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Electroporation of a multivalent DNA vaccine cocktail elicits a protective immune response against anthrax and plague

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ABSTRACT

Electroporation of DNA vaccines represents a platform technology well positioned for the development of multivalent biodefense vaccines. To evaluate this hypothesis, three vaccine constructs were produced using codon-optimized genes encoding *Bacillus anthracis* Protective Antigen (PA), and the *Yersinia pestis* genes LcrV and F1, cloned into pVAX1. A/J mice were immunized on a prime-boost schedule with these constructs using the electroporation-based TriGrid Delivery System. Immunization with the individual pDNA vaccines elicited higher levels of antigen-specific IgG than when used in combination. DNA vaccine effectiveness was proven, the pVAX-PA titers were toxin neutralizing and fully protective against a lethal *B. anthracis* spore challenge when administered alone or co-formulated with the plague pDNA vaccines. LcrV and F1 pVAX vaccines against plague were synergistic, resulting in 100% survival, but less protective individually and when co-formulated with pVAX-PA. These DNA vaccine responses were Th1/Th2 balanced with high levels of IFN-γ and IL-4 in splenocyte recall assays, contrary to complimentary protein Alum vaccinations displaying a Th2 bias with increased IL-4 and low levels of IFN-γ. These results demonstrate the feasibility of electroporation to deliver and maintain the overall efficacy of an anthrax-plague DNA vaccine cocktail whose individual components have qualitative immunological differences when combined.

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1. Introduction

Vaccination is widely recognized as one of the most desirable approaches to disease prevention. The low cost of proactive immunization compared to reactive treatment of sick patients, who can spread disease, has been a major driving force for the development of vaccines [1]. Currently, there are a number of common vaccine modalities including protein subunit, attenuated live or killed pathogen, and culture supernatants. However, these technologies have limitations when applied to *Bacillus anthracis* and *Yersinia pestis*, providing the impetus for the investigation of novel vaccine approaches.

B. anthracis, the causative agent of anthrax, has a high mortality rate but is a vaccine preventable infectious disease. Biothrax™, the currently licensed vaccine for anthrax prevention, is an aluminum-salt adsorbed vaccine produced using a filtered culture supernatant of the toxinogenic unencapsulated strain of *B. anthracis* V770-NP1-R [2] primarily containing the immunizing protective antigen (PA). Although this FDA-licensed vaccine is in use today, there are concerns arising from vaccine compliance due to the complicated and lengthy immunization regimen and the associated injection site reactogenicity and systemic side effects [3].

In contrast, plague vaccine candidates have traditionally been based on either live attenuated *Y. pestis* strains, such as EV76, or formaldehyde-killed whole cell preparations, such as the previously available United States Pharmacopoeia (USP) plague vaccine. Unfortunately, both vaccines have an unacceptably high incidence of reactogenicity at the site of infection as well as a risk of systemic side-effects [4,5]. The USP plague vaccine also is prone to eliciting short-term immunity, necessitating annual boosters, and was also found to have questionable efficacy during pneumonic plague infections [4,6,7]. For these reasons, neither plague vaccine is currently licensed in the USA.

Abbreviations: PA, protective antigen; LF, lethal factor; EF, edema factor; pDNA, plasmid deoxyribonucleic acid; BCA, bicinchoninic acid; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TDS, trigrid delivery system; EP, electroporation; TNA, toxin neutralization activity.

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Nucleic acid based, or plasmid DNA (pDNA) vaccines, are an attractive vaccination strategy that is well positioned for a multi-agent immunization targeting both anthrax and plague. This interest is the result of their low cost, ease of production, excellent stability profile, and minimal risk of deleterious interactions when formulating multivalent vaccines [8]. Despite these promising attributes, development of pDNA immunization has suffered from insufficient potency in the clinical setting [9]. There is general consensus that a key factor contributing to the suboptimal potency is the relative inefficiency of uptake of pDNA to its intracellular site of action following conventional methods of administration [10]. To overcome this hurdle, pDNA immunization technologies have been developed emphasizing improved delivery methods such as electroporation (EP) to enhance the intracellular uptake of pDNA vaccines. Use of EP as a potent method for intracellular gene delivery has been found to increase both humoral and cellular immune responses up to 100-fold [11–15] compared to conventional injection of the pDNA vaccines.

While pDNA vaccines expressing anthrax PA, and V antigen (V) or the Fraction 1 Capsular Antigen (F1) from *Y. pestis* have been shown to elicit protective humoral responses [16–18], the combination of all three pDNAs has not been fully evaluated. In this report, we investigated the potential of the EP-based TriGrid Delivery System (TDS) mediated DNA immunization to co-deliver a previously described pDNA vaccine expressing PA [16] and two additional constructs expressing V and F1. The objectives of this work were to (1) characterize the humoral responses and Th1/Th2 balance of this type of vaccination relative to protein vaccination, (2) determine if there is synergy or interference between each of the DNA vaccine components when they are co-formulated, and (3) demonstrate the protective efficacy of a multi-agent/multivalent anthrax and plague DNA vaccine relative to recombinant protein vaccination.

2. Materials and methods

2.1. Plasmid DNA vaccine construction

In vivo mammalian expression of the prokaryotic genes encoding *B. anthracis* PA (GenBank accession no. AAA22637.1) and *Y. pestis* V (GenBank accession no. NC009595) and F1 (GenBank accession no. NC006323.1) was enhanced through in silico codon optimization using established human codon biases (GeneArt, Invitrogen, Carlsbad, CA). This process ensures proper transcription and translation while capitalizing on the degeneracy of the genetic code to replace rare prokaryotic codons with synonymous rare human codons to maintain the wild-type amino acid sequence [19]. Extracellular transport of each gene product was signaled by fusing a human tissue plasminogen activator (tPA) leader sequence to the five prime end of each full length gene sequence [19]. The resulting synthetic, full length genes were cloned downstream and in frame of the human cytomegalovirus immediate/early promoter (CMVp) located upstream and in-frame of the multi-cloning site in pVAX1 (Invitrogen). Three vaccine constructs, pVAX-PA, pVAX-V, and pVAX-F1, were verified by sequencing and encode the complete, i.e., full length, protein sequence for each target antigen.

2.2. Animal immunization procedures

Plasmid DNA (pDNA) vaccinations were performed by intramuscular (i.m.) electroporation using the TriGrid™ Delivery System (Ichor Medical Systems, San Diego, CA) as reported previously [14]. Briefly, seven week old female A/J mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized by intraperitoneal (i.p.) injection of a ketamine/xylazine cocktail (80 mg/kg-bw mixed with 20 mg/kg-bw) then shaved and aseptically swabbed at the injection site. A loss of motor reflexes and deep pain response were evaluated by a toe pinch before any further immunization procedures were performed. DNA was suspended in calcium- and magnesium-free injectable PBS (Invitrogen, Carlsbad, California) and injected using 3/10 cc 30 G insulin syringes (BD, Franklin Lakes, NJ) inserted into the center of a TriGrid™ electrode array with 2.5 mm electrode spacing. DNA solutions composed of two or more pDNA vaccines were gently mixed in 1.5 ml Eppendorf tubes prior to loading the syringes and electroporation. Each mouse received a total of 9 μg of pDNA in a volume of 20 μl but no more than 3 μg of any given pDNA vaccine (Supplemental Table 1). To equalize the total amount of DNA injected, groups receiving one or two constructs received an additional 6 or 3 μg of the pVAX plasmid without a gene insert (pVAX-Empty). Immediately following i.m. injection of the pDNA into one tibialis anterior (TA) muscle, the site of injection was stimulated with an electrical pulse that had an amplitude of 250 V per cm of electrode spacing for 40 ms over a 400 ms interval. At the end of the cycle the integrated TriGrid Delivery System, including the insulin syringe, was removed and the animal allowed to recover under a warming lamp. Sham vaccinated animals received 9 μg of the pVAX-Empty plasmid by TDS. Mice were vaccinated twice, on days 0 and 28; with the first injection in the mouse's right TA and the second in the left TA.

Protein vaccinations and Biothrax™ (as a positive control for survival during anthrax challenge) were administered i.p. using a standard tuberculin syringe mounted with a 26 G needle (BD). Full length recombinant PA (rPA), V (rV) and F1 (rF1) were expressed and purified in-house using a hepta-his tag in conjunction with high-affinity chromatography, cation exchange, and gel filtration chromatography. Endotoxin was removed with a 0.1% triton X-114 extraction and found to be less than 13 EU/mg of protein as measured with the Kinetic-QCLR chromogenic LAL assay (Lonza, Walkersville, MD). The final purified proteins were visualized with SDS–PAGE and quantified with the BCA Protein Assay kit (Pierce, Rockford, IL). Recombinant proteins were mixed 1:1 with Imject Alum (Alum, Pierce) at a final concentration of 0.1 μg/μl so that each 100 μl injection contained a total of 10 μg of each protein. Intraperitoneal injections were performed aseptically just caudal to the umbilicus and lateral to the midline. Mice were vaccinated on days 0 and 28; with the first injection on the mouse's right and the second on the left.

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "*Guide for the Care and Use of Laboratory Animals*" Institute of Laboratory Animal Resources, commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996. All animal procedures were approved by NMRC's IACUC and conducted in the WRAIR/NMRC vivarium, an AALAC accredited facility.

2.3. Characterization of mouse IgG responses

Murine anti-PA, anti-V, and anti-F1 IgG concentrations were quantified in all animals of each group ($N = 18$ per group) by ELISA as described previously [20]. Briefly, Immulon-IV HBX microtiter plates (Thermo Labsystems, Franklin, MA) were coated overnight at 4 °C with 1 μg/ml rPA, 5 μg/ml rV, or 1 μg/ml rF1 prepared in 0.2 M carbonate buffer (pH 7.4). Each plate included doubling dilutions of purified mouse IgG as a positive control (Sigma, St. Louis, MO). Mouse serum samples and quantitative standard curves composed of purified mouse anti-PA, anti-V, or anti-F1 IgG produced in-house were serially diluted and added to the plate after blocking and washing. Bound antigen-specific antibodies were detected using a mouse-specific horseradish peroxidase-conjugated anti-IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:12,800 and incubated in the wells for 1 h at 37 °C.