

# Combining Anthrax Vaccine and Therapy: a Dominant-Negative Inhibitor of Anthrax Toxin Is Also a Potent and Safe Immunogen for Vaccines

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**Anthrax is caused by the unimpeded growth of *Bacillus anthracis* in the host and the secretion of toxins. The currently available vaccine is based on protective antigen (PA), a central component of anthrax toxin. Vaccination with PA raises no direct immune response against the bacilli and, being a natural toxin component, PA might be hazardous when used immediately following exposure to *B. anthracis*. Thus, we have sought to develop a vaccine or therapeutic agent that is safe and eliminates both secreted toxins and bacilli. To that end, we have previously developed a dually active vaccine by conjugating the capsular poly- $\gamma$ -D-glutamate (PGA) with PA to elicit the production of antibodies specific for both bacilli and toxins. In the present report, we describe the improved potency of anthrax vaccines through the use of a dominant-negative inhibitory (DNI) mutant to replace PA in PA or PA-PGA vaccines. When tested in mice, DNI alone is more immunogenic than PA, and DNI-PGA conjugate elicits significantly higher levels of antibodies against PA and PGA than PA-PGA conjugate. To explain the enhanced immunogenicity of DNI, we propose that the two point mutations in DNI may have improved epitopes of PA allowing better antigen presentation to helper T cells. Alternatively, these mutations may enhance the immunological processing of PA by altering endosomal trafficking of the toxin in antigen-presenting cells. Because DNI has previously been demonstrated to inhibit anthrax toxin, postexposure use of DNI-based vaccines, including conjugate vaccines, may provide improved immunogenicity and therapeutic activity simultaneously.**

The deadly threat posed by the potential use of anthrax spores as a biological weapon in terrorism and warfare urgently calls for effective means of protection against anthrax. Anthrax is an acute infectious disease caused by the spore-forming gram-positive bacterium *Bacillus anthracis* (2, 9, 16). The infection is initiated by the entry of *B. anthracis* spores into the host through abrasions in the skin, ingestion, or inhalation. Once in the host, anthrax spores are taken up as inert particles by local macrophages, where they germinate and transform into vegetative bacilli (16). Bacilli are then released by macrophages into regional lymph nodes and start replicating extracellularly within the lymphatic system, causing regional hemorrhagic lymphadenitis. Subsequently, bacilli spread to the bloodstream and continue multiplying rapidly, reaching concentrations as high as  $10^8$  bacilli per ml of blood and secreting large quantities of toxins (9, 10). Extensive bacteremia and toxemia develop within a few days of infection and rapidly kill the host (1, 22).

The virulence of *B. anthracis* is attributed to two major factors: its poly- $\gamma$ -D-glutamic acid (PGA) capsule and a tripartite toxin (8, 9, 28). PGA is weakly immunogenic (23) and antiphagocytic (29, 30). The encapsulation of bacilli by PGA disguises the bacilli from the host immune surveillance and

prevents phagocytosis by the host. We have previously hypothesized that full immunity to *B. anthracis* will require an immune response to PGA (9). Humoral immunity to PGA should provide a very early barrier to the replication of PGA-encapsulated bacilli. To this end, we have recently developed a PGA-based conjugate vaccine that elicits high levels of protective antibodies to PGA (23). This vaccine and another analogous vaccine induce antibodies that can opsonize PGA-encapsulated bacilli and facilitate their killing by host complement or phagocytes (23, 25).

*B. anthracis* releases three discrete, nontoxic proteins, namely, protective antigen (PA), lethal factor (LF), and edema factor (EF). The complexing of either LF or EF with PA forms lethal and edema toxins, respectively (8). Upon release, PA molecules bind to host cell receptors (5, 24) and the cell-bound portion self-assembles to heptamers (13). LF and EF bind competitively to PA heptamers and are subsequently transported by PA to the cytosol, where LF and EF exert their enzymatic activities and damage the cells. Given its central role in anthrax toxin action, PA is the major immunogen in the anthrax vaccines that are currently licensed for human use in the United States (2, 12). Antibodies to PA neutralize anthrax toxin by blocking adherence of PA to host cells, binding of LF/EF to PA, or assembly of PA heptamer.

Postexposure vaccines based on PA alone are limited in several respects. First, since PA is a natural component of anthrax toxin, it may not be safe to administer PA-based vac-

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cine to people who have been or are suspected of having been exposed to anthrax recently. Although postexposure vaccination could offer protection from later onset of anthrax disease, administered PA could participate in toxin formation and would therefore be unsafe in these situations. Furthermore, anthrax spores may remain in the host for an extensive period of time, i.e., up to 60 days (10), and it is unclear whether PA-based vaccines have any direct effect on bacilli or spores or simply protect host antibacterial immune defenses from the effect of the toxin. In theory, the best vaccine against anthrax would be one which prevents bacteremia before the elaboration of toxins. With this goal, we and others have developed improved, dually active anthrax vaccine by conjugating PGA to PA (7, 23). PGA-PA conjugates induce the production of protective antibodies against both the capsular PGA and the toxin component PA and hence confer simultaneous protection against both anthrax bacilli and secreted toxins. In the present study, we describe significant improvements in safety and immunogenicity of anthrax vaccines by the replacement of PA with a dominant-negative inhibitor (DNI) of anthrax toxin.

DNI is a translocation-deficient mutant of PA with double mutations of K397D and D425K (26). This mutant co-oligomerizes with wild-type PA and potently blocks the translocation process, thereby inhibiting toxin action. DNI can assemble with PA molecules into heptamers that can still bind LF/EF. However, chimeric DNI/PA heptamers are not capable of transporting LF or EF into the cell cytosol, thus preventing the cell damage caused by LF/EF (26). DNI interferes with the intoxication process and provides immediate protection against anthrax toxins *in vivo* (26). Aside from the therapeutic potential of DNI, we explored the immunogenicity of DNI and DNI-based PGA conjugate vaccines. As shown in the present study, DNI is a stronger immunogen than PA. DNI and DNI-PGA could therefore be used as a two-in-one therapy/vaccine for anthrax. Conjugation of DNI to PGA significantly boosts the antibody response to PGA, and thus we propose that DNI could serve as an excellent safe, general-purpose carrier for poor immunogens.

#### MATERIALS AND METHODS

**Preparation of PA and DNI.** Recombinant wild-type PA and mutant DNI were cloned into the pET-22b(+) expression vector (Stratagene, La Jolla, CA), transformed into *Escherichia coli* BL21\*(DE3) (Novagen, Madison, WI), and expressed as periplasmic proteins (3, 27). Bacterial cultures were grown at 37°C in LB broth containing 100 µg/ml of carbenicillin until the optical density at 600 nm reached 0.6 to 0.8. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside at 30°C for 4 h. Cells were harvested by centrifugation, and periplasmic proteins were extracted from the cells as follows. To each gram of cell pellet, 4 ml of buffer containing 20% sucrose, 20 mM Tris · HCl, and 1 mM EDTA (pH 8.0) was added, and the suspension was stirred gently at room temperature for 15 min. After centrifugation, cell pellets were resuspended in 5 mM MgSO<sub>4</sub> at 4°C for 15 min. The mixture was then diluted with 20 mM Tris · HCl (pH 8.0), and proteins in the supernatant were collected after centrifugation. PA or DNI was then purified on anion-exchange and gel filtration columns. Purity and molecular size of PA and DNI were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

**Isolation and degradation of PGA.** PGA was prepared from *Bacillus licheniformis* ATCC 9945a as previously described (19, 23). Briefly, highly mucoid colonies were selected from E medium agar plates and cultured in liquid E medium at 37°C for 96 h. Each liter of E medium contained 20.0 g L-glutamic acid, 12.0 g citric acid, 80.0 g glycerol, 7.0 g NH<sub>4</sub>Cl, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.5g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.04 g FeCl<sub>3</sub> · 6H<sub>2</sub>O, 0.15 g CaCl<sub>2</sub> · 2H<sub>2</sub>O, and 0.104 g MnSO<sub>4</sub> · H<sub>2</sub>O. The viscous bacterial culture was centrifuged at 6,500 × g for 20 min, and the PGA-containing supernatant was collected. PGA was precipitated from the su-

pernatant with 3 volumes of ethanol at 4°C overnight. Crude PGA samples were collected by centrifugation and dialyzed against deionized water. The PGA solution was acidified to pH 1.5 with 6 M HCl and immediately precipitated with 3 volumes of 1-propanol at -20°C. PGA was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified PGA was then dissolved in water, dialyzed extensively, and lyophilized. The purity of PGA was verified by UV-Vis scanning from 190 to 300 nm, and its structure was verified by <sup>1</sup>H nuclear magnetic resonance spectroscopy.

Because the average molecular mass of native PGA is rather large (~500 kDa), its conjugation with DNI or PA could result in an insoluble gel. PGA was therefore degraded to an average molecular size of ~10 kDa. The degradation was accomplished by alkaline hydrolysis of 10 mg of PGA with 1 ml of 2 M NaOH at 80°C for 80 min. The molecular sizes of PGA and degradation products were monitored by a Superose 12 column that had been calibrated with dextran standards (Amersham Biosciences, Piscataway, NJ). PGA fragments corresponding to a molecular mass of ~10 kDa were collected from the gel filtration column, dialyzed against water, and lyophilized.

**Synthesis of PGA-protein conjugates.** PGA-PA and PGA-DNI conjugates were synthesized in parallel by the same procedure as follows. To 1 mg of DNI in 0.5 ml phosphate-buffered saline (PBS; pH 7.0), 0.5 mg of degraded PGA was added. After PGA had dissolved, 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added, and the solution was mixed on a rotary shaker at 25°C for 4 h. The conjugation process was monitored by SDS-PAGE (9). The conjugates were purified on a PD10 column (Amersham Biosciences), verified by SDS-PAGE, and stored at -20°C until use. For enzyme-linked immunosorbent assay (ELISA) measurement of PGA-specific antibodies, a conjugate of PGA to bovine serum albumin (BSA) was prepared by the same chemical method.

**Mouse immunization.** Each vaccine candidate was evaluated in a group of eight female BALB/c mice, 6 to 8 weeks of age (Jackson Laboratory, Bar Harbor, ME). Mice were immunized three times by intraperitoneal injection at 2-week intervals. For antibody analysis, sera were obtained before (preimmune) and 1 week after each immunization. Doses of 10 µg (protein content) of DNI, PA, PGA-DNI, and PGA-PA were tested. Each dose was dissolved in 50 µl of PBS and adsorbed to an equal volume of Al(OH)<sub>3</sub> gel adjuvant (equivalent to 0.18 mg/dose). As a negative control, a group of mice were injected with PBS-Al(OH)<sub>3</sub> on the same immunization schedule.

**Antibody measurement.** Serum levels of anti-PA and anti-PGA immunoglobulin G (IgG) and IgM were determined by ELISA. Immulon 96-well Maxisorp plates (Nalge Nunc, Rochester, NY) were coated with 2.5 µg/ml of PA or PGA-BSA conjugate in 0.1 M carbonate buffer (pH 9.6) at 4°C for 16 h. Serum samples were diluted 1:500 with Tris-buffered saline (50 mM Tris · HCl, 0.15 M NaCl, pH 7.4) and 5% fetal calf serum and tested in duplicate. The plates were incubated at 37°C for 1.5 h and then washed three times. Bound IgG or IgM antibodies were detected with alkaline phosphatase-labeled goat anti-mouse IgG or rat anti-mouse IgM (Southern Biotech, Birmingham, AL). The plates were developed with *p*-nitrophenylphosphate (Sigma), and their absorbances were determined at 405- and 630-nm wavelengths. Antibody isotypes were determined similarly, except that alkaline phosphatase-labeled anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotech) was used as the secondary antibody. Specific immunoglobulin concentrations were determined by comparison with standard curves. Standard curves were obtained for each plate using goat F(ab')<sub>2</sub> anti-mouse Ig as the capturing agent and known concentrations of mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 as standards.

**Competitive ELISA inhibition.** Similar to the measurement of anti-PA IgG described above, microtiter plates were coated with 2.5 µg/ml of PA at 4°C for 16 h and then blocked with 100 µg/ml of BSA at 30°C for 1 h. Serum samples were mixed with PA inhibitor in 1:4 serial dilutions and then added to the microtiter wells. After plates were incubated at 37°C for 1 h and washed three times, bound IgG was detected as described above. The percentage of inhibition was calculated.

**Mouse challenge and protection.** To verify the protective activity of DNI and PGA-DNI conjugate as anthrax vaccine, we tested whether immunization with these agents protected mice against challenge with lethal toxin. Two weeks after the third vaccination, each mouse was injected through its tail vein with a mixture of 48 µg of PA and 20 µg of LF (List Biologicals, Campbell, CA), the equivalent of approximately four times the 50% lethal dose of lethal toxin in mice. Without exception, unprotected mice died within 36 h. Protected mice were monitored closely for 2 weeks.

**Statistical analysis.** The Mann-Whitney test was employed to assess the statistical significance of differences between independent sample groups. A two-sided probability value of *P* < 0.05 was considered statistically significant.

## RESULTS

**DNI is more potent than PA as an anthrax vaccine.** PA, a central component of anthrax lethal and edema toxins, is the principal antigen of the currently licensed anthrax vaccine in the United States. However, being a functional part of the anthrax toxins, PA should not be administered during or shortly after anthrax infection. This limitation is an especially serious concern during a large-scale public anthrax attack when vaccination of a population is desired to prevent further infection but administration of PA is not safe without knowing whether individuals have been exposed to anthrax. However, DNI could be safely administered in this situation. DNI could act therapeutically to inhibit ongoing intoxication or as a vaccine to prevent intoxication in a future infection. The potential for using DNI as a surrogate anthrax vaccine depends on whether DNI retains the antigenicity of PA and displays immunogenicity of similar or even greater magnitude than PA.

To evaluate the potential of DNI for vaccine use, we immunized groups of mice with DNI or PA for comparison. Each mouse was immunized with 10  $\mu\text{g}$  of DNI or PA three times at 2-week intervals. Serum samples were obtained before vaccination and 7 days after each vaccination. Levels of PA-specific IgG and IgM were measured by quantitative ELISA. Both DNI and PA induced high levels of anti-PA IgG (Fig. 1A). Primary immunization with DNI and PA elicited  $0.28 \pm 0.15$  (mean  $\pm$  standard error) and  $0.018 \pm 0.004$   $\mu\text{g}/\text{ml}$  of PA-specific IgG, respectively. After the second injection (the first boost), anti-PA IgG concentrations increased to  $23.6 \pm 3.0$  and  $14.6 \pm 2.9$   $\mu\text{g}/\text{ml}$  in DNI- and PA-immunized mice, respectively. After the third immunization (the second boost), anti-PA IgG levels in DNI-immunized mice increased dramatically, to a range of 138.0 to 367.6  $\mu\text{g}/\text{ml}$ , with a mean value of 271.5, whereas PA induced 36.3 to 79.2  $\mu\text{g}/\text{ml}$  of anti-PA IgG, with a mean value of 64.2. The IgG responses to DNI are statistically significantly higher than those to PA, with  $P$  values of 0.01, 0.038, and 0.0002 after one, two, and three immunizations, respectively. Furthermore, anti-PA IgG produced by either DNI or PA is predominantly IgG1, which is consistent with our previous results (23). Also consistent with our previous findings are the low levels of IgM in mice immunized with DNI or PA (23). The above results illustrate that DNI is highly efficient and more potent than PA in eliciting specific antibodies.

To verify whether DNI retains the antigenicity of PA, we conducted competitive ELISA inhibition experiments to test whether DNI inhibits the specific binding between PA and PA-specific antibodies from PA-immunized mouse sera (Fig. 1B). For this test, microtiter wells were coated with native PA. DNI displayed an inhibition curve nearly identical to that of native PA, whereas the negative control BSA did not inhibit the binding at all. Similar to PA, DNI was able to completely inhibit the binding between PA and PA-specific antibodies. This finding confirms that DNI has virtually the same antigenicity as PA.

**DNI is a potent immunogenic carrier.** Immunization with PA or DNI will induce antibodies that specifically bind PA and neutralize anthrax toxins. Hence, the most direct mode of action of PA or DNI vaccine will be to confer protection against anthrax toxins. To prevent the propagation of *B. an-*

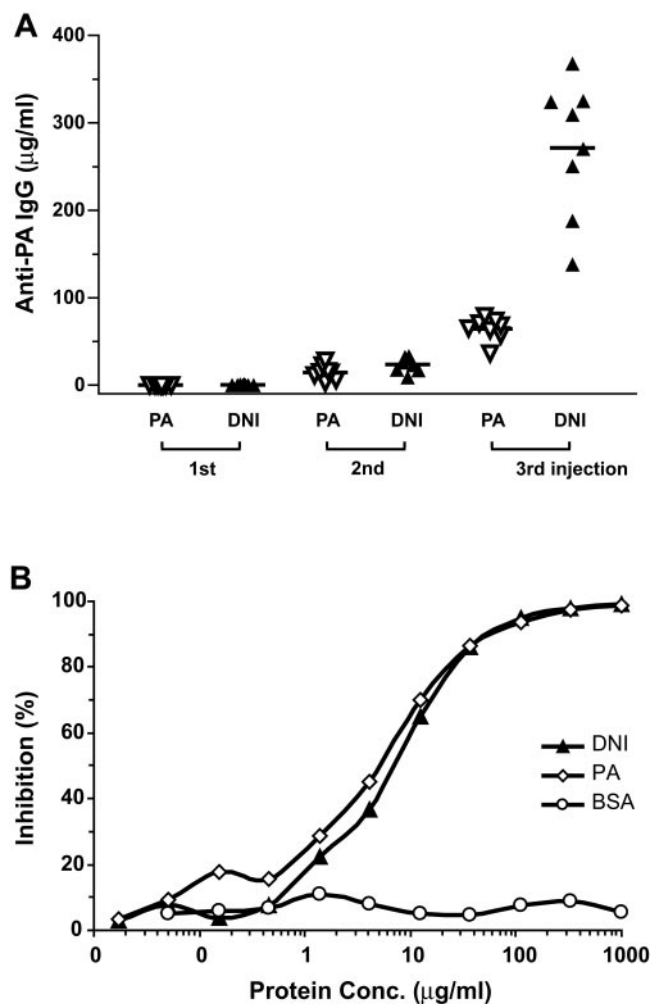


FIG. 1. (A) Serum levels of anti-PA IgG in individual mice after immunization with three doses of DNI and PA. Each point represents one mouse, and bars indicate the mean values for eight mice each. Group means ( $\pm$  standard errors) are, from left to right,  $0.018 \pm 0.004$ ,  $0.28 \pm 0.15$ ,  $14.6 \pm 2.9$ ,  $23.6 \pm 3.0$ ,  $64.2 \pm 4.6$ , and  $271.5 \pm 27.2$   $\mu\text{g}/\text{ml}$  anti-PA IgG, respectively. The first to third injections are the injection after which sera were obtained. (B) Competitive ELISA inhibition demonstrating the antigenic specificity of DNI and PA.

*thraxis* bacteria that produce toxins in the first place, we have recently developed a dually active anthrax vaccine (DAAV) by conjugating PGA to PA to elicit antibodies to both the capsular PGA of bacilli and PA. PA was used in DAAV both as an antigen and as a carrier to enhance the immunogenicity of PGA. To take advantage of the enhanced immunogenicity of DNI over PA, we evaluated whether DNI can replace PA in DAAV to boost the humoral response to PGA.

We prepared two sets of conjugates, PGA-DNI and PGA-PA, by the same chemical method and with the same PGA-to-protein ratio of 1:2 (wt/wt). We then immunized two groups of mice with PGA-DNI or PGA-PA conjugates using the same dose, injection route, immunization schedule, and adjuvant. Sera from immunized mice were analyzed for anti-PGA and anti-PA. Overall, PGA-PA and PGA-DNI conjugates induced strong IgG responses against PA (Fig. 2A) and PGA (Fig. 2B).

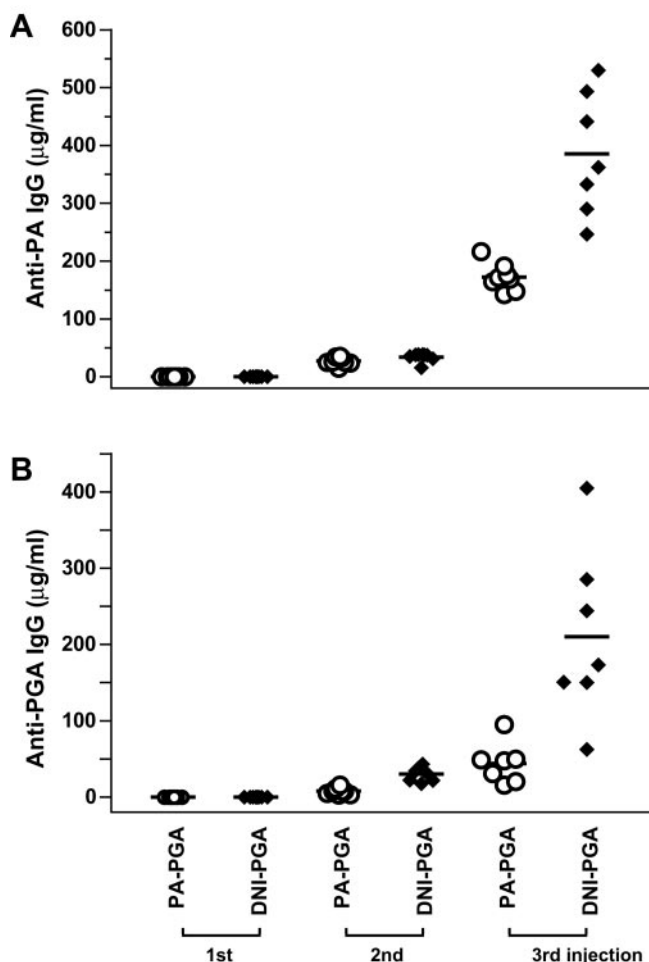


FIG. 2. Serum levels of IgG specific for PA (A) and PGA (B) in mice immunized with PA-PGA or DNI-PGA conjugate. Each point represents one mouse, and bars indicate the mean values for eight mice each. Group means ( $\pm$  standard errors) are, from left to right, (A)  $0.001 \pm 0.002$ ,  $0.002 \pm 0.004$ ,  $27.4 \pm 2.4$ ,  $33.9 \pm 2.8$ ,  $172.6 \pm 8.4$ , and  $385.3 \pm 40.1$   $\mu\text{g/ml}$  anti-PA IgG, respectively, and (B)  $0.001 \pm 0.005$ ,  $0.001 \pm 0.002$ ,  $7.8 \pm 1.5$ ,  $30.3 \pm 3.2$ ,  $44.2 \pm 10.0$ , and  $210.1 \pm 42.2$   $\mu\text{g/ml}$  anti-PGA IgG, respectively. The first to third doses are the doses after which sera were obtained.

After the second and the third injections, anti-PA IgG increased from  $27.4 \pm 2.4$  (mean  $\pm$  standard error) to  $172.6 \pm 8.4$   $\mu\text{g/ml}$  (6.3-fold) in mice immunized with PA-PGA. In mice immunized with DNI-PGA, anti-PA IgG levels increased 11.4-fold from  $33.9 \pm 2.8$   $\mu\text{g/ml}$  after the second dose to  $385.3 \pm 40.1$   $\mu\text{g/ml}$  after the third dose. The anti-PGA IgG response followed a similar trend. After the second injection, anti-PGA IgG reached  $7.8 \pm 1.5$  (mean  $\pm$  standard error) and  $30.3 \pm 3.2$   $\mu\text{g/ml}$  in mice immunized with PA-PGA and DNI-PGA, respectively. After the third injection, anti-PGA IgG in mice

immunized with PA-PGA and DNI-PGA increased 5.6- and 6.9-fold to  $44.2 \pm 10.0$  and  $210.1 \pm 42.2$   $\mu\text{g/ml}$ , respectively. Both anti-PGA and anti-PA IgG levels were significantly higher in mice immunized with DNI-PGA than in those immunized with PA-PGA after each injections (all  $P < 0.05$ ). The enhancement in specific IgG response was most pronounced after three injections. The above result demonstrates that DNI can be used in conjugate vaccines for two purposes: (i) as potentially a safer alternative to PA and (ii) as an immunogenic carrier to enhance the immunogenicity of capsular PGA.

**Vaccination with DNI or DNI-PGA protects mice against lethal toxin challenge.** To test whether immunization with DNI or DNI-PGA conjugate confers protection against anthrax toxins, we challenged groups of mice with a high dose of lethal toxin. Each mouse was injected through its tail vein with a mixture of 48  $\mu\text{g}$  of PA and 20  $\mu\text{g}$  of LF, which is equivalent to four times the 50% lethal dose of anthrax lethal toxin in mice. All mice immunized with DNI, DNI-PGA conjugate, or PA survived the lethal toxin challenge (Table 1). Without exception, unimmunized mice died within 36 h of challenge. Unprotected mice were characterized by minor initial hyperactivity, which was soon followed by body swelling and pronounced moribund lethargy. Protected mice progressed through a short phase of reduced activity to full recovery. It should be noted that existing experimental mouse models of anthrax infection suggest that the bacillar capsule is also an important virulence factor. The role of capsular antigens is not assessed in the present intoxication model. Taken together, the results of the lethal toxin challenge indicate that vaccination with either DNI or DNI-PGA conjugate affords protection against anthrax toxins similar to that provided by PA. We conclude that DNI could thus be used effectively to replace PA in anthrax vaccines.

DISCUSSION

The strong immunogenicity and retained antigenicity of DNI suggest that DNI is a promising and potentially safer candidate for use in anthrax vaccine than PA. Moreover, vaccination with DNI can be boosted more significantly than that with PA (Fig. 1A). While anti-PA IgG levels increased 11.5-fold from the second to the third injections in DNI-immunized mice, this increase was only 4.4-fold in PA-immunized mice. Given that the currently licensed PA-based vaccine, AVA, requires six immunizations over an 18-month period with subsequent yearly boosts (2, 12), the strong booster effect of DNI may reduce the number of immunizations needed and thus could offer another significant advantage as an anthrax vaccine in comparison to PA.

DNI, a double mutant of PA, is a dominant-negative inhibitor of anthrax toxins and by itself provides antitoxic therapy (16). The present study reveals that DNI is also a potent immunogen that can be used in anthrax vaccines either by itself

TABLE 1. Protection of immunized mice against challenge with lethal toxin<sup>a</sup>

Immunization group:	DNI	DNI-PGA	PA	PA-PGA	Control
Protection (no. surviving/no. challenged):	8/8	8/8	8/8	8/8	0/10

<sup>a</sup> Each mouse was challenged intravenously with 55  $\mu\text{g}$  of PA and 20  $\mu\text{g}$  of LF.

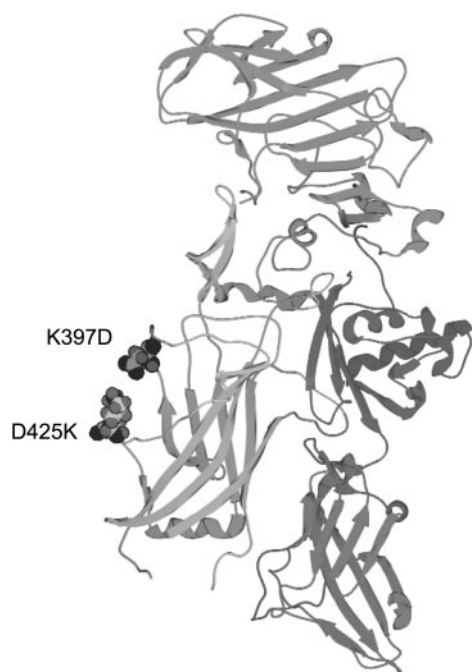


FIG. 3. Molecular model of DNI. The model is modified from the crystal structure of PA. Mutated residues K397D and D425K are illustrated as space-filling models.

or after conjugation with PGA. It is intriguing to ask why these mutations in PA significantly improve the immunogenicity of DNI. PA consists of 735 amino acid residues with a molecular mass of 86 kDa (8). DNI is a mutant of PA with two mutations: Lys<sup>397</sup>→Asp<sup>397</sup> and Asp<sup>425</sup>→Lys<sup>425</sup> (Fig. 3) (26, 27). To explain the enhanced immunogenicity of DNI, we propose two possible mechanisms: a change in immunogenic epitopes affecting presentation and/or a change in cellular fate of DNI affecting antigen processing.

**Change in peptide epitopes?** One possibility is that the mutations in DNI produce a change in peptide epitopes that can bind major histocompatibility complex (MHC) class II molecules and T-cell receptors. If one assumes that the peptides that bind to MHC class II molecules and T-cell receptors are

predominately 12-mers, two mutations would give rise to a total of 24 differential potential epitopes between DNI and PA (Table 2). These peptides differ in their primary sequences. The K397D and D425K mutations reverse the charges on residues 397 and 425, respectively. These changes in charge patterns or specific peptide sequences could allow DNI peptides to fit better than the corresponding PA peptides into the peptide-binding grooves of MHC class II molecules. Such DNI-derived peptides might be presented more efficiently and thereby stimulate helper T cells more effectively. The potential improvement in presentation may explain the increased immunogenicity of DNI compared to PA. It should be noted that this epitope hypothesis is at this point speculative and awaits experimental verification.

**“Endosomal trapping” and more efficient antigen processing of DNI?** A second possible explanation for the enhanced immunogenicity of DNI compared to PA is that DNI might undergo more efficient processing by antigen-presenting cells compared to PA. PA molecules undergo several transformations during the intoxication process (Fig. 4) (8). Upon release, the 83-kDa PA molecules bind to host cellular receptors (24) and are subsequently cleaved N terminally by furin into 20-kDa and 63-kDa (PA<sub>63</sub>) fragments. The cell-bound PA<sub>63</sub> molecules then self-assemble into ring-shaped (PA<sub>63</sub>)<sub>7</sub> heptamers to which LF, EF, or both bind subsequently (21). The heptamers undergo receptor-mediated endocytosis, and in the acidic endosome compartment, (PA<sub>63</sub>)<sub>7</sub> changes its conformation from a ring-shaped core to a membrane-inserting β barrel. This conformational transformation allows (PA<sub>63</sub>)<sub>7</sub> to form an integral-membrane, ion-conductive pore in the endosomal membrane (4, 15, 18). LF and EF then shuttle across the endosomal membrane through the (PA<sub>63</sub>)<sub>7</sub> channel into the cytosol. Native PA is folded into four functionally independent domains, each of which is required for a specific step in the intoxication process (13, 20, 24). Mutated residues 397 and 425 in DNI are located in domain II, the pore-forming domain (17). It has been suggested that these two mutations inhibit the essential conformational change of (PA<sub>63</sub>)<sub>7</sub> from a ring-shaped core to a β barrel and thus prevent the heptamer from inserting itself into the endosomal membrane (27). Consequently, these mutations inhibit the translocation of LF or EF to the cytosol and prevent cytotoxicity (26, 27).

TABLE 2. Sequence comparison of and possible T-cell peptide epitopes of DNI and PA<sup>a</sup>

DNI peptide containing D397	DNI charge pattern	PA charge pattern	DNI peptide containing K425	DNI charge pattern	PA charge pattern
GKNOTLATIKAD	++-	+++	KNLAPIALNAQK	++	+ -
KNQTLATIKADE	+++ -	++++ -	NLAPIALNAQKD	+ -	- -
NOTLATIKADEN	+- -	+- +	LAPIALNAQKDF	+ -	- -
QTLATIKADENQ	+- -	+- +	APIALNAQKDFS	+ -	- -
TLATIKADENQL	+- -	+- +	PIALNAQKDFSS	+ -	- -
LATIKADENQLS	+- -	+- +	IALNAQKDFSST	+ -	- -
ATIKADENQLSQ	+- -	+- +	ALNAQKDFSSTP	+ -	- -
TIKADENQLSQI	+- -	+- +	LNAQKDFSSTPI	+ -	- -
IKADENQLSQIL	+- -	+- +	NAQKDFSSTPIT	+ -	- -
KADENQLSQILA	+- -	+- +	AQKDFSSTPITM	+ -	- -
ADENQLSQILAP	- -	- +	QKDFSSTPITMN	+ -	- -
DENQLSQILAPN	- -	- +	KDFSSTPITMNY	+ -	- -

<sup>a</sup> DNI sequence: <sup>386</sup>GKNOTLATIKADENQLSQILAPNNYPSKLNAPIALNAQKDFSSTPITMNY<sup>436</sup>. PA sequence: <sup>386</sup>GKNOTLATIKAKENQLSQILAPNNYPSKLNAPIALNAQKDFSSTPITMNY<sup>436</sup>.

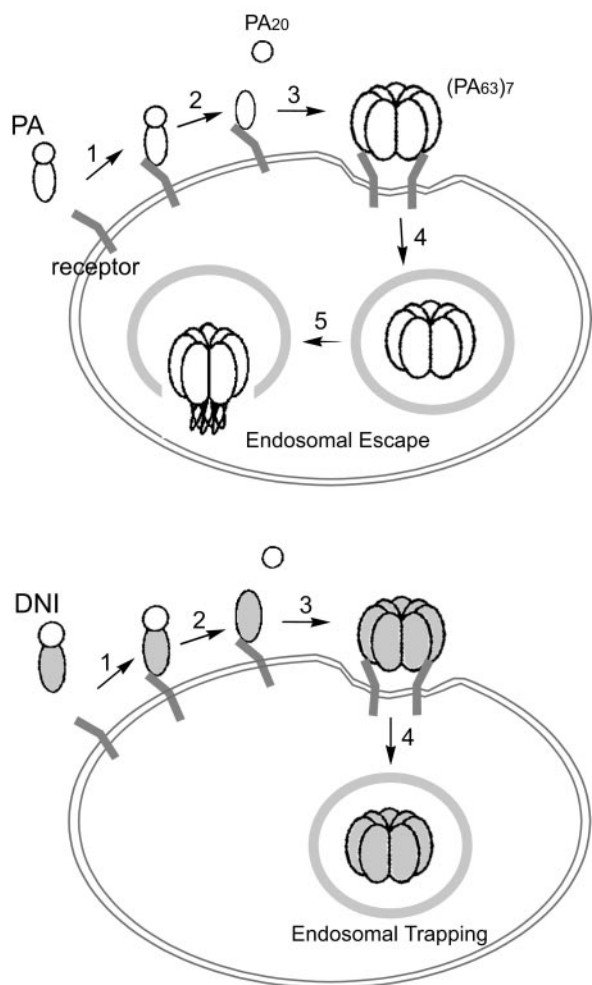


FIG. 4. Models illustrating the different cellular fates of PA and DNI. (Top) Upon binding cellular receptors (step 1), PA is cleaved (step 2). The small fragments diffuse away, and the cell-bound fraction self-assembles into heptameric cores termed  $(PA_{63})_7$  (step 3). The heptamers then undergo receptor-mediated endocytosis (step 4). Once inside the acidic endosomal compartment, the PA heptamers change conformation and insert themselves into the endosomal membrane (step 5). (Bottom) DNI, similar to PA, enters the endosome (steps 1 to 4). However, DNI heptamers do not undergo the necessary conformational changes for insertion into the membrane and therefore remain trapped inside the endosome. The part of DNI that carries the two point mutations is shown in grey.

To elicit T-cell help in antibody production, protein antigens are processed in the endosomes of antigen-presenting cells. The peptides from degraded protein antigens are loaded onto MHC class II molecules and transported to the cell surface. Thus, the insertion of PA pores in the endosomal membrane may disrupt the integrity of the endosome and thus affect the function of the endosome, perhaps leading to altered vesicular trafficking of endocytic PA molecules. Altered vesicular trafficking of PA may affect its delivery to specialized endosomal compartments where antigenic processing of PA occurs. It is also possible that PA pore formation might simply change the pH or ionic environment inside the lumen of the endosome and thus affect the processing/presentation machinery. Furthermore, PA may disrupt or otherwise “leak” out of the en-

dosome and simply enter the cytosol (as is seen for the LF and EF subunits) and thus escape efficient endosomal processing in that way. In contrast, DNI heptamers do not undergo the essential conformational change to insert themselves into the endosomal membrane and block translocation of LF and EF into the cytosol (18). Thus, DNI is expected to be trapped in the endosome and undergo normal vesicular trafficking (Fig. 4). Hence, the translocation-deficient mutant DNI may concentrate in the endosome; that is, DNI may be more prone to endosomal trapping than PA, and this difference in localization may increase in the generation of processed DNI peptides suitable for binding to MHC class II molecules. If such a mechanism explains the enhanced immunogenicity of DNI, it is apparent that similar strategic mutations might be introduced into other toxin immunogens to improve their immunogenicity through this endosomal trapping mechanism.

**Postexposure use of DNI vaccines.** The symptoms and incubation period of human anthrax vary depending on the route of transmission. In inhalational anthrax, the most lethal form of anthrax, the reported incubation period ranges from 1 to 43 days (14). Data from animal studies suggest that anthrax spores persist in the host for several weeks postinfection and that antibiotics can prolong the incubation period for developing disease (2). Studies with nonhuman primates indicate that inhaled spores do not immediately germinate within the alveolar recesses but reside there potentially for weeks until taken up by alveolar macrophages. Spores then germinate and begin replication within the macrophages (11). Development of anthrax disease can be prevented as long as a therapeutic level of antibiotic is maintained to kill germinating bacilli. After antibiotics are discontinued, disease will develop if the nongerminated spores germinate in the absence of a protective immune response (2). In previous animal studies, treatment with antibiotics for 5 or 10 days, beginning 1 day after anthrax spore aerosol challenge, was protective during drug therapy, but animals died after the antibiotic was discontinued (11). Longer antibiotic treatment, e.g., for 30 days, might be necessary to ensure full recovery. However, antibiotic treatment cannot protect against relapse or subsequent exposure to anthrax. Long-term protection was achieved only by combining antibiotic therapy with postexposure vaccination (10).

In such situations, vaccination with DNI-PGA conjugate or DNI would be ideal, whereas administration of PA may be potentially dangerous because it may combine with trace amounts of LF or EF to cause toxicity. During the tragic anthrax attack in the United States in the fall of 2001, some groups of individuals underwent a 60-day regimen of antibiotic prophylaxis. Statistical analysis shows that this preventative measure might have saved many lives (6). In the event of an anthrax attack, the incubation periods will vary among individuals. The timely postexposure administration of DNI as both a conjunctive therapy and prophylactic vaccine would be expected to save lives.

In summary, our study demonstrates significant potential for the use of DNI as a replacement for PA in anthrax vaccines. The replacement of PA with DNI offers several distinct advantages, the major ones being the enhanced immunogenicity and safety of DNI in postexposure situations. In addition, DNI is a potent immunogenic carrier in formulating dually active anthrax vaccines against bacilli and toxins, as illustrated by DNI-

PGA conjugate vaccine. DNI may emerge as an attractive immunologic carrier for haptens in general. Moreover, in the event of anthrax infection, the administration of DNI can serve not only as an antitoxic therapy as an immediate response but also as a prophylactic vaccine to prevent late-onset or future anthrax infection.

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