

Botulinum neurotoxin: a deadly protease with applications to human medicine

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Botulinum neurotoxins (BoNTs) are some of the most potent biological toxins to humans. They are synthesized by the gram-positive, spore-forming bacterium *Clostridium botulinum*. BoNT is secreted from the bacterium as a ~150 kDa polypeptide which is cleaved by bacterial or host proteases into a ~50 kDa light chain and a ~100 kDa heavy chain disulfide-linked protein. The light chain of the toxin contains the catalytic domain that blocks acetylcholine release from neurons and results in flaccid muscle paralysis. Four serotypes of this pathogen have thus far been associated with human foodborne contamination. Due to their potent toxicity, botulinum neurotoxins pose bioterrorism concerns and are listed as select agents. Ironically, BoNTs, also known as BOTOX®, are used quite liberally among the population for cosmetic reasons. Less well known are the many medical uses of BoNT, such as treatment for strabismus, cervical dystonia, and an ever-increasing list of medical ailments. In this review, BoNTs will be explored from perspectives as human poisons or medicines. New technologies to identify and neutralize the effects of the toxin will also be presented and discussed.

Keywords *Clostridium botulinum*; botulinum neurotoxins; detection technologies; medical applications

A brief historical perspective on human botulism

The history of botulism is an interesting story of a double-edged sword, in this case a bacterial toxin associated with contaminated foods, its use as a biological weapon, and ironically its use as intervention for a number of medical problems and even for cosmetic purposes. A detailed description of human botulism was reported as late as the eighteenth century when an outbreak in south western German spurred then District medical officer Justinus Kerner (1786-1862) to write and publish the first detailed and accurate description of disease symptoms [1,2]. He collected data over a five-year period (1817 through 1822) and suggested the cause of disease to be a biological poison. He coined the phrase “sausage poison” and “fatty poison” to describe botulism since it was often associated with improperly handled meat. In 1895 another outbreak in the small Belgian village of Ellezelles advanced understanding of the disease by identifying the associated pathogen as *Clostridium botulinum*. In the 1970s, Drs. Alan B. Scott and Edward J. Schantz pioneered the use of botulinum toxins for medical treatment when they carried out a number of ground-breaking animal studies and demonstrating that the toxin may be effective for treatment of strabismus. In 1989, the US Food and Drug Administration approved the use of BOTOX for cosmetic purposes. Research continues to better delineate properties of the toxin, its mechanism of action, and its potential use in human medicine [1,2].

BoNT as a human poison

1.1 Biological action of botulinum neurotoxins: molecular properties and pathogenesis.

Botulinum neurotoxins (BoNTs) are among the most toxic substances to humans. A single gram of crystallized toxin disseminated and subsequently inhaled can lead to the deaths of over 1 million people [3]. These substances are produced by the ubiquitously distributed, gram-positive, strictly anaerobic, spore-forming bacterium *Clostridium botulinum*. BoNT is synthesized as a ~150 kD protein that is subsequently processed by a clostridial trypsin-like protease into two polypeptides held together by a single disulfide bond; similar to other known bacterial A-B dimeric toxins. A ~100 kD fragment, known as the heavy chain, facilitates binding of the toxin to specific host cell receptors. The second fragment, also known as the light chain (LC), is composed of a ~50 kD fragment and contains protease activity. To date, seven different botulinum serotypes have been identified, indicated by letters A through G. Serotypes A, B, and F are encoded on the chromosome; serotypes C, D, and E are encoded by bacteriophage; and G encoded by plasmid. Over 98.5% of cases of botulism are caused by serotypes A, B, and E; while serotypes C and D [3] are rare and usually associated with animals. The incredible toxicity associated with these molecules can be attributed to their enzymatic properties.

There are at least two different schools of thought with regard to mechanisms of binding to host cell receptors and entry through the host intestinal epithelium [4,5]. One pathway implicates a role for a group of hemagglutinin (HA) proteins facilitating binding; the other suggests a major role of the toxin itself in binding [5]. It is still unclear which hypothesis is correct. Interestingly, toxin produced by a mutant strain of *C. botulinum* did not lose the ability to bind

host receptors [5]. These findings however do not preclude the possibility of multiple mechanisms associated with binding. The cellular target for these toxins is the neuron. On the target host cell surface, specific receptors for the heavy chain of BoNTs have been identified; botulinum toxin A binds to glycoprotein SV2; serotype F binds SV2 and gangliosides; and serotype G binds synaptotagmin I and II. And even beyond binding the host cells, it is still an enigma as to how these toxins are eliminated from nerve endings [4,5].

Whichever mechanism(s) is/are utilized to bind host cells, binding is followed by receptor-mediated endocytosis and subsequent translocation of the light chain into the cytoplasm occurs (Figure 1). Routine trafficking through receptor mediated endocytosis incurs a lowering of vesicular pH in order to mediate uncoupling of receptor-ligand interactions [4,5]. A membrane protein that pumps hydrogen ions into vesicles mediates the lowering of the pH to 4.5. It is thought that botulinum toxin enters through a similar mechanism. Using lysomotrophic amines, which prevent the lowering of intravesicle pH, a delay in the onset or inhibition of paralysis suggests that the toxin is most likely trafficked through intravesicular pathways associated with receptor-mediated endocytosis [5]. This pathway is not unique to botulinum toxins; such pathways are also utilized by other dimeric bacterial exotoxins. The translocation of the toxin into the cytoplasm occurs somewhere along the internalization pathway, either by being directly released or by escape of the toxin from vesicles. Regardless of whichever mechanism is utilized, the toxin (specifically the catalytic domain of the enzyme) winds up in the cytoplasm. Here, the light chain, depending to which toxin serotype one refers, the target can be a SNARE protein -- either SNAP-25 or VAMP2. Toxin serotypes A and E target SNAP-25 and serotypes B, D, E, and G target VAMP2, and serotype C targets syntaxin. These SNARE proteins are necessary for pre-synaptic vesicle trafficking in neurons. This strict target specificity toward SNAREs is fascinating given that conserved residues in the catalytic sites have been observed in LCs of serotypes A, B and E. Crystallographic analyses show differences on the external surface of the protein; however, differences, either major or minor, in the catalytic domains that might explain the high level of specificity toward host target SNAREs have been elusive [4,5].

Given the pathogenesis of botulism is linked to the proteolytic activity of the light chain, a number of structure-based studies have helped to identify some of its biochemical properties and its binding to its cellular substrate [4,5]. The LC belongs to the Zn²⁺-dependent metalloprotease group of enzymes and hydrolyzes its target protein SNAP25 only 9 amino acids from its N-terminus. Recombinant production of light chain from the different serotypes has advanced our understanding of enzyme-substrate kinetics and site directed mutagenesis has helped to analyze the role of different amino acids in proteolytic activity and stability. Furthermore, these studies have allowed for the large-scale production of these light chains in the absence of their heavy chain partners. Bacterial and yeast expression systems have allowed for production of specific parts of the toxin, often making it safer to work with botulinum toxins, since handling of the highly toxic bacterium can be avoided [4,5].

At least three main pathways leading to intoxication have been described [6]. These are food intoxication (still the most common mode worldwide), wound intoxication, and infant botulism. Recently, two other means of transmission were described; these were called “hidden” and “inadvertent” botulism. The former, also called intestinal colonization botulism, refers to cases where adults harboring the clostridial spores experience *in vivo* growth and toxin production. This is also referred to as the adult version of infant botulism. Inadvertent botulism refers to those cases that involve accidents in handling the toxin in laboratory settings, or medical errors, as well as bioterrorism or biowarfare situations. In all of these cases the pathology is associated with the action of the toxin, whether it be acquired via food, wound, or *in vivo* germination of clostridial spores in cases of infant botulism [6]. The most common symptoms associated with botulism are visual impairment, specifically blurred vision, diplopia, and photophobia [6].

Due to the small number of clinical cases of botulism each year, rapid identification and diagnosis are often difficult and missed [5]. Time is absolutely critical to block the activity of the toxin. Intoxication can be reduced with the use of an equine-derived antitoxin, although diagnosis is often late. However, these antibodies cannot reverse toxin already bound to receptors and internalized; explaining why time is so critical for treatment. To date, there are no approved vaccinations for the general public, although there is an experimental vaccine available for those individuals who handle the toxin in laboratory settings [5].

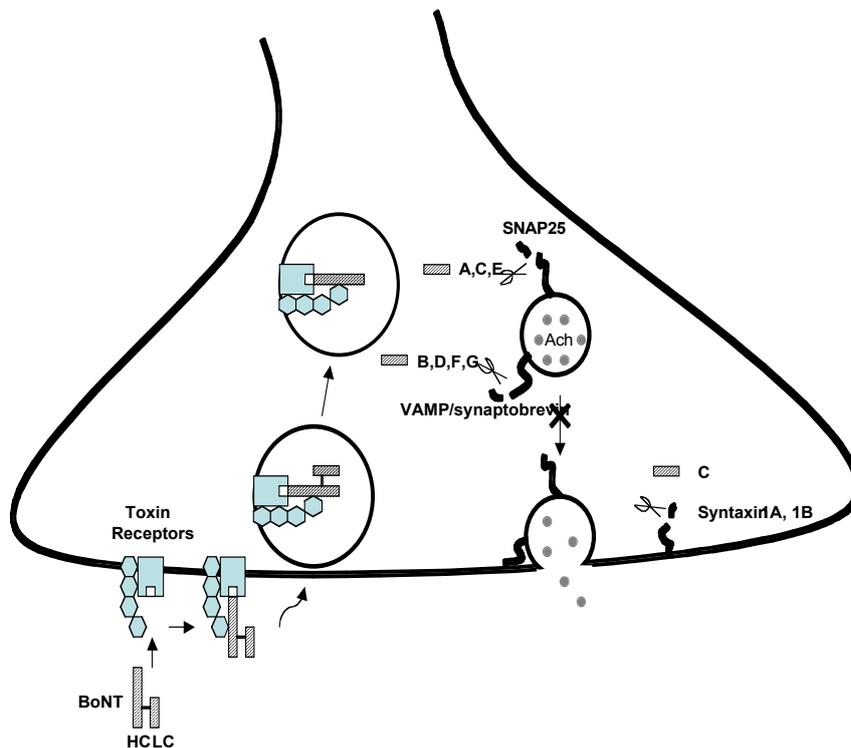


Figure 1. Cellular mode-of-action of botulinum neurotoxins. Upper-case letters (A, B, C, D, E, F, and G) indicate serotype-specific toxins.

1.2 Treatment of intentional or unintentional intoxications with botulinum toxins

Treatment of foodborne botulism can require 2 to 8 weeks of mechanical ventilation, and some documented cases of infant botulism have required an average one-month stay in the hospital [6-8]. Further, paralysis can last up to 7 months. Time-to-disease associated with aerosol exposure to the toxin is still unclear. In a study with monkeys, administering 4 to 7 monkey LD50 dosages resulted in a time-to-disease between 12 to 80 hours. In another study of three documented human cases, disease set in 72 hours post exposure. One proposed mode of intentional intoxication put forth in the past was adulteration of the water supply. However, adulteration of water supplies now is highly unlikely given that current standard water treatments can inactivate the toxin, and a relatively large inoculation size would be required. Also, to date, there have been no documented cases of waterborne botulism reported. As recent as 2008, 153 cases of botulism were reported to the US Centers for Disease Control and Prevention [7]. Of these 153 cases, 18 were associated with food (18%), 111 were associated with infant botulism (73%), 23 were wound associated (15%), and 1 was of unknown etiology (1%). In the cases of food intoxication, toxin serotype A was responsible for 10 (56%), serotype E was responsible for 6 (33%), and unknown serotype was responsible for 2 cases (11%). In this report, the median age was 57 years (range being between 3 to 87 years).

Recently, there have been a small number of food recalls in the US with confirmed contamination with botulinum toxin [7]. The most extensive recall in recent years occurred in 2007 when a number of products produced by a specific company, including hot dog chili sauce, various types of dog food, chili with meat, and corned beef hash were found to contain the toxin. In the past year there have been suspected cases of botulism in dried fish that had not been eviscerated. In most cases the mode of contamination was thought to be due to exsporulation of spores and subsequent toxin production by vegetative cells. In 1994, a large outbreak of botulism in the US occurred where baked potatoes were left wrapped in aluminum foil for days prior to its preparation in a Greek dish [7].

With the events of September 11, 2001 in the United States, the focus on the possibility of using botulinum toxin in bioterrorism became a greater concern. The potential adulteration of food and water supplies became possible modes of harming large populations of people. Intense focus was centered on mechanisms of contamination and means of rapid detection. One such scenario, which could also represent the worse possible situation, would be the contamination of the food supply, such as milk [9]. To understand the dynamics of such a possible scenario, a rigorous mathematical model of such a theoretical contamination scenario has been described [9]. The authors of the study call this the "cow to consumer" model and suggest that established processing of milk could lead to greater dilution of toxin. Calculation of a minimum amount of toxin required to cause a large number of deaths could not be determined in the study, but assuming that terrorists could acquire large amounts of the toxin (and the authors make this assumption in developing their mathematical model) then a standard milk processing facility could still give rise to large numbers of

dead individuals despite downstream processing that would dilute toxin. Their calculations indicate that an in-process testing step could potentially reduce the risk associated with botulism contamination, and could cost less than one cent per gallon. The authors also recommend that rigorous methods to inactivate the toxin using pasteurization procedures should be developed in the event of adulteration of the milk supply [9].

Unintentional contamination of laboratory workers who handle botulinum toxin have also been documented. To reduce this risk, all laboratory personnel in the United States who handle the toxin must work in biosafety level two (BSL-2) facilities [10]. Clinical settings in the United States are also outfitted as BSL-2 facilities, allowing for the handling of potential samples containing the clostridial cells or tainted with the toxin.

To prevent foodborne botulism, proper refrigeration at temperatures below 3°C (38°F) will prevent growth of *C. botulinum*. The organism is also sensitive to high salt concentrations and low pH. The toxin is heat labile, and thorough cooking of food will destroy enzymatic activity [7].

1.3 Detection technologies

Successful treatment of botulism requires rapid and sensitive methods of detection and diagnosis. Clinical laboratory testing of serum specimens, culturing of tissue samples removed from wounds, and culturing and toxicity testing of stool samples are routine methods to identify botulism. In one such study from 1975 through 1988, out of 309 cases collected by the CDC, 37% of sera samples were positive and 23% of stool samples were positive for botulism. Of the battery of tests carried out on these specimens, at least one test was positive for 65% of patients [11]. The mouse toxicity and neutralization assay is the gold standard, and the only acceptable test available to confirm cases of botulism [12,13]. ELISA methods have been in development and used in conjunction with the mouse bioassay or other sensitive methods for the detection of botulism [14-16]. One advantage of ELISA is that it can be completed in one day, while mouse bioassays require several days. Other similar methods include an ELISA-HRP method and an enzyme amplified protein microarray immunoassay. Other laboratory tests often used to confirm botulism include a lipase reaction, gram stain, observation of spores, and testing oxygen sensitivity (clostridia are strict anaerobes) [11].

Despite the high level of sensitivity associated with mouse bioassays in the detection of botulism, limitations include a high level of possible error, high costs and time associated with performing animal assays, the lack of standardization, the requirement for highly technical expertise, and the use of a large number of animals [12].

In research settings, a number of new and more sensitive methods of detecting the neurotoxins have been developed. These include exploiting our understanding of the target SNARE proteins of these toxins. For instance, for the light chain of BoNT/A, which targets SNAP-25, short peptides based on the cleavage site (called SNAPtides) in SNAP-25 have been used to detect small quantities of toxin in a variety of food matrices [18]. Peptides based on VAMP have been used to assay for the presence of BoNT/B activity.

A cell-based approach using primary rat spinal cord cells (the RSC assay) show similar levels of sensitivity to the mouse bioassay; and recent studies show that the RSC assay also has high reproducibility and a low error rate [19]. This assay may provide an equally sensitive and rapid method for the detection of neurotoxin to that of the animal assay with lower costs and error than mouse bioassays.

Molecular biological detection methods include real-time PCR assays for BoNT/A, B, E, and F serotypes. In one validation study, PCR had sensitivities of 100-1000 fg total DNA (25-250 genome equivalents) which translate to 10^3 to 10^4 cells per milliliter [21, 22]. In another study, a 48-hour enrichment of the specimen allowed the detection of *clostridia* in a sample of suspected foie gras. Other PCR-based methods include immuno-PCR and liposome-based PCR. A recently described method called ALLISA (assay with a large immunosorbent surface area) utilizes a matrix bound with toxin-specific antibodies to “capture” toxin molecules out of samples and in effect concentrates them for greater detection sensitivity. This method allows for the detection of attomolar levels of toxin and is four to five orders of magnitude more sensitive than the mouse bioassay and is also considerably faster [18].

Mass spectrometry has also been used to detect serotypes A, B, E, and F in samples [23-26]. This method, also known as endopep-MS, is based on the high specificity of the neurotoxin protease activity on its cellular substrates. Cleaved peptide products are then identified by mass spectrometry. Multiplexing by combining two or more serotypes did not show cross reactivity. This method has been successfully used to monitor botulinum toxin in environmental samples.

Another area of study applied to toxin biology is engineering antibody specificity [27]. The development of antibodies that could aid in detecting toxins and neutralizing them could be extremely valuable. Using yeast display, antibody gene diversity libraries, and a dual selection strategy, Garcia-Rodriguez and colleagues identified single antibodies with the capabilities of binding two epitopes, in this case, two different subtypes of type A botulinum neurotoxin [27]. Binding to BoNT/A1 was less than that of BoNT/A2, but X-ray co-crystal structures provided a basis for the increased cross-reactivity and identified specific amino acids different between the two subtypes. These data suggest how antibodies could be engineered to bind two different antigens and possibly be fine-tuned for different specificity and cross-reactivity.

Microarray technology has also been developed for detecting expression of genes encoding different botulinum toxins [22]. A focused oligonucleotide microarray using 62 probes targeted against variable regions of different

botulinum serotypes as well as toxin gene cluster components has facilitated detection of serotypes A through G [28]. Paramagnetic beads have also been utilized to detect toxin in biological samples [29].

Table I – Summary of different methods used to detect BoNT in food samples.

Method	Level of Sensitivity (fg/mL)	Reference
Mouse bioassay	6,000	[13]
Cell-based assay	Not-yet assayed for food samples	
ELISA	60,000 (10 pg/ml)	[15,16]
Multiplex-PCR	10 ⁻¹ to 10 ⁻² spores/g	[20]
Immuno-PCR	50	[22]
Liposome-PCR	0.02	[21]
ALLISA	0.5	[14]
Fluorogenic substrates	Not-yet extensively tested in food samples	
Mass spectrometry	320,000	[23-26]

2. Uses of BoNT as medical treatments

2.1 Medical uses of BoNT

BoNT has been used in three areas of medicine: dermatology, ophthalmology, and neurology [31-32]. These include ocular disorders, disorders of the digestive tract, dystonia and hemifacial spasm, gastrointestinal and urogenital disorders, and management of pain. And more recently, botulinum toxin has been used for cosmetic purposes. Advantages of using BoNT for treatment include rare systemic side effects; and any adverse effects are usually transient. This is most likely due to the high degree of substrate specificity of the toxin. Disadvantages include the need for repeated injections, overcoming difficulties with patient compliance, and the fact that BoNT acts locally; often requiring multiple injections for treatments involving large muscles. Interestingly, a more recent study by Cady and colleagues report the experimental use of onabotulinumtoxin A for treatment of chronic migraines [31].

2.2 Current medical uses of BoNTs

Physician Alan Scott pioneered the first medical applications of botulinum toxin A in the 1980s [2]. As part of his initial strategy, he carried-out local injections to alleviate muscle spasms associated with strabismus. Subsequent clinical trials led to US Food and Drug Administration approval in 1989 for use in ophthalmological uses. In the early 1990s, the use of botulinum toxin in dermatology began, with injections used to remove frown lines, crow's feet, forehead lines, and brow ptosis. Clinical studies published in 2010 demonstrate additional uses of botulinum toxin for treatment of other diseases; including neuropathic itch, neuropathic pain, temporomandibular joint dislocation, benign prostatic hyperplasia, chronic anal fissures, and palmar hyperhidrosis; as well as long-term analyses of these uses of botulinum toxin [32].

3. Future applications of botulinum toxin in research and medicine

The widespread distribution of SNAP25 and the wide diversity of toxin receptors are now being exploited for therapeutic applications. As discussed above, the sensitivity of detection remains the major hurdle in developing methods for determining the presence of toxins in samples. Methods for rapid diagnosis in complex matrices of food or biological samples remain a top priority of many research laboratories interested in botulism. The development of extremely sensitive methods of toxin detection will also set the stage for further development of similar assays for other microbial toxins. Given the infrequent episodes of botulism and the potential medical benefits of botulinum toxin, the development and use of a vaccine for the general public is not a viable option. Instead, development of therapeutics such as small molecule inhibitors of the enzyme is moving forward [4-6]. Modified peptide inhibitors that compete with the toxin for binding to cellular substrates or in neutralizing toxin that is cell bound have been explored. In the course of study of therapeutics for botulism, the use of botulinum toxin as vehicles to target inhibitors to neurons for other neurological disorders have also been explored and the use of therapeutic antibodies have also been sought after as options [33]. On the other hand, new uses for this deadly protease continue to emerge as clinicians find medical applications of using the toxin for certain diseases. The future holds great promise for the development of new diagnostics, detection systems, treatments, and medical uses of one of the potent substances known to humans.

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