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Individual and bivalent vaccines against botulinum neurotoxin serotypes A and B using DNA-based Semliki Forest virus vectors

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ABSTRACT

We evaluated individual and bivalent replicon vaccines against *Clostridium botulinum* neurotoxin serotypes A (BoNT/A) or B (BoNT/B). The DNA replicon vaccine (pSCARSBHc) encoding the Hc domain of BoNT/B (BHc) induced better responses and protection against BoNT/B mouse challenge than conventional DNA vaccine. The dual-expressing DNA vaccine (pSCARSA/BHc) protected similarly to a DNA replicon vaccine mixture (pSCARSAHc + pSCARSBHc). Additionally, recombinant SFV particles, VRP-AHc or VRP-BHc, protected mice from high-dose BoNT/A or BoNT/B challenge, respectively. Mice given either dual-expressing VRP-A/BHc or mixture of VRP-AHc and VRP-BHc were protected from challenge with serotype A/B mixtures. These data justify further testing in other animals or humans.

1. Introduction

The *Clostridium botulinum* neurotoxins (BoNTs) produced by *C. botulinum* are among the most potent toxins affecting humans and can be divided into seven serotypes (A–G) which possess similar structures but are antigenically distinct. Human botulism is commonly associated with toxin serotypes A, B, E and F, of which serotypes A and B are the most poisonous. Each 150 kDa BoNT molecule, consisting of a heavy (100 kDa) and a light (50 kDa) chain connected by a single disulfide bond, contains three functional domains: an N-terminal catalytic domain (light chain), an internal heavy chain translocation domain (H_N domain, 50 kDa) and the C-terminal heavy chain receptor-binding domain (Hc domain, 50 kDa) [1,2]. The carboxyl-terminal 50 kDa domain of the heavy chain (Hc domain), which alone is nontoxic, mediates the binding to target neurons and has demonstrated the ability to elicit protective immune responses in animals challenged with homologous botulinum neurotoxin [3–5]. The Hc domains of BoNTs produced in *Escherichia coli* and *Pichia pastoris* have been shown to elicit protective immune responses in mice and other animals [6–14]. DNA vaccines encoding the Hc domains of serotypes A and F have been described as next generation botulinum vaccines [15–18]. Candidate vaccines against BoNT serotypes A and C were also developed by using a Venezuelan equine encephalitis (VEE) virus replicon vector [19,20].

In a recent study, we developed a plasmid DNA replicon vaccine (pSCARSAHc) encoding the Hc domain of BoNT/A (AHc) by using a Semliki Forest virus (SFV) replicon vector. The pSCARSAHc vaccine induced stronger immune responses and greater protection than a conventional DNA vaccine against BoNT/A in mice [21]. In this current study, we also developed a plasmid DNA replicon vaccine for BoNT serotype B and evaluated its immunogenicity and protective capability against toxin challenge in a mouse model. To develop a single vaccine to protect against both BoNT serotypes A and B, we created and evaluated a dual-expression plasmid DNA replicon vaccine that expressed the AHc and BHc domains of BoNT serotypes A and B, respectively. In addition, recombinant SFV replicon particles (VRP) using DNA-based vectors expressing the Hc domains of BoNT serotypes A and B were developed. Finally, we evaluated the protective capability of these individual and bivalent vaccines using the DNA-based SFV vectors against challenge with BoNT serotypes A and B in mice.

2. Materials and methods

2.1. DNA vaccine construction

A plasmid DNA replicon vaccine (pSCARSAHc) encoding the Hc domain of *C. botulinum* neurotoxin serotype A (AHc) with an amino terminal fusion to a signal peptide of Ig κ was described previously (Fig. 1a) [21]. The plasmid DNA replicon vaccine (pSCARSBHc) encoding the Hc domain of *C. botulinum* neurotoxin serotype B (BHc) and a dual-expression plasmid DNA replicon vaccine (pSCARSA/BHc) encoding the Hc domains of the *C. botulinum* neurotoxin serotypes A and B (AHc and BHc) were

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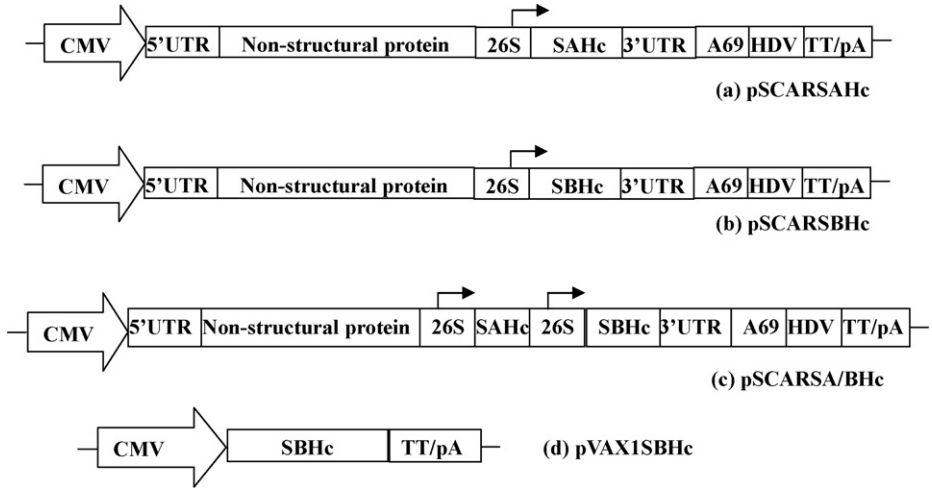

Fig. 1. Schematic diagrams of plasmids used for DNA vaccination. The individual transcriptional control elements comprising the DNA-based replicon vaccine plasmids based on SFV replicon (pSCARSAHc, pSCARSBHc and pSCARSA/BHc) and the conventional DNA vaccine plasmid (pVAX1SBHc) are indicated. CMV, cytomegalovirus immediate early (CMV IE) enhancer/promoter. TT/pA, BGH transcription termination and polyadenylation signal. HDV, HDV antigenomic ribozyme sequence. 26S, the subgenomic promoter of SFV. AHc and BHc, two completely synthetic genes encoding the Hc domains of BoNT serotypes A and B, respectively. S, Ig κ leader sequence. pSCARSA/BHc, a dual-expression plasmid DNA replicon vaccine that expressed the Hc domains of BoNT serotypes A and B under two separate 26S promoters.

also constructed in this study. In brief, the following primers were used for PCR amplification of the BHc gene from pGEM-BHc (containing a completely synthetic gene encoding the Hc domain of *C. botulinum* neurotoxin serotype B, amino acids 853 through 1291, ~50 kD) [22]: forward primer, F-BHcN (*Nde* I), 5′-GCCGCATATGTTCAACAAATACAACTCCGAAATC-3′; and reverse primer R-BHcN (*Nsi* I), 5′-TGACATGCATTTACTCGGTCCAACCCTCGTCTT-3′ (underlined sequences indicate restriction enzyme recognition sites). The PCR products were digested with NdeI and NsiI to excise the BHc DNA fragment and subcloned into a pSCAR DNA replicon expression vector [21,23] (cut with the same restriction enzymes). The resulting recombinant plasmid pSCARSBHc contains the BHc gene fused with an Ig κ signal peptide sequence (Fig. 1b). In order to create a plasmid DNA replicon vaccine co-expressing both AHc and BHc, pSCARSBHc was digested by MscI and SpeI and the 2.1 kb DNA fragment containing both the 26S promoter and BHc gene was purified and inserted into pSCARSAHc which was digested with SmaI and SpeI; the resulting plasmid pSCARSA/BHc expressed each of the AHc and BHc antigens under separate 26S promoters (Fig. 1c). To generate a conventional DNA vaccine encoding the same antigen, the DNA fragment encoding BHc was isolated by digesting pSCARSBHc with BamHI and SpeI, and then inserting it into the vector pVAX1 to create pVAX1SBHc (Fig. 1d).

All plasmids were prepared and purified using Endofree Mega-Q kits (Qiagen Ltd.) for transfection and immunization. BHK-21 cells were transfected with pSCARSBHc, pSCARSA/BHc, or pVAX1SBHc, and expression of AHc or BHc was analyzed as described previously [21,24].

2.2. Preparation of DNA-based recombinant Semliki Forest virus replicon particles (VRP)

Recombinant Semliki Forest virus replicon particles (VRP) were prepared as described [24–26]. Briefly, BHK cells were co-transfected with DNA-based replicon expression vectors (i.e., pSCAR-LacZ, pSCARSAHc, pSCARSBHc, or pSCARSA/BHc) and helper vector (pSHCAR), and the VRP were harvested between 24 and 36 h after transfection. VRP were concentrated and purified by centrifugation, and the virus pellets were resuspended in TNE buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1 mM EDTA). The VRP were then filtered through a 0.22 μm membrane and stored at −70 °C until use. Before use, VRP were activated *in vitro* with chymotrypsin, which renders them infectious by cleavage of the spike protein. For titer determination, BHK-21 cells were infected with serial dilutions of activated virus and monitored for expression of antigen by X-gal staining (VRP-lacZ) or immunofluorescence assay (IFA) (e.g., VRP-AHc, VRP-BHc or VRP-A/BHc) as described previously [24,25]. This procedure typically yielded VRP titers of 2.5×10^7 infectious particles/ml.

2.3. Vaccinations

Specific pathogen-free female Balb/c mice 6 weeks of age were randomly assigned to different treatment groups and vaccinated with DNA or VRP. For DNA vaccination, groups of eight mice were injected with 10 μg of plasmid DNA replicon vaccines or conventional DNA vaccine intramuscularly (bilaterally in the quadriceps) in a total volume of 0.1 ml four times with 2-week intervals between each injection. As a negative control, mice were vaccinated with 10 μg of pSCAR or pVAX1 as above. For VRP vaccination, 5×10^5 or 5×10^6 infectious particles (one infectious unit = 1 IU) of activated VRP-AHc, VRP-BHc, VRP-A/BHc or VRP-lacZ (negative control) in a total volume of 0.2 ml were inoculated subcutaneously twice or three times with 2-week intervals in between. Blood from all groups was collected via the tail vein before each vaccination or neurotoxin challenge and the serum isolated for rAHc or rBHc antibody reactivity. Mice from all groups were challenged i.p. with different dosages of pure BoNT serotypes A and B (BoNT serotype A from strain 62A and BoNT serotype B from strain Okra) diluted in 20 mM sodium phosphate buffer (pH 6.5) containing 0.2% (w/v) gelatin (Wako, Japan) 3 weeks after the last vaccination. The mice were observed for 1 week after challenge, and survival was determined for each vaccination group.

2.4. Antibody titer measurements

Sera from mice in the different treatment groups were screened for anti-rAHc or rBHc antibodies by ELISA. ELISA plates (Corning Incorporated, Corning, NY) were coated overnight at 4 °C with 100 μl rAHc or rBHc (2 μg/ml) [14,22]. Plates were washed with PBST (PBS containing 0.5% Tween-20) between all incubations. Serum samples were serially diluted at 1:2 increments beginning at 1:100 and 100 μl was added to each well for 1 h at 37 °C. After washing, 100 μl of a 1:2000 dilution of goat anti-mouse