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Presentation of peptides from *Bacillus anthracis* protective antigen on Tobacco Mosaic Virus as an epitope targeted anthrax vaccine

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ABSTRACT

The current anthrax vaccine requires improvements for rapidly invoking longer-lasting neutralizing antibody responses with fewer doses from a well-defined formulation. Designing antigens that target neutralizing antibody epitopes of anthrax protective antigen, a component of anthrax toxin, may offer a solution for achieving a vaccine that can induce strong and long lasting antibody responses with fewer boosters. Here we report implementation of a strategy for developing epitope focused virus nanoparticle vaccines against anthrax by using immunogenic virus particles to present peptides derived from anthrax toxin previously identified in (1) neutralizing antibody epitope mapping studies, (2) toxin crystal structure analyses to identify functional regions, and (3) toxin mutational analyses. We successfully expressed two of three peptide epitopes from anthrax toxin that, in previous reports, bound antibodies that were partially neutralizing against toxin activity, discovered cross-reactivity between vaccine constructs and toxin specific antibodies raised in goats against native toxin and showed that antibodies induced by our vaccine constructs also cross-react with native toxin. While protection against intoxication in cellular and animal studies were not as effective as in previous studies, partial toxin neutralization was observed in animals, demonstrating the feasibility of using plant-virus nanoparticles as a platform for epitope defined anthrax vaccines.

1. Introduction

Anthrax lethal toxin (LT) is the main pathogenicity factor of *Bacillus anthracis*. It consists of two toxin components called protective antigen (PA) and lethal factor (LF). PA is an 83 kDa protein (PA83) binds cellular receptors [1,2]. Once bound, host furin cleaves a 20 kDa fragment from the N-terminus of PA83, thus activating the 63 kDa protein, PA63 [3]. This enables the formation of PA oligomers that bind LF [3,4]. PA oligomers form pores in the endosomal membrane allowing LF to translocate to the cytoplasm [5–7]. Once in the cytoplasm, LF cleaves mitogen activated protein kinase kinases and causes apoptosis [8].

Immunity to anthrax is conferred by an antibody response against PA [9–11]. A single FDA approved vaccine, called Anthrax Vaccine Adsorbed (AVA), is made using attenuated *B. anthracis* culture adsorbed to aluminum hydroxide [12]. AVA is administered at 0, 1 and 6 months, boosters at 12 and 18 months and annual boosters thereafter [13]. This vaccine requires improvements for rapidly invoking longer-lasting neutralizing antibody responses with fewer doses from a well-defined formulation.

One way to improve vaccine effectiveness may be to target defined neutralizing antibody epitopes on the PA toxin utilizing an immunogenic antigen display platform. Crowe et al. identified 13 linear epitopes that bound antibodies from AVA vaccinated donors [14]. Antibodies against three of these epitopes (PA 152–171, PA 232–247 and PA 628–637) were shown to provide partial levels of protection against LT challenge in macrophages and mice on their own. Each epitope has also been associated with important functions throughout the process of cellular invasion by anthrax LT. The PA 152–171 epitope contains the furin cleavage site $_{164}$RKKR$_{167}$ [15]. Mutations at amino acids 240 and 245 within the PA 232–247 epitope have been shown to reduce LT toxicity by at least 100-fold in cellular assays [16]. The PA 232–247 sequence is also in close proximity to the proposed LF binding site on the PA oligomer [17]. Finally, the location of the PA 628–637 epitope resides in the cellular receptor binding region [18].

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Tobacco Mosaic Virus (TMV) is an attractive antigen display platform and has been tested in a variety of small animals [19]. TMV is a rod-shaped virus approximately 18 nm × 300 nm comprising an RNA genome encapsidated by 2130 self-assembling coat protein monomers. The size and repetitive surface of TMV is optimal for efficient uptake by antigen presenting cells for $CD4^+$ T-cell priming and for potent cross-linking of B-cell receptors. Plants expressing TMV can be grown in GMP environments and scaled-up making this system good for producing a consistent, well-defined vaccine product [20].

In this study, we have investigated the vaccine potential of the PA 152–171, PA 232–247 and PA 628–637 epitopes genetically expressed on the surface of TMV in *Nicotiana benthamiana* and tested their ability to induce anthrax LT neutralizing antibodies that could protect mice from anthrax spores.

2. Materials and methods

2.1. TMV expression and virus purification

TMV coat protein U1 strain genes were synthesized (Genewiz Inc.) with sequences for PA 152–171, PA 232–247 and PA 628–637 epitopes [14], optimized for TMV codon specificity, at the C-terminus. Modified coat protein genes were cloned into the plasmid pDN15-TCS using AvrII and NotI (NEB). pDN15-TCS contains TMV replicase and movement protein genes with the multiple cloning site, 5′ UTR and Ribozyme sequences from pJLTurbo [21] under T7 promoter transcriptional control. Infectious TMV RNA was transcribed from plasmid templates using the mMessage mMachine T7 Transcription Kit (Life Technologies) according to the manufacturer's directions. Three-month old *Nicotiana benthamiana* plants were inoculated with T7 transcribed RNA as described in [22] and incubated at 24 °C/16 h light and 21 °C/8 h dark for 2-weeks. Modified viruses were purified from leaf tissue as in [23] with slight modifications in the extraction buffer composition (50 mM Sodium Acetate, 0.1% Sodium Metabisulfite (w/v), 0.01% Beta-Mercaptoethanol (v/v), pH 5.0) and omission of chloroform extraction. TMV-PA 232–247/TMV-PA 628–637 combination vaccine contained a mixture of TMV-PA 232–247 and TMV-PA 628–637 such that the concentration of each alone was 0.25 mg/mL but added together was 0.5 mg/mL. Purified viruses were quantified using BCA (Pierce).

2.2. SDS–PAGE, Western blot and ELISA

For SDS–PAGE, purified TMV virus extracts were prepared 1:1 in Laemmli Blue with beta-mercaptoethanol, boiled and run on 4–20% Mini-Protean TGX gels (BioRad) in 1× Tris/Glycine/SDS buffer and stained with BioSafe Coomassie G-250 stain (BioRad) according to manufacturer's direction.

Western blot analysis was performed as in [24] with the following modifications. Primary antibodies were diluted in blocking buffer at 1:1000 (Goat anti-PA polyclonal, List Biological Laboratories) or 1:200 (Rabbit anti-TMV polyclonal, Agdia). Secondary antibodies were diluted at 1:3000 (Goat anti-Rabbit HRP, BioRad) or 1:2000 (Rabbit anti-Goat HRP, Life Technologies). Opti-4CN (BioRad) reagent was added for 5-min for detection. Images were acquired using a 5-megapixel camera and processed using Adobe Photoshop CS4. We utilized the software ImageJ [http://imagej.nih.gov/ij/] to digitally quantify the relative abundance of TMV-PA band in comparison to the PA83.

For Indirect ELISA analysis, 96-well plates were coated with 1 μg PA83 (List Biological Laboratories) or purified TMV virus in 100 mM Bicarbonate/Carbonate buffer (0.03 M Na_2CO_3/0.07 M $NaHCO_3$, pH 9.6) overnight at 4 °C. Primary antibodies were diluted in 5% non-fat milk in PBS and incubated overnight at 4 °C. Secondary antibody dilutions were incubated at room temperature for 2-h. OPD substrate was used (Sigma) according to manufacturer's instruction, developed for 30-min, and read at 450 nm.

For serum antibody analysis, mouse serum samples were diluted 3-fold beginning at 1:100 in 5% non-fat milk in PBS. Goat anti-mouse IgG HRP (BioRad) was used at 1:3000 for secondary antibody detection. End-point titers were determined as the last dilution at which the OD450 was above the cutoff. Cutoff values, determined for each dilution, were the upper prediction limit at a 95% confidence level using the Student *t*-distribution derived from pre-immune serum samples ($n = 16$) [25]. Data was analyzed using Microsoft Excel.

2.3. Reverse transcription-PCR and sequencing

Ten milligrams of viral extracts were precipitated as previously stated. Pellets were resuspended in a solution of 0.1 M NaCl, 10 mM Tris–Cl pH 7.6, and 1 mM EDTA at approximately 20 mg/mL and extracted with two volumes phenol:chloroform (1:1, pH 8.0). Samples were purified with RNA Clean and Concentrator™ spin columns (Zymo Research) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV Reverse Transcriptase H-Point Mutant (Promega) with 1 μg of extracted RNA and 125 ng of random hexamers (Invitrogen, P/N 58875) according to the manufacturer's instructions. PCR reactions were performed on TMV cDNA with OneTaq DNA polymerase (NEB), using TMV Coat RT-PCR Forward and Reverse primers 5′-GATCTTACAGTATCACTACTCCATCTC-3′ and 5′-CGCTTTATTACGTGCCTGC-3′. DNA products were gel-purified and verified by sequencing (Laragen).

2.4. Vaccination and spore challenge

All animal experiments were performed in accordance with IACUC standards. Seven-week-old female C57BL/6J mice (Jackson Laboratories) received three intraperitoneal injections two weeks apart on days 1, 15 and 29 with 50 μg TMV, TMV-PA 232–247, TMV-PA 628–637 or a 1:1 mixture of TMV-PA 232–247/TMV-PA 628–637. Serum was harvested from anesthetized mice (four mice per group) in the bleed groups by taking blood samples from the retro-orbital sinus one day before each vaccination (day 0, 14, 28). Sterne strain *B. anthracis* spores (a gift from Dr. Chris Cote, USAMRIID) for animal challenges were prepared in sterile water and heat shocked at 65 °C for 30-min. Mice were injected intraperitoneally with 100 μL of 2.5×10^8 cfu/mL spores on day 35 (five mice per group). Challenged mice were observed twice/day and euthanized when symptoms were observed. Mice in the serum harvest groups were euthanized and their serum obtained through cardiac puncture on day 35. Kaplan–Meier survival analysis was performed in GraphPad Prism and *p*-values generated using the Log-rank test.

2.5. RAW264.7 macrophage LT treatment assay

Five-thousand RAW264.7 cells/well were plated in half-area 96-well tissue culture plates and incubated overnight in DMEM supplemented with 10% Fetal Bovine Serum and 1% penicillin-streptomycin. The next morning, mouse serum samples pooled by vaccine group were prepared with or without LT in growth-media and incubated 1-h at room temperature. PA toxin concentrations were varied from 62.5 to 250 ng/mL while LF toxin was used at a constant 500 ng/mL. Positive control serum was obtained from a mouse that survived challenge from *B. anthracis* Sterne strain spores and was found to have neutralizing anti-PA antibodies. Following incubation, 50 μL/well samples were added to cells and incubated 24-h at 37 °C/5% CO_2. Cellular survival was assessed by

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