The immunogenicity of *Bacillus anthracis* capsule (poly-γ-D-glutamic acid [PGA]) conjugated to recombinant *B. anthracis* protective antigen (rPA) or to tetanus toxoid (TT) was evaluated in two anthrax-naive juvenile chimpanzees. In a previous study of these conjugates, highly protective monoclonal antibodies (MAbs) against PGA were generated. This study examines the polyclonal antibody response of the same animals. Preimmune antibodies to PGA with titers of >10^5 were detected in the chimpanzees. The maximal titer of anti-PGA was induced within 1 to 2 weeks following the 1st immunization, with no booster effects following the 2nd and 3rd immunizations. Thus, the anti-PGA response in the chimpanzees resembled a secondary immune response. Screening of sera from nine unimmunized chimpanzees and six humans revealed antibodies to PGA in all samples, with an average titer of 10^5. An anti-PA response was also observed following immunization with PGA-rPA conjugate, similar to that seen following immunization with rPA alone. However, in contrast to anti-PGA, preimmune anti-PA antibody titers and those following the 1st immunization were ≤300, with the antibodies peaking above 10^5 following the 2nd immunization. The polyclonal anti-PGA shared the MAb 11D epitope and, similar to the MAbs, exerted opsonophagocytic killing of *B. anthracis*. Most important, the PGA-TT–induced antibodies protected mice from a lethal challenge with virulent *B. anthracis* spores. Our data support the use of PGA conjugates, especially PGA-rPA targeting both toxin and capsule, as expanded-spectrum anthrax vaccines.

*Bacillus anthracis*, the causative agent of anthrax, has two obligatory virulence factors: the toxins and the capsule. The toxins consist of the protective antigen (PA), the lethal factor (LF), and the edema factor (EF). PA is the cell receptor-binding component common to the lethal and edema toxins (1). The capsule is composed of poly-γ-D-glutamic acid (γ-D-PGA). Although licensed PA-based anthrax vaccines are safe and effective, expanding protection by including additional antigens in the vaccine would be desirable as defense against bioterrorism (2, 3). Given the capsule’s role in virulence, induction of antcapsular antibodies has been recommended (4-10).

The capsule, present in vegetative *B. anthracis*, is encoded by the capBCADE operon located on plasmid pXO2 (11–14). Strains that lack pXO2 and capsule are highly attenuated (15–17) and have been used as vaccines to prevent anthrax in domesticated animals for >50 years and in some countries have been used in humans as well (18). The capsule of *B. anthracis* contributes to the organism’s virulence by its antiphagocytic action (13, 19–21). The γ-D-PGA is poorly immunogenic and acts as a T-cell independent antigen (21, 22), but γ-D-glutamic acid peptides conjugated to carrier proteins such as PA, bovine serum albumin (BSA), or tetanus toxoid (TT) are highly immunogenic in mice, guinea pigs, rabbits, and monkeys (4–9).

To further evaluate PGA-based conjugates as vaccine candidates, we immunized chimpanzees with PGA-TT or PGA-recombinant protective antigen (rPA) and monitored both anti-PGA and anti-PA antibody responses. We also determined the protection afforded by the PGA-TT–induced antibodies in a mouse inhalational model following a challenge with virulent *B. anthracis* spores. We found that IgG anti-PGA antibody is protective and therefore suggest that PGA-rPA conjugates be developed as expanded-spectrum anthrax vaccines.

**MATERIALS AND METHODS**

**Antigen and sera.** *B. anthracis* γ-D-PGA purified from the culture supernatants, synthetic γ-D-PGA peptide conjugates of rPA, and TT were described previously (4). The γ-D-PGA from *Bacillus subtilis* was a gift from Vedan Enterprise Corporation, Taiwan (23). Sera from treatment-naive human volunteers were purchased from Millennium Biotech, Inc.

**Immunization.** Two anthrax-naive juvenile chimpanzees (~6 years of age) were immunized intramuscularly (i.m.) with alum-adsorbed PGA peptide conjugates shown to induce high-level antibody responses in mice (4). Chimpanzee AOA006 received PGA bound to TT, and chimpanzee AOA007 received PGA coupled to rPA. The chimpanzees were injected with 25 μg PGA in the conjugate 3 times at 6-week intervals. Chimpanzees 1603 and 1609 (also ~6 years of age) were previously immunized with 50 μg of alum-adsorbed rPA 3 times at 2-week intervals (24). The immunized chimpanzees were bled weekly. The housing and care of the chimpanzees were in compliance with all relevant guidelines and requirements.
in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal study protocols involving chimpanzees (LID 26, LID 64) were approved by the Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases and the Animal Care and Use Committee of the facility housing the animals.

Preparation of polyclonal anti-PGA antibodies. Remaining sera (after antibody assays) collected from weekly bleedings of chimpanzee AOa006 immunized with PGA-TT were pooled and concentrated—10-fold using ammonium sulfate precipitation (25% to 45%), caprylic acid precipitation, and Amicon Ultra-15 centrifugal filters. The anti-PGA concentration of this preparation was measured by enzyme-linked immunosorbent assay (ELISA), using mouse monoclonal antibody (Mab) anti-human IgG and rat anti-mouse for detection and a 1-mg/ml solution of Mab D11 as the standard (25).

Antibody assays by ELISA. Serum antibody titers were measured by ELISA. Briefly, 96-well Nunc-Immuno plates (Thermo, Milford, MA) were coated with 100 μl of purified antigen (rPA or PGA) at a concentration of 4.5 to 5 μg/ml in phosphate-buffered saline (PBS), pH 7.4. Coated plates were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with 3% nonfat dry milk in PBS for 2 h at 37°C. Serial 3-fold dilutions of each serum were made beginning at 1:100 and incubated in the coated plates for 2 h at room temperature (RT). After washing, the binding of antibodies to the antigen was detected by incubation with goat anti-human IgG (Fab1), conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA; 1:5,000 dilution) for 1 h at RT. Color was developed with tetramethylbenzidine solution (TMB) (KPL, Gaithersburg, MD). ELISA titers were calculated using the reciprocal of the absorbance value that was 3-fold higher than the average of the background absorbance. Antibody titers were assigned a value of <100 when ELISA was negative at the starting dilution (1:100). PGA antibody titers were measured twice, and geometric mean titers (GMTs) were calculated and plotted.

Anti-PGA and anti-PA antibody levels in unimmunized chimpanzees and humans were measured by the ELISA method described above, except that serial 3-fold dilutions of each serum were made beginning at 1:10. A secondary antibody conjugate alone was included in all the ELISAs as a negative control. Data were analyzed with GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA). Groups were compared and analyzed with an unpaired t test. Differences were considered significant if the P value was <0.05.

Cross-reactivity of anti-PGA monoclonal and polyclonal antibodies. Nunc-Immuno 96-well plates were coated with 100 μl per well of purified γ-γ-PA or γ-γ-PA of rPGA at a concentration of 4.5 to 5 μg/ml in PBS, pH 7.4. Coated plates were washed and blocked as described above. Plates were then incubated with 100 μl of 3-fold serially diluted anti-γ-PGA MABS 4C and 11D starting with 1 μg/ml or of chimpanzee or human serum beginning at a 1:10 dilution. After washing, antibodies bound to the antigen were detected by incubation with goat anti-human IgG (Fab1)1 conjugated with horse-radish peroxidase (Jackson ImmunoResearch; 1:5,000 dilution) for 1 h at RT. Color was developed with TMB. The absorbance values at representative concentrations were plotted.

Blocking of polyclonal antibody binding to γ-PA by MAB 11D Fab. We showed previously that the γ-PA MABs 4C and 11D compete for binding to PGA, indicating that they recognize the same or overlapping epitopes (25). A blocking experiment was performed to assess the specificity of the polyclonal antibodies for this epitope. The ability of MAB 11D Fab to block binding of the homologous IgG, 4C MAB IgG, and the polyclonal sera to γ-PA was examined by ELISA. Briefly, 96-well Nunc-Immuno plates were coated with 100 μl of γ-PA at a concentration of 2.5 μg/ml and incubated overnight at 4°C. Wells were washed with PBS-T and blocked with 3% nonfat milk in PBS for 2 h at 37°C. The plates were incubated with 100 μl of 11D Fab at a concentration of 10 μg/ml for 2 h at RT. The incubation with the same amount of irrelevant anti-PA Fab W1 was included as a control. Plates were washed with PBS-T, then incubated for 0.5 h at RT with 3-fold serially diluted test IgGs (4C and 11D IgGs and chimpanzee serum). Plates incubated with 3-fold serially diluted test IgGs without preincubation with anti-PGA 11D Fab served as references. The binding of the test IgGs to γ-PA was detected with horse radish peroxidase-conjugated goat anti-human IgG Fc (1:5,000), and the color was developed with TMB substrate (KPL). The degree to which the 11D Fab blocked the binding of the test IgGs was determined by the following ratio: % blocking = (1 – test IgG bound after incubation with 11D Fab/test IgG bound after incubation with 3% milk) × 100%.

Opsonophagocytic killing ofB. anthracis. As described previously for the γ-γ-PGA MABS (25), the in vitro opsonophagocytic bactericidal activity of the polyclonal PGA-TT–induced antibodies was measured by their ability to kill encapsulatedB. anthracis cells in the presence of complement and human polymorphonuclear leukocytes (PMNs). PGA-TT–induced antibodies and B. anthracis Ames A34 strain (pXO1−, pXO2−) bacteria were used. Assays with all components except antibodies, complement, or PMNs served as controls. The opsonophagocytic titer was calculated as the reciprocal of the antibody dilution yielding 50% bacterial killing by the Reed-Muench method (26). The antibody dilutions were In transformed and plotted, and a linear regression curve was applied to determine the relationship between antibody concentration and the opsonophagocytic killing (GraphPad Prism, version 5.0).

Mouse protection studies. A murine inhalational anthrax model was used to assess the protection afforded by polyclonal antibodies (PBs) compared to that by the 11D MABs (27). The experiments were performed in a CDC-registered, specific pathogen-free select agent animal biosafety level 3 facility at the University of New Mexico Health Sciences Center (UNMHSC) under animal protocol number 09-100158-HSC. Groups of 6 to 8 female BALB/c mice (ages 9 to 10 weeks) were injected intraperitoneally (i.p.) with various amounts of anti-PGA MAB or with PBs 18 h before challenge. Control animals were treated with vehicle alone (Dulbecco’s PBS [DPBS]). For pulmonary challenge with the Ames strain of B. anthracis (pXO1−, pXO2−), the mice were inoculated intratracheally (i.t.) with 1 × 104 spores per mouse (~10 times the 50% lethal dose [LD50]) in 50 μl DPBS as previously described (25). The mice were monitored for survival and clinical signs twice daily for 2 weeks following the challenge, and the results were evaluated statistically by Kaplan-Meier and log-rank (Mantel-Cox) tests (GraphPad Prism, version 4.0, San Diego, CA). All animal protocols for mouse studies were approved by the UNMHSC Institutional Animal Care and Use Committee.

RESULTS

Anti-PGA and anti-PA antibody responses in chimpanzees immunized with conjugated γ-PA. Serum titers of antibodies to PGA and PA were determined before and after immunization of chimpanzees with either PGA-rPA or PGA-TT conjugate. As shown in Fig. 1, titers of 2 × 105 of PGA antibodies were already detectable before immunization. Background binding was not detected with the 2nd antibody conjugate alone. Titers of 1 × 105 were elicited within 1 to 2 weeks following the 1st immunization. There were no booster responses following the 2nd and 3rd immunizations. Titers of 1 × 105 of PGA antibodies were still detectable 33 weeks after the 3rd immunization. Overall, the two conjugates induced similar levels of anti-PGA.

The PA antibody response was monitored in chimpanzee AOa007, which was immunized with PGA-rPA. In contrast to the anti-PA response, anti-PA antibody titers before immunization were <1:100, and only a small anti-PA response (to 1:300 titer) was observed following the 1st immunization (Fig. 2). The 2nd immunization induced an antibody response with a maximal titer of 7 × 105. The 3rd immunization induced little further increase in antibody titer. Anti-PA was still detectable with an antibody titer of ~1 × 105 at week 33 after the 3rd immunization.
To evaluate a possible effect of linking PGA to rPA on the anti-PA response, anti-PA levels induced by PGA-rPA were compared to those induced by rPA alone in chimpanzees immunized in a previous experiment (24). Despite different immunization schemes for PGA-rPA (3 times, 6 weeks apart) and for rPA (3 times, 2 weeks apart), the anti-PA response to PGA-rPA was similar to that for rPA alone (Fig. 2). A maximal antibody titer of $7.3 \times 10^4$ was elicited by the conjugate and rPA. However, we found that the anti-PA concentration declined faster in the conjugate-immunized chimpanzee than in the rPA-immunized chimpanzees, resulting in an ~10-fold difference in antibody titer at the end of the experiment (data not shown). In addition, the anti-PGA and anti-PA IgGs from the immunized chimpanzees were quantified by ELISA for anti-PA IgG (28), and the result revealed a similar pattern (data not shown).

To investigate whether preimmune PGA antibodies are common in chimpanzees and humans, we tested sera from an additional nine chimpanzees and six humans (Fig. 3). Antibodies to
PGA were detected in all of the samples tested, with an average titer of $1 \times 10^2$. For comparison, we measured the anti-PA antibodies in these samples. Anti-PA was not detected in any samples when the starting dilution of 1:100 was used, but anti-PA antibodies with titers in the range of 1:10 to 1:90 were detected when the starting dilution was decreased to 1:10 (Fig. 3). Anti-PGA titers were significantly higher than those of anti-PA, according to the unpaired $t$ test.

The origin of the preimmune PGA antibodies is not known. A possible source is the $\gamma$-DL-PGA present in other, nonpathogenic bacteria. We tested our anti-PA MAbs and representative nonimmune chimpanzee and human sera for binding to $\gamma$-D-PGA produced by $B$. anthracis and $\gamma$-DL-PGA produced by $B$. subtilis. We found that, while both our anti-PA MAbs (4C and 11D) and chimpanzee and human sera reacted with $\gamma$-D-PGA, they also reacted with $\gamma$-DL-PGA (Fig. 4).

**MAbs and polyclonal antibodies react with the same or a closely related epitope.** The ability to block the binding of IgG anti-PGA to PGA by the MAb 11D Fab was examined using ELISA. The 11D Fab blocked the binding to PGA by the homologous IgG, as well as that of MAb 4C and the polyclonal IgG, in a dose-dependent manner, with the maximal blocking achieved for homologous IgG, as well as that of MAb 4C and the polyclonal IgG, respectively (Fig. 5A). The blocking is specific to anti-PGA Fab because the irrelevant anti-PA Fab did not block binding by all the IgGs tested. This finding indicates that MAB 11D targets an immunodominant epitope.

**Opsonophagocytic killing of $B$. anthracis by the polyclonal antibodies.** The opsonophagocytic bactericidal activity of the PAbs was similar to that of MAbs 4C and 11D (25), with an LD$_{50}$ of 1:1,600 (corresponding to 0.625 $\mu$g/ml of total IgG) (Fig. 6). Linear regression analysis showed a strong correlation between antibody concentration and opsonophagocytic bactericidal activity based on the high calculated regression coefficient ($R^2 = 0.9913$).

**Protection of mice from pulmonary challenge with virulent $B$. anthracis spores.** BALB/c mice were pretreated with the polyclonal sera at doses of 1, 0.3, or 0.1 mg per mouse or with the 11D MAb at doses of 0.1 or 0.03 mg per mouse and challenged 18 h later with $1 \times 10^4$ spores ($\approx 10$ LD$_{50}$). The survival results showed that pretreatment with either the PAb anti-PGA or the MAB 11D provided significant protection against the pulmonary challenge with $B$. anthracis Ames strain spores at all doses tested ($P \leq 0.05$ for all Ab-treated groups, compared to the vehicle control group) (Fig. 7). A higher percent survival was observed for treatment with 11D MAB than for treatment with PABs at the 0.1-mg dose, although the difference was not statistically significant according to Kaplan-Meier or log-rank analysis ($P > 0.1$).

**DISCUSSION**

Chimpanzees are phylogenetically closer to humans than to mice, guinea pigs, rabbits, and monkeys and have been considered a preferred animal model for some studies (29). Chimpanzees were used to study a number of human pathogens such as hepatitis A, B, C, D, and E virus; respiratory syncytial virus; and Norwalk virus and showed serological and biochemical characteristics similar to those of humans following experimental infection (30–34). In a previous study, we prepared and evaluated MAbs to $\gamma$-D-PGA, the capsule of $B$. anthracis, from bone marrow-derived lymphocytes of chimpanzees immunized with $\gamma$-D-PGA peptides conjugated to TT or rPA (25). We demonstrated that these MAbs were bactericidal and highly protective against an inhalational challenge of mice with fully virulent $B$. anthracis spores. In the current study,
we compared the PAbs of the same animals to the MAbs and expanded on their characterization. Our study is limited by the fact that only two chimpanzees were immunized with /H9253-D-PGA conjugates, each to a different carrier, but we believe that some principles can be derived.

Antibody measurements revealed preexisting PGA antibodies in chimpanzees and humans. These antibodies were PGA specific because no binding of the test sera to BSA, milk, or rPA was detected, neither did the secondary antibody; goat anti-human IgG (Fab)\(_2\) antibodies bind to the PGA. The blocking experiments indicated that the epitope specificity of the PGA conjugate-induced antibodies is identical or closely related to that of the MAbs. The detection of preexisting PGA antibodies, but not PA antibodies, is also not due to the higher affinities of anti-PGA antibodies compared with those of anti-PA antibodies, as our previous studies showed that anti-PA antibodies had much higher affinities than anti-PGA antibodies (24, 25). Furthermore, the detection in humans of anti-PGA antibodies is consistent with unpublished data from Thomas Kozel’s lab, University of Nevada, showing that 33 human donors from the Reno, Nevada, area had anti-PGA antibodies with a median IgG titer of 1:2,400 and a median IgM titer of 1:12,000 (personal communication). The origin of these antibodies is not clear, but they are possibly the result of exposures to PGA-like structures produced by nonpathogenic bacteria, such as the bacillus species, that are known to produce /H9253-DL-PGA. The response of the chimpanzees to PGA conjugate vaccination seemed anamnestic, with maximal levels achieved after a single injection without further booster effects. These anti-PGA antibodies were shown by their susceptibility to blocking by a Fab from MAb D11 to bind to the same or an overlapping epitope. Thus, both the MAb and the PAb represent a general antibody response, which is likely directed to the oligo-\(\gamma\)-D-Glu sequence used as the immunogen. The fact that the characteristics of the MAb and PAb are similar indicates that the MAb is not a selected rarity but represents the general antibody response.

Preimmune PA antibodies in the chimpanzees were detected

FIG 5 Anti-PGA MAb 11D blocks the binding of polyclonal serum to \(\gamma\)-D-PGA. (A) Representative blockage of different concentrations of chimpanzee serum by MAb 11D Fab. (B) Reactivity of MAb 4C and 11D IgGs and the polyclonal serum at concentration of 28 ng/ml against \(\gamma\)-D-PGA in the presence or absence of 11D Fab. Reactivity of tested IgGs with \(\gamma\)-D-PGA was measured as described in Materials and Methods. Each symbol represents the mean of triplicate wells from a single experiment. Bars represent the optical density at 450 nm (OD\(_{450}\)) average. Student’s \(t\) test was used to compare differences in blocking assays with unblocked control. Statistical evaluation was done with the GraphPad Prism, version 5.0. \(P\) values of \(\leq 0.0002\) were considered significant and are denoted by stars. Percent blocking by 11D Fab was calculated and is indicated.

FIG 6 Correlation between antibody concentration and opsonophagocytic bactericidal activity of IgG PGA polyclonal antibodies. The concentrated polyclonal antibodies collected from immunized chimpanzees were analyzed for opsonophagocytic activity. This is representative of three experiments with similar results. The results were analyzed by linear regression. Numbers in the \(x\) axis representing opsonophagocytic titers (reciprocal of total IgG dilution) are ln transformed.

FIG 7 Survival of mice pretreated with either \(\gamma\)-D-PGA–specific PAb or MAb 11D. Groups of mice (6 mice/1-mg-dose group and 8 mice/group at all other doses) were treated i.p. with the designated dose of PAb or MAb 11D at 18 h prechallenge. Control mice received the vehicle alone. The mice were then infected i.t. with \(1 \times 10^4\) B. anthracis (Ames strain) spores. The survival of mice was monitored twice daily for 2 weeks postchallenge. Treatment with either anti-\(\gamma\)-D-PGA PAb or MAb at all doses provided significant protection against infection with B. anthracis compared with that in the vehicle control group (\(P < 0.05\) for all groups).
only at titers of <1:100. Immunization with the PGA-PA conjugate generated low PA antibody levels following the 1st immunization, while high levels were achieved after booster injections. In these respects, the antibody responses to PA with PA alone or with PGA-PA were similar.

The anti-PA PABs, like the previously described MAbs, were effective in opsonizing and killing vegetative, capsule-producing B. anthracis. More significant, the anti-PA PAlbodies protected mice from infection with virulent B. anthracis in an inhalational mouse challenge assay. We inferred from these findings that immunization of humans with γ-PA conjugated to an acceptable carrier will provide protection from infection.

The work described here supports the continued development of a candidate, improved, broad-spectrum anthrax vaccine, PGA-rPA. The currently licensed vaccines (PA based) are derived from partially purified culture supernatants of Sterne-type strains. These are likely to be replaced soon by vaccines containing highly purified PA or PA variants slightly modified to facilitate production and improve stability (35). While effective and relatively safe in the animal models used to achieve FDA approval, these vaccines may not have sufficient strength to protect humans exposed to high doses in a bioterror event. For this reason, we and others have sought to generate vaccines containing additional immunogens. Because the PGA capsule is an essential virulence factor, antibodies to PG are expected to contribute to protection against infection. Free PGA is a poor immunogen, but its administration as a conjugate to protein carriers induces IgG that is opsonophagocytic and protective in certain animal models, as shown previously and confirmed here.

We showed previously that the candidate PGA-rPA vaccine elicits an additional protective immune response that directly kills the organism, while the antibody response to the rPA carrier neutralizes the toxin, as occurs with the current vaccines. In a postexposure situation, use of such a vaccine and/or humanized PGA-specific MAbs may supplant or reduce the duration of the currently recommended 2-month regimen of antibiotics (36), which had poor patient compliance when used in 2001 (37). A PGA-rPA vaccine may also be preferred over a simple PA vaccine in the postexposure treatment regimen because of the evidence presented here of the very rapid anti-PA response. Furthermore, a rapid anti-PA response would be advantageous when an exposure (e.g., a warfare or bioterror event) is viewed as imminent and rapid protection is needed.

Improved vaccines that contain multiple immunogens are also anticipated when it is anticipated that exposure to an engineered pathogen may occur. B. anthracis strains are easily made resistant to one or more antibiotics, in which case, antibodies to key virulence factors are essential for protection. However, a single MAb to a protein can be circumvented by mutation of the protein epitope. This is of less concern with antibodies to a simple immunogen like PGA, where modifications to the structure would destroy its functional activity. These considerations argue that vaccines containing multiple immunogens will generally be preferred because they have expanded scope and potency. Based on these factors and the data presented here, we suggest that a broad-spectrum PGA-rPA vaccine will be preferred to the current PA-only vaccines for all clinical indications. Similarly, the data here argue that the chimpanzee-human anti-PA MAbs described in our previous reports constitute a valuable addition to prophylactic and postexposure treatment regimens.

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