the manufacturing process that involves various steps, including fermentation, crystal precipitation, purification, water removal, and final vacuum drying. Despite this journey, and across a wide range of pH exposure in solutions, the final product remains as an intact 900 kDa complex. Internal testing has taken the pH as high as 8.0% and 88% of the 900 kDa structure remained intact (Evolus, data on file).

Do Accessory Proteins Have a Role in Botulinum Therapy?

In 1999, the first research was published suggesting that NAPs have a role beyond gastrointestinal protection and may potentiate the endopeptidase activity of BoNT/A.

The study measured BoNT/A activity using both in vitro and in vivo assays against SNAP25. As expected, under nonreducing conditions, free BoNT/A demonstrated very little activity, averaging 4% SNAP25 cleavage. In contrast and unexpectedly, BoNT/A complexed with NAPs was very active, with an average cleavage of 66% (16.5-fold greater). Similar results were demonstrated using the in vivo bovine brain synaptosome SNAP25 assay. Under reducing conditions, free BoNT/A averaged 18% cleavage, while the BoNT/A complex cleaved 80% (fourfold greater). The researchers concluded that NAPs play an active role in

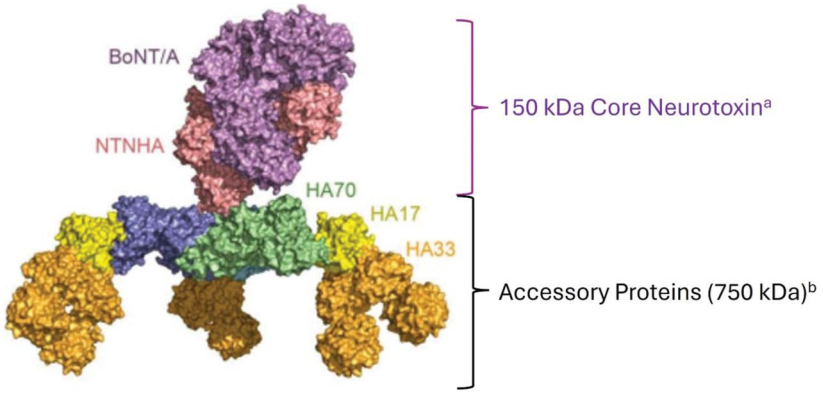

Figure 1. Structure of the botulinum toxin type A complex. [a]Composed of the 50 kDa light chain and the 100 kDa HC. [b]The accessory proteins are the proteins in the BoNT/A complex other than the core neurotoxin and include NTNHA, HA70 (HA50 + HA20), HA17, and HA33 (i.e., Hn-33). BoNT/A, botulinum neurotoxin type A; HA/Hn, hemagglutinin; HC, heavy chain; L-PTC/A, large progenitor toxin complex type A; NTNHA, nontoxic nonhemagglutinin. This figure was adapted from the stereo view of L-PTC/A in surface representation as published in Lee and colleagues (2013) (available at: https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1003690#s5). The figure is licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

BoNT/A activity, rather than a role limited solely to gastrointestinal protection of the toxin, and that the enhanced protease activity of the BoNT/A complex was likely a result of a conformational interaction between the BoNT/A and NAPs. Also of interest, the investigators noted that the BoNT/A complex remained intact and did not dissociate during the experimental conditions.[9]

Another study focused on the NAP hemagglutinin-33 (Hn-33). Hn-33 represents the largest fraction (25%) among the NAPs in BoNT/A, but interestingly it is not found in BoNT type E (BoNT/E). The study described a series of experiments testing the in vitro and in vivo endopeptidase activities of both BoNT/A and BoNT/E as the free neurotoxins and with Hn-33 added.[12] Similar to the previous study,[9] both BoNT/A and BoNT/E demonstrated virtually no activity (4% and 2% cleavage, respectively) under nonreducing conditions, but increased 21-fold (85% cleavage) and 25-fold (50% cleavage), respectively, when preincubated with Hn-33.[12] Under reducing conditions, free BoNT/A and BoNT/E had more enzymatic activity (22% and 10% cleavage, respectively). Preincubation with Hn-33 once again dramatically increased the amount of SNAP25 cleavage by 4.5-fold (99% cleavage) for BoNT/A and 8.5-fold (85% cleavage) for BoNT/E and in half of the standard 30-minute assay reaction time. A similar pattern occurred when using an in vivo rat brain synaptosome SNAP25 assay, although the cleavage values were lower. This likely reflects the fact that the neurotoxin complex needs to be taken up into the synaptosomes and, thus, has more limited access to SNAP25 than in the in vitro assay.[12]

The researchers suggested that the increase in the endopeptidase activity in the presence of Hn-33 may be a result of Hn-33 interacting with BoNT/A to expose the active site, which is occluded by a 56-amino acid residue belt in its nonreduced form. Botulinum neurotoxin/E is a BoNT that targets a different cleavage point and has different kinetics, with a shorter onset and duration, than BoNT/A. Despite not having an NAP comparable to Hn-33 in its complex, thus being foreign to BoNT/E, it is worthy to note that Hn-33 had an impact on increasing the activity of BoNT/E.[2,12]

Clinical Data Correlation

These bench top data suggest that the molecular weight of a BoNT/A complex has a role in its efficacy, but is there a clinical correlation between clinical efficacy and molecular weight? Comparisons could be drawn across the various toxins, but most studies are not head-to-head, have various limitations, and can demonstrate conflicting relative results.

One study of interest, which was performed without industry support, compared the relative performance of 3 commercial BoNT/A formulations representing the spectrum of molecular weights (150, 900 kDa, and in-between) to treat glabellar lines. This study assessed toxin performance using a sensitive computer assessment assay measuring Dynamic Strain Reduction (DSR) in the glabellar area. The act of frowning is a form of strain at the glabellar complex. As BoNT takes effect, the muscles relax and there is less strain and this decrease in strain corresponds with an increase in clinical efficacy; therefore, measuring DSR is another way of assessing glabellar lines at maximum frown. This method is unbiased by human interpretation and represents a continuous (as opposed to dichotomous) measurement that has more sensitivity. In a technique known as speckle-tracking photogrammetry, DSR assessment involves speckling of the subject's forehead and measuring the relative distance between speckles after activation of the underlying muscles using a camera that captures the dynamic contractions. The data are then entered into a computer and the average strain reduction, or relaxation, is calculated.[13]

A total of 75 patients were randomized to 1 of 3 toxin groups, representing the spectrum of BoNT/A molecular weights. Treatment of the glabellar region was with either 20 U OnaA (900 kDa), 20 U of IncA (150 kDa), or 60 U of AboA (intermediate weight). Of note, 60 U of AboA represents 20% more drug than approved for the glabellar indication. After baseline was established, patients returned for DSR assessment on Days 4, 14, and 90 after injection. At Day 4, the 900 kDa group had the greatest strain reduction (42.1%) compared with the intermediate weight group (39.4%) and 150 kDa group (19.8%). The strain reduction with 900 kDa and intermediate weight complexes were both statistically superior compared with the 150 kDa group. The pattern was the same at Day 14, except that the strain reduction in the 900 kDa group (66.1%) was statistically superior to both the intermediate weight group (51.4%) and the 150 kDa group (42.8%). On Day 90, the strain reduction in the 900 kDa group (43.5%) continued to be statistically superior to the 150 kDa group (25.3%), with the intermediate weight group again having an intermediate result (38.4%).[13] While recognizing the limitations of a single study and that further investigations are required, it is of interest that an independent study using a sensitive efficacy assay that excludes human interpretation has observed a positive correlation between efficacy results and the amount of complexed NAPs, which corresponds with toxin molecular weight.

Another potential implication around the concept of neurotoxins staying intact is around diffusion and field of effect. In gel electrophoresis, smaller BoNT molecules migrate further than larger ones. Anhidrosis studies have demonstrated that 900 kDa molecules, PraA and OnaA, have narrower fields of effect than AboA (~500 kDa).[14] However, another study reported that IncA (150 kDa) has the same anhidrosis radius of effect compared with OnaA.[15] The study detailed earlier using DSR to assess efficacy suggests that IncA units are less potent than OnaA units.[13] If this is correct, that potencies are not equal yet the radius of spread was equal, it would imply that the smaller 150 kDa structure (IncA) spread more. A limitation of the anhidrosis model is that it is injected in a different tissue plane and targets sweat glands instead of muscle and neuromuscular junctions. A consensus panel, based on their clinical observations with all of the toxins, noted that the field of effect of the 900 kDa PraA may be more precise than the other BoNT/As, supporting that larger structures may spread less.[16]

Accessory Protein Immunogenicity

Nontoxin accessory proteins have been deemed as a potential immunologic liability; however, these publications are often written by conflicted authors.[17] Concerns that NAPs may stimulate the production of neutralizing antibodies have been expressed, but the argument seems to contradict itself—if NAPs have no role in mediating efficacy, then how can a neutralizing antibody against a protein that does nothing neutralize BoNT activity? An independent systematic review of BoNT/A immunogenicity that was performed across multiple indications over a time period from 2000 to 2020 concluded that there were no significant differences in neutralizing antibody formation between the 3 formulations reviewed, which represented 3 different molecular weights of 900 kDa (OnaA), 150 kDa (IncA), and intermediate weight (AboA).[18] There also seems to be no correlation between molecular weight and immunogenicity according to the FDA-published product instructions. PrabotulinumtoxinA-xvfs, a 900 kDa BoNT/A, demonstrated no general antibody or neutralizing antibody formation in their glabellar line registration studies.[3] IncobulinumtoxinA, a 150 kDa BoNT/A, reported no neutralizing antibody formation and there is no mention of general antibody formation after exposure.[7] In contrast, another 150 kDa molecule after exposure demonstrated a high level of general antibody formation at 2% (0.8% against the 150 kDa BoNT component, 1.2% against the RTP004 excipient peptide).[6]

Conclusion

Evolutionarily speaking, producing NAPs is an enormous cellular energy cost to an organism, but there may be a reason for this expenditure. This review provides an argument that NAPs may have an active role in potentiating the endopeptidase activity of BoNTs beyond the role of simply protecting BoNT in the gastrointestinal tract to allow for its systemic distribution in botulism. Evidence exists that NAPs, including Hn-33, increased the activities of not only BoNT/A but also BoNT/E, and the intactness of the BoNT complex may explain why the diffusion and field of effect is narrower in BoNT/A complexes of larger sizes.

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