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Enhanced efficacy of DNA vaccination against botulinum neurotoxin serotype A by co-administration of plasmids encoding DC-stimulating Flt3L and MIP-3α cytokines

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ABSTRACT

Targeting antigens encoded by DNA vaccines to the key antigen-presenting cells by chemotactic or growth factors, is an effective strategy for enhancing the potency of DNA vaccinations. Here, we report the effects of chemotactic or growth factors on a DNA vaccine against botulinum neurotoxin serotype A (BoNT/A) in a mouse model. We demonstrated that mice immunized with DNA constructs encoding the Hc domain of BoNT/A (AHc) fused with DC-stimulating Flt3L or MIP-3α cytokines failed to elicit an enhanced or efficacious AHc-specific humoral or protective response in mice. However, the potency of DNA vaccination was significantly modulated and enhanced by co-administration of AHc-expressing DNA with pFlt3L or pMIP-3α, which generated strong immune and protective responses against BoNT/A. Moreover, the enhanced potency was further boosted by co-administration of AHc-expressing DNA with the combination of pFlt3L and pMIP-3α in mice, but not with the Flt3L-MIP-3α fusion molecule, which indicated that co-immunization with both pFlt3L and pMIP-3α could synergistically enhance AHc-specific immune and protective responses against BoNT/A. In summary, our findings indicate that co-administration of plasmids encoding antigen and cytokine rather than administration of plasmids encoding cytokine-antigen fusion is effective to enhance the potency of AHc-expressing DNA vaccine.

1. Introduction

Although naked DNA vaccines often work well in mice model, the efficiency is restricted in larger animals and humans, which hinders the practical application of DNA vaccines. Co-expression of cytokines and other immunomodulatory molecules has been described as an effective strategy to overcome this problem [1,2]. Targeting antigens encoded by DNA vaccines to the key antigen-presenting cells (APCs), especially dendritic cells (DCs), by chemotactic and growth factors, is an effective strategy for enhancing the potency of DNA vaccines [3,4]. Chemokines, function as immunologic mediators, play a critical role in regulating the trafficking of leukocytes, including DCs. Expression of chemokines or other immunostimulatory cytokines at the site of vaccination may recruit immature DCs to the site of injection, which is beneficial to capture DNA vaccines and their expression products to generate the enhanced immune responses [3,5].

Among of these chemokines, macrophage inflammatory protein (MIP)-3α is the only chemokine ligand for CCR6, which is expressed on immature DCs, can trigger adaptive immunity by attracting immature DCs to the site of inflammation [6,7]. Additionally, fms-like tyrosine kinase 3 ligand (Flt3L), a potent DC-specific growth factor, has the unique ability to expand and mature DCs in mice and humans [3,4,8,9]. Previously, DNA vaccines elicited lower humoral and protective responses against botulinum neurotoxins (BoNTs) when compared with those elicited by conventional subunit protein vaccines in a mouse model [10–12]. Therefore, it was necessary to improve the potencies of BoNTs DNA vaccines for future clinical studies. In this study, Flt3L and MIP-3α cytokines were evaluated for the potential to enhance the efficiency of DNA vaccination against botulinum neurotoxin serotype A (BoNT/A). We hypothesized that these DC-stimulating chemotactic and growth factors as molecular adjuvants of DNA vaccines

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may target antigen to immune cells, and enhance immune responses and protective efficacy against BoNT/A in a mouse model.

2. Materials and methods

2.1. Constructions of DNA plasmids

A plasmid pVAX1SAHc expressing the Hc domain of BoNT/A (AHc) has been described in previous study [13]. A pABK-HIV-MF plasmid containing the *Flt3L* and *MIP-3α* fusion gene (*Flt3L-MIP-3α*) has been described in previous study [14]. A series of plasmid DNA vectors that expressed these two molecular adjuvants were constructed by inserting them into pVAX1 as previously described [13,15]. Briefly, the *Flt3L*, *MIP-3α* and *Flt3L-MIP-3α* genes amplified by PCR were inserted into pVAX1 to produce recombinant pFlt3L, pMIP-3α, and pFlt3L-MIP-3α, respectively. The recombinant pFlt3L-AHc and pMIP-3α-AHc plasmids were constructed by fusing the AHc to C-terminus of Flt3L and MIP-3α with G4SG4S small linker, respectively. All plasmids were prepared and purified using Endo-free Mega-Q kits (Qiagen, Hilden, Germany) for transfection and immunization.

The *in vitro* expression of AHc, Flt3L or MIP-3α in DNA-transfected cells was detected by immunoblots as previously described [14,15]. Briefly, BHK-21 cells were seeded into six-well tissue culture plates at 2×10^5cells/well and grown to 70–80% confluence, then were transfected with complexes of each plasmid DNA and Lipo-fectamine 2000 (Invitrogen, CA, USA) following the manufacturer's instructions. These transfected cells were harvested with lysis buffer after 24 h post-transfection. The lysates from approximately 1×10^5 cells were separated by SDS-PAGE, transferred onto membranes, and probed by Western blots using hyperimmune mouse anti-AHc serum [15], mouse monoclonal Flt3L antibody (F-6, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse monoclonal MIP-3α Antibody (530, Santa Cruz Biotechnology, Inc.), respectively.

2.2. Vaccinations and challenge

Eight-week-old specific pathogen-free female Balb/c mice were purchased from the Beijing Laboratory Animal Center (Beijing, China) and randomly assigned to different treatment groups ($n = 8$ per group). DNA dosage used in the immunized groups was optimized by a series of preliminary experiments. Mice were i.m. immunized with 30 μg of each DNA plasmid in a total volume of 0.1 ml three times with 3-week intervals between each injection. As a negative control, mice were vaccinated with 30 μg of pVAX1 using the same protocol. Blood from all groups was collected via the tail vein before each vaccination or neurotoxin challenge. Mice from all groups were challenged i.p. with 10^3 or 10^4 50% mouse lethal dose (LD_{50}) of active BoNT/A 4 weeks after the last vaccination. The strain 62A BoNT/A (BoNT/A1) was obtained from National Institutes of Food and Drug Control, Beijing, China. Potency of BoNT/A1 was 1.88×10^6 LD_{50} per mg toxin. The mice were observed for 1 week after challenge, and survival was recorded for each group. 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.

2.3. Detection of antibody responses

Anti-AHc antibodies of sera from mice in the different groups were detected by ELISA as previously described [13,15]. Briefly, ELISA plates (Corning Incorporated, Corning, NY, USA) were coated overnight at 4 °C with 100 μl recombinant AHc (2 μg/ml). Serum samples were diluted 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 IgG-HRP (Santa Cruz Biotechnology, Inc.) was added and incubated for 30 min at 37 °C. Then, anti-AHc reactivity was visualized by adding 100 μl of citrate buffer (pH 5.0) containing 0.04% (w/v) of o-phenylenediamine and 0.02% (v/v) hydrogen peroxide for 5 min at 37 °C. The reaction was stopped with 50 μl of 2 M H_2SO_4 and the absorbance was read at 492 nm using a Thermo Labsystems (Franklin, MA, USA) microplate reader. Data represent the mean + SD ($n = 8$). The individual isotype of IgG (IgG1 and IgG2a) antibody responses were further determined using HRP-conjugated goat anti-mouse IgG1 and IgG2a antibodies (Santa Cruz Biotechnology) at a dilution of 1:2000. The IgG1/IgG2a ratios of specific-AHc sera antibodies in different immunization groups were determined to evaluate the type of response to immunization. Results for each group represent the average ratio ± SEM.

2.4. BoNT/A neutralization assay

Anti-AHc sera from mice in the different groups were pooled for the BoNT/A neutralization assay as previously described [13,16]. Briefly, mixtures of serial dilutions of sera in phosphate buffer (50 mM Na_2HPO4) containing 1% gelatin with 100 LD_{50} of BoNT/A (BoNT serotype A from strain 62A) were incubated 0.5 h at room temperature and the mixtures were injected i.p. into Balb/c mice using a volume of 500 μl/mouse (four mice in each group). The mice were observed for one week, and survival was determined for each group. The concentration of neutralizing antibody in the sera was calculated relative to a National Institutes of Food and Drug Control botulinum serotype A antitoxin reference standard (540 IU/ampoule) and neutralizing antibody titers of sera were reported as international units per milliliter (IU/ml).

2.5. Lymphocyte proliferative responses

Spleens were removed from the immunized mice at 4 weeks after the last vaccination and resuspended in RPMI 1640 at 2×10^6 cells/ml as previously described [15,16]. Splenocytes were plated in 96-well flat-bottomed plates (100 μl/well). Subsequently, medium with or without AHc (10 μg/ml) was added (100 μl/well) and mixed. After 72 h of incubation, the proliferative responses were detected using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. The proliferation stimulation index represents the fold difference between stimulated and un-stimulated cells for each sample.

2.6. Statistical analysis

Differences in the humoral and cellular immune responses were analyzed statistically by ANOVA one-way variance test or using the Student's t test for the analysis of differences between groups. Fisher's exact test was used to determine statistical differences in survival between the treatment groups. For all tests only data resulting in P values <0.05 were regarded as statistically significant.

3. Results

3.1. Expression of recombinant proteins

A series of plasmid DNA vectors encoding these Flt3L and MIP-3α two molecular adjuvants were constructed as DNA vaccines against BoNT/A. Expression of recombinant proteins was confirmed in BHK-21 cells transfected with these different DNA constructs, but