A dually active anthrax vaccine that confers protection against both bacilli and toxins

Gi-Eun Rhie[†‡], Michael H. Roehrl[§], Michael Mourez[†], R. John Collier[†], John J. Mekalanos[†], and Julia Y. Wang[¶‖]

Departments of [†]Microbiology and Molecular Genetics and [§]Biological Chemistry and Molecular Pharmacology, and [¶]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

Contributed by John J. Mekalanos, July 17, 2003

Systemic anthrax is caused by unimpeded bacillar replication and toxin secretion. We developed a dually active anthrax vaccine (DAAV) that confers simultaneous protection against both bacilli and toxins. DAAV was constructed by conjugating capsular poly-γ-D-glutamic acid (PGA) to protective antigen (PA), converting the weakly immunogenic PGA to a potent immunogen, and synergistically enhancing the humoral response to PA. PGA-specific antibodies bound to encapsulated bacilli and promoted the killing of bacilli by complement. PA-specific antibodies neutralized toxin activity and protected immunized mice against lethal challenge with anthrax toxin. Thus, DAAV combines both antibacterial and antitoxic components in a single vaccine against anthrax. DAAV introduces a vaccine design that may be widely applicable against infectious diseases and provides additional tools in medicine and biodefense.

Recent events dramatically highlight the universal threat posed by the potential use of anthrax in terrorism and warfare (1). Anthrax is a highly lethal infectious disease caused by the spore-forming bacterium *Bacillus anthracis* (2). After entering the host, anthrax spores germinate inside macrophages, which transport the bacteria to regional lymph nodes. Released bacilli then multiply extracellularly, secrete high levels of exotoxins, and spread systemically in the blood stream, where they reach up to 10^9 organisms per milliliter. Systemic anthrax progresses rapidly from nonspecific initial symptoms to death with little opportunity for therapeutic intervention (1, 2). An effective and safe prophylactic approach to anthrax would be highly desirable, especially in the context of public health. Currently, the only human vaccine available in the United States, anthrax vaccine adsorbed, is primarily directed at anthrax toxins but does not directly target the bacilli (3). Because anthrax involves a dual process of bacterial replication and toxin production, we sought to develop a dually active anthrax vaccine (DAAV) that confers simultaneous protection against both bacilli and toxins.

We chose the two major virulence factors of *B. anthracis* as target antigens for DAAV, namely, the capsular poly-γ-D-glutamic acid (PGA) and the essential toxin component, protective antigen (PA) (4). The weakly immunogenic and antiphagocytic PGA capsule disguises the bacilli from immune surveillance in a similar manner to the role of capsular polysaccharides in protecting pathogens, such as pneumococci and meningococci (5, 6). Encapsulated *B. anthracis* strains grow unimpeded in the infected host, whereas isolates lacking the capsule are phagocytized and are virtually avirulent (7, 8). Anthrax toxins are formed by PA, lethal factor (LF), and edema factor, which are secreted separately as nontoxic monomers (9). The binding of LF or edema factor to PA results in the formation of active lethal toxin or edema toxin, respectively (10). Because of its ability to elicit a protective immune response against both anthrax toxins, PA is the target antigen of existing anthrax vaccine (3). However, we reasoned that a vaccine based on both PGA and PA would allow direct targeting of bacillar growth, as well as inhibiting toxin activity, making it more effective than a vaccine based on PA alone. PGA is an attractive antigen because it consists of D-glutamic acid residues linked by γ peptide bonds, and thus bears no resemblance to mammalian host molecules.

DAAV was designed as a PGA–PA conjugate vaccine to optimize the immunogenicity of both components, especially of the poorly immunogenic PGA (11), because covalent linking of epitopes often significantly enhances a specific immune response (12). Recombinant PA from *Escherichia coli* and PGA from *Bacillus licheniformis* ATCC 9945a were prepared, and conjugates were synthesized by coupling carboxyl groups of PGA to the amines of PA by carbodiimide-mediated condensation. We present results for two sets of conjugates with 1:2 and 1:1 (wt/wt) PGA-to-PA ratios, designated DAAV-1 and DAAV-2, respectively.

Materials and Methods

Strain, Inoculation, and Culture Methods. *B. licheniformis* ATCC 9945a was obtained from the American Type Culture Collection. Highly mucoid colonies were selected and grown aerobically in Erlenmeyer flasks with E broth (13). The formulation of E medium in $g \cdot liter^{-1}$ was as follows: L-glutamic acid, 20.0; citric acid, 12.0; glycerol, 80.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.104. Cultures were incubated at 37°C and shaken at 250 rpm for 96 h.

Purification of PGA. PGA was purified from culture supernatant following the procedure described by Perez-Camero *et al.* (14) with slight modifications. The highly viscous bacterial culture was centrifuged at 4°C (6,500 × *g*, 20 min) to remove bacteria. The supernatant was collected and precipitated with 3 vol of ethanol at 4°C overnight. PGA precipitate was collected by centrifugation and dialyzed against deionized water. PGA solution was acidified to pH 1.5 with 6 M HCl and immediately precipitated with 3 vol 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 and structure of PGA were verified by UV-vis scanning from 190 to 300 nm and by ^1H NMR spectroscopy.

Degradation of PGA. The average molecular mass of native PGA was ≈500 kDa as determined on a Superose 12 (Amersham Biosciences) column that had been calibrated with dextran standards. Because of the large size of PGA, direct conjugation to PA would result in an insoluble gel, which is undesirable for vaccine formulation. Therefore, we degraded PGA by using an ultrasonic processor (Tekmar, Cincinnati) operating at 20 kHz (14). PGA solution (6.5 $mg \cdot ml^{-1}$ in PBS) was placed in an ice

Abbreviations: DAAV, dually active anthrax vaccine; DTA, diphtheria toxin A chain; LF, lethal factor; LF_N, PA-binding domain of LF; PA, protective antigen; PGA, poly-γ-D-glutamic acid.

[‡]On leave within the International Postdoctoral Program of the International Vaccine Institute, Seoul 151-600, Korea.

[‖]To whom correspondence should be addressed. E-mail: julia_wang@rics.bwh.harvard.edu.

bath to maintain the system at room temperature. The PGA solution was irradiated for 1.5 h, and the average molecular size of PGA was decreased to 100 kDa, as determined by the dextran-calibrated Superose 12 column.

Purification of PA. Recombinant wild-type PA was expressed in *E. coli* BL21* (DE3) from a pET-22b expression vector as described (15). PA was purified from periplasmic proteins by means of Q Sepharose and Superdex 200 (Amersham Biosciences) columns. Purity and molecular size of PA were verified by SDS/PAGE analysis.

Synthesis of PGA–Protein Conjugates. Either 1.0 mg (for preparation of DAAV-1) or 0.5 mg (DAAV-2) of PA in 0.6 ml of PBS (pH 7.0) was mixed with 0.5 mg of degraded PGA. Five milligrams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma) was added, and the mixture was stirred for 4 h at room temperature. The conjugates were purified on a PD-10 (Amersham Biosciences) column, verified by SDS/PAGE analysis, and stored at −20°C until use. Similarly, PGA was conjugated to BSA at a ratio of 1:1 (wt/wt) for use in antibody measurements.

Mouse Immunization. Groups of female BALB/c mice at 6–8 weeks of age (The Jackson Laboratory) were immunized by i.p. injection on days 0, 14, and 28. DAAV-1 was tested at 10- and 20-μg doses, and DAAV-2 was tested at 2-, 10-, and 20-μg doses (doses refer to PA content). PA and PGA were tested at 20-μg doses. Unconjugated PGA–PA mixture included 20 μg of PGA and 20 μg of PA. Each dose was dissolved in 50 μl of PBS and adsorbed to an equal volume of $Al(OH)_3$ gel adjuvant (equivalent to 0.187 mg per dose). PBS/$Al(OH)_3$ was used as a negative control. Sera were obtained before immunization (preimmune) and 1 week after each vaccination.

Quantification of Antibodies to PA and PGA. Serum levels of anti-PA and anti-PGA IgG and IgM were determined by enzyme-linked immunosorbent assays (ELISAs) (12). In brief, Immulon 96-well Maxisorp plates (Nalge Nunc) were coated with 2.5 $\mu g \cdot ml^{-1}$ PA or PGA–BSA conjugate in 0.1 M carbonate buffer (pH 9.6) at 4°C for 16 h. Serum samples were serially diluted in Tris-buffered saline (TBS) (50 mM Tris·HCl/0.15 M NaCl, pH 7.4) containing 5% (vol/vol) FCS with 0.05% Brij and titrated 2-fold across the plates. The plates were incubated at 37°C for 1.5 h. After washing, bound IgG or IgM antibodies were detected with 1 $mg \cdot ml^{-1}$ alkaline phosphatase-labeled goat anti-mouse IgG or rat anti-mouse IgM (Southern Biotechnology Associates) at 1:2,000 dilutions. The plates were developed with *p*-nitrophenyl phosphate (Sigma), and their absorbances were determined at 405- and 630-nm wavelengths. Antibody isotypes were determined in a similar manner, except that alkaline phosphatase-labeled anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates) was used as the secondary antibody. Standard curves were obtained for each plate by using goat $F(ab')_2$ anti-mouse Ig as the capturing agent and known concentrations of mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 as standards. Specific Ig concentrations were determined by comparison with the standard curves.

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

Immunoelectron Microscopy. Immunoelectron microscopy was performed to determine whether antibodies raised against PGA–PA conjugates bind to PGA capsules of *B. licheniformis* 9945a. *B. licheniformis* 9945a cells were grown in E medium broth at 37°C overnight. Drops of bacterial solution (5 μl) were placed on a piece of Parafilm, and a 200-mesh Formvar carbon-coated copper grid (Electron Microscopy Sources, Fort Washington, MA) was placed on top of each drop for 1 min. The grids were blocked by placing them on a 5-μl drop of 0.5% fish skin gelatin in PBS with 0.1% Tween 20. The grids were then incubated with immune mouse sera (at dilutions of 1:5) for 20 min at room temperature and washed with PBS/Tween. Subsequently, the grids were incubated with rabbit anti-mouse antibody (at a dilution of 1:5) for 20 min and washed with PBS/Tween. Gold-labeled protein A (20-nm gold particles) was applied at a dilution of 1:10 to detect bound antibodies. After incubation for 20 min at room temperature, the grids were washed four times with deionized water. Photographs were taken on a JEOL 1200 EX electron microscope at magnifications of 10,000- to 25,000-fold. Preimmune mouse sera were tested as controls by using the same procedure.

Antibody-Specific and Complement-Mediated Bacteriolysis. To evaluate the functional specificity of anti-PGA sera against bacilli, we adapted a complement-mediated bactericidal assay with slight modifications (16). In brief, pooled sera from groups of immunized mice were serially diluted in PBS. Samples of 30 μl of *B. licheniformis* 9945a ($\approx$100 colony-forming units) in PBS were incubated with 50 μl of diluted serum and 20 μl of human complement (diluted 1:4 in PBS) at 37°C for 1 h on a platform shaker. Sera from mice immunized with 10-μg doses of DAAV-1 and DAAV-2 were tested. Preimmune sera were tested analogously as negative reference controls. Bacteria were also cultured with sera or complement alone as controls. Samples of incubation mixture (50 μl) were plated on 5% sheep-blood agar plates. After the plates had been incubated at 37°C overnight, the colonies were counted, and the percentage of bacterial killing was calculated for each antibody titration by comparison with the number of colonies on preimmune reference plates. All tests were performed in four replicates.

The bacteriolysis mediated by complement and antibodies was confirmed by electron microscopy. For preparation of samples for electron microscopy, 30-μl samples of *B. licheniformis* 9945a ($\approx 3.7 \times 10^7$ colony-forming units) in PBS were incubated with 20 μl of undiluted serum and 50 μl of undiluted human complement at 37°C for 30 min on a platform shaker. As negative controls, samples of *B. licheniformis* were incubated with undiluted preimmune sera and complement. Bacteria were also incubated with sera or complement alone. After incubation, reaction mixtures were centrifuged, and pellets were fixed for 1 h in a mixture of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed in 0.1 M cacodylate buffer, and fixed with 1% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide [$K_4Fe(CN)_6$] for 1 h. Pellets were then washed in water, stained with 1% aqueous uranyl acetate for 30 min, dehydrated in alcohol (5 min in 70%, 5 min in 90%, twice for 5 min in 100%), and then embedded in TAAB Epon (Marivac Canada, St. Laurent, QC, Canada). Ultrathin sections ($\approx$60 nm) were cut on a Reichert Ultracut-S Microtome, transferred onto copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX transmission electron microscope.

Inhibition of PA Cytotoxicity. To evaluate the ability of PA-specific antibodies to inhibit the biological activity of PA, we performed PA-mediated cytotoxicity experiments with CHO (Chinese hamster ovary) K1 cells (17, 18). Sera obtained from mice immunized with DAAV-1, DAAV-2, or PA were normalized with respect to their concentrations of anti-PA IgG and serially diluted. Control sera from mice immunized with PBS or PGA were serially diluted based on the group that had the lowest concentration of anti-PA IgG. Confluent CHO K1 cells in a 96-well plate were incubated with 0.1 nM PA, 0.1 nM LF_N-diphtheria toxin A chain

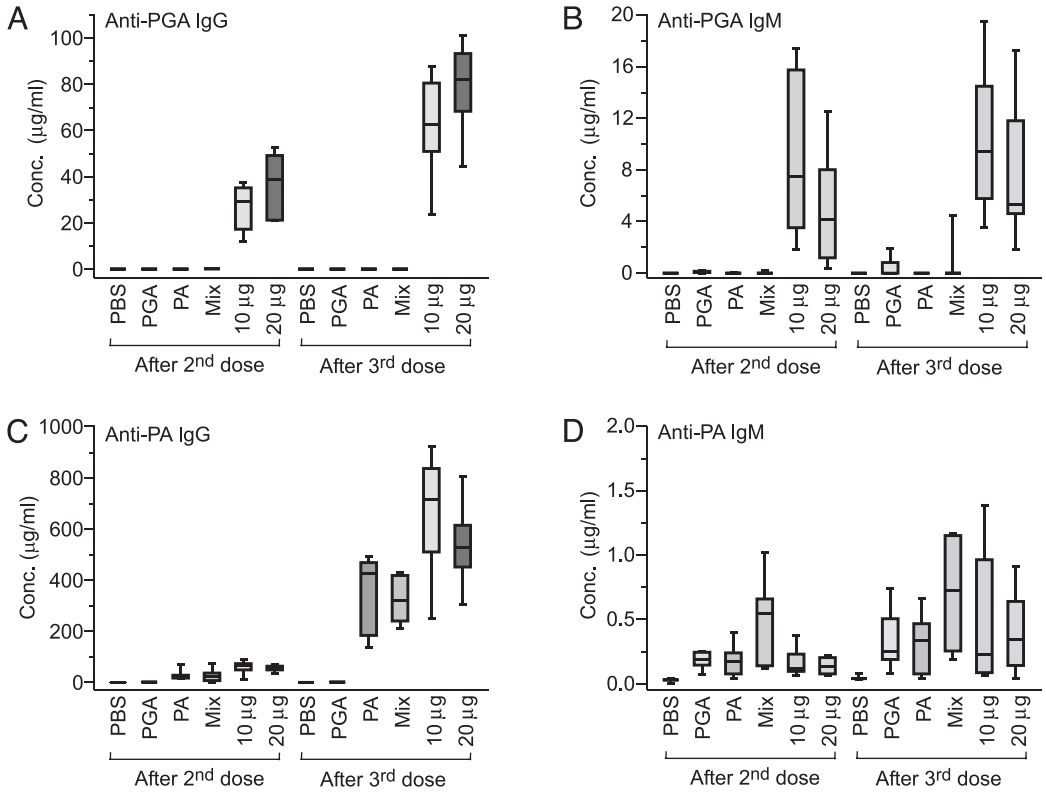


Fig. 1. Specific antibody response to DAAV-1 immunization in mice. (*A* and *B*) Anti-PGA IgG (*A*) and IgM (*B*). Anti-PGA IgG concentrations after the third dose were significantly higher than those after the second ($P = 0.002$, 10-μg group; $P = 0.007$, 20-μg group). (*C* and *D*) Anti-PA IgG (*C*) and IgM (*D*). After three doses, DAAV-1 induced significantly more anti-PA IgG than did PA alone ($P = 0.02$, 10-μg group vs. PA; $P = 0.01$, 20-μg group vs. PA). Note that all anti-PA IgM concentrations are very low and close to the detection limit. No significant difference exists between 10- and 20-μg groups. Anti-PGA and anti-PA responses were >98% IgG1. Control immunization agents included PBS, PGA, PA, and unconjugated PGA–PA mixture (Mix). Boxes represent 25th to 75th percentiles of eight data points, and bars indicate minimum, median, and maximum values.

(DTA), and diluted serum at 37°C for 4 h. LF_N-DTA is a fusion protein of the N-terminal PA-binding domain of LF to the DTA chain. The DTA moiety catalyzes ADP-ribosylation of elongation factor 2 within the cytosol and inhibits protein synthesis. Cytotoxicity was measured by the decrease of [^{3}H]leucine uptake by cells. Inhibition of cytotoxicity was expressed as the percentage of incorporated radioactivity relative to control (radioactivity recovered from cells incubated without LF_N-DTA). Experiments were performed in duplicates and repeated three times.

Protection of Mice Against Lethal Toxin Challenge. To verify the protective activity of PGA–PA conjugates as anthrax vaccine, we tested whether immunization with DAAVs protects mice against challenge with lethal toxin. Two weeks after the third dose of vaccination, each mouse was challenged through its tail vein with a mixture of 48 μg of PA and 20 μg of LF (List Biological Laboratories, Campbell, CA), the equivalent of approximately 4 times the LD_{50} of lethal toxin in mice (19). Without exception, unprotected mice died within 24 h. Protected mice were monitored closely for at least 2 weeks. Unprotected mice were characterized by minor initial hyperactivity, which was soon followed by pronounced moribund lethargy, whereas protected mice progressed through a short phase of reduced activity to full recovery.

Histopathologic Analysis. Mouse organs were fixed in Bouin's fixative for 2 days and embedded in paraffin. Five-micrometer thin sections were cut and stained with hematoxylin and eosin. Macroscopically and histologically, unprotected mice had hypoplastic spleens and necrotic intestinal lesions. Other major organs, such as kidneys, liver, lungs, heart, and brain, appeared normal.

Results and Discussion

After three immunizations in mice, DAAV-1 induced high levels of serum anti-PGA IgG and booster injections significantly enhanced the IgG response (Fig. 1*A*). Anti-PGA IgM levels were lower and there was no booster effect (Fig. 1*B*). In contrast, PGA alone elicited virtually no specific IgG and barely detectable levels of IgM. Thus, although PGA by itself displays poor immunogenicity that could not be improved by coadministration in an unconjugated PGA–PA mixture, covalent linkage to an immunogenic carrier significantly enhanced immunogenicity.

DAAV-1 induced high anti-PA IgG concentrations after three immunizations (Fig. 1*C*). Comparing effects of second and third immunizations, booster doses increased specific IgG by ≈10-fold over 14 days. PA alone and PGA–PA mixture induced significantly lower titers than those elicited by DAAV-1. PA-specific IgM levels remained barely detectable (<1 μg/ml) in all groups (Fig. 1*D*). Overall, the conjugation of PGA and PA in DAAV-1 synergistically enhanced the immunogenicity of both PGA and PA.

The antibody response pattern after DAAV-2 immunization resembled that after DAAV-1 immunization, except for significantly lower specific IgG levels (Fig. 2). With 2-, 10-, and 20-μg doses and after three injections, DAAV-2 elicited median values of 18, 38, and 37 μg/ml anti-PGA IgG, respectively. Notable amounts of anti-PGA IgM were detected after two doses, rising very slightly to median values of 8–14 μg/ml after three doses.

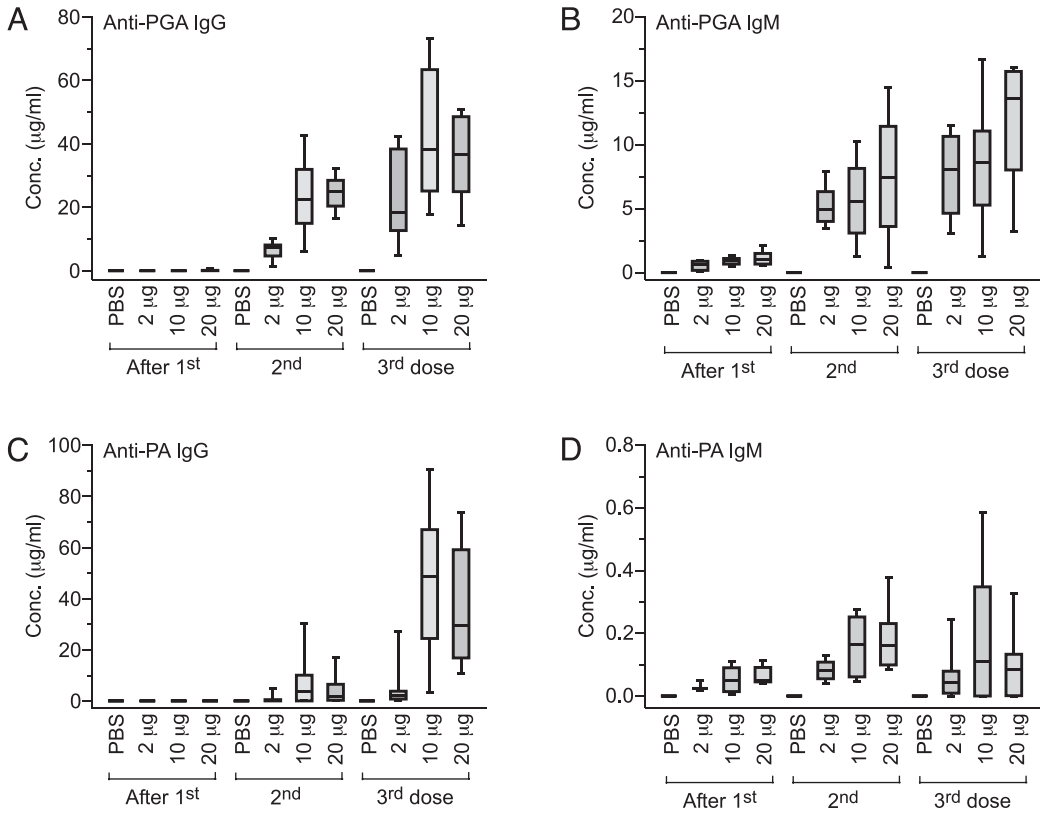


Fig. 2. Specific antibody response to DAAV-2 immunization in mice. Anti-PGA IgG (*A*), anti-PGA IgM (*B*), anti-PA IgG (*C*), and anti-PA IgM (*D*) are shown. Both anti-PGA and anti-PA IgG display booster responses. With respect to IgG, the 10- and 20-μg doses are superior to the 2-μg dose ($P < 0.05$). The maximum attainable IgG concentrations are ≈2-fold (anti-PGA) and ≈10-fold (anti-PA) below those attained with DAAV-1. Anti-PGA and anti-PA responses were >98% IgG1. PBS served as a negative control. Boxes represent 25th to 75th percentiles of eight data points, and bars indicate minimum, median, and maximum values.

DAAV-2 also induced 2, 49, and 30 μg/ml anti-PA IgG after three 2-, 10-, and 20-μg doses, respectively.

DAAV-1 appears to be a more promising vaccine candidate than DAAV-2, because DAAV-1 induced about twice as much anti-PGA IgG as did DAAV-2, and the anti-PA IgG potency of DAAV-1 was >14-fold superior to that of DAAV-2. This difference is likely due to the lower degree of modification of PA by the coupling to PGA in DAAV-1. The amount of PGA coupled to PA may alter natural epitopes and affect PA immunogenicity. These results demonstrate that the structural parameters of PGA–PA conjugates are critical determinants for the induction of PGA- and PA-specific antibodies.

To evaluate the dual functional potency of DAAVs against bacilli and toxins, we first examined the ability of PGA-specific antibodies to bind encapsulated bacilli and facilitate killing. Because of the inaccessibility of virulent *B. anthracis* and the hazards associated with working with this pathogen, we used *B. licheniformis* 9455a in our tests. Encapsulated *B. licheniformis* is surrounded by a thick coat of fibrillar PGA (Fig. 3*A*) that is chemically and immunologically identical to that of *B. anthracis* (20, 21). As shown by immunoelectron microscopy, antibodies to PGA from mice immunized with DAAV-1 or DAAV-2 bound specifically to the capsule of *B. licheniformis* and completely coated the bacterial cells (Fig. 3*A*). Preimmune sera or sera from mice immunized with PBS did not react with the PGA capsule at all.

After binding to the capsule, antibodies can mediate the killing of bacteria by means of complement-driven lysis and/or opsonophagocytosis (22). We performed bactericidal assays to test the potency of PGA-specific antibodies in activating complement and killing bacilli (16). Sera from DAAV-immunized mice exerted potent bactericidal activity (Fig. 3*B*). Bactericidal potency correlated with serum dilution and anti-PGA Ig concentration. Furthermore, electron microscopic analysis confirmed the lysis of bacilli (Fig. 3 *C* and *D*). Whereas capsular PGA adheres to *B. anthracis*, *B. licheniformis* sheds significant amounts of its capsule (4, 14). Therefore, future experiments will need to analyze the effects of PGA-specific antibodies on *B. anthracis* directly. These findings demonstrate that DAAV-induced antibodies to PGA can kill bacilli by antibody-specific, complement-dependent mechanisms. The antibacterial component of DAAVs could thus potentially block replication of the bacilli at an early phase of the infection and preempt the secretion of large amounts of toxins.

We next evaluated whether DAAV vaccination could protect against anthrax toxins. PA, the required core component of anthrax toxins, translocates both LF and edema factor into the cytosol of host cells. Because blocking PA prevents anthrax toxicity, we tested the ability of DAAV-induced antibodies to neutralize PA (17, 18). We exposed CHO cells to PA and LF_N-DTA, a fusion protein of PA-binding domain of LF and DTA (17, 18). PA-mediated translocation of LF_N-DTA into the cytosol causes inhibition of protein synthesis and cell death, whereas blocking of PA prevents translocation and protects cells. Sera from mice immunized with DAAV-1, DAAV-2, or PA efficiently rescued cells (Fig. 4*A*). In contrast, preimmune sera or sera from mice immunized with PBS did not inhibit cytotoxicity. At concentrations ≥1 μg/ml, anti-PA IgG afforded complete protection. Furthermore, anti-PA IgG induced by DAAV-1, DAAV-2, or PA displayed very similar concentration-dependent protection curves. These findings indicate that anti-PA IgGs elicited by DAAVs or PA have the same functional potency to neutralize PA.

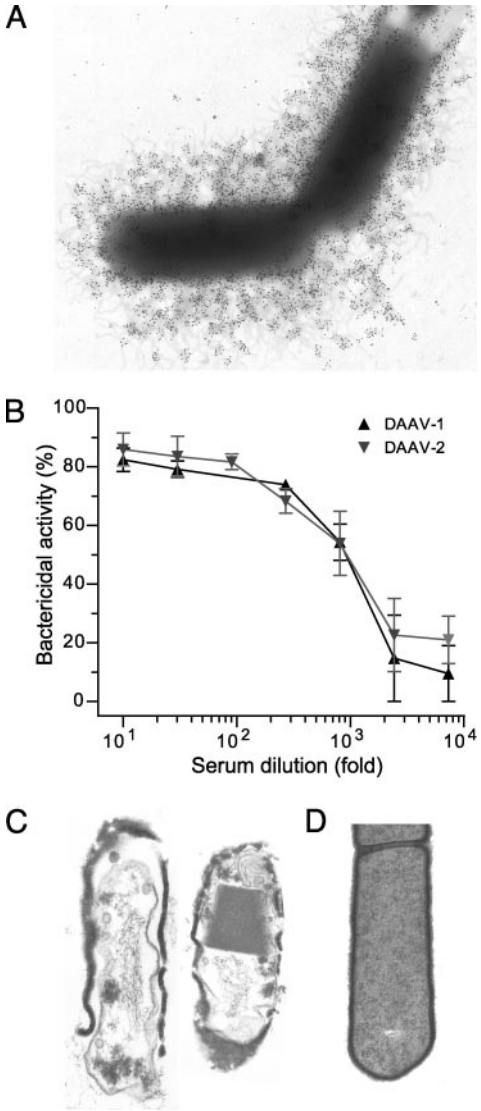
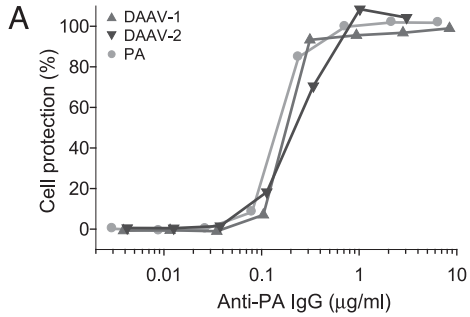


Fig. 3. Opsonizing and bacteriolytic activities of antibodies to PGA. (*A*) Immunoelectron micrograph showing the binding of PGA-specific antibodies to *B. licheniformis* 9455a capsule. Antibodies were from mice immunized with DAAV-1 and were detected with gold-labeled protein A particles. (×40,000.) (*B*) Bactericidal activity of PGA-specific antibodies against *B. licheniformis* 9945a. Bacilli were incubated with complement and serial dilutions of sera from DAAV-immunized mice. Percentage killing was calculated relative to growth of bacilli incubated with control preimmune serum and complement. (*C*) Electron micrograph of two bacilli lysed with anti-PGA Ig and complement. (×40,000.) (*D*) Electron micrograph of a viable control bacillus. (×40,000.)



B

Immunization group	Protection against LeTx challenge (%)
DAAV-1	100 (5/5)
DAAV-2	100 (6/6)
PA	100 (6/6)
PGA	0 (0/6)
PBS	0 (0/8)

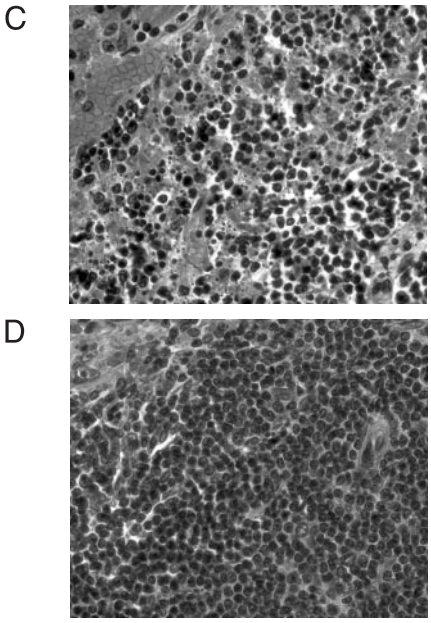


Fig. 4. Protective activities of antibodies to PA. (*A*) Correlation between levels of anti-PA IgG and protection of CHO cells against PA-mediated cytotoxicity; 0% and 100% levels correspond to cells incubated without serum or without LF_N-DTA, respectively. Preimmune sera or sera from mice immunized with PBS did not inhibit cytotoxicity. (*B*) Protection of mice immunized with DAAVs or control agents against lethal toxin (LeTx) challenge. Surviving mice were monitored for at least 2 weeks. (*C*) Histologic spleen specimen from an unprotected mouse challenged with LeTx. The animal had died 24 h after challenge. Note the marked reduction in cell density and the numerous pyknotic nuclei of cells undergoing apoptosis. (*D*) Spleen specimen from a DAAV-protected mouse 24 h after challenge with LeTx. The microscopic appearance is normal. (*C* and *D*, ×40.)

To directly verify the effectiveness of DAAVs in protection against anthrax, we challenged immunized mice with anthrax lethal toxin. Ten days after the third immunization, each mouse was given a mixture of 48 μg of PA and 20 μg of LF through its tail vein, corresponding to about 4 times the LD_{50} of lethal toxin (19). Without exception, control mice immunized with PBS or PGA died within 24 h (Fig. 4*B*). In contrast, all mice immunized with DAAV-1, DAAV-2, or PA survived the lethal challenge.

Unprotected mice had hypoplastic spleens and experienced extensive splenic cell apoptosis and a sharp decrease in cell density, whereas DAAV-protected mice had normal spleens (Fig. 4 *C* and *D*). *In vitro*, lethal toxin induces apoptosis by cleaving mitogen-activated protein kinase kinases (23, 24). Our analysis illustrates this specific pathologic damage caused by lethal toxin *in vivo*.

In conclusion, we demonstrated that DAAVs are a class of promising conjugate anthrax vaccines that can offer simultaneous protection against both bacilli and toxins. PGA–PA conjugation has several advantages. Conjugation can synergistically augment the protective humoral response to both PGA and PA. Despite the complex life cycle of *B. anthracis*, systemic anthrax disease is caused by the massive extracellular replication of bacilli and the secretion of toxins. We envision that our

IMMUNOLOGY

DAAVs may prevent or stop the disease by eliminating bacteria by means of anti-capsular antibodies early in the sequence of anthrax infection, well before severe bacteremia and toxemia take place. In addition, antibodies to PA provide a parallel line of defense against residual toxins. DAAVs embody the paradigm of combining both antibacterial (i.e., prophylactic) and antitoxic (i.e., therapeutic) components into a single vaccine. This concept may find broad application in combating infectious disease.

Note Added in Proof. During the review of this paper, a publication by Schneerson *et al*. (25) came to our attention that reports the synthesis of PGA–PA conjugates by using different chemical methods. The findings thus confirm independently the two groups' common conclusions concerning the utility of such conjugates as vaccines that dramatically improve the immunogenicity of PGA.

We thank Maria Ericsson and Louise Trakimas for expert assistance with immunoelectron microscopy, Roderick Bronson for histopathologic advice, Yong-Hoon Choi for molecular modeling of PA, and Ruth Pimental and Amanda Ganong for technical assistance. This work was supported by National Institutes of Health Grant R21 AI053369 (to J.Y.W.) and a postdoctoral fellowship from the International Vaccine Institute, Seoul (to G.-E.R.). J.Y.W. acknowledges the Channing Laboratory and Dr. Dennis Kasper for support.

1. Inglesby, T. V., O'Toole, T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Friedlander, A. M., Gerberding, J., Hauer, J., Hughes, J., *et al.* (2002) *J. Am. Med. Assoc.* **287,** 2236–2252.
2. Dixon, T. C., Meselson, M., Guillemin, J. & Hanna, P. C. (1999) *N. Engl. J. Med.* **341,** 815–826.
3. Joellenbeck, L. M., Zwanziger, L. L., Durch, J. S. & Strom, B. L., eds. (2002) *The Anthrax Vaccine: Is It Safe? Does It Work?* (Natl. Acad. Press, Washington, DC).
4. Mock, M. & Fouet, A. (2001) *Annu. Rev. Microbiol.* **55,** 647–671.
5. Ezzell, J. W. & Welkos, S. L. (1999) *J. Appl. Microbiol.* **87,** 250.
6. Leppla, S. H., Robbins, J. B., Schneerson, R. & Shiloach, J. (2002) *J. Clin. Invest.* **110,** 141–144.
7. Welkos, S. L. (1991) *Microb. Pathog.* **10,** 183–198.
8. Welkos, S. L., Vietri, N. J. & Gibbs, P. H. (1993) *Microb. Pathog.* **14,** 381–388.
9. Lacy, D. B. & Collier, R. J. (2002) *Curr. Top. Microbiol. Immunol.* **271,** 61–85.
10. Mogridge, J., Cunningham, K. & Collier, R. J. (2002) *Biochemistry* **41,** 1079–1082.
11. Goodman, J. W. & Nitecki, D. E. (1967) *Immunology* **13,** 577–583.
12. Wang, J. Y., Chang, A. H., Guttormsen, H. K., Rosas, A. L. & Kasper, D. L. (2003) *Vaccine* **21,** 1112–1117.
13. Birrer, G. A., Cromwick, A. M. & Gross, R. A. (1994) *Int. J. Biol. Macromol.* **16,** 265–275.
14. Perez-Camero, G., Congregado, F., Bou, J. J. & Munoz-Guerra, S. (1999) *Biotechnol. Bioeng.* **63,** 110–115.
15. Benson, E. L., Huynh, P. D., Finkelstein, A. & Collier, R. J. (1998) *Biochemistry* **37,** 3941–3948.
16. Jennings, H. J., Gamian, A. & Ashton, F. E. (1987) *J. Exp. Med.* **165,** 1207–1211.
17. Milne, J. C., Blanke, S. R., Hanna, P. C. & Collier, R. J. (1995) *Mol. Microbiol.* **15,** 661–666.
18. Mourez, M., Kane, R. S., Mogridge, J., Metallo, S., Deschatelets, P., Sellman, B. R., Whitesides, G. M. & Collier, R. J. (2001) *Nat. Biotechnol.* **19,** 958–961.
19. Price, B. M., Liner, A. L., Park, S., Leppla, S. H., Mateczun, A. & Galloway, D. R. (2001) *Infect. Immun.* **69,** 4509–4515.
20. Mesnage, S., Tosi-Couture, E., Gounon, P., Mock, M. & Fouet, A. (1998) *J. Bacteriol.* **180,** 52–58.
21. Makino, S., Uchida, I., Terakado, N., Sasakawa, C. & Yoshikawa, M. (1989) *J. Bacteriol.* **171,** 722–730.
22. Prodinger, W. M., Wurzner, R., Erdei, A. & Dierich, M. P. (1999) in *Fundamental Immunology*, ed. Paul, W. E. (Lippincott–Raven, Philadelphia), Vol. 1, pp. 967–995.
23. Park, J. M., Greten, F. R., Li, Z. W. & Karin, M. (2002) *Science* **297,** 2048–2051.
24. Popov, S. G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Popova, T., Wu, A., Alibek, D., Bailey, C. & Alibek, K. (2002) *FEBS Lett.* **527,** 211–215.
25. Schneerson, R., Kubler-Kielb, J., Liu, T. Y., Dai, Z. D., Leppla, S. H., Yergey, A., Backlund, P., Shiloach, J., Majadly, F. & Robbins, J. B. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 8945–8950.