Toxicon 101 (2015) 63–69

Contents lists available at ScienceDirect

Toxicon

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

Activity of botulinum neurotoxin type D (strain 1873) in human neurons

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ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form
24 April 2015
Accepted 29 April 2015
Available online 30 April 2015

Keywords:
Botulinum neurotoxin
BoNT/D
Botulinum neurotoxin D
Clostridium botulinum type D

ABSTRACT

Botulinum Neurotoxin type D (BoNT/D) causes periodic outbreaks of botulism in cattle and horses, but is rarely associated with human botulism. Previous studies have shown that humans responded poorly to peripheral injection of up to 10 U of BoNT/D. Isolated human pyramidalis muscle preparations were resistant to BoNT/D, whereas isolated human intercostal muscle preparations responded to BoNT/D similarly as to other BoNT serotypes. I*n vitro* data indicate that BoNT/D does not cleave human VAMP1 efficiently, and differential expression of the VAMP 1 and 2 isoforms may be responsible for the above observations. Here we examined sensitivity of cultured human neurons derived from human induced pluripotent stem cells to BoNT/D. Our data indicate that BoNT/D can enter and cleave VAMP 2 in human neurons, but at significantly lower efficiency than other BoNT serotypes. In addition, BoNT/D had a short duration of action in the cultured neurons, similar to that of BoNT/E. *In vivo* analyses indicated a slower time to death in mice, as well as a later onset and shorter duration of action than BoNT/A1. Finally, examination of BoNT/D activity in various rodent and human cell models resulted in dramatic differences in sensitivity, indicating a unique cell entry mechanism of BoNT/D.

1. Introduction

Botulinum Neurotoxin type D (BoNT/D) has been mainly associated with botulism in cattle and horses, at times leading to large outbreaks in cattle herds with a mortality rate above 80% and large associated economic losses (Lindstrom et al., 2010). Botulism in cattle caused by BoNT/D has been reported mostly in South Africa, Europe and Canada, but also in USA and Israel (Lindstrom et al., 2010). Studies investigating the epidemiology of BoNT/D-producing Group III *Clostridium botulinum* in the environment (Lindstrom et al., 2010; Rodloff and Kruger, 2012; Kruger et al., 2012) showed periodic isolation from cattle feeds, animal remains and cows' gastrointestinal contents. This indicates potentially widespread presence of BoNT/D producing *C. botulinum* in the environment, as is the case with other *C. botulinum* strains, and thus potential exposure of humans to this toxin. However, BoNT/D very rarely causes human botulism. In fact, there is only one published report of BoNT/D identified in naturally occurring human botulism (Demarchi et al., 1958). This report described a mild botulism outbreak in Moundou (Tchad) in January 1958 involving 8 people who ate raw ham. Two of them ate very little and had no symptoms, four had very mild early botulism symptoms accompanied by extensive diarrhea and vomiting, and recovered completely the following day. One person had these symptoms for 10 days but required no specialized treatment, and one person had symptoms for 1 month with recovery beginning at 2 1/2 weeks after the ham consumption. Treatment of this last patient with anti-BoNT/A and/B antitoxin did not affect the symptoms, and analysis of extracts from the contaminated ham as well as cultures grown from the ham showed that only anti-BoNT/D serum and not anti-A, B, C, or E sera protected mice against botulism symptoms, confirming that the outbreak was caused by BoNT/D (Demarchi et al., 1958). A subsequent study isolated *C. botulinum* strain 1873, which produces BoNT/D, from the contaminated ham (Prevot and Sillioc, 1959).

Botulinum neurotoxins have been classified into seven immunologically distinct serotypes (A-G) (Gimenez and Gimenez, 1995). BoNTs are the causative agent of human and animal botulism, which is characterized by flaccid paralysis that may last for several weeks or months and up to a year, depending on the serotype and dose (Johnson et al., 2008). The toxins exert their pathology by

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http://dx.doi.org/10.1016/j.toxicon.2015.04.015
0041-0101/

selectively entering neuronal cells and cleaving soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins (Schiavo et al., 2000, 1995; Rossetto et al., 2014), which are VAMP1 and VAMP2 in the case of BoNT/D (Rossetto et al., 2014). Due to the high potency of botulinum neurotoxins combined with the lack of efficacious countermeasures, particularly after entry into neuronal cells, BoNTs are considered a potential bioterrorism threat (Arnon et al., 2001; Kman and Nelson, 2008). On the other hand, BoNT serotype A (and to a lesser extent B), are being used as powerful pharmaceuticals to treat a variety of disorders (Dressler, 2012). Since BoNT/A, B, E, and F cause almost all naturally occurring human botulism cases, countermeasure efforts as well as novel pharmaceuticals usually focus on these four serotypes. However, it is unknown whether humans would be susceptible to a bioterrorist attack using BoNT/D or derivatives thereof, and whether BoNT/D may present an alternative potential biopharmaceutical.

Scientific evidence on the susceptibility of humans to BoNT/D is unclear. A recent *in vitro* study showed that the BoNT/D light chain (LC) efficiently cleaved neuronal human VAMP 2 but inefficiently cleaved the other neuronal human VAMP isoform, VAMP 1 (Yamamoto et al., 2012). Inefficient and slow proteolysis of rat VAMP1 by BoNT/D has also been reported (Yamasaki et al., 1994; Pellizzari et al., 1997). This may be the reason underlying the recent observation in an experimental electrophysiology study in humans that up to 10 mouse LD50 Units of BoNT/D was poorly effective in inducing local paralysis in humans after injection into the extensor digitalis brevis muscle (Eleopra et al., 2013). In addition, a study using isolated human pyramidalis muscle indicated that the neuromuscular junction in this human muscle preparation was resistant to BoNT/D (Coffield et al., 1997). However, in another study, human intercostal muscle was found to be similarly sensitive to BoNT/D as to BoNT/A, B, and E (Anderson et al., 2009). The human botulism outbreak attributed to BoNT/D also indicates that humans can be susceptible to intoxication by BoNT/D (Demarchi et al., 1958), although the symptoms appear to be much milder than in botulism caused by BoNT/A or/B.

In this study we examined the activity of BoNT/D in human neurons and analyzed the onset and duration of action in mice. Our data indicate that BoNT/D can enter and cleave VAMP 2 in human neurons, but at significantly lower efficiency than BoNT/A1, and that it has a short duration of action similar to that of BoNT/E. In addition, dramatically differential sensitivity of various neuronal cell populations and a slow time to death in mice indicate potentially distinct pharmacological properties of this toxin compared to BoNT/A1.

2. Materials and methods

All the work described in this manuscript was approved by the University of Wisconsin–Madison Institutional Biosafety Committee. All animal experiments were approved by and conducted according to guidelines by the University of Wisconsin Animal Care and Use Committee.

2.1. Botulinum neurotoxins

BoNT/A1 was isolated from *C. botulinum* strain Hall A hyper as previously described (Malizio et al., 2000). The specific activity in mice was determined to be 1.25×10^8 mouse LD_{50} Units (U)/mg. BoNT/D toxin was isolated from *C. botulinum* strain D 1873 using a method similar to that used in BoNT/A purification with the following modifications. The ammonium sulfate precipitated material from DEAE chromatography at pH 5.5 was collected by centrifugation and resuspended in 20 mM NaOAc buffer pH 6.0. The sample was applied to a p-aminobenzyl 1-thio-β-D-galactopyranoside agarose affinity column (pABTG agarose) that tightly binds the hemagglutinin of the toxin complex (Moberg and Sugiyama, 1978). The toxin was eluted from the column by addition of 20 mM Tris buffer pH 8.0 containing 0.15 M NaCl. Specific activity in mice was determined to be 1.15×10^8 mouse LD_{50} Units/mg. BoNT/B1 was isolated from *C. botulinum* strain as previously described (Prabakaran et al., 2001), and the specific activity in mice was determined to be 3.13×10^8 mouse LD_{50} Units (U)/mg.

2.2. Neuronal cell models

Several neuronal cell models were used in this study. The iCell Neurons and media were purchased from Cellular Dynamics Inc. (Madison, WI) and seeded and maintained according to company instructions. HIP Neurons and media were provided by Globalstem (Gaithersburg, MD) and were prepared as previously described (Whitemarsh et al., 2013). Primary rat (strain Sprague Dawley) and mouse (strain C57/BL6 or ICR, as indicated) spinal cord cells, hippocampal cells, or cortical cells were all prepared as described previously (Pellett et al., 2010, 2007) and seeded into 0.01% poly-L-ornithine (SIGMA) and 8.3 μg/cm^2 matrigel (BD Biosciences) coated 96-well Techno Plastic Products (TPP) plates at a density of 50,000 cells/well. The cells were allowed to mature for at least 18 days before use unless otherwise noted. The primary cells were seeded and maintained in serum free culture medium (Neurobasal® medium supplemented with 2% B27, 2 mM glutamax, and 100 units/mL penicillin/streptomycin (all from Invitrogen)).

2.3. Cell-based assays

The cells were exposed to serial dilutions of BoNT/D in 50 μL of the respective media for 48 h. The extracellular solution containing BoNT/D was removed, cells were lysed in 1X lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and analyzed by Western immunoblot for VAMP2 cleavage as previously described using a monoclonal anti-VAMP2 antibody from Synaptic Systems (Göttingen, Germany) (Pellett et al., 2007; Whitemarsh et al., 2012). VAMP2 and syntaxin bands were quantified by densitometry using a Foto/Analyst FX system and TotalLab Quant software (Fotodyne). Data plots and EC50 values (four parameters – variable slope) were generated using PRISM 6 software, and statistical significance was determined by PRISM 6 software using an Extra-sum-of-squares F-test with an α-value of 0.05.

For the duration of action assay, iCell Neurons were exposed to 800 mouse LD50 Units (U) of BoNT/D in 50 μL of modified culture media containing 56 mM KCl and 2.2 mM $CaCl_2$ (cell stimulation media) for 10 min, followed by complete removal of the toxin, washing of the cells two times with 300 μL of culture media, and further incubation of the cells in culture media at 37 °C in a CO_2 incubator. Four replicate samples were harvested every 2–3 days, and analyzed for VAMP2 cleavage as above.

To determine onset of action in human neurons, iCell Neurons were exposed to 10,000 U of BoNT/D in 50 μL of either culture media or cell stimulation media for 0–8 h, and triplicate samples were harvested every hour for analysis of VAMP2 cleavage. To further analyze activity dependent uptake, iCell Neurons were exposed to serial dilutions of BoNT/D in 50 μL of either cell stimulation media or culture media for 10 min, followed by complete removal of the toxin, washing of the cells two times with 300 μL of culture media, and further incubation of the cells in culture media at 37 °C in a CO_2 incubator for 16 h.

2.4. In vivo analysis of BoNT/D activities in mice

Groups of 5 female ICR mice (weight between 18 and 22 g) were