Toxicon 67 (2013) 63–70

Contents lists available at SciVerse ScienceDirect

Toxicon

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

Recombinant proteins that trigger production of antibodies recognizing botulinum neurotoxin while not possessing sequences of this toxin

Alexey Zdanovsky*, Maria Zdanovskaia

Alpha Universe LLC., 1202 Ann Street, Madison, WI 53713, USA

ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 11 February 2013
Accepted 22 February 2013
Available online 13 March 2013

Keywords:
Botulinum
Neurotoxin
Epitope
Mimetics
Chimera
Antibody

ABSTRACT

Sequences mimicking epitopes of pathogens can be used as effective tools for fast development and characterization of pathogen-specific antibodies. To demonstrate this, we used phage displays and isolated a number of short peptides mimicking epitopes of botulinum neurotoxin serotype A (BoNT/A). Presented data suggest that some of these peptides mimic linear epitopes while others mimic structural epitopes of BoNT/A. All tested peptides retained their ability to be recognized by BoNT/A-specific antibodies even when transferred into new carrier proteins. We demonstrated how such new hybrids can be used for fast conversion of pathogen-specific serums into panels of mono-epitope specific antibodies. We also demonstrated that hybrid proteins carrying multiple isolated peptides can be used as a substitute for BoNT/A in immunization studies and triggers production of BoNT/A-specific antibodies.

1. Introduction

Pathogen-specific antibodies are the most efficient means that can be used both for detection and inactivation of those pathogens. However, production of such antibodies often requires handling large quantities of dangerous pathogens or lengthy and expensive work related to development of derivatives of these pathogens that are safe to handle, but still able to trigger production of pathogen-specific antibodies.

Botulinum neurotoxins (BoNT) may serve as an example of pathogens posing such problems. These neurotoxins are the most potent toxins known to mankind (Schantz and Johnson, 1992). Currently, seven different serotypes of these toxins are known: A, B, C, D, E, F and G (BoNT/A, /B, /C, /D, /E, /F and /G, respectively). The diversity becomes even more dramatic when the existence of different subtypes of the same serotype is taken into account (Kalb et al., 2012; Smith et al., 2007). Even within the same serotype, antibodies produced using one subtype may be relatively ineffective in controlling the other subtypes (Smith et al., 2005).

The standard approach for producing antibodies against these neurotoxins requires growth of anaerobe *Clostridium botulinum*, *Clostridium argentiensis* and *Clostridium baratii* followed by purification and handling of proteins classified as category A biothreat agents. Prior to use for immunization, these proteins have to be inactivated by treatment with formalin or similar crosslinking agents. The first attempts to generate non-toxic recombinant derivatives of BoNTs that could be used for production of BoNT-specific antibodies began more than 17 years ago (Clayton et al., 1995). The main goal of these attempts was to generate a substitute to a pentavalent botulinum toxoid vaccine that was produced more than 30 years ago via chemical inactivation of native toxins. A recent notice of the CDC's discontinuation of this toxoid vaccine substantially raised

* Corresponding author. Tel./fax: +1 608 661 0861.
E-mail addresses: alexeyzd@tds.net, alexeyzd@alphayou.com (A. Zdanovsky).

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.02.011

the importance of such efforts (CDC, 2011). Until recently, the main focus of different research groups involved in these efforts was on carboxy-terminal regions of BoNTs (Yu et al., 2009; Chen et al., 2010; Mustafa et al., 2011). These regions form receptor-recognizing domains and were considered to carry the majority of epitopes that trigger production of protective antibodies. Recently, this paradigm started shifting (Takahashi et al., 2009) signaling that additional regions of BoNTs may be required for development of a potent equivalent of the pentavalent toxoid vaccine. Epitopes from other parts of BoNT molecules are even more important for the purpose of detection and identification of these neurotoxins and their derivatives. However, recombinant production of polypeptides with sizes close to that of BoNTs (~150 kDa) is rather challenging and often results in low yield of such polypeptides.

It has been demonstrated, though, that out of the whole polypeptide, only a few amino acid residues are involved in the formation of structures that trigger production of antibodies (Atassi et al., 2008; Zdanovsky et al., 2012). Such structures can be formed by linear stretches of amino acid residues (linear epitopes) or by residues brought together as a result of the specific 3D structure (structural epitopes). Previously, we demonstrated that multiple short epitope-forming peptides originating from different serotypes of botulinum neurotoxins (BoNT) can be combined on special carrier proteins and retain not only their ability to be recognized by corresponding serotype-specific antibodies, but can also trigger production of such antibodies (Zdanovsky et al., 2012). This method for constructing new immunogens, however, requires lengthy isolation of epitope-forming sequences and is limited to only linear epitopes.

The peptide display approach allows isolation of short peptides targeted at any chosen protein of interest, including antibodies (Jiang et al., 2005; Pincus et al., 1998; Smith, 1985). Often, these peptides interact with grooves on the molecules' surfaces that function as binding regions for other molecules. Thus, peptide displays can be used for isolation of peptides that mimic structures of other molecules, including linear and even structural epitopes of antigens.

Herein, we describe the use of the phage display library for identification of peptides mimicking epitopes of BoNT/A (mimetics). Our results demonstrate that, similar to peptides encoding real epitopes, these mimetics can be assembled on a carrier protein and in this form retain the ability to interact with corresponding antibodies, as well as trigger production of antibodies that recognize botulinum neurotoxin.

2. Materials and methods

2.1. Cells and plasmids

Escherichia coli DH5αF′ cells were used as a host in the course of all genetic engineering efforts. *E. coli* strain BL21(λDE3) was used for expression of assembled hybrid sequences controlled by bacteriophage T7 promoter (Studier and Moffatt, 1986). *E. coli* AU-DH10, which is a derivative of strain DH5αF′ carrying F′-factor with tetracycline resistance marker, was used for propagation of components of MD12™ phage display. *E. coli* strain ER2738, provided as part of the Ph.D.-12™ Phage Display Peptide Library Kit (New England BioLabs, Inc.) was used as suggested by the manufacturer to propagate the Ph.D.-12™ Phage Display.

Plasmid pParaBAD-T7HisbioRAP2 was priorly constructed in our laboratory (Zdanovsky et al., 2012). It was used as a carrier for sequences encoding mimetics isolated from the Ph.D.-12™ Phage Display. After treatment with restriction endonucleases *Not*I and *Sfi*I, mimetic-encoding sequences were cloned into *Eco*52I and *Sfi*I sites of plasmid pParaBAD-T7HisbioRAP2 to generate plasmids encoding Receptor Associated Protein (RAP) derivatives carrying His-tag and a peptide that can serve as a substrate for biotin ligase on the N-terminus, and a BoNT/A mimetic on the C-terminus.

Plasmid pSChoB17 was priorly constructed in our laboratory on the basis of plasmid pET28b and encodes subunit B of cholera toxin fused with signal peptide of *E. coli* heat-labile enterotoxin. The sequence encoding this chimera is flanked at the 5′-end with the recognition sequence for endonuclease *Xba*I and at the 3′-end with recognition sequences for endonucleases *Acc*65I and *Pst*I. These sites were used to insert sequences encoding mimetics of BoNT/A into the plasmid and, following recombination of the resulting constructs, into plasmid pETSChoB-BoNT/Amimetic1-8, which encodes eight different mimetics, as will be described in the Results section.

Plasmid pParaBAD-T7HisbioRAP-BoNT/Amimetic1-8 was constructed by joining a big fragment, generated by subsequent treatment of plasmid pParaBAD-T7HisbioRAP-A-Zn (Zdanovsky et al., 2012) with endonuclease *Sal*I, T4 DNA-polymerase, and endonuclease *Xho*I, and small fragment, generated by subsequent treatment of plasmid pETSChoB-BoNT/Amimetic1-8 with endonuclease *Pst*I, T4 DNA-polymerase and endonuclease *Xho*I.

2.2. DNA modifying enzymes

All restriction endonucleases, as well as T4 DNA-polymerase, Dream Taq™ DNA-polymerase, and rapid DNA ligation kit, were supplied by Fermentas Life Sciences.

2.3. Oligonucleotides

All oligonucleotides were synthesized at Integrated DNA Technologies Corporation. Sequences of BoNT/A mimetics isolated from the Ph.D.-12™ phage display were determined using oligonucleotide -28 gIII (5′-GTATGGGATTTTGCTAAACAAC) and those isolated from the MD12™ were determined using oligonucleotide VII-N5 (5′-GCGCCTGGTCTGTACACCGTTCATCTG). Sequences encoding chosen mimetics from the first display were amplified using primers PhD-5 (5′-ATCTGCGGCCGCTCTTAGTGGTACCTTTCTATTCTC) and PhD-3 (5′-TGCAGGCCGGCGCGGCCTTAACAGTTTCGGCCGAACCTC), and those isolated from the second display were amplified with primers MD12-5 (5′-AAAAGATCTGGTACCCGGGTCTGGGGGCAGCGGT) and MD12-3 (5′-TGTCTGCAGCTGTACATCTGCGGCCGCCGA).

2.4. Antibodies

Rabbit serum raised against BoNT/A was kindly provided by Dr. Vertiev (Moscow, Russia). This sera was used in