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Research paper

Immuno-detection of cleaved SNAP-25 from differentiated mouse embryonic stem cells provides a sensitive assay for determination of botulinum A toxin and antitoxin potency

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ABSTRACT

Botulinum toxin type A is a causative agent of human botulism. Due to high toxicity and ease of production it is classified by the Centres for Disease Control and Prevention as a category A bioterrorism agent. The same serotype, BoNT/A, is also the most widely used in pharmaceutical preparations for treatment of a diverse range of neuromuscular disorders. Traditionally, animals are used to confirm the presence and activity of toxin and to establish neutralizing capabilities of countermeasures in toxin neutralization tests. Cell based assays for BoNT/A have been reported as the most viable alternative to animal models, since they are capable of reflecting all key steps (binding, translocation, internalization and cleavage of intracellular substrate) involved in toxin activity. In this paper we report preliminary development of a simple immunochemical method for specifically detecting BoNT/A cleaved intracellular substrate, SNAP-25, in cell lysates of neurons derived from mouse embryonic stem cells. The assay offers sensitivity of better than 0.1 LD50/ml (3 fM) which is not matched by other functional assays, including the mouse bioassay, and provides serotype specificity for quantitative detection of BoNT/A and anti-BoNT/A antitoxin. Subject to formal validation, the method described here could potentially be used as a substitute for the mouse bioassay to measure potency and consistency of therapeutic products.

1. Introduction

Botulinum neurotoxins (BoNT/s) are considered the most lethal toxins (Gill, 1982). They induce prolonged muscle paralysis and block muscle function through inhibition of neuronal transmitters from peripheral cholinergic nerve endings (Rossetto, et al., 2014). Although they are highly toxic and the causative agents of botulism, BoNTs have been used for over twenty years in many medical interventions to treat muscle hyperactivity, such as dystonia and spastic conditions, exocrine gland hyperactivity, such as hyperhidrosis, and pain disorders (Dressler, 2012). A number of licensed products are manufactured globally and for many years the in vivo mouse bioassay has been applied to determine toxin activity per vial, monitor lot to lot variability and to define shelf life. Animals are still used for assessment of toxin potency by many manufacturers, for clinical diagnosis, and for potency testing of antitoxins (Sesardic, 2012). The mouse bioassays have considerable ethical and safety limitations and alternative non-animal methods have been sought as replacements for a number of years (Sesardic and Gaines Das, 2008; NIH Publication, 2008; Sesardic, 2012; Adler et al., 2010).

To date, seven serologically distinct botulinum toxin serotypes (A–G) have been identified. They all comprise a 150 kDa single-chain progenitor toxin which is subsequently activated by protease to generate a disulphide bond-linked structure containing a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC). The HC contains two functional domains that are both required for toxin binding and uptake into the nerve cells which involves a dual-receptor mechanism involving gangliosides, such as GT1b, and a protein receptor. Once the toxin is internalized, the inter-chain disulphide bond is broken by reducing conditions within the endocytic vesicles. This activates and releases the endopeptidase light chain (LC) which specifically cleaves one of three proteins involved in neurotransmitter release (Pirazzini et al., 2017).

Abbreviations: BoNT/A, botulinum neurotoxin A; SNAP-25, synaptosomal protein of molecular mass 25 kDa; EB, embryoid bodies; ESDN, embryonic stem cell-derived neurons; mESC, mouse embryonic stem cells; HC, heavy chain; LC, light chain; LHn/A, recombinant endopeptidase fragment of BoNT/A LC and the N-terminus of the HC; ELISA, enzyme linked immunosorbent assay; WB, Western blotting; PBS, phosphate buffered saline; CM, culture medium; RA, retinoic acid; LIF, leukemia inhibitory factor; GMEM, Glasgow's Minimal Essential Medium; Shh, Sonic Hedgehog; FD, final differentiation; NGF-β, nerve growth factor beta; BDNF, brain-derived neurotrophic factor; RT, room temperature

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The endopeptidase activity of BoNT LC is highly specific so that each toxin has a unique substrate/target bond combination. Whereas the LCs of botulinum toxin serotypes A, E and C1 all cleave SNAP-25 (synaptosomal associated protein of molecular mass 25 kDa), they target this protein at distinct sites (Q197-R198, R180-I181, and R198-A199, respectively) (Schiavo et al., 1993; Vadakkanchery et al., 1999; Rossetto et al., 2014; Pirazzini et al., 2017).

Once the molecular mechanism of BoNT/s was unravelled in the early 1990′s, it became possible to develop immunoassays designed to detect the endopeptidase activity of the BoNT LCs. This approach required production of a recombinant or synthetic substrate (SNAP-25 for BoNT/A and BoNT/E or VAMP2 for BoNT/B) and a specific neoepitope antibody, recognising only a peptide sequence that becomes exposed upon toxin-mediated cleavage of the substrate. Taking this approach, serotype specific assays have been developed for several BoNT/s (Ekong et al., 1997; Jones et al., 2008; Jones et al., 2009; Sesardic et al., 2004; Simon et al., 2015). However, endopeptidase assays can suffer from unwanted matrix interference from albumin and human serum (Jones et al., 2011; Simon et al., 2015), cannot detect changes to the binding and translocation domains of the toxin, and can detect free LC activity in the absence of a functional binding domain, thus potentially giving false positive results (Sesardic, 2012). Furthermore, these methods are not considered to be indicators of stability. While these endopeptidase assays are suitable for monitoring lot-to-lot consistency following validation for a given toxin product, they cannot be considered as a full replacement for the mouse LD50 potency test (NIH Publication, 2008, Adler et al., 2010).

To circumvent the limitations of the first generation of in vitro endopeptidase assays, second generation methods were developed that require both functional binding to toxin receptor or antibody and catalytic endopeptidase activity for detection of BoNT/A and BoNT/B (Evans et al., 2009; Liu et al., 2012; Rosen et al., 2016). However, the concept of biochemical assays which mimic only two steps in the botulinum intoxication process is also considered limited as these assays do not reflect all the events required for toxin activity in vivo, such as the internalization step and, particularly, the persistence of toxin within the cell, which are known to contribute to toxin potency (Keller and Neale, 2001).

Cell based bioassays for BoNT/s are superior to other in vitro models because they are capable of reflecting all major steps of botulinum toxin action (Pellet, 2013) and they are therefore considered to offer the most realistic strategy towards complete replacement of the mouse LD50 test (NIH Publication, 2008; Adler et al., 2010). Primary cells have been used for BoNT research for many years, and the initial studies for cell based methods focused on neurons derived from rat (Keller et al., 2004), mouse (Dong et al., 2007) and chicken embryos (Nuss et al., 2010). However, these studies did not offer sensitivity comparable to the mouse bioassay since only nM rather than low pM concentrations of BoNT/A could be detected. The first highly sensitive cell based assay for detection of BoNT/A was developed using primary rat embryonic spinal cord cells. Comparable sensitivity to the mouse bioassays was reported using detection of cleaved SNAP-25 quantified by Western blotting (Pellet et al., 2010). The approach to use primary rat spinal cord cells for detection and quantification of botulinum neurotoxin antibodies was also reported as an opportunity to replace the mouse toxin neutralization test (Hall et al., 2004). Still, it was recognised that primary cells do not provide an easily standardized and robust model that would be preferred for routine laboratory use, and they also require animals for the supply of cells.

Immortalized cell lines can offer the desired standardization properties, but they are typically at least 1000-fold less sensitive for BoNT/A compared to the mouse bioassay (Purkiss et al., 2001; Yowler et al., 2002, Pellet, 2013), even after lengthy differentiation protocols and addition of GT1b to improve sensitivity (Rasetti-Escargueil, et al., 2011). The notable exception is the cancer derived SiMa cell line (human neuroblastoma cell line) which, with an EC50 of ~1 pM (~35 LD50/ml), offers a sufficiently sensitive and robust method for potency testing of BoNT/A in Botox® (Fernandez-Salas et al., 2012).

The availability of neurons derived from pluripotent stem cells has provided a new platform for highly sensitive detection of botulinum neurotoxins (Kiris et al., 2011; McNutt et al., 2011, Pellet et al., 2011; Whitemarsh et al., 2012; Beske et al., 2015; Jenkinson et al., 2017). In addition to the source of cells, development of cell based assays is highly dependent on the use of a relevant and quantitative endpoint to measure the botulinum toxin activity. Measurement of neurotransmitter release has been applied in assay development studies (Keller and Neale, 2001; Rasetti-Escargueil et al., 2011; Hall et al., 2004) and, while it is an important endpoint and research tool, it is considered less useful for routine quality control purposes because it is relatively nonspecific and highly dependent on cell type (Pellet, 2013). Most recently, blockade of synaptic transmission in a network culture of neurons derived from human and rodent stem cells has been described as a functionally relevant readout for BoNT intoxication (Beske et al., 2015, 2016; Jenkinson et al., 2017). However, this approach relies on the use of specialized whole cell electrophysiology equipment or multielectrode arrays which is not applicable in a routine control laboratory setting and does not offer high throughput capability. Western blotting (Pellett et al., 2007, 2015, 2017, Whitemarsh et al., 2012; McNutt et al., 2011), quantitative immunofluorescence (Dong et al., 2004; Gilmore et al., 2011; Kiris et al., 2011) or ELISA (Nuss et al. 2010; Pellett et al., 2017) are methods that are all based on detection of intracellular substrates after cleavage by BoNT/s which is considered to be the most specific endpoint and can be applied to any neuronal cell type.

In this study, we report preliminary development of a simple capture ELISA method for detection of BoNT/A activity in neurons derived from mouse embryonic stem cells. Our results suggest that the assay is likely to be suitable for testing the activity of therapeutic and commercial grade BoNT/A toxins and could also be used to measure the potency of antitoxin to BoNT/A. The method described here is more sensitive than the mouse bioassay for detection of BoNT/A and other cell based assays that have been developed for this toxin serotype.

2. Materials and methods

2.1. Toxins

Purified type BoNT/A haemagglutinin (HA) free toxin purchased from Metabiologics Inc., (Madison, USA, @ 2.3×10 (Dong et al., 2007) LD50/mg, 1 mg/ml from Hall strain) was used. A working stock was prepared at 20,000 LD50/ml (87 ng/ml) in Gelatine (0.2% w/v) Phosphate (50 mM di-sodium hydrogen orthophosphate) Buffer (GPB, pH 6.5) as previously described (Jones et al., 2008). All stocks of aliquoted toxins were stored at − 80 °C prior to use.

Two formulations of therapeutic BoNT/A toxin for injection, from the same manufacturer, containing different concentrations of BoNT/A as active component were also used. Recombinant LHn/A fragment, with an LD50 of 0.3 mg/mouse, was a generous gift from Syntaxin Ltd, and was used as reported previously (Liu et al., 2012). All BoNT/A used in this study were sub-type A1. Purified BoNT/B1 and BoNT/E3 were purchased from Metabiologics (USA) and working stocks of 20,000 and 12,560 mouse LD50/ml, respectively were prepared in GPB buffer and stored at − 80 °C before use.

2.2. Antibodies

BoNT/A cleavage site-specific polyclonal anti-peptide antibody against SNAP-$25_{190–197}$ was made in rabbits as previously described (Ekong et al., 1997; Jones et al., 2008) and affinity purified against the immunizing peptide. The same antibody was also custom made by Bio Trend (Chemikalin GmbH, Köln, Germany) and purchased affinity purified to capture BoNT/A cleaved SNAP-25 from cell lysates.

Two separate polyclonal detection antibodies (sheep anti-SNAP-