Botulinum Neurotoxins

Chanokporn Sukonpan

Abstract

It is fascinating that one of the most awareness bioterrorism agents as botulinum neurotoxin (BoNT) becomes a great benefit to cosmetic application and to patients who suffer from agony muscle spasm. This amazing toxin is produced as a harmless single chain 150 kDa peptide by *Clostridium botulinum*, a spore-forming bacillus, under an anaerobic condition. However, the natural activation process turns this na¿ve protein into dicain polypeptide which is the most toxic substance known to human being. Its toxicity is resulted from a selective blockage to neurotransmitter release from the nerve terminal. The structure and the detail mechanism of action of this deadly toxin are described in this article.

Keywords : botulinum neurotoxin, BoNT, biological waepon, Botox®, Myobloc®, Dysport®, Neurobloc®, CS-BOT®, anti wrinkle agent, botulism.

Botulinum neurotoxin (BoNT) is one of the most interesting proteins. Due to its extreme toxicity, virulence and limited treatment options, this toxin had been developed to be a biological weapon by both Allied and Axis troops since the World War II. Even though this project was officially ceased by the international agreement under the 1972-BWC (the Biological and Toxin Weapons Convention), some countries such as Iraq and the former Soviet Union still produced a large amount of BoNT. Hence, it is not wonder why the US-CDC (The Centers for Disease Control and Prevention of The United State) has placed BoNT and *Clostridium botulinum* in the category A of the bioterrorism agents.

Interestingly, even though BoNT is considered to be one of the highest awareness bioterrorism agents, it was the first bacterial toxin that was approved by US-FDA (The United State Food Drug Administration) for treatment of pain and muscle spasm. Currently, this deadly toxin becomes popular as an effective anti-aging agent in the cosmetic world under the name of Botox®. This article will review mainly on structure and mechanism of action.

Botulinum neurotoxin (BoNT)

Botulinum neurotoxin (BoNT) has been recognized as the most poisonous toxin known. This deadly protein is mainly produced by a gram-positive, strictly anaerobic bacillus *Clostridium botulinum*. C. botulinum is classified as a single species but consists of at least three genetically distinguishable groups of organisms. These are alike in their abilities to produce neurotoxins with similar pharmacological activities. This microorganism can be divided in to seven subspecies according to the anti-genicity of the toxin it produces. Alphabets A through G are used to name all seven serotypes of the proteins as well as to call the bacillus that makes the toxin.

Among the seven serotypes, serotypes A, B and E have been known to be the three major strains causing a bilaterally symmetric descending neuroparalytic syndrome or botulism in humans. As the deadliest toxin known, the serotype A or BoNT/A has the LD_{50} (in mouse) of 0.2 picomoles (about 0.4 ng/Kg body weight) which is about 15,000 and 100 billion times more toxic than the nerve agents and cyanide, respectively. The lethal effect occurs after the toxins specifically destroy SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) that are essential for neurotransmitter release. A flaccid muscle paralysis and, eventually, respiratory collapse are the results of this event (Arnon et al., 2001; Humeau, Doussau, Grant, & Poulain, 2000; Montecucco & Schiavo, 1995; Pellizzari, Rossetto, Schiavo, & Montecucco, 1999; B. R. Singh, 2000).

Structure of Botulium Neurotoxin (BoNTs)

All of the seven serotypes of BoNTs share similar domain structures and functions. They are translated in bacterial cytosol, as single 150 KDa peptide chain protoxins with three distinct 50 KDa domains (Figure 1). After the bacterial cell lysis and the protoxin release into the culture media, proteolytic enzymes from tissue or bacterial cell will change the protoxin to a dichain holotoxin by cleaving one Lys-Ala peptide bond in a disulfide loop at about one-third from the N-terminus to generate two active domains, the light chain (L chain; M_r = 50 KDa) and the heavy chain (H chain; M_r = 100 KDa), leaving only one disulfide bond and non-covalent protein-protein interactions holding them together (Figure 1).

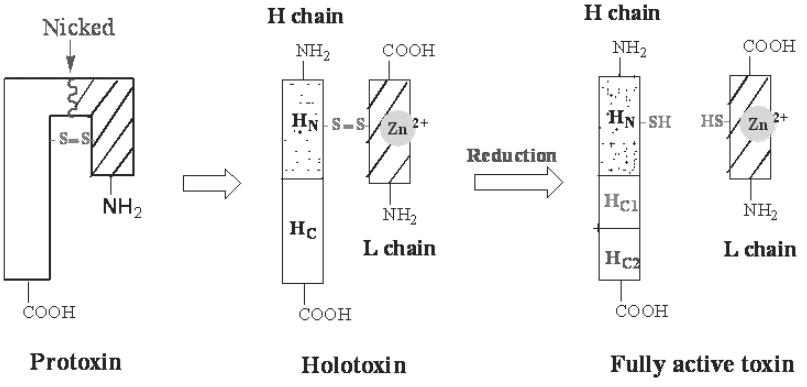

Fig. 1 Scheme of structure and mechanism of activation of Clostridium neurotoxins. This figure is modified from Montecucco & Schiavo, 1995.

The holotoxin functions as a neurotoxin delivery system. The 100 KDa heavy chain works as a Trojan horse. It carries a frail enzyme across membranes passing many rigorous systems in the body before specifically releases it into the neuronal cytosol of a presynaptic nerve. Under a reducing condition of the cytosol, the only disulfide bridge in the holotoxin is destroyed. Then, a fully active zinc-metalloprotease is free to selectively attack SNARE

proteins that are essential in exocytosis. Lacking an excellent carrier as the heavy chain, the light chain is a harmless metalloenzyme (Humeau, Doussau, Grant, & Poulain, 2000; Lacy, Tepp, Cohen, DasGupta, & Stevens, 1998; Montecucco & Schiavo, 1995; B. R. Singh, 2000).

Using papain hydrolysis, the H chain can be further hydrolysed into two 50 KDa domains, the C-terminus part (H_C) and the N-terminus part (H_N). The crystal structures of BoNT/A and BoNT/B provided more information about function of each subunits. With exception to the N-terminus end of the H_C, the folding structures of the C-terminus subunits of these toxins are identical even though the overall homology of the amino acids in the H domains was poor (Figure 2). This evidence suggested that, except for the N-terminus end, all other subunits are responsible for the same function in both BoNT/A and BoNT/B. The C-terminus of H_N domain functions as a receptor binding domain. It is responsible to selectively bind to BoNT receptor on cell surface around NMJ. The C-terminus peptide HC is a translocation domain that involves in movement of the L chain across the endosomal membrane. Moreover, the difference in folding of the very N-terminus end suggested that there were two different binding sub-domains that folded with respect to the two different receptors; an identical folding of the C-terminus half and a different folding structure on the N-terminus side. The later evidence indicates that the C-terminus subunits responsible for the primary low affinity binding with the ganglioside receptors (non-selective binding) and the N-terminus side specifically and tightly binds to the particular protein receptors at the nerve terminal (Lacy & Stevens, 1998; Lacy, Tepp, Cohen, DasGupta, & Stevens, 1998; Lalli et al., 1999; Swaminathan & Eswaramoorthy, 2000). The arrangement and the function of dichain subunit in BoNTs are analogy to those in diphtheria toxin and anthrax toxin complex (Humeau, Doussau, Grant, & Poulain, 2000; Montecucco, Papini, & Schiavo, 1994; Montecucco & Schiavo, 1994, , 1995; G. Schiavo, Rossello, & Montecucco, 1994; G. Schiavo, Rossetto, O. and Monteccuco, C., 1994; B. R. Singh, 2000).

Molecular Targets of Botulinum Neurotoxins (BoNTs)

BoNT is a unique enzyme. It has highly target selectivity. Each serotype attacks only one specific target peptide bond on a particular fusion protein. Except for BoNT/C1, BoNTs cleave only one specific peptide bond on one particular SNARE proteins (VAMP or synaptobrevin of the synaptic vesicle, SNAP-25 and Syntaxin in the presynaptic membrane) without hydrolysing other identical peptide bonds in other part on the substrate (Montecucco & Schiavo, 1995). BoNT/A and /E cleave SNAP-25, whereas BoNT/B, /D, /F, and /G attack VAMP protein. The specific cleavage sites of each toxin are shown in Table 1 (Humeau, Doussau, Grant, & Poulain, 2000; Montecucco & Schiavo, 1995).

Table 1 A molecular target and a particular peptide bond cleaved by each BoNT (Montecucco & Schiavo, 1995; Rossetto et al., 1994)

Toxin	Molecular Target	Peptide bond cleavage P_3 - P_2 - P1↔ P_1'- P_2'- P_3'
BoNT/A	SNAP-25	Ala-Asn-Gln↔Arg-Ala-Thr
BoNT/B	VAMP	Ala-Ser-Gln↔Phe-Glu-Thr
BoNT/C	syntaxin	Thr-Lys-Lys↔Ala-Val-Lys
BoNT/D	VAMP	Asp-Gln-Lys↔Leu-Ser-Glu
BoNT/E	SNAP-25	Ile-ASp-Arg↔Ile-Met-glu
BoNT/F	VAMP	Arg-Asp-Gln↔Lys-Leu-Ser
BoNT/G	VAMP	Thr-Ser-Ala↔Ala-Lys-Leu

The exquisite high substrate selectivity of clostridium neurotoxins is due to the double recognition of substrate by the enzyme active site. To provide optimal proteolytic activity, clostridium neurotoxins need both the zinc-binding motif (HEXXH + E) and the SNARE motif (á-helix amphiphilic motifs on the N-terminus side of scissile bond) (Pellizzari et al., 1996; Washbourne, Pellizzari, Baldini, Wilson, & Montecucco, 1997).

The SNARE motifs are conserved nine amino acid residues existing in multiple copies which are present in all three SNARE proteins or in soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptors. There are two copies of SNARE motifs in VAMP (V1 and V2) and in syntaxin (X1 and X2), as well as four copies in SNAP-25 (S1, S2, S3 and S4) (Rossetto et al., 1994; Washbourne, Pellizzari, Baldini, Wilson, & Montecucco, 1997).

Pathogenic Mechanism of Botulinum Neurotoxins (BoNTs) : A Highly Specific Denervating Agent

One of the main reasons for the use of BoNT as a therapeutic agent is its high selectivity to presynaptic neurons of neuromuscular junctions (NMJ). This characteristic comes from its mechanism of action. As mentioned earlier, the arrangement of protein subunits and their functions in BoNT are similar to those in diphtheria toxin and anthrax toxin complex. It is not wonder why these deadly proteins share the same pathogenesis mechanisms. Their intoxication mechanisms can be divided into four consecutive stages including neurospecific recognition, receptor-mediated internalization, membrane translocation and target modification (Figure 2) (Montecucco, Papini, & Schiavo, 1994; Montecucco, Schiavo, Tugnoli, & deGrandis, 1996; G. Schiavo, Rossello, & Montecucco, 1994).

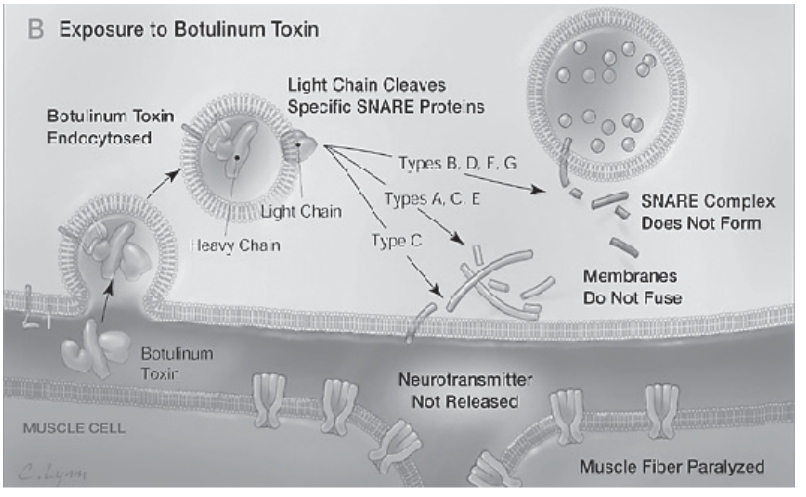

Fig. 2 Four consecutive steps of mechanism of action of Botulinum Neurotoxins (BoNT) at nerve terminal and cytosolic activity. At beginning, BoNT binds selectively to its dual receptors on the presynaptic membrane of NMJ (a neurospecific recognition step) and, subsequently, internalization via receptor-mediated mechanism (a receptor-mediated internalization step). A membrane translocation process is started in the late endosome when the pH in the late endosome drops to pH around 5. In acidic medium, the conformation of BoNT was changed. This modification promotes the membrane penetration and translocation of the light chain through the endosomal membrane (a membrane translocation step). The reducing condition and neutral pH of the cytosol let the light chain (Lc) leave its carrier and refold in the form that is ready to attack its particular substrate (SNARE proteins) (a target modification step). The result from these four consecutive steps is the inhibition of acetylcholine (Ach) release in NMJ and, subsequently, causes a flaccid paralysis (Montecucco, Papini, & Schiavo, 1994); (G. Schiavo, Rossello, & Montecucco, 1994); (Montecucco, Schiavo, Tugnoli, & deGrandis, 1996). (This picture came from Arnon et al., 2001).

However, the actual mechanisms of receptor-mediated endocytosis and membrane-translocation of BoNT are not known yet. The “double receptor” model proposed by Montecucco has been widely used. Applying this model to the NMJ surface, there were two different types of receptors that bound to the H_C with different affinity : a low affinity receptor (K_D in nM range) and a high affinity receptor (K_D in sub-nM range). Later experiments revealed that the primary low affinity receptors were polysialogangliosides in GT1b series including GT1b, GD1b and GQ1b binding in “Lock and key” fashion (Kozaki, Kamata, Watarai, Nishiki, & Mochida, 1998; Montecucco, 1986a, , 1986b; A. K. Singh, Harrison, & Schoeniger, 2000; B. R. Singh, 2000). This non-specific binding to the lipid receptors plays an important role in promoting a proximity effect or the alteration in toxin conformation, and leads to tight-binding to certain types of high affinity receptors. The specific high affinity receptors for BoNT serotype A, B and E (BoNT/A, /B and /E) were later found to be integral proteins for small synaptic vesicles (SSVs) or Synaptogamins (Li & Singh, 1998; Nishiki et al., 1996). Montecucco and others believed that these tight-binding receptors were dictated the location of action of BoNTs. Moreover, this tight-binding also triggers the internalization process of BoNT-receptor complex (Montecucco, Papini, & Schiavo, 1994; Montecucco & Schiavo, 1995). The later evidence indicates that the C-terminus section is responsible for the primary low affinity binding with the ganglioside receptors (non-selective binding) and the N-terminus side specifically and tightly binds to the particular protein receptors at the nerve terminal.

Following the receptor-mediated internalization, the receptor-BoNTs complexes are packed in the endosome of the final target cell. Processed by the ATP–dependent proton pump, the acidic pH (pH ~ 4.5-5.0) of the late endosome promotes the dissociation of the receptor-toxin complexes and releases BoNTs within the endosome. Furthermore, the low pH promotes the conformation change of BoNTs from a water-soluble form to a more

lipid soluble form. The increase in hydrophobicity enables the toxins to penetrate to the hydrophobic core of the phospholipid bilayer and directs the light chain across the endosome wall (Montecucco & Schiavo, 1995; B. R. Singh, 2000).

Until now, there is very little knowledge about the actual translocation mechanism of BoNTs. However, there have been two proposed models for explaining the translocation mechanism (Montecucco & Schiavo, 1994, , 1995). The "tunnel model" was the first proposed model. According to this model, after undergoing the conformational change in acidic medium, the four á-helix-H_N domains could insert to form an ion channel within the membrane (Busath & Singh, 1999; Li & Singh, 2000). The unfolded light chain moves through this hydrophilic channel without exposure to any hydrophobic surface, and then it refolds and regains its hydrophilicity in the neutral pH of the cytosol (Lacy & Stevens, 1998; B. R. Singh, 2000). This model is supported by three-dimensional imaging from electron microscope of the pore formation in phospholipid bilayer by BoNT/B (Schmid, Robinson, & Dasgupta, 1993). However, with this model, there still is a question how can the 50 KDa light chain pass through 8 Å-pore. The other model, the "cleft model", two toxin subunits incorporate into the phospholipid bilayer and form a cleft. According to this model, the cleft wall was constructed with a hydrophilic H_N surface on one side and a hydrophobic (lipid bilayer) wall on the other. Undergoing a molten globe formation, the structure of light chain was more flexible and could move across this amphiphilic hole. Again, in the aqueous medium with neutral pH of cytosol, the light chain regains its tertiary structure and its hydrophilicity (Montecucco & Schiavo, 1994, , 1995). Recently, Singh and co-workers reported that, at the low pH (pH 4.7), the secondary structure of the light chain of BoNT/A was still retained even at denaturating temperatures (> 55°C); its hydrophobic surface was more accessible to a non-polar dye as ANS (1-anilinonaphthalenesulfonate) as well. This result suggested the formation of the molten globe structure of the light chain and the

possibility of the cleft model (Li & Singh, 2000). However, the existence of zinc at the active site during the translocation is still questioned.

More robust evidences to reveal the actual mechanism of receptor-mediated endocytosis and membrane-translocation mechanism of BoNTs are needed. Nevertheless, many scientists try to exploit this delivery system. Combined with liposome technology, the nontoxic heavy chain of BoNT can be used as a carrier for directing therapeutic agents or neuronal probing reagents into certain parts of nervous system without encountering the blood brain barrier (BBB) (Johnson, 1999; Simpson, 2000). This application of BoNT's heavy chain is presently under investigation.

Following translocation across the membrane, the remaining disulfide bridge was reduced and let the light chain free. In the cytosol, the light chain refolds itself and regains its active structure. Then, the fully active enzyme specifically attacks and hydrolysed the fusion proteins at a particular peptide bond. This inhibits formation of a SNARE complex which is essential for membrane fusion and neurotransmitter release.

Botulism

Botulism can be identified as a bilaterally symmetric descending neuroparalytic illness caused by BoNTs. It is one of an old sickness that has been known since ancient time. The word "botulism" that means "sausage poisoning" in Latin was used as it name since this illness was frequently occurred after eating spoiled sausage. In 1895, during a big outbreak of botulism in Ellezelles, Germany, a German microbiologist, Emile van Ermengen could isolate a spore-forming anaerobic bacillus association with this outbreak. He also named the bacteria he discovered *Clostridium botulinum* (Arnon et al., 2001; Mohanty S, 2001; G. Schiavo, Rossetto, & Montecucco, 1994).

C. botulinum is a strictly anaerobic bacillus (Figure 3). It can live in broad range of temperature (10°C to 48°C). Therefore, it can

be found in soil and mud throughout the world. Even in mud and sand in the gulf of Thailand, there was a reports shown the present of *C. botulinum* serotypes D and E in the samples from Hua-Hin area (Tanasukran, 1979). Moreover, this microorganism can produce a heat-resistant spore that makes it can survive in even higher temperature. Particularly, the spore of *C. botulinum* serotypes A could survive in 100°C for 25 minutes (Arnon et al., 2001; Mohanty S, 2001). If some spores have been left from improper canned-food processing, they can grow and generate the deadly neurotoxin into the food under anaerobic condition. Therefore, later, the food-borne botulism becomes well known as "canned food poisoning". The recent outbreak of food-borne botulism in Nan province Thailand from eating home-canned bamboo shoots was good example (U.S. Department of Health&Human services, 1999).

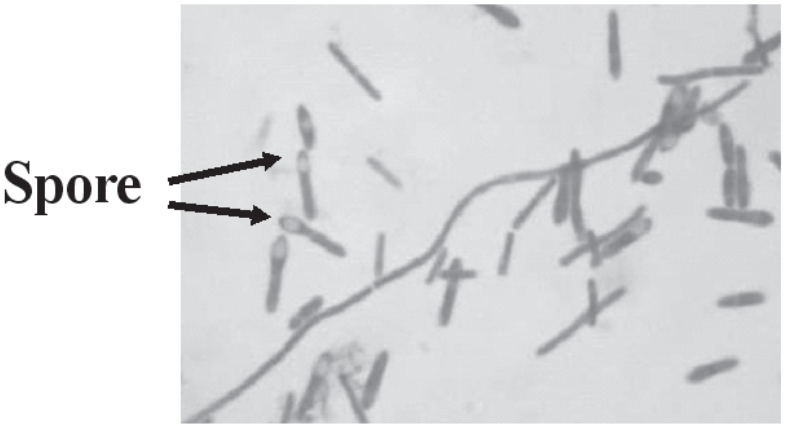

Fig. 3 *C. botulinum* is a strictly anaerobic bacillus (Todar, 2005).

Besides the food-borne botulism which is the most frequently found, there are other forms of botulism including infant botulism, wound botulism, adult infectious botulism (adult intestinal colonization), inadvertent botulism and inhaled botulism. For the first three types, the toxins are produced in the human body by the living bacteria whereas, for the remaining kinds, the patients get the

toxin directly from the environment. Even though there are many forms of botulism, the major clinical features of them are similar. As mentioned earlier, this disease is a bilaterally symmetric descending paralysis. Hence, the symptoms start from the upper part of the body including pupil fixed and dilation, blurred or double vision, dry mouth, dysphonia (difficulty speaking) and dysphagia (difficulty swallowing). The evenly paralysis is then progress respectively to lower part of the body including paralysis of both arms, paralysis of thorax muscles and diaphragm and, lastly, paralysis of both legs. Without good supportive treatment, the patient will be death from respiratory failure (Arnon et al., 2001; Franz et al., 1997; Mohanty S, 2001).

BoNT as Biological weapon

The United States Centers for Disease Control (CDC) classifies potential biological weapons into 3 categories from A through C according to their virulence. According to CDC criteria, BoNTs have been classified as one of the greatest concerns for biological agents along with *Bacillus anthracis* (Anthrax), *Variola major* (smallpox), *Yersinia pestis* (plague) and Ebola virus (Arnon et al., 2001; Darling R.g., 2002; Niiler, 2002). This concern is attributed to their extremely toxicity, high degree of contagiousness and limited treatment options. As a biological weapon, there is an evidence suggesting that Japanese terrorists tried to used BoNTs in downtown Tokyo and a US military base at least 3 times during the period of 1990 to 1995 (Arnon et al., 2001). After finding anthrax spores in the US Senate and US postal service, the awareness of bioterrorism has increased dramatically in the US.

Since victims can be easily exposed to BoNTs as a biological weapon via inhalation and food contamination, BoNTs have tremendous mass destructive power. One gram of high purity BoNTs in aerosol form could kill more than 1 million people. Due to BoNTs slow onset of action, timely recognition of exposure makes it difficult to stop the outbreak and prevention. Typically, the

intoxication signs appear after 12-36 hours or as long as 14 days depending upon exposure. However, the neurological symptoms from botulism are difficult to differentiate from other CNS diseases, like myasthenia gravis or stroke and often are misdiagnosed. Generally, the neurological effects from botulism will last from a couple months up to 12 months depending on the serotype of the neurotoxins. During the intoxication period, the patient needs prolonged supportive care that costs a lot of money (Arnon et al., 2001; Caya, 2001; Franz et al., 1997).

Another issue concerning BoNT is that it is easy to obtain from nature. *Clostridium spp*. are strictly anaerobic bacteria that are found in soil worldwide and in malprocessed foods. With certain microbiological techniques and aerosol generating equipment, terrorists can produce enough lethal toxins for a biological weapon of mass killing. In 1995, the Iraq government admitted that they had 19,000 liters of concentrated BoNTs in their possession. This amount is about three times of the required amount of toxin needed to kill all human beings in the entire world by an aerosol exposure (Arnon et al., 2001; Caya, 2001; Franz et al., 1997; Niiler, 2002).

Medical Applications of BoNT

Fascinatingly, these powerful biological agents became the first biological toxins to be approved as therapeutic agents in humans. At 0.05% of the lethal oral dose, BoNT/A have been used clinically as effective local chemical denervation agents in various neuromuscular disorders including strabismus, blepharospasm and other facial nerve disorders. Botox® (BoNT/A, Allergan, USA) has been introduced in the market as an effective muscle relaxant since 1989. Recently, the US Food and Drug Administration (FDA) has approved Myobloc® (BoNT/B, Elan, USA) as an therapeutic agent and also approved to use BoNT/A in cosmetic (Caya, 2001; P. Setler, 2000; P. E. Setler, 2001) Since then, Botox® has been famous as a magic anti-wrinkle agent. Regarding to the data from

American Society for Anesthetics Plastic Surgery (ASAPS), the demand of Botox® and Myobloc® for cosmetic purpose in the US dramatically increase for 2,446 % with in 5 years.

These medicines are also well known in Europe and Asia. In Europe, BoNT/A and /B have been widely used under the name of Dysport® (BoNT/A, Speywood pharmaceutical, UK) (Bigalke, Wohlfarth, Irmer, & Dengler, 2001)and Neurobloc® (BoNT/B, Elan, UK) as well as In Japan, BoNT/A is sold under the name CS-BOT®.

Botox® seems to be an ideal drug for severe neuromuscular disorders because of its high potency, few side effects and long duration of action. Besides the treatment of overactive nerve disorders, Botox® has been used for non-surgical face-lifts. This application makes BoNT/A and /B well known and increases the demand of these toxins in the commercial market. Although the side effects from the therapeutic use of BoNTs are very rare and mild, there have been some documented incidents of botulism overdosing due to improper administration (Arnon et al., 2001; Klein, 2001; Klein & Elson, 2000). Moreover, the immunological resistance to the toxin is another factor that should be aware of. This resistance leads to higher dose of administration and it increases the incidence of serious side effects. Regarding to this awareness as well as the popularity of these toxins, the other serotypes which have inferior properties including less effectiveness and shorter duration have been tested and are developed into therapeutic agents.

Conclusion

All three subunits of BoNTs comprise a wonderful natural design for a toxin delivery system. They work together with a high precision and effectiveness. It is no wonder that BoNTs are recognized as the most potent toxins known.

Additionally, it is amazing that the most toxic protein known could be used as effective therapeutic agents, particularly in knifeless cosmetic surgery. Due to very high target selectivity,

BoNT shows extremely high effectiveness with mild and rare side effects. BoNT is also an excellent chemical denervation agent and widely used in many neurological research programs. Moreover, the heavy chain of BoNTs is potential to be developed for use as carriers for drugs targeting the nervous system.

Even though BoTNs have been widely used as therapeutic agents, research tools, and as bioterrorist weapons over the last five decades, there are still some missing pieces of puzzle that lead many scientists to investigate how they work (mechanism of action) and how they could be controlled (selective inhibitors). All of these, features along with the threat of using BoNTs as a biological weapon, compel many scientists to investigate BoNT. The problems of BoNT intoxication from misuse and potential bioterrorist threats have led to programs for discovering effective antidotes to these lethal toxins. However, there is no effective antidote for BoNTs currently available. The only treatment approved for BoNT detoxification is the infusion of a trivalent equine antitoxin to neutralize the toxin in circulation. Unfortunately, the antiserum is most effective only in the early stages of intoxication. It does not work at all when the toxin gets into the nerve terminal. The use of metal chelators might be an alternative treatment, but the subsequent high doses of metal chelators such as TEPN (N,N,N,N-tetrakis-(2-pyridylmethyl) ethylenediamine)) prolonged life minimally. At effective concentrations to BoNT, TEPN has side effects such as ataxia, convulsion and death (Adler, Dinterman, & Wannemacher, 1997). Only a supportive treatment keeps patients alive until all toxins are metabolized which may take up to 12 months. These factors inspire many researchers to find an effective way to counteract the clostridium neurotoxin metalloproteases.

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