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Enhanced effects of DNA vaccine against botulinum neurotoxin serotype A by targeting antigen to dendritic cells

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ABSTRACT

As dendritic cells (DCs) play a critical role in priming antigen-specific immune responses, the efficacy of DNA vaccines may be enhanced by targeting the encoded antigen proteins to DCs. In this study, we constructed a DC-targeted DNA vaccine encoding the Hc domain of botulinum neurotoxin serotype A (AHc) fused with scDEC, a single-chain Fv antibody (scFv) specific for the DC-restricted antigen-uptake receptor DEC205. Intramuscular injections of mice with the DC-targeted DNA vaccine (pVAX1-scDEC-AHc) stimulated more DCs to mature than the non-targeted DNA vaccine (pVAX1-SAHc) in the splenocytes. The DC-targeted DNA vaccine could induce more DCs maturation at the site of inoculation. The DC-targeted DNA vaccine induced stronger AHc-specific humoral immune responses, lymphocyte proliferative responses and protective potency against BoNT/A in mice than did pVAX1-SAHc. Moreover, the DC-targeting DNA vaccine provided effective protection after only two inoculations. In summary, these results showed that the DC-targeted fusion DNA vaccine could generate strong immunity, indicating that maturation of DCs induced by pVAX1-scDEC-AHc may be helpful for priming and boosting immune responses. Thus, we propose that the strategy of targeting antigen to DCs *in vivo* via DEC205 can enhance effectively the potency of DNA vaccines against BoNTs or other pathogens in an animal model.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a vital role in priming antigen-specific immune responses [1–3]. Immature DCs can respond to a number of stimuli and subsequently undergo a maturation process which leads to up-regulation of co-stimulatory, adhesion and MHC molecules that prime T lymphocytes, thereby effectively activating immune responses to prevent pathogenic invasion [1,3,4]. DEC205, a transmembrane protein that is highly expressed on DCs, belongs to the family of C-type lectins, including CD206, macrophage mannose receptor and phospholipase A2 receptor [5–7]. DEC205-specific antibodies fused with antigens can be targeted to DCs *in vivo*. This fusion protein antigen has been shown to induce DCs maturation and enhance the efficiency of antigen delivery, presentation and generate strong T-cell immunity in mice, rendering it an ideal candidate vehicle for vaccines against different pathogens [8,9].

Botulinum neurotoxins (BoNTs), which are produced by the Gram-positive, anaerobic bacterium *Clostridium botulinum*, are the most toxic substances known. Among the seven serological serotypes (A-G) of botulinum neurotoxins, BoNT/A, BoNT/B, BoNT/E and BoNT/F are associated with human botulism, with BoNT/A causing the highest mortality rate [10–13]. The BoNT/A protein is secreted by *C. botulinum* A and has a molecular mass of ~150 kDa. It consists of a 100-kDa heavy chain (HC) coupled with a 50-kDa light chain (LC) by a disulfide bond. The C-terminal half of the heavy chain (AHc, 50 kDa), which is nontoxic, mediates the binding of BoNT/A with neurons and is able to induce protective immune responses against natural BoNT/A in animals [13–15].

In previous studies, the Hc antigen of BoNT/A expressed in *Escherichia coli* or *Pichia pastoris* as a subunit vaccine has been shown to induce protective immune responses in mice and other animals [16,17]. In addition, DNA vaccines encoding the nontoxic AHc have been studied as the next generation of botulinum vaccines [13,18–20]. DNA vaccines can induce both antigen-specific cellular and humoral immune responses, but their efficacy has proven to be limited [1]. Therefore,

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new strategies must be developed to increase the potency of DNA vaccines against BoNT/A [1,21,22]. In this work, we explored and evaluated effects of a DC-targeted DNA vaccine encoding the AHc-conjugated anti-DEC205 antibody (scDEC-AHc) fusion protein, which can target the recombinant fusion antigen to DCs *in vivo* with high-efficiency for antigen processing and presentation [23,24]. Our results showed that the pVAX1-scDEC-AHc vaccine led to more DCs to mature at the site of injection and in spleen tissues compared with the pVAX1-SAHc vaccine. We also showed that the pVAX1-scDEC-AHc DNA vaccine induced stronger protective immune responses against natural BoNT/A in mice than the pVAX1-SAHc vaccine. These studies described that targeting antigens, especially to the DCs through the DEC205 receptor, is an effective strategy for enhancing the potency of DNA vaccines [2,6,25,26].

2. Materials and methods

2.1. Construction of DNA vaccine

To generate the pVAX1-scDEC-AHc DNA vaccine vector, the *scDEC* gene encoding the variable regions of the heavy (scDEC-H) and light (scDEC-L) chains was synthesized according to published sequences [4]. The *scDEC* DNA fragment was cloned into the pVAX1-SAHc vector (a conventional DNA vector encoding the Hc domain of botulinum neurotoxin serotype A (AHc) that was constructed and maintained in our lab) using the *BamH* I and *EcoR* I restriction sites [27]. The resulting recombinant plasmid pVAX1-scDEC-AHc encodes the *AHc* gene fused with the *scDEC* gene. All plasmids were prepared and purified using Endofree Mega-Q kits (Qiagen, Hilden, Germany) for transfection and immunization.

2.2. Western blot analysis

To evaluate expression of the scDEC-AHc fusion protein, 293Ft cells (1×10^6 cells/well) were transfected with complexes of each plasmid DNA and 293fectin™ Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. These transfected cells were harvested with lysis buffer at 36 h post-transfection, and both the cell lysate and supernatant were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and then incubated with mouse anti-AHc serum antibodies (1:1000) at 4 °C [19]. After washing in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 30 min and incubating with goat anti-mouse IgG-HRP (1:4000; ZSGB-BIO, Beijing, China) for 1 h at room temperature, the membranes finally were washed in TBS-T for 30 min and exposed to Super Signal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA, USA).

2.3. Immunization of mice and challenge with BoNT/A

Specific pathogen-free female Balb/c mice (Beijing Laboratory Animal Center, Beijing, China) at 6–8 weeks of age were randomly assigned to different treatment groups (8 mice per group). Mice were intramuscularly (i.m.) injected with 30 μg of plasmid pVAX1-scDEC-AHc in a total volume of 0.1 ml in combination with electrical pulses. Mice were injected with 30 μg of pVAX1 or pVAX1-SAHc plasmid as described above as controls. The same method and amounts of DNA were used for boosting two times at 3-week intervals. Three weeks after the third vaccination, mice were challenged with an active preparation containing 10^3 or 10^4 LD_{50} of BoNT/A by i.p. injection, and survival was monitored for 7 days.

To further assess the immune protective response against botulinum neurotoxin, mice were i.m. injected with 30 μg of DNA vaccine as described above (8 mice per group). Three weeks after the first or second vaccination, eight mice of each group were challenged with an active preparation containing 10^3 LD_{50} of BoNT/A by i.p. injection, and survival was monitored for 7 days. All animal procedures were conducted with the approval of the Beijing Institute of Biotechnology Institutional Animal Care and Use Committee and were in full compliance with the Committee's guidelines.

2.4. Flow cytometry

To analyze the phenotype of splenocytes from the immunized mice, mice (n = 4) were immunized three times with the DC-targeted DNA vaccine. At three weeks after the last immunization, spleens from the immunized mice were mashed through a 400 mesh strainer and re-suspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (ExCell Bio, Shanghai, China). After low-speed centrifugation, the cell suspension was treated with Tris-NH_4Cl (pH 7.2) and resuspended in PBS. The cells (1×10^6 in 100 μl volume) were incubated with 0.5 μg phycoerythrin (PE)-labeled anti-mouse CD80 (BD Biosciences, San Jose, CA, USA) and 0.5 μg fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11c (BD Biosciences) for 30 min in the dark at 4 °C. Cells were washed with PBS and fixed with PBS containing 2% paraformaldehyde in 400 μl volume. Samples from splenocytes were analyzed by 2-color flow cytometry with CD11c + and CD80 + gating to determine the maturation state of DCs. The fluorescence profile of each sample (at least 10,000 cells) was analyzed on FACS-Calibur flow cytometer (BD Biosciences). Samples from naive mice were utilized as negative controls. Data were recorded and analyzed with BD FACSDiva 8.0.1 software.

2.5. Histopathology and immunohistochemistry

Three weeks after the last immunization, the injected mouse muscles were excised and fixed with 4% paraformaldehyde immediately. The muscle tissues were embedded using paraffin and cut into 5 μm sections. Fixed sections were stained with hematoxylin and eosin (H & E) to assess the recruitment of inflammatory cells.

Five-micrometer paraffin sections were dewaxed, rehydrated with deionized water and treated with 3% H_2O_2 for 10 min to block endogenous peroxidase. Antigens in the sections were restored using a microwave oven in 0.01 M citrate buffer (pH 6.0) for a total of 30 min. The nonspecific binding was blocked with rabbit serum or goat serum for 2 h at room temperature and then incubated with purified anti-mouse CD83 (BD Biosciences) overnight at 4 °C. Sections were washed in PBS and incubated with goat anti-rat IgG-HRP (Jackson Immunoresearch, Lancaster, PA, USA) for 30 min at 37 °C. The slides were washed with PBS three times, and DAB substrate (ZSGB-BIO, Beijing, China) was used for color development. After color development, the slides were counterstained with hematoxylin (ZSGB-BIO) and mounted with a cover glass.

2.6. Serum antibody titer measurements

To determine AHc-specific antibody responses of immunized mice, serum samples were collected three times at 3 weeks after each immunization and stored at −20 °C before assaying. ELISA plates were coated overnight at 4 °C with purified recombinant AHc (0.2 μg/well) [19]. Plates were washed with PBS-T (PBS, 0.05% Tween-20) and blocked with PBS containing 2% albumin bovine V (BBI Life Sciences, Shanghai, China). Serum samples were two-fold serially diluted beginning at the 1:100 dilutions, and plates were incubated with serum diluents (100 μl/well) for 1.5 h at 37 °C. After washing with PBS-T, total antibody titers were measured using 1:2000 dilution of goat anti-mouse IgG-HRP (Jackson Immunoresearch) for 45 min at 37 °C. The HRP-conjugated goat anti-muse IgG1 and IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for measuring IgG1 and IgG2a isotype antibody titers (1:2000; 100 μl/well), respectively. After washing, antibody responses were visualized by adding 50 μl of citrate buffer