ABSTRACT: Botulinum neurotoxin type-A (BoNT/A) is very effective in the therapy of a wide range of human syndromes characterized by hyperactivity of peripheral cholinergic nerve terminals. Little diffusion of this toxin from the site of injection is commonly observed, but even minor changes in this property would greatly affect the validity of the treatment. Different pharmacological formulations of BoNT/A are available, and they may have different diffusion characteristics due to protein complex size, product format, and pharmacological properties. Here we assessed the extent of diffusion of three commercial preparations of BoNT/A: Botox (Allergan), Dysport (Ipsen), and Xeomin (Merz Pharmaceuticals) using a novel and highly sensitive test based on neural cell adhesion molecule (N-CAM) expression in muscle. N-CAM is a membrane glycoprotein that accumulates on muscle fibers after denervation and is not expressed in untreated adult muscle. This allows fine monitoring of the functional diffusion of this toxin, and the sensitivity of this assay is emphasized by the use of the mouse model because of the small muscle dimensions. The results presented here indicate that there is no significant difference between Botox, Dysport, and Xeomin with respect to diffusion into adjacent muscles in the mouse leg.

Muscle Nerve : 000–000, 2009

ASSAY OF DIFFUSION OF DIFFERENT BOTULINUM NEUROTOXIN TYPE A FORMULATIONS INJECTED IN THE MOUSE LEG

LUCA CARLI, PhD, CESARE MONTECUCCO, FP, and ORNELLA ROSSETTO, PhD

Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, Padova, Italy

Accepted 9 February 2009

Botulinum neurotoxins (BoNTs) are bacterial metalloproteases that affect acetylcholine release at cholinergic nerve terminals. They cleave key proteins of the neuroexocytosis apparatus, causing persistent inhibition of neurotransmitter release.[22,23] Over time, BoNTs effects are reversed following toxin degradation and replacement of the cleaved target proteins.[17,24]

BoNT/A is the BoNT serotype (out of the seven known and named from A to G) employed in the treatment of some human diseases characterized by hyperactivity of cholinergic nerve terminals because of its long duration of action.[18] In addition, BoNT/A inhibits acetylcholine release at autonomic nerve terminals that innervate glands and smooth muscles, and it is currently used to treat various forms of hyperhydrosis.[20] At present, three different BoNT/A formulations are available in Europe: Botox (Allergan, Irvine, California), Dysport (Ipsen, Slough, UK), and Xeomin (Merz Pharmaceuticals, Greensborough, North Carolina). BoNT therapy has a remarkable record of safety, and this depends partly on the toxin's ability to remain relatively localized at the site of injection. Diffusion to nearby muscles is thought to underlie rare side effects of this therapy, such as ptosis, following injection into extraocular muscle and dysphagia following injection into the sternocleidomastoid muscle.[5] These undesired effects are related to local diffusion of BoNT.[6,26] Safety issues may become increasingly important as high BoNT/A doses are used to treat conditions such as cerebral palsy or spasticity, and symptoms of generalized weakness have been described in some BoNT-treated patients.[4,27]

Different formulations of botulinum toxin type A may have different migration/diffusion characteristics owing to their different compositions. In fact, Botox and Dysport consist of the botulinum neuro-

Additional Supporting Information may be found in the online version of this article.

Abbreviations: Ach, acetylcholine; BoNT, botulinum neurotoxin; DAS, digit abduction score; EDL, extensor digitorum longus muscle; N-CAM, neural cell adhesion molecule; TA, tibialis anterior muscle

Key words: botulinum toxin type A; neuromuscular junction; N-CAM; diffusion

Correspondence to: O. Rossetto; e-mail: ornella.rossetto@unipd.it

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.21343

toxin (150 kDa) and of nontoxic complexing proteins that produce conglomerates of 900 kDa (Botox) or 300–900 kDa (Dysport). In contrast, Xeomin contains only the active 150 kDa neurotoxin, free of complexing proteins. It has been claimed that, because of the size of the complexes, the diffusion of neurotoxin into adjacent tissues is slower in the high molecular weight complex compared with the lower molecular weight complexes or with free neurotoxin.[2] This possibility has not been analyzed in vivo. Therefore, owing to its paramount importance, we undertook an ad hoc study to compare the diffusion of the above-mentioned BoNT/A formulations in vivo. We used the mouse as an animal model because its small muscle size increases the sensitivity of the assay. As a readout of diffusion, we chose the neural cell adhesion molecule (N-CAM) expression in mouse hindlimb muscles after different time periods from toxin injection. N-CAM can be detected with high sensitivity and spatial resolution by histological and Western blot analyses. In fact, N-CAM is present on the surface of embryonic myotubes, but it is no longer present as development proceeds. It is nearly absent from adult muscle, but denervation of adult muscle induces the reappearance of N-CAM.[8] Paralysis of skeletal muscle by BoNT/A injection is sufficient to activate N-CAM expression.[19] We found that BoNT/A injected intramuscularly into the tibialis anterior (TA) muscle of mice exhibited limited diffusion to muscles adjacent to the site of injection, and diffusion of the three different commercial preparations of BoNT/A is so similar as to be indistinguishable. Moreover, this study qualifies N-CAM as a good and general marker for the study of BoNT diffusion in vivo.

MATERIALS AND METHODS

Animals. Swiss-Webster adult male CD1 mice weighing 30 g were used. Mice were housed in groups of six, and food and water were provided ad libitum. They were maintained on a 12-h light/dark photoperiod for 4 days before the start of experiments. All experiments were performed in accordance with Italian animal care guidelines, law no. 116/1992.

Injection Protocol. The vials of vacuum-dried *C. botulinum* type A neurotoxin preparations (Botox from Allergan, Dysport from Ipsen, and Xeomin from Merz) were reconstituted with 0.9% sodium chloride to a concentration of 10 U/ml (Botox and Xeomin) or 40 U/ml (Dysport). Before toxin injection, mice were anesthetized with 15 mg/kg of Zoletil (Laboratoires Virbac, Carros, France) and 3 mg/kg of xylazine (Laboratoires Calier, Barcelona, Spain). In order to inject 0.25 U Botox, 1.0 U Dysport, and 0.25 U Xeomin (1:4:1 ratio) the toxin injection volume was always 25 μl in the TA of each hindlimb (using a different formulation on each side). In another set of experiments, each mouse was injected with one of the three toxin formulations in one hindlimb muscle, while the contralateral muscle was injected with carrier alone and used as a control.

Digit Abduction Scoring Assay. After toxin injection in the mouse hindlimb, functional recovery was monitored using the digit abduction score (DAS) assay.[1] Briefly, mice were suspended by the tail to elicit a characteristic startle response in which the animal extends its hindlimbs and abducts its hind digits. Following neurotoxin injection, the degree of digit abduction was scored on a five-point scale (0, normal, to 4, maximal reduction in digit abduction and leg extension) by an observer who was masked to treatment.

Surgical Procedures. Mice were anesthetized with 25 mg/kg Zoletil and 5 mg/kg xylazine before a single incision of the skin was made to expose the sciatic nerve at the femoral-head level. The nerve was cut, and a 5 mm portion of the nerve was removed to prevent regeneration. Finally the skin was sutured and disinfected with iodine solution. Denervated muscles are TA, soleus, plantaris, and gastrocnemius. No denervation was made for quadriceps femoris.

Immunohistochemistry. Seven, 14, 21, 30, and 60 days after drug injection, M. tibialis anterior, M. soleus, M. gastrocnemius, and M. quadriceps femoris were dissected from both hindlimbs and frozen in isopentane precooled in liquid nitrogen. Ten-μm thick cryosections were fixed in chilled methanol for 10 min, followed by incubation with phosphate-buffered saline (PBS) containing 10% bovine serum albumin (BSA) for 1 h to block nonspecific binding (blocking solution). Sections were incubated for 1.5 h at room temperature with polyclonal antibody to N-CAM (Chemicon International, Temecula, California) diluted 1:500 in blocking solution.

Following three 10-min washes with PBS, sections were overlaid with goat antirabbit IgG conjugated to Alexa-555 or Alexa-488 (Invitrogen, La Jolla, California) diluted 1:200 in blocking solution. After 1 h, sections underwent three 10-min washes in PBS and were mounted with antifading agent (90% glycerol, n-propyl gallate 3% p/v in PBS). Images were col-

lected with a video-confocal microscope (VI.CO. Nikon).

Protein Isolation and Western Blot Analyses. After dissection, muscles were frozen in isopentane precooled in liquid nitrogen and homogenized in a liquid nitrogen-cooled-cryomortar (PBI International, Milan, Italy). Tissue powder was resuspended in lysis buffer composed of 25 mM Hepes pH 7,4, 150 mM NaCl, 1% nonidet P-40, sodium deoxycholate 0.25%, glycerol 1%, 1 mM EDTA, and protease inhibitors (Protease inhibitor cocktail tablets, Roche, Indianapolis, Indiana). The samples were stirred for 30 min in a rotating wheel and centrifuged at 13,000 rpm for 15 min (two cycles). After the protein content determination of the supernatants by the BCA-protein assay kit (Pierce, Rockford, Illinois), they were frozen in liquid nitrogen and stored at −80°C until use. Samples were loaded (10 μg/lane) in 4%–12% polyacrylamide gels and subjected to electrophoresis, and the proteins were transferred onto nitrocellulose membranes (BA 85, Whatman, Clifton, New Jersey). These membranes were incubated overnight with 5% nonfat dried milk (Santa Cruz Biotechnology, Santa Cruz, California) in PBS containing 0.05% Tween 20 (PBST) prior to a 3-h incubation at 4°C with the anti-N-CAM polyclonal antibody diluted 1:3,000 in PBST containing 5% nonfat milk. This primary antibody was detected by horseradish peroxidase-conjugated antirabbit antibody (Calbiochem, La Jolla, California) diluted 1:2,000 in PBST containing 5% nonfat dried milk and developed by enhanced chemiluminescence (ECL Plus, Amersham, Arlington Heights, Illinois). Membranes were also probed with antitubulin monoclonal antibody (1:1,000, Sigma, St. Louis, Missouri), which serves as an internal standard for protein quantification. Densitometric analysis was performed using "Quantity one" image analysis software (Biorad, Hercules, California).

Statistical Analysis. Descriptive statistics of means and standard deviations (±SD) were calculated. The *t*-test was used post-hoc to determine where differences existed. Significance was established at $P < 0.05$.

RESULTS

Comparison of the BoNT Type A Products on Muscle Weakening. In order to compare the tissue diffusion of the three formulations of BoNT/A, we tested the biological strength of toxin batches by the DAS assay.[1] To this end, we determined the dose of each toxin formulation that caused a loss of the toe spread reflex with a score of 2, i.e., the intramuscular median efficacy dose after 2 days from the TA muscle injection (IM ED_{50}). The doses of 0.25 U for Botox and Xeomin and of 1 U for Dysport were chosen after a dose–response study showed there was no difference between Botox and Xeomin, whereas a 4-fold higher dose of Dysport was needed to achieve the same effect. This result is similar to what has been found in previous studies, which include some performed in humans.[7,16,21] Accordingly, in all subsequent experiments a dose ratio Botox/Dysport/Xeomin 1:4:1 was used.

Histochemical Staining of N-CAM- in BoNT/A-Injected Animals. In order to assess the diffusion of BoNT/A, N-CAM staining was performed. As shown in Figure 1, N-CAM is already localized on the surface of the muscle fibers of the injected TA muscle 7 days postinjection with Botox (Fig. 1a); its staining is well-defined and is identical to that obtained after surgical section of the sciatic nerve (Fig. 1c), showing that the amount of toxin injected was appropriate to produce a very effective chemical blockade comparable to the surgical one. There was no detectable staining of the TA of the contralateral limb (Fig. 1b) or of the muscle injected with saline (Fig. 1d). These results indicate that the staining of N-CAM in the TA muscle of the mouse is a sensitive and reliable readout of the blockade of the skeletal nerve terminals by BoNT/A.

Diffusion of the Muscle Effects of BoNT/A. On this basis, we proceeded to evaluate the extent of diffusion of different BoNT/A preparations by examining the effect of BoNT on muscles located at different distances from the site of injection. In particular, sections of the soleus muscle, which is close to the injected TA muscle, of the gastrocnemius muscle, which is next to the soleus, and of the even more distant quadriceps femoris muscle (QF) which is located in a rostral position, were observed. Figure 2 shows that the soleus muscle was stained in addition to the injected TA muscle, whereas none of the fibers of gastrocnemius or QF were stained. The panels shown in Figure 2 are representative of many sections ($n = 9$) made from each of the different injected animals ($n = 8$). The staining was indistinguishable for the Botox, Dysport, or Xeomin formulations of BoNT/A.

These data show that positive N-CAM staining is already evident at day 7 after injection of each of the three toxin formulations, suggesting that the toxin diffuses in the anatomical area adjacent to the site of

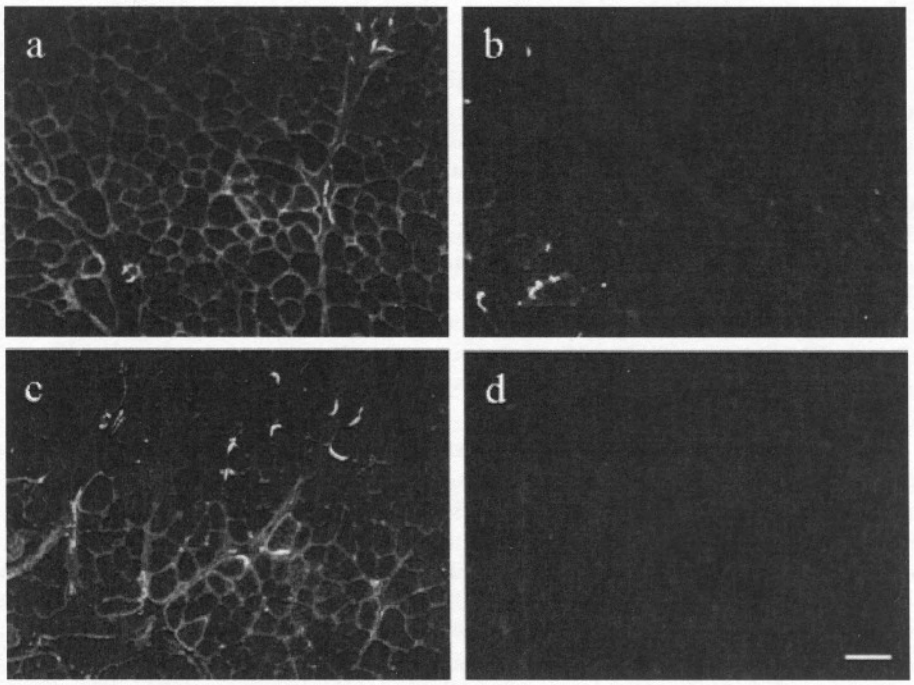

FIGURE 1. Indirect immunofluorescent N-CAM staining of tibialis anterior muscle cryostat sections showing 7-day Botox-injected muscle **(a)**, contralateral muscle **(b)**, 7 day mechanically-denervated muscle **(c)** and saline injected muscle **(d)**. Scale bar = 50 μm.

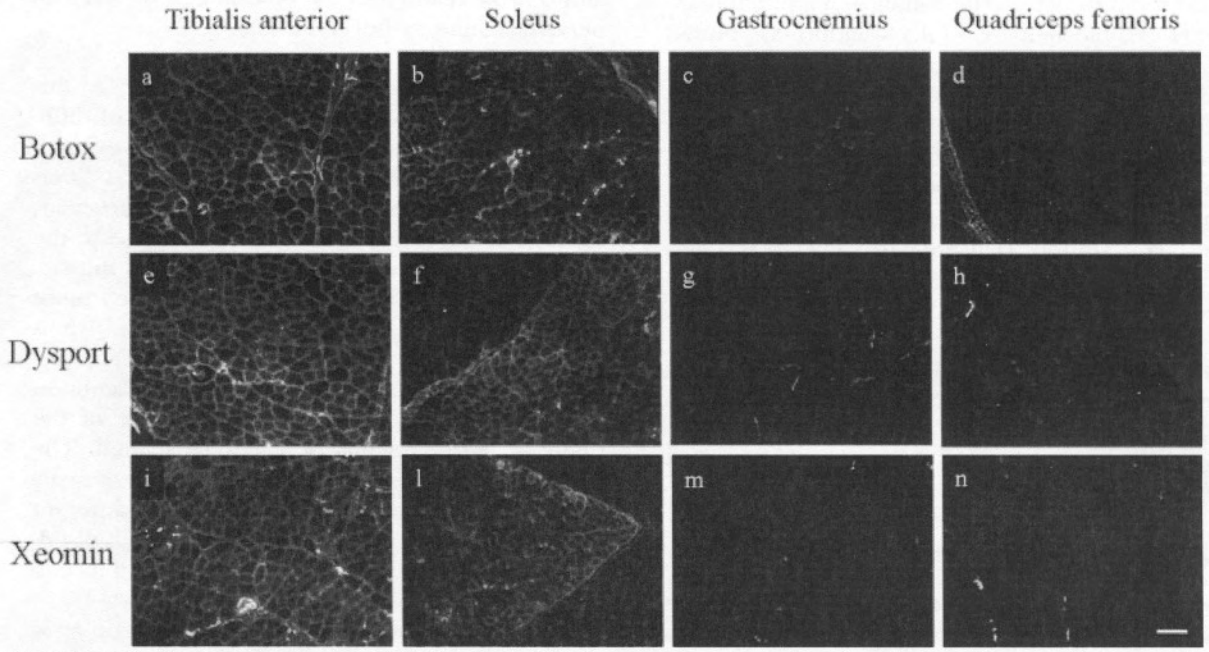

FIGURE 2. Indirect immunofluorescence N-CAM staining of tibialis anterior muscles 7 days after injection with Botox **(a)**, Dysport **(e)** Xeomin **(i)**, soleus **(b,f,l)**, gastrocnemius **(c,g,m)** and quadriceps femoris **(d,h,n)** muscles of ipsilateral injected leg. Staining in **d** is due to nonspecific binding to connective tissue. Scale bar = 100 μm.

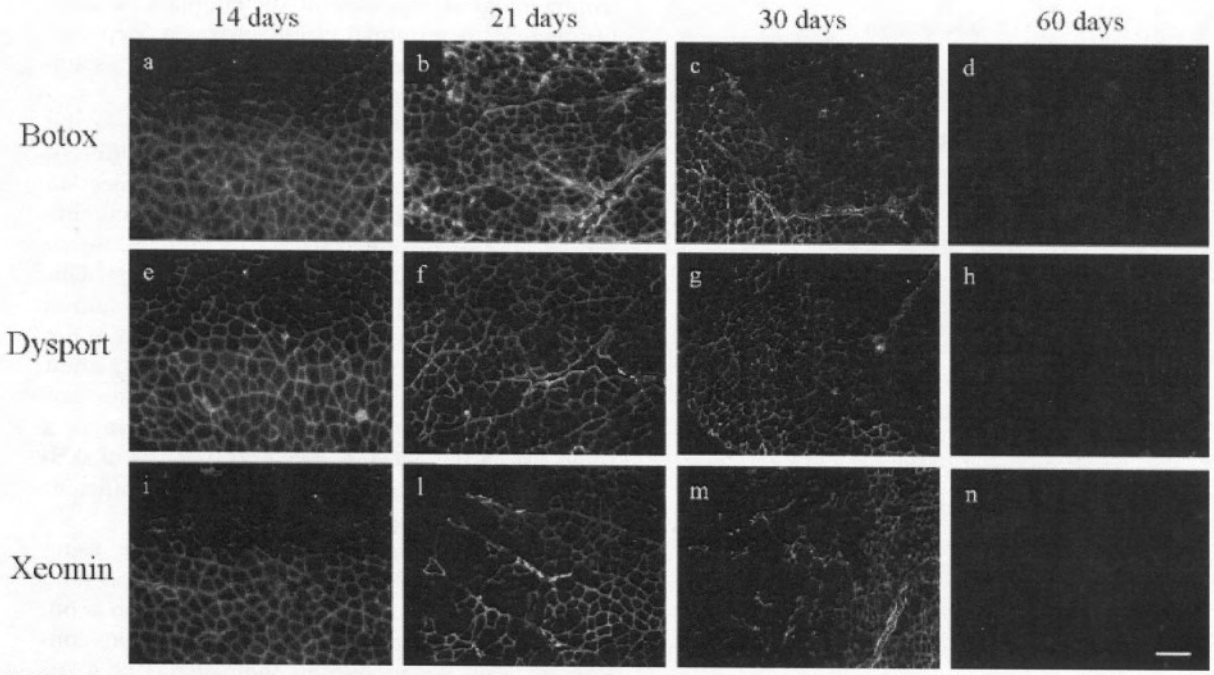

FIGURE 3. Indirect immunofluorescence N-CAM staining of tibialis anterior muscles 14, 21, 30, and 60 days after Botox **(a–d)**, Dysport **(e–h)** or Xeomin injection **(i,l–n)**. Scale bar = 100 μm.

injection. However, there was no N-CAM staining on the contralateral, noninjected side (Fig. 1b), indicating that BoNT/A does not have distant effects.

The level of N-CAM on TA muscle increased at 14 and 21 days postinjection (Fig. 3) and remained detectable even after 30 days from injection, although some fibers became negative for N-CAM staining at this timepoint. However, by day 60 the level of N-CAM staining had dropped to barely detectable levels in the injected TA muscle, which became nearly as negative to N-CAM staining as the distant muscles (Suppl. Figs. 1S, 2S). No difference was found as well in the time course of the decrease of N-CAM staining of muscles injected with the three different formulations. In other words, Botox, Dysport, and Xeomin injection provided an effect on TA N-CAM muscle staining that decreased over time in a similar way.

Western Blot Analysis of BoNT Diffusion. The level of the expression of N-CAM in different muscles after TA injection with the three different formulations of BoNT/A was assessed quantitatively by Western blot analysis. Figure 4 shows the mean and SD of the relative intensities obtained by scanning the bands of Western blots of muscle lysates after different time periods from injection of Botox, Dysport, or Xeomin. The data obtained were normalized using tubulin as standard of loaded material and plotted as intensity units relative to the basal value of the non injected muscle. Figure 4A shows the N-CAM levels of expression in the TA muscles 7, 14, 21, 30, and 60 days after injection of Botox, Dysport, or Xeomin. These data confirm the results obtained by immunohistochemistry with considerable expression of N-CAM in the TA 7 days postinjection with all formulations, which increased at days 14 and 21 postinjection. At 30 days postinjection N-CAM expression started to decrease, and at day 60 it was barely detectable in all cases (Fig. 4A). The low level of expression of N-CAM found in the gastrocnemius (Fig. 4B) and quadriceps femoris (Fig. 4C) muscles again indicates the limited diffusion of BoNT in the three formulations considered here. Small differences among the bars obtained with the three BoNT formulations are not statistically significant ($P \geq 0.05$). Similar to data obtained by immunohistochemistry, no staining was detected by Western blot in any of the muscles of the contralateral hindlimb (not shown).

DISCUSSION

The phenomenon of BoNT diffusion has been considered in humans[12–15] and animals using both direct and indirect methods,[3,21,28] which included elec-

(BoNT/A) Assay

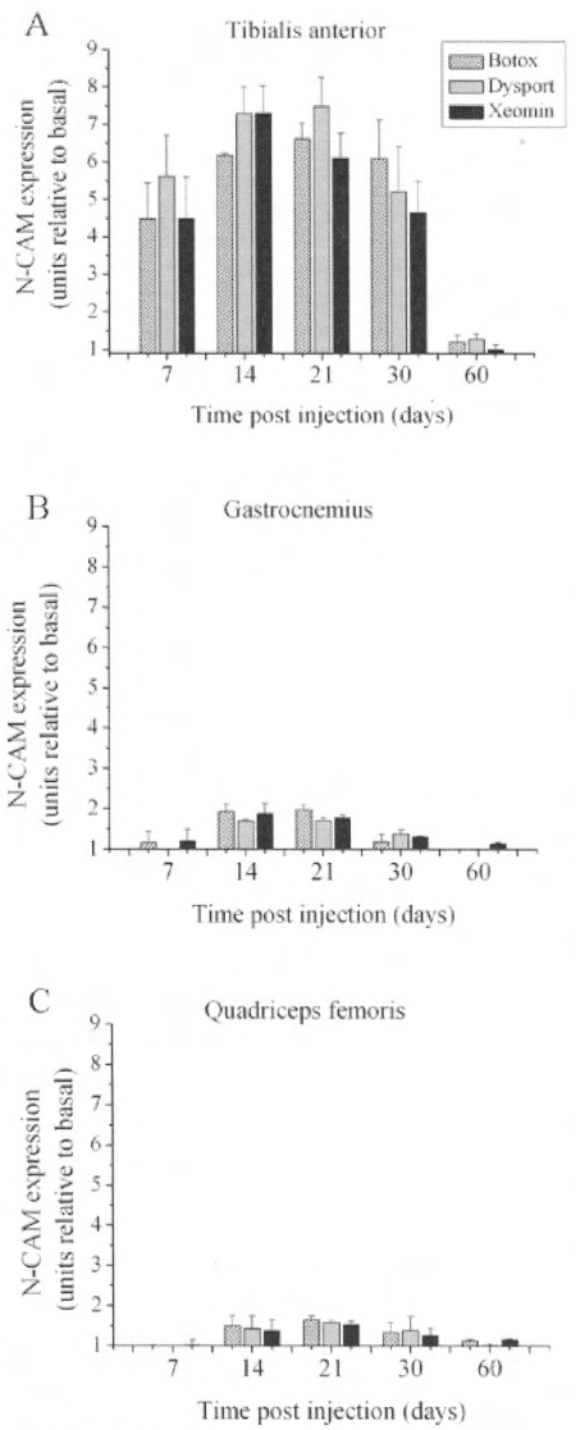

FIGURE 4. N-CAM expression measured by quantitative Western blot analysis. Densitometric analysis of immunoblotting for N-CAM on protein extracts from BoNT/A-injected tibialis anterior **(A)**, gastrocnemius **(B)**, and quadriceps femoris **(C)** muscles of the injected limb prepared at the timepoints from injection of Botox, Dysport, or Xeomin, indicated on the abscissa. Data are means ± SD.

trophysiological assessment of endplate activity, histological determination of glycogen depleted muscle, or of the acetylcholine esterase staining.[5,6,10,12,25]

Here, N-CAM staining was used to assess the diffusion of the biological activity of three different BoNT/A formulations from the point of injection along the mouse hindlimb. The mouse leg was chosen because of the small size of its muscles, which maximizes the possibility of detecting diffusion. This assay relies on regional chemodenervation and is also effective in reflecting the biologic activity of the different preparations of BoNT/A. In fact, adult mouse muscle does not express N-CAM on the sarcolemma. However, after denervation there is a rapid rise in the N-CAM level, and paralysis of skeletal muscle by botulinum toxin injection is sufficient to activate N-CAM expression.[19]

The major finding of this study is that, using equipotent doses of toxin which cause a similar time course of paralysis, no difference of diffusion is observed between the three BoNT/A formulations considered here. We monitored their effects by toxin injection into mouse TA muscle as a function of time. Strong staining of N-CAM was detected in cryostat sections of the injected muscle 7 days after injection, with weaker staining of the soleus muscle and no staining of the gastrocnemius and the quadriceps femoris muscles. These findings indicate that the majority of the neurotoxin remained close to the injection site, and a limited amount of toxin reached adjacent muscles. Our results are consistent with observations of BoNT/A diffusion from injection sites across muscle fascia made in previous studies using Botox.[5,6,26,28] No staining was detected in any of the muscles of the contralateral hindlimb, clearly indicating that there is no systemic spread of the toxin with any of the three toxin formulations.

The time course of N-CAM expression in the TA-injected muscle correlates well with the known timing of reinnervation.[9] There is considerable expression of N-CAM in the TA 7 days postinjection with all formulations, which increased at days 14 and 21 postinjection. At 30 days postinjection N-CAM expression started to decrease, and at day 60 it returned to control levels.

This is the first study that compares three commercial BoNT/A preparations in terms of diffusion properties and with a high spatial definition. Taken together, the present results indicate that there is no significant difference between Botox, Dysport, and Xeomin, and therefore between complexed or free forms, with respect to diffusion into adjacent muscles. This result is consistent with recent findings

that the 150-kDa neurotoxin molecule is released from the 900-kDa complex in less than a minute when exposed to physiological pH values.[11] This time interval is markedly shorter than the time required by BoNT/A to perform the four steps required to block the nerve terminal,[22,24] which can be estimated in terms of hours. To this, one has to add the time required to observe the weakening of muscle contraction, which is estimated in days. The present data are therefore fully consistent with the present knowledge on the biochemistry and cell intoxication of BoNT and with a separation of the active molecule from the accessory proteins taking place immediately after injection.

The containment of diffusion is a desirable goal for botulinum toxin injection therapy. The data presented here demonstrate that N-CAM staining can be used to measure the diffusion of different toxin formulations or at different toxin doses in a very reliable and sensitive mode.

Supported by research grants from Telethon-Italia GGP06133, the University of Padova, and Fondazione Cariparo Project "Physiopathology of the synapse: neurotransmitters, neurotoxins and novel therapies," and Biotech 3bis.

REFERENCES

1. Aoki KR. A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon 2001;39: 1815–1820.
2. Aoki KR, Ranoux D, Wissel J. Using translational medicine to understand clinical differences between botulinum toxin formulations. Eur J Neurol 2006;13(Suppl 4):10–19.
3. Arezzo JC, Litwak MS, Caputo FA, Meyer KE, Shopp GM. Spread of paralysis to nearby and distant noninjected muscles in a monkey hand model: comparison of bont-B and bont-A. In: Brin LC, Hallet M, Jankovic J, editors. Scientific and therapeutic aspects of botulinum toxin. Philadelphia: Lippincott Williams & Wilkins; 2002. p 123–133.
4. Bakheit AM, Ward CD, McLellan DL. Generalised botulism-like syndrome after intramuscular injections of botulinum toxin type A: a report of two cases. J Neurol Neurosurg Psychiatry 1997;62:198.
5. Borodic GE, Joseph M, Fay L, Cozzolino D, Ferrante RJ. Botulinum A toxin for the treatment of spasmodic torticollis: dysphagia and regional toxin spread. Head Neck 1990;12: 392–399.
6. Borodic GE, Ferrante R, Pearce LB, Smith K. Histologic assessment of dose-related diffusion and muscle fiber response after therapeutic botulinum a toxin injections. Mov Disord 1994;9:31–39.
7. Brin MF, Blitzer A. Botulinum toxin: dangerous terminology errors. J R Soc Med 1993;86:493–494.
8. Covault J, Sanes JR. Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralyzed skeletal muscles. Proc Natl Acad Sci U S A 1985;82:4544–4548.
9. De Paiva A, Meunier FA, Molgó J, Aoki KR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci U S A 1999;96:3200–3205.
10. Dodd S, Rowell B, Vrabas I, Arrowsmith R, Weatherill P. A comparison of the spread of three formulations of botulinum neurotoxin A as determined by effects on muscle function. Eur J Neurol 1998;5:181–186.
11. Eisele KH, Taylor HV. Dissociation of the 900 kda neurotoxin complex from C. botulinum under physiological conditions. Toxicon 2008;51:10.
12. 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;46:1158–1160.
13. Eleopra R, Tugnoli V, Quatrale R, Rossetto O, Montecucco C. Different types of botulinum toxin in humans. Mov Disord 2004;19(Suppl 8):S53–59.
14. Flynn TC, Clark RE2. Botulinum toxin type B (Myobloc) versus botulinum toxin type A (Botox) frontalis study: rate of onset and radius of diffusion. Dermatol Surg 2003;29:519–522; discussion, 522.
15. Hsu TSJ, Dover JS, Arndt KA. Effect of volume and concentration on the diffusion of botulinum exotoxin A. Arch Dermatol 2004;140:1351–1354.
16. Jost WH, Blümel J, Grafe S. Botulinum neurotoxin type A free of complexing proteins (Xeomin) in focal dystonia. Drugs 2007;67:669–683.
17. Meunier FA, Schiavo G, Molgó J. Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. J Physiol Paris 2002;96:105–113.
18. Montecucco C, Molgó J. Botulinal neurotoxins: revival of an old killer. Curr Opin Pharmacol 2005;5:274–279.
19. Moore SE, Walsh FS. Nerve dependent regulation of neural cell adhesion molecule expression in skeletal muscle. Neuroscience 1986;18:499–505.
20. Naumann M, Jost W. Botulinum toxin treatment of secretory disorders. Mov Disord 2004;19(Suppl 8):S137–141.
21. Rosales RL, Bigalke H, Dressler D. Pharmacology of botulinum toxin: differences between type A preparations. Eur J Neurol 2006;13(Suppl 1):2–10.
22. Rossetto O, Seveso M, Caccin P, Schiavo G, Montecucco C. Tetanus and botulinum neurotoxins: turning bad guys into good by research. Toxicon 2001;39:27–41.
23. Rossetto O, Morbiato L, Caccin P, Rigoni M, Montecucco C. Presynaptic enzymatic neurotoxins. J Neurochem 2006;97: 1534–1545.
24. Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. Physiol Rev 2000;80:717–766.
25. Shaari CM, George E, Wu BL, Biller HF, Sanders I. Quantifying the spread of botulinum toxin through muscle fascia. Laryngoscope 1991;101:960–964.
26. Shaari CM, Sanders I. Quantifying how location and dose of botulinum toxin injections affect muscle paralysis. Muscle Nerve 1993;16:964–969.
27. Simpson DM, Gracies J, Graham HK, Miyasaki JM, Naumann M, Russman B, et al. Assessment: botulinum neurotoxin for the treatment of spasticity (an evidence-based review): report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology 2008; 70:1691–1698.
28. Yaraskavitch M, Leonard T, Herzog W. Botox produces functional weakness in non-injected muscles adjacent to the target muscle. J Biomech 2008;41:897–902.