Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Short communication

An *in vitro* cell-based potency assay for pharmaceutical type A botulinum antitoxins

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ARTICLE INFO

Article history:
Received 24 June 2017
Received in revised form 30 October 2017
Accepted 7 November 2017
Available online xxxx

Keywords:
Clostridium botulinum
Botulinum antitoxin
Neutralization
Potency
In vitro assay

ABSTRACT

Botulism therapy relies on passive immunization with antitoxin. The mouse neutralization test is the only pharmacopeia assay to measure the potency of antitoxin preparations. Herein, we present an *in vitro* cell-based assay for the measurement of pharmaceutical type A antitoxin potency. Accuracy, reproducibility and compatibility with the mouse bioassay were demonstrated using different batches of standard antitoxin and toxin preparations. The established assay may substantially reduce the use of laboratory animals in the process of pharmaceutical antitoxin production.

1. Introduction

Botulinum neurotoxins (BoNTs), produced mainly by strains of *Clostridium botulinum*, are considered the most lethal toxins in nature, with an estimated human median lethal dose of 1 ng/kg body weight [1–4]. Seven BoNT serotypes are known (A–G), of which types A, B, and E are responsible for most cases of human botulism [5]. All BoNT serotypes act via similar mechanisms on their target nerve cell [6]: initial binding of the C-terminal portion of the toxin heavy chain to receptors on the presynaptic cell surface followed by internalization into the nerve ending and translocation to the cytosol by the N-terminal portion of the heavy chain [7]. Inside the nerve terminal, the toxin light chain, which is a zinc-dependent endopeptidase, cleaves one of the three soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), thereby preventing the release of acetylcholine [6]. Each BoNT serotype has a specific cleavage site on its target SNARE protein. Serotype A (BoNT/A), which is associated with the most severe human botulism cases, cleaves the 25 kDa synaptosomal associated protein (SNAP-25) [6].

The only current approved therapeutic for botulism in adults is antitoxin collected from hyper-immune horses [8–10]. The potency of anti-botulinum pharmaceutical antitoxins is determined by the pharmacopeia mouse neutralization assay (PMNA) [11]. In this assay, a pre-calibrated fixed toxin dose (test dose) is incubated separately with serial dilutions of the tested antitoxin sample, and with serial known concentrations of a standard antitoxin (calibrated according to the World Health Organization (WHO) international standard antitoxin) as a reference. The mixtures are then injected to mice and survival is monitored for 96 h. The use of a high toxin dose ($\geq$1000 $MsLD_{50}$) together with adequate antitoxin dilutions leads to a cut-off survival profile in which complete and zero survival are obtained in adjacent antitoxin dilutions. The potency of the tested antitoxin is then determined by comparing the cut-off point (transition from survival to mortality) of the tested antitoxin with that obtained with the standard antitoxin.

Abbreviations: BoNT, botulinum neurotoxin; SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SNAP-25, 25 kDa synaptosomal associated protein; WHO, World Health Organization; $MsLD_{50}$, mouse 50% lethal dose; IIBR, Israel Institute for Biological Research; IU, international units; W/V, weight/volume; BSA, bovine serum albumin; SAT, standard antitoxin; TOX, toxin test dose; HIP, hyper-immune plasma; DS, drug substance; PMNA, pharmacopeia mouse neutralization assay.

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https://doi.org/10.1016/j.vaccine.2017.11.015
0264-410X/

The PMNA has several drawbacks. It is time consuming, labor intensive, requires trained personnel, and above all requires a large number of laboratory animals per sample (n = 28). Consequently, efforts to develop alternative *in vitro* methods have been made [12–16]. However, most of these suggested alternative assays measure only one of the three steps of toxin action (binding, internalization or enzymatic activity) [17]. On the other hand, cellular assays measure all three steps of intoxication and therefore have the highest potential for the authentic simulation of natural BoNT intoxication and correlation with the PMNA. Indeed, several cell-based assays that measure BoNT activity have been developed, including those that aim to measure neutralizing antibodies [18–29]. However, none of the results obtained by the cell-based BoNT/A neutralization tests have been further confirmed by the PMNA. In the current study, we present an *in vitro* cell-based potency assay for therapeutic BoNT/A-equine antitoxins. This assay is fully compatible with the pharmacopeia assay design and was able to accurately determine the potency of pharmaceutical antitoxin preparations as compared to the PMNA.

2. Methods

2.1. Ethics statement

All animal experiments were performed in accordance with the Israeli law and were approved by the Ethics Committee for Animal Experiments at the Israel Institute for Biological Research.

2.2. Toxins and antitoxins

Clostridium botulinum A strain was obtained from the Israel Institute for Biological Research (IIBR) collection (strain A198). Sequence analysis revealed compliance of the neurotoxin gene with serotype 62A (GenBank Accession Number M30196) of *C. botulinum* type A1 [30]. The toxin test dose and antitoxins were prepared as described previously [16,31]. Horse anti BoNT/A antitoxins were collected and purified from hyper-immune horses immunized against BoNT/A. Fc fragment was removed by pepsin digestion [32]. Purified $F(ab)'_2$ antitoxin neutralizing activity was determined according to the European Pharmacopeia. Standard antitoxins (SAT1 and SAT2 with potency of 330 and 300 IU/ml, respectively) were calibrated against the World Health Organization (WHO) international standard antitoxin according to the pharmacopeia [11]. Equine hyper-immune plasma (HIP) represents an anti-BoNT/A horse IgG preparation, and pharmaceutical drug substance (DS) is a purified $F(ab')_2$ preparation.

2.3. Cell-based potency assay

The potency assay consisted of two steps: (1) *in vitro* neutralization of BoNT/A with antitoxin followed by (2) measurement of residual BoNT/A activity in a cell culture system modified from Fernandez-Salas et al. [23]. To this end, a toxin test dose (≥1000 $MsLD_{50}$) that was previously calibrated according to the pharmacopeia reference bioassay method was incubated for 1 h at 25 °C with 0.02–0.14 International units (IU) per ml of standard antitoxin in 0.02 IU/ml dilution intervals. According to the WHO standardization, 1 IU of antitoxin neutralizes at least 10^4 $MsLD_{50}$ of toxin [33]. Toxin-antitoxin mixtures were then added in quadruplicate to differentiated SiMa cells (5×10^4 per well). Differentiation was conducted in poly-D-lysine coated 96 well plates containing Minimum Essential Medium with Earls salts and Glutamax (MEM), serum-free, supplemented with 25 μg/ml GT1b for 2 days, as previously described [23]. Medium was removed and replaced with MEM containing toxin and antitoxin samples. Following incubation for 24 h at 37 °C, medium was replaced with fresh MEM and cells were incubated for an additional 24 h at 37 °C. Cells were then lysed with buffer containing 0.1% Triton X-100 and each of the lysates was analyzed in quadruplicates for cleaved SNAP-25 using specific ELISA. Briefly, 96 well plates (Maxisorp, Nunclon) were coated with a synthetic mouse-human chimera monoclonal antibody (100 ng/well) that specifically recognizes BoNT/A-cleaved SNAP-25. This antibody was generated in an expression system [34] based on the V-gene sequences published by Fernandez-Salas et al. [35]. Plates were blocked with TSTA buffer (2% (W/V) bovine serum albumin (BSA), 0.9% NaCl, 0.05% Tween 20 in 50 mM Tris (pH 7.6)). Following blocking and washing, plates were incubated with cell lysates (50 μl, in quadruplicate) for 1 h at 37 °C. After an additional wash, plates were incubated for 1 h at 37 °C with polyclonal rabbit anti-human SNAP-25 (Sigma, MO, USA), diluted 1:4000 in blocking solution supplemented with 1% naïve mouse and human sera. Plates were washed and then incubated with HRP-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories Inc., PA, USA), diluted 1:4000 in sera supplemented blocking solution. The colorimetric reaction was developed using SureBlue substrate (Sera Care, MA, USA) and stopped with 1 N H_2SO_4. Absorbance was measured at 450 nm with a Spectramax M3 reader (Molecular Devices, CA, USA).

2.4. Mouse potency assay

The PMNA was conducted according to the European Pharmacopoeia [11], and was performed in CD1 mice as previously described [16,31].

3. Results and discussion

In the PMNA, toxin-antitoxin mixtures containing 0.1 IU/ml antitoxin or less fail to protect mice, whereas mixtures containing 0.12 IU or above are expected to confer full protection (n = 4 per dilution) [11]. Our goal was to apply the cut-off signal pattern to the cellular assay to allow determination of the potency of pharmaceutical antitoxins. In terms of a cell assay, protection is reflected by the absence of SNAP-25 cleavage. Thus, in the calibrated cellular assay, protection would have been indicated by the last antitoxin dilution that prevents SNAP-25 cleavage, while the subsequent dilution would not be able to provide complete protection. Hence, for a proof of concept, the cellular assay signal pattern was evaluated using two batches of standard antitoxins (SAT1 and SAT2, Fig. 1A) and two batches of toxin test dose TOX1 (Fig. 1A) and TOX2 (Fig. 1B) that were previously calibrated according to the mouse pharmacopeia assay. The assay "cut-off" representing complete protection was defined as at least 90% inhibition of the toxin signal. A clear and precise cut-off point value of 0.04 IU/ml was obtained. This value was reproducible over all tested batches of toxin test doses and standard antitoxins. To further validate assay performance, two antitoxin preparations, equine hyper-immune plasma (HIP) and a pharmaceutical antitoxin drug substance (DS), were measured for their potency. The results were compared to those determined by the PMNA. Fig. 2 reveals a clear cut-off pattern for both samples. When back calculating the antitoxin dilution at the cut-off point, the potency of the HIP and DS antitoxin preparations was determined to be 500 IU/ml and 650 IU/ml, respectively (Fig. 2). Identical results were obtained using the PMNA.

Passive immunization with antitoxin is currently the only accepted treatment for botulinum intoxication [8–10]. The PMNA is the only approved and standardized method to measure the potency of pharmaceutical botulinum antitoxin preparations [11]. In order for a cell-based potency assay to be applied as an

Please cite this article in press as: Torgeman A et al. An *in vitro* cell-based potency assay for pharmaceutical type A botulinum antitoxins. Vaccine (2017), https://doi.org/10.1016/j.vaccine.2017.11.015