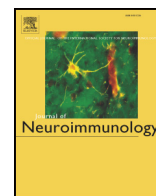




Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Reduction of established antibody responses against botulinum neurotoxin A by synthetic monomethoxypolyethylene glycol peptide conjugates

M. Zouhair Atassi^{a,b,*}, Masooma Naqvi^a, Behzod Z. Dolimbek^a, K. Roger Aoki^c

^a Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX 77030, United States

^b Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030, United States

^c The Neurotoxin Research Program, Department of Biological Sciences, Allergan, Inc., Irvine, CA 92612, United States

ARTICLE INFO

Article history:

Received 25 October 2013

Received in revised form 23 April 2014

Accepted 28 April 2014

Available online xxxx

Keywords:

Antibodies

Botulinum neurotoxin A

Monomethoxypolyethylene glycol

Mouse protection assay

Synaptosome binding

Synthetic peptides

Tolerization

ABSTRACT

In cervical dystonia, injection of botulinum neurotoxin (BoNT) A or B into affected neck muscle reduces symptoms but may elicit anti-toxin antibodies (Abs) that block responsiveness to treatment. Previously, we localized the BoNT/A and BoNT/B sites that bind mouse or human blocking Abs. We also reported that site-specific auto-Abs can be suppressed by a monomethoxypolyethylene glycol (mPEG)-epitope conjugate. So we elicited here anti-toxin Abs in outbred mice by immunization with sublethal-suboptimal doses of active BoNT/A and determined the efficacy of selected mPEG-epitopes in reducing established anti-BoNT/A Abs. We tested in outbred mice four synthetic mPEG-N^α-epitopes [N8 (residues 547–565), N25 (785–803), C15 (1051–1069), C31 (1275–1296)] of BoNT/A in tolerance against ongoing anti-toxin Abs. After short immunizations, tolerization with an mPEG-peptide reduced Abs to correlate peptide and caused varying Ab reductions to the other 3 peptides. Anti-N8 Abs were unaffected by mPEG-N25 tolerization, but mPEG-N8 and mPEG-N25 caused drop in anti-BoNT/A Abs. After long immunization with BoNT/A, tolerization with mPEG-N8 lessened anti-N8 Abs. Anti-C15 Abs decreased by tolerization with mPEG-C15 or any other mPEG-peptide. Anti-N25 Abs were not altered by mPEG-N25, but decreased after tolerization with mPEG-C15. Anti-C31 Abs disappeared on day 474 by tolerization with mPEG-C31 or mPEG-N8, mPEG-N25 or mPEG-C15. When an Ab response returns, a decrease can be re-established by re-administering the correlate mPEG-peptide. The method may be beneficial for extending BoNT treatment in immunoresistant patients.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Botulinum neurotoxins (BoNTs) are protein toxins produced by *Clostridium botulinum* in seven immunological serotypes (A through G). BoNTs are the most toxic substances known. Action of BoNT on the peripheral nervous system causes decline of acetylcholine release at the neuromuscular junction and so leads to local muscle relaxation. This effect on motor neuron activity has been constructively exploited for treatments of an array of clinical conditions linked to involuntary muscle spasm and contractions, and has found increasing usefulness

in cosmetic and other therapeutic applications. It is used in treatment of cervical dystonia (Jankovic, 2004, 2006; Jankovic et al., 2006), gastrointestinal motility disorders (Gui et al., 2003), dermatology (Diamond and Jankovic, 2006), migraine headache (Silberstein, 2001; Binder et al., 2002), otolaryngology, head and neck practice (Yin et al., 2001), strabismus (Kowal et al., 2007), ophthalmology (Dutton and Fowler, 2007), facial rejuvenation (Fagien and Raspaldo, 2007), prostate gland (Galosi, 2010), oral and maxillofacial surgery (Majid, 2010), spasticity and cerebral palsy (Meholjic and Madjar, 2010) and clinical and cosmetic practice (Carruthers et al., 2013). (For reviews see Atassi and Oshima, 1999; Turton et al., 2002; Dressler, 2012).

The clinical benefit, obtained by injection of a sublethal dose of BoNT (usually type A or type B) into the affected muscle, is not permanent and the toxin needs to be re-injected every 3–6 months. Multiple injections of BoNT/A have been reported (Gordon and Barron, 2006) to improve periods of clinical responses. The various BoNTs have different durations of action which have been correlated with the half-lives of blockade of transmitter release yielded inhibition (Foran et al., 2003). The repeated administration of injections and frequency of injections causes some patients to develop blocking anti-BoNT antibody (Ab) responses that

Abbreviations: Ab, antibody; BoNT/A, botulinum neurotoxin serotype A; BSA, bovine serum albumin; H-chain, the heavy chain (residues 449–1296) of BoNT/A; H_C, peptide numbers preceded by C indicate peptides of the C-terminal domain (residues 855–1296) of the H-chain of BoNT/A; H_N, peptides denoted by N indicate peptides of the N-terminal domain (residues 449–859) of the H-chain of BoNT/A; mPEG, monomethoxypolyethylene glycol; MPA, mouse protection assay; PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.20; snps, synaptosomes.

* Corresponding author at: Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, United States.

E-mail address: matassi@bcm.edu (M.Z. Atassi).

<http://dx.doi.org/10.1016/j.jneuroim.2014.04.015>
0165-5728/© 2014 Elsevier B.V. All rights reserved.

Please cite this article as: Atassi, M.Z., et al., Reduction of established antibody responses against botulinum neurotoxin A by synthetic monomethoxypolyethylene glycol peptide conjugates, J. Neuroimmunol. (2014), <http://dx.doi.org/10.1016/j.jneuroim.2014.04.015>

inhibit the normal action of BoNT and prevent its effectiveness in such patients (Göschel et al., 1997; Atassi and Oshima, 1999; Atassi, 2004, 2006; Jankovic, 2004, 2006; Jankovic et al., 2006). The development of blocking Abs in BoNT/A treatment is quite low (1–2%). Nevertheless, with the growing use of the toxin in increasing numbers of clinical conditions it would be extremely beneficial to be able to eliminate, or substantially reduce, the levels of the Ab responses in patients who develop immunoresistance to the toxin so they can continue to be helped by the treatment.

Cervical dystonia (CD) is associated with involuntary contractions of neck muscles which generate pain and cause the head to twist or turn to one side or to tilt forward or backward. Symptoms of CD can be alleviated by injecting the affected muscle with a BoNT. BoNT/B appears to be more likely to elicit blocking Abs in patients than BoNT/A. In a study of 100 patients with CD, a third of the patients who were negative for BoNT/B Abs at baseline became positive for BoNT/B Abs during a 42-month follow up which included about 5 treatment visits per patient (Jankovic et al., 2006). So treatment with BoNT/A elicits antibody responses much less frequently than BoNT/B.

We have recently completed localization of the sites on BoNT/A (Dolimbek et al., 2007; Atassi et al., 2011; Dolimbek et al., 2011a, 2011b) and BoNT/B (Dolimbek et al., 2008; Atassi et al., 2008, 2012) to which blocking Abs are directed in CD patients who, while receiving treatment with a given toxin, become unresponsive to the correlate toxin. In previous work we reported (Atassi et al., 1992) that epitope-specific auto-Ab responses in experimental autoimmune myasthenia gravis can be suppressed by a monomethoxypolyethylene glycol (mPEG) conjugate of a myasthenogenic synthetic peptide. The purpose of the current work was to determine the usefulness of this epitope-specific suppression in substantially reducing ongoing anti-BoNT/A Ab responses. We selected four antigenic sites on the heavy (H) chain of BoNT/A. Each synthetic peptide was conjugated to mPEG, at its N^α-amino group (Atassi and Manshouri, 1991) and each mPEG-peptide conjugate was tested for its ability to decrease ongoing anti-toxin Ab responses in mice.

2. Materials and methods

2.1. Animals, botulinum neurotoxin, synthetic peptides and mPEG derivatives

Six to eight week old outbred (ICR) mice (weight 23–25 g) were obtained from the Center for Comparative Medicine, Baylor College of Medicine. Active pure BoNT/A was purchased from Metabionics (Madison, WI) as a solution (0.25 mg/ml) in 0.01 M phosphate buffer, pH 7.2 containing 0.15 M NaCl (PBS) with 20% glycerol to avoid freezing and stored at –20 °C.

Coupling of monomethoxypolyethylene glycol (mPEG) (molecular weight 5000, Aldrich) to the N-terminal of the peptides was done as described (Atassi and Manshouri, 1991). Peptides N8, N25, C15 and C31 (Fig. 1) were prepared by solid-phase peptide synthesis as described (Atassi et al., 1996; Atassi and Dolimbek, 2004). To 0.18 mM peptide-resin, with the peptide still attached to the resin support and

with its N^α-group deprotected, 0.6 mM mPEG succinate, 0.6 mM 1-hydroxybenzotriazole monohydrate (Peptides International, Louisville, KY) and 0.6 mM N,N'-diisopropylcarbodiimide (Fisher Scientific, Waltham, MA) in 10 ml of a mixture of dichloromethane and dimethylformamide (1:1, vol/vol) were added and reacted until the ninhydrin reaction for the N^α-amino group became negative (3–4 days) (Kaiser et al., 1970). The mPEG-peptide conjugates were cleaved from the resin as described (Albericio et al., 1990) and the conjugate was separated from any uncoupled peptide by Sephadex G-50 F chromatography.

2.2. Tolerization with mPEG-peptides after a short immunization period with BoNT/A

Antibodies against active BoNT/A were elicited in outbred (ICR mice). To stimulate Ab responses under conditions that approximate those in CD patients being treated with active BoNT/A, 7–8 week old mice (55 mice, 24–25 g each) were each injected in the right hind footpad with sublethal doses ($0.70 \times \text{LD}_{100} = 10 \text{ pg}$ in $10 \mu\text{l}$ PBS) of active BoNT/A in PBS. The mice were given two boosters of 11 and 12 pg at 2 weeks and 4 weeks respectively, one in the left footpad and the last in the right footpad. Test bleeds were obtained from the mice two weeks after the last injection. The antisera were divided into five groups (each from 11 mice) so that the anti-BoNT/A Ab titers of the five groups were similar. The antisera were divided into $50 \mu\text{l}$ aliquots and kept frozen until use.

Four of the mouse groups were each tolerized with one of the mPEG-peptides. Tolerization was initiated by injection with an mPEG-peptide ($30 \mu\text{g}$ in $20 \mu\text{l}$ PBS) into each mouse intraperitoneally (i.p.) followed, at 4 days and 8 days later, by similar injections of the same mPEG-peptide. Three days after the last injection of mPEG-peptide, the mice were each given, in the left hind footpad, a booster injection of active BoNT/A (13 pg in $10 \mu\text{l}$ PBS). Fourteen days later test bleeds were obtained from the mice. The fifth group was not tolerized with an mPEG-peptide but was otherwise subjected to BoNT/A booster injections and used as a control. The antisera of mice from each group were mixed, divided into $50 \mu\text{l}$ aliquots and kept frozen until use.

2.3. Tolerization of long established antibody responses

Tolerization with mPEG-peptides after a long establishment of Ab response was also investigated. For this, 55 mice were injected in the footpad with active BNTA (10 pg in $10 \mu\text{l}$ PBS). During an immunization period of 75 weeks, the mice received similar booster injections every two weeks. Test bleeds were taken periodically to determine the titer of the Abs and their *in vivo* protective (toxin blocking) activity. Ten test bleeds were obtained at days (1) 36, (2) 112, (3) 267, (4) 290, (5) 363, (6) 393, (7) 407, (8) 453, (9) 474 and (10) 523. The mice were divided into 5 groups of 11 mice each that had Ab titers of similar levels. For tolerization, mice of groups 1 to 4 were each injected intraperitoneally (i.p.) with mPEG-N8, mPEG-N25, mPEG-C15, or mPEG-C31 ($30 \mu\text{g}$ in $20 \mu\text{l}$ PBS) on days 412, 415 and 419 and again on days 509, 512 and 516. Group 1 received no mPEG-peptide and was used as a control for untolerized Abs.

2.4. Solid phase radioimmunoassay

The synthetic peptides (1 mg) were each dissolved in 0.1 ml DMF and the solution was diluted with 0.9 ml PBS to give a stock solution of 1 mg/ml, and from this a working solution containing $50 \mu\text{g}/\text{ml}$ of PBS was prepared. For measurement of Ab levels, $50\text{-}\mu\text{l}$ aliquots of the peptide ($2.5 \mu\text{g}$) or active BoNT/A ($0.5 \mu\text{g}$) in PBS were introduced into the wells of flexible 96-well flat bottom polyvinyl chloride plate (Becton Dickinson, New Jersey). The plates were covered and left overnight at room temperature. The wells were washed 5 times with PBS after which they were blocked with $100 \mu\text{l}$ of 1.0% BSA in PBS and the plates incubated for 2 h at 37 °C. The plates were washed 5 times with PBS. The prediluted test antisera (1:250 in $50 \mu\text{l}$ 0.1% BSA in PBS) were added to

Peptide	Seq. position in BoNT/A	Amino Acid Sequence
mPEG-N8	547–565	mPEG-KYTMFHYLRAQEFEGKSR
mPEG-N25	785–803	mPEG-NKFLNQCSVSYSYLMNSMIPY
mPEG-C15	1051–1069	mPEG-NNIMFKLDGCRDTHRYIWI
mPEG-C31	1275–1296	mPEG-SRTLGCSEWEIFVDDGWGERPL

Fig. 1. Peptides of BoNT/A H-chain tested in the current studies. For the tolerization studies, mPEG was coupled to each peptide at its N-terminal amino group (Atassi and Manshouri, 1991). These peptides were selected because mouse and human anti-BoNT/A antisera have high to medium Ab levels against these regions (Atassi and Dolimbek, 2004; Dolimbek et al., 2007; Atassi and Dolimbek, 2004).

the wells and the plates were allowed to stand overnight at room temperature after which the plates were washed again 5 times with PBS. An aliquot (50 μ l) of rabbit anti-mouse (IgG + IgM) Abs (Accurate Chemical & Scientific Corporation, New York, NY) which had been prediluted (1:500 vol/vol) in 0.1% BSA in PBS was added into the wells. The plates were incubated at 37 °C for 2 h, washed 5 times with PBS and then Protein A (Sigma, Saint Louis, MO) (^{125} I-labeled, 200,000 cpm in 50 μ l of 0.1% BSA in PBS) was added. The plates were allowed to stand at room temperature for 2 h, washed and dried and the wells were cut out and placed individually into clean tubes and counted for radioactivity in a gamma-counter (1277 Gamma Master, LKB, Finland). Experiments were done in three replicate analyses which gave the average of Ab binding in the antisera of each group of 10–11 mice.

2.5. Synaptosomes binding assay

Synaptosomes (snps) were isolated from mouse brain as described (Whittaker, 1959) with small changes (Dolimbek et al., 2012). Active BoNT/A (10 μ g) was labeled with ^{125} I by the chloramine T method (Hunter and Greenwood, 1962), and free ^{125} I was removed from ^{125} I-labeled BoNT/A by filtering through a Sephadex G-25 M column [in Ringer's buffer containing 120 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 4 mM MgCl₂, 5 mM Tris, and 0.5% BSA, pH 7.0]. The labeled toxin was stored at 4 °C and used within one week of preparation.

Amounts of snps (4 μ l of a 105.0 μ g/ml of snp protein) were incubated (37 °C, 20 min) in duplicates with ^{125} I-labeled active BoNT/A (50,000 cpm) under isotonic conditions in 100 μ l of Ringer's buffer. The reaction mixture was centrifuged (1.5 ml plastic tubes, 20,000 g, room temperature, 3 min), followed by three washes on the centrifuge with 0.5 ml of Ringer's isotonic buffer. After washing the bottoms of the tubes, containing spots of the snp pellet, were cut out, transferred to clean glass tubes and their radioactivity measured in an Automatic Gamma Counter (1277 Gammamaster, LKB-Wallac, Turku, Finland).

2.6. Mouse protection assay

The antisera of mPEG-peptide-tolerized and control intolerized mice were tested in a mouse protection assay (MPA) for their ability to protect mice against challenge with a lethal dose of BoNT/A. The LD₁₀₀ of the present BoNT/A preparation to which no mouse survived was 13.0 pg/mouse. Different dilutions of an mPEG-peptide-tolerized or control antisera (in 0.5 ml of 0.5% BSA in PBS) were mixed with $5 \times 1.05 \times$ LD₁₀₀ of active BoNT/A (68.00 pg) in 0.5 ml of 0.5% BSA/PBS and incubated for 1 h at 37 °C and then placed on ice. Five mice were injected i.v. in the tail with 0.2 ml of the mixture each, per dilution. The control mice received 13.0 pg of active BoNT/A in 0.1 ml 0.5% BSA in PBS. The mice were examined 3 times daily for 6 days. The percent mice that survived the toxin challenge, if any, relative to the total mice per group was determined. The percent decrease in protection in tolerized groups was determined from the titers of Abs relative to the Ab titer observed in the control (group 5).

3. Results

3.1. Stimulation of mouse antibodies against active BoNT/A

We found that immunization of mice with a sublethal, and immunogenically suboptimal, dose of active BoNT/A without adjuvant did in fact stimulate anti-BoNT/A Ab responses even after two booster injections. This enabled us to examine the effect of treatment with various mPEG-peptides on the Ab responses. The levels and activities of the Abs were studied after a short immunization period, and also after a long immunization period, and tolerization.

3.2. mPEG-peptide tolerization after short term immunization with BoNT/A

After three injections with BoNT/A over a period of 30 days, the mice produced Abs against the active toxin. Then the mice received three injections, 3–4 days apart, of a given mPEG-peptide after which they were bled and the Ab titers and activities determined. The Abs so obtained were not able to block the binding of ^{125} I-labeled BoNT/A to snps. The protective activity of the Abs was also checked by a mouse protection assay. The Abs had no protective activity in mice, even when tested undiluted, against a lethal dose (LD₁₀₀ $\times$ 1.05) of BoNT/A. Therefore, we examined the effect of treatment with a given mPEG-peptide on the levels of Abs that bound to BoNT/A, the correlate peptide as well as to each of the other non-correlate peptides. The results are summarized in Table 1. Tolerization with mPEG-N8 after a short establishment of the anti-BoNT/A Ab responses caused, by the second bleed, over 12.2% reduction in Ab level to N8. Similarly, tolerization with each of the mPEG-peptides caused some decrease in Ab levels to the correlate peptide (Ab decrease to correlate peptide after: mPEG-N25 tolerization, about 10%; mPEG-C15, 18%; mPEG-C31, 10.7%). However, we found that each mPEG-peptide caused varying levels of reduction in Ab levels to the other 3 peptides, except for mPEG-N25 that had no effect on Ab levels to N8. The results are summarized in Table 1. In addition, mPEG-N8 and mPEG-N25 caused significant declines in Ab levels that bind to BoNT/A whereas the other mPEG-peptides have no measurable effect.

3.3. mPEG-peptide tolerization after long-term immunization with BoNT/A

Mice were immunized and boosted every 2 weeks with active BoNT/A in PBS. Test bleeds were taken at days 36, 112, 267, 290, 363, 393 and 407 and Abs in each test bleed were checked for snp inhibition and for in vivo ability to block BoNT/A toxicity in mice. None of these antisera had any effect on the binding of the toxin to snps or any in vivo blocking activity by MPA. The antisera, however, had significant anti-BoNT/A Ab titers. So we decided to examine whether the mPEG-peptide treatment will cause a decrease in the levels of such long-term established Ab responses.

Each group of mice received a 3-injection treatment of an mPEG-peptide, after which two test bleeds were taken at days 453 and 474. This was followed by a second 3-injection treatment with the same mPEG-peptide and a final bleed at day 523 after the first immunization. The effect of tolerization by a given mPEG-peptide on the Ab levels to the correlate peptide is summarized in Fig. 2. Tolerization with mPEG-N8 caused a small decrease (by 9.5%) in anti-N8 Ab levels in the 8th (453 day) and by 33% in the 9th (474 day) bleed. Tolerization with mPEG-N25 had no effect on the levels of Abs that bind to N25. Tolerization with mPEG-C15 caused the levels of the Abs that bind C15 to decrease by 12% and 16% in the 8th and 9th test bleeds respectively. Finally tolerization with mPEG-C31 caused the Ab levels to go down 78% and 100% in the 8th and 9th test bleeds.

We also determined the effect of tolerization with a given mPEG-peptide on the Ab responses that bind to the other peptide regions. We found that Abs that bind to C15 and C31 are substantially reduced by toleration with any of the other three non-correlate mPEG-peptides.

Table 1

Reduction in antibody levels to BoNT/A and peptides after tolerization with mPEG-peptide (test bleed 2).

mPEG-peptide	Percent decrease in Ab levels to BoNT/A and peptides				
	BoNT/A	N8	N25	C15	C31
mPEG-N8	18.2	12.24 ^a	28.7	29.1	31.3
mPEG-N25	16.7	0	9.9 ^a	13.4	30.9
mPEG-C15	0.0	22.3	9.0	18.0 ^a	34.0
mPEG-C31	0.0	53.9	46.5	65.6	10.7 ^a

^a These values are expressed in percent reduction of short-term established Ab levels to the peptide after treatment with the correlate mPEG-peptide.

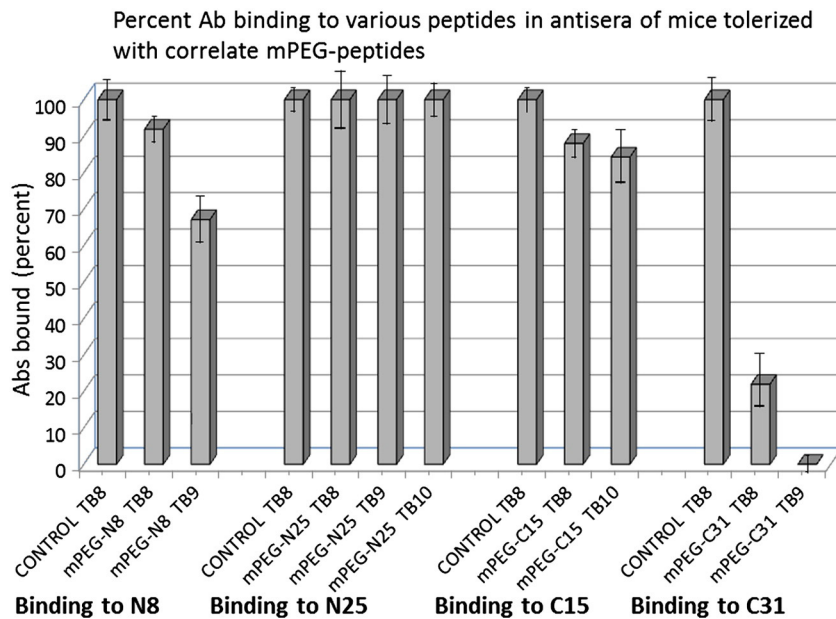


Fig. 2. Percent Ab binding to various peptides in antisera of mice tolerized with correlate mPEG-peptides. The results show the effect of tolerization with each mPEG-peptide on the level of Abs directed against the correlate unmodified peptide. Tolerization with mPEG-N8 reduced anti-N8 Ab levels by 9.5% in test bleed 8 (453 day) and 33% in the test bleed 9 (474 day). Tolerization with mPEG-N25 had no effect on the Ab levels to N25. Tolerization with mPEG-C15 reduced the Ab levels that bind to C15 by 12% and 16% in test bleeds 8 and 9, respectively. Tolerization with mPEG-C31 effected reduction in the Ab levels by 78% and 100% in test bleeds 8 and 9, respectively.

The effect of treatment by the other three mPEG-peptides on the Ab levels to peptide C15 (Fig. 3) was mPEG-N8 (37%), mPEG-N25 (4.6%) and mPEG-C31 (15%), whereas tolerization with mPEG-C15 caused Abs to peptide C15 to go down by 66%. The tolerogenic effect on anti-C31 Abs (Fig. 4) caused Abs to decrease, for example in test bleed 8, mPEG-N8 (40%), mPEG-N25 (60%), and mPEG-C15 (56%) (compare to 80% reduction of anti-C31 Abs after toleration with mPEG-C31). There was little or no effect on anti-N8 or anti-N25 Abs by tolerization with the other three non-correlate mPEG-peptides.

4. Discussion

Treatment of CD patients with BoNTs A or B stimulates in a few patients Ab responses that could render the patient unresponsive to

the treatment. This immunoresistance occurs more in treatment with BoNT/B and much less with BoNT/A (Jankovic et al., 2007). Patients receive a very minute dose of active toxin. The mean treatment dose of BoNT/A (onabotulinumtoxinA) complex per session for CD patients is approximately 236 U or 11.8 ng (Aoki, 2002). Also, CD patients who were injected with BoNT/B mounted higher responses to BoNT/A than patients treated with BoNT/A only (Oshima et al., 2011). Frequent injections (>2.1/year) were associated with a significantly higher T-cell response to BoNT/A. The reason some (about 1–2%) mount blocking Ab responses appears to be linked to the patient's MHC. The current study is designed to investigate the likelihood of manipulating an ongoing anti-BoNT/A Ab response previously established in outbred mice by immunization with active BoNT/A.

We have previously localized the antigenic regions on BoNT/A that are recognized by mouse (Atassi and Dolimbek, 2004; Dolimbek et al., 2005) and by human blocking Abs of cervical dystonia patients who became immunoresistant to BoNT/A (Dolimbek et al., 2007; Atassi et al., 2011). We selected four of the immunodominant epitopes and examined the possibility of reducing the Ab levels against these regions as a potential tool for lowering immunoresistance caused by blocking Abs. The four antigenic regions on BoNT/A (Fig. 1) were synthesized with monomethoxypolyethylene glycol (mPEG) linked to their N-termini (Dolimbek et al., 2011a, 2011b). The mPEG-peptides were able to reduce Ab responses, before their establishment, against the correlate regions and the antisera were less protective in mice than the antisera of untolerized controls (Dolimbek et al., 2011a, 2011b). This selective tolerization method might be potentially useful for clinical applications to immunoresistant patients (Dolimbek et al., 2011a, 2011b). However, as mentioned, those studies examined the effect of animal pre-tolerization on the level and in vivo protective activity of Abs produced by subsequent immunization (Dolimbek et al., 2011a, 2011b). The current work is designed to investigate tolerization effects of the aforementioned mPEG-peptides on pre-established and ongoing Abs. Such conditions will be more comparable to the patient situation.

The mice were, therefore, immunized with sublethal BoNT/A doses that were gradually increased in boosters by 30% per mouse. The mice mounted Ab responses, which even after 17 months of immunizations remained non-blocking. A similar result was obtained in a second

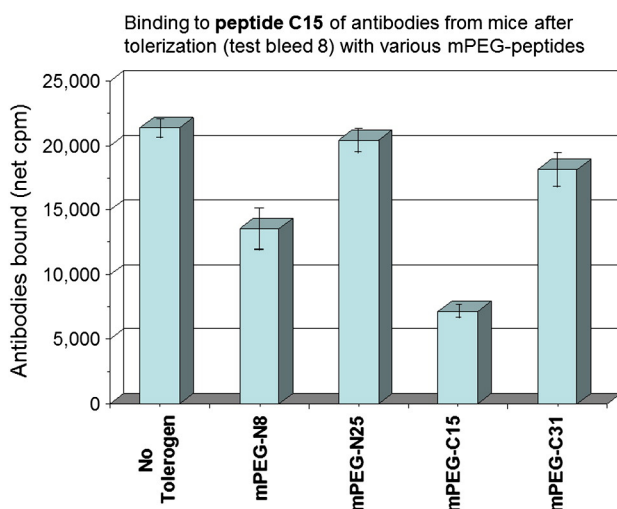


Fig. 3. Binding to peptide C15 of Abs from mice after tolerization (test bleed 8) with various mPEG-peptides. The results were obtained from test bleed 8 (day 453 after the first immunization). They show the net cpm bound to C15 (corrected for non-specific binding to BSA or unrelated peptide) in the absence of any tolerization or after tolerization with mPEG-N8, mPEG-N25, mPEG-C15 or mPEG-C31.

Binding to peptide C31 of Abs from mice after tolerization (test bleeds 8 and 9) with various mPEG-peptides

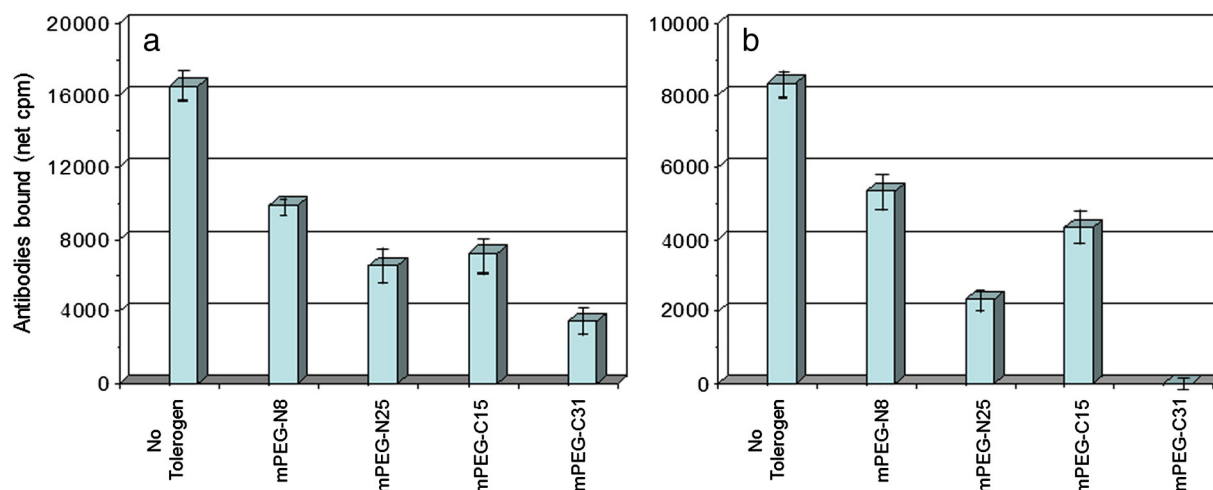


Fig. 4. Binding to peptide C31 of Abs from mice after tolerization with various mPEG-peptides. (a) Test bleed 8 (day 453 after the first immunization); (b) test bleed 9 (day 474 after the initial immunization). The results show the binding of Abs to peptide C31 without tolerization or after tolerization with mPEG-N8, mPEG-N25, mPEG-C15 or mPEG-C31. They are expressed in net cpm bound to peptide C31 after correction for non-specific binding to unrelated protein or peptide.

attempt with a second group of mice. This was not unexpected because the antigen dose was so extremely suboptimal and it was injected into the mice without adjuvant. We investigated the effect of tolerization on the levels of Abs to these epitopes, based on the idea that a decrease of Ab levels will cause a decrease in the blocking activity of the antisera.

The choice of the four peptides (Fig. 1) for this study was based on: (a) Strong Ab responses are mounted against region in peptide N8 in BoNT/A-immunized outbred mice antisera (Atassi and Dolimbek, 2004) but are infrequent in antisera of BoNT/A-treated CD patients and high in hyperimmune human antisera (Atassi and Dolimbek, 2004; Dolimbek et al., 2007). (b) Abs against region N25 are very high in outbred mice (Atassi and Dolimbek, 2004) and also in immunoresistant BoNT/A-treated CD patients (Dolimbek et al., 2007) and in hyperimmune BoNT/A immunized human sera (Atassi and Dolimbek, 2004). Peptide N25 (residues 785–803) overlaps with peptide N26 (799–817), which binds to snps (Maruta et al., 2004). When peptide N25 was administered with a lethal dose ($LD_{100} \times 1.05$) of BoNT/A in vivo into mice it delayed their mortality substantially, while an equimolar mixture of peptides N25 and N26 actually protected about 40% of the mice that were challenged with a lethal dose of the toxin (Atassi et al., 2010). (c) Abs against region C15 are high in BoNT/A-immunized outbred mice, medium in BoNT/A-treated CD patients (Atassi and Dolimbek, 2004; Dolimbek et al., 2007) and high in hyperimmune human sera (Atassi and Dolimbek, 2004). A mixture of peptides C15 (residues 1051–1069) and C16 (residues 1065–1083) administered with toxin delayed mouse mortality substantially (Atassi et al., 2010). (d) The region of peptide C31 elicits strong and medium levels of Abs in BoNT/A-immunized outbred mice or in BoNT/A-treated CD patients, respectively (Atassi and Dolimbek, 2004; Dolimbek et al., 2007). Peptide C31 binds to snps (Maruta et al., 2004), but the free peptide was unable to provide a significant protection in vivo (Atassi et al., 2010). We therefore considered that the mPEG-derivatives of these peptides would be appropriate for investigating the effect of tolerization on the anti-BoNT/A Ab responses to the correlate regions.

We had previously found (Oshima and Atassi, 2000) in experimental autoimmune myasthenia gravis (EAMG) that pretreatment of the mice with mPEG-derivative of the myasthenogenic peptide $\alpha 125$ –148 of the alpha-chain of acetylcholine receptor suppressed EAMG. Suppression of the autoimmune disease was due to T cells participation (Oshima and Atassi, 2000). Transfer of T cells from mPEG-peptide pretreated mice

lowered, in recipient mice, Ab induction (after cell transfer) and Ab production (after two acetylcholine receptor injections) toward alpha-chain peptides. Treatment of B6 mice, which do not develop EAMG, with mPEG-peptide did not change the capability of their antigen-presenting cells to present peptide $\alpha 146$ –162 to peptide-specific B6 T cells.

In short-term established Ab responses, tolerization with each mPEG-peptide reduced the Ab levels from 10% (mPEG-N25) up to 18% (mPEG-C15). But each mPEG peptide also impacted, to considerable extents, the Ab levels to the other peptides (Table 1). In the case of the long-term ongoing Ab responses tolerization with each mPEG-peptide, except for mPEG-N25, reduced the Ab levels to the correlate peptide region. But Abs to region C15 were also reduced by tolerization with mPEG-N8 or mPEG-C31 but not by mPEG-N25. On the other hand, anti-C31 Abs were reduced by tolerization with mPEG-N8, mPEG-N25 or mPEG-C15. The reason why tolerization with one epitope causes reduction of the Ab levels to other epitopes probably stems from the fact that certain BoNT/A antigenic regions are adjoining in the 3-D structure and comprise one recognition feature on the surface of the molecule (Dolimbek et al., 2007). Thus, C15 and C31 occupy a large surface area on native BoNT/A and it is highly probable that suppression of Ab responses against one part of this region causes Ab binding to the other part of this surface to also decline. A similar condition is encountered with N8 and N25. It has been shown that T helper cells against one antigenic site often provide help to B cells that make Abs to the same site recognized by the T cells, but they also may provide help to B cells that produce Abs against other epitopes (Rosenberg et al., 1996; Rosenberg and Atassi, 1997; Hamajima and Atassi, 1998). Since the effect of tolerization with an mPEG-peptide acts initially at the T cell recognition level (Oshima and Atassi, 2000), it is quite likely that suppressed T cells provide less help to B cells that produce Abs against other toxin epitope(s).

The down-regulation of the Ab responses against a given region causes the lower Ab response to be less protective against in vivo challenge with toxin thus causing the 50% protection to be attained at higher concentrations (i.e. lower dilution) of the antisera. The ability to establish Ab suppression after a prolonged immunization suggests that this process would be feasible for clinical application. Although we could not generate blocking Abs by the current immunization protocol, nevertheless the procedure should be useful for lowering the blocking

Ab levels in patients who become immunoresistant to BoNT/A treatment and hence allow the treatment to be effective again in those patients.

In conclusion, we have shown that administration in vivo of mPEG-derivatives of peptides representing antigenic sites recognized by mouse (and human) anti-BoNT/A Abs reduces the levels of Ab responses to the correlate antigenic region on the toxin and causes a decline in the responses to other sites. Suppression of the Ab response if it fades can be revived by re-administration of the correlate mPEG-peptide. The method could be useful for extending the treatment of immunoresistant patients with a BoNT. The method should also be of general therapeutic usefulness, such as modulating auto-Abs in an autoimmune disease and in allergic reactions. mPEG-derivatives are non-toxic, and pegylated protein drugs have been approved for human use by the Food and Drug Administration.

Acknowledgments

This study was supported by an unrestricted grant from Allergan Foundation and by the Welch Foundation (grant Q007), and the award to M.Z.A. of the Robert A. Welch Chair of Chemistry.

References

- Albericio, F., Kneib-Cordonier, N., Biancalana, S., Gera, L., Masada, R.I., Hudson, D., Barany, G., 1990. Preparation and application of the 5-(4-(9-fluorenylmethyloxycarbonyl)aminoethyl-3,5-dimethoxyphenoxy)-valeric acid (PAL) handle for the solid-phase synthesis of C-terminal peptide amides under mild conditions. *J. Org. Chem.* 55, 3730–3743.
- Aoki, K.R., 2002. Immunologic and other properties of therapeutic botulinum toxin serotypes. In: Brin, M.F., Hallett, M., Jankovic, J. (Eds.), *Scientific and Therapeutic Aspects of Botulinum Toxin*. Lippincott Williams & Wilkins, Philadelphia.
- Atassi, M.Z., 2004. Basic immunological aspects of botulinum toxin therapy. *Mov. Disord.* 19, S68–S84.
- Atassi, M.Z., 2006. On the enhancement of anti-neurotoxin antibody production by subcomponents HA1 and HA3b of *Clostridium botulinum* type B 16S toxin-haemagglutinin. *Microbiology* 152 (Pt 7), 1891–1895.
- Atassi, M.Z., Dolimbek, B.Z., 2004. Mapping of the antibody-binding regions on the HN-domain (residues 449–859) of botulinum neurotoxin A with antitoxin antibodies from four host species. Full profile of the continuous antigenic regions of the H-chain of botulinum neurotoxin A. *Protein J.* 23, 39–52.
- Atassi, M.Z., Manshouri, T., 1991. Synthesis of tolerogenic monomethoxypolyethylene glycol and polyvinyl alcohol conjugates of peptides. *J. Protein Chem.* 10, 623–627.
- Atassi, M.Z., Oshima, M., 1999. Structure, activity, and immune T and B cell recognition of botulinum neurotoxins. *Crit. Rev. Immunol.* 19 (3), 219–260.
- Atassi, M.Z., Ruan, K.H., Jinnai, K., Oshima, M., Ashizawa, T., 1992. Epitope-specific suppression of antibody response in experimental autoimmune myasthenia gravis by a monomethoxypolyethylene glycol conjugate of a myasthenogenic synthetic peptide. *Proc. Natl. Acad. Sci. U. S. A.* 89, 5852–5856.
- Atassi, M.Z., Dolimbek, B.Z., Hayakari, M., Middlebrook, J.L., Whitney, B., Oshima, M., 1996. Mapping of the antibody-binding regions on botulinum neurotoxin H-chain domain 855–1296 with antitoxin antibodies from three host species. *J. Protein Chem.* 15, 691–700.
- Atassi, M.Z., Dolimbek, B.Z., Jankovic, J., Steward, L.E., Aoki, K.R., 2008. Molecular recognition of botulinum neurotoxin B heavy chain by human antibodies from cervical dystonia patients that develop immunoresistance to toxin treatment. *Mol. Immunol.* 45 (15), 3878–3888 (Epub 2008 Aug 3).
- Atassi, M.Z., Dolimbek, B.Z., Steward, L.E., Aoki, K.R., 2010. Inhibition of botulinum neurotoxin A toxic action in vivo by synthetic synaptosome- and blocking antibody-binding regions. *Protein J.* 29 (5), 320–327 (Jul). Erratum in: *Protein J.* 29 (6):453–455.
- Atassi, M.Z., Dolimbek, B.Z., Jankovic, J., Steward, L.E., Aoki, K.R., 2011. Regions of botulinum neurotoxin A light chain recognized by human anti-toxin antibodies from cervical dystonia patients immunoresistant to toxin treatment. The antigenic structure of the active toxin recognized by human antibodies. *Immunobiology* 216 (7), 782–792 (Epub 2010 Dec 25).
- Binder, W.J., Brin, M.F., Blitzer, A., Pogoda, J.M., 2002. Botulinum toxin type A (BOTOX) for treatment of migraine. *Dis. Mon.* 48, 323–335.
- Carruthers, A., Kane, M.A., Flynn, T.C., Huang, P., Kim, S.D., Solish, N., Kaeuper, G., 2013. The convergence of medicine and neurotoxins: a focus on botulinum toxin type A and its application in aesthetic medicine—a global, evidence-based botulinum toxin consensus education initiative: part I: botulinum toxin in clinical and cosmetic practice. *Dermatol. Surg.* 39 (3 Pt 2), 493–509.
- Diamond, A., Jankovic, J., 2006. Botulinum toxin in dermatology — beyond wrinkles and sweat. *J. Cosmet. Dermatol.* 5, 169.
- Dolimbek, G.S., Dolimbek, B.Z., Aoki, K.R., Atassi, M.Z., 2005. Mapping of the antibody and T cell recognition profiles of the HN domain (residues 449–859) of the heavy chain of botulinum neurotoxin A in two high-responder mouse strains. *Immunol. Invest.* 34, 119–142.
- Dolimbek, B.Z., Aoki, K.R., Steward, L.E., Jankovic, J., Atassi, M.Z., 2007. Mapping of the regions on the heavy chain of botulinum neurotoxin A (BoNT/A) recognized by antibodies of cervical dystonia patients with immunoresistance to BoNT/A. *Mol. Immunol.* 44, 1029–1041.
- Dolimbek, B.Z., Steward, L.E., Aoki, K.R., Atassi, M.Z., 2008. Immune recognition of botulinum neurotoxin B: antibody-binding regions on the heavy chain of the toxin. *Mol. Immunol.* 45 (4), 910–924 (Epub 2007 Sep 25).
- Dolimbek, B.Z., Aoki, K.R., Atassi, M.Z., 2011a. Reduction of antibody response against botulinum neurotoxin A by synthetic monomethoxypolyethylene glycol-peptide conjugates. *Immunol. Lett.* 137 (1–2), 46–52. <http://dx.doi.org/10.1016/j.imlet.2011.02.002> (Jun 30, Epub Feb 13).
- Dolimbek, B.Z., Steward, L.E., Aoki, K.R., Atassi, M.Z., 2011b. Regions of recognition by blocking antibodies on the light chain of botulinum neurotoxin A: antigenic structure of the entire toxin. *Immunobiology* 216 (6), 698–706 (Epub 2010 Nov 4).
- Dolimbek, B.Z., Steward, L.E., Aoki, K.R., Atassi, M.Z., 2012. Location of the synaptosome-binding regions on botulinum neurotoxin B. *Biochemistry* 51 (1), 316–328 (Epub 2011 Dec 29).
- Dressler, D., 2012. Clinical applications of botulinum toxin. *Curr. Opin. Microbiol.* 15 (3), 325–336.
- Dutton, J.J., Fowler, A.M., 2007. Botulinum toxin in ophthalmology. *Surv. Ophthalmol.* 52 (1), 13–31.
- Fagien, S., Raspaldo, H., 2007. Facial rejuvenation with botulinum neurotoxin: an anatomical and experiential perspective. *J. Cosmet. Laser Ther.* 9 (Suppl. 1), 23–31.
- Foran, P.G., Mohammed, N., Lisk, G.O., Nagwaney, S., Lawrence, G.W., Johnson, E., Smith, L., Aoki, K.R., Dolly, J.O., 2003. Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. *J. Biol. Chem.* 278, 1363–1371.
- Galosi, A.B., 2010. Clinical and experimental applications of botulinum toxins in humans: the prostate gland as example. *Anal. Quant. Cytol. Histol.* 32 (4), 183–185 (Aug).
- Gordon, M.F., Barron, R., 2006. Effectiveness of repeated treatment with botulinum toxin type A across different conditions. *South. Med. J.* 99, 853–861.
- Göschel, H., Wohlfarth, K., Frevert, J., Dengler, R., Bigalke, H., 1997. Botulinum A toxin therapy: neutralizing and nonneutralizing antibodies—therapeutic consequences. *Exp. Neurol.* 147, 96–102.
- Gui, D., Rossi, S., Runfola, M., Magalini, S.C., 2003. Review article: botulinum toxin in the therapy of gastrointestinal motility disorders. *Aliment. Pharmacol. Ther.* 18, 1–16.
- Hamajima, S., Atassi, M.Z., 1998. B-cell activation in vitro by helper T cells specific to a protein region that is recognized both by T cells and by antibodies. *Immunol. Invest.* 27, 121–134.
- Hunter, W.M., Greenwood, F.C., 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* 194, 495–496.
- Jankovic, J., 2004. Botulinum toxin in clinical practice. *J. Neurol. Neurosurg. Psychiatry* 75, 951–957.
- Jankovic, J., 2006. Botulinum toxin therapy for cervical dystonia. *Neurotox. Res.* 9, 145–148.
- Jankovic, J., Hunter, C., Dolimbek, B.Z., Dolimbek, G.S., Adler, C.H., Brashear, A., Comella, C. L., Gordon, M., Riley, D.E., Sethi, K., Singer, C., Stacy, M., Tarsy, D., Atassi, M.Z., 2006. Clinico-immunologic aspects of botulinum toxin type B treatment of cervical dystonia. *Neurology* 67 (12), 2233–2235.
- Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 34, 595–598.
- Kowal, L., Wong, E., Yahalom, C., 2007. Botulinum toxin in the treatment of strabismus. A review of its use and effects. *Disabil. Rehabil.* 29 (23), 1823–1831.
- Majid, O.W., 2010. Clinical use of botulinum toxins in oral and maxillofacial surgery. *Int. J. Oral Maxillofac. Surg.* 39 (3), 197–207.
- Maruta, T., Dolimbek, B.Z., Aoki, K.R., Steward, L.E., Atassi, M.Z., 2004. Mapping of the synaptosome-binding regions on the heavy chain of botulinum neurotoxin A by synthetic overlapping peptides encompassing the entire chain. *Protein J.* 23, 539–552.
- Meholjic, A., Madjar, D., 2010. Application of botulinum toxin in treatment of spasticity and functional improvements for children suffering from cerebral palsy. *Med. Arh.* 64 (6), 359–361.
- Oshima, M., Atassi, M.Z., 2000. T cells of mice treated with mPEG-myasthenogenic peptide conjugate are involved in protection against EAMG by stimulating lower pathogenic antibody responses. *Autoimmunity* 32 (1), 45–55.
- Oshima, M., Deitiker, P.R., Jankovic, J., Duane, D.D., Aoki, K.R., Atassi, M.Z., 2011. Human T-cell responses to botulinum neurotoxin. Responses in vitro of lymphocytes from patients with cervical dystonia and/or other movement disorders treated with BoNT/A or BoNT/B. *J. Neuroimmunol.* 240–241, 121–128 (Dec 15, Epub 2011 Nov 12).
- Rosenberg, J.S., Atassi, M.Z., 1997. Intersite helper function of T cells specific for a protein epitope that is not recognized by antibodies. *Immunol. Invest.* 26, 473–489.
- Rosenberg, J.S., Oshima, M., Atassi, M.Z., 1996. B-cell activation in vitro by helper T cells specific to region alpha 146–162 of *Torpedo californica* nicotinic acetylcholine receptor. *J. Immunol.* 157, 3192–3199.
- Silberstein, S.D., 2001. Review of botulinum toxin type A and its clinical applications in migraine headache. *Expert Opin. Pharmacother.* 2 (10), 1649–1654.
- Turtton, K., Chaddock, J.A., Acharya, K.R., 2002. Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *Trends Biochem. Sci.* 27, 552–558.
- Whittaker, V.P., 1959. The isolation and characterization of acetylcholine-containing particles from brain. *Biochem. J.* 72, 694–706.
- Yin, S., Stucker, F.J., Nathan, C.O., 2001. Clinical application of botulinum toxin in otolaryngology, head and neck practice (brief review). *J. La State Med. Soc.* 153 (2), 92–97.