Protein- and DNA-based anthrax toxin vaccines confer protection in guinea pigs against inhalational challenge with *Bacillus cereus* G9241

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This is a straight-forward approach to examine the ability to protect guinea pigs from a spore challenge with the ‘anthrax like’ *Bacillus cereus* strain G9241 with DNA and protein-based anthrax toxin vaccines.

Keywords
inhalation anthrax; DNA vaccine; recombinant protein vaccine; immunization.

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Abstract
In the past decade, several *Bacillus cereus* strains have been isolated from otherwise healthy individuals who succumbed to bacterial pneumonia presenting symptoms resembling inhalational anthrax. One strain was indistinguishable from *B. cereus* G9241, previously cultured from an individual who survived a similar pneumonia-like illness and which was shown to possess a complete set of plasmid-borne anthrax toxin-encoding homologs. The finding that *B. cereus* G9241 pathogenesis in mice is dependent on *pagA1*-derived protective antigen (PA) synthesis suggests that an anthrax toxin-based vaccine may be effective against this toxin-encoding *B. cereus* strain. Dunkin Hartley guinea pigs were immunized with protein- and DNA-based anthrax toxin-based vaccines, immune responses were evaluated and survival rates were calculated after lethal aerosol exposure with *B. cereus* G9241 spores. Each vaccine induced seroconversion with the protein immunization regimen eliciting significantly higher serum levels of antigen-specific antibodies at the prechallenge time-point compared with the DNA–protein prime-boost immunization schedule. Complete protection against lethal challenge was observed in all groups with a detectable prechallenge serum titer of toxin neutralizing antibodies. For the first time, we demonstrated that the efficacy of fully defined anthrax toxin-based vaccines was protective against lethal *B. cereus* G9241 aerosol challenge in the guinea pig animal model.

Until recently, *Bacillus anthracis* and *B. cereus* were only distinguished by pathogen status as they share a close phylogeny and both belong to the *B. cereus sensu lato* group (Kolstø et al., 2009). *B. anthracis*, the etiologic agent of anthrax, is an obligate pathogen in many animals including humans. *B. cereus* exposure conversely is usually restricted to nonsevere infections often via ingestion of contaminated food, with serious illness restricted to immunocompromised hosts. However, several recent cases of fatal *B. cereus* infections have been documented in otherwise healthy individuals. In these cases, strains of *B. cereus* were isolated from blood and tissue samples from five metal workers who succumbed to a pneumonia-like illness with symptoms resembling inhalational anthrax (Miller et al., 1997; Hoffmaster et al., 2006; Avashia et al., 2007; Wright et al., 2011). Of the five patients, two died in Louisiana in 1996 and 1997 and three died in Texas (two in 2003 and one in 2011). One of the *B. cereus* strains was indistinguishable from another strain, *B. cereus* G9241, which had previously been isolated from another Louisiana welder who survived a pulmonary anthrax-like disease in 1994 (Hoffmaster et al., 2004). Subsequent genomic characterization of *B. cereus* G9241 revealed the presence of two mega plasmids, pBCXO1 and pBC218. pBCXO1 is highly syntenic with a 99.6% similarity to the *B. anthracis* virulence plasmid pXO1 and encodes a complete set of *B. anthracis* toxin homologs: protective antigen (PA, *pagA1*), lethal factor (LF, *lef1*), and edema factor (EF, *cya1*). pBCXO1 also encodes a homolog of AtxA (*atxA1*) that is known to function in *B. anthracis* as a gene regulator modulating toxin and poly-γ-D-glutamic acid
capsule (PDGA) expression (Hammerstrom et al., 2011) and possesses, unlike \textit{B. anthracis} pXO1, a functional hasACB operon that directs synthesis of a hyaluronic acid (HA) capsule (Oh et al., 2011). pBC218 lacks synteny to any known \textit{B. cereus} plasmid, and although harboring genes for additional copies of PA as well as a truncated LF and AbxA, the pBC218-encoding proteins exhibit only 60%, 36%, and 78% amino acid identities, respectively, compared with the \textit{B. anthracis} homologs (Hoffmaster et al., 2004). pBC218 also possesses the bpsX-H operon that directs synthesis of BPS, a second polysaccharide-based capsule (Oh et al., 2011).

To date, the relative virulence of \textit{B. cereus} G9241 spores compared to \textit{B. anthracis} strains has been evaluated in rabbit and mouse models. In New Zealand rabbits, \textit{B. cereus} G9241 is essentially avirulent for exposure via the subcutaneous route or attenuated 100-fold for inhalation-mediated infection compared with the corresponding routes and doses of fully virulent \textit{B. anthracis} Ames (Wilson et al., 2011). In the mouse model, \textit{B. cereus} G9241 infection in both immune-competent C57BL/6 mice and complement-deficient A/J mice is lethal and elicits an anthrax-like disease progression. Challenge studies in both mouse strains indicate \textit{B. cereus} G9241 is less virulent than \textit{B. anthracis} Ames, with 50% lethal dose (LD$_{50}$) values 50-fold to 400-fold greater for intranasal and subcutaneous delivery, respectively (Wilson et al., 2011). In contrast, compared to \textit{B. anthracis} Sterne, an attenuated strain that lacks the PDGA capsule-encoding virulence plasmid pXO2, \textit{B. cereus} G9241 LD$_{50}$ values were either similar for subcutaneous challenge or c. fivefold greater for intranasal delivery (Wilson et al., 2011). Mortality rates in mice infected with G9241 strains that were cured for one or both of the virulence plasmids, deleted for the pBCXO1-borne PA encoding gene (\textit{pagA1}) or HA-encoding operon (\textit{hasACB}) or the pBC218-borne BPS encoding operon (\textit{bpsX-H}), indicate expression of toxin and both capsules is necessary for full virulence (Oh et al., 2011; Wilson et al., 2011). In subcutaneous challenge studies with plasmid-cured strains using A/J mice, only a \textit{B. cereus} G9241 pBCXO1-\textit{pagA1} derivative caused mortality at a dose $> 3$ logs higher than the wild-type LD$_{50}$ value (Wilson et al., 2011). C57BL/6 mice challenged intraperitoneally with 30× LD$_{50}$ wild-type equivalents of \textit{B. cereus} G9241 variants either deleted for the toxin subunit PA or one or both of the HA and BPS capsules exhibited attenuated phenotypes with mortality rates of 50% (\textit{hasACB} and \textit{bpsX-H}) or 60% (\textit{hasACB} and \textit{bpsX-H}), compared with the wild-type parent strain (Oh et al., 2011). Deletion of both capsules (\textit{hasACB} and \textit{bpsX-H}) rendered \textit{B. cereus} G9241 spores avirulent (Oh et al., 2011).

Given the recent fatalities and wide geographical distribution of \textit{B. cereus} G9241-mediated anthrax-like illness, the identification of effective medical countermeasures against future similar cases is urgent. The finding that \textit{B. cereus} G9241 pathogenesis in mice is dependent on \textit{pagA1}-derived PA synthesis suggests an anthrax vaccine formulated using the PA subunit may be efficacious against exposure to a toxin-encoding \textit{B. cereus} strain (Oh et al., 2011). Currently, most anthrax vaccines are toxin-based. The FDA-licensed anthrax vaccine BioThrax®, also known as AVA (Anthrax Vaccine Adsorbed, Emergent Biosolutions, Rockville, MD), contains a PA-aluminum hydroxide-adsorbed precipitate derived from a \textit{B. anthracis} pXO1+ pXO2+ culture supernatant, and rPA-based next-generation anthrax vaccines are currently undergoing clinical trials (Brown et al., 2010; Bellanti et al., 2012). In addition, vaccine-engineered plasmids expressing PA and LF can protect A/J mice against lethal challenge with aerosolized \textit{B. anthracis} spores (Albrecht et al., 2012a, b). Recent work has also demonstrated that immunization with formalin-inactivated spores derived from nontoxicigenic, nonencapsulated \textit{B. cereus} G9241 strains (pBCXO1−, pBC218−) containing the immunodominant spore protein BclA (dcG9241) or a deletion mutant (dcG9241 \textit{bclA}) conferred significant protection in mice against spore challenge with \textit{B. anthracis} Ames or Ames \textit{bclA} (Vergis et al., 2013).

The viability of using an anthrax PA-based vaccine as a prophylactic against \textit{B. cereus} G9241 infection was recently demonstrated (Oh et al., 2013). AVA-immunized C57BL/6 mice exhibited robust protection against exposure to \textit{B. cereus} G9241 spores via both the intraperitoneal (100% survival at 37× LD$_{50}$) and aerosol (90% survival at 30× LD$_{50}$) routes of infection. Consequently, we decided to extend the above observations by evaluating the protective efficacy of PA and LF recombinant protein- and DNA-based anthrax toxin subunit vaccines in a second aerosol-based larger animal model. In this study, we utilized Dunkin Hartley guinea pigs as our animal model against challenge with \textit{B. cereus} G9241 spores.

DNA and recombinant protein PA and LFn (first 254 amino acids of LF) subunit vaccines were prepared as described elsewhere (Albrecht et al., 2012a) with the following modifications. DNA sequences encoding PA and LFn were codon-optimized for human expression using NMRC-patented methodology (Goldman & Albrecht, 2011). The optimized genes were then synthesized by Gene Oracle Inc. (Mountain View, CA) and transferred to the DNA vaccine plasmid pDNAVACCultra2 (Nature Technology Corporation, Lincoln, NE) to produce pPAho and pLFnho. The DNA vaccines were administered intramuscularly at 100 µg plasmid per vaccination event (100 µL of vaccine plus 100 µL of adjuvant were combined, divided in half, then injected into each quadriceps). The lipid-based adjuvant was formulated by combining PE (1,2-Diphytanoyl-sn-Glycero-3-Phosphoethanolamine) and DDAB (Dimethyldioctadecylammonium bromide) at a PE-to-DDAB ratio of 4:1 (Avanti Polar Lipids Inc., Alabaster, AL). rPA and rLFn were administered subcutaneously at 50 µg per vaccination event with Imject® Alum adjuvant (200 µg protein plus 200 µL adjuvant). Groups of guinea pigs (\textit{n} = 11 for groups treated with rPA, rLFn, pPAho + rPA; \textit{n} = 10 for group treated with pLFnho + rLFn) were vaccinated on days 0, 14, and 28; for each DNA vaccination regimen, a protein boost of 50 µg of the corresponding recombinant protein was administered subcutaneously on Day 14 in addition to the DNA vaccine. Control groups (\textit{n} = 6) were given either empty pDNAVaccultra2 vector or saline. On Day 42, a six-jet Collison nebulizer (BGI, Waltham, MA) in conjunction with a CH Technologies
nose-only inhalation exposure system (Westwood, NJ) was used to aerosol challenge each group of guinea pigs with a target dose of $2.5 \times 10^7$ B. cereus G9241 spores [500 × LD$_{50}$ B. anthrax Ames equivalents (Savransky et al., 2013)]. Each total-inhaled dose was determined from impinger sample concentration, sampling parameters, and exposure duration. Mean-calculated inhaled doses as colony-forming units (CFU) for each group were as follows: rPA, 2.35 $\times 10^7$ CFU; rLFn, 2.54 $\times 10^7$ CFU; pPAho-rPA, 2.55 $\times 10^7$ CFU; pLFnho-rLFn, 2.52 $\times 10^7$ CFU; saline, 2.49 $\times 10^7$ CFU and empty vector, 2.59 $\times 10^7$ CFU.

Serum from blood samples collected via the cranial vena cava was used in ELISAs and toxin neutralization assays to determine antigen-specific IgG titers (for sera obtained on days 0, 41, 44, 49, and 56) and the ability to neutralize anthrax toxin (for sera obtained on Days 41 and 56), respectively, as previously described (Albrecht et al., 2007).

Consistent with previous work in guinea pigs and other animal models (Price et al., 2001; Reuveny et al., 2001; Galloway et al., 2004; DuBois et al., 2007; Albrecht et al., 2012b; Cote et al., 2012), immunization with each of the PA groups a Day 14 of the DNA vaccination regimen, a 50 µg protein boost of the corresponding subunit vaccine was also administered. Negative controls were either saline or empty vector. Anti-PA and anti-LF serum titers were determined for the days indicated for the protein (a) and DNA–protein prime-boost (b) vaccination schedules. Prechallenge (Day 41) and end of study (Day 56) TNA serum titers (c). Percentage of guinea pig survival rate on Day 56 after challenge on Day 42 with a target dose of $2.5 \times 10^7$ B. cereus G9241 spores (d).

Table 1 TNA serum fractions compared with total antigen-specific IgG levels for day 41 and day 56

<table>
<thead>
<tr>
<th>Vaccine treatment</th>
<th>Day 41</th>
<th>Day 56</th>
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<tbody>
<tr>
<td>rPA</td>
<td>51 (684 : 1329)</td>
<td>81 (1557 : 1923)</td>
</tr>
<tr>
<td>rLFn</td>
<td>9 (79 : 874)</td>
<td>20 (225 : 1137)</td>
</tr>
<tr>
<td>pPAho-rPA</td>
<td>6 (4 : 72)</td>
<td>42 (957 : 2291)</td>
</tr>
<tr>
<td>pLFnho-rLFn</td>
<td>ND</td>
<td>3 (90 : 2828)</td>
</tr>
</tbody>
</table>

ND, Not detected.
and LF vaccines induced seroconversion (Fig. 1a and b). However, the antigen-specific IgG synthesis profiles for the protein and DNA–protein prime-boost vaccination regimens were different. On Day 41, the prechallenge time-point, anti-PA IgG, and anti-LF IgG levels were 18- and 43-fold greater for the recombinant protein vaccines, respectively, compared with each corresponding DNA vaccine. For the postchallenge protein vaccination time-points, with the exception of a modest rise in the anti-PA IgG level observed at the study’s conclusion on Day 56, anti-PA and anti-LF IgG levels did not significantly change (P = 0.05). In contrast, while antigen-specific IgG responses elicited from the DNA–protein prime-boost vaccinations at Day 44 remained low, by Day 49 and Day 56, anti-PA and anti-LF IgG serum titers had increased significantly to levels similar or exceeding those found for the corresponding protein vaccines, suggesting that the DNA–protein prime-boost vaccination schedule had induced a memory response in those guinea pigs that survived.

Previous findings in rabbits, guinea pigs, and mice have demonstrated that the toxin neutralizing antibody (TNA) fractions of anti-PA and anti-LF serum titers constitute a major component of protective immunity against anthrax and can function as a surrogate marker for protection (Pitt et al., 1999, 2001; Reuveny et al., 2001; Staats et al., 2007; Chun et al., 2012). In parallel with the results observed for total antigen-specific IgG levels, the TNA serum fraction elicited by each vaccine was also significantly different and ranged immediately prior to challenge at Day 41 from 51% and 9% for rPA and rLF, respectively, to 6% for the pPAHo-rPA prime-boost vaccination schedule (Fig. 1c and Table 1). At the prechallenge time-point, there was no detectable TNA response from the pLFhno-rLFn prime-boost series of vaccinations. Guinea pig survival rate subsequent to aerosol challenge on Day 42 with B. cereus G9241 spores was associated with the presence of detectable prechallenge levels of serum TNA titers (Fig. 1c and d). After pathogen challenge, all guinea pigs in the control groups that received either saline or empty DNA vector succumbed within 3–4 days, a time to death interval c. 1 day more than that determined for guinea pigs aerosol challenged with an equivalent dose of B. anthracis Ames spores (Savransky et al., 2013). Protein and DNA–protein vaccinated animals conversely showed either partial (i.e. 30% for the animals administered pLFhno-rLFn, P < 0.05) or complete protection (in the groups administered rPA, rLF, or pPAHo-rPA, P < 0.0001).

End of study TNA serum titers were also elevated, compared with prechallenge levels, with a detectable TNA response now also present in the group vaccinated with pLFhno-rLFn. Final percentage of TNA serum titers, compared with total antigen-specific IgG levels, were 81% and 20% for rPA and rLFn, respectively, and 42% and 3% for pPAHo-rPA and pLFhno-rLFn, respectively (Fig. 1c and Table 1). The data from this study suggest that even though the TNA serum titers generated by the rLFn and pPAHo-rPA vaccination regimens were ninefold (79 μg mL⁻¹) and 171-fold (4 μg mL⁻¹) lower, respectively, than that for the rPA (684 μg mL⁻¹) vaccination schedule, they were still sufficient to confer complete protection from challenge and suggest a threshold serum TNA level to achieve robust protection against B. cereus G9241 spore challenge for this study’s experimental conditions. Recently, it has been demonstrated that mice immunized with the FDA-approved AVA vaccine are protected against challenge with B. cereus G9241 spores (Oh et al., 2013). Here, we demonstrate for the first time that fully defined DNA- and protein-based anthrax toxin vaccines can also confer protection against lethal aerosol challenge with B. cereus G9241 spores in Dunkin Hartley guinea pigs.

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