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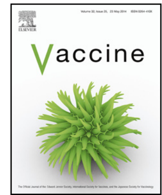

Epitope-focused peptide immunogens in human use adjuvants protect rabbits from experimental inhalation anthrax

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ABSTRACT

Background: Anthrax represents a formidable bioterrorism threat for which new, optimized vaccines are required. We previously demonstrated that epitope-focused multiple antigenic peptides or a recombinant protein in Freund's adjuvant can elicit Ab against the loop neutralizing determinant (LND), a cryptic linear neutralizing epitope in the 2ß2–2ß3 loop of protective antigen from *Bacillus anthracis*, which mediated protection of rabbits from inhalation challenge with *B. anthracis* Ames strain. However, demonstration of efficacy using human-use adjuvants is required before proceeding with further development of an LND vaccine for testing in non-human primates and humans.
Methods: To optimize the LND immunogen, we first evaluated the protective efficacy and immune correlates associated with immunization of rabbits with mixtures containing two molecular variants of multiple antigenic peptides in Freunds adjuvant, termed BT-LND(2) and TB-LND(2). TB-LND(2) was then further evaluated for protective efficacy in rabbits employing human-use adjuvants.
Results: Immunization of rabbits with TB-LND(2) in human-use adjuvants elicited protection from Ames strain spore challenge which was statistically indistinguishable from that elicited through immunization with protective antigen. All TB-LND(2) rabbits with any detectable serum neutralization prior to challenge were protected from aerosolized spore exposure. Remarkably, rabbits immunized with TB-LND(2) in Alhydrogel/CpG had significant anamnestic increases in post-challenge LND-specific Ab and neutralization titers despite little evidence of spore germination in these rabbits.
Conclusions: An LND-specific epitope-focused vaccine may complement PA-based vaccines and may represent a complementary stand-alone vaccine for anthrax.

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1. Introduction

While over a decade has passed since spores of *Bacillus anthracis* were sent through the U.S mail resulting in 22 infections including 5 fatal cases of inhalation anthrax, efforts continue to be directed toward improving our preparedness for bioterrorist threats with weaponized anthrax [1–6].

We have previously shown that immunization with epitope-focused immunogens can elicit Ab specific for a linear determinant in the 2β2–2β3 loop of protective antigen (PA) which can mediate protection of rabbits from spore challenge with *B. anthracis* Ames strain [7,8]. This protective neutralizing epitope, referred to as the loop neutralizing determinant (LND), is found within a segment of PA critical to the translocation of edema and lethal factor into cells [9–11]. Mutagenesis of sequences within the LND abrogates lethal toxin (LeTx) toxicity, thereby rendering this neutralizing epitope potentially less vulnerable compared to other neutralizing epitopes in PA to malicious re-engineering in a manner meant to circumvent the efficacy of PA-specific antibody [12–14]. Unexpectedly, antibodies to the LND are virtually absent in rabbits and non-human primates immunized with PA, and are undetectable in sera from a large cohort of healthy adults who were immunized with AVA in a phase 4 clinical trial [7,15] (Oscherwitz J, Quinn C.P. and Cease K.B., in preparation). Since the LND specificity, therefore, is non-overlapping with the neutralizing antibody specificities elicited by AVA or other PA-based vaccines, the elicitation of this specificity could complement these vaccines.

Our prior work established that the elicitation of LND-specific Ab can protect rabbits from an Ames strain spore challenge; however, these proof-of-concept studies all used Freund's adjuvant for

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immunizations, and critical prerequisites remain before further development of an LND-based vaccine including: demonstration that protective levels of LND-specific antibody can be elicited employing human-use adjuvants; characterization of the immune correlates of protection using such adjuvants, and; comparison of these immune correlates with the correlates identified when using Freund's adjuvant. Toward this end, we postulated that immunization of rabbits with a mixture of two LND-specific multiple antigenic peptides (MAPs), each with an independent heterologous universal helper T cell epitope, would enhance the likelihood of stimulating a diverse outbred animal population and increase the likelihood of achieving protective immunity when employing human-use adjuvants. We therefore proceeded to first evaluate the immunogenicity and protective efficacy in rabbits of two molecular variants of LND MAP mixtures using Freund's adjuvant. The most immunogenic and efficacious combination of LND-MAPs was then further evaluated for immunogenicity and protective efficacy in rabbits using human-use adjuvants.

2. Methods

2.1. Synthetic peptides

The synthesis of peptides in a branched chain configuration on a lysine backbone was first described by Tam et al. to potentiate immune responses to peptide immunogens [16,17]. MAPs used in these studies were synthesized commercially (Biosynthesis, Lewisville TX and Sigma-Genosys, The Woodlands, TX). Two MAPS contained the 16-residue LND B cell epitope (a.a., 304–319, HGNAEVHASFFDIGGS) from the 2β2–2β3 loop of PA (Genbank Accession: P13423) synthesized collinearly at either the N- or C-terminus of the T* helper T cell epitope, a well-characterized universal T cell epitope from the circumsporozoite protein of *Plasmodium falciparum* (EYLNKIQNSLSTEWSPCSVT) [18,19]. A third MAP contained the LND B cell epitope synthesized collinearly at the C-terminus of the P30 helper T cell epitope from tetanus toxin (FNNFTVSFWLRVPKVSASHLE) [20,21]. The P30 helper epitope is also a universal helper T cell epitope. We and others have shown these epitopes are capable of supporting the induction of humoral responses in outbred animals, including humans [22]. An irrelevant B cell epitope from alpha-hemolysin, a β-pore-forming exotoxin from *Staphylococcal aureus* (GNVTGDDTGKIGGLIG) was synthesized collinearly with T* for use as a negative control immunogen and in vitro reagent.

2.2. Animals and vaccinations

For rabbit experiments employing Freund's adjuvant, Female New Zealand white (NZW) rabbits (Covance Research Products, Denver, PA) weighing approximately 2.5 kg were immunized on day 0 with a mixture of two MAPs (125 μg per MAP) in CFA and boosted 4 times at two-week intervals with the MAPs in IFA. For studies using human-use adjuvants, NZW rabbits were injected with TB-LND(2) or PA83 (List Labs, Campbell, CA) according to the above schedule, and employed Alhydrogel (Brenntag AG, Germany), the only adjuvant currently approved in the U.S. for use in humans, alone mixed 1:1 with immunogen in PBS, or Alhydrogel mixed with either 100 μg of monophosphoryl lipid A (Sigma Biochemicals, St. Louis, MO), 50 μg CpG (Cell Sciences, Canton, MA) or 100 μg QS-21 (Agenus Inc., Lexington, MA). These three immunopotentiators were chosen for the current study since each has shown promise in human studies. MPL is a component of the licensed Cervarix vaccine and both MPL and QS-21 are constituents of AS01® [23]. Since controlling for potential non-specific activity associated with each adjuvant combination in the human use adjuvant studies was not possible, we instead employed CFA/IFA for priming and boosting immunizations, respectively, with the irrelevant control MAP. All immunizations were given s.c. except those with Alhydrogel/CpG which is typically administered i.m. in rabbits [24]. Rabbits were bled 10–14 days after the final immunization. All rabbits were cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and protocols were approved by Institutional Animal Care and Use Committees of The University of Michigan, Covance Research Products and Battelle Memorial Institute, Columbus OH.

2.3. Enzyme-linked immunosorbent assay

Antibody responses were assessed by ELISA as described [7]. Antibody titers were determined from serial two-fold dilutions of serum and represent the reciprocal dilution at the EC_{50} established using nonlinear regression to fit a variable slope sigmoidal equation to the serial dilution data. For inhibition studies, serum samples were pre-incubated with the T*-containing LND MAP at 32 μM (2×) for 30 min at RT prior to evaluation in the standard ELISA. An irrelevant T*-containing MAP was used as a control. The lower limit of quantitation for the ELISA was a reciprocal dilution of 16. Samples with antibody titers below this limit were assigned a value of 8.

2.4. Toxin neutralization assay

The ability of antibody to block LeTx cytotoxicity in vitro was assessed using the RAW264.7 cell line (American Type Culture Collection, Manassas, VA) as described [20]. The reciprocal of the effective dilution protecting 50% of the cells from cytotoxicity (ED_{50}) [25], was determined for each serum by using nonlinear regression using Prism 5.0. The standard TNA assay has a lower limit of quantification of 16. Samples with TNA below this limit were assigned a value of 8. For inhibition studies, serum samples were pre-incubated with the T*-containing LND MAP at 32 μM (2×) in complete medium for 30 min at RT prior to evaluation in the standard TNA. An irrelevant T*-containing MAP was used as a control.

2.5. Aerosol spore challenges

In the first of two separate spore challenges, the positive and negative control and naïve rabbits were shared with another previously reported study, and both challenges were performed at Battelle Biomedical Research Center (Studies: 851-G006008, 1021-G006377, Columbus, OH) as described [18]. Following challenge, clinical observations were performed for 14 days and moribund animals were euthanized. Deaths were recorded on the day the animal was found dead or was euthanized.

2.6. Statistical analysis

The Kruskal–Wallis and Dunn's Multiple Comparison Test were used for comparing titers from more than two groups, and the Wilcoxon matched-pairs signed rank test was used for comparing pre- and post-challenge titers from individual rabbits. Student's *t* test was used to compare peptide inhibition between adjuvant groups. The Kaplan–Meier method was used to plot survival data, and differences in survival were compared using the Mantel–Cox log-rank test. For all statistical analysis, a p value of <0.05 was considered significant.

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