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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 2β2–2β3 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 Freund's 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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Conflict of interest: J.O and K.B.C are co-inventors on a patent application related to these studies filed by the University of Michigan and the Department of Veterans Affairs. All authors report no conflicts of interest.

Keywords

Epitope; Antibody; Inhalation anthrax; Neutralization; Peptide; Vaccine

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 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 2 β 2–2 β 3 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 μ g 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 μ g of monophosphoryl lipid A (Sigma Biochemicals, St. Louis, MO), 50 μ g CpG (Cell Sciences, Canton, MA) or 100 μ g 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 EC₅₀ 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 (ED₅₀) [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.

3. Results

3.1. Immunogenicity and protective efficacy of LND MAPs in rabbits

We postulated that immunizing rabbits with two MAPs, one containing the P30 helper epitope from tetanus toxin and the other the T* helper epitope from the circumsporozoite protein of *P. falciparum*, each separately linked to the LND peptide sequence, would reduce the likelihood of non-responses and thereby improve the protective efficacy of the MAPs.

Both helper epitopes had been previously determined to elicit complementary T cell stimulation in inbred mice [18,20]. Since the molecular orientation of T and B cell epitopes within MAPs can impact immunogenicity [18], and pairs of helper epitopes may compete [26], we first compared the immunogenicity and protective efficacy in Freund's of the T*-containing LND-MAP in two separate molecular orientations combined in each case with a P30-containing LND-MAP (Fig. 1).

Groups of female New Zealand White (NZW) rabbits ($n = 7$) were immunized 5 times at two-week intervals with either the BT-LND(2) or the TB-LND(2) formulated in CFA for priming immunizations and IFA for booster immunizations. The positive control group ($n = 7$) received PA, the negative control ($n = 9$) received an irrelevant T*-containing MAP and a naïve group ($n = 6$) was not immunized. To minimize the number of rabbits killed in this study, all three control groups were shared with a study published previously [18]. Approximately 3 weeks after their final immunization, all rabbits were challenged with aerosolized *B. anthracis* Ames strain spores and were observed for 14 days. Both groups of rabbits immunized with the LND-MAPs and the PA control were completely protected from challenge ($p < 0.0001$ compared to naïve, Table 1 and supplementary Fig. 1) [18]. All rabbits immunized with the TB-LND(2) and the BT-LND(2) developed high titers of Ab against the LND, and all the TB-LND(2)-immunized rabbits, and 6 out of 7 BT-LND(2)-immunized rabbits had detectable serum neutralizing Ab prior to challenge. Analysis of survivor sera obtained 14 days after challenge revealed that all TB-LND(2)- and PA-immunized rabbits and 8 out of 9 of the BT-LND(2)-immunized rabbits had undetectable serum LF-specific IgM or IgG suggesting minimal or no vegetative outgrowth [18]. Levels of neutralization in the post-challenge sera of TB-LND(2)-immunized rabbits only were significantly higher than their pre-challenge levels ($p = 0.031$, Table 1). These increases in neutralization were mediated exclusively by LND-specific Ab as all neutralization in the post-challenge sera from this group was completely inhibitable with LND-peptide in vitro (not shown). Neutralization in the post-challenge sera of PA-immunized rabbits was significantly lower compared to pre-challenge levels ($p = 0.016$).

Supplementary Fig. 1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.11.042>.

3.2. Immunogenicity and protective efficacy of TB-LND(2) using human use adjuvants

Based on the immunogenicity and protective efficacy of TB-LND(2) in the aerosol challenge, we proceeded to evaluate the protective efficacy of TB-LND(2) using human use adjuvants. Groups of rabbits ($n = 9$) were immunized with TB-LND(2) employing one of four adjuvant formulations: Alhydrogel (ALOH) alone, or ALOH mixed with either Monophosphoryl Lipid A (MPL), QS-21, or CpG. To enable comparisons with the prior study using Freund's, we employed an identical immunization regimen of 5 immunizations at two-week intervals. Positive and negative control groups were immunized with PA in ALOH or an irrelevant T*-containing MAP in Freund's adjuvant, respectively. Two weeks after the final immunizations, all rabbits were challenged with aerosolized *B. anthracis* Ames strain spores and were observed for 14 days. All rabbits (100%) immunized with TB-LND(2) in ALOH/MPL (actual exposure: 167 ± 21 LD₅₀s) and PA (actual exposure: $196 \pm$

17 LD₅₀s), eight out of nine rabbits (89%) in ALOH/CpG (actual exposure: 143 ± 25 LD₅₀s) and ALOH/QS-21 (actual exposure: 160 ± 18 LD₅₀s) and 6 out of 9 (66%) rabbits in the ALOH only group (actual exposure: 177 ± 25 LD₅₀s) were protected from spore challenge (for all TB-LND(2) groups and the PA group, $p < 0.0001$ compared to naïve (actual exposure: 216 ± 17 LD₅₀s) and irrelevantly immune controls (actual exposure: 186 ± 72 LD₅₀s, Fig. 2 and supplementary Table). There were no significant differences in survival between the groups of rabbits receiving the TB-LND(2) combined with the different adjuvant formulations, or between the different adjuvant groups and the group receiving PA ($p = 0.125$).

Supplementary Table related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.11.042>.

To evaluate surrogates of protection, pre-challenge sera were analyzed by ELISA and in the TNA (Fig. 3, supplementary Table). There were no significant differences in the Ab or neutralization titers elicited across all adjuvant combinations with geometric mean Ab titers (GMTs) of 6051, 4526, 4146 and 2843 and neutralization GMTs of 777, 538, 518 and 395 for the ALOH, ALOH/MPL, ALOH/QS-21 and ALOH/CpG groups, respectively. The presence of detectable serum neutralization pre-challenge did predict survival, as with only one exception, all rabbits with detectable serum neutralization prior to challenge were survivors (Fig. 3B, filled circles).

Evaluation of survivor sera revealed that 22 out of 31 survivor rabbits immunized with TB-LND(2) (71%) lacked detectable de novo LF-specific IgM or IgG suggesting little or no vegetative outgrowth in these animals, and none of the positive control rabbits immunized with PA developed such antibody (Fig. 4A). Despite this lack of evidence of spore germination in most rabbits immunized with TB-LND(2), post-challenge Ab and neutralization titers increased significantly compared to pre-challenge levels among rabbits in the ALOH/QS-21 ($p = 0.008$ for Ab, $p = 0.023$ for TNA) and ALOH/CpG groups ($p = 0.039$ for Ab and TNA, Fig. 4B and C). In the ALOH/QS-21 group, the increases in PA-specific Ab comprised both LND-specific and de novo non-LND PA-specific Ab, as only 35% and 40% of the post-challenge Ab detected in the ELISA and TNA, respectively, were inhibitable with LND peptide. By comparison, the increased Ab and neutralization titers detected in the post-challenge sera of ALOH/CpG rabbits (Fig. 4B and C) were comprised of significantly more LND-specific Ab compared to the ALOH/QS-21 group, with 70% and 85% of the Ab detected by ELISA and in the TNA, respectively, being inhibitable with LND peptide ($p = 0.001$, Fig. 5A and B). Post-challenge serum Ab and neutralization titers in rabbits immunized with PA were unchanged compared to their pre-challenge levels. There were no significant differences in the post-challenge geometric mean Ab and neutralization titers between the respective adjuvant groups.

4. Discussion

Immunization of rabbits with two molecular variations of LND MAPs in Freund's adjuvant completely protected rabbits from a 200 LD₅₀ – targeted challenge with spores of *B. anthracis* Ames strain and spore germination appeared to be minimal or absent in both

groups of rabbits. These findings parallel earlier challenge studies employing MAP or recombinant protein immunogens which elicited relatively high levels of LND-specific neutralizing Ab and protected rabbits from inhalation challenge [8,18]. As in these and other studies, the findings are consistent with the notion that overall, the presence of relatively high levels of neutralization at the time of inhalation challenge leads to neutralization of LeTx to an extent that few signs of PA or LF antigen production are detectable post-challenge [27]. Despite these findings, however, post-challenge survivor sera from rabbits immunized with TB-LND(2) in Freund's adjuvant had significantly increased levels of neutralization compared to pre-challenge levels, despite unchanged levels in post-challenge Ab titers, and all of the neutralization in the post-challenge sera was inhibitable in vitro with LND peptide. This seemingly paradoxical finding may reflect effects associated with production of very low levels of PA derived from spore germination in vivo that while incapable of stimulating detectable de novo immunity may nevertheless be sufficient to engage and stimulate memory B cells displaying LND-specific IgG [8,28,29,30].

Employing an identical immunization protocol as in the first challenge, immunization of rabbits with TB-LND(2) in ALOH with or without immunopotentiating agents protected rabbits from inhalation challenge with an efficacy that was statistically indistinguishable compared to immunization with PA. Not surprisingly, LND-specific antibody and neutralization titers elicited using human use adjuvants were markedly lower prior to aerosol challenge than those observed using Freund's adjuvant in the first challenge, and in previous studies using Freund's; however, like those results, the presence of any detectable neutralization in vitro prior to challenge predicted survival. The sole exception in this and prior challenges was the single rabbit immunized with TB-LND(2) in ALOH which, despite high levels of serum neutralization prior to challenge (neutralization ED₅₀ = 1421), died six days after spore exposure. This exception notwithstanding, the finding that any level of serum neutralization, including relatively low levels among some rabbits, predicts protection from high dose aerosol spore challenge is a departure from prior data evaluating the protective efficacy of PA-immunization in rabbits where the presence of low and even intermediate levels of serum neutralization was not protective [31,32].

While no significant differences in protective efficacy were detected among the different adjuvant groups, analysis of survivor sera from rabbits immunized with TB-LND(2) in ALOH/QS-21 and ALOH/CpG revealed significant increases in antibody and neutralization titers compared to their pre-challenge levels. Since the LND-specific Ab can be inhibited in vitro in both the ELISA and TNA, we were able to parse out the constituent Ab specificities responsible for these increases in the post-challenge titers. In the ALOH/QS-21 group, these increases were comprised of predominantly de novo production of non-LND PA-specific neutralizing antibody. In contrast, the increases in antibody and neutralization titers among rabbits immunized with TB-LND(2) in ALOH/CpG primarily reflect anamnestic immunity as the inhibition studies showed that these responses were predominantly comprised of LND-specific antibody. These findings indicate that the LND peptide sequence, a.a. 304–319 in PA, contains a helper T cell epitope in rabbits, since the remainder of the LND MAP sequence is completely heterologous with respect to PA. Anamnestic LND-specific immunity has been identified in a prior rabbit challenge, and data suggests that the LND sequence may be recognized by human T cells [18,20,33]. The high prevalence of

anamnestic immunity in the ALOH/CpG group is likely attributable to the use of CpG which acts on TLR9 receptors to stimulate the TH1 subset of helper and memory T cells [34–36].

The elicitation of LeTx neutralizing Ab is considered a critical requirement for anthrax vaccines [37]. The data in the current study demonstrate that the presence of LND-specific neutralizing antibody elicited with human use adjuvants is highly predictive of survival from spore challenge in rabbits. Since antibody to this epitope is not significantly represented among the repertoire of Abs elicited in rabbits, non-human primates and humans immunized with PA-based vaccines, an LND-based vaccine may hold promise for complementing PA vaccines through elicitation of this uncommon but protective neutralizing specificity. In addition, a stand-alone LND-based vaccine may find use in post-exposure scenarios and in the unlikely but theoretically possible scenario of bioterrorist attempts to circumvent the primary neutralizing specificities which serve as the humoral basis of protection with PA-based vaccines [38,39].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Diagrammatic representation of LND MAP immunogens. Shown are diagrammatic representations of the individual MAPs which comprise the respective immunogen mixtures. As shown, BT-LND(2) is comprised of two LND MAPs: one containing the LND linked to the N-terminus of the T* helper T cell epitope and the second containing the LND peptide linked to the C-terminus of the P30 helper T cell epitope. TB-LND(2) contains the identical P30-containing LND MAP along with a second MAP containing the LND peptide linked to the C-terminus of the T* helper T cell epitope. Immunogens are not drawn to scale.

Fig. 2.

Immunization with LND MAPs in human use adjuvants protects rabbits from spore challenge with *B. anthracis* Ames strain. Shown are Kaplan–Meier survival curves from groups of rabbits ($n = 9$) immunized with either TB-LND(2) formulated in different adjuvants, PA83 in ALOH or an irrelevant MAP in CFA and then challenged with aerosolized spores of *B. anthracis* Ames strain. An unimmunized naïve group of rabbits ($n = 6$) was also challenged. All naïve and irrelevant control rabbits died by day 4 and 6, respectively. All groups of rabbits immunized with TB-LND(2) and the PA controls were significantly protected from challenge compared to the naïve and irrelevantly immunized groups ($p < 0.0001$, Log Rank test). There were no significant differences in survival between the individual groups immunized with TB-LND(2) in the different adjuvant formulations, or between these groups and PA-immunized rabbits.

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Fig. 4.

Analysis of postchallenge sera for LF-specific IgM and IgG, and for PA-specific Ab and neutralization. Survivor rabbit sera obtained 14 days after spore challenge was analyzed by ELISA for reactivity with immobilized LF (A). The lower limit of quantification for the ELISA is a reciprocal dilution of 16. Samples with titers below this level were assigned a value of 8. Each circle represents a data point from an individual rabbit and horizontal bars represent geometric means. Also shown are pre- and post-challenge antibody and neutralization titers from individual rabbits which survived challenge. Pre-challenge sera obtained 10 days prior to challenge and post-challenge survivor sera obtained 14 days after challenge were analyzed by ELISA (B) and in the TNA (C). Rabbits in the ALOH/QS-21 group had significantly increased antibody and neutralization titers post-challenge (EC_{50} Ab

= 25,094, ED₅₀ neutralization = 7096) compared to pre-challenge (EC₅₀ Ab = 4146, ED₅₀ neutralization = 518, $p = 0.008$ for Ab, $p = 0.023$ for TNA, Wilcoxon matched-pairs signed-rank test). Rabbits in the ALOH/CpG group also demonstrated significant increases in both antibody and neutralization titers detectable in post-challenge sera (EC₅₀ Ab = 14,216, ED₅₀ neutralization = 8649) compared to pre-challenge levels (EC₅₀ Ab = 2843, ED₅₀ neutralization = 395, $p = 0.039$ for Ab and TNA, Wilcoxon matched-pairs signed-rank test). The differences between pre- and post-challenge serum antibody and neutralization titers among survivor rabbits in the ALOH and ALOH/MPL groups and the PA (positive control) group were not significant. There were no significant differences in the post-challenge geometric mean Ab and neutralization titers between the respective adjuvant groups.

Fig. 5.

In vitro inhibition of post-challenge Ab and TNA responses with LND peptide. Shown are the % reductions in antibody binding (A) and neutralization (B) for survivor rabbits from the ALOH/QS-21 and ALOH/CpG groups following in vitro incubation with inhibitory concentrations of the LND peptide as described in the Methods. The percent reductions in antibody binding and neutralization detectable in survivor sera of ALOH/CpG rabbits was significantly greater than the reductions determined in the ALOH/QS-21 group ($p = 0.001$ for Ab and neutralization, Student's t test). A positive control LND-specific antisera and negative control PA-specific antisera were inhibited 100% and 0% respectively in both the Ab and neutralization inhibition assays (not shown). Percent reductions in antibody binding were determined through use of the following formula: % Reduction = $(EC_{50}$ titer with irrelevant peptide - EC_{50} titer with LND peptide) / EC_{50} titer with irrelevant peptide $\times 100$. Percent reductions in neutralization were determined through use of the following formula: % Reduction = $(ED_{50}$ titer with irrelevant peptide - ED_{50} titer with LND peptide) / ED_{50} titer with irrelevant peptide $\times 100$.

Table 1

Group	Immunogen	Survival	Antibody titer (EC50)		Neutralization titer (ED50)		Detectable LF IgG/IgM Post-challenge	Inhaled spore dose (LD50 ± SD)
			Pre-challenge	Post-challenge	Pre-challenge	Post-challenge		
1	BT-LND(2)	7/7 ^a	38,427	32,949	1813	4876	1/7	204 ± 43
2	TB-LND(2)	7/7 ^a	57,185	51,381	4764	8,751 ^b	0/7	195 ± 46
3 ^c	PA	7/7 ^a	82,651	76,336	1954	836 ^d	0/7	196 ± 44
4 ^c	Control MAP	0/9	ND	ND	ND	ND	ND	186 ± 52
5 ^c	Naïve	0/6	ND	ND	ND	ND	ND	202 ± 45

Pre- and post-challenge titers shown are geometric mean titers.

^a $p < 0.0001$, compared to Naïve, Mantel–Cox log-rank test.

^b $p = 0.031$, Wilcoxon matched-pairs signed rank test.

^c Control groups reported previously.

^d $p = 0.016$, Wilcoxon matched-pairs signed rank test.