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Original article

A new neutralizing antibody against botulinum neurotoxin B recognizes the protein receptor binding sites for synaptotagmins II

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Abstract

Botulinum neurotoxins (BoNts) pose a biological hazard to humans and a serious potential bioweapon threat. Given the safety concern regarding the currently used equine antitoxin therapy for botulism, it is imperative to develop agents that are effective binding inhibitors. The aim of this study was to identify a novel neutralizing antibody against botulinum neurotoxin B (BoNtb) that recognizes the protein receptor binding sites for synaptotagmins II. This antibody showed significant dose-dependent protection against lethal toxin challenge *in vivo* at an intraperitoneal (i.p.) dose 10 times the half lethal dose (LD50). We proved that the efficacy of SC12 was based on its counteraction on the recognition and binding of BoNtb to target cells, resulting from the combination of antibody with the high affinity (KD: 1.34 nM) to protein receptor binding sites of BoNt by targeting a 25-mer dominant antigenic site on Hcc region (residues 1253–1277). The structure of the site targeted by this antibody overlaps the pocket-like protein receptor binding sites located at the distal tip of toxin molecule. Information gained from this study will facilitate the development of potent inhibitors that prevent the binding of BoNts with its receptors.

Keywords: Botulinum neurotoxins; Synaptotagmins II; Antigenic epitope peptide; Neutralizing antibody; Receptor binding sites

1. Introduction

Botulinum neurotoxins (BoNts) are the etiological agents responsible for botulism, a disease characterized by peripheral neuromuscular blockade and a characteristic flaccid paralysis in humans. Seven immunologically distinct serotypes of the neurotoxin (A–G) are produced and secreted by the strains of the gram-positive, sporulating, anaerobic bacillus *Clostridium botulinum*, as well as by the neurotoxigenic strains of *Clostridium butyricum* and *Clostridium baratii* [1,2]. These toxins are the most lethal substances known, with an estimated lethal intravenous dose of 1–5 ng kg^{-1} in humans [3,4], and they are listed as category A (highest priority) bioterror agents by the Centers for Disease Control and Prevention in many countries because of their extreme potency, lethality, ease of production, ease of transportation, and the need for prolonged medical care on exposure to these agents [3]. These toxins may be dispersed in the form of aerosols [4,5], and consequently, represent a serious threat for exposure [6,7]. Moreover, BoNts are now established as biotherapeutic agents for a range of physical ailments and cosmetic treatments [5,8,9], thus increasing their misuse and adverse effects [10]. Reports have described the use of currently available antitoxins or antibodies to treat BoNts toxicity by blocking these toxins before they enter the neurons. Thus, another treatment option

Abbreviations used: BoNtb, botulinum neurotoxin serotype B; Hc, C-terminus of the heavy chain; *Vh*, variable region of heavy chain; *Vl*, light variable region of light chain; MBP, maltose binding proteinscFv, single-chain variable fragment; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Syt II, Synaptotagmins II; LD50, the median lethal dose; KD, equilibrium constant; RBD, receptor binding domain; 3-D, three dimensions; CDRs, complementary determinant regions; PDB, protein database.

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is to develop therapeutic antibodies that serve as post-exposure prophylactic therapeutics.

The mode of action of BoNts involves 3 steps: (1) binding to receptors on the presynaptic membranes via the C-terminus of the heavy chain (Hc); (2) translocation of the light chain into the cytosol via the N-terminus of the heavy chain (Hn); and (3) cleavage of one or more key components in the synaptic vesicle docking and fusion protein complex by the zinc protease activity of the light chain. Structurally, BoNts consist of 3 functional domains: catalytic, translocation, and binding. The occurrence of BoNt poisoning depends on binding of the Hc protein to its receptor, especially to specific protein receptors such as synaptotagmins I and II (Syt I, II) or synaptic vesicle glycoprotein (SV) [11−16]. In some recent studies on the Syt II toxin receptor or ganglioside, the C-terminus of Hc (Hcc) was identified as the receptor binding domain (RBD) [17−19]. Theoretically, an effective blockade of Hc receptor binding can be achieved by using antibodies targeting the RBD of BoNts.

In previous study, it has been proven that the Hc proteins of toxins of different serotypes (A−B), which are safe and effective substitutes for toxoids, could induce protective immunity *in vivo* against BoNt [20−22]; this result leads to a hypothesis that neutralizing antibodies could be selected from Hc-immunized phage-display library. To further confirm which sites or areas contain dominant antigenic epitopes, we designed and chemically synthesized 8 mimic peptides that contained possible antigenic epitopes from the Hc region (residues 845−1291) of BoNt serotype B (BoNtb) by using 3-D structure-based bioinformatic prediction methods [23,24]. In peptide-specific enzyme-linked immunosorbent assay (ELISA) with the 8 peptides, BP7 and BP8 were more easily recognized and their binding with antitoxins of BoNtb was better than that of the others. Furthermore, peptides BP4, BP7, and BP8 showed marked protection against toxin challenge at minimum lethal dose in mice with 25.0% (2 survivals of 8 tested animals), 37.5% (3 survivals of 8 tested animals), and 50.0% (4 survivals of 8 tested animals) survival rate (data not published), respectively; thus, suggesting that these peptides might contain neutralizing epitopes.

In order to identify neutralizing antibodies that could disrupt the interaction of BoNtb with its protein receptor Syt II, we employed the combination of *in vitro* screening against BoNt, kinetics analysis, cellular-binding inhibiting assays, and *in vivo* toxicity assays. In this study, an Hc-immunized phage-display library was constructed, and consequently, several anti-BoNtb antibody clones were screened *in vitro*. Of the antibody clones, a strain of anti-BP8 antibody with high affinity was selected, and it displayed dose-dependent protection against BoNtb in *in vivo* toxicity assays. Epitopes peptide scan defined the binding sites of the neutralizing antibody. The kinetic characteristics of antigen−antibody molecular interaction were analyzed by SPR technology. Results from this study provide important information for further optimizing the design and development of therapeutic antibodies and inhibitors against botulism.

2. Materials and methods

2.1. Plasmids and strains, protein and peptides

The *C. botulinum* strain that could produce BoNtb was provided by the State Key Laboratory of Pathogens and Biosecurity, Beijing, PR China. The BoNtb was purified by a method described previously [25].

We designed and synthesized 8 peptides that contained possible antigenic epitopes from the Hc region (residues 845−1291) of BoNtb by using 3-D structure-based bioinformatic prediction methods [23,24]. The protein of Hc fragment of BoNtb was expressed and purified in our laboratory by using recombinant genetic engineering technology [26].

The strain of Syt II^+ PC12 cells that expressed the specific protein receptor of BoNTb, Syt II, was obtained by transfecting the PC12 cells with pcDNA3.1-Syt II plasmid [27]. The pcDNA3.1-Syt II plasmid was kindly provided by Dr. Min Dong of the University of Wisconsin.

2.2. Construction and screening of anti-BoNtb single-chain fragment of variants (scFv) phage-display library

The single-chain fragment of variants (scFv) phage-display library was constructed using a recombinant phage antibody system (RPAS), including mouse scFv and expression module with phagemid pCANTAB-5E according to the protocols supplied by the manufacturer and previous description [28,29]. The library size = clone numbers × transformed rate mg of target DNA. The recombinant phagemid was rescued with helper phage M13KO7. The supernatant containing the recombinant antibody phage was collected by centrifugation for the enrichment of antigen-binding phage. The specific binding clones were screened by phage-ELISA. Monoclonal phage stocks were prepared in microtiter plates according to the RPAS protocol. The ELISA-positive clones were selected for further experiments.

2.3. Expression and purification of SC12 scFv-fusion protein

Primers were designed as P01 (5′-gtcctcgcaactgcgaattcatggcccaggts-3′) and P02 (5′-gagtcattctaagcttgcccgttttat-3′) to amplify the *scFv* gene expressed by positive clones of SC12. The purified *scFv* gene was digested using the restriction enzymes *EcoR*I and *Hind*III (New England Biolabs Co.) and then cloned into pMalc2x (New England Biolabs) to construct the recombinant plasmid; the pMal-SC12 was then transformed into *Escherichia coli* 2566. SC12 fusion protein was expressed by isopropyl β-D-1-thiogalactopyranoside (IPTG)-induction and purified by using an amylose resin column (New England Biolabs).

2.4. Selection of antigenic peptides for SC12

For the identification of antigenic domain or sites targeted by the antibody SC12, a peptide-base ELISA was performed in