Toxicon 121 (2016) 30–35

Contents lists available at ScienceDirect

Toxicon

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

Neurotoxins from *Clostridium botulinum* (serotype A) isolated from the soil of Mendoza (Argentina) differ from the A-Hall archetype and from that causing infant botulism

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ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
10 August 2016
Accepted 11 August 2016
Available online 13 August 2016

Keywords:
Botulism
Neurotoxins
Clostridium botulinum
Infant botulism
SNAREs

ABSTRACT

The type A of neurotoxin produced by *Clostridium botulinum* is the prevalent serotype in strains of Mendoza. The soil is the main reservoir for *C.botulinum* and is possibly one of the infection sources in infant botulism. In this study, we characterized and compared autochthonous *C. botulinum* strains and their neurotoxins. Bacterial samples were obtained from the soil and from fecal samples collected from children with infant botulism. We first observed differences in the appearance of the colonies between strains from each source and with the A Hall control strain. In addition, purified neurotoxins of both strains were found to be enriched in a band of 300 kDa, whereas the A-Hall strain was mainly made up of a band of ~600 kDa. This finding is in line with the lack of hemagglutinating activity of the neurotoxins under study. Moreover, the proteolytic activity of *C. botulinum* neurotoxins was evaluated against SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) proteins from rat brain. It was observed that both, SNAP 25 (synaptosomal-associated protein 25) and VAMP 2 (vesicle-associated membrane protein) were cleaved by the neurotoxins isolated from the soil strains, whereas the neurotoxins from infant botulism strains only induced a partial cleavage of VAMP 2. On the other hand, the neurotoxin from the A-Hall strain was able to cleave both proteins, though at a lesser extent. Our data indicate that the *C.botulinum* strain isolated from the soil, and its BoNT, exhibit different properties compared to the strain obtained from infant botulism patients, and from the A-Hall archetype.

1. Introduction

Clostridium botulinum (Cb) is a spore-forming Gram positive bacillus that produces botulinum neurotoxins (BoNT). BoNT are responsible for the fatal neuroparalytic disease botulism. BoNT enter nerve terminals and cause a prolonged neurotransmitter exocytosis blockade, resulting in an impairment of muscle contraction and autonomic nerve functions (reviewed by Zhang et al., 2010). To date, two forms of the disease have been described: the one caused by the preformed toxin, including botulism caused by contaminated food intake, and the toxo-infection which is caused by bacterial colonization of the intestine and the subsequent *in-situ* release of BoNT. The latter category includes the infant botulism (IB), which is one of the most frequent forms of botulism worldwide affecting infants under one year of age (Cox and Hinkle, 2002; Koepke et al., 2007; Fenicia and Anniballi, 2009). Spores of BoNT-producing clostridia are present in the environment and can be found in the dust both domiciliary and peridomiciliary, where the soil appears to be the most important source of contamination (Thompson et al., 1980).

Seven serotypes (A-G) of BoNT have been identified based upon their antigenicity (Arnon et al., 1979). Each serotype is produced by a different strain of *Clostridium botulinum (Cb)*, with all exhibiting a high amino-acid sequence homology (Hill et al., 2007; reviewed by Peng Chen et al., 2012). Four serotypes (A, B, E and rarely F) are known to cause human botulism (reviewed by Hambleton, 1992; Montal, 2010; Peng Chen et al., 2012). In turn, several subtypes have been identified from these serotypes (Smith et al., 2005; Arndt et al., 2006; Carter et al., 2009; Umeda et al., 2009; Jacobson et al.,

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http://dx.doi.org/10.1016/j.toxicon.2016.08.010
0041-0101/

2011; Peng Chen et al., 2012). Each BoNT is synthesized as a harmless single polypeptide chain with a molecular mass of ~150 kDa (protoxin). The inactive precursor protein is cleaved by proteases into two active domains, a 50-kDa light chain (LC) and a 100 kDa heavy chain (HC) linked by an interchain disulfide bond (holotoxin). After reduction of the disulfide bond, the fully active toxin is generated. However, to reach their targets (peripheral nerves), toxins need to cross the epithelial barrier of the digestive tract. To this end, BoNTs associate with non-toxic, non-hemagglutinin (NTNH) and some also with hemagglutinin components (NTH). This association gives rise to three complex forms; a protein of 300 kDa (12S) associated with NTNH (toxin M), a protein of 600 kDa (16S) associated with both NTNH and NTH components (toxin L), and a 19S complex, which is thought to be a 16S dimer (toxin LL) (reviewed by Fujinaga, 2010). The *Cb* serotype A, (subtype A1 and A5) produces the three types of complexes (Carter and Peck, 2015), while serotypes B, C and D only produce the 12S and 16S complexes. In turn, the subtype A2-A4, and the serotypes E and F, only produce the 12S complex (Sakaguchi, 1982; Oguma et al., 1999; Poulain et al., 2008; Carter and Peck, 2015).

It is believed that the component NTNH confers proteolytic resistance to BoNTs in the gastrointestinal tract, whereas NTH may play a role in the toxin internalization by the intestine epithelial cells (Fujinaga et al., 1997).

Although the lethal doses of BoNTs in humans are not known, they are assumed to be very similar to those of mice, except for BoNT/D, which is not harmless to humans (Coffield et al., 1997). Arnon et al. (2001) have estimated a LD_{50} of 1 ng/kg in humans.

BoNTs cause flaccid paralysis through an acetylcholine release blockade at the neuromuscular junction in peripheral α-motor neurons. This blockade is accomplished through the cleavage of SNARE proteins (reviewed by Anhert-Hilga et al., 2013) by the LC zinc metalloprotease domain. Thus, serotypes A, C, and E cleave SNAP25, while serotypes B, D, F, and G cleave VAMP-2, and serotype C also cleaves syntaxin 1a (Schiavo et al., 2000; Zhang et al., 2010).

Epidemiological and molecular studies have correlated the prevalence of clostridia producing different botulinum neurotoxin serotypes with geographical regions, and with the presence of disease. Thus, in Argentina the serotype A is prevalent and mostly linked to IB (Lúquez et al., 2005), being the principal route of entry through ingestion of spores from the soil.

In order to identify the soil (a *Cb* reservoir) as the main infection source for IB in the province of Mendoza (Argentina), we characterized and compared *Cb* strains and their respective neurotoxins in bacterial isolates obtained from the soil and those obtained from the fecal samples of IB patients.

2. Materials and methods

2.1. Obtention of native strains of C. botulinum

Ten native strains of *Cb* were isolated, five from fecal samples obtained from infants with botulism (IB*Cb*) and five from soil samples (S*Cb*) of different areas in the province of Mendoza (Argentina) (Bianco et al., 2008; 2009). Strain A-Hall was used for comparison. These strains were maintained lyophilized until used. Strains were resuspended in chopped-meat medium (Giménez and Ciccarelli, 1970) under anaerobic conditions. After 48 h incubation at 35 °C, the purity of strains was assessed on 4% agar plates and egg yolk agar under anaerobic conditions and 1.5% agar (Difco, USA) under aerobic conditions (Dezfulian et al., 1981).

In order to check the mobility of *Cb*, selected strains were subcultured in 1.5% agar for 24–48 h at 35 °C and swarming observation, and subsequently subcultured in 4% agar under anaerobic conditions (48 h at 35 °C) to study the colony morphology (Cato et al., 1986). The colonies formed were photographed with a Nikon COOLPIX 5600 digital camera mounted on a Zeiss Stemi 1000 stereoscopic magnifier.

2.2. Obtention and purification of BoNT from the native strains of Cb

Strains isolated from soil and IB feces were maintained in chopped-meat medium broth with reseeding every 24 h. To obtain the toxin, an aliquot of medium was inoculated into 250 ml of toxin production medium (10 mM Na_2HPO_4, pH 7.2, containing 24% triptycase, 5% proteose peptone, 5% yeast extract, and 5% glucose) and incubated under anaerobic conditions at 35 °C for 96 h. The broth was then cleared by centrifugation at 10,000*g* at 4 °C for 30 min, and the proteins were precipitated with ammonium sulfate (60% saturation) for 24 h at 4 °C with continuous gentle stirring (Sakaguchi, 1982). Crude toxin was then pelleted at 10,000 *g* at 4 °C. Pellets were then dissolved in phosphate buffer (0.03 M, pH 6.8) and then dialyzed for 24 h against 2 mM phosphate buffer (pH 6.8), with three changes.

2.3. Toxicity of BoNTs in mice

The potency of BoNTs was evaluated by intraperitoneal inoculation (ip) of female Swiss-Webster mice weighting 18–22 g each (n = 10), and the LD_{50} was estimated according to Reed and Muench (1938). Animal handling was carried out in accordance with local and national regulations for laboratory animal use. The protocol was approved by ICCULA (Institutional Commission for the Care and Use of Laboratory Animals). Specific activity of the BoNTs was valued as the toxic activity per mg protein.

2.4. Hemagglutination assay

The hemagglutination assay was carried out with the native (from S*Cb* and IB*Cb)* or archetype BoNTs. The assay was performed according to Miyata et al. (2012), with slight modifications. Briefly, 2-fold serial dilutions (starting at 0.5 μg/μl) of either the purified native BoNTs or the archetype BoNT were prepared in phosphate buffered saline (PBS: 0.15 M NaCl, 20 mM NaH_2PO_4, pH 7.0) in 96-well microtitre plates. Then, an equal volume of pre-washed human erythrocytes (O-positive) suspended in PBS (2.5% v/v) was added to each well. After incubation for 2 h at room temperature, the hemagglutination titre was expressed as the reciprocal value of the maximal dilution (2^n) that produced agglutination. All the assays were carried out in duplicate in three independent experiments.

2.5. Electrophoresis

SDS-PAGE was performed under non-reducing conditions according to Laemmli (1970), using 6% polyacrylamide gels. Samples (~25 μg protein) of either the native (S*Cb* and IB*Cb*) or the A-Hall toxins were loaded on the gel and run for 90 min at 25 mV. Protein bands were visualized with Coomassie Brilliant Blue. The molecular mass was determined using a HMW Calibration Kit (Amersham GE Healthcare).

2.6. Proteolytic activity of BoNTs

Rat brain was homogenized (1/5 w/v) in buffer H (50 mM Hepes pH 7.1, containing 5 mM NaCl, 0.1% Tween 20, 10 μM $ZnCl_2$ and 5 mM DTT) with a Teflon pestle homogenizer at 4 °C. Homogenates were centrifuged at 1000*g* for 10 min at 4 °C and the post-nuclear fraction was recovered from the supernatant. For evaluating the