We previously showed that a multiple antigenic peptide (MAP) vaccine displaying amino acids (aa) 304 to 319 from the 2β2-2β3 loop of protective antigen was capable of protecting rabbits from an aerosolized spore challenge with *Bacillus anthracis* Ames strain. Antibodies to this sequence, referred to as the loop-neutralizing determinant (LND), are highly potent at neutralizing lethal toxin yet are virtually absent in rabbit and human protective antigen (PA) antisera. While the MAP vaccine was protective against anthrax, it contains a single heterologous helper T cell epitope which may be suboptimal for stimulating an outbred human population. We therefore engineered a recombinant vaccine (Rec-LND) containing two tandemly repeated copies of the LND fused to maltose binding protein, with enhanced immunogenicity resulting from the p38/P4 helper T cell epitope from *Schistosoma mansoni*. Rec-LND was found to be highly immunogenic in four major histocompatibility complex (MHC)-diverse strains of mice. All (7/7) rabbits immunized with Rec-LND developed high-titer antibody, 6 out of 7 developed neutralizing antibody, and all rabbits were protected from an aerosolized spore challenge of 193 50% lethal doses (LD50) of the *B. anthracis* Ames strain. Survivor serum from Rec-LND-immunized rabbits revealed significantly increased neutralization titers and specific activity compared to prechallenge levels yet lacked PA or lethal factor (LF) antigenemia. Control rabbits immunized with PA, which were also completely protected, appeared sterilely immune, exhibiting significant declines in neutralization titer and specific activity compared to prechallenge levels. We conclude that Rec-LND may represent a prototype anthrax vaccine for use alone or potentially combined with PA-containing vaccines.

*Recombinant Vaccine Displaying the Loop-Neutralizing Determinant from Protective Antigen Completely Protects Rabbits from Experimental Inhalation Anthrax*

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We previously showed that a multiple antigenic peptide (MAP) vaccine displaying amino acids (aa) 304 to 319 from the 2β2-2β3 loop of protective antigen was capable of protecting rabbits from an aerosolized spore challenge with *Bacillus anthracis* Ames strain. Antibodies to this sequence, referred to as the loop-neutralizing determinant (LND), are highly potent at neutralizing lethal toxin yet are virtually absent in rabbit and human protective antigen (PA) antisera. While the MAP vaccine was protective against anthrax, it contains a single heterologous helper T cell epitope which may be suboptimal for stimulating an outbred human population. We therefore engineered a recombinant vaccine (Rec-LND) containing two tandemly repeated copies of the LND fused to maltose binding protein, with enhanced immunogenicity resulting from the p38/P4 helper T cell epitope from *Schistosoma mansoni*. Rec-LND was found to be highly immunogenic in four major histocompatibility complex (MHC)-diverse strains of mice. All (7/7) rabbits immunized with Rec-LND developed high-titer antibody, 6 out of 7 developed neutralizing antibody, and all rabbits were protected from an aerosolized spore challenge of 193 50% lethal doses (LD50) of the *B. anthracis* Ames strain. Survivor serum from Rec-LND-immunized rabbits revealed significantly increased neutralization titers and specific activity compared to prechallenge levels yet lacked PA or lethal factor (LF) antigenemia. Control rabbits immunized with PA, which were also completely protected, appeared sterilely immune, exhibiting significant declines in neutralization titer and specific activity compared to prechallenge levels. We conclude that Rec-LND may represent a prototype anthrax vaccine for use alone or potentially combined with PA-containing vaccines.

*Bacillus anthracis* is a Gram-positive, spore-forming bacterium that naturally infects wildlife, livestock, and, less frequently, humans. Since 2001, when spores of *B. anthracis* sent through the U.S. mail resulted in infection in 22 individuals, including 5 fatal cases of inhalation anthrax, significant efforts have been directed toward reevaluating our preparedness for possible bioterrorist threats, including weaponized anthrax. This has included renewed efforts to more critically evaluate the anthrax vaccine currently approved in the United States, BioThrax, as well as continued development of new, alternative vaccines for anthrax (1–6). We previously showed that immunization of rabbits with a multiple antigenic peptide (MAP), which display multiple copies of a target sequence extending from a branched lysine core, was capable of eliciting antibody specific for a linear determinant in the 2β2-2β3 loop, which mediated high-titer neutralization of lethal toxin (LeTx) in *vitro* (7, 8) and protection of rabbits from a targeted aerosol challenge of 200 50% lethal doses (LD50) of *B. anthracis* Ames strain in *vivo* (9). The target of the antibody, referred to as the loop-neutralizing determinant (LND), is a critical molecular structure of PA involved in the translocation of edema and lethal factors (LFs) (10–12). Mutations or deletions in the linear sequences comprising the LND, especially those involving the F313-F314, have been shown to completely abrogate the cytotoxicity of LeTx in *vitro*, suggesting that a vaccine which effectively targets the LND would be relatively resistant to circumvention (13–16). Remarkably, antibodies to the LND appear to be virtually absent in PA-immunized rabbits and may be present only at functionally insignificant levels in human AVA serum (8, 17). Thus, the LND appears to be immunologically silent in animals immunized with PA. Development of vaccines capable of eliciting protective immunity against this cryptic epitope, therefore, could be strategic in the event of malicious attempts to circumvent neutralizing specificities elicited by BioThrax or other PA-based vaccines.

While the LND MAP immunogen was efficacious in protecting outbred rabbits from experimental inhalation anthrax, the presence of only a single heterologous helper T cell epitope in the MAP may be suboptimal for stimulation of a diverse genetic human population. Moreover, there are technical challenges associated with the purification and GMP scale-up of MAPs for development of a clinical-grade vaccine. Recombinant proteins, however, can be engineered to contain many helper T cell epitopes and have well-demonstrated paths leading to clinical application (18). We therefore developed a recombinant protein targeting the LND for assessment as a potential anthrax vaccine. The construction and expression of the recombinant protein was facilitated by our use of pBMX-7, a vector we engineered through modification of the pMAL-c2 vector and which we designed to facilitate the construction and expression of modular peptide component immunogens, including those with tandemly repeated epitopes (19). Naturally occurring tandem repeat sequences exist in the CS protein of all species of the genus *Plasmodium* (20, 21), in a conserved antigenic
epitope of *Trypanosoma cruzi* (22), and in the 120-kDa surface protein, WI-1, of *Blastomyces dermatitidis* (23). Indeed, all of these naturally occurring tandem repeat sequences have been shown to be immunodominant B cell epitopes. We and others have shown, using recombinant proteins, that the presence of tandemly repeated sequences can potentiate the immunogenicity of both B and T cell epitopes (19,24–27). Recombinant proteins constructed in pBMX7 are expressed as a fusion with maltose-binding protein (MBP), which facilitates purification through its affinity for maltodextrin-containing moieties. While MBP can be cleaved from the recombinant protein following purification, it effectively stimulates helper T cell epitopes across multiple major histocompatibility complex (MHC)-disparate strains of inbred mice and, therefore, when retained, can be an effective source of cognate T cell help (28). Such T cell stimulation is particularly critical for the induction of antibody responses against discrete peptide targets, like the LND, since these short sequences are often devoid of intrinsic helper T cell epitopes (7).

To evaluate a recombinant vaccine targeting the LND, we molecularly constructed a plasmid encoding a fusion protein containing two copies of the LND peptide sequence (amino acids [aa] 305 to 319) co-linearly in the C terminus of three copies of the p38/P4 helper T cell epitope from *Schistosoma mansoni*, which in turn is fused to the C terminus of MBP (29). The purified recombinant protein, termed Rec-LND, was first assessed for immunogenicity in mice and rabbits and was then evaluated for efficacy in protecting rabbits from a lethal aerosol spore challenge with *B. anthracis* Ames strain.

**MATERIALS AND METHODS**

Recombinant proteins and synthetic peptides. Rec-LND was constructed using the BMX7 vector (19). This vector was developed as a high-copy-number plasmid into which synthetic DNA inserts, bearing standard, complementary, nonpalindromic, 4-base, 5’ overhangs, are directionally ligated for the construction and expression of uni- and multideterminant tandem repeat sequences. pBMX7 was derived from modifications to the p-Mal vector (NEB, Carlsbad, CA) as previously described (19). Rec-LND encodes two copies of the synthetic DNA insert (sense, 5’-CGCGGGCCACGCCAGCTGACGGCGCAGCAGCCAGCCGAGGCCGAC, encoding a 15-aa peptide (aa 305 to 319; GNAEVHASFFDGVCHG)) and an irrelevant 14-mer control peptide (DAEFRHDSGYEVHH). All synthetic peptides were synthesized commercially (Sigma-Genosys, The Woodlands, TX) and were of high-performance liquid chromatography (HPLC) purity to greater than 90% purity. Recombinant MBP (NEB, Carlsbad CA) was used as a test antigen in the lymph node proliferation assays.

**Animals and vaccinations.** Approximately 12- to 16-week-old female C57BL/6, C3H, SJL, and BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for the mouse experiments. For proliferation assays, mice (*n* = 5) were immunized once subcutaneously at the base of the tail with 12 nmol of peptide or 40 µg of the Rec-LND in an emulsion with complete Freund’s adjuvant (CFA). For antibody studies, mice were immunized subcutaneously (s.c.) on days 0, 14, and 28 with 40 µg of the Rec-LND in Alhydrogel (Brenntag Biosector, Denmark) mixed with 10 µg of monophosphoryl lipid A (Sigma Biochemicals, St. Louis, MO) per dose. For rabbit experiments, female New Zealand White (NZW) rabbits (Covance Research Products, Denver, PA) were immunized on day 0 with 250 µg of the Rec-LND or with control PA83 (List Biological Laboratories, Inc., Campbell, CA) in an emulsion with CFA and were then boosted 4 times at 2-week intervals with 125 µg of the Rec-LND or PA83 in an emulsion with incomplete Freund’s adjuvant (IFA). For assessment of antibody responses, mice and rabbits were bled 10 to 14 days after the final immunization. Mice and rabbits were cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care, International, and all animal procedures were approved by an Institutional Animal Care and Use Committee.

**Enzyme-linked immunosorbent assay.** Antibody responses were assessed by enzyme-linked immunosorbent assay (ELISA) essentially as described previously (8). For analysis of antibodies specific for PA, wells of microtiter plates (Immulon 2; Thermo Labsystems, Franklin, MA) were coated overnight at 4°C with 100 ng of PA in a 0.05 M carbonate buffer, pH 9.5. Bound antibody was detected with secondary biotinylated antibody specific for either mouse or rabbit IgG or for rabbit IgM (Southern Biotechnology, Birmingham, AL) followed by streptavidin-alkaline phosphatase and 4-nitrophenylphosphate (Roche, Indianapolis, IN). Absorbance at 405 nm minus absorbance at 650 nm was determined using an ELISA reader (Emax microplate reader; Molecular Devices, Menlo Park, CA). Antibody titers were determined from serial 2-fold dilutions of serum and represent the reciprocal dilution at the EC<sub>50</sub> 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 quantitation for the ELISA was 16.

**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 previously (8). For neutralization studies, 110 ng/ml PA was used along with 300 ng of LE. The reciprocal of the effective dilution at which 50% of the cells are protected from cytotoxicity (ED<sub>50</sub>) (31) 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 toxin neutralization assay (TNA) has a lower limit of quantification of 16. For some assays, modified TNA was employed with a lower limit of quantification of 2. Samples with TNA titers below these lower limits of quantification were assigned values of 16 or 2, respectively.

**Antibody affinity.** For determinations of the concentration of peptide capable of inhibiting 50% of binding (IC<sub>50</sub>) for each serum, serial 2-fold dilutions were performed with the LND linear peptide, aa 304 to 319, starting at 32 µM (2×) in dilution solution (phosphate-buffered saline [PBS], 2% bovine serum albumin [BSA], 0.05% Tween 20). Each experimental serum was added to the diluted peptides in duplicate at a predetermined dilution of two times the reciprocal IG<sub>50</sub> or ED<sub>50</sub> titer for the ELISA-based or TNA-based assays, respectively. The serum-peptide mixtures were allowed to equilibrate at room temperature for 2 h and then overnight at 4°C. The following day, the serum–peptide mixtures were analyzed in either the standard ELISA (ELISA-based IC<sub>50</sub> or TNA-based IC<sub>50</sub>) or in the TNA (TNA-based IC<sub>50</sub>), and the peptide IC<sub>50</sub> were determined for each serum using nonlinear regression. Peptide IC<sub>50</sub> were considered to represent good approximations of the antibody dissociation constant (*K*<sub>d</sub>) (32). An irrelevant 14-mer peptide was used as a specificity control in both assays.

**T cell proliferation assay.** Ten days after immunization, para-aortic and inguinal lymph nodes were aseptically removed from individual mice and single-cell suspensions were prepared. Pooled lymph node cells, representing five mice per group, were cultured with test antigens in 96-well
and C57BL/6 mice detectable T cells in C3H mice. Lymph node proliferative responses in BALB/c, C3H, SJL, and spore concentration data from contemporaneous air sampling. One aerosol LD50 of Ames spores in NZW rabbits is 1.05 x 10⁵ spores (33). Aerosol challenge doses are listed as the mean LD50 ± standard deviations (SD). Following challenge, animals were observed for a period of 14 days. Clinical observations were noted, and moribund animals were euthanized. Deaths were recorded on the day the animal was found dead or was euthanized. Animals surviving to day 14 were anesthetized, terminalized, and euthanized.

Statistical analysis. For determination of ELISA EC₅₀ and TNA ED₅₀ titers, four-parameter logistic regression was used to fit variable slope sigmoidal equations to the serial dilution data. The Kruskal-Wallis and Dunn’s multiple comparison tests were used for comparing EC₅₀ antibody titers involving more than two groups. The Wilcoxon matched-pair signed-rank test was used for comparing pre- and postchallenge antibody, neutralization, specific activity, and IC₅₀ data. The Kaplan-Meier method was used to plot survival data, and statistical differences in survival curves between groups were compared using the Mantel-Cox log-rank test. For all statistical analysis, a P value of <0.05 was considered significant. All statistical analysis was performed using GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Molecular construction and immunogenicity testing of Rec-LND. In previous work, we demonstrated that MBP is an excellent carrier protein, capable of stimulating T cells in 3 out of the 4 common inbred mouse strains, C57BL/6, SJL, and BALB/c, which we use to evaluate helper T cell activity, but found that it is relatively poor at stimulating T cells in the C3H strain (28). To overcome this nonresponsiveness in C3H, we identified the p38/P4 epitope from Schistosoma mansoni as a candidate helper T cell epitope for inclusion in the recombinant vaccine construct, since it has been shown to robustly stimulate T cells in the H-² mice (29). First, we evaluated the ability of the p38/P4 peptide to stimulate T cells in the four strains of mice (Fig. 1). As shown, T cells from C3H mice immunized with the p38/P4 peptide exhibited strong T cell proliferation in vitro in the presence of the p38/P4 epitope, while T cells from C57BL/6, SJL, and BALB/c mice immunized with the p38/P4 peptide exhibited little or no proliferation to the peptide in vitro, confirming the MHC class II restriction of this peptide to H-². Next, we successively cloned the DNA encoding three tandemly repeated copies of the p38/P4 epitope and two tandemly repeated copies of the LND peptide sequence (aa 305 to 319) into the pBMX7 vector to yield the Rec-LND fusion protein (Fig. 2). While cloning into the BMX7 always yields fusion proteins with MBP at the N terminus, we chose to engineer Rec-LND with three copies of the p38/P4 epitope N-terminal to the LND sequence, since previous work with LND MAPs suggested this orientation would be more immunogenic (9). The copy numbers for the p38/P4 and LND epitopes were established empirically. Following verification of the sequence fidelity of the construct, and expression and purification of Rec-LND, we immunized C3H mice to evaluate whether the p38/P4 epitope, now as part of the Rec-LND protein, retained the ability to stimulate T cells. As shown in Fig. 3, T cells from C3H mice immunized with Rec-LND demonstrated strong proliferation to the p38/P4 peptide at all four test antigen doses. T cells specific for the MBP component of Rec-LND exhibited strong restimulation at only the highest test antigen dose, and consistent with previous studies, the LND peptide sequence did not stimulate T cells in mice (7, 9).

We next immunized mice from each of four common H-² disparate strains with Rec-LND to evaluate antibody responses. All four strains of mice produced LND-specific antibody following three immunizations with the Rec-LND mixed with Alhydrogel/MPL adjuvant, demonstrating that Rec-LND was immuno-

![FIG 2 Diagrammatic representation of Rec-LND. Each of the two LND repeats represents aa 305 to 319 from PA of B. anthracis. The LND repeats are expressed at the C terminus of 3 copies of the p38/P4 peptide from the p38 egg antigen of Schistosoma mansoni. The LND and p38/P4 repeats are expressed linked to the C terminus of MBP.](image-url)
genic in mice and exhibited broad MHC restriction (Fig. 4). Antibody responses elicited in the C3H mice were significantly higher than the responses observed in the BALB/c and SJL mice (P/H11005 0.008).

Assessment of the immunogenicity and protective efficacy of Rec-LND in rabbits. Pilot studies were performed which confirmed the immunogenicity of Rec-LND in outbred rabbits (not shown). We therefore proceeded to a definitive study aimed at evaluating whether immunization with Rec-LND could protect rabbits from a lethal challenge with aerosolized \textit{B. anthracis} Ames strain spores. For this study, 7 female NZW rabbits were immunized with the Rec-LND 5 times at 2-week intervals using CFA for priming immunizations and IFA for booster immunizations. A positive-control group received PA administered using the same immunization regimen. Though exclusively a research adjuvant, Freund’s adjuvant was employed in these studies using a 5-injection protocol to allow direct comparison of the protective efficacy of Rec-LND with the MAP-LND vaccine tested previously in the \textit{B. anthracis} Ames strain aerosol challenge model and for economic and animal use considerations, to enable the positive control PA group to be utilized for both studies (9).

The antibody and neutralization titers of the rabbit sera obtained approximately 10 days after their 5th and final immunization are shown in Fig. 5. As shown, all rabbits immunized with the Rec-LND developed high-titer antibody responses by ELISA (geometric mean titer [GMT] = 35,946), and 6 out of 7 of the rabbits developed neutralizing responses in the TNA (GMT = 802). Despite high LND-specific antibody titers, one rabbit had undetectable neutralization in the TNA. All rabbits immunized with PA developed PA-specific antibody (ELISA GMT = 82,651) capable of neutralizing LeTx \textit{in vitro} (TNA GMT = 1,954).

Approximately 3 weeks after their final immunization, all rabbits were exposed to a targeted aerosol dose of 200 LD50 of \textit{B. anthracis} Ames spores and were observed for 14 days. The Kaplan-Meier survival curves are shown in Fig. 6. All rabbits immunized with the Rec-LND (actual exposure, 193 ± 36 LD50) as well as all rabbits immunized with PA (actual exposure, 196 ± 44 LD50) were protected from spore challenge (P < 0.0001 compared to naïve controls). Mean time to death for age-matched naïve control rabbits (n = 6) was 3.33 ± 0.52 days (actual exposure, 202 ± 25 LD50).

Analysis of sera obtained 14 days after challenge revealed that...
Postchallenge antibody titers (GMT = 76,336) from PA-immunized rabbits were not significantly different from the prechallenge levels; however, their postchallenge neutralization titers (GMT = 836) were significantly lower than their prechallenge levels (P = 0.016; Fig. 7). Postchallenge Rec-LND-immunized rabbit antibody titers (GMT = 20,003) declined slightly, though significantly, compared to their prechallenge levels (P = 0.031), yet despite the declines in antibody titer, postchallenge serum from Rec-LND-immunized rabbits demonstrated significant increases in neutralization (GMT = 1,931) compared to prechallenge levels (P = 0.031). The single Rec-LND rabbit which had LND-specific antibody but undetectable neutralization prechallenge also had no detectable neutralization in the postchallenge serum.

The data displayed in panels A to D of Fig. 7 were used to calculate the pre- and postchallenge serum-specific activities (Fig. 7E and F), operationally defined as the quotient of the ED_{50} TNA titers and the EC_{50} antibody titers × 100. This is functionally equivalent to the proportion of PA-specific antibody participating in neutralization (7). The specific activity of the PA group declined significantly from a mean of 2.47% prechallenge to a mean of 1.27% postchallenge (P = 0.016), whereas specific activity for the Rec-LND group increased significantly following challenge from a mean of 11.09% prechallenge to 38.36% postchallenge (P = 0.031).

**Determination of peptide-specific antibody affinity in the pre- and postchallenge sera of rabbits immunized with Rec-LND.** To evaluate whether the significant increases in the neutralization observed in the postchallenge sera of the Rec-LND rabbits might be explained through increases in the LND-specific antibody affinity, we used peptide inhibition to determine the peptide concentration resulting in 50% inhibition (IC_{50}), since we have previously shown that the LND-specific antibody is completely inhibitable with peptide (7, 8). The IC_{50}s, in turn, are good approximations of the aggregate affinity (K_{a}) of the LND-specific antibodies (34). We used both the ELISA and TNA for these determinations so as to selectively measure both overall LND-specific antibody affinity and the affinity of only the neutralizing antibody subset.

An ELISA-based assay, employed previously, yielded IC_{50} determinations for all LND-peptide-specific antibodies. As shown in Table 1, LND-specific antibody from both the pre- and postchallenge sera were found to be highly avid in the ELISA-based assay but did not differ significantly between the pre- and postchallenge...
sera \((P = 0.81)\). Peptide-specific IC\(_{50}\)\(s (K_d)\) ranged from 0.90 to 7.23 nM, with geometric mean IC\(_{50}\) of 3.61 nM and 3.54 nM in the pre- and postchallenge sera, respectively. The ELISA-based IC\(_{50}\) demonstrated some correlation with neutralization titers \((R^2 = 0.26;\) Fig. 8A\), consistent with an earlier report using a MAP version of the LND vaccine \((7)\).

Since the specific activity of the sera induced to Rec-LND is below 100\%, a significant proportion of antibody, though inhibitable with peptide, does not participate in neutralization. Therefore, to better resolve the affinities of only antibodies participating in neutralization, we also employed an assay for TNA-based IC\(_{50}\) determinations, which yields the concentration of peptide capable of inhibiting 50\% of the binding of antibodies participating in neutralization. IC\(_{50}\) data from the TNA-based assay also highlighted the very high-affinity antibody elicited with Rec-LND (Table 1). The geometric mean IC\(_{50}\) of the postchallenge sera, 2.31 nM, was lower (higher affinity) than the prechallenge IC\(_{50}\) of 3.75 nM, but the differences did not reach statistical significance \((P = 0.28)\). Unlike the ELISA-based results, however, there was considerably more divergence among the TNA-based IC\(_{50}\) from anti-sera with high and low neutralization titers. This is reflected in the greater range of the TNA-based IC\(_{50}\) data, 0.42 nM to 171.80 nM, compared to the ELISA-based data, and in the considerably higher correlation between the TNA-based IC\(_{50}\) data and neutralization \((R^2 = 0.79;\) Fig. 8B).

**DISCUSSION**

In this study, we developed a recombinant vaccine for use in eliciting immunity against the LND of PA. Immunization with Rec-LND completely protected rabbits from a targeted challenge with 200 LD\(_{50}\) of aerosolized spores of *B. anthracis* Ames strain. Spore germination appeared to be minimal or absent in Rec-LND-immunized rabbits as evidenced by the absence of *de novo* LF-specific or non-LND PA-specific antibody in their postchallenge sera. These findings parallel an earlier study employing MAP versions of an LND vaccine \((9)\). Rabbits immunized with PA also had no evidence of significant *de novo* LF-specific antibody or increases in PA-specific antibody in their postchallenge sera. The results suggest that, overall, both the Rec-LND- and PA-immunized rabbits had serum levels of neutralization at challenge which were capable of completely neutralizing LeTx.

Rec-LND-immunized rabbits, unlike rabbits immunized with PA, demonstrated a significant increase in their neutralization titers postchallenge compared to prechallenge levels, despite overall significant declines in their postchallenge antibody titers. This resulted in a significant increase in specific activity for this group. The increases in neutralization and specific activity identified in the postchallenge sera of the Rec-LND group is remarkable, though its basis is unclear. We hypothesized that the increases in neutralization in the postchallenge sera might be secondary to

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**TABLE 1 Peptide IC\(_{50}\) data from analysis of pre- and postchallenge sera from Rec-LND-immunized rabbits**

| Rabbit | Prechallenge | Postchallenge |
|--------|--------------|--------------|
|        | TNA titer | ELISA-based | TNA-based | TNA titer | ELISA-based | TNA-based |
|        | ED\(_{50}\) | IC\(_{50}\) (nM) | IC\(_{50}\) (nM) | ED\(_{50}\) | IC\(_{50}\) (nM) | IC\(_{50}\) (nM) |
| 758    | 3,882      | 2.56        | 4.56       | 17,955    | 3.88        | 0.51       |
| 759    | 6,538      | 3.89        | 0.42       | 18,181    | 4.20        | 2.29       |
| 760    | 7,757      | 5.49        | 0.63       | 11,616    | 3.09        | 0.87       |
| 761    | 227        | 7.23        | 171.80     | 958       | 6.00        | 55.54      |
| 762    | 2          | 0.97        | ND\(^a\)   | 2         | 0.90        | ND         |
| 763    | 6,611      | 4.99        | 0.54       | 6,758     | 4.66        | 0.71       |
| 764    | 362        | 4.21        | 24.70      | 2,041     | 5.43        | 3.76       |
| Geometric mean | 802 | 3.61        | 3.75       | 1,931     | 3.54        | 2.31       |

\(^a\) ND, not done.

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increases in the affinity of the LND-specific antibody as a consequence of the challenge. The affinities of the neutralizing antibody subset did trend higher in the postchallenge sera than prechallenge levels, though the difference did not reach statistical significance. Interestingly, however, the affinities of the neutralizing antibody subset were found to be highly correlated to TNA titers. As might be expected, this correlation was considerably stronger than the correlations determined using an ELISA-based assay in the current and previous studies [7]. as the ELISA-based method assesses the affinities for all LND-specific antibodies, some of which do not participate in neutralization.

The refinement of antibody specificity offers a second, though not mutually exclusive, hypothesis for explaining the increase in specific activity in the postchallenge sera. The antibody repertoire in the postchallenge sera may be more refined as a consequence of the spore challenge and contain a higher proportion of antibody with the precise LND fine specificity responsible for neutralizing LeTx. In either case, an increase in antibody affinity or a refinement of the LND-specific antibody repertoire might have occurred as a consequence of the stimulation of memory B cells bearing high-affinity, LND-specific surface IgG, by PA on the surface of spores, with the consequent stimulation and production of neutralizing antibody by those cells [35].

The antibody elicited by Rec-LND demonstrated strikingly high affinities using both the ELISA- and TNA-based inhibition assays compared to peptide immunogens. We found the affinity of the prechallenge LND-specific antibody induced through immunization with Rec-LND to be approximately an order of magnitude higher than the affinity of LND-specific antibody induced through immunization of rabbits with an LND MAP vaccine using an identical immunization protocol [7]. It is likely that the induction of such high-affinity antibody with Rec-LND is at least partly attributable to the abundant helper T cell epitopes present in the MBP component of Rec-LND, and possibly also to the potent helper T cell activity associated with the p38/P4 epitope from Schistosoma mansoni. Early seminal work with hapten-carrier conjugates has shown that covalently linked sources of helper T cell epitopes contribute to the affinity of antibody induced to haptenic structures [36]. Though devoid of helper epitopes for a number of mouse strains [7], the LND peptide sequence itself may also contain helper T cell epitopes in rabbits and humans [7, 9, 37], which could potentially contribute to the affinity of LND-specific antibody in rabbits. Finally, as has been demonstrated with other tandem-repeat-containing MBP fusion proteins, the display of B cell epitopes as tandem repeats can lead to increased carrier protein-specific T cell stimulation. This effect is likely secondary to the more efficient uptake of the fusion proteins by B cells as a consequence of the high-affinity binding of the tandem repeat sequences to the BCR [19].

Despite the abundant evidence that PA-specific antibody must be capable of neutralization in order to mediate protection from aerosol spore challenge [38–41], one surviving Rec-LND rabbit (actual exposure: 171 LD_{50} ± 35) had LND-specific antibody but no detectable neutralization in the pre- or postchallenge serum. Like the other Rec-LND rabbits, the postchallenge serum from this rabbit did not demonstrate immunological evidence of spore germination and vegetative cell outgrowth. In addition to testing in the standard TNA, pre- and postchallenge sera from this rabbit were also repeatedly tested in a modified TNA designed for increased sensitivity, yet in all cases the sera had no detectable neutralization. Interestingly, this rabbit was found to have the highest-affinity LND-specific antibody among all of the Rec-LND-immunized rabbits in both the prechallenge (IC_{50} = 0.97 nM) and postchallenge (IC_{50} = 0.90 nM) sera using the ELISA-based peptide inhibition assay. Since PA or PA-like antigens are present on the surface of spores [42], and PA-specific antibody has been demonstrated to decrease both spore viability and the rate of spore germination in vitro [35, 43, 44], it is possible that the survival of this rabbit was mediated through the binding of LND-specific antibody to B. anthracis spores in vivo. We are not aware, however, of any direct evidence to date that protection from inhalation spore challenge with B. anthracis can be mediated exclusively through the binding to PA on the spore surface. An alternative, though nonmutually exclusive, hypothesis would be that the very-high-affinity nonneutralizing antibody can sequester PA in vivo in a manner not modeled by the TNA but that nonetheless can confer protection.

The current vaccine for anthrax in the United States, BioThrax, has been shown experimentally to protect rabbits and nonhuman primates from inhalation anthrax [39, 45]. Yet, efforts are under way to develop new and more effective PA-targeted vaccines, as well as vaccines directed toward other B. anthracis antigens [2, 5, 46, 47]. These efforts have been motivated in part by the intensive immunization protocol required with BioThrax and the findings in some studies of reactogenicity among vaccinees [48, 49]. Of perhaps greater concern, however, is the finding that in a recent study, a large percentage of BioThrax vaccinees were found to lack levels of neutralizing antibody which would be predicted to confer protection from inhalation anthrax [50]. Explanations for such potential vaccine failures range from the normal variability of the humoral immune response to the weak adjuvancing properties of Alhydrogel. It has also been suggested that whole PA may not be an ideal antigen for the elicitation of neutralizing antibody against LeTx [51, 52]. This is supported by studies detailing efforts to develop MAbs against PA which have revealed that only a few regions of PA are responsible for eliciting the preponderance of the neutralizing specificities [51, 53–55]. Conceptually, this insight is aligned with the findings in the current study that the specific activity of PA is quite low, ranging from 1 to 3%. Despite the production of large amounts of PA-specific antibody in PA-immunized rabbits, the overall fraction of antibody participating in the neutralization of LeTx is strikingly low. We considered the possibility that the low specific activity observed could be due to the adjuvant formulation employed since we have observed almost identical specific activities in an independent cohort of rabbits immunized with PA in Freund’s adjuvant. However, a retrospective analysis of sera from rabbits immunized with recombinant adenovirus-expressing PA63 [5] yields a specific activity of approximately 5%, suggesting that low specific activity may in fact be a general characteristic of the antibody response to PA in rabbits. If such findings are not species specific and are true for humans as well, it reinforces the concern that some BioThrax vaccinees, especially those in whom PA antibody titers are low, will lack adequate levels of neutralizing antibody for protection against inhalation anthrax. In such individuals, the utility of an alternative vaccine, like Rec-LND, may theoretically convert a low-responder, nonprotected individual into a vaccinee with protective levels of neutralizing antibody. Though we have not yet evaluated combined immunization of Rec-LND with PA,
there is a theoretical basis for such a combination since rabbit and human PA antisera do not contain antibody against the LND.

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