Botulinum B Toxin as an Alternative to Botulinum A Toxin: A Histologic Study

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Summary: Histochemical effects of botulinum B toxin were studied on fibers from longissimus dorsi muscle in Albino rabbits and compared to effects produced by botulinum A toxin. Acetylcholinesterase staining, muscle fiber size analysis, and ATPase staining indicated botulinum B toxin produced a denervation gradient and field similar to that produced by botulinum A toxin. At 5 weeks postinjection with botulinum B toxin, analysis showed muscle fiber size variability, and diffuse acetylcholinesterase fiber staining comparable to botulinum A toxin at the injection site. Muscle sections taken at 4.0 cm for analysis showed statistically significant decreased fiber size variability and contraction of acetylcholinesterase staining pattern for both immunotypes. In addition, the denervation reflected by histochemical staining and fiber size analysis appeared transient and lasted for approximately 3 months for both immunotypes. These findings suggest botulinum B toxin produces pharmacologic effects on innervation of striated muscle similar to botulinum A toxin. Because immunologic tolerance has been demonstrated after therapeutic botulinum A toxin injections, further clinical studies need to be conducted with other immunotypes of toxin with no cross-reactivity to type A.

Key Words: Movement disorders—Botulinum B toxin—Botulinum A toxin—Animal Studies—Blepharospasm.

Botulinum A toxin has been used in clinical studies to treat a number of segmental movement disorders, including blepharospasm, hemifacial spasm, spasmodic torticollis, spasmodic dysphonia, and regional hand dystonias (1–10).

Botulinum neurotoxins are produced by certain strains of the bacterial species Clostridium botulinum and Clostridium baratti (11). The toxins are classified into seven serotypes A–G. The botulinum neurotoxins comprise a family of pharmacologically similar poisons that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. Type A botulinum toxin is the serotype currently approved by the FDA for use in clinical practice.

The essential pharmacologic properties of a point injection of botulinum A toxin to striated muscle are (a) the blockade of acetylcholine release; (b) diminished muscle fiber contractility followed by muscle fiber atrophy, which becomes prominent after several weeks (12–14); and (c) a reversal of denervation and muscle atrophy after 10–15 weeks associated with collateral axonal sprouting and contraction of acetylcholinesterase fiber staining (12–15). The regional denervation effect and sequential reversibility of the therapy has been most useful in modulating treatment of regional movement disorders, such as essential blepharospasm (1–5).

The effects of botulisum toxin are temporary, therefore most patients have received treatment with botulinum A toxin on multiple occasions over many years (3,14,16). Repeated injections have led to lack of effectiveness in some patients, and sensiti-
zation to botulinum A toxin protein has been associated with its use in others (17–21). Neutralizing antibodies to botulinum A toxin have been demonstrated using the standard mouse assay, and sensitization appears to be related to frequent injections at higher doses (16–21). Neutralizing antibodies as determined with the mouse assay appears to render the therapy ineffective (17,20,21). Due to sensitization, other immunotypes of botulinum toxin need to be considered for clinical application.

This paper reports the histopathologic effects following botulinum B toxin injection into striated muscle and compares these findings to those produced by botulinum A toxin.

METHODS AND MATERIALS

The following is an outline for culture production of botulinum B toxin (18). The B toxin used in these experiments was prepared from CDC culture 208 (“bean strain” [origin British culture collection] NCTC-7273). This organism provided the source for the B toxoid preparation formulation for the pentavalent vaccine.

The culture media consisted of 15 g of tryptocase (BBL) with 5 g of yeast extract diluted in quantity sufficient to 1,000 ml with normal saline. The pH was adjusted to 7.2 with sodium hydroxide (solution A). Another solution consisting of 20% glucose was autoclaved for 15 min at 121°C (solution B). Ten milliliters of solution A was placed in 200 ml of solution B (solution C). A 24 h culture of the organism was made with CMG media (BBL). Five milliliters of the CMG-botulinum B toxin culture were used to inoculate solution C. Toxicity determinations were made over 3 days: day 1—10,000 Mouse LD 50/ml; day 2—100,000 Mouse LD 50/ml; and day 3—100,000 Mouse LD 50/ml (1 IU = one LD 50 for white mouse).

3 N sulfuric acid was added to the flasks after 3 days, which develops the “mud,” a suspension with stable biologic B toxin activity.

This preparation was diluted in normal saline containing 5% glycerin and 5% gelatin in acetate buffer adjusted to pH of 4.7.

Specimens taken from longissimus dorsi of 2–3 kg albino rabbits were immediately placed in cold (4°C) formol-calcium (Baker’s solution) and fixed for 6–12 h at 4°C. Muscle specimens were then cryoprotected in gum sucrose solution for 3 h. The muscle was oriented both in cross and longitudinal plane on a specimen chuck in OCT compound (Tissue Tek) and frozen in a cryostat. Cut tissue sections (10 µm) were adhered to gel coated slides, air dried for 2 min, and subsequently stained for acetylcholinesterase activity (Geneser-Jensen and Blackstad, 1971) (22). Enzyme histochemistry for myofibrillar ATPase activity (Brooke, Kaiser, 1969) (22) and NADH activity (Scarpelli, Hess, Pearse, 1958) (22) was also conducted on the specimens. Sections for acetylcholinesterase activity were incubated in a solution containing 13 ml of maleic buffer (1.96 g maleic acid, 0.8 g NaOH, 10.8 ml 1 N NaOH, 200 ml distilled water), 10 mg acetylthiocholine iodide, 2 ml 0.03 M cupric sulfate, 1 ml 0.1 sodium citrate, and 0.5 M potassium ferricyanide for 1 h at 37°C. Contiguous cryostat sections were stained either with hematoxylin and eosin or with Gomori trichrome stain to assess normal tissue morphology.

Alternatively, fresh skeletal muscle tissue was flash frozen in isopentane, and cooled to −160°C using liquid nitrogen. Serial cut sections (10 µm) were stained with hematoxylin and eosin or Gomori trichrome to identify any tissue alterations. Enzyme histochemistry for acetylcholinesterase activity was used to quantify endplate structures and assess for denervation.

Histologic measurements were made with the bioquant II system. Fiber size variation comparisons were generated using standard deviation and variance values counted from at least 200 fiber diameters. Also, an F ratio test was conducted to compare fiber size variability.

RESULTS

Five Weeks After Point Injection of Botulinum B Toxin (Dose = 15 IU/kg)

Using the fiber size variability analysis as an indication of denervation, a marked degree of fiber size variability was demonstrated at the injection site 5 weeks after the injection of botulinum B toxin (fiber size diameter median = 44.4 microns; variance = 493; standard deviation = 22.2) (Fig. 1A). When compared to untreated control values (fiber size diameter average = 37.95, variance = 78.6, standard deviation = 8.9), the fiber size variation was significantly greater than controls (F ratio = 4.32 P < 0.01).

When comparing a muscle biopsy 4 cm from the injection point, there appeared to be a significant diminution in fiber size variability (median fiber
FIG. 1.  
A: Large degree of fiber diameter variability is noted at the injection site (H & E, original magnification ×10). The line on the right hand site of the field is fixation artifact. B: Variation in fiber size decreases at increasing distance from the injection site (photo demonstrates fiber cross-section at 40 mm from the injection site, the line on the superior aspect of the field is fixation artifact).
diameter = 58, variance = 278, standard deviation = 16.4) (Fig. 1B). Fiber size variability at 4 cm was significantly different from the injection point (F ratio = 1.77, P < 0.05) indicating regional denervation was more pronounced at the injection site than at 4 cm. Fiber size variability at 4 cm was, however, still significantly greater than fiber variability within control specimens (F ratio = 2.4, p < 0.01) indicating a denervation process even at this distance from the point injection.

Additionally, the spread of cholinesterase was most prominent at the injection site (Fig. 2A). At 4 cm from the injection site, there was a substantial diminution of acetylcholinesterase spread approaching normal intensity (Fig. 2B).

In control specimens, myofibrillar ATPase activity at pH = 9.4 demonstrated type 1 and type 2 fibers. The number or percent ratio of type 1/type 2 fibers was 3.5% of the total. Type 1 fibers were evenly distributed throughout the muscle specimens in the saline injected control tissue. At the injection site there was marked variation of muscle fiber size effecting both fiber types (Fig. 3A). The pattern of fiber typing was altered in that there were now small groups of type 1 fibers, suggesting denervation and renervation. The ratio of type 1/type 2 fibers increased significantly with type 1 fibers representing 22% of the total population. Distally, 4.5 cm away from the injection site, there was much less fiber size variability. The percentage of type 1 fibers was reduced (10.7% of the total), although still not normal (Fig. 3B).

The NADH activity equally demonstrated alterations in the fiber size as well as the fiber typing. In addition, the method identified changes in the intermyofibrillary network consistent with denervation at the injection sites.

Fourteen Weeks After Point Injection of Botulinum Toxin B

There was significantly less acetylcholinesterase staining when comparing the injection site at 14 weeks versus 5 weeks. There were minimal differences in acetylcholinesterase activity at 14 weeks compared to controls. Fiber size variability appeared not to be significantly different from control variability 14 weeks after injection (average diameter = 29.5 microns; variance = 75.7; standard deviation = 8.7; F ratio = 0.7; P = NS).

Furthermore, there was no difference in the fiber size variability or acetylcholinesterase staining pattern when comparing the injection site with muscle tissue 4 cm from the injection after 13 weeks (fiber diameter = 28.1; variance = 54; s = 7.4; F ratio = 0.47, P = NS).

In summary, at 14 weeks both acetylcholinesterase and fiber size analysis did not appear to indicate significant denervation.

Botulinum A Toxin Diffusion Gradient Data After 5 Weeks (Dose = 2–3 IU/kg)

There was considerable fiber size variation at the site of injection associated with the spread of acetylcholinesterase staining on muscle fibers in three animals studied (median diameter = 27.3 microns; s = 14.55; ν = 212; F ratio = 2.5; P < 0.01). At 15 mm from the injection site, similar fiber size variability and cholinesterase spread were noted (median diameter = 30.7; s = 12.9; ν = 166; F = 1.98, p < 0.01). At 40 mm there was considerable contraction of the acetylcholinesterase staining pattern as well as more uniform muscle fiber diameter sizes (median diameter = 24.9; s = 9.7; ν = 93; F = 1.11; P = NS). At 45 mm, the acetylcholinesterase staining pattern and muscle fiber size variations were similar to controls (median diameter = 30.6; s = 6.4; ν = 41; F = 0.49; P = NS).

At the same saline injection site and 15 mm intervals, Table 1 outlines control values for fiber size variability and acetylcholinesterase staining pattern.

**DISCUSSION**

**Basic Science and Pharmacology of Botulinum B Toxin Compared to Botulinum A Toxin**

Botulinum type A and B neurotoxins exhibit a number of functional, structural, and mechanistic similarities. Both produce chemical denervation at the neuromuscular junction that is thought to occur through a three-step process resulting in the irreversible inhibition of normal neurotransmitter release (23,24). Both species have a molecular weight of approximately 150,000 daltons and the active form of the toxin exists as a dichain molecule consisting of a light (Mr −50,000 DA) and heavy (Mr −100,000 DA) chain linked by a disulfide bond (25,26).

Despite the general similarities between the A and B toxins, closer examination of these species reveals very significant differences. All of the neu-
FIG. 2.  A: Acetylcholinesterase staining pattern is diffuse at the injection associated with high degree of fiber size variability. Both histologic parameters indicate substantial denervation at the injection site. B: At 40 mm from the injection site there is marked decrease in acetylcholinesterase staining pattern associated with decreased fiber size variability. The biologic effects of the point injection of botulinum B toxin have diminished at this distance from the point injection (original magnification ×10).
FIG. 3. A: ATPase stain at pH 9.4 indicates a predominance of type II fibers. Type I fiber grouping was noted at the injection site compared to the type I fiber distribution at 40 mm from the injection site (B) (original magnification ×10). B: Type I fibers are evenly distributed throughout the muscle specimen at 40 mm from the injection site.
rotoxins produced by *Clostridium botulinum* are immunologically distinct, which suggests very significant differences in the amino acid sequences of these toxins. Analysis of the partial amino acid sequences for the A and B types has revealed greater homologies between the primary and secondary structure for the light chains than the heavy chains. The degree of primary structure homology is only 20% for the light chains versus 40% for the heavy chains (27). Although similar in secondary and tertiary structure, it is believed that differences in the conformation of the neurotoxins at or near the active site may be responsible for the differences in the neurotoxicity of the toxins (28).

Electrophysiological studies have demonstrated that the botulinum toxins affect different steps in the neurotransmitter release process. Botulinum B toxin affects synchronization of quantal transmitter release whereas botulinum A toxin does not (29). Similarly, differences exist with regard to the reversibility of the inhibition of calcium-dependent release of neurotransmitter. Introduction of calcium into nerve terminals using a calcium ionophore produces the release of transmitter from synaptic vesicles poisoned by botulinum A toxin more readily than those poisoned by botulinum B toxin (30). At the neuromuscular junction aminopyridine was also more effective at reversal of inhibition produced by botulinum A toxin (31). Asatryan and Dolly (30) have recently demonstrated that microtubule-dissociating drugs were effective in blocking the inhibitory effects of botulinum B on neurotransmitter release and ineffective against the A toxin.

The differences in the toxic and neurophysiological effects of the type A and B toxins may be related to the putative existence of two distinct receptor (receptor) sites for these species (29). Examination of the effect of botulinum A toxin on 125 I-botulinum B toxin binding to neuronal membranes showed a very weak interaction involving both the high and low affinity sites for toxin binding.

**Clinical Significance**

Botulinum A toxin has been useful in the treatment of regional movement disorders. When injected into muscles, the denervation effect appears to be contained within a definitive field from the injection site, and is temporary in nature.

The diffusion of biologic activity away from the injection site has important consequence to clinical application. Complications associated with therapeutic injection of botulinum toxin in striated muscle appear to result from unwanted spread of the toxin into contiguous muscular structures causing sympotmatology. Ptosis and diplopia have been known to occur following eyelid injections, while dysphagia has been associated with botulinum injections to the sternomastoid muscle (15,32). Regional diffusion may also be important for homogeneous effects on an injected muscle. The number and distance between injections may be important in saturating a muscle's innervation zone (33). A fully saturated innervation zone presumably will create a greater denervation effect (33).

The contained region of denervation produced by low dose injections prevents disseminated weakness, a potential complication that may be encountered with very high doses of botulinum A toxin. Containment of biologic effect is, of course, an essential pharmacologic property of therapeutic botulinum injections, which can be assessed by using the histologic analysis outlined in this study. Since regional and reversible denervation are universal effects of therapeutic botulinum A toxin injections for any clinical application, assessing these properties for botulinum B toxin will be important prior to instituting clinical trials with this immunotype. Recently, further pharmacologic investigation with other immunotypes has been cited as a future goal of the botulinum toxin-clinical application technology (National Institute of Health Consensus Conference on Therapeutic Application of Botulinum Toxin, November 1990).

Botulinum B toxin has a different amino acid sequence than botulinum A toxin. As the chemical composition is different than the A toxin, so is the antigenicity of the molecule. It has been long recognized that the cross reactivity of B toxin with A toxin antibodies is extremely small (33-37). Additionally, the receptor site for botulinum B toxin

<table>
<thead>
<tr>
<th>Distance from Injection Point (mm)</th>
<th>Fiber Diameter (microns)</th>
<th>Variability</th>
<th>Cholinesterase Staining Pattern</th>
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<tbody>
<tr>
<td>Injection point</td>
<td>37.2</td>
<td>v = 84</td>
<td>focal</td>
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<tr>
<td>15 mm</td>
<td>45</td>
<td>v = 68</td>
<td>focal</td>
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<tr>
<td>30 mm</td>
<td>31.9</td>
<td>v = 35</td>
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<td>45 mm</td>
<td>28.5</td>
<td>v = 70.1</td>
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<td>60 mm</td>
<td>41</td>
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The number or percent ratio of type 1/type 2 fibers was 3.5% of the total in control specimens.

*Ophthalmic Plast Reconstr Surg, Vol. 9, No. 3, 1993*
on the presynaptic membrane is thought to be chemically distinct from the A toxin as has been outlined in the first section of this discussion.

The literature contains reports of negative antibody production in patients treated with botulinum A toxin (1,2). However, other reports show that antibody production is real. In a clinical study performed by Tsui (38), 32 patients with spasmodic torticollis who received repeated injections of botulinum A toxin were tested for the presence of circulating antibodies. The results showed that 4 of 32 patients (12.5%) produced antibodies after 2-9 months of treatment. The remaining 28 patients remained seronegative for up to 30 months. The doses were the same for both the seropositive and the seronegative groups. Other authors have also demonstrated antibody formation in patients treated with botulinum toxin for spasmodic torticollis (20,21).

The dose used for the treatment of spasmodic torticollis is usually between 150-300 IU compared to 40-100 IU for blepharospasm. The incidence of antibody formation in blepharospasm patients after multiple botulinum injection over many years still remains unknown, although the work of Gonnering and associates with the assistance of Hathaway (19,39) has suggested this is not a problem in the short term. Recently, a patient with blepharospasm has been shown to develop antibodies to botulinum A toxin after repetitive eyelid injections (unpublished data).

Antibodies have been shown to neutralize the beneficial effect of botulinum toxin in a clinical setting (40). A patient immunized with botulinum A toxoid demonstrated circulating antibodies using the mouse assay. The toxoid was administered for occupational safety purposes. Subsequently, he developed spasmodic dysphonia, and vocal cord injections with botulinum A toxin were attempted. No benefit was obtained presumably because of the presence of circulating neutralizing antibodies (40,41). Antibodies have been demonstrated to occur at botulinum dose levels used to treat blepharospasm in several patients (40-100 IU) (40). More clinical study is clearly needed on the incidence of antibody formation in patients with chronic movement disorders treated with repeated injections of botulinum A toxin over many years to more exactly assess the incidence of sensitization.

Since the B toxin is immunologically distinct and there has been a definite incidence of sensitization occurring with repeated injections of botulinum A toxin, the potential pharmacologic properties of botulinum B toxin were investigated with respect to regional and sequential effects. Additionally, botulinum B toxin may have a different receptor than the A toxin, which could have future therapeutic significance. The method of determining regional denervation that has been helpful for botulinum A toxin has included acetylcholinesterase staining and fiber size variability analysis (15). Upon muscle tissue analysis with acetylcholineesterase histochemistry shortly after injection, patients noted to have had injections of botulinum toxin for blepharospasm were found to demonstrate substantial spread of acetylcholinesterase on muscle fibers (14,15). Furthermore, patients having muscle biopsies within 2 months of botulinum A toxin injection appear to have a higher degree of fiber size variability than controls (13). When biopsies were taken 4 months after injection, cholinesterase staining became indistinguishable from controls and fiber size variability returned to normal (13). The histologic sequence demonstrated on those orbicularis specimens using botulinum A toxin, was reproduced on animal muscle specimens using botulinum B toxin in this study.

In summary, this study has demonstrated that botulinum B toxin is capable of producing regional denervation from a point injection and has a reversibility similar to botulinum A toxin. Therefore, it is anticipated that the regional denervation effect that has been pharmacologically useful in the A toxin will be noted with the application of B toxin.

There may be therapeutic significance to these findings, because immunologic resistance has occurred after repeated injections of botulinum A toxin for the treatment of segmental dystonia. Further studies will be necessary to establish the stability, purity, and biologic activity consistency of botulinum B toxin. Because botulinum B toxin is immunologically distinct, it may have differing biologic effects at the cellular level and may also be useful as adjuvant therapy for patients resistant or refractory to botulinum A toxin.

REFERENCES


40. Shalans E, University of Wisconsin, personal communication.

41. Hatheway C, Center for Disease Control, personal communication.