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Additional Conjugation Methods and Immunogenicity of *Bacillus anthracis* Poly-γ-D-Glutamic Acid–Protein Conjugates

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The capsule of *Bacillus anthracis*, composed of poly-γ-D-glutamic acid (γDPGA), is an essential virulence factor of *B. anthracis*. The capsule inhibits innate host defense through its antiphagocytic action. γDPGA is a poor immunogen, but when covalently bound to a carrier protein, it elicits serum antibodies. To identify the optimal construct for clinical use, synthetic γDPGAs of different lengths were bound to carrier proteins at different densities. The advantages of the synthetic over the natural polypeptide are the homogeneous chain length and end groups, allowing conjugates to be accurately characterized and standardized and their chemical compositions to be related to their immunogenicities. In the present study, we evaluated, in addition to methods reported by us, hydrazone, oxime, and thioether linkages between γDPGA and several proteins, including bovine serum albumin, recombinant *Pseudomonas aeruginosa* exotoxin A, recombinant *B. anthracis* protective antigen (rPA), and tetanus toxoid (TT). The effects of the dosage and formulation on the immunogenicities of the conjugates were evaluated in mice. All conjugates were immunogenic. The optimal γDPGA chain length of 10 to 15 amino acids and the density, an average of 15 mol γDPGA per mol of protein, were confirmed. The thioether bond was the optimal linkage type, and TT and rPA were the best carriers. The optimal dosage was 1.2 to 2.5 μg of γDPGA per mouse, and adsorption of the conjugates onto aluminum hydroxide significantly increased the antibody response to the protein with a lesser effect on anti-γDPGA levels.

Anthrax, a potentially lethal human disease, is a zoonotic infection that under natural conditions is contracted by humans directly or indirectly from animals. The causative organism, *Bacillus anthracis*, exists in vegetative or spore forms, the latter being the infecting agent. A veterinary vaccine based on a capsule-negative, toxin-positive strain (Sterne) is available and is routinely used throughout the world (25). In addition, anthrax vaccine adsorbed, containing the protective antigen, is licensed and used by veterinarians, animal by-product handlers, and the U.S. Army (2, 5). Anthrax is endemic in countries that do not immunize domesticated animals (9, 12, 24). Because of the ease of cultivating the organism and improved technology of spore preparation, *B. anthracis* is a potential bioterrorism agent. In 2001, *B. anthracis* spores were used successfully via the U.S. mail, though few people were affected. A major bioterrorism attack may be airborne, with a number of spores far exceeding a natural exposure, causing inhalation anthrax and affecting a large number of people, including children. These facts warrant devising an improved anthrax vaccine.

The addition of components other than those of anthrax toxin to improve vaccine-induced protection has been considered (22). The capsule, composed of poly-γ-D-glutamic acid (γDPGA), is an essential virulence factor and antiphagocytic, and antibodies to this polypeptide have been shown to be opsonophagocytic and protective in mice (3, 10, 22). γDPGA by itself is a poor immunogen and does not induce booster responses, probably because of its simple homopolymeric structure, similar to those of capsular polysaccharides; it is a T-cell-independent antigen and of D-amino acid composition (7). These immunologic properties can be overcome by covalent binding of the T-cell-independent antigen to immunogenic proteins (22). Because of the success in inducing protective levels of antibodies in infants against systemic infection with capsulated pathogens, we developed conjugates of γDPGA with several carrier proteins, including bovine serum albumin (BSA), recombinant *B. anthracis* protective antigen (rPA), and recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA). Unlike γDPGA alone, these conjugates were immunogenic in mice, with booster responses upon reinjection. Conjugate-induced antibodies were opsonophagocytic (22, 27). This study describes additional synthetic schemes in an attempt to develop the most immunogenic conjugates.

MATERIALS AND METHODS

Analyses. Amino acid analysis was done by gas-liquid chromatography–mass spectrometry (GLC-MS) after hydrolysis with 6 N HCl at 150°C for 1 h and derivatization to *N*-heptafluorobutyryl *R*-(−)-isobutyl esters and assay with a Hewlett-Packard apparatus (model HP 6890) with an HP-5 0.32- by 30-mm glass capillary column with temperature programming at 8°C/min from 125 to 250°C in the electron ionization (106-eV) mode (22). The number of peptide chains in the conjugates was calculated by the ratio between L- and D-glutamic acids. The protein concentration was measured by the method of Lowry et al. (16), free ε amino groups by Fields' assay (6), benzaldehyde groups by colorimetric reaction with 2-hydrazinopyridine (Solulink, San Diego, CA), hydrazide was measured as reported previously (23), and thiolation was measured by release of 2-pyridylthio groups (A_{343}) (1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis used 14% gels according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Double immunodiffusion was performed in 1.0% agarose gels in phosphate-buffered saline (PBS) with rabbit anti-γDPGA, rabbit anti-BSA (Sigma, St. Louis, MO), goat

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anti-exotoxin A (List Biological Laboratories, Inc.), anti-tetanus toxoid (obtained from W. Vann, FDA, Bethesda MD), and anti-*B. anthracis* protective antigen (obtained from S. Leppla, NIH/NIAID, Bethesda, MD). Aluminum hydroxide was used as Alhydrogel (Staten Serum Institut, Copenhagen, Denmark).

MALDI-TOF. Mass spectra were obtained with an OmniFlex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) instrument (Bruker Daltonics) operated in the linear mode. Samples for analysis were desalted, and 1 μl was mixed with 20 μl of sinapinic acid matrix made in 30% CH_3CN and 0.1% trifluoroacetic acid. Then, 1 μl of the mixture was dried on the sample stage and placed in the mass spectrometer.

Antigens. BSA (66.5 kDa; Sigma, St. Louis, MO) was dialyzed against pyrogen-free water, sterile filtered, and freeze-dried. rPA (83 kDa) from *B. anthracis* and rEPA (67 kDa) from *P. aeruginosa* were prepared and characterized (8, 21). Tetanus toxoid (TT) (150 kDa) was obtained from Merieux, Lyon, France.

γDPGA was purified from the culture supernatant of *B. anthracis* strain A34 toxin-negative by cetavlon precipitation, acidification to pH 1.5, precipitation with ethanol, and passage through a 2.5- by 100-cm Sephacryl S-1000 column in 0.2 M NaCl (26). Its structure was confirmed by ^1H nuclear magnetic resonance and ^{13}C nuclear magnetic resonance, and its enantiomeric composition was determined by GLC-MS spectroscopy. γDPGA peptides were synthesized by the method of Merrifield (AnaSpec, San Jose, CA). Peptides were divided into groups depending on the types of linkages through which they were bound to proteins: (i) thioether linkage, NAc-γDPGA$_{10}$-Gly$_3$-L-Cys-CONH$_2$ (γDPGA$_{10}$-Cys) or NBrAc-Gly$_3$-γDPGA$_{10}$-COOH (Br-γDPGA$_{10}$); (ii) hydrazone linkage, 4-formylbenzoyl-Gly$_3$-γDPGA$_{10}$-COOH (CHO-γDPGA$_{10}$), NAc-γDPGA$_{10}$-Gly$_3$-CO-NH-NH-CO-(CH$_2$)$_4$-CO-NH-NH$_2$ (γDPGA$_{10}$-AH), or NAc-γDPGA$_{15}$-CO-NH-NH-CO-(CH$_2$)$_4$-CO-NH-NH$_2$ (γDPGA$_{15}$-AH), where AH is adipic acid hydrazide; and (iii) oxime linkage, 4-formylbenzoyl-Gly$_3$-γDPGA$_{10}$-COOH (CHO-PGA$_{10}$).

Conjugation. (i) Thioether linkage. First, protein was bromoacylated using succinimidyl 3-(bromoacetamido)propionate (SBAP) (Pierce, Rockford, IL) and reacted with peptides equipped with a terminal cysteine residue as reported previously (22) (protein/*S*-Cys-Gly$_3$-γDPGA$_{10}$-NAc, protein/*S*-Cys-γDPGA$_{10}$-NAc, or protein/*S*-Cys-γDPGA$_{15}$-NAc).

Second, protein was derivatized with an *N*-hydroxysuccinimide ester of 3-(2-pyridyldithio)propionic acid (SPDP) (Pierce, Rockford, IL) and reacted with bromoacylated peptide (22) (protein-*S*/Gly$_3$-γDPGA$_{10}$-COOH).

Third, sulfhydryl groups were introduced into the protein using 2-iminothiolane (ITL) (ScienceLab, Houston, TX). Protein (20 mg) was derivatized with 2.8 mg ITL in 1.5 ml of buffer A (PBS, 0.1% glycerol, 0.005 M EDTA, pH 7.4) for 60 min. Next, a solution of Br-γDPGA$_{10}$ (35 mg) in 300 μl of 1 M K_2HPO_4 was added while the pH was maintained at 7.4 with the addition of 0.2 N NaOH at room temperature. After 90 min, the reaction mixture was passed through a Sepharose CL-6B column (1 by 120 cm) and eluted with 0.2 M NaCl. Fractions reacting with anti-protein and anti-γDPGA were pooled (protein-ITL/Gly$_3$-γDPGA$_{10}$-COOH).

(ii) Hydrazone linkage. First, protein was derivatized with succinimidyl 4-formylbenzoate (SFB) (Solulink, San Diego, CA). To a solution of protein (30 mg) in 1.2 ml buffer A, SFB (7.5 mg) in 100 μl dimethyl sulfoxide was added and reacted for 2 h at pH 7.4. The product 4-formylbenzoyl–protein was passed through a Sephadex G-50 column (1 by 100 cm) in 0.2 M NaCl. Protein-containing fractions were pooled and assayed for the presence of benzaldehyde, antigenicity, and protein concentration. To 4-formylbenzoyl–protein (20 mg) in 1.25 ml buffer A, a solution of 15 mg of AH-γDPGA$_{10}$ or AH-γDPGA$_{15}$ in 200 μl 1 M K_2HPO_4 was added. The pH of the reaction mixture was adjusted to 7.4, and the mixture was incubated overnight at room temperature and then passed through a Sepharose CL-6B column as described above. Fractions reacting with anti-protein and anti-γDPGA were pooled and assayed (protein-SFB/AH-Gly$_3$-γDPGA$_{10}$-NAc or protein-SFB/AH-γDPGA$_{15}$-NAc).

Second, derivatization of protein with adipic acid dihydrazide using a water-soluble carbodiimide was done as reported previously (23). The incorporation of hydrazide residues was 2 to 5% per protein. To protein-AH (20 mg) in 1.3 ml buffer A, a solution of 20 mg of CHO-γDPGA$_{10}$ in 200 μl 1 M K_2HPO_4 was added. The pH was adjusted to 7.4, and the solution was incubated overnight at room temperature and then passed through a Sepharose CL-6B column as described above. Fractions reacting with anti-protein and anti-γDPGA were pooled and assayed (protein-AH/SFB-Gly$_3$-γDPGA$_{15}$-COOH).

(iii) Oxime linkage. BSA was first bromoacylated with SBAP and then reacted with *O*-(3-thiolpropyl)hydroxylamine, a heterobifunctional aminooxy-thiol linker (11). To 20 mg of aminooxylated protein (protein-ONH$_2$) in 1.3 ml buffer A, a solution of 20 mg of CHO-PGA in 200 μl 1 M K_2HPO_4 was added. The pH was adjusted to 7.4, and the mixture was incubated overnight at room temperature and then passed through a Sepharose CL-6B column as described above. Fractions reacting with anti-protein and anti-γDPGA were pooled and assayed (protein-ONH$_2$/SFB-Gly$_3$-γDPGA$_{10}$-COOH).

Immunization. All animal experiments were approved by the National Institute of Child Health and Human Development (NICHD) Animal Care and Use Committee. Five- to 6-week-old female NIH general-purpose mice were immunized subcutaneously three times at 2-week intervals with 2.5 μg γDPGA as a conjugate in 0.1 ml PBS. Groups of 10 were exsanguinated 7 days after the second or third injection (23). Controls received PBS.

Antibodies. Serum immunoglobulin G (IgG) antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (23). Nunc Maxisorb plates were coated with *B. anthracis* γDPGA, 20 μg/ml PBS, or 4 μg protein/ml PBS (determined by checkerboard titration). The plates were blocked with 0.5% BSA (or with 0.5% HSA for assay of BSA conjugates) in PBS for 2 h at room temperature. An MRX Dynatech reader was used. Antibody levels were calculated relative to standard sera: for γDPGA, a hyperimmune murine serum (22); for PA, a monoclonal antibody containing 4.7 mg antibody/ml (15); for BSA and rEPA, a pool of highest-titer sera obtained from mice immunized three times and assigned a value of 100 EU. The results were computed with an ELISA data-processing program provided by the Biostatistics and Information Management Branch, CDC (19). IgG levels are expressed as geometric means (GM).

Statistics. The Bonferroni multiple-comparison test was used for different groups of mice.

RESULTS

Characterization of conjugates. The conjugation methods used in the study for binding γDPGA to protein carriers are illustrated in Fig. 1. The purity of the conjugates and the absence of free protein were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by MALDI-TOF spectroscopy. The molar ratio of γDPGA to protein in conjugates, calculated by GLC-MS analysis, was based on the ratio of D-glutamic acid to L-glutamic acid in the conjugate and by the increase in molecular mass, measured by MALDI-TOF, of the conjugate compared to that of the protein derivatized with the appropriate linker.

Antibodies to γDPGA. Conjugates varied in γDPGA chain length, density on the protein, carrier proteins, and types of linkage between γDPGA and the protein. Additional variables were the formulation and the dosage injected into mice. All conjugates reacted with anti-γDPGA and anti-protein sera with a line of identity. All conjugates were highly immunogenic in mice; most immune sera precipitated *B. anthracis* γDPGA by double immunodiffusion (results not shown).

To identify the most immunogenic conjugates, we compared the immunogenicities of new constructs with those reported previously (22). Our previous results showed that protein conjugates of 10-mers of γDPGA were more immunogenic than the conjugates of 5- or 20-mers (22). Here, we have used 10-mers with three glycine residues (Gly$_3$-γDPGA$_{10}$) at the end linked to protein as before and 10-mers (γDPGA$_{10}$) and 15-mers (γDPGA$_{15}$) without glycine to avoid the generation of antibodies to the glycine linker. There were no statistical differences in the anti-γDPGA levels between the conjugates of the three peptides after three injections (Table 1). For example, the two conjugates TT/Cys-γDPGA$_{10}$ and TT/Cys-γDPGA$_{15}$, having the same average density of γDPGA on the protein (16 chains), gave similar responses, irrespective of the peptide chain length (after the second injection, the TT/Cys-γDPGA$_{10}$ GM was 6,868 and the TT/Cys-γDPGA$_{15}$ GM was 6,547; after the third injection, the TT/Cys-γDPGA$_{10}$ GM was 6,667 and the TT/Cys-γDPGA$_{15}$ GM was 7,688). Conjugate TT/Cys-Gly$_3$-γDPGA$_{10}$, with an average density of 11 γDPGA chains per protein, induced a lower level of antibodies after the sec-

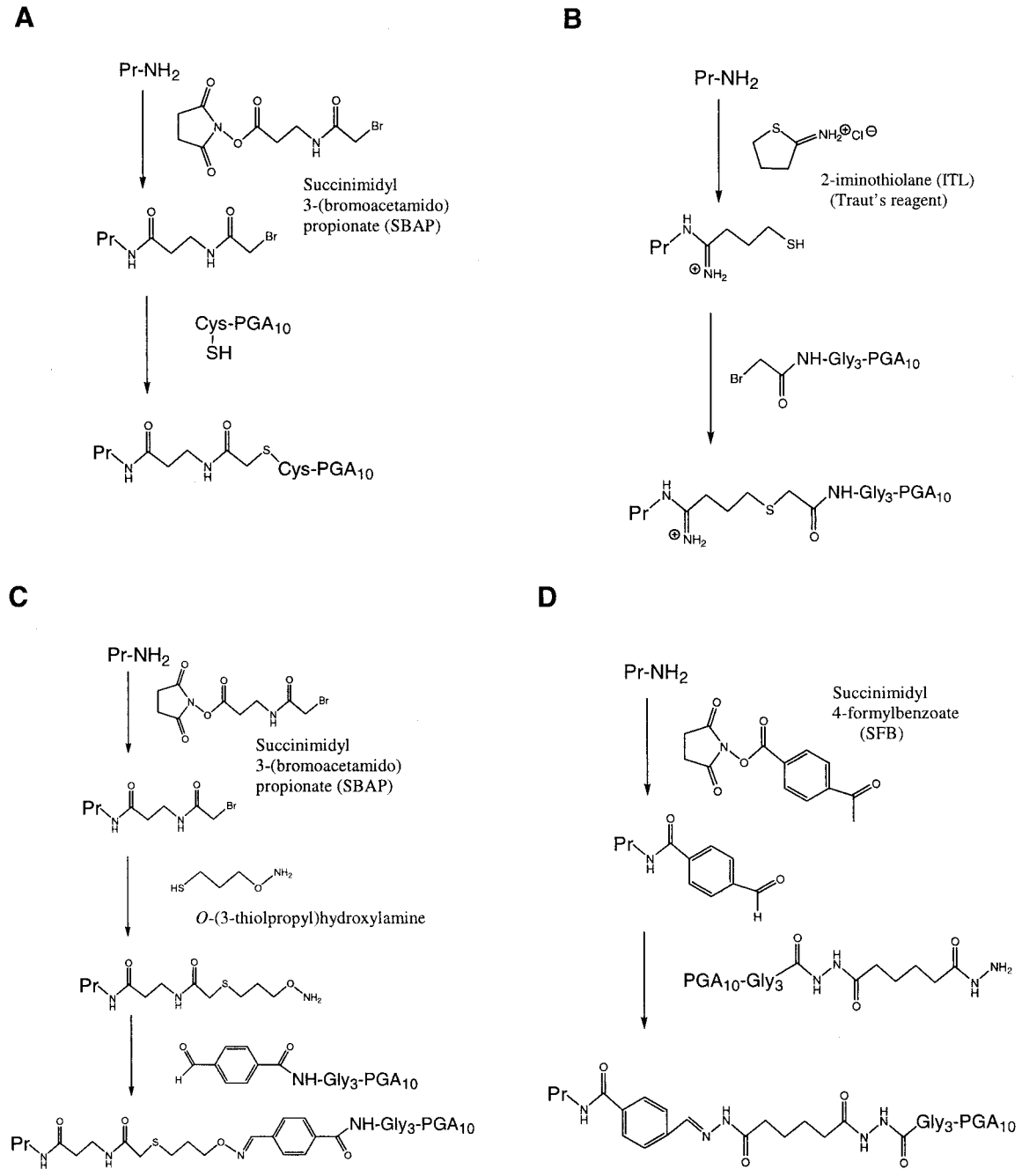

FIG. 1. Examples of linkage types used for conjugation of poly-γ-D-glutamic acid (γDPGA) peptides to protein carriers. (A and B) Thioether linkage. (C) Oxime linkage. (D) Hydrazone linkage.

ond injection (GM = 421; $P < 0.001$), but after the third injection, the GM antibody level was similar to that of the conjugate without the glycine linker (GM = 7,762).

Table 1 summarizes the influence of the γDPGA chain density on the protein on the conjugate's immunogenicity. The best responses were achieved with an average of 10 to 15 chains per protein. The antibody level induced by TT/Cys-$\gamma DPGA_{15}$ with 16 chains per protein (GM = 7,688) was significantly higher than that induced by TT-CHO/AH-$\gamma DPGA_{15}$ with 37 chains per protein (GM = 2,322; $P < 0.001$). Similarly, conjugate rPA-CHO/AH-$\gamma DPGA_{15}$ with 11 chains (GM = 5,225) induced a higher level of antibodies than the conjugate with an average of 5 chains per protein (GM = 315; $P < 0.001$) or rPA-CHO/AH-Gly_3-$\gamma DPGA_{10}$ with 29 chains per protein (GM = 1,256; $P < 0.001$). There were no statistical differences between rPA-ITL/Br-Gly_3-$\gamma DPGA_{10}$ with either 8 or 18 chains per protein and rPA/Cys-Gly_3-$\gamma DPGA_{10}$ with 15 chains per protein.

A comparison of different linkage methods, including those tested previously (22), is presented in Table 2. The best results

TABLE 1. Density/immunogenicity relationships of conjugates prepared with 10- and 15-mers of γDPGA bound to TT or rPA[a]

Conjugate	γDPGA (mol)/ protein (mol)	Protein/γDPGA (wt/wt)	Anti-γDPGA (EU)		Anti-rPA (μg/ml)	
			2nd injection	3rd injection	2nd injection	3rd injection
TT/Cys-Gly_3-γ$DPGA_{10}$	11	1:0.10	420	7,800	NT[b]	NT
TT/Cys-γ$DPGA_{10}$	16	1:0.16	6,900	6,700	NT	NT
TT/Cys-γ$DPGA_{15}$	16	1:0.22	6,500	7,700	NT	NT
TT-CHO/AH-γ$DPGA_{15}$	37	1:0.48	1,900	2,300	NT	NT
rPA-CHO/AH-γ$DPGA_{15}$	5	1:0.12	120	320	0.2	4
rPA-CHO/AH-γ$DPGA_{15}$	11	1:0.26	3,600	5,200	0.1	13
rPA-CHO/AH-Gly_3-γ$DPGA_{10}$	29	1:0.45	210	1,300	0.1	2
rPA-AH/CHO-Gly_3-γ$DPGA_{10}$	22	1:0.34	1,000	2,500	0.3	28
rPA/Cys-Gly_3-γ$DPGA_{10}$	15	1:0.24	2,100	4,000	37	170
rPA-ITL/Br-Gly_3-γ$DPGA_{10}$	8	1:0.13	2,300	3,700	4.7	61
rPA-ITL/Br-Gly_3-γ$DPGA_{10}$	18	1:0.29	2,400	3,000	0.2	41

[a] Five- to 6-week-old NIH general-purpose mice ($n = 10$) were injected subcutaneously with 2.5 μg of γDPGA as a conjugate 2 weeks apart and exsanguinated 7 days after the second or third injection. IgG anti-γDPGA and anti-rPA were measured by ELISA, and the results are expressed as the geometric means.
[b] NT, not tested.

were obtained by thioether-linked conjugates created between thiolated protein with SPDP and bromoacylated γDPGA or, conversely, bromoacylated protein and SH-γDPGA. These antibody levels were significantly higher over thioether-linked conjugates created using 2-iminothiolane and over hydrazone-linked conjugates (rPA-SH/Br-Gly_3-γ$DPGA_{10}$ [GM = 7,206] versus rPA-ITL/Br-Gly_3-γ$DPGA_{10}$ [GM = 3,726; $P < 0.05$]; rPA-SH/Br-Gly_3-γ$DPGA_{10}$ versus rPA-CHO/AH-γ$DPGA_{15}$ [GM = 3,004; $P < 0.01$]; rPA-SH/Br-Gly_3-γ$DPGA_{10}$ versus rPA-AH/CHO-Gly_3-γ$DPGA_{10}$ [GM = 2,478; $P < 0.001$]; rPA-Br/Cys-Gly_3-γ$DPGA_{10}$ [GM = 5,822] versus rPA-CHO/AH-γ$DPGA_{15}$ [GM = 3,004; $P < 0.05$]; and rPA-Br/Cys-Gly_3-γ$DPGA_{10}$ versus rPA-AH/CHO-Gly_3-γ$DPGA_{10}$ [GM = 2,478; $P < 0.05$]). There were no statistically significant differences between the best thioether-linked conjugate (GM = 7,206) and the aminooxy-linked conjugate (GM = 4,257), but the higher level of antibodies and the ease with which the thioether bond is formed (one less step is required) make the first the better choice.

The effects of the carrier proteins on immunogenicity are summarized in Table 3. Among all carriers, rPA and TT conjugates produced the best responses to γDPGA (rPA and TT versus BSA [$P < 0.001$]; rPA and TT versus rEPA [$P < 0.01$]; and rEPA versus BSA [no statistical difference]). Formulation of the vaccine with aluminum hydroxide significantly increased the antibody response to the carrier protein with minimal significant effect upon the response to γDPGA (Table 4). In only one case (TT/Cys-γ$DPGA_{15}$) did the aluminum hydroxide significantly ($P < 0.01$) increase the response to γDPGA, rendering it the best conjugate, inducing 14,950 EU. The optimal dose of conjugated γDPGA was between 1.2 and 2.5 μg per mouse (Table 5).

Antibody to the protein carrier. The antibody levels were calculated in ELISA units relative to a standard for each protein, arbitrarily assigned a value of 100 EU for BSA and rEPA and μg/ml for rPA. Therefore, the comparison is possible only within each type of carrier. The responses to protein varied among the conjugates, since the dosage was always based on the amount of γDPGA in the conjugate. Overall, the highest levels were induced by conjugates prepared by thioether linkage between bromoacylated protein and peptide containing terminal cysteine formulated with aluminum hydroxide. The antibody response was directly related to the dosage of the carrier protein.

DISCUSSION

The pathogenicity of *B. anthracis* requires two essential virulence factors: the tripartite toxin and the γDPGA capsule. The licensed vaccine against anthrax (Anthrax Vaccine Adsorbed) contains the protective antigen, the binding compo-

TABLE 2. Conjugation method/immunogenicity relationships of conjugates prepared with a 10-mer of γDPGA bound to rPA[a]

Conjugate	γDPGA (mol)/ protein (mol)	Protein/γDPGA (wt/wt)	Anti-γDPGA (EU)		Anti-rPA (μg/ml)	
			2nd injection	3rd injection	2nd injection	3rd injection
Thioether linkage						
rPA-SH/Br-Gly_3-γ$DPGA_{10}$	14	1:0.22	4,400	7,200	0.3	66
rPA-Br/Cys-Gly_3-γ$DPGA_{10}$	22	1:0.35	2,200	5,800	2	210
rPA-ITL/Br-Gly_3-γ$DPGA_{10}$	8	1:0.13	2,300	3,700	4.7	61
Oxime linkage						
rPA-$CONH_2$/CHO-Gly_3-γ$DPGA_{10}$	20	1:0.32	2,000	4,300	0.4	25
Hydrazone linkage						
rPA-CHO/AH-γ$DPGA_{15}$	15	1:0.36	2,200	3,000	0.2	6
rPA-AH/CHO-Gly_3-γ$DPGA_{10}$	22	1:0.34	1,000	2,500	0.3	28

[a] Five- to 6-week-old NIH general-purpose mice ($n = 10$) were injected subcutaneously with 2.5 μg of γDPGA as a conjugate 2 weeks apart and exsanguinated 7 days after the second or third injection. IgG anti-γDPGA and anti-rPA were measured by ELISA, and the results are expressed as the geometric means.

TABLE 3. Protein carrier/immunogenicity relationships of conjugates prepared with a 10-mer of γDPGA bound to BSA, rPA, rEPA, or TT[a]

Conjugate	γDPGA (mol)/ protein (mol)	Anti-γDPGA (EU)		Anti-protein[c]	
		2nd injection	3rd injection	2nd injection	3rd injection
BSA/Br-Gly_3-γ$DPGA_{10}$	18	1,900	1,800	5	19
rEPA/Br-Gly_3-γ$DPGA_{10}$	9	930	3,200	0.6	6
rPA/Br-Gly_3-γ$DPGA_{10}$	14	4,400	7,200	1	96
rPA/Cys-Gly_3-γ$DPGA_{10}$	21	5,500	7,500	0.1	2.2
TT/Cys-Gly_3-γ$DPGA_{10}$	11	420	7,800	NT[b]	NT

[a] Five- to 6-week-old NIH general-purpose mice ($n = 10$) were injected subcutaneously with 2.5 μg of γDPGA as a conjugate 2 weeks apart and exsanguinated 7 days after the second or third injection. IgG anti-γDPGA and anti-rPA were measured by ELISA, and the results are expressed as the geometric means.
[b] NT, not tested.
[c] EU for anti-BSA and anti-rEPA and μg/ml for anti-rPA.

TABLE 5. Dose/immunogenicity relationships of conjugates prepared with a 10-mer of γDPGA bound to rPA[a]

Conjugate	γDPGA (mol)/ rPA (mol)	Dose (μg/mouse)	Anti-γDPGA (EU)		Anti-rPA (μg/ml)	
			2nd injection	3rd injection	2nd injection	3rd injection
rPA/Cys-Gly_3-γ$DPGA_{10}$	22	0.31	490	3,500	0.3	9
		0.63	980	4,900	0.6	37
		1.25	2,300	6,200	2	120
		2.5	2,200	5,800	2	200
		20	NT[b]	3,700	NT	440

[a] Five- to 6-week-old NIH general-purpose mice ($n = 10$) were injected subcutaneously with 2.5 μg of γDPGA as a conjugate 2 weeks apart and exsanguinated 7 days after the second or third injection. IgG anti-γDPGA and anti-rPA were measured by ELISA, and the results are expressed as the geometric means.
[b] NT, not tested.

nent of the toxin complex (14). The protective effects of the anticapsular antibodies were investigated recently (3, 10, 22).

We have described the preparation of conjugates of synthetic γDPGA peptides with several carrier proteins bound through thioether linkages (22). Peptides of different lengths were used, at an average of 5 to 32 mol of peptide per mol of protein. Unlike γDPGA alone, these conjugates were immunogenic in 5- to 6-week old general-purpose mice when injected at a dosage and schedule relevant for use in humans, and the antibodies induced opsonophagocytic killing of *B. anthracis*. This finding was confirmed by others (27).

The conjugation techniques, including the types of chemical linkages between the hapten and the protein, as well as the peptide/sugar chain length and the density on the protein, influence the serum antibody response to both components (4, 13, 17, 18, 20). Therefore, we studied several constructs for human use by employing additional methods and formulations of the γDPGA conjugates. Previously, we created thioether bonds between the protein and the γDPGA by introduction of a bromoacyl group (SBAP) or a thiol group (SPDP) into the protein. The activated protein was then bound to a peptide with a thiol or bromoacetyl group at its N or C terminus, respectively. In the present study, we tested (i) thioether bonds generated by derivatization of the protein with thiol groups using 2-iminothiolane, followed by binding to bromoacetylpeptide; (ii) hydrazone bonds generated by derivatizing the proteins with adipic acid dihydrazide, followed by reaction with benzaldehyde-derivatized peptides or, alternatively, a benzaldehyde group was introduced into the protein using SFB and the formylated protein bound to a hydrazide-derivatized peptide; (iii) oxime bonds created by derivatization of the protein with SBAP, which was then coupled to an aminoxy-thiol linker; the aminooxylated protein was then bound to 4-formylbenzoyl-γDPGA. The immunogenicities of these conjugates in mice were similar to those previously prepared: the optimal density was ~15 mol γDPGA per mol protein with a peptide chain length of 10 or 15 amino acids. Tetanus toxoid and rPA were better carriers than BSA or rEPA. The most successful and reproducible linkage was formed by introduction of bromoacetyl groups onto the lysine residues of the protein, followed by conjugation with γDPGA equipped with a terminal cysteine residue. A dosage of 1.2 to 2.5 μg of conjugated γDPGA per mouse gave the best γDPGA response. Aluminum hydroxide should be included in the formulation if a high response to rPA or to another carrier protein is needed.

The choice of an anthrax conjugate to be further studied can be evaluated by (i) its immunogenicity, (ii) its yield, and (iii)

TABLE 4. Formulation/immunogenicity relationships of conjugates prepared with a 10-mer of γDPGA bound to rPA or TT[a]

Conjugate	γDPGA (mol)/ rPA (mol)	Dose (μg/mouse)[b]	Anti-γDPGA (EU)		Anti-protein (μg/ml)	
			2nd injection	3rd injection	2nd injection	3rd injection
rPA/Cys-Gly_3-γ$DPGA_{10}$	22	2.5	2,200	5,800	2	210
		2.5 + al	3,500	6,200	80	280
rPA/Cys-Gly_3-γ$DPGA_{10}$	13	1	NT[c]	2,900	NT	61
		1 + form	NT	2,600	NT	23
		1 + al	NT	4,000	NT	260
		1 + al & form	NT	3,300	NT	300
rPA-ITL/Br-Gly_3-γ$DPGA_{10}$	18	2.5	2,400	3,000	0.2	41
		2.5 + al	5,600	5,400	250	680
TT/Cys-γ$DPGA_{10}$	16	2.5	6,900	6,700	NT	NT
		2.5 + al	8,310	NT	NT	NT
TT/Cys-γ$DPGA_{15}$	16	2.5	6,500	7,700	NT	NT
		2.5 + al	7,800	15,000	NT	NT

[a] Five- to 6-week-old NIH general-purpose mice ($n = 10$) were injected subcutaneously with 2.5 μg of γDPGA as a conjugate 2 weeks apart and exsanguinated 7 days after the second or third injection. IgG anti-γDPGA and anti-rPA were measured by ELISA, and the results are expressed as the geometric means.
[b] al, aluminium hydroxide (Alhydrogel); form, formaldehyde treatment according to the methods of Porro et al. (19a) and Nencioni et al. (17a).
[c] NT, not tested.

the ease of preparation. Based on our studies, a highly immunogenic and simple-to-prepare investigational vaccine, inducing antibody to either one γDPGA only (TT/Cys-γDPGA$_{15}$) or two anthrax virulence factors, γDPGA and PA (*r*PA/Cys-Gly$_3$-γDPGA$_{10}$), can be prepared and considered for clinical testing.

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