Expert Opinion on Investigational Drugs

Review

Central & Peripheral Nervous Systems

The therapeutic use of botulinum toxin

Valeria Tugnoli, Roberto Eleopra, Cesare Montecucco & Domenico De Grandis

Since Alan Scott's research, botulinum toxin (BoNT) has been used in several diseases or conditions characterised by muscular overactivity. BoNT acts on either neuromuscular or autonomic cholinergic junctions. Seven different serotypes are known, with antigenic specificity and different therapeutic profiles. BoNT is made up of a heavy chain, involved in binding and membrane translocation, and a light chain, involved in blocking neuroexcytosis. Each serotype shares a specific acceptor on the presynaptic membrane of a cholinergic junction. The available BoNT preparations differ in toxicity, purity and stability. Injection of the neurotoxin produces several modifications at a neuromuscular junction. Axonal sprouting, muscular fibre atrophy, and new end-plates are the most evident histological events after BoNT treatment. They appear to be reversible in untreated muscles. Diffusion can occur at first by haematogeneous or local BoNT spread. Several factors, such as dose, volume, site of injection, muscle size, and muscular fascia, can influence the amount of diffusion and possible side-effects. After prolonged BoNT treatment patients can become unresponsive. Antibodies directed against BoNT have been observed with ELISA or mouse bioassay. Different serotypes have been used to treat non-responder patients. Novel toxins with lower immunogenicity and prolonged clinical efficacy are required for more effective treatment.

Keywords: *botulinum toxin, cholinergic synapse, dystonia, neuromuscular junction*

Exp. Opin. Invest. Drugs (1997) ***6****(10):1383-1394*

1. Introduction

Though physiologists have utilised BoNT to study neuromuscular blockage without provoking nerve injury for a long time, the use of BoNT in human pathology has only recently taken place. In the 1950s, Brooks hypothesised that BoNT could be used to reduce activity in muscle disorders [1], but it was only after Alan Scott's research [2,3] that BoNT injection into extra-ocular muscles was carried out, as an alternative treatment to strabismus surgery [3].

BoNT was selected from several blocking agents due to its functional and predictable effects, which lasted from weeks to months, producing a reversible and quantifiable paralysis. Nevertheless, in clinical practice variable results are frequently observed, thereby forcing clinicians to modify the therapeutic strategy for each individual patient. In recent years, several papers

have reported data on the therapeutic application of BoNT.

The pharmacological approach is generally inappropriate for treating muscular overactivity diseases because of excessive side-effects. However, since 1980, BoNT, the most potent natural poison, has become an important drug for the treatment of muscular overactivity conditions and other human diseases, representing a new challenge to the neurologist.

BoNT treatment demands that the clinician be very familiar with the chemical and functional properties of the neurotoxin in order to be able to choose the correct treatment, and understand the possible side-effects and the varying responses. Moreover, it is essential that both pharmacologists and pathologists learn from previous clinical results in order to improve present day BoNTs and develop new, more effective preparations.

2. BoNT structure and mechanism of action

BoNT acts selectively on cholinergic junctions, both neuromuscular and autonomic, at a presynaptic level, inhibiting acetylcholine release [4,5]. The specific site of action causes the characteristic symptoms of botulism poisoning, with the effects on muscular function and on the autonomic system, similar to those observed after BoNT injections.

BoNT is produced by anaerobic *Clostridium* spp. (botulinum, butyricum and barati) in seven different serotypes (indicated with letters from A to G). BoNT A, B, E, and sporadically BoNT/F, are responsible for human disease [6]. Up to now. only one case of BoNT C intoxication has been described in humans [7].

Molecular genetic techniques have allowed the elucidation of the amino acid sequences of all the BoNT serotypes [8]. They vary in length from the 1251 residues of BoNT/E to the 1297 residues of BoNT/G. All the BoNTs are produced as single chains and are accumulated in the bacterial cytosol until they are released by cell lysis as complexes with non-toxic protein components of different sizes. These non-toxic accessory proteins protect the toxins during their passage through the acid and proteolytic environment of the stomach [8-10]. In a mildly alkaline solvent (such as that of intestine), the complex dissociates and releases the toxin, which has to be proteolytically cleaved (nicked) at a single site to be activated. Hence, the active form of BoNTs is a disulfide-linked dichain protein consisting of a heavy chain (100 kDa), responsible for the binding and entry into neurones, and a light chain (50 kDa), which is a metalloprotease involved in blocking neuroexocytosis [11]. The crystallographic structure of BoNT/A should be resolved in the near future. The protein is made of three distinct domains: the light chain and the two domains of the heavy chain. This is in agreement with a previous suggestion [12]. The amino terminal domain is made of α-helices involved in membrane translocation, whereas the carboxyl terminal domain is a lecitin-like domain, which binds to the receptor [13].

The toxic mechanism consists of four steps:

- binding
- internalisation
- membrane translocation
- intracellular poisoning

2.1 Binding

Binding of BoNTs to the neuronal receptors, located on the presynaptic membrane of cholinergic terminals, has not yet been demonstrated. It is possible that the seven serotypes of BoNT recognise different receptors. A wealth of data indicates that negatively charged oligosaccharides present on lipids and proteins are involved in BoNT binding. The effective binding occurs quickly in neuromuscular isolated preparations [14], at very low BoNT concentration (picomolar or less), even without the stimulus of high temperature or electricity. It is one of the most potent neurotoxins and inhibiting its action in pathology and is internalised within a few hours, necessitating rapid treamtent to inhibit its pathological action. Only a small amount of BoNT injected in human treatment is required to obtain effective neuromuscular block, while a portion is probably lost in body fluids, without reaching the target.

Gangliosides [15,16] and, more recently, sialoglycoproteins [17] are considered important binding components. Different quantitative or qualitative receptor variability in the human population could be a possible factor in explaining some different responses observed in BoNT treatment.

2.2 Internalisation

BoNT internalisation after specific receptor binding occurs by endocytosis. This process is very common in cellular events, is energy dependent [18,19], and determines the entrapment of the neurotoxin inside the lumen of intracellular vesicles, from which at least the light chain has to escape and to enter the cytosol.

2.3 Membrane translocation

Membrane translocation is not yet fully understood. The passage into the cytosol can occur only after acidification of the endosome lumen by a proton pump [20]. The N-terminal half portion of the heavy chain is

the segment most involved in this process. The structure of the neurotoxin is modified with the exposure of hydrophobic segments. This modification can allow the formation of channels which permit the translocation of the light chain into the cytosol.

2.4 Intracellular poisoning

The light chain terminal of BoNT is a metalloprotease (zinc endopeptidase) [21], which is very specific for one of the three protein components of the neuroexocytosis apparatus. BoNT/A and E cleave SNAP-25; BoNT B-D-F-G cleave VAMP, and BoNT/C cleaves SNAP 25 and syntaxin. The neurotoxins that recognise the same target catalyse the hydrolysis of different protein sites [22,23]. The cleavage of VAMP or SNAP-25 or syntaxin at single sites results in their permanent inactivation and, hence, a persisting block of acetylcholine release. Nonetheless, since VAMP, SNAP-25 and syntaxin play different roles in neuroexocytosis and are replaced by newly synthesised molecules at different rates, different times of recovery from neuromuscular paralysis caused by different BoNT serotypes are observed. Moreover, the different light chains of the seven BoNTs can persist in a functional form in the neuronal cytosol for different time periods. It can be anticipated that the employment of different BoNTs can be optimised for the treatment of different pathologies and that chimeric toxins will prove to be very valuable [24].

3. Botulinum toxin preparation

In 1928 Snipe and Sommer of the Hooper Foundation at the University of California described the possibility of purifying BoNT/A from culture broth; by acidification to pH 3.5, 90% BoNT/A could be obtained as a precipitate [25]. In 1946, Lamanna *et al.* first described the crystalline form of BoNT/A [26,27]. In the same period, Mueller obtained high levels of BoNT/A from the A Hall strain [28]. Improved methods were developed with the aim of obtaining purified BoNT [29]. With the discovery of the therapeutic potential of BoNT, efforts were made to improve the preparation of this toxin. Standards of quality, safety, stability and efficacy were required [30]. Added enzymes, or other substances such as animal meat, synthetic solvents and resins, which could contaminate the final product, had to be avoided. Furthermore, some speculated that the latter precautions were not sufficient to ensure complete safety in the final product [24]. Different laboratories have described their own methods to produce BoNT for human injections. Different methods have been applied to purification. Hambleton *et al.*, in Porton Down, UK, described precipitation with antichaotropic and ion exchange chromatography [31]. Schantz *et al.*, in Wisconsin, USA, applied successive re-precipitation with different substances to obtain 60 - 70 mg of crystals from 12 l of culture [30].

The crystalline form of BoNT/A consists of 900,000 kDa, which is formed by 150,000 KDa toxic protein and non-toxic macromolecules. Different methods, such as light absorption or electrophoresis, are described with the aim of verifying the purity of the BoNT preparation, without achieving universal agreement on the best test [24]. The two forms can be separated at alkaline pH [32]. The non-toxic components are made up of proteins which may or may not precipitate erythrocytes (haemagglutinins and non-haemagglutinins). They have an important role in stabilising secondary and tertiary BoNT structures, maintaining the BoNT toxic shape. The heavy complex (900,000 KDa) shows resistance and more toxicity in comparison to pure neurotoxin in alkaline environments, while pure BoNT shows greater toxicity when injected directly into muscles. Very little is known about the role of the non-toxic proteins in human treatment, especially in their possible inflammatory effects, in delaying BoNT action, or their possible contribution to immunogenic response.

In 1984, the US FDA licensed BoNT/A as an orphan drug named OCULINUM, and in 1989 as a full drug, named BOTOX, by Allergan, USA. In the same period in the UK, BoNT/A was distributed for research and only in 1991 was it registered as DYSPORT, presently manufactured by Speywood. In Japan a new BoNT/A preparation, named CS-BOT, by Chiba Serum Institute was used in humans [33].

3.1 Toxicity

The commercially available preparations of BoNT/A are different regarding both dosage and potency. To standardise the data for different drugs, the potency is defined by the biological mouse assay, in IU (International Unit), as the quantity of BoNT required to kill for 50% of a group of 18 - 20 female Swiss-Webster 20 - 30 g white mice by ip. injection. Unfortunately, a distinct variation was observed due to the different bioassay tests utilised by the different manufacturers. Data showed that the potency of the BoNT/A preparations varied between 0.075 - 0.032 ng for 1 mouse LD_{50} [34]. The different results were attributed to different responses in mice, also some variations in environmental and technical properties [24]. From a study in monkeys, the dose for human intoxication has been calculated at about 3000 IU.

To date, two commercial preparations are available; they are produced in vials of 100 IU or 40 ng for BOTOX (Allergan, USA) and 500 IU or 12.5 ng for DYSPORT (Speywood, UK). As the potency and cost

of a single vial is similar for both BoNT/A preparations, the relative potency between American and British products is 3 - 5:1. New methods of quantification of neuromuscular block in specific neuromuscular junctions *in vitro*, by electrophysiology, have stimulated recent interest [35-37]. *In vivo*, a neurophysiological test on the extensor digitoris brevis muscle of a toe (EBD) is recommended to test individual sensitivity and the temporal profile for the different serotypes of BoNT [38,39]. The method, simple and not time-consuming, correlates well to clinical BoNT effect, in non-responder patients. The clinical evaluation of the denervation field produced by injection of a small dosage of BoNT in the frontalis muscle has also been performed [40].

3.2 Storage and stability

The BoNT used in the clinic is a crystalline preparation, type A of American or British production, dispensed in vials, freeze-dried. The preparation is stable for months to years if frozen or refrigerated, while it loses more than 40% of its toxicity if reconstituted at -6°C for more than 6 h [41]. The loss of potency, related to the low concentrations routinely used, is supplemented by the addition of animal or human albumin. The pH value is also an important variable; the stability observed in acidic conditions diminishes significantly if the pH is raised above 7, probably because of the activation of proteases. This does not occur if the toxin is highly purified or if it is pure. Some researchers have studied the loss of potency of BoNT quantitatively, reconstituted with saline solution, for routine use. The interest in solving this question is derived from the necessity of using small quantities of BoNT in some diseases, such as spastic dysphonia, for a limited population, without discarding the unused toxin, so as to limit the high cost of treatment. A lot of data suggest that the stability of the different commercial preparations is not similar, probably in relation to the different association of non-toxic substances [41]. Immediate refreezing (-20 to -70°C) or refrigerating (+4°C) has led researchers to different conclusions, such as no drop in clinical effect [42,43] or a reduction of 69.8% of BoNT/A toxicity if utilised after 2 weeks [41]. Partial inactivation, due to bad storage, can cause the formation of toxoids, which are without clinical effect, but potentially immunogenic. More complex is the storage of pure BoNTs, as they result in particularly unstable, if are not treated with proteins, such as albumin. In this way, long-term storage is possible at high concentrations [42], while no significant data are available for low concentrations, such as those usually adopted in human treatment [45,46].

4. Biological effects

4.1 Histological features

At first, some caution for the use of BoNT treatment stemmed from a fear of inducing irreversible alterations in muscles but now histochemical studies have been carried out in animals and humans [47-51]. After BoNT/A injection, several modifications in muscles and neuromuscular structures occur, always with a typical temporal profile. Firstly, synaptic vesicles accumulate at the presynaptic membrane due to neuromuscular blockage and the inhibition of discharging neurotransmitters in synaptic space [51-53]. The contact between the axons and the muscle fibre persists. Growth factors are released by functional denervated muscles determining several modifications of terminal axonal arborisation [54-56].

Terminal and, rarely, collateral sprouting from distal nodes of Ranvier are observed 7 - 10 days after BoNT injection [57]. The axon direction follows the basal lamina which maintains its integrity. Muscular fibres show atrophy, which begins 1 - 2 weeks after injection, and lasts up to 4 - 6 weeks, showing a significant size variability [58]. These aspects lead to specific neurophysiological features (fibrillation potentials and positive sharp waves, polyphasicity, impaired maximal effort pattern), which can be studied with the aim of detecting and quantifying BoNT activity *in vivo*.

The muscular membrane changes its properties in a similar way to that observed in denervation [59]. The abnormal spread of acetylcholinesterase and of acetylcholine receptors along the fibre in extra-junctional sites appears. New small end-plates are constituted, generally reached by thin axons. These phenomena are more rapid in slow-twitch than in fast-twitch muscles [60]. During the effect of neuromuscular blockage the muscular fibre can be innervated by another motor axon at a different end-plate region; the original end-plate expands and becomes discontinuous [50].

These aspects are transient and decrease with time after BoNT injections, until the muscle recovers its original structure and dimensions [61]. The histological features of a muscle four months after the treatment with BoNT/A are similar to those of untreated muscle [55,58]; the atrophy is reversible, but some neuromuscular and motor axon abnormalities can be observed in muscles treated for a long time [51].

4.2 Distant effects

After BoNT injection, cholinergic blockage and related functions are also shown at sites distant from the first treated muscle. The hypothesised mechanisms of diffusion are the following:

- spread through tissue by local diffusion along a concentration gradient
- retrograde axonal transport to anterior horn cells
- haematogeneous diffusion
- diffusion through muscle fibres to spindle structures
- trans-synaptic diffusion to contralateral motor neurones or centrally to the brain

Each of the above explains events frequently observed in BoNT treatment.

The local diffusion near the treated muscle, across fascia structures and within other muscular groups, is responsible for side-effects related to the involuntary paralysis of muscles that are close anatomically to the one originally treated. This effect has been studied in animal models by the detection of denervation fields. Acetylcholinesterase staining estimation along muscle fibres [62] and glycogen depletion variability in muscle fibres blocked by BoNT have been utilised experimentally to quantify the extension of the denervation field [63,64]. In patients treated for haemifacial spasm at orbicularis oculi level, a common diffusion to untreated muscles in the lower facial portion, also of trigeminal innervation, has been identified in a neurophysiological study [65]. While only a few diffuse clinical effects of small significance, such as flu-like symptoms and asthenia [66-68], dysautonomia [69], urinary bladder post-micturition residue [70] and gallbladder dysfunction [71], have been described in patients treated with standard BoNT doses, generally for dystonia, several subclinical effects have been detected by neurophysiological tests.

An abnormal increase of jitter in distant muscles measured by single fibre electromyography (SFEMG) [72-76] and abnormal cardiovascular reflexes [77,78] have documented the systemic effects on neuromuscular and autonomic synapses after BoNT injections, probably by haematogeneous diffusion.

By autoradiographic methods BoNT has been observed in anterior horn cell bodies of the ipsilateral and contralateral spinal cord, by retrograde axonal transportation and trans-synaptic diffusion [79,80], but, up to now, no direct central effects have been demonstrated.

A reduction of reflex activity, through the direct blocking of neuromuscular transmission in spindle gamma loops and successive effects on muscular tone and afferent input, has been shown in animals and humans [81-83] and support BoNT results in dystonia and spasticity [84,85].

5. Clinical features of BoNT treatment

5.1 Therapeutic indications

Since the first report of BoNT administration in strabismus and dystonia, a large number of new applications have been reported in the literature. They include neuromuscular blockage in conditions or diseases characterised by muscular overactivity (**Table 1**).

More recently the use of BoNT in some autonomic diseases with increased cholinergic activity, such as achalasia, rhinorrhea, hyperhydrosis or Frey's syndrome, have been reported (**Table 2**). Some conditions that show improvement with BoNT treatment have a proven application, while other conditions show sporadic amelioration which must be confirmed in larger populations [86].

5.2 Administration (dose, site, muscle, concentration)

A clinician must decide on the following when treating a patient with BoNT:

- in which muscle(s) should injection take place
- the sites of injection
- the dosage of BoNT which should be used for each muscle
- the serotype that should be used
- the dilution of the serotype employed

Each of these represents a variable that can affect the clinical outcome, and is frequently based on a single-centre experience. The amount of data in the recent literature is invaluable material for reciprocal comparison.

Muscles are generally identified by clinical examination, often supported by neurophysiological analysis. Electromyography (EMG) is used both to identify the specific patterns of muscular activity and for direct injection through the recording electrode [87,88]. Various results have been obtained with different studies quantifying the advantages of EMG in BoNT treatment [87].

Clinical experience shows that the dose used is related to the muscle dimension [89]. For example, the dose utilised in the cervical muscle can be 10 times greater than the one utilised in the facial district. From animal models the concentric denervation field has been quantified in relation to the amount of BoNT; with a low dose the diameter was of 30 mm with a gradient inversely related to the point of injection, while with a larger dose the whole muscle is blocked [51]. With high doses more side-effects were observed due to diffusion to adjacent muscles.

Table 1: A list of conditions and diseases caused by muscular overactivity where BoNT therapy has been used. Bold indicates that BoNT treatment has been proven to be beneficial. Not bold indicates that initial positive results must be confirmed in larger populations.

Blepharospasm
Hemifacial spasm
Aberrant facial nerve regeneration
Myokymia
Medical tarsorrhaphy (ptosis)
Strabismus
Oromandibular dystonia
Laryngeal dysphonia
Cervical dystonia
Focal hand dystonia
Occupational cramps
Limb dystonia
Spasticity
Bruxism
Nystagmus
Tremor
Facial wrinkles
Muscle-contraction headache
Myoclonus
Tics
Myofascial pain
Sphincter dyssynergy
Anismus, anal fissure

Table 2: A list of autonomic diseases characterised by cholinergic overactivity where BoNT therapy has been used. These conditions, only recently treated with BoNT therapy, must be verified in larger populations.

Palmer hyperhydrosis
Ascellar hyperhydrosis
Rhinorrea
Achalasia
Hypertrophic pyloric stenosis
Dysphagia (spasm or dystonia of upper oesophageal sphincter)
Frey syndrome
Crocodile-tears syndrome

The site of injection in animal models has quite a different role to that in humans. In experiments, application on the end plate site produes the most evident paralysis [64], while a 50% reduction of effectiveness was noted if the application was only 0.5 cm away from this site. At other sites, several clinical studies have demonstrated that multiple injections reduce complications, such as district hypotrophy or eccentric toxin spread [89], improving clinical results, probably due to a more homogeneous diffusion along the treated muscle. Multiple site administration is more effective than single-site or motor-point BoNT administration [90]. Different techniques can be utilised for muscles with spread or narrow end-plate regions [91].

An increase in BoNT dilution by the use of a greater volume of drug, maintaining a constant toxin dose, produces a greater diffusion along the muscle [64] and a greater incidence of side-effects [92-94]. All the above must be considered in order to optimise treatment for each clinical condition, individual patient and even at different times in the same subject.

5.3 Assessment of response: clinical and instrumental tests

One of the most difficult aspects of BoNT treatment is the impossibility of predicting the clinical outcome. Each treatment is considered an individual case, often characterised by different clinical results. Variability in the response to the toxin or the temporal profile of the response is frequently observed. Fluctuations in original disease can be mistaken for incorrect treatment, e.g., a decrease in muscular basal activity can influence the BoNT effect [38]. The specific characteristics of the neurotoxin used and the method of storage can alter further the clinical results, as can the choice of different technique strategy.

Understanding the pattern of the disease before and after BoNT treatment is essential in order to understand the real factors responsible for effective therapy. Video-recordings, clinical scores, neurophysiological examinations or electromagnetic tracking systems have been used to assess the course of the treatment [95-105].

5.4 Side-effects (diffusion)

The success of BoNT treatment is also related to the absence of side-effects. Few reports of systemic symptoms, such as diffuse asthenia in ALS patients or in high dose spasticity treatment [66] or brachial plexus immune-mediated disease [107], have been reported after BoNT administration, and these only sporadically. More frequently, the common side-effects are related to the spread of the neurotoxin to the muscles or

cholinergic vegetative systems located near the treated regions, by local diffusion.

Although no teratogenic properties of BoNT have been demonstrated, use in pregnancy is discouraged. Nevertheless, BoNT treatment during pregnancy has not shown any complications [108].

5.5 Resistance to BoNT

Patients who have undergone BoNT therapy, can become unresponsive (secondary non-responder). Clinical modification of the original disease, inappropriate choice of muscle, or BoNT dosage may be responsible, along with antibodies directed against the drug.

There have only been a few reports of patients who show no response to first time treatment with BoNT/A (primary non-responder). The cause for this is at present not known (previous immunisation has been hypothesised) [109]. Repeated BoNT/A treatments, particularly with large doses [110] and with high frequency of administration [111], can lead to immune-mediated resistance.

There are two distinct methods that can be employed to study BoNT immune-resistance: enzyme immunosorbent assay (ELISA) and mouse bioassay. The first method detects circulating antibodies directed against both the neurotoxin itself and the associated proteins. The presence of these antibodies does not always correlate with clinical tolerance, because only those antibodies that neutralise the biological effect of BoNT are responsible for the lack of response [112-115]. Even if a higher statistical presence of antibodies in patients with tolerance to BoNT is observed, a clear correlation between antibody titre and clinical resistance has not been documented. Furthermore, ELISA appears to be poorly specific due to the occurrence of cross-reactions between different serotypes [116-119]. Systems utilising monoclonal antibody reagents have been employed to improve specificity, but they often lack sensibility [119]. At present, mouse bioassay is the most effective test. An increasing dilution of patient serum is injected into animals with a test dose of neurotoxin. The neutralisation of BoNT's toxic effects by patient antibodies is evaluated. Even if difficult and expensive, this is the only reliable method that can document an immune-mediated resistance against BoNT [120], perhaps underestimating the real incidence of sensitisation [121]. New alternative bioassays have recently been proposed [119,122].

Some observations indicate that immunogenicity against BoNT can be transient and not absolute [123]. This is an interesting point, which might explain the need to increase the dosage in some patients, who are partial non-responders, or for the re-use BoNT after a time of suspension. However, in our clinic, the patients who have become non-responders, according to a neurophysiological test, maintain resistance to BoNT. The percentage of patients who carry antibodies and tolerance to BoNT are different in several reports. Generally they vary from 0 - 10% [110].

6. The clinical use of different BoNTs

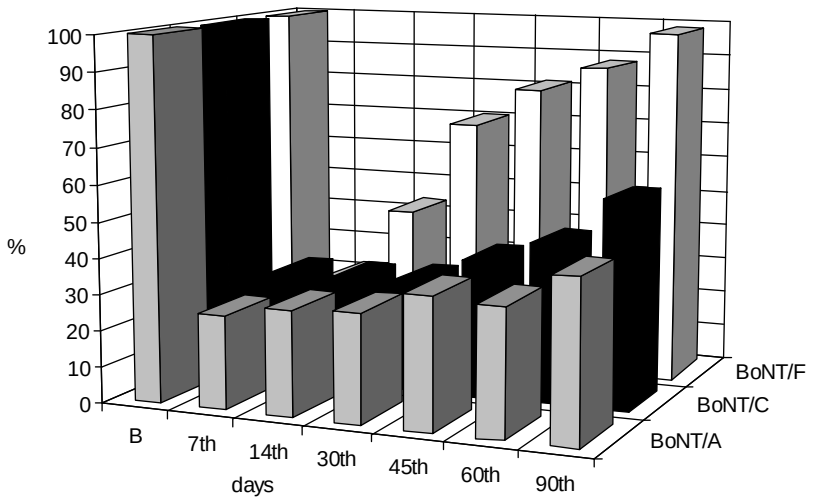
BoNT/A is widely used. Other BoNT serotypes have been used in animal models to compare biological effects with the better understood BoNT/A. In rats, the paralysis produced by the same doses of BoNT/A and other serotypes are quite different, the former being more active [124,125].

Clinicians have used other BoNT serotypes in both animals and humans due to tolerance observed in patients treated for a long time with high doses of BoNT. Clinical applications of BoNT/F, B and C have been performed [60,108,127-130]. For BoNT/F and B, the results have shown a good response, similar to that obtained by BoNT/A, but the effects have lasted for a shorter period of time. For BoNT/C the profile is longer than for F or B, and very similar to that of the A serotype. In **Figure 1,** the different temporal profiles and the amount of neuromuscular blockage for different serotypes F and C (obtained from Wako, Japan and tested in BALB/C mice) have been compared to BoNT/A (Botox/Allergan) with neurophysiological testing [38].

The variability in clinical effects observed can be related to different sites of action (double only for BoNT/C) of the BoNT tested or to their different active life, or to the different periods of time necessary for the re-synthesis of the specific targets (VAMP, SNAP-25 and syntaxin).

Previously, the other serotypes were used in patients who were non-responders to BoNT/A; now, a more ambitious application is being undertaken. Taking advantage of the biological differences of the serotypes, the BoNT serotype with the 'correct' properties can be selected to treat a particular condition. For example, the use of BoNT with a short active life can be applied for the treatment of transient situations, such as in surgical complications or in addition to physical rehabilitation. BoNT/B and F, which act on cholinergic autonomic structures [69,130], can be used to treat specific applications, such as in hyperhydrosis. At present, multiple association of different neurotoxin serotypes does not appear to be more useful than single toxin serotype application. Synthesis of complexes with different neurotoxin properties, chimeric toxins, is being attempted [24,86].

Figure 1: Temporal profile of neuromuscular blockage obtained by small injections of different BoNTs (A, C, F) into the extensor digitoris brevis muscle, calculated by neurophysiological tests. BoNT/A and BoNT/C profiles are similar. BoNT/F shows a more rapid effect with early recovery of the original conditions, these aspects correspond to clinical observations in patients treated with BoNT/F.



7. Expert opinion

Since the first application of BoNT/A in strabismus, many more conditions and diseases have been treated with this neurotoxin. In particular, BoNT treatment has been used in neurological conditions where no relief is obtained with 'classical' drugs.

At present, BoNT treatment is considered safe and effective in focal dystonia and hemifacial spasm, while the use of this neurotoxin in other indications requires further proof. As BoNT treatment is symptomatic and multiple administration is required, each new patient must be considered as an individual case. In this way, possible side-effects or specific disorders provoked by BoNT are tolerated by each patient.

Excessive variability in clinical response after each treatment is currently an obstacle to satisfactory patient management. Products with improved stability and fixed toxic properties are needed. Despite the fact that studies on the long-term effects of BoNT underline the safety and efficacy of treatment, non-responder patients and antibody production have been reported.

The 'intelligent' application of various BoNT serotypes, not only in secondary non-responder patients, but also in situations which require different results with regard to temporal profile or amount of cholinergic blockage, or different efficacy, for example, in neuromuscular or vegetative systems, will be a further significant improvement. The synthesis of new complexes, combining different portions of the well-known BoNTs, which could be differentiated according to any requirement, could be used for 'personalised' treatments. Furthermore, the search for novel neurotoxins with lower immunogenicity and prolonged clinical efficacy must be pursued in order to reduce side-effects and increase tolerability.

Bibliography

1. SCHANTZ EJ: **Historical perspective.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M (Eds.), New York, Marcel Dekker, Inc. (1994) xxiii-xxvi.

2. SCOTT AB: **Botulinum toxin injection of eye muscles to correct strabismus.** *Trans. Am. Ophthalmol. Soc.* (1981) **79**:734-770.

3. SCOTT AB: **Botulinum toxin injection of eye muscles as an alternative to strabismus surgery.** *Ophthalmology* (1989) **87**:1044-1049.

4. SIMPSON LL: **The origin, structure, and pharmacological activity of botulinum toxin.** *Pharmacol. Rev.* (1981) **33**:155-188.

5. SIMPSON LL: **The action of clostridial toxins on storage and release of neurotransmitters.** In: *Natural and Synthetic Neurotoxins.* Harvey A (Ed.), San Diego, Academic Press (1993) 278-317.

6. HATHEWAY CL: **Botulism: the present status of the disease.** *Curr. Top. Microbiol. Immunol.* (1995) **195**:55-75.

7. OGUMA K, YOKOTA K, HAYASHI S *et al.*: **Infant botulism due to *Clostridium botulinum* type C toxin.** *Lancet* (1990) **336**:1449-1450.

8. MINTON NP: **Molecular genetics of clostridial neurotoxins.** *Curr. Top. Microbiol. Immunol.* (1995) **195**:161-191.

9. SAKAGUCHI G: ***Clostridium botulinum* toxins.** *Pharmacol. Ther.* (1983) **19**:165-194.

10. SHONE CC, TRANTER HS: **Growth of Clostridia and preparation of their neurotoxins.** *Curr. Top. Microbiol. Immunol.* (1995) **195**:143-160.

11. SCHIAVO G, ROSSETTO O, SANTUCCI A, DSGUPTA BR, MONTECUCCO C: **Botulinum neurotoxins are zinc proteins.** *J. Biol. Chem.* (1992) **267**:23479-23483.

12. MONTECUCCO C, SCHIAVO G: **Tetanus and botulinum neurotoxins: a new group of zinc-endopeptidases.** *Trends Biochem. Sci.* (1993) **18**:324-327.

13. STEVENS R: *International Conference on the Molecular Genetics and Pathogenesis of the Clostridia*. Seillac, France (1997).

14. DOLLY JO, BLACK J, WILLIAMS RS, MELLING J: **Acceptors for botulinum toxin reside on motor nerve terminals and mediate its internalisation.** *Nature* (1984) **307**:457-460.

15. SIMPSON LL, RAPPORT MM: **Ganglioside inactivation of botulinum toxin.** *J. Neurochem.* (1971) **18**:1341-1343.

16. SIMPSON LL, RAPPORT MM: **The binding of botulinum toxin to membrane lipids: phospholipids and proteolipid.** *J. Neurochem.* (1971) **18**:1761-1767.

17. BAKRY N, KAMATA Y, SIMPSON LL: **Lectins from *Triticum vulgaris* and *Limax flavus* are universal antagonists of botulinum neurotoxin and tetanus toxin.** *J. Pharmacol. Exp. Ther.* (1991) **258**:830-836.

18. SIMPSON LL: **Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction.** *J. Pharmacol. Exp. Ther.* (1980) **212**:16-21.

19. SIMPSON LL, KAMATA Y, KOZAKI S: **Use of monoclonal antibodies as probes for the structure and biological activity of botulinum neurotoxin.** *J. Pharmacol. Exp. Ther.* (1990) **255**:227-232.

20. SIMPSON LL,COFFIELD JA, BAKRY N: **Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A_2 neurotoxins.** *J. Pharmacol. Exp. Ther.* (1994) **269**:256-262.

21. BARRETT AJ: **Proteolytic enzymes: aspartic and metallopeptidases.** In: *Methods in Enzymology.* Barrett AJ (Ed.), Academic Press (1995) 248.

22. MONTECUCCO C, SCHIAVO G: **The structure and function of tetanus and botulinum neurotoxins.** *Quart. Rev. Biophys.* (1995) **28**:423-472.

23. ROSSETTO O *et al.*: **SNARE motif and neurotoxin recognition.** *Nature* (1995) **372**:415-416.

24. DASGUPTA BR: **Structures of *Botulinum neurotoxin*, its functional domains, and perspectives on the crystalline type A toxin.** In: *Therapy with Botulinum toxin*. Jankovic J, Hallett M (Eds.), New York, Marcell Dekker, Inc. (1994) 15-39.

25. SNIPE PT, SOMMER H: **Studies on *Botulinum toxin*. 3. Acid preparation of botulinum toxin.** *J. Infect. Dis.* (1928) **43**:152-160.

26. LAMANNA C, EKLUND HW, MCELROY OE: **Botulinum toxin (type A): including a study of shaking with chloroform as a step in the isolation procedure.** *J. Bacteriol.* (1946) **52**:1-13.

27. LAMANNA C, CARR CJ: **The botulinal, tetanal, and enterostaphylococcal toxins: a review.** *Clin. Pharmacol. Ther.* (1967) **8**:286-332.

28. LEWIS KH, HILL EV: **Practical media and control measures for producing highly toxic cultures of *Clostridium botulinum* type A.** *J. Bacteriol.* (1947) **53**:213-230.

29. DUFF JT, WRIGHT GG, KLERER J, MOORE DE, BIBLER RH: **Studies on immunity to toxins of *Clostridium botulinum*. I. A simplified procedure for isolation of type A toxin.** *J. Bacteriol.* (1957) **73**:42-47.

30. SCHANTZ EJ, JOHNSON EA: **Properties and use of botulinum toxin and other microbial neurotoxins in medicine.** *Microbiol. Rev.* (1992) **56**(1):80-99.

31. HAMBLETON P: ***Clostridium botulinum* toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use.** *J. Neurol.* (1992) **239**:16-20.

32. DASGUPTA BR, BOROFF DA: **Separation of toxin and hemagglutinin from crystalline toxin of *Clostridium botulinum* type A by anion exchange chromatography and determination of their dimensions by gel filtration.** *J. Biol. Chem.* (1968) **243**:1065-1072.

33. MEZAKI T, KAJI R, HAMANO T *et al.*: **Optimisation of botulinum treatment for cervical and axial dystonias: experience with a Japanese type A toxin.** *J. Neurol. Neurosurg. Psychiat.* (1994) **57**:1535-1537.

34. SCHANTZ EJ, KAUTTER DA: **Standardization assay for *Clostridium botulinum* toxins.** *J. Assoc. Anal. Chem.* (1978) **61**:96-99.

35. LOMNETH R, SUSKIW JB, DASGUPTA BR: **Response of the chick ciliary ganglion, iris neuromuscular preparation to botulinum neurotoxin.** *Neurosci. Lett.* (1990) **113**:211-216.

36. SIMPSON LL: **Kinetic suites on the interaction between toxin type A and the cholinergic neuromuscular junction.** *J. Pharmacol. Exp. Ther.* (1980) **212**:16-21.

37. CLARK AW, BANDYOPADHYAY S, DASGUPTA BR: **The plantar nerves-lumbrical muscles: a useful nerve-muscle preparation for assaying the effects of *Botulinum neurotoxin*.** *J. Neurosci. Meth.* (1987) **19**:285-295.

38. ELEOPRA R, TUGNOLI V, DE GRANDIS D: **The variability in the clinical effect induced by botulinum toxin type A: the role of muscle activity in humans.** *Mov. Disord.* (1997) **12**:89-94.

39. SLOOP RR, ESCUTIN RO, MATUS JA, COLE BA, PETERSON GW: **Dose-response curve of human extensor digitorum brevis muscle function to im. injected botulinum toxin type A.** *Neurology* (1996) **46**(5):1382-1386.

40. BORODIC GE, PEARCE B, DUANE D, JOHNSON E: **Antibodies to botulinum toxin.** *Neurology* (1995) **45**:204.

41. GARTLAU MG, HOFFMAN HT: **Crystalline preparation of botulinum toxin type A (Botox): degradation in potency with storage.** *Otoryngol.-Head Neck Surg.* (1993) **108**:135-140.

42. WILLIAMS RS, TSE CK, DOLLY JO, HAMBLETON P, MELLING J: **Radioiodination of botulinum neurotoxin type A with retention of biological activity and its binding to brain synaptosomes.** (1983) **131**:437-445.

43. GREENE P: **Clinical trials for cervical dystonia 2.** In: *NIH Consensus Development Conference on Clinical Use of Botulinum Toxin Program* (1990) **53**:6.

44. COLE BA, SLOOP RR, ESCUTIN RO: **Effect on refreezing or refrigerating reconstituted Botulinum A toxin on human muscle paralysis.** *Neurology* (1995) **45**(Suppl. 4A):457.

45. SIMPSON LL, DASGUPTA BR: **Botulinum neurotoxin type E: studies on mechanism of action and on structure-activity relationship.** *J. Pharmacol. Exp. Ther.* (1983) **224**:135-140.

46. TSE CK, DOLLY JO, HAMBLETON P, WRAY D, MELLING J: **Preparation and characterization of hogeneous neurotoxin type A from *Clostridium botulinum*. Its inhibitory action on neuronal release of acetylcholine in the absence and presence of β-bungaro toxin.** *Eur. J. Biochem.* (1982) **122**:493-500.

47. DUCHEN LW: **An electron microscopic study of the changes induced by botulinum toxin in the motor end plates of slow and fast skeletal muscle fibres of the mouse.** *J. Neurol. Sci.* (1971) **14**:47-60.

48. DIAZ J, MOLGÒ J, PÉCOT-DECHAVASSINE M: **Sprouting of frog motor nerve terminals after long-term paralysis by botulinum type A toxin.** *Neurosci. Lett.* (1989) **96**:127-132.

49. SPENCER RF, MCNEER KW: **Botulinum toxin paralysis of adult monkey extraocular muscle.** *Arch. Ophthalmol.* (1987) **105**:1703-1711.

50. ALDERSEN K, HOLDS JB, ANDERSON RL: **Botulinum-induced alterations of nerve-muscle interactions in the human orbicularis oculi following treatment for blepharospasm.** *Neurology* (1991) **41**:1800-1805.

51. BORODIC GE, FERRANTE RJ, PEARCE LB, ALDERSON K: **Pharmacology and histology of the therapeutic application of Botulinum toxin.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M (Eds.), New York, Marcell Dekker, Inc. (1994) 119-157.

52. KAO I, DRACHMAN D, PRICE DL: **Botulinum toxin: mechanism of presynaptic blockade.** *Science* (1976) **193**:1256.

53. MONTECUCCO C, SCHIAVO G: **The structure and function of tetanus and botulinum neurotoxins.** *Quart. Rev. Biophys.* (1995) **28**:423-472.

54. PESTRONK A, DRACHMAN DB, GRIFFIN JW: **Effect of Botulinum toxin on trophic regulation of acetylcholine receptors.** *Nature* (1976) **264**:787-789.

55. YEE WC, PESTRONK A: **Mechanisms of postsynaptic plasticity: remodeling of the junctional acetylcholine receptor cluster induced by motor nerve terminal outgrowth.** *J. Neurosci.* (1987) **7**:2019-2024.

56. ANGAUT-PETIT D, MOLGO J, COMELLA JX, FAILLE L, TABTI N: **Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features.** *Neuroscience* (1990) **37**:799-808.

57. HOLDS JB, ALDERSON K, FOGG SG, ANDERSON RL: **Terminal nerve and motor end plate changes in human orbicularis muscle following botulinum A toxin injection.** *Invest. Ophthalmol. Vis. Sci.* (1990) **31**:178-181.

58. BORODIC GE, FERRANTE R: **Histological effects of repeated botulinum toxin over many years in human orbicularis oculi muscle.** *J. Clin. Neuro.-Ophthalmol.* (1992) **12**:121-127.

59. MILEDI R: **The acetylcholine sensitivity of muscle fibers after complete and partial denervation.** *J. Physiol.* (1960) **151**:1-23.

60. MOYER E, SETLER PE: **Botulinum toxin type B: experimental and clinical experience. Comparative muscle paralytic efficacy.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M (Eds.), New York, Marcell Dekker (1994) 76-78.

61. HARRIS CP, ALDERSON K, NEBEKER J, HOLDS JB, ANDERSON RL: **Histological features of human orbicularis oculi treated with botulinum A toxin.** *Arch. Ophthalmol.* (1991) **109**(3):393-395.

62. BORODIC GE, JOSEPH M, FAY L, CAZZOLINO D, FERRANTE R: **Botulinum A toxin for the treatment of spasmodic torticollis. Dysphagia and regional spread.** *Head Neck* (1990) **12**:392-398.

63. SHAARI CM, GEIROGE E, WU BL, BILLER HF, SANDERS I: **Quantifying the spread of botulinum toxin through muscle fascia.** *Laryngoscope* (1991) **9**:960-964.

64. SHAARI CM, SANDERS I: **Quantifying how location and dose of botulinum toxin injections affect muscle paralysis.** *Muscle Nerve* (1993) **16**:964-969.

65. ELEOPRA R, TUGNOLI V, CANIATTI L, DE GRANDIS D: **Botulinum toxin treatment in the facial muscles of humans: evidence of an action in untreated near muscles by peripheral local diffusion.** *Neurology* (1996) **46**(4):1158-1160.

66. BRIN MF, FAHN S, MOSCOWITZ C *et al.*: **Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm.** In: *Dystonia*. Fahn S, Marsden CD (Eds.), New York, Raven Press (1988) 559-608.

67. LORENTZ IT, SUBRAMANIAM SS, YANNIKAS C: **Treatment of idiopathic torticollis with botulinum toxin A: a double-blind study on 23 patients.** *Mov. Disord.* (1991) **6**:145-150.

68. NIX WA, BUTLER IF, ROONTGA S, GUTMANN L, HOPF HC: **Persistent unilateral tibialis anterior muscle hypertrophy with complex repetitive discharges and myalgia: report of two unique cases and response to botulinum toxin.** *Neurology* (1992) **42**:602-606.

69. TUGNOLI V, ELEOPRA R, DE GRANDIS D: **Acute cholinergic failure in patient treated with botulinum toxin type F for cervical dystonia.** *Electroenceph. Clin. Neurophysiol.* (In Press).

70. SCHNIDER P, BERGER T, SCHMIED M, FERTL L, AUFF F: **Increased residual urine volume after local injection of botulinum A toxin.** *Nervenarzt* (1995) **66**(6):165-467.

71. SCHNEIDER P, BRICHTA A, SCHMIELD M, AUFF E: **Gallbladder dysfunction induced by botulinum A toxin.** *Lancet* (1993) **342**:811-812.

72. LANGE DJ, BRIN MF, WARNER CL *et al.*: **Distant effects of local injection of botulinum toxin.** *Muscle Nerve* (1987) **10**:552-555.

73. LANGE DJ, BRIN MF, FAHN S *et al.*: **Distant effects of locally injected botulinum toxin: incidence and course.** In: *Dystonia*. Fahn S, Marsden CD (Eds.), New York, Raven Press (1988) 609-613.

74. LANGE DJ, RUBIN M, GREENE PE *et al.*: **Distant effects of locally injected botulinum toxin: a double-blind study of single fiber EMG changes.** *Muscle Nerve* (1991) **14**:672-675.

75. OLNEY RK, AMINOFF MG, GELB DJ *et al.*: **Neuromuscular effects distant from the site of botulinum neurotoxin injection.** *Neurology* (1988) **38**:1780-1783.

76. SANDERS DB, MASSEY EW, BUCKLEY EC: **Botulinum toxin for blepharospasm: single fiber EMG studies.** *Neurology* (1986) **36**:545-547.

77. GIRLANDA P, VITA G, NICOLOSI C, MILONE S, MESSINA C: **Botulinum toxin therapy: distant effects on neuromuscular transmission and autonomic nervous system.** *J. Neurol. Neurosurg. Psychiat.* (1992) **55**(9):844-845.

78. CLAUS D, DRUSCHKYA A, ERBGUTH F: **Botulinum toxin: influence on respiratory hearth rate variation.** *Mov. Dis.* (1995) **10**:574-579.

79. WIEGAND H, ERDMANN G, WELLHONER HH: **I^{125}-labeled botulinum A neurotoxin: pharmacokinetics in cats after im. injection.** *Naunyn-Schmiedebergs Arch. Pharmacol.* (1976) **292**:161-165.

80. BLACK JD, DOLLY JO: **Interaction of I^{125}-labeled botulinum neurotoxins with nerve terminals. I: Ultrastructural autoradiographic localization and quantitation of distinct membrane receptors for types A and B on motor neurons.** *J. Cell Biol.* (1986) **103**:521-534.

81. FILIPPI GM, ERRICO P, SANTARELLI R, BAGOLINI B, MANNI E: **Botulinum A toxin effects on rat jaw muscle spindles.** *Acta. Otolaryngol. (Stockholm)* (1993) **113**:400-404.

82. MANNI E, BAGOLINI B, PETTOROSSI VE, ERRICO P: **Effect of botulinum toxin on extraocular muscle proprioception.** *Doc. Ophthalmol.* (1989) **72**(2):189-198.

83. ROSALES RL, ARIMURA K, TAKENAGA S, OSAME M: **Extrafusal and intrafusal muscle effects in experimental botulinum toxin-A injection.** *Muscle Nerve* (1996) **19**(4):488-496.

84. COSGROVE AP, GRAHAM HK: **Botulinum toxin prevents the development of contractures in the hereditary spastic mouse.** *Dev. Med. Child Neurol.* (1994) **36**:379-385.

85. COSGROVE AP, CORRY IS, GRAHAM HK: **Botulinum toxin in the management of the lower limb in cerebral palsy.** *Dev. Med. Child Neurol.* (1994) **36**:386-396.

86. MONTECUCCO C, SCHIAVO G, TUGNOLI V, DE GRANDIS D: **Botulinum neurotoxins: mechanism of action and therapeutic applications.** *Mol. Med. Today* (1996) **2**:418-424.

87. COMELLA CL: **Electromyography-assisted botulinum toxin injections for cervical dystonia.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M, Marcell Dekker, Inc. (1994) 289-298.

88. GEENEN C, CONSKY E, ASHBY P: **Localizing muscles for botulinum toxin treatment of focal dystonia.** *Can. J. Neurol. Sci.* (1996) **23**:194-197.

89. JANKOVIC J, BRIN MF: **Therapeutic use of botulinum toxin.** *New Engl. J. Med.* (1991) **324**(17):1168-1194.

90. BORODIC GE, FERRANTE R, PEARCE BL, SMITH K: **Histologic assessment of dose-related diffusion and muscle fiber response after therapeutic botulinum-A toxin injections.** *Mov. Disord.* (1994) **9**(1):31-39.

91. BORODIC GE, WEIGENER A, FERRANTE R, YOUNG R: **Orbicularis oculi innervation zone and implications for botulinum A toxin therapy for blepharospasm.** *Ophthalmol. Plast. Reconstr. Surg.* (1991) **7**:54-59.

92. SHAARI C, SANDERS I: **Assessment of the biological activity of botulinum toxin.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M (Eds.), Marcell Dekker, Inc. (1994) 159-170.

93. BLACKIE JD, LEES AJ: **Botulinum toxin treatment in spasmodic torticollis.** *J. Neurol. Neurosurg. Psychiat.* (1989) **52**:355-363.

94. PATRINELY JR, WHITING AS, ANDERSON RL: **Local side-effects of botulinum toxin injections.** In: *Advances in Neurology: Facial Dyskinsias*. Jankovic J, Tolosa E (Eds.), New York, Raven Press (1988) **49**:439-499.

95. MARSDEN CD, SCHACHTER M: **Assessment of extrapyramidal disorders.** *Br. J. Clin. Pharmacol.* (1981) **11**:129-151.

96. LANG AE, SHEEHY MP, MARSDEN CD: **Anticholinergics in adult-onset dystonia.** *Can. J. Neurol. Sci.* (1982) **9**:313-319.

97. FAHN S: **Assessment of the primary dystonias.** In: *Quantification of Neurologic Deficit*. Munsat TL (Ed.), Butterworth, London (1989) 241-270.

98. TSUI JKC, EISEN A, STOESSL AJ, CALNE DB: **Double-blind study of botulinum toxin in spasmodic torticollis.** *Lancet* (1986) **2**:245-247.

99. JANKOVIC J, SCHWARTZ K: **Botulinum toxin injections for cervical dystonia.** *Neurology* (1990) **40**:277-280.

100. CONSKY ES, BASINKI A, BELLE L, RANAWAYA R, LANG AE: **The Toronto Western Spasmodic Torticollis Rating Scale (TWSTRS): assessment of validity and inter-rater reliability.** *Neurology* (1990) **40**(Suppl. 1):445.

101. ANSARI KA, WEBSTER DD: **Quantitative measurements in spasmodic torticollis.** *Dis. Nerv. Syst.* (1974) **35**:44-47.

102. ROGERS GW, ROOTES M, LYE RH: **Computer/video analysis of movement disorders.** *J. Audiovis. Media Med.* (1985) **8**:101-107.

103. DYKSTRA D, ELLINGHAM C, BELFIE A *et al.*: **Quantitative measurement of cervical range of motion in patients with torticollis treated with botulinum A toxin.** *Mov. Disord.* (1993) **8**:38-42.

104. GELB DJ, YOSHIMURA DM, OLNEY RK, LOWENSTEIN DH, AMINOFF MJ: **Change in pattern of muscle activity following botulinum toxin injections for torticollis.** *Ann. Neurol.* (1991) **29**:370-376.

105. TSUI JK, EISEN A, CALNE DB: **Botulinum toxin in spasmodic torticollis.** *Adv. Neurol.* (1988) **50**:593-597.

106. MEZAKI T, KAJI R, KOHARA N, KIMURA J: **Development of general weakness in a patient with amyotrophic lateral sclerosis after focal botulinum toxin injection.** *Neurology* (1996) **46**(3):845-846.

107. GLANZMAN RL, GELB DJ, DRURY I, BROMBERG MB, TRUONG DD: **Brachial plexopathy after botulinum toxin injections.** *Neurology* (1990) **40**:1143.

108. MOSER E, LIGON KM, SINGER C, SETHI KD: **Botulinum toxin A (Botox) therapy during pregnancy.** *Neurology* (1997) **48**:A399.

109. GREENE PE, FAHN S: **Use of botulinum toxin type F injections to treat torticollis in patients with immunity to botulinum toxin type A.** *Mov. Disord.* (1993) **8**:479-483.

110. JANKOVIC J, SCHWARTZ K: **Response and immunoresistance to botulinum toxin injections.** *Neurology* (1995) **45**:1743-1746.

111. GREENE P, FAHN S, DIAMOND B: **Development of resistance to botulinum toxin type A in patients with torticollis.** *Mov. Disord.* (1994) **9**:213-217.

112. TSUI JK, WONG NLM, WONG E, CALNE DB: **Production of circulating antibodies to botulinum A toxin in patients receiving injections for dystonia.** *Ann. Neurol.* (1988) **23**:181.

113. HAMBLETON P, COHEN HE, PALMER BJ, MELLING J: **Antitoxins and botulinum toxin treatment.** *Br. Med. J.* (1992) **304**(6832):959-960.

114. GREENE P, FAHN S: **Development of antibodies to botulinum toxin type A in torticollis patients treated with botulinum toxin injections.** *Mov. Disord.* (1992) **7**(Suppl. 1):134.

115. JANKOVIC J: **Botulinum toxin in movement disorders.** *Curr. Opin. Neurol.* (1994) **7**:358-366.

116. DEZFULIAN M, BARTLETT JG: **Detection of *Clostridium botulinum* type A toxin by enzyme-linked immunosorbent assay with antibodies produced in immunologically tolerant animals.** *J. Clin. Microbiol.* (1984) **19**:645-648.

117. DEZFULIAN M, HATHEWAY CL, YOLKEN RH, BARTLETT JG: **Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* type A and type B toxins in stool samples from infants with botulism.** *J. Clin. Microbiol.* (1984) **20**:379-383.

118. DUANE DD: **Non-comparability of *in vivo* and *in vitro* Botulinum toxin A antibody measurement techniques in treated cervical dystonia patients?** *Neurology* (1997) **48**:A339.

119. SHONE C, WILTON-SMITH P, APPLETON N *et al.*: **Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* toxin is comparable to the mouse bioassay.** *Appl. Environ. Microbiol.* (1985) **50**:63-67.

120. HATHEWAY CL, DANG C: **Immunogenecity of the neurotoxins of Clostridium botulinum.** In: *Therapy with Botulinum Toxin*. Jankovic J, Hallett M (Eds.), Marcell Dekker, Inc., New York (1994) 93-108.

121. BORODIC G, JOHNSON E, GOODNOUGH M, SCHANTZ E: **Botulinum toxin therapy, immunologic resistance, and problems with available materials.** *Neurology* (1996) **46**(1):26-29.

122. SESARDIC D, MCLELLAN K, EKONG TA, DAS RG: **Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin.** *Pharmacol. Toxicol.* (1996) **78**(5):283-288.

123. GREENE PE, FAHN S: **Use of botulinum tozin type F injections to treat *torticollis* in patients with immunity to botulinum toxin Type A.** *Mov. Discord.* (1993) **8**:479-483.

124. SELLIN LC, KAUFFMAN JA, DASGUPTA BR: **Comparison of the effects of botulinum neurotoxins types A and E at the rat neuromuscular junction.** *Med. Biol.* (1983) **61**:120-125.

125. KAUFFMAN JA, WAY JF, SIEGEL LS, SELLIN LC: **Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction.** *Toxicol. Appl. Pharmacol.* (1985) **79**:211-217.

126. LUDLOW CL *et al*: **Therapeutic use of type F botulinum toxin.** *New Engl. J. Med.* (1992) **326**:349-350.

127. ELEOPRA R, TUGNOLI V, ROSSETTO O, MONTECUCCO C, DE GRANDIS D: **Botulinum neurotoxin serotype C: a novel effective botulinum toxin therapy in human.** *Neurosci. Lett.* (1997) **224**:91-94.

128. TSUI JK, HAYWARD M, MAK EK, SCHULZER M: **Botulinum toxin type B in the treatment of cervical dystonia: a pilot study.** *Neurology* (1995) **45**(11):2109-2110.

129. SHEEN GL, LEES AJ: **Botulinum toxin F in the treatment of torticollis clinically resistant to botulinum toxin A.** *J. Neurol. Neurosurg. Psychiat.* (1995) **59**(6):601-607.

130. JENZERG, MUMENTHALER M, LUDIN HP, ROBERT F: **Autonomic dysfunction in botulism B: a clinical report.** *Neurology* (1975) **25**:150-153.

Valeria Tugnoli[1†], Roberto Eleopra[1], Cesare Montecucco[2] & Domenico De Grandis[1]

[†] Author for correspondence

[1†] Neurological Department, St Anna Hospital, Corso Giovecca 203, 44100 Ferrara, Italy

(Tel: +39 532 295264; Fax: +39 532 295588)

[2]Biomedical Science Department, University of Padua, Padua, Italy