Chapter 2
Molecular Structure, Mode of Action, Immunology, Safety, and Side Effects of BoNTs

Introduction

This chapter describes the molecular structure of botulinum neurotoxins (BoNTs), the intricacies of its mode of action on cholinergic synapses, the immunological aspects of BoNTs, and the safety profile of botulinum neurotoxins.

Structure of Botulinum Neurotoxin and Its Mode of Action

Botulinum neurotoxin (BoNT) is produced by clostridium botulinum, a gram-positive anaerobic bacillus that is widely present in nature. There are now eight serotypes of BoNTs designated as A, B, C, D, E, F, G, and X. In recent years, several subtypes have been described such as A1, A2, A3… for the A serotype [1]. Among these serotypes, only types A, B, C, D, and F cause botulism in humans. The source of infection is usually contaminated food as the spores of these bacteria can survive for a long time in the environment. Although each serotype has a distinct molecular structure, there is significant homology between different toxins as well as between BoNTs and tetanus toxin [2].

Currently, only serotypes A (A1) and B are used in clinical practice. After intramuscular injection, these serotypes exert their therapeutic action within a few days which usually lasts for 3–4 months. Serotype E has a faster onset (usually within 24 h) and shorter duration of action (2–4 weeks); the latter may be desirable for analgesic indications [3].

Botulinum neurotoxin complex is composed of a toxin core with a molecular weight of 150 kDa, a size that is constant among different toxin serotypes. The toxin is embedded into a larger nontoxic protein complex, the size of which varies among different toxins; for instance, it is 900 kDa for onabotulinumtoxinA (botox) and

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700 kDa for rimabotulinumtoxinB (Myobloc). This protein protects the toxin when exposed to deactivating factors such as stomach acid, high temperature, and proteases. The nontoxic protein complex (NAPS) includes hemagglutinin proteins (HA proteins) and non hemagglutinin proteins. The non-hemagglutinin protein part of the protein complex includes specific antigenic proteins which are the source of antibody formation against the toxin after BoNT therapy.

The core toxin is composed of a heavy chain (HC) and a light chain (LC); the two chains are linked by a single disulfide bond. The light chain has one domain (catalytic domain) and is the active moiety of the toxin; its function is exerted inside the cell and on a specific set of proteins (SNARE proteins) through a zinc-activated protease. The heavy chain has two distinct domains HC and HN. These domains help the toxin molecule attach itself to cell membrane (end of peripheral axon, HC-binding domain), enter the peripheral nerve terminal, and later free the light chain from the core toxin structure in order for it to exert its catalytic function (translocation domain HN).

In clinical practice and for most indications, botulinum toxins are introduced through intramuscular injection; for some pain indications, they are injected subcutaneously, however. After injection, within minutes, the core toxin disassociates itself from the surrounding protein complex and is activated (nicked) [4]. The type A toxin is naturally activated by its own protease [5], whereas activation of other BoNTs takes place by exposure to the tissue proteases. After activation, the toxin quickly reaches the peripheral synapses of cholinergic neurons probably through lymphatics or blood. There, the toxin blocks the release of acetylcholine from presynaptic vesicles through five intricate sequential steps:

1. Receptor binding: Upon reaching the cholinergic synapse (neuromuscular junction or autonomic synapse), the HC domain (binding domain) of the toxin's heavy chain (Fig. 2.1) attaches the core toxin complex to two specific cell membrane receptors, namely, ganglioside and synaptic vesicle (SV) receptors [1, 6, 7]. The polysialoganglioside (PSG) receptor is abundant on the presynaptic membrane. The SV is present on the wall of synaptic vesicles (Fig. 2.2). This dual attachment is believed to be important since attachment to PSG receptor facilitates attachment of the toxin to SV receptor, which acts as a channel to let the toxin to endocytose and reach inside the presynaptic part of the cholinergic axon [8]. BoNTs A and B attach to different segments of SV receptor. The type A toxin attaches to the region known as SV2 which is a glycoprotein. The SV attachment site for type B toxin is called synaptotagmin (Syt1/Syt2) and contains calcium sensors (Fig. 2.2).
2. Internalization: After entry into the cell (presynaptic part of the axon), the toxin is visualized mainly inside presynaptic vesicles (mouse model).
3. Translocation: Inside the presynaptic vesicles is an acidic milieu caused by a specific proton pump that keeps a PH gradient across vesicle membrane in order to move the neurotransmitters into the vesicle. This acidic environment weakens the HC bond of the toxin resulting in the formation of a translocation channel that moves the light chain of the toxin from inside of the vesicle to the side of the ves-

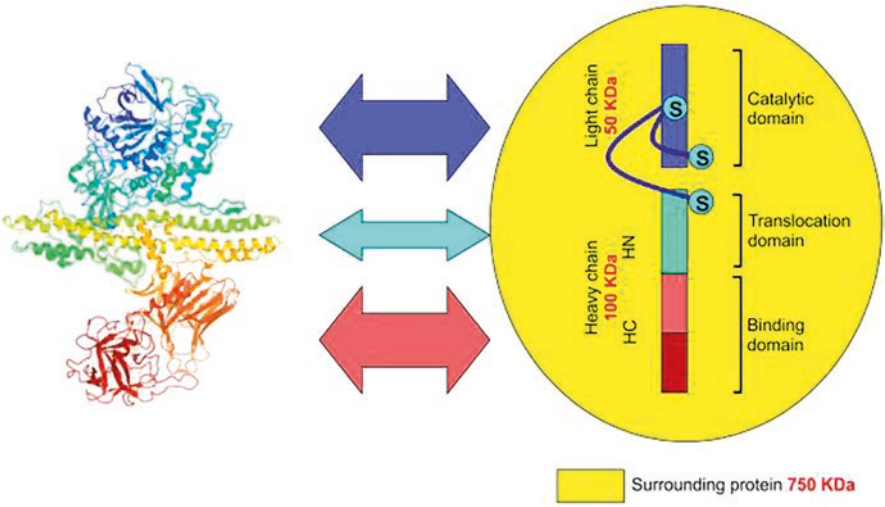

Fig. 2.1 The molecular structure of botulinum toxin. From Choudhury et al., Botulinum Toxin. An update on pharmacology and newer product developments. Toxins, 2021. Reproduced under creative commons distribution. Courtesy of PMC and Toxins

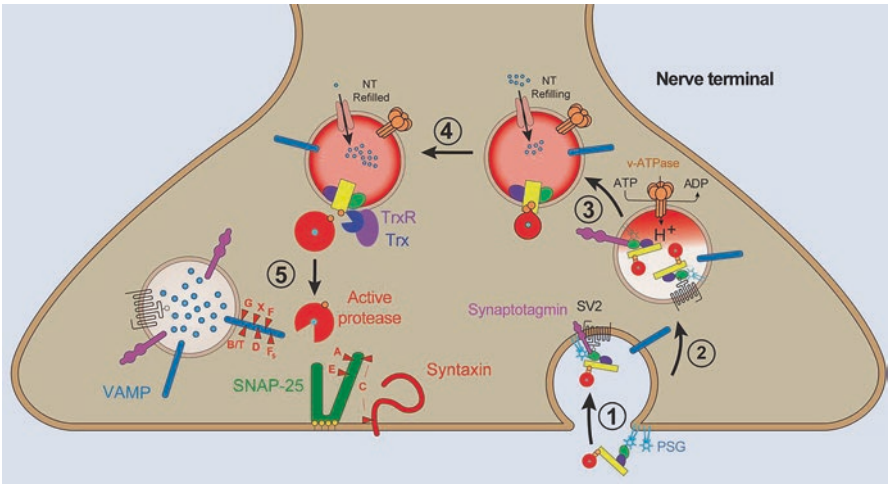

Fig. 2.2 Mechanisms of action of BoNTs. From Rossetto O, Pirazzini M, Fabris F, Montecucco C. Botulinum Neurotoxins: Mechanism of Action. Handb Exp Pharmacol. 2021;63:35–47. Reproduced with permission of publisher (Springer)

icle membrane facing the cytosol. The partially unfolded light chain and disulfide bond between two chains are now subject to the function of cytosolic enzymes.

4. Reduction of disulfide bond: At this stage, the disulfide bond that connects HC and LC is reduced and broken releasing the light chain into the cytosol to target SNARE proteins. The cytosolic enzyme that performs this function is NADPH–Thioredoxin reductase–thioredoxin system. It has been shown in animals that inhibition of this enzyme can prevent BoNT intoxication [9].
5. Cleavage of SNARE proteins by the light chain of BoNT: SNARE proteins are a set of proteins that are present in cholinergic synapses, and their function is to fuse the synaptic vesicles to the membrane of the nerve terminal, causing its rupture and release of the neurotransmitter into the synapse. The light chain of botulinum toxin, via its zinc-activated protease, inactivates SNARE proteins, hence preventing vesicle fusion and neurotransmitter release. BoNTs A and E cleave the SNARE protein named SNAP25 which is located on the nerve terminal membrane, whereas BoNTs B, D, F, and G cleave SNARE protein VAMP which is located on the vesicle membrane itself (Fig. 2.2). Inhibition of SNARE proteins in the neuromuscular synapses results in muscle paralysis, and in autonomic synapses, causes loss of gland secretions (saliva, tear). The effect on pain transmitters is discussed in the next chapter (Chap. 3) of this book. Since the action of light chain's protease is zinc dependent and zinc deficiency is not uncommon in western countries, it has been suggested that adding zinc to the dietary regimen (especially when zinc deficiency is suspected) might enhance the therapeutic efficacy of BoNTs [10].

Currently, four types of BoNTs are used widely in clinical practice with the following FDA designations and trade names:

- OnabotulinumtoxinA (onaA)—Trade name: Botox, Allergan Inc., Irvine, CA.
- AbobotulinumtoxinA (aboA)—Trade name: Dysport, Ipsen Biopharm LTD, Wrexham, UK.
- IncobotulinumtoxinA (incoA)—Trade name: Xeomin, Merz Pharmaceuticals LLC, Greensboro, NC.
- RimabotulinumtoxinB (rimaB)—Trade name: Myobloc in the United States and Neurobloc in Europe, Solstice Neurosciences, Inc., San Francisco, CA.

OnaA is provided in vials of 50, 100, and 200 units, incoA in vials of 50 and 100 units, aboA in vials of 300 and 500 units, and RimaB in vials of 2500, 5000, and 10,000 units. A newly FDA-approved toxin (2019) for dermatological indication (glabellar lines), prabotulinumtoxinA (Jeuveau), is provided in vials of 100 units. Although, as emphasized by FDA, units of various BoNTs are not interchangeable, in randomized comparator clinical trials (RCTs), an approximate unit equivalence is sometimes used (1 onaA unit = 1 incoA = 2.5–3 aboA = 40–50 units of rimaB).

All FDA-approved type A toxins (onaA, aboA, incoA, and praA) need to be diluted with preservative-free saline before use. The commonly used dilutions are in 1–2 cc of 0.9% saline. AbotulinumtoxinA was initially approved for 1 cc dilution, but a recent study has proven that 2 cc dilution is also effective [11]. RimabotulinumtoxinA is provided in an already diluted form per vial. Among these

toxins, incoA does not need refrigeration, but all other toxins do. After mixing, gentle shaking is recommended for onaA, but inverting the vial is not recommended for incoA, and the manufacturer recommends gentle shaking and multiple inversions of the vial. All manufactures recommend using the prepared solution of the toxin within 4–24 h after mixing the powder inside of the vial with saline. Liu et al. [12], however, have shown that prepared solution of onabotulinumtoxinA kept up its efficacy up to 6 weeks if kept refrigerated at 39.2°F (4 °C); if frozen, its efficacy lasted up to 6 months. Structure, formulation, pharmacokinetic, and pharmacological properties of botulinum neurotoxins are presented in Table 2.1 [13].

Diffusion and Spread of Neurotoxins

Ramirez-Castaneda et al. [13] have provided a detailed review of diffusion, spread, and migration of the botulinum neurotoxins. Overall, their conclusion was that in most clinical conditions, the diffusion of the toxin is limited, a factor that accounts for its relative safety in clinical practice.

A variety of factors could potentially influence diffusion of the injected BoNTs into the adjacent muscles. The total dose, number of injected sites, volume of injected solution, type of toxin, state of muscle pathology, and status of muscle activity after injection are all potential contributors to the extent of toxin diffusion. Detailed information about these factors is still scarce and evolving, however. Although several studies indicate that local injection of the BoNTs causes abnormal electrophysiological changes in distant and even sometimes contralateral muscles [14–16], these changes do not seem to have meaningful clinical implications since significant weakness of distant muscles rarely occurs after local injection and contralateral weakness has not been convincingly documented in clinical settings.

Animal studies suggest that the extent of diffusion after BoNT injection is dose dependent. In one study [17], injection of 1 unit of onaA into longissimus dorsi muscle of the rabbit demonstrated marked reduction of diffusion gradient beyond 15–30 mm from the site of injection, whereas injection of 5–10 units caused an effect within the entire muscle. The extent of diffusion in this study was defined through acetylcholine esterase staining. As the four commonly used neurotoxins in the United States and Europe (onaA, aboA, incoA, and rimaA) have different molecular sizes (Table 2.1), one would think that toxins with smaller molecular weight (for instance, incoA with total 150 kDa and practically no additional protein) would diffuse more readily and more extensively in the injected tissue. A study in mice, however, has demonstrated that all three type A toxins (onaA, aboA, and incoA) possess a very similar diffusion pattern, regardless of their molecular weight, after injection into the tibialis anterior muscle; most of the toxins remained close to the site of the injection and did not spread to the adjacent muscles [18]. The investigators used neural cell adhesion molecule (N-CAM) as a measure of BoNT diffusion. This molecule is present in embryonic muscle tissue but disappears in adult

Table 2.1 Properties of botulinum neurotoxins

Non-proprietary name: BoNT type	OnabotulinumtoxinA	Abobotulinumtoxin A	Incobatulinumtoxin A	Pimabotulinumtoxin B
Company	Alergan, Inc., Irvine, CA, USA	Ipeen Biopharm Ltd., Wrexham, UK	Merz Pharmaceuticals GmbH, Frankfurt, Germany	Europe/US WorldMeds, Louisville, KY, USA
Trade name	Botox	Dysport	Xeomin	NeuroBioc/Myobioc
Machanism of action	Cleaves SNAP 25	Cleaves SNAP 25	Cleaves SNAP 25	Cleaves VAMP
Molecular weight, kD	900	500–900	150	700
Dosage form	Spray-dried powder	Freeze-dried powder	Freeze-dried power	Sterlie solution
Shef life, mo	36	24	36	24
Storage temperature, °C	<8	<8	<25	<8
pH value after reconstitution	7.4	7.4	7.4	5.6
Excipients	500 mg HSA and 0.9 mg NaCl in 100-U vial	125 mg HSA and 2.5 mg lactese in 500-U vial	1000 mg HSA and 4.7 mg sucrose in 100-U vial	0.5 mg/cc HSA; 0.01 M sodium succinate; 0.1 M NaCl; and SWI in 2500-U, 5000-U, and 10,000-U vials
Units per vial	50, 100, 200	300, 500	50, 100	2500; 5000; 10,000
Recommended volume of reconstitution	Maximum, 10 mL	Maximum, 1 mM	Maximum, 8 mL	0.5 mL; 1 mL; 2 mL
Total protein, ngivial	rv5	rv5	rv0.6	rv50
Antigenic protein load, ng/vial	rv0.8	Unknown	rv0.6	rv10.7
Biologic activity	100 MU-A/vial	500 MU-I/vial	100 MU-M/vial	1.0/2.5/10.0 kMU-E/vial
Specfic activity, Uing	20	40	167	75–125

From Ramirez-Castaneda. Movement Disorders, 2013. Reproduced by permission from publisher (Wiley)
BoNT botulinum toxin, *SNAP* soluble N-ethylmaleimide sensitive fusion protein (NSF) attachment protein, *VAMP* vesicle-associated membrane protien, *HSA* human serum albumin, *NaCl*, sodium chloride, *SWI* sterle water for injection, *MU-A* mouse units in the Alergen mouse lethality assay, *MU-I* mouse units in the Ipsen mouse lethality MU-M mouse units in the Merz mouse lethality assay, *kMu-E*, kilo-mouse unit equivalents.

muscle; it gets activated and reappears after the muscle paralysis caused by intramuscular BoNT injection.

Limited evidence in human suggests that larger volumes of the toxin may increase the diffusion of the toxin within injected and adjacent muscles. In one study, the effect of toxin volume was investigated in 10 human volunteers by injecting two different volumes of onaA (2 units/0.1 cc and 2 unit/0.02 cc) into the forehead muscles [19]. In 9 of the 10 patients, the side of the forehead which received the larger volume (and lower concentration) showed a more extensive diffusion effect. In a randomized, prospective study of 13 patients with spasticity [20], however, the investigators found no difference in efficacy between 50 and 100 units/cc dilutions of onaA preparations. In another double-blind, placebo-controlled study [21], comparing the effect of onaA and rimaB volume in 18 patients with hyperhidrosis, the increased volume of the toxin preparation increased the anhidrotic field for both toxins. The injection of rimaB, however, caused a larger anhidrotic area compared to an equal injected volume of onaA. Increasing injected volume of onaB also demonstrated more diffusion. The conversion ratio in this study was 1 onaA = 75 rimaB. Since the toxins are not truly interchangeable, different ratios have been used in clinical trials between onaA and onaB (from 1:40 to 1: 75); currently, 1:40 is an acceptable ratio [22], and one could argue that the higher dose of B toxin used in this study might have influenced the results. There is a need for larger controlled studies to discern the effect of volume and toxin type on diffusion of different BoNTs.

The effect of a single intramuscular injection versus multiple injections as a factor influencing the diffusion of BoNTs has not been thoroughly studied. Ramirez-Castenada et al. [13] state that multiple point injections along the length of affected muscle retain the biological effect of the toxin within the targeted muscle better than the single injections.

Immunology of BoNTs

The nontoxic protein complex (NAP) of BoNT structure is the main source of antigen formation after BoNT injection. The molecular structure and protein ratios within the nontoxic protein complex of BoNT have been described recently and consist of NBP (124 kDa), HC (90 kDa), LC (53 kDa), NAP-53 (50 kDa), NAP-33 (36 kDa), NAP-22 (24 kDa), and NAP-17 (17 kDa) [23]. Indirect ELISA analysis of BoNT/A and its associated proteins has shown that the BoNT/A protein complex antigen has a 32-fold higher titer than BoNT/A antigen itself, and most of this antigenicity is related to the NAP-33 protein component. In fact, activity of NAP-33 is equal to all the rest of the proteins in the NAP complex combined. The immune response to the botulinum neurotoxins is probably under genetic control, and the major histocompatibility of the host controls the appearance of blocking antibodies and emergence of immunoresistance [24].

The types of antibodies most associated with nonresponsiveness to BoNTs are neutralizing antibodies (nABs). This issue is particularly important when large doses of toxin may be needed such as for patients with severe spasticity or for some patients with advanced cervical dystonia (CD). Most studies of neutralizing antibodies in humans have been conducted with onabotulinumtoxinA (onaA) and in patients with CD. In regard to onaA, development of neutralizing antibodies (nABs) and loss of clinical response have been significantly reduced since the introduction of the new onaA formulation (1997), which contains only 5 ng (rather than 25 ng) of the old formulation in toxin's complex proteins. In one study [25], none of the 119 patients who had received the new onaA formulation developed neutralizing antibodies (nABs) compared to 9.5% among the 130 patients for whom the old formulation of toxin was used for treatment of cervical dystonia. In a prospective, open-label clinical trial, Brin et al. [26] investigated the development of nABs in 326 toxin-naïve patients who had an average of 9 injection sessions over a mean period of 2.5 years. All patients received the new formulation of onaA with a dose per session ranging from 148 to 213 units. Four of 326 subjects (2%) developed neutralizing antibodies against the toxin; three of these four (0.9% of 326) became eventually unresponsive to treatment which is documented by using the frontalis antibody test (FTAT). In another study [27], neutralizing antibodies to onaA were found in 32 of 191 patients (17%) with CD who had at least one to two injections of the old formulation of onaA (containing 25 ng of NAPs). These patients were then enrolled first in an open label and then in a double-blind, placebo-controlled clinical trial using the new toxin over a period of 2 years. One hundred and fourteen patients had antibody assessment both at the entry and at the exit time. Two of 114 patients (1.5%) developed new neutralizing antibodies; both patients, however, remained responsive to BoNT treatment during the course of the study.

These data indicate that with new formulation of onaA (used since 1997), only a small number of treated patients develop neutralizing antibodies and also a small number (less than 1%) manifest clinical unresponsiveness. As commented in a major recent review [1], botulinum neurotoxins, in general, seem to be poor antigens particularly when compared with their cousin molecule, the tetanus toxin. The relation of unresponsiveness to nAB titer and evolution of unresponsiveness over time is complex and deserves clarification through further investigations.

Regarding rimaB, an earlier communication based on data from a small number of patients with cervical dystonia (CD) had shown a high rate of nAB titers (in mouse assay) corresponding to unresponsiveness after 9 rimaB injection cycles in 44% of the studied population [28]. In a review paper [29], authors scrutinized the data of 4 large-scale RCTs conducted on rimaB efficacy in CD (1134 patients) in which nAB levels were provided. Authors found neutralizing antibodies in over 20% of patients, but there was no difference between nAB^+ and nAB− patients in regard to efficacy and continued responsiveness. They concluded that the presence

of neutralizing antibodies has no meaningful clinical significance in patients treated with rimaB. These issues suggest existence of major immunological differences between the two toxins, the importance of which deserves further exploration.

Chen and Dashtipour [30] summarized the relative immunogenicity of different BoNTs based on the total NAPs of each toxin:

> The total protein content (150 kD) toxin including nABs/ 100 units for ABO, INCO, ONA, and RIMA are 0.87, 0.44, 5, and 2.2 ng, respectively. Assuming that a dose equivalency ratio of INCO:ONA is 1:1, the total protein load with INCO (0.44 ng/100 units) would be at least 10-fold less than that of ONA (5 ng/100 units). If the dose equivalency ratio of ABO: ONA is 2:1–3:1, then the total protein load with ABO would be 2–3-fold less than that of ONA for each clinical dose. Thus, theoretically, INCO would carry a lower risk of immunogenicity, followed by ABO, ONA, and RIMA.

There is evidence that some cross-reactivity exists between type A and type B toxins. The first toxin could prime the immune response to stimulate the production of neutralizing antibodies to the second serotype faster than in a naïve individual devoid of antitoxin antibodies [31].

Overall, the above-mentioned data indicate low impact of immunogenicity in the current practice with all four commonly used FDA-approved BoNTs. Many factors influence the development of neutralizing antibodies and the clinical immune response; among these factors are the genetic makeup of the individual and prior exposures to toxins with similar homology, manufacturing process, toxin source, and perhaps the presence of denatured toxin acting as toxoid. Anatomic sites of injection may also be important in the development of immunogenicity, such as in the neck region, which is rich in lymph nodes [1]. Since immunogenicity increases with the dose of the toxin and frequency of administration, it is prudent to avoid excessive dosing and short intervals of application.

Most botulinum toxin clinics in the United States use a brief clinical test for defining unresponsiveness rather than measuring neutralizing antibodies through the cumbersome mouse immune-assay test. The most widely used clinical test is the frontalis antibody test (FTAT) in which the BoNT is injected at two points into frontalis muscle on one side (usually two 10 units for onaA). The injected side is then compared with the uninjected side in 10–14 days. If the BoNT is still effective, the frontalis muscle on the injected side flattens and contracts less compared to the uninjected side. Alternatively, one could use the response of the abductor digiti minimi (ADM) for this purpose. Injection of 15–20 units of onaA or other toxin in a comparable dose into ADM weakens this muscle sufficiently to limit abduction of the little finger. Finally, the response to toxin can be measured also electrophysiologically by recording the change in amplitude of compound muscle action potential (CMAP) in EMG which should show substantial reduction if the toxin is active. FTAT and ADM tests are easy to perform, and in complex cases, one could use both tests to ensure responsiveness to the injected BoNT.

Side Effects of BoNTs

The brochure of FDA-approved BoNTs carries a black box indicating a potential for serious side effects including major disability and even death. This is due to the fact that BoNT is one of the most potent natural toxins and when inappropriately used can be lethal. Absolute contraindications include hypersensitivity to BoNTs and presence of local infection. In practice and in experienced hands, however, BoNT therapy is generally safe, and most side effects are mild and transient. Pain at the site of injection, small local bleeding, and local infection may occur. Local injection of rimaB may cause more pain (compared to A toxins) due to the acidity of the solution; the pH of rimaB solution is 5.6 compared to the alkaline pH of BoNT-As (>7). Mild transient dysphagia after injection of the neck muscles in cervical dystonia occurs in 15% to 20% of patients which is often ignored by the patient and is not mentioned until asked. Chronic cough and upper respiratory tract infection rarely develops with deep neck injections.

Acute hypersensitivity reaction to BoNTs is extremely rare. Theoretically, presence of human albumin in the toxin carries a small risk of slow virus disease. No such case has ever been documented with BoNT treatment over three decades and including millions of patients. Patients with neuromuscular disorders are at risk of deterioration and increased severity of symptoms. BoNT treatment is not recommended in patients with myasthenia gravis or patients taking drugs which are known to significantly impair neuromuscular transmission (e.g., aminoglycosides, neuromuscular blockers). BoNT therapy is also currently not recommended in pregnancy due to the paucity of information in this area. Several new studies, however, have shown that BoNT treatment of pregnant women is safe probably due to low systemic absorption of the toxin and very low toxin transfer through the placenta [32, 33].

In a large multicenter study that included 214 patients, the side effects of onabotulinumtoxinA injection at a mean dose of 241.3 units (range, 95–360 units) were compared with the placebo (saline) [27]. The screened side effects included neck pain, back pain, dysphagia, rhinitis, headache, hypertonia, increased pain, flu symptoms, increased cough, muscle weakness, and sinus infection. Only incidence of rhinitis was significantly higher in the toxin group ($P < 0.05$).

In my nearly 30 years of experience with BoNT therapy, more than half of which included two full days per week of injecting a large number of patients, I have never witnessed a serious side effect requiring hospitalization. Among thousands of injections for cervical dystonia, I had two patients with moderate dysphagia that required close watch for several weeks; both fully recovered. In both cases, there was bilateral injection of anterior neck muscles, and the total dose exceeded 300 units. Despite my positive experience, which is shared by many others, one should not lose sight of the fact that botulinum neurotoxin is one of the most potent toxins in nature. Therefore, clinicians who are engaged in this practice should always pay close attention to proper dosing and dilution. In the case of muscular injection, familiarity with muscle anatomy is essential to avoid injecting the wrong muscles.

Serious side effects should be referred to emergency department immediately and dealt with aggressively since time is essential. Fortunately, with the availability of modern intensive care units, most intoxicated patients when detected early survive with proper and maintained support of respiration.

In the area of pain treatment, which is the subject of this book, logically the patients should experience less side effects than patients with spasticity or dystonia due to a lower dose of the used toxin. Also, injections are usually subcutaneous or intradermal with a lower potential of spreading to vital structures. The literature on BoNT therapy in chronic migraine and studies published on several human pain disorders supports lower incidence of side effects with BoNT treatment in pain disorders (Chaps. 4 to 19 of this book).

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