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Research paper

An improved method for development of toxoid vaccines and antitoxins

Russell G.A. Jones [a,*], Yvonne Liu [a], Peter Rigsby [b], Dorothea Sesardic [a]

[a] Division of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, UK
[b] Biostatistics Section, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, UK

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ABSTRACT

Botulinum neurotoxins are the most potent toxins known and causative agents of human botulism. Treatment comprises of administering purified polyclonal antitoxin or the prophylactic use of a vaccine containing formaldehyde inactivated toxoid. Whilst formaldehyde inhibits toxin activity, it induces so many structural changes in the molecule that immunisation often results in low levels of neutralising antibodies. We describe here for the first time a simple, less time consuming, novel method for producing a non-toxic toxoid that is structurally and antigenically more similar to the native toxin. Toxin is chemically inactivated by alkylation with iodoacetamide in the presence of reversibly denaturing conditions. This reduces neurotoxic activity by at least 7-orders of magnitude to undetectable levels. Following immunisation, *in vivo* neutralising antibody levels were 600-times higher than those produced with formaldehyde toxoid, despite generating equivalent ELISA antitoxin binding titres. These studies demonstrate that the new toxoid retains more of the native toxins structure and critical epitopes responsible for inducing life-saving neutralising antibody. Toxoid produced by the new method should substantially improve both antitoxin and vaccine production and be applicable to other toxins and immunogens.

1. Introduction

Seven types or serotypes (A to G) of botulinum toxin are currently known. Each serotype is composed of a heavy and a light chain linked together by disulphide bonds. The C-terminal end (H_C) of the heavy chain is responsible for binding to specific pre-synaptic neuronal cell receptors and the N-terminal end (H_N) facilitates internalisation. Once inside the cell, the light chain transverses the membrane of the endocytolic vesicles and cleaves specific proteins within the cytoplasm, essential for the docking and fusion of neurotransmitter containing vesicles at the nerve terminal (Schiavo et al., 2000). Subsequently, the extra-cellular release of neurotransmitter into the neuromuscular junction is blocked and results in a flaccid muscular paralysis.

Although rare, the life-threatening, paralysing disease botulism can be caused by the ingestion of food contaminated with either toxin (food-borne botulism) or botulinum spores (infant or intestinal botulism). Another form of botulism is caused by spore contamination of traumatised tissue (wound botulism). More recently the threat of botulinum toxin used as a biological weapon, which would cause inhalational botulism, has also been identified. Naturally occurring botulism in humans is more frequently associated with serotypes A, B and E and specific treatment available in the form of intravenously administered antitoxin or the prophylactic use of a vaccine (Shapiro et al., 1998; Mayers et al., 2001; Hibbs et al., 1996).

Current vaccines and antitoxins are produced using formaldehyde inactivated toxin. Formaldehyde (HCHO) inactivation is a prolonged procedure typically involving dialysis against low concentrations of formaldehyde (0.2–0.6%) over 7 or more days at 30 °C, resulting in a highly cross-linked toxoid (Wright et al., 1960; Sugiyama et al., 1974; Torii et al., 2002; Kobayashi et al., 2005). The reaction is complex and involves the formation of methylene (–CH_2–) bridges with indole, phenol, or

Abbreviations: BoNT/B, Botulinum type B toxin; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis.

* Corresponding author. Fax: +44 1707 641054.
E-mail address: rjones@nibsc.ac.uk (R.G.A. Jones).

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imidazole rings, or amide, ε-NH_2, or guanidine groups, with the numerous lysine residues principally being cross-linked (type A and B toxins contain 103 and 117 lysine residues respectively; Sathyamoorthy and Dasgupta, 1988; Petre et al., 1996). However, the complete removal of toxicity is so difficult that the currently available botulinum A–E pentavalent toxoid vaccine distributed by the CDC (Centers for Disease Control and Prevention, Atlanta, USA) contains 0.22% formaldehyde as a stabiliser despite its associated side effects (Dubuisson and Dennis, 1977; Moghaddam et al., 2006). Bacterial toxins treated with formaldehyde can be highly immunogenic when total antibody binding levels are measured (Nencioni et al., 1991); however, only low levels of neutralising antibodies are produced with resultant minimal protection (Fiock et al., 1963; Siegel, 1988; Nencioni et al., 1991). Botulinum type B toxoid is known to be one of the less efficacious immunogens (Torii et al., 2002) and, following an initial course of three doses of the pentavalent (A–E) vaccine, mean neutralising responses of just 0.49 and 0.08 IU/ml against type A and B toxins respectively are produced in humans (Siegel, 1988).

Formaldehyde toxoid is capable of absorbing out all the antitoxoid neutralising antibody, but it is known to be incapable of fully absorbing out antitoxin neutralising antibodies (Heimsch et al., 1970). This indicates that formaldehyde inactivation produces major conformational changes of the toxin, making the toxoid immunogenically less toxin-like and lacking certain neutralising epitopes. The production of therapeutic botulinum antitoxins typically involves immunising large animals such as horses or sheep with high doses of botulinum toxoid made by formaldehyde detoxification (Mayers et al., 2001). In the past, following a period of toxoid immunisation, animals were subsequently immunised with large doses of toxin to produce highly potent antitoxins suitable for therapeutic purposes (Bowmer, 1963). Due to the risk of potentially fatal needle stick injuries with high toxin doses, Health and Safety and Insurance constraints have now made it virtually impossible to boost large animals on a commercial scale with high toxin doses suitable to obtain potent antitoxins. A 'mild' method of inactivating botulinum toxin without causing gross conformational changes to the molecule would, therefore, potentially provide a non-toxic immunogen retaining more of the native toxins critical antigenic components for inducing neutralising antibodies.

Highly efficacious new immunogens are therefore required both to allow the use of lower protein doses in vaccines while providing greater protection and for the rapid attainment of hyperimmune serum to use in therapeutic antitoxins with resultant improved safety characteristics. As a consequence, many attempts have been made in the past to try and fully inactivate the highly potent botulinum neurotoxins using non-formaldehyde inactivation methods, but with limited success (Gerwing et al., 1966; Beers and Reich, 1969; Knox et al., 1970; Bhattacharyya and Sugiyama, 1989; De Paiva et al., 1993; Simpson et al., 2004; Woody and Dasgupta, 1989). For instance, although a 92%, but always incomplete inactivation of type B toxin following iodoacetamide alkylation treatment alone has been described by Beers and Reich (1969), there are contradictory reports of a 50% (Knox et al., 1970) and 0% (De Paiva et al., 1993) reduction in toxicity for type A toxin. Type B toxin contains 10 cysteine residues of which two or four are cross-linked by a disulphide bridge, compared to 9 cysteines on type A toxin of which 4 are disulphide linked (Antharavally et al., 1998; Antharavally and Dasgupta, 1998; De Paiva et al., 1993). The disulphide bond, linking the heavy and light chains, is known to be critical for membrane translocation and therefore for neurotoxicity and an approximately 1000-fold, but again incomplete inactivation, has previously been reported following reduction and alkylation or carboxymethylation of botulinum type A toxin (De Paiva et al., 1993). Reduction alone followed by formaldehyde (0.5%) treatment in the presence of a reducing agent has however been shown to cause a marked decrease in the production of neutralising antibody relative to conventional toxoid produced with formaldehyde alone (Sugiyama et al., 1974).

We have, therefore, developed a new, non-formaldehyde, non-cross-linking method of inactivating toxins which is described here for the first time. As formaldehyde inactivated toxoid of botulinum serotype B is known to be one of the least protective immunogens, it was, therefore, selected as a model toxin.

2. Materials and methods

2.1. Toxin, formaldehyde toxoid, antitoxin standard and animals

Concentrated botulinum toxin was safely handled within a class I safety cabinet. Purified, haemaglutinin free Botulinum type B toxin (Okra strain) with an activity of 8×10^7 mouse LD_{50}/mg and formaldehyde inactivated toxoid were obtained at 1 mg/ml from Metabiologics Inc (Madison, Wisconsin, USA). The WHO 2nd International Standard for botulinum type B antitoxin (BUSB) was obtained from NIBSC, UK, at 31 International Units (IU)/ampoule (Jones et al., 2006b). All animal work was performed to UK, Home Office and institutional guidelines.

2.2. New toxin alkylation procedure

One volume of botulinum toxin (at 1 mg/ml) was mixed with three volumes of freshly prepared alkylating buffer (66.67 mM Tris, 1.33 mM EDTA, 2.67 M NaCl, 2.67 M urea, pH 8.0 containing 266.7 mM Iodoacetamide), and incubated for 3 h at 37 °C in the dark to unfold and alkylate the exposed residues. The material was then dialysed at room temperature against 1 L phosphate buffer (150 mM $Na_2HPO_4.2H_2O$, 2 M NaCl, pH7.0) utilising a 10 kDa nMWCO dialysis membrane with two subsequent further changes of dialysis buffer and the resultant toxoid removed and stored at 4 °C. Protein concentrations of supernatants were determined using the BCA (Pierce) protein assay.

2.3. Hemidiaphragm assay

Left phrenic nerve-hemidiaphragm preparations were isolated from 18 to 30 g male, MF1 (Harlan, UK) out-bred white mice (Jones et al., 1999). Each was bathed in 6 ml Gelatine-Krebs buffer (Gelatine 0.2%, NaCl 118 mM, KCl 4.8 mM, KH_2PO_4 1.19 mM, $NaHCO_3$ 25 mM, $MgSO_4.7H_2O$ 1.2 mM, D-glucose $C_6H_{12}O_6$ 11.1 mM, $CaCl_2.2H_2O$ 2.54 mM) gassed (95% O_2 +- 5% CO_2) and maintained at 37 °C. Indirect stimulation (via the nerve) was applied at supramaximal voltage (approximately 3 V, 1Hz, 0.2 ms) and muscle contractions recorded using an isometric force transducer linked to a bridge amplifier and PowerLab (ADInstruments, UK) chart recorder.