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Anthrax vaccine recipients lack antibody against the loop neutralizing determinant: a protective neutralizing epitope from *Bacillus anthracis* protective antigen

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Abstract

Background—Epitope-focused immunogens can elicit antibody against the loop neutralizing determinant (LND), a neutralizing epitope found within the 2β2-2β3 loop of protective antigen (PA), which can protect rabbits from high-dose inhalation challenge with *Bacillus anthracis* Ames strain. Interestingly, data suggests that this epitope is relatively immunosilent in rabbits and non-human primates immunized with full length PA.

Methods—To determine whether the LND is immunosilent among humans vaccinated with PA, we screened antisera from AVA- or placebo-vaccinees from a clinical trial for antibody reactive with the LND.

Results—AVA-vaccinee sera had significant PA-specific antibody compared to placebo-vaccinee sera; however, sera from the two cohorts were indistinguishable with regard to the frequency of individuals with antibody specific for the LND.

Conclusions—AVA-vaccinees have a low frequency of antibody reactive with the LND. As with rabbits and non-human primates, the elicitation of LND-specific antibody in humans appears to require immunization with an epitope-focused vaccine.

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Conflict of Interest

J.O and K.B.C are co-inventors on a patent application related to the LND filed by the University of Michigan and the Department of Veterans Affairs.

All authors report no conflicts of interest.

Keywords

AVA; epitope; vaccine; protective antigen; antibody

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, research continues to be directed towards improving our preparedness for possible bioterrorist threats including weaponized anthrax. This has included efforts to critically evaluate and optimize the anthrax vaccine currently approved in the U.S., BioThrax®/AVA (Anthrax Vaccine Adsorbed) as well as efforts to develop new and stable alternative vaccines, and therapeutic interventions for use in post-exposure scenarios [1].

Untreated inhalation anthrax has a high fatality rate. The primary virulence factors of *B. anthracis* include the two protein exotoxins, lethal toxin (LeTx) and edema toxin. The enzymatically active components of these toxins, lethal and edema factor, respectively, bind protective antigen (PA) at the cell surface leading ultimately to well-described cellular dysfunction and intoxication [2, 3]. Humoral immunity to PA, the basis of the current vaccine, can successfully mediate protection from lethal challenges in animal models of inhalation anthrax and this protection is correlated with the ability of PA-specific antibodies (Abs) to neutralize LeTx in vitro in the toxin neutralization assay (TNA) [4-8]. Until 2012, individuals at risk of exposure to anthrax underwent a vaccination schedule with AVA consisting of subcutaneous (*s.c.*) priming immunizations at 0, 2, and 4 weeks, and 6, 12 and 18 months followed by yearly boosts thereafter. As evidence accumulated demonstrating that reductions in this intensive immunization schedule were not associated with significant decreases in the elicitation of PA-specific Ab, the recommended immunization regimen was reduced to 3 intramuscular (*i.m.*) immunizations over 6 months with boosters at months 12 and 18 followed by annual boosters [9]. While efforts continue to evaluate whether or not further changes in the vaccine booster schedule are feasible, the current schedule for AVA remains burdensome. Effective anthrax vaccines that provide long lasting immunity with a minimal number of doses and a shorter priming period are needed.

We have previously shown that immunization with epitope-focused immunogens using either research adjuvants like Freund's or AIOH-containing human-use adjuvants can elicit Ab specific for a linear determinant in the 2β2-2β3 loop of PA which can mediate protection of rabbits from aerosolized spore challenge with *B. anthracis* Ames strain [10-12]. This epitope, referred to as the loop neutralizing determinant (LND), appears to be a functionally critical target for Ab, as relatively low serum titers of LND-specific Ab are capable of protecting rabbits from high dose aerosol challenge. This sensitivity may be related, in part, to the location of the LND which is found within a critical molecular structure of PA involved in translocating edema and lethal factor into cells, and mutagenesis of sequence within the LND has been shown to completely abrogate LeTx toxicity [13, 14]. The LND epitope, therefore, may also be less vulnerable compared to other protective neutralizing epitopes in PA to intentional re-engineering in a manner meant to circumvent the efficacy of

the protective antibody specificities elicited in vaccinees [15]. Surprisingly, however, antibodies to the LND appear to be virtually absent in rabbits and non-human primates immunized with PA, and were undetectable in pooled standardized samples of antisera from AVA-vaccinated humans including AVR801 [11, 16]. Consequently, since the LND specificity appears to be non-overlapping with the neutralizing antibody specificities elicited by AVA or other PA-based vaccines, the elicitation of this specificity could complement the neutralizing specificities elicited through immunization with PA-based vaccines. To ascertain whether LND-specific Ab is elicited in humans vaccinated with AVA, we evaluated antisera from vaccinees who received AVA in the context of a previously reported clinical trial [9].

Materials and Methods

Vaccinee samples

This study was performed on 247 samples from a previously reported clinical trial (CDC AVRP 281; ClinicalTrials.gov Identifier: NCT00119067) [9]. The serum samples were comprised of 209 samples from AVA-vaccinees and 38 samples from saline controls, all of whom received either the original licensed schedule first 4 immunizations administered *s.c.* at 0, 2 and 4 weeks and 6 months, or one of two modified regimens consisting of either the first 4 immunizations administered *i.m.*, or only the 0 and 4 week and 6 month immunizations administered *i.m.* [9]. The CDC study and all study amendments were reviewed by the CDC's Institutional Review Board (IRB) and by the local site IRBs. All samples were coded and deidentified prior to this study and did not represent research involving human subjects as determined by the VA Ann Arbor Healthcare System Human Studies Committee.

Proteins and peptides

Immobilized PA83 (LIST Biological Laboratories, Inc.) was used in the ELISA to establish titers of PA-reactive Ab in the clinical samples. LND-specific Ab was detected through use of an immobilized recombinant protein displaying two tandemly-repeated copies of a.a. 299-327 (single letter code: HTSEVHGNAEVHASFFDIGGSVSAGFSNS) of PA fused to maltose binding protein as employed previously [16, 17]. An MBP fusion protein that lacked the LND sequence, but that was otherwise identical, was used as a control. A synthetic peptide (Sigma-Genosys, The Woodlands, TX) displaying a.a. 304-319 (single letter code: HGNAEVHASFFDIGGS) synthesized collinear with the P30 epitope from tetanus toxin was used to inhibit LND-specific Ab in the TNA as described [17].

Enzyme-linked immunosorbent assay

Antibody responses were assessed by ELISA essentially as described [17]. Antibody titers were determined from serial two-fold dilutions of individual serum samples and represent the reciprocal dilution at the EC₅₀ established using nonlinear regression to fit a variable slope sigmoidal equation to the serial dilution data using Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The lower limit of detection (LOD) for the ELISA was a reciprocal dilution of 8; titers below this limit were assigned a value of 4. For performance of the ELISAs, investigators were blinded as to whether serum samples were obtained from AVA-

or saline-vaccinated individuals. A positive control rabbit LND-specific antisera was used in each assay and throughout the study. The antisera was raised through immunization of rabbits with multiple antigenic peptides as described[18].

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 [17]. For neutralization studies, 110 ng/ml PA (LIST Laboratories, Campbell, CA) was used along with 300 ng of LF. The reciprocal of the effective dilution protecting 50% of the cells from cytotoxicity (ED₅₀), was determined for each serum by using nonlinear regression to fit a variable slope sigmoidal equation to the serial dilution data set using Prism 5.0. The standard TNA assay has a lower limit of detection of 16; titers below this limit were assigned a value of 8. For inhibition studies, 15 serum samples with among the highest PA-specific Ab titers among the AVA-vaccinee cohort were selected for study. Because of the number of samples and conditions, neutralization was analyzed over two successive studies. Serum samples were pre-incubated with 32 μ M (2X) linear synthetic peptide containing a.a. 304-319 of PA synthesized collinear with the P30 epitope from tetanus toxin, or with the P30 epitope alone (control) in complete medium for 30 minutes at RT prior to evaluation in the standard TNA as described [11].

Statistical Analysis

ELISA EC₅₀ and TNA ED₅₀ titers were determined using four parameter non-linear regression to fit variable slope sigmoidal equations to serial dilution data. The Mann-Whitney *U* test was used to compare serum antibody titers between AVA- and saline-immunized individuals and Fisher's exact test was used to compare the frequencies of PA-, LND and control-specific responses above the LOD in the two sera cohorts. The Wilcoxon matched-pairs signed rank test was used to compare the serum lethal toxin neutralization titers of AVA-immunized individuals in the presence or absence of inhibitory LND peptide. All statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego CA).

Results

We evaluated individual serum samples obtained from 247 healthy adults who in the course of a phase 4 clinical trial, were vaccinated with either AVA (n=209) or saline (n=38) by the *s.c.* or *i.m.* route. All serum samples were first evaluated for reactivity with immobilized PA by ELISA. Geometric mean PA-specific EC₅₀ titers were 755.4 among all the AVA-vaccinee sera evaluated (GMT *i.m.* route= 814.2; GMT *s.c.* route= 550.1, *p*=0.151) and 9.2 in the placebo-vaccinees (*p*< 0.0001, figure 1, columns A and B). When evaluated for reactivity with a recombinant protein displaying the LND sequence (figure 1, columns C and D) [16, 17], both the AVA-vaccinee and placebo-vaccinee sera demonstrated very low and indistinguishable Ab titers reactive with the LND sequence (GMT AVA vaccinees= 8.1; GMT placebo vaccinees = 6.7, *p*= 0.442), while a control LND-specific rabbit antisera reacted strongly with the LND sequence (not shown). Geometric mean LND-specific responses in the sera of individuals immunized with AVA by the *i.m.* route were 8.1, while

those immunized with AVA by the s.c. route were 7.9 ($p = 0.649$). The antibody titers on control-recombinant protein were also not significantly different between the two cohorts ($p = 0.559$, figure 1, columns E and F). The percentage of LND-specific responses at or above the LOD did not differ between the AVA- and placebo-vaccinee sera (35.4% and 31.6%, respectively, $p = 0.649$, table 1) nor were there significant differences in the respective frequencies of reactivity at or above the LOD with the control immunogen (23.9% and 21.1, respectively, $p = 0.8362$, table 1).

We have previously shown that LND-specific neutralization can be completely and specifically inhibited in vitro following incubation of LND-specific antisera with synthetic peptides displaying the LND peptide sequence [11, 16, 17]. We therefore utilized peptide inhibition to further assess whether AVA-vaccinee sera exhibited neutralizing activity that was mediated by LND-specific Ab. For these studies, 15 serum samples with among the highest PA-specific Ab titers among the AVA-vaccinee cohort were selected for study and serum neutralization was determined over two successive assays, with each serum sample assessed both with or without pre-incubation with inhibitory concentrations of an LND peptide prior to assessment in the TNA. Neutralization titers of the AVA-vaccinee sera were unchanged regardless of whether or not the antisera were pre-incubated with the LND peptide ($p = 0.250$, figures 2a,2b). Neutralizing activity in control rabbit LND antisera was completely inhibited following incubation with the LND peptide, but not a control peptide, prior to assessment in the TNA (figures 2a,2b).

Discussion

These studies demonstrate that in a large cohort of individuals immunized with AVA, vaccinees developed significant antibody titers specific for PA when compared to the PA-specific titers observed in placebo-vaccinees who were immunized with saline only, has shown previously by Marano et al [9]. But despite the presence of significant PA-specific antibody titers, AVA-vaccinee sera demonstrated no greater frequency of LND-specific Ab than the frequency of such Ab observed in the sera of placebo vaccinees. Moreover, serum neutralization among AVA-immunized individuals with the highest PA-specific antibody titers in the cohort studied were unaffected by pre-incubation with inhibitory concentrations of an LND peptide prior to analysis in the TNA. Since similar concentrations of LND peptide completely inhibit LND-mediated neutralization[17], the results demonstrate that AVA-vaccinee sera lack detectable neutralizing activity attributable to LND-specific Ab.

Overall, the data support the conclusion that among individuals immunized with AVA, and likely other PA-based vaccines, the LND epitope within the 2 β 2-2 β 3 loop of PA appears sub-dominant to the point of being immunosilent compared to other epitopes within the PA molecule. These data are consistent with previously reported findings that LND-specific Ab is present at very low level or undetectable levels in rabbits and non-human primates immunized with PA, or in pooled standardized sera from AVA-immunized individuals including AVR801 [11, 16]. The most obvious and important implication of this finding is that immunization with an efficacious LND-focused vaccine, which appears required for elicitation of LND-specific Ab, may generate a protective neutralizing specificity that would be complementary to the specificities elicited through immunization with AVA or other PA-

based vaccines[19, 20]. Additionally, since the LND epitope includes the critical Phe-Phe at a.a. 312-313 of PA, and these sequences have been demonstrated to be required for LeTx function[13, 14], the protective LND Ab specificity may be more resistant to intentional circumvention than other protective antibody specificities elicited by PA-based vaccines. Thus an LND-focused vaccine might represent a potentially important adjunctive combination or stand-alone vaccine for enhancing protection against *B. anthracis* bearing wild type PA, and for countering efforts to engineer vaccine-resistant strains of *B. anthracis*[15]. While to our knowledge, LND-focused immunogens have not yet been tested in combination with PA-based vaccines, the results from the current study suggest that such dual vaccination could theoretically broaden the PA-specific antibody repertoire among vaccinees in a manner leading to more robust vaccine-mediated protection.

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- Anthrax vaccine recipients lack antibody against the LND.
- AVA and LND-vaccines elicit non-overlapping protective Ab specificities for anthrax.
- An epitope-focused LND vaccine would compliment PA-based vaccines
- The elicitation of LND Ab requires vaccination with an epitope-focused vaccine.

Figure 1. Analysis of vaccinee sera by ELISA

Sera from 247 individuals who received immunizations with either AVA (n= 209) or saline (placebo) (n= 38) were analyzed for reactivity with a panel of immobilized antigens by ELISA as described in the methods. Antibody titers of AVA-vaccinee sera (GMT= 755.4, column A, circles) with immobilized PA83 were significantly higher than the titers of placebo-vaccinee sera (GMT= 9.2, column B, squares, $p < 0.0001$, Mann Whitney). When analyzed for reactivity with an immobilized recombinant protein displaying the LND peptide sequence, there were no significant differences in GMTs between the AVA-vaccinee

sera (GMT= 8.1) and placebo-vaccinee sera (GMT= 6.7)($p= 0.4417$, Mann Whitney, columns C and D). AVA- and placebo-vaccinee sera also did not have significantly different antibody titers reactive with a control recombinant protein (column E and F). Control rabbit LND antisera demonstrated reactivity with PA and LND plate coats but not with the control recombinant protein (not shown). Each symbol represents the result for an individual serum sample and horizontal lines represent geometric means. The lower limit of detection in the ELISA is 8; individual serum titers below the lower limit were assigned a value of 4.

Figure 2.

Lethal toxin neutralization titers among AVA-vaccinees determined in the presence or absence of an inhibitory LND peptide. Individual serum samples from 15 AVA-vaccinated individuals (closed circles) with among the highest PA-specific titers in the cohort were analyzed over two successive TNAs, as shown in panels A and B, in each case with and without prior incubation with an LND peptide as described in the methods. There were no significant differences between the neutralization titers determined in the presence or absence of the LND peptide ($p=0.250$, Wilcoxon matched-pairs signed rank test). In each neutralization assay, toxin neutralization was also determined for a control LND-specific rabbit antiserum (open circles) without prior incubation with peptides, or with prior incubation in the presence of an LND or control peptide. The lower limit of detection in the TNA is 16; titers below this level were assigned a value of 8.

Table 1

Analysis of sera from AVA- and Placebo-immunized subjects for reactivity with immobilized PA, LND, and Control test antigen at or above the Limit of Detection (LOD) by ELISA.

Test Antigen Immunogen	PA		LND		Control	
	AVA	Placebo	AVA	Placebo	AVA	Placebo
% LOD	95.22	36.84	35.41	31.58	23.92	21.05
Significance ^a	$p < 0.0001$		$p = 0.7141$		$p = 0.8362$	

^aFisher's Exact test