Toxicon 107 (2015) 50–58

Contents lists available at ScienceDirect

Toxicon

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

Review

Molecular basis of immunogenicity to botulinum neurotoxins and uses of the defined antigenic regions

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ARTICLE INFO

Article history:
Received 5 June 2015
Accepted 11 June 2015
Available online 16 June 2015

Keywords:
Botulinum neurotoxin
Antibodies
Epitopes
Synaptosomes
Tolerization
Monomethoxypolyethylene glycol peptides

ABSTRACT

Intensive research in this laboratory over the last 19 years has aimed at understanding the molecular bases for immune recognition of botulinum neurotoxin, types A and B and the role of anti-toxin immune responses in defense against the toxin. Using 92 synthetic 19-residue peptides that overlapped by 5 residues and comprised an entire toxin (A or B) we determined the peptides' ability to bind anti-toxin Abs of human, mouse, horse and chicken. We also localized the epitopes recognized by Abs of cervical dystonia patients who developed immunoresistance to correlate toxin during treatment with BoNT/A or BoNT/B. For BoNT/A, patients' blocking Abs bound to 13 regions (5 on L and 8 on H subunit) on the surface and the response to each region was under separate MHC control. The responses were defined by the structure of the antigen and by the MHC of the host. The antigenic regions coincided or overlapped with synaptosomes (SNPS) binding regions. Antibody binding blocked the toxin's ability to bind to neuronal cells. In fact selected synthetic peptides were able to inhibit the toxin's action *in vivo*. A combination of three synthetic strong antigenic peptides detected blocking Abs in 88% of immunoresistant patients' sera. Administration of selected epitopes, pre-linked at their N^{α} group to monomethoxyployethylene glycol, into mice with ongoing blocking anti-toxin Abs, reduced blocking Ab levels in the recipients. This may be suitable for clinical applications. Defined epitopes should also be valuable in synthetic vaccines design.

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1. Introduction

Botulinum neurotoxins (BoNTs) are protein toxins produced by the Gram-positive, obligate anaerobic bacterium *Clostridium botulinum*. The toxins are expressed as an inactive single polypeptide chain which is cleaved post-translationally into two polypeptide chains, a 100 kDa heavy (H) subunit and a light (L) 50 kDa subunit, that are linked by a disulfide bond (Kumaran et al., 2008). The L subunit is a Zn^{2+} endopeptidase (Schiavo et al., 1992; Fu et al., 1998). There are eight different serotypes (A through H) of BoNT (Baldwin et al., 2008; Barash and Arnon, 2014; Dover et al., 2014) and a number of subtypes for each serotype.

The toxin is the most lethal natural substance identified. Its effectiveness stems from its extraordinary specificity for nerve endings. It binds first through its H chain using both the H_C and the H_N domains (Ayyar et al., 2015) to the membrane of the nerve ending on the presynaptic neuromuscular junctions, then the L chain is translocated and set free in the cytosol where its enzymatic action causes proteolysis of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex proteins [VAMP (vesicle-associated membrane protein), Syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa)]. Cleavage of SNARE proteins prevents fusion of synaptic vesicles (containing the neurotransmitter, acetylcholine) onto the plasma membrane, and thus blocks release of the neurotransmitter and causes paralysis (Bajjalieh, 1999; Schiavo et al., 2000). BoNTs A and E cleave SNAP-25 (Vaidyanathan et al., 1999), while BoNT/B (and D, F and G) cleave VAMP at particular peptide bonds (for a recent review of BoNT's action see Aoki et al., 2010). BoNT/C is uniquely able to cleave both, SNAP-25 and syntaxin (Williamson et al., 1996; Osen-Sand et al.,

Abbreviations: BoNT, botulinum neurotoxin; Ab, antibody; CD, cervical dystonia; MPA, mouse protection assay; SNPS, synaptosomes; L subunit, residues 1–453 of BoNT/A; H chain, the heavy chain (residues 449–1296) of BoNT/A; H_N, the N-terminal domain (residues 449–859) of the BoNT/A H chain; H_C, the C-terminal domain (residues 855–1296) of the H chain; N1–N29, synthetic H_N domain peptides; C1–C31, synthetic H_C domain peptides; mPEG, monomethoxypolyethylene glycol.

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http://dx.doi.org/10.1016/j.toxicon.2015.06.003
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1996).

Understanding how BoNTs work has opened up the way to a variety of uses in many therapeutic, pharmaceutical, and cosmetic applications (Atassi and Oshima, 1999; Benedetto, 1999; Turton et al., 2002; Jankovic et al., 2009). However, the detail of the full mechanism of action is still to be revealed. Injection of extremely minute sublethal doses of toxin into the affected muscle causes its temporary relaxation. But in any clinical application, the toxin's action declines in weeks to months after injection (depending on the toxin serotype used) and therefore the toxin would need to be re-injected every 3–6 months. Repeated administration of the toxin may result in the stimulation of anti-toxin Ab responses that can block the action of the toxin. Increasing the toxin dose would also of course boost the Ab responses even more. And switching to another toxin serotype would mostly provide brief benefit for only a few injections before blocking Abs against the second serotype then appear (Jankovic et al., 2006).

In the last 19 years we have carried out intensive research to identify the submolecular regions of BoNT serotypes A and B that are involved in binding to synaptosomes (SNPS) (Maruta et al., 2004; Dolimbek et al., 2012) and the sites that are recognized by anti-toxin T lymphocytes (Oshima et al., 2011a, b; Atassi et al., 2012a,b; 2014; Ayyar et al., 2015) and by Abs of human, mouse, horse and chicken. We then used this information to lower anti-toxin blocking Ab responses and to design anti-toxin synthetic vaccines. In this article, the molecular bases of immune recognition of BoNTs A and B are summarized followed by use of these epitopes in reducing the anti-toxin Ab response.

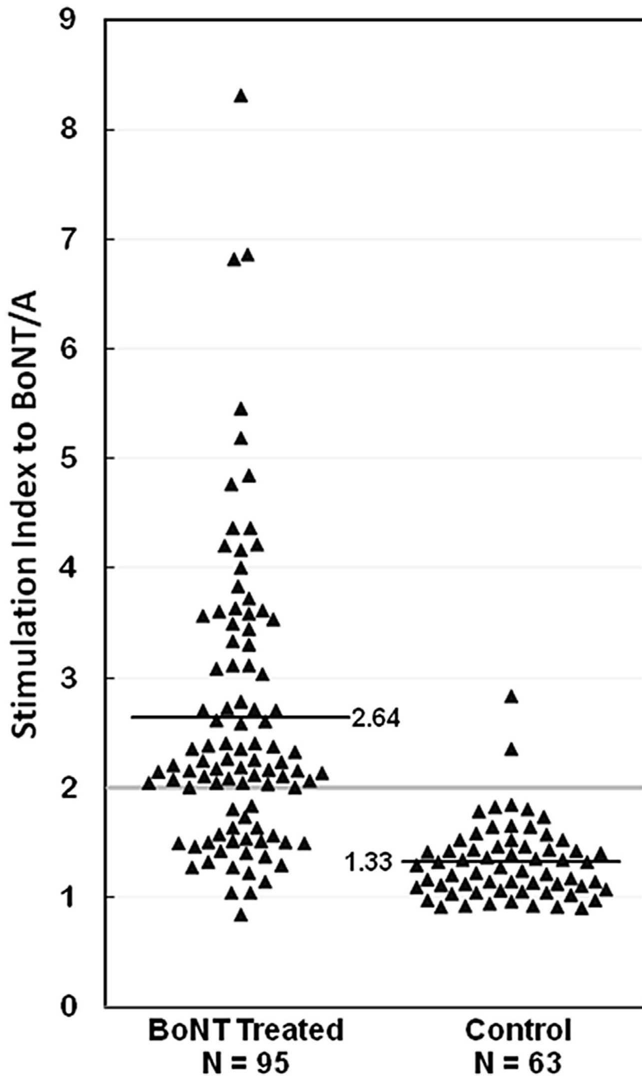

Fig. 1. T-cell responses to BoNT/A of PBL from 95 patients treated with BoNT and from 63 control subjects who never received the toxin. Average of SI: treated patient, 2.64 ± 1.33 (geometric mean 2.38 + 1.34/−0.86); control, 1.33 ± 0.35 (geometric mean 1.29 + 0.34/−0.27); $P < 10^{-20}$. Positive rate (SI≥2.0): patient, 68/95 = 72%; control, 2/63 = 3%; $P < 10^{-18}$. Figure is from Oshima et al., 2011b. Journal of Neuroimmunology 237, 66–72.

1.1. T lymphocyte responses in BoNT treated patients

To date, the T lymphocyte response studies in humans have been at the whole molecule level. No information is yet available about the submolecular features recognized on a BoNT molecule by T lymphocytes in humans. However information on the molecular features recognized by T cells in high responder mouse strains has been reported (Oshima et al., 1997, 1998, 2014; Dolimbek et al., 2005; Atassi et al., 2012b; Atassi and Oshima, 1999; Rosenberg, 1997).

We determined the T-cell responses against BoNT/A and against tetanus toxin (TeNT) of peripheral blood lymphocytes (PBLs) from 95 BoNT-treated patients and 63 untreated controls (Oshima et al., 2011a). The patients included 80 cervical dystonia (CD) cases and 15 cases of additional movement disorders. Most (68%) of the treated patients and only 3% of the controls mounted positive T cell responses (Fig. 1). In the treated patients, the T-cell responses were not different for those who were still clinically responsive and those who had developed unresponsiveness to BoNT treatment. BoNT-treated patients gave significantly higher *in vitro* cross-reactive T-cell responses to TeNT than did the controls (Oshima et al., 2011a). The T-cell responses to BoNT/A were significantly higher in CD patients than in patients with different movement disorders (Oshima et al., 2011b). A higher cross-reactive T-cell response to TeNT was observed in CD relative to untreated controls. Furthermore, CD patients who were treated with BoNT/B had higher T cell responses to BoNT/A than those treated only with BoNT/A (Oshima et al., 2011b). Repeated BoNT/A injections (more than 2.1/year) in CD resulted in a higher anti-BoNT/A T-cell response (Oshima et al., 2011b).

Table 1
Locations of the major antigenic regions of BoNT/A recognized by blocking Abs from CD patients treated with BoNT/A.

Peptide	Position	Primary structure	Antibody response
L-chain			
L11	141–159	DGSYRSEELNLVIIGPSAD	Very Strong
L14	183–201	TQYIRFSPDFTFGFEESLE	Strong
L18	239–257	PNRVFKVNTNAYYEMSGLE	Strong
L27	365–383	TYLNFDKAVFKINIVPKVN	Very weak
L29	393–411	RNTNLAANFNGQNTEINNM	Very weak
H-chain			
N4	491–509	EENISLDLIQQYYLTFNFD	Very weak
N16	659–677	SGAVILLEFIPEIAIPVLG	Very weak
N22	743–761	TKAIINYQYNQYTEEEKNN	Very weak
N25	785–803	NKFLNQCSVSYLMNSMIPY	Very strong
C10	981–999	GEIIWTLQDTQEIKQRVVF	Very strong
C15	1051–1069	NNIMFKLDGCRDTHRYIWI	Medium
C20	1121–1139	KYVDVNNVGIRGYMYLKGP	Medium
C31	1275–1296	SRTLGCSWEFIPVDDGWGERPL	Medium

Table summarizes structures within which reside the major antigenic regions recognized by human blocking Abs from BoNT/A-treated CD patients who developed immunoresistance to the treatment. The L-chain regions are from Atassi et al. (2011). The H-chain Ab binding regions are from Dolimbek et al. (2007).

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