Anthrax LFn-PA Hybrid Antigens: Biochemistry, Immunogenicity, and Protection Against Lethal Ames Spore Challenge in Rabbits[#]

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Abstract

We describe a novel hybrid anthrax toxin approach that incorporates multiple components into a single vaccine product. The key domains of protective antigen (PA) and lethal factor (LF) that may be critical for inducing protective immunity are combined into one recombinant molecule. Two LF N-terminal domain-PA hybrids, one with wild-type PA and another with furin cleavage-minus PA, were expressed in *E. coli* and purified in a native form. Both the hybrids bind to the extracellular domain of the host receptor, CMG2; the wild-type hybrid can be cleaved by furin exposing the LF interacting domain, allowing it to oligomerize into lethal toxin as well as translocation pore-like complexes. The hybrid antigens are immunogenic in Dutch-belted rabbits, eliciting strong PA-specific and LF-specific antibodies. However, the lethal toxin neutralizing antibody titers are 3-7 times lower than those elicited by PA-alum. The hybrid antigens conferred 100% (6/6) protection in rabbits challenged intranasally with a 100 LD_{50} dose of *Bacillus anthracis* Ames strain spores.

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INTRODUCTION

Bacillus anthracis, the etiologic agent of anthrax, has been high on the list of potential biological warfare and bioterrorist agents due to the ease of preparation and dissemination of its spores. Of the two major virulence factors, the poly-gamma-D-glutamic acid capsule and the tripartite anthrax toxin, the toxin has been widely targeted for development of anthrax vaccines [1,2].

The anthrax toxin is composed of three components, protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa). These proteins are nontoxic as monomers but assemble into potent toxin complexes through a series of protein-protein interactions [1]. PA binds to the host cell receptors, either the capillary morphogenesis protein 2 (CMG2) or the tumor endothelial marker 8 (TEM8) [3,4], and the N-terminal 20 kDa fragment (PA20) is cleaved off by the furin protease [5,6]. The receptor-bound PA63 (63 kDa) assembles into a heptameric pre-pore ring structure. Up to 3 molecules of LF and/ or EF then bind to the heptamer through the N-terminal domain (LFn or EFn) to form lethal toxin (LTx) and edema toxin (ETx). The toxin complexes are internalized *via* receptor-mediated endocytosis where the pre-pore is converted to pore in the acidic environment of the endocytic vesicle. Translocation of LF (metalloprotease specific for mitogen-activated protein kinase) and EF (adenylate cyclase) through the pore into the cytosol triggers a cascade of cellular metabolic changes leading to cytotoxicity and potentially cell death [7-9].

As protein-protein interactions between PA and LF are central to anthrax toxicity, antigen formulations that induce high titers of antibody (Ab) that can block these interactions are essential for an effective anthrax vaccine. The human anthrax vaccines licensed in the USA (AVA) and the UK (AVP) consist of PA as the principal component with varying amounts of LF and EF [10,11]. While PA was well documented to induce LTx neutralizing Abs and protective immunity in animal models [12-14], the contributions by LF (or EF) have been sparsely investigated. Recent reports suggest that inclusion of LF in vaccine formulations might provide additional protective benefit [15-19].

We have been exploring novel approaches such as bacteriophage T4 display and transcutaneous immunization to develop multicomponent single product anthrax vaccines [20,21]. Here we describe the construction of LFn-PA, a hybrid recombinant in which the key domains of PA and LF are engineered into one antigen. The hybrid domains fold into the respective functional states and assemble into oligomeric intermediates of the anthrax toxin pathway. In a Dutch-belted rabbit model, the hybrid antigens elicit high titers of PA- and LF-specific IgG Abs, LTx neutralization titers, and provide 100% protection against intranasal challenge with Ames strain spores.

METHODS

Bacteria and Plasmids

The T7 expression plasmid pET-28b (Kan^r) (EMD Biosciences, Inc) was used for construction of recombinant PA and LFn hybrid clones. Plasmids pPA26 [22], pYS5-PAU7 (the cleavage site $_{164}$RKKR$_{167}$ of PA was replaced by PGG, which cannot be cleaved by furin or other proteases) [23] and pLF7 (LF$_{E687C}$) [24] were used as templates for PCR amplification of the PA, PA cleavage-minus (PA$_{CM}$), and LFn, respectively. *Escherichia coli* strains, XL10-Gold and codon-plus BL21 (DE3) RIPL (Stratagene), were used as host strains for initial transformation and subsequent IPTG-induced over-expression of hybrid proteins, respectively.

Construction of LFn-PA and LFn-PA_{CM} Hybrid Genes

PCR-based splicing-by-overlap-extension (SOE) was used to construct gene fusions [25]. As shown in Fig. (1A), LFn, the N-terminal PA binding domain of LF [amino acids (aa) 1-262; [26] was fused to the N-terminus of PA or PA_{CM} with a four aa linker, SASA, in the middle. The primers, pl that corresponds to nucleotides (nt) 100-120 of LF gene [5′-*CCATCA*GCTAGCGCGGGCGGTCATGGTGATGTA-3′] and p2 that corresponds to the LFn-PA junction sequence (5′-AATAACCGGTTCTCCTGTTTAACTT**AGCACTTGCTGA**CCGTTGATCTTTAAGTTCTTCCAAG-3′; of this sequence, nt 1-26 are complementary to nt 88-113 of the PA gene and nt 27-38 correspond to the SASA linker, and nt 39-63 are complementary to nt 864-888 of LF gene) were used to amplify the 837-bp LFn fragment plus the 26 nt 3′-end corresponding to the N-terminus of PA gene. A second PCR was performed using oligonucleotides p3 that corresponds to the LFn-PA junction sequence (5′-CTTGGAAGAACTTAAAGATCAACGG**TCAGCAAGTGCT**GAAGTTAAACAGGAGAACCGGTTATT -3′; of this sequence, nt 1-25 correspond to nt 864-888 of the LF gene, nt 26-37 corresponding to the linker and nt 38-63 correspond to nt 88-113 of the PA gene) and p4 that corresponds to nt 2268-2292 of the PA gene [5′-*CGC*GGATCCTTATCCTATCTCATAGCCTTTTTTAGAA -3′) to amplify the 2251-bp PA or PA_{CM} gene plus the 25-bp 5′-end corresponding to the 3′-end of LFn gene (underlined sequences correspond to *Nhe* I or *BamH* I restriction sites; bolded sequences correspond to SASA linker; italicized sequences correspond to 5′-tags that allow restriction enzyme digestion at the ends of the PCR amplified DNA). The two PCR products were mixed and a third PCR was performed to "stitch" the fragments through overlap extension. The LFn-PA and LFn-PA_{CM} fusion products were then amplified using the end primers, p1 and p4, gel-purified, digested with *Nhe*I and *BamH*I, and inserted into the pET28b vector. This results in in-frame fusion of the 18 aa hexa-histidine sequence at the N-terminus of LFn-PA hybrids. The recombinants were transformed into the non-expression strain, *E. coli* XL10-Gold (Stratagene), and the sequence of the hybrid clones was ascertained by DNA sequencing. The clones were then transformed into the expression strain, *E. coli* codon-plus BL21(DE3) RIPL.

Protein Purification

Highly purified recombinant proteins were prepared in our laboratories for binding assays as well as immunizations, as described below. For ELISA and LTx neutralization assays, purified PA and LF were purchased from List Biologicals Laboratories, Inc., Campbell, CA.

PA and LF (the mutant LF_{E687C} was used in all binding assays) were purified from *B. anthracis* strain BH445, as described previously [27,28]. Briefly, the culture filtrate was loaded onto the Phenyl Sepharose column and eluted with a linear gradient from 1.5 M to 0 M ammonium sulfate. The proteins were then loaded onto Q Sepharose Fast Flow anion exchange column and eluted with a linear gradient of 0 to 0.5 M NaCl. The pooled fractions were further purified by Superdex 75 gel filtration.

The PA63 heptamer was prepared by trypsin cleavage of PA and purified as per the procedures described previously [29]. The purified soluble human furin (aa 1 - 604) [30] was a gift from Dr. Iris Lindberg, University of Maryland Medical School, Baltimore, MD. The purified extracellular domain of the anthrax toxin receptor CMG2 (aa 40 - 218) was a gift from Robert Liddington, Burnham Institute, LaJolla, CA [31].

For purification of LFn-PA hybrids, a 2-liter bacterial culture expressing LFn-PA or LFn-PA_{CM} was induced with 1 mM IPTG for 3 h. The cells were harvested by low speed centrifugation (6,000 × *g*, 15 min, 4°C) and resuspended in 50 ml PBS buffer (pH 7.4)

containing the protease inhibitor cocktail (Roche Diagnostics). The cells were lysed using a French-press (Aminco, Inc.), and the lysate was centrifuged at $10,000 \times g$ for 15 min. The pellet was washed twice with HisTrap binding buffer (50 mM Tris-HCl, pH 8, 20 mM imidazole and 300 mM NaCl), and the protein was solubilized with denaturing buffer (8 M Urea, 50 mM Tris-HCl, pH 8, 20 mM imidazole and 300 mM NaCl). The protein was bound to the HisTrap column (AKTA-prime, GE Healthcare) and renatured by a decreasing urea gradient (8-0 M) and eluted with 20-500 mM linear imidazole gradient. The peak fractions were desalted by passing through the Hiprep10/26 column and concentrated by Amicon Ultra-4 centrifugal filtration (5 kDa cut-off; Millipore). The proteins were then purified by gel filtration on Hi-Load 16/60 Superdex 200 column (prep-grade) (AKTA-FPLC, GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 8 and 100 mM NaCl. Any denatured and/or aggregated protein eluted in the void volume was discarded; only the peak LFn-PA monomer fractions that elute as a symmetrical peak were collected, concentrated, and stored at −80°C.

Furin Cleavage of PA and LFn-PA Hybrids

The purified PA or LFn-PA were treated with furin using a furin:PA ratio of 1:500 (w/w) in 20 μL buffer containing 50 mM HEPES pH 7.5, 2 mM $CaCl_2$, 0.5 mM EDTA, and 0.2% β-octylglucoside. The reactions were performed at 37°C for 45 min and terminated by the addition of phenyl methyl sulfonyl fluoride to 1 mM. Cleavage of 83 kDa PA or 115 kDa LFn-PA to 63 kDa nicked PA (nPA) was quantified by SDS-PAGE and laser densitometry.

Formation of SDS-Resistant Pore-like Complexes

PA or LFn-PA was cleaved by furin as described above in the presence of equimolar amounts of LF. SDS sample buffer was then added and the samples were boiled for 5 min. Formation of SDS-resistant complexes was analyzed by SDS-PAGE.

Binding Assays

The PA-binding activity of LFn-PA hybrids was determined by incubating 1.5 μg of LFn-PA hybrids (or LF control) with 2.5 μg of furin-cleaved nPA at room temperature for 30 min. The nPA oligomerizes into heptamers, more efficiently in the presence of LF or LFn [32].

The receptor-binding activity of LFn-PA was determined by incubating 1.2 μg of CMG2 with 1.3 μg of PA or 1.2 μg of LFn-PA hybrids in furin cleavage buffer at room temperature for 30 min. The molar ratios of CMG2 to PA or LFn-PA were 4:1 and 6:1 respectively.

The above samples were electrophoresed on a native 4-12% gradient polyacrylamide gel and stained with Coomassie blue G-250 (Bio-Rad Laboratories, Inc.).

Rabbit Immunizations

Six to seven week old female Dutch-belted rabbits (Myrtle's Rabbitry) were used to inject the antigens intramuscularly into the caudal thigh muscle using a 26 gauze needle. Alternate thighs were used for subsequent injections. Six rabbits /group were used for LFn-PA and LFn-PA$_{CM}$ antigens and 5 rabbits/group were used for PA and naïve controls. The rabbits were immunized at weeks 0, 4, and 8 with equimolar amounts of either PA (10 μg), LFn-PA (15 μg), or LFn-PA$_{CM}$ (15 μg), adsorbed to Alhydrogel® (0.3 mg alu minum/injection; Brenntag Biosector). The rabbits were bled at 2-week intervals and individual serum samples were analyzed for PA-specific and LF-specific IgG antibodies (Abs) and for LTx neutralizing Abs as described below.

Antigen-Specific ELISA

Sera from individual rabbits were assayed by ELISA for the presence of PA-specific and LF-specific IgG. Briefly, 96-well flat-bottomed Nunc Maxisorp plates (VWR International, Inc.) were coated with either 0.1 μg/well of PA or LF (List Biological Laboratories, Inc; wild-type LF was used in all ELISA and LTx neutralization assays), blocked with 0.5% casein in PBS, washed and incubated for 1 h at 37°C with the test serum followed by incubation with horseradish peroxidase-labeled, affinity-purified goat anti-rabbit IgG (The Binding Site) and the substrate (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; KPL). Plates were read at 405 nm on a SpectraMax 250 plate reader (Molecular Devices). The data are expressed as end point titers defined as the highest dilution that yielded an optical density reading greater than or equal to twice that of the background values. The titers were calculated after subtracting the mean absorbance of duplicate wells lacking antigen from wells containing antigen.

LTx Neutralization Assay

LTx neutralizing Ab titers were determined by the ability of the sera to neutralize the cytotoxicity of LTx in a J774A.1 macrophage cell line as described previously [33,34]. A 4-parametric sigmoid regression curve was used to determine the dilution of antisera that resulted in 50% reduction in toxicity (ED_{50}) of anthrax LTx. Each plate contained control wells that had toxin only (no antisera) and no toxin and no antisera. Polyclonal rabbit anti-PA Ab was used as a positive control.

Lethal *B. anthracis* Challenge

Rabbits were challenged under BSL-3 conditions at week 10 with 100 LD_{50} (1×10^7 CFU) of *B. anthracis* Ames strain spores by the intranasal route as described previously [35]. Briefly, rabbits were anesthetized and suspended vertically by the upper incisors. They were instilled with spore solutions (50 μl/ naris) over 2-3 minutes followed by 50 μl PBS to wash any non-adherent spores from the nasal cavity into the lungs. Rabbits were monitored twice daily for 14 days for signs of disease.

RESULTS

Construction and Characterization of LFn-PA Hybrid Clones

The LFn domain (aa 1-262) of LF [26], which interacts with the nPA heptamer, was fused to either the full-length wild-type PA (LFn-PA) or the furin cleavage-minus mutant, PA_{CM} (LFn-PA_{CM}) (Fig. 1A). The recombinant hybrid proteins were over-expressed in *E. coli*. Appearance of a new band after IPTG induction corresponding to the molecular weight (mol. wt.) of the fusion protein (115 kDa) demonstrated the production of the recombinant LFn-PA hybrid proteins (Fig. 1B, lanes 2 and 4 labeled with an arrow). Most of the over-expressed protein was partitioned into the insoluble fraction.

The LFn-PA hybrid proteins were purified by the denaturation-renaturation method (see Materials and Methods). The purified proteins migrated as a sharp band upon native-PAGE, suggesting proper folding (Fig. 1C, lanes 2 and 3). The purified proteins also migrated as one predominant species upon SDS-PAGE, corresponding to the expected mol. wt. of the hybrid protein (Fig. 1D, lanes 3 and 5). The LFn-PA was cleaved by furin into 2 fragments, a 50 kDa N-terminal LFn-PA20 and a 63 kDa C-terminal PA63 (Fig. 1D, lane 4), whereas no cleavage was evident with the LFn-PA_{CM} (Fig. 1D, lane 6).

The LFn Domain of the LFn-PA Hybrids Binds to the PA63 Heptamer

The *in vitro* binding experiments show that the LFn domain of the hybrid proteins forms high mol. wt. LTx-hybrid complexes with PA63 heptamer. These complexes (Fig. 2A, lanes 4 and 5) migrate slower than the LTx complexes formed with LF (lane 3), which are reported to have a stoichiometry of $(PA63)_7-(LF)_{1-3}$ [36,37]. This is consistent with the presence of additional PA mass attached to each LFn domain interacting with the PA63 heptamer.

The Furin-Cleaved LFn-PA Self-Assembles into LTx-Like Complexes

The LFn-PA20 and PA63 fragments produced by furin cleavage of LFn-PA self-assembled (SA) into a LTx-SA complex through interactions between the exposed LFn binding surface of PA63 and LFn-PA20 (Fig. 2B, lane 2), suggesting that both the PA63 and LFn domains of LFn-PA are functional. Considering that all the PA63 was in the complex (note that no PA63 band is seen in lane 2), it should have a stoichiometry of $(PA63)_7-(LFn-PA20)_{1-3}$.

LFn-PA Hybrid Forms Pore-Like Complexes

A small fraction of the LTx complexes formed in solution are SDS-resistant. These are thought to mimic the intermediates of acid-mediated pre-pore to pore conversion in the anthrax toxin translocation pathway [1,38,39]. To determine whether LFn-PA can form such complexes, furin-cleaved LFn-PA or the PA control was incubated with LF. Both formed high mol. wt. SDS-resistant complexes. Unlike the single complex band visualized in the PA sample (Fig. 2C, lane 1), multiple bands were observed in the LFn-PA sample (lane 2). This is consistent with the expectation that a combination of LFn-PA20, LF, and LFn-PA (uncleaved) would be part of these complexes.

The PA Domain-4 of LFn-PA Binds to the CMG2 Receptor

The extracellular soluble domain of the cellular receptor, CMG2, was incubated with LFn-PA, LFn-PA$_{CM}$, or LTx-SA complexes, all having the intact PA domain-4, and the complexes formed were analyzed by native PAGE. The affinity for CMG2 binding to PA is very high (*Kd*: ~2nM) [40]; consequently, at the molar ratios used, essentially all the PA complexed with CMG2 (Fig. 3, lane 3). All three hybrids, like the control PA and PA63 (Fig. 3, compare lanes 3 and 5 to lanes 2 and 4), bind to the CMG2 receptor, as evident by the disappearance of the hybrid band(s) and appearance of higher mol. wt. complex bands (Fig. 3, compare lanes 7, 9, and 11 with lanes 6, 8, and 10, respectively).

Immunogenicity of LFn-PA Hybrid Antigens

The immunogenicity of LFn-PA hybrids was tested in our recently established Dutch-belted rabbit model [35]. The rabbits were immunized by intramuscular route with equimolar amounts of PA, LFn-PA, or LFn-PA$_{CM}$ absorbed to Alhydrogel® and the PA- and LF-specific Ab responses as well as the LTx neutralization titers were determined. All the groups elicited high levels of PA-specific end point Ab titers (Fig. 4); the LFn-PA hybrids (*E. coli*-produced) induced titers of 3.8 million (LFn-PA$_{CM}$; p = 0.016 compared to PA) and 4.6 million (LFn-PA), whereas the PA (*B. anthracis*-produced) induced titers of 9 million (black bars, left axis). The LFn-PA hybrids also induced high LF-specific Ab titers in the range of 0.4 to 1 million (open bars, left axis). In addition, the LFn-PA constructs induced LTx neutralization titers (ED$_{50}$) of 500-1300 at week-10, whereas PA gave a titer of about 3500 (gray bars, right axis).

Rabbits Immunized with LFn-PA or LFn-PA$_{CM}$ are Completely Protected against Ames Spore Challenge

The LFn-PA and LFn-PA$_{CM}$ immunized Dutch-belted rabbits were challenged intranasally with a 100 LD$_{50}$ dose of *B. anthracis* Ames strain spores at week-10 post-immunization. All naïve control rabbits died by day 3 whereas both the LFn-PA and LFn-PA$_{CM}$ immunized rabbits, as well as the control PA-immunized rabbits, showed 100% survival (Fig. 5).

DISCUSSION

The anthrax vaccines licensed in the US (AVA) and UK (AVP) are cell-free culture supernatants of *B. anthracis* strains V770-NP1-R and Sterne 34F$_2$ adsorbed to either pre-formed aluminum hydroxide or alum precipitate, respectively [41,42]. The principal component of the vaccines that is thought to provide protective immunity against anthrax is the PA [12,13,43-45], although contribution by the variable amounts of LF and EF present in these vaccines is possible [10,11]. More recent efforts have been directed towards the development of pure recombinant PA-alum vaccines that can confer equally good protection. It is however unclear whether the anthrax vaccines would benefit from the addition of certain LF epitopes that could elicit additional protective responses. We have explored a novel approach in which the key domains of the anthrax lethal toxin are combined into a single product vaccine.

Monoclonal Abs with LTx neutralization capacity have been helpful in defining the domains that may be critical for an effective anthrax vaccine [1,2]. These domains include: i) PA domain-4, which is required for the attachment of PA to the host cell receptor(s); ii) PA domain-1b, which is necessary for interaction with LFn domain; iii) PA domain-2, which is involved in oligomerization of PA; and iv) LFn domain, which interacts with PA63 heptamer forming the LTx complex. We have constructed a single recombinant antigen, LFn-PA, which includes all four of these domains. Additionally, we have constructed a second antigen, LFn-PA$_{CM}$, which lacks the furin cleavage site and therefore could generate stable or alternative intermediates for antigen presentation. A series of *in vitro* binding assays demonstrated that the functionality of all four domains in the LFn-PA hybrids was comparable to that of their respective native domains. Specifically, the LFn-PA hybrid binds to the host receptor, exposes the LF interacting surface through furin cleavage, and oligomerizes into pore-like complexes. Additionally, the furin cleaved PA63 and LFn-PA20 fragments can self-assemble, as well as further interact with CMG2 receptor, thus mimicking the native LTx complexes.

Both the LFn-PA hybrid antigens generated robust PA-specific Ab titers that were comparable to that of PA-alum. In addition, the hybrids induced strong LF-specific Ab titers, which could be considered an advantage because there is evidence that LF Abs alone can confer protection against anthrax [15,16]. While the hybrids also elicited LTx neutralization titers, these responses were 3-7 times lower than that of PA. Thus, the inclusion of LFn in the vaccine formulation as a fusion protein appears to be a disadvantage with respect to the abundance of LTx neutralization titers. However this is not to say that LFn does not contribute to the LTx neutralization titers as the assay cannot differentiate contributions of individual domains, nor do our results imply that immunization with LFn or LF would not enhance LTx neutralization titers in a different context. One possible explanation for the weaker LTx neutralization response by the LFn-PA hybrids may be that self-assembly of the furin-cleaved LFn-PA20 to PA63, or the presence of additional LFn-PA20 mass in the cleavage-minus mutant, interfered with the optimal exposure of the neutralization epitope(s) present in PA domain-1b. Alternatively, antigen processing of the hybrid complexes may be altered generating qualitatively different responses. Another contributing factor may be that potential differences exist between the *E. coli*-produced

hybrid antigens vs. the naturally secreted *B. anthracis* PA; PA modification by deamidation and its conformational sensitivity have been previously reported [46,47], although their effect on the quality of Ab responses is unknown.

Both the hybrid antigens conferred 100% protection against a high 100 LD_{50} intranasal challenge with Ames strain spores using our newly established Dutch-belted rabbit model. All control animals died within 3 days of the challenge, demonstrating that the hybrid vaccines are efficacious in providing protection against the most virulent form of anthrax. These data are consistent with the hypothesis that both the Ag-specific Ab titers, as well as the LTx-neutralization Abs, are likely correlates of protection in the Dutch-belted rabbits as was previously reported in the New Zealand white rabbits [12,13,48].

CONCLUSION

The key domains of the anthrax lethal toxin are combined into a single recombinant vaccine. The LFn-PA hybrid antigens thus constructed retain the biochemical functions of the respective domains, immunogenicity in rabbits, and provide complete protection against intranasal Ames strain spores challenge.

Acknowledgments

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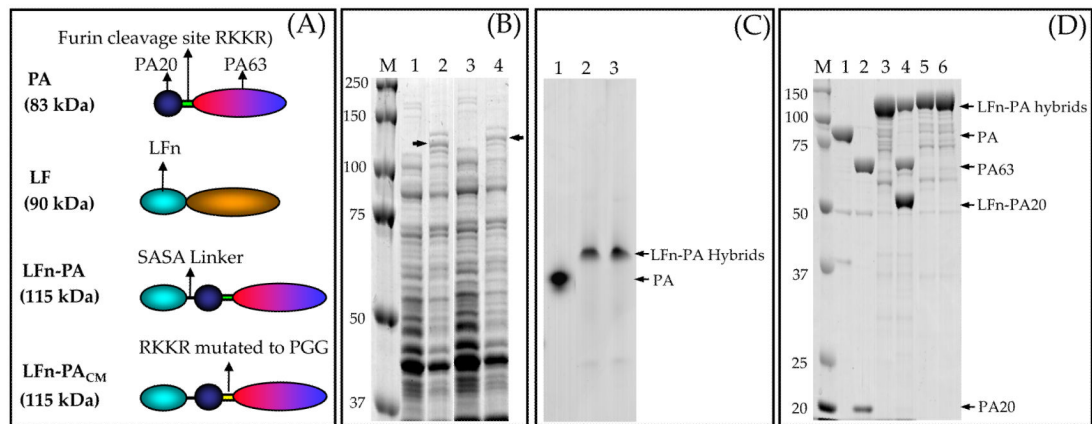
Fig. (1).
Construction and characterization of the LFn-PA anthrax toxin hybrid recombinants. (**A**). Schematic of the constructs. The N-terminal PA binding domain of LF (LFn, blue) was fused to the N-terminus of PA or PA$_{CM}$ mutant. The N-terminus of LFn is fused to hexahistidine tag present in the vector. The PA and LF domains, cleavage sites, and linker are shown. The C-terminal domain-4 of PA is shaded in blue. (**B**). Over-expression of the recombinant LFn-PA hybrid proteins. The *E. coli* recombinants were induced with IPTG for 2.5 h and subjected to electrophoresis on 10% SDS-polyacrylamide gel. The gels were stained with Coomassie Blue G250 and destained with a solution containing 10% acetic acid and 10% methanol. Lanes: 1 and 3, before induction; and 2 and 4, after induction; of LFn-PA and LFn-PA$_{CM}$, respectively. The arrows point to the new bands appeared after induction. (**C**). Native PAGE (4-12% gradient gel) of purified PA (lane 1), LFn-PA (lane 2), and LFn-PA$_{CM}$ (lane 3). (**D**). SDS-PAGE of purified PA (lane 1), LFn-PA (lane 3), LFn-PA$_{CM}$ (lane 5) and after treatment with furin (lane 2, PA; lane 4, LFn-PA; lane 6, LFn-PA$_{CM}$). Lanes M in panels A and D, mol. wt. standards.
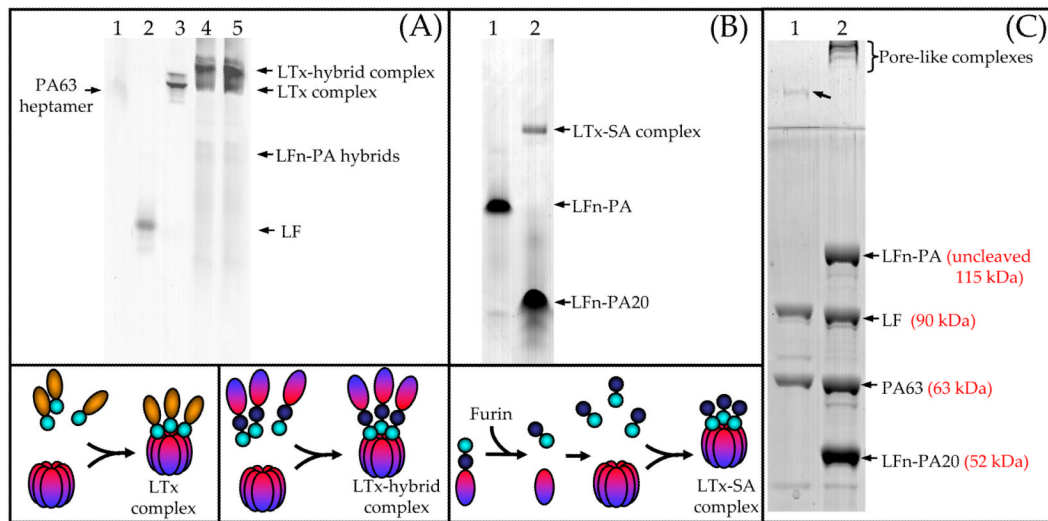
Fig. (2).
The LFn-PA hybrid domains retain their binding functions. (**A**). The LFn domain of the hybrids bind to the PA63 heptamer. Lanes: 1, PA63 heptamer; 2, LF; 3, LF + PA63 heptamer; 4, LFn-PA + PA63 heptamer; 5, LFn-PA$_{CM}$ + PA63 heptamer. (**B**). Furin-cleaved LFn-PA self-assembles into an LTx-hybrid complex. Lanes: 1, LFn-PA; 2, LFn-PA + furin; (**C**). Cleaved LFn-PA forms SDS-resistant pore-like complexes. Lanes: 1, LF + furin-cleaved PA; 2, LF + furin-cleaved LFn-PA. In the lower panels, the components used in the binding assays and the complexes formed are shown as schematics as per Fig. 1(A). See Materials and Methods for details on binding assays. The samples in panels **A** and **B** were electrophoresed in a native 4-12% linear gradient polyacrylamide gel containing no SDS or β-mercaptoethanol, and the samples in panel C were electrophoresed on a 10% SDS-polyacrylamide gel. The gels were then stained with Coomassie blue G-250 (panels **A** and **B**) or R (panel **C**). Note that the PA63 heptamer migrates as a diffused band in the unbound form (panel **A**, lane 1) and as a sharp band upon binding to LF (or LFn) (panel **A**, lane 3). This is most likely because the heptamer is in a conformationally flexible state unless bound to its binding partner.
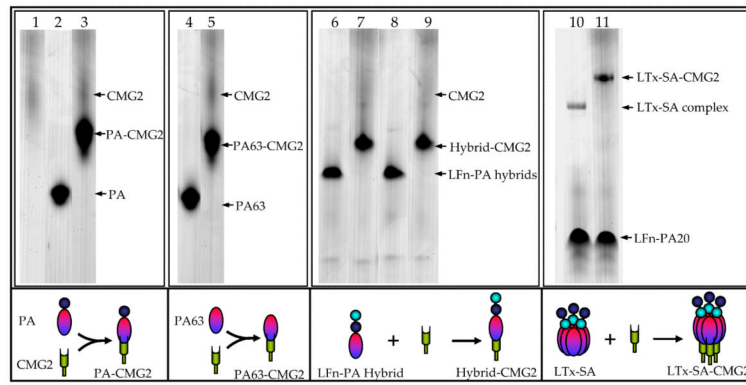
Fig. (3).
The LFn-PA hybrids bind to the soluble extracellular domain of the host receptor, CMG2. The PA components used in the binding reaction with CMG2 are shown as schematics in the lower panels. The samples after the respective treatments were subjected to electrophoresis on native 4-12% linear gradient polyacrylamide gel. The gels were stained with Coomassie blue G-250. Note that CMG2 migrates as a broad smear (lane 1), which might be because the soluble (membrane-free) protein is in a conformationally flexible state unless bound to PA. The proportion of LFn-PA20 vs. LTx-SA/CMG2 complexes (lanes 10 and 11) is different because some of the complexes that are quite large and heterogeneous either precipitated or did not enter the native gel.
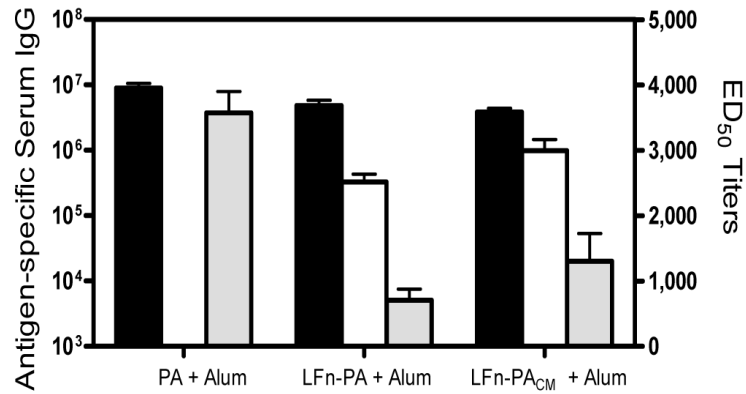
Fig. (4).

Immunogenicity of LFn-PA constructs in Dutch-belted rabbits. Rabbits (6 rabbits/group for LFn-PA and LFn-PA$_{CM}$ and 5 rabbits/group for PA) were immunized with the antigens as described in Materials and Method. Serum PA-specific (black bars; left axis) and LF-specific (open bars; left axis) IgG titers were determined by ELISA at week 10 following the primary immunization. LTx neutralizing Ab titers (ED$_{50}$; gray bars; right axis) were determined using the mouse macrophage-derived J774A.1 cells and colorimetric cytotoxicity assay [33]. The bars represent arithmetic mean antibody titers ±SEM.
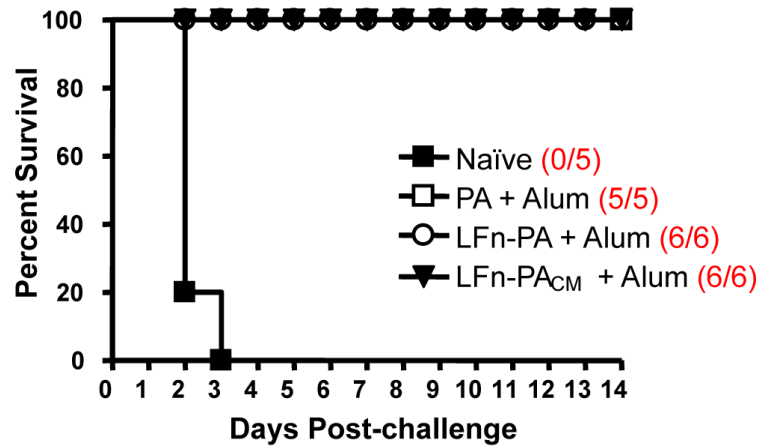
Fig. (5).
Survival curve of Dutch-belted rabbits challenged intranasally with *B. anthracis* Ames strain spores. Rabbits (6 rabbits/group for LFn-PA and LFn-PA$_{CM}$ and 5 rabbits/group for PA) were immunized with the antigens as described in Materials and Methods. Rabbits were challenged with 100 LD$_{50}$ of *B. anthracis* Ames strain spores intranasally at week 10 following the primary immunization. Rabbits were evaluated twice a day for the first week and then daily.