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# Binding of the von Willebrand Factor A Domain of Capillary Morphogenesis Protein 2 to Anthrax Protective Antigen Vaccine Reduces Immunogenicity in Mice

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**ABSTRACT** Protective antigen (PA) is a component of anthrax toxin that can elicit toxin-neutralizing antibody responses. PA is also the major antigen in the current vaccine to prevent anthrax, but stability problems with recombinant proteins have complicated the development of new vaccines containing recombinant PA. The relationship between antigen physical stability and immunogenicity is poorly understood, but there are theoretical reasons to think that this parameter can affect immune responses. We investigated the immunogenicity of anthrax PA, in the presence and absence of the soluble von Willebrand factor A domain of the human form of receptor capillary morphogenesis protein 2 (sCMG2), to elicit antibodies to PA in BALB/c mice. Prior studies showed that sCMG2 stabilizes the 83-kDa PA structure to pH, chemical denaturants, temperature, and proteolysis and slows the hydrogen-deuterium exchange rate of histidine residues far from the binding interface. In contrast to a vaccine containing PA without adjuvant, we found that mice immunized with PA in stable complex with sCMG2 showed markedly reduced antibody responses to PA, including toxin-neutralizing antibodies and antibodies to domain 4, which correlated with fewer toxin-neutralizing antibodies. In contrast, mice immunized with PA in concert with a nonbinding mutant of sCMG2 (D50A) showed anti-PA antibody responses similar to those observed with PA alone. Our results suggest that addition of sCMG2 to a PA vaccine formulation is likely to result in a significantly diminished immune response, but we discuss the multitude of factors that could contribute to reduced immunogenicity.

**IMPORTANCE** The anthrax toxin PA is the major immunogen in the current anthrax vaccine (anthrax vaccine adsorbed). Improving the anthrax vaccine for avoidance of a cold chain necessitates improvements in the thermodynamic stability of PA. We address how stabilizing PA using sCMG2 affects PA immunogenicity in BALB/c mice. Although the stability of PA is increased by binding to sCMG2, PA immunogenicity is decreased. This study emphasizes that, while binding of a ligand retains or improves conformational stability without affecting the native sequence, epitope recognition or processing may be affected, abrogating an effective immune response.

**KEYWORDS** anthrax, antigen processing, immunization, protein stability

One of the most studied immunogens is the *Bacillus anthracis* protective antigen (PA), a four-domain 83-kDa protein that is the cell-binding part of the anthrax toxin, a three-component AB toxin that is critical for anthrax pathogenesis. PA is also the major immunogenic component of the current anthrax vaccine (anthrax vaccine

adsorbed [AVA]) and can provide protective immunity against anthrax infection. Numerous studies on the immune response to PA, either as part of AVA or as a recombinant expressed protein, have identified linear B- and T-cell epitopes in all four domains of PA, as well as conformational epitopes targeted by monoclonal antibodies (1–6). Indeed, one of the current postexposure treatment options for inhalational anthrax includes a derivative of mouse monoclonal antibody 14B7, which targets domain 4 (Anthim [obiltoxaximab]) (7–10); the other (Anthrasil) is a polyclonal antibody directed against PA that is derived from the plasma of individuals immunized with AVA (11).

Previous experiments (12, 13) showed that the stability of full-length PA toward chemical denaturants, pH, temperature, and proteolysis could be improved if the soluble von Willebrand factor A domain (VWA) of receptor capillary morphogenesis protein 2 (sCMG2) (a host cellular receptor for PA) was bound. In addition, several studies showed that the 63-kDa form of PA was more stable to pH when bound to sCMG2 (14–16). The binding constant for PA and sCMG2 is ~300 pM, an affinity that is dependent on a $m$etal-$i$on-$d$ependent $a$dhesion $s$ite (MIDAS) within sCMG2 (17, 18). The binding interface between PA and sCMG2 is known (19) and is dependent on domain 4 of PA, which contains a critical aspartic acid (D683) in the small loop of domain 4 located in the region between amino acids 671 and 721, for coordination to the metal ion at the interface, as well as interactions within domain 2. The domain $2\beta_3$-$2\beta_4$ loop binds in a groove on the surface of CMG2 and is critical for pH-dependent interactions with sCMG2 (14, 15, 18).

The relationship between antigen stability and immunogenicity is poorly understood, but stabilizing conformational epitopes may improve the ability to elicit immune responses that primarily result in antibodies that recognize native protein configurations. Protein antigens can be recognized by B-cell receptors on naive B cells through discontinuous or conformational determinants, as well as linear determinants. In addition to B cells, professional antigen-presenting cells (APCs) take up antigens that are then processed intracellularly to present peptides bound to major histocompatibility complex I (MHC-I) or MHC-II, which are recognized by T cells for adaptive immunity (20). Protein stability and conformation thus become important factors in recognition of configuration and processing of antigens. If an antigen is too stable, however, it may resist processing and cause a reduced T cell response (21). Antigen stability is also of practical importance in vaccine formulations, and improvements in protein stability can increase the long-term storage of vaccines. The thermal stability of PA is seemingly related to the immunogenicity of PA as a vaccine; incubation of PA at high temperatures has been shown to reduce the induction of toxin-neutralizing antibodies (TNAs), the primary markers of protective immunity to anthrax (22). It is presumed that conformational determinants are primarily affected by exposure to elevated temperatures. In fact, recombinant PA vaccines have been plagued by problems of antigen stability, which have interfered with the development of next-generation vaccines against anthrax (23).

Because sCMG2 stabilizes PA and addition of sCMG2 to a vaccine formulation would ensure a more stable native conformation of PA without resorting to mutagenesis, we decided to evaluate the effect of sCMG2 on PA immunogenicity. While sCMG2 increases the stability of PA, we realize that complexation of PA with sCMG2 could result in masking of critical epitopes contained in domain 4, which is one target for neutralizing antibodies that block the binding of PA to cells as the first step in intoxication (24). Furthermore, studies have shown that, when PA is injected intravenously or intraperitoneally into mice, it is fairly rapidly cleaved to the 63-kDa form of PA (PA$_{63}$) by a serum protease and is cleared from the bloodstream as either the PA$_{63}$ or PA$_{83}$ form (25–27). Clearance is dependent on binding to the receptor, since utilization of a PA mutant containing three substitutions that prevented specific receptor binding (D683A, L685E, and Y688K) reduced the rate of clearance of PA$_{83}$ (25). Clearance in that case might have been due to circulating myeloid cells, which have been shown to contain CMG2 on their surfaces, resulting in uptake of PA and specific targeting of PA to myeloid cells
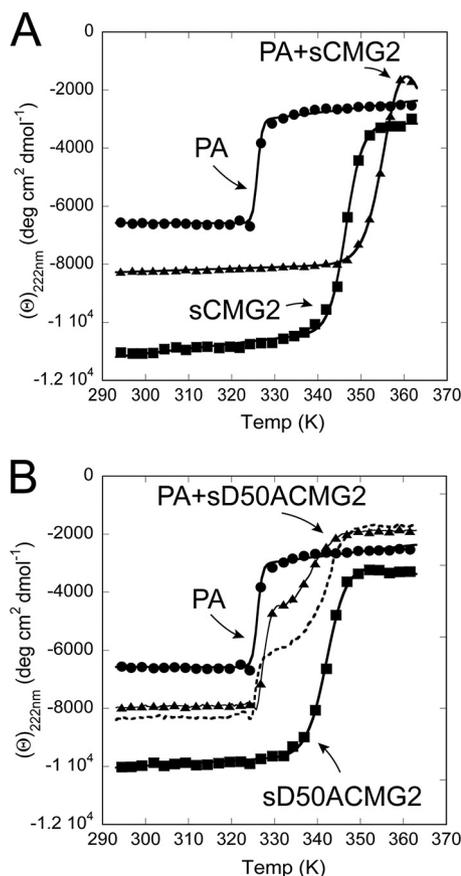
**FIG 1** Temperature-dependent CD analysis of PA (●), PA plus sCMG2 (▲), and sCMG2 alone (■) (A) and PA (●), PA plus sD50ACMG2 (▲), and sD50ACMG2 alone (■) (B). Solid lines through the curves in panels A and B are fits to a two-state thermal unfolding transition. In panel B, the data for PA plus sD50ACMG2 were not fit to a two-state transition, and the line through the data is meant to help guide the eye. PA (10 μM) and complexes (15 μM [1:1.5]) were recorded at 222 nm using a temperature ramp of 1°C per minute in a 0.5-mm-path-length cell. In panel B, the sum (dashed line) represents the normalized sum of the CD molar ellipticity of PA alone and sD50ACMG2 alone.

(28). We have carried out an immunization study of PA, injecting PA subcutaneously into BALB/c mice, either alone or in complex with a 1.5-fold excess of VWA of sCMG2. As a control, we used the D50A mutant of sCMG2, which does not bind to PA (29). We evaluated the immune response with enzyme-linked immunosorbent assays (ELISAs) of isolated serum toward PA, domain 4 of PA, and sCMG2, evaluated the presence of TNAs at peak titer (week 7), and also reevaluated the thermal stability of the complex of PA, PA plus sCMG2, and PA plus sD50ACMG2 using circular dichroism (CD) measurements. We offer an interpretation of our results that takes into account previous work that focused on understanding the relationship between protein stability and immunogenicity.

## RESULTS

**Stability of PA in the presence of sCMG2 using CD.** The stability of PA when bound to sCMG2 has been shown to be significantly increased (12–14), using a number of parameters. For the proteins studied here, we recapitulated the effect of sCMG2 on stability to temperature using CD spectroscopy. Figure 1 shows the effects of sCMG2 (Fig. 1A) and a nonbinding mutant of sCMG2 (sD50ACMG2) (Fig. 1B) on the thermal stability of PA. The calculated $T_m$ of PA alone was 52.9 ± 0.04°C, and addition of sCMG2 led to an increase in the global $T_m$ to 83.3 ± 0.1°C (Fig. 1A). The $T_m$ of sCMG2 alone was 73.5 ± 0.07°C. We observed no evidence of separate transitions in the complex, indicating that both proteins are stabilized through complex formation. If complexation did
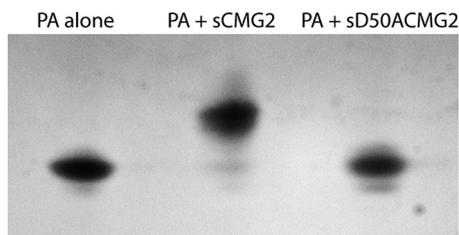
**FIG 2** Native PAGE of lyophilized PA and receptor complexes. PA (120 $\mu$M [10 mg/ml]), PA plus sCMG2 (120 and 180 $\mu$M [1:1.5]), and PA plus sD50ACMG2 (120 and 180 $\mu$M) samples were lyophilized in a sucrose-mannitol-phosphate buffer (34 mg/ml mannitol, 10 mg/ml sucrose, and 0.73 mg/ml Na$_2$HPO$_4$) containing 130 $\mu$M magnesium chloride. A batch of lyophilized sample kept at Wichita State University was resuspended in 100 $\mu$l of water, further diluted using 20 mM Tris (pH 8)-0.15 M NaCl with 5% glycerol, and applied to a premade gradient (4 to 20%) native PAGE gel (Bio-Rad).

not occur, then we would expect to observe two separate transitions. In Fig. 1B, we observed two separate transitions for PA plus sD50ACMG2, indicating that the two are unfolding independently of one another. However, when we compared the complex (PA plus sD50ACMG2) to a sum of the individual PA and sD50ACMG2 transitions, the transitions did not match that of the complex (Fig. 1B). The $T_m$ of sD50ACMG2 was 69.2 $\pm$ 0.1°C, significantly lower than that of sCMG2, but the $T_m$ of the sD50ACMG2 in the complex of PA plus sD50ACMG2 (Fig. 1B) was 65.1 $\pm$ 0.3°C, lower than that of sD50ACMG2 alone.

**Immunogenicity of PA in the presence of sCMG2.** We immunized BALB/c mice with purified preparations of PA, PA and a 1.5-fold excess of sCMG2, or PA and a 1.5-fold excess of sD50ACMG2. We utilized lyophilized preparations of these proteins, in part to ensure that all proteins were in a similar conformational state at the start of the process of immunization and were not influenced by exposure to aqueous buffer during storage and shipment, up to the time directly prior to immunization. However, the first two vaccinations, at the start and at week 2, were performed with vaccine rehydrated directly prior to immunization, while the last immunization, at week 4, was performed with protein rehydrated at week 2 and retained at 4°C for 2 weeks prior to use. Lyophilization/rehydration of the proteins did not affect the ability of PA to remain bound to sCMG2, as assessed using a standard gel-shift assay (Fig. 2), indicating that PA and sCMG2 were in a conformation that permitted the binding of PA to sCMG2 through a native domain 4 configuration and suggesting that both proteins were in an overall native configuration. We noted that there was a small amount of free PA in the PA plus sCMG2 complex that had not undergone the gel shift, despite the PA/sCMG2 ratio of 1:1.5. This could be due to the procedure for lyophilization, in which reabsorption of water could have occurred during storage and handling, leading to some partial denaturation (30).

All immunizations were performed in the absence of adjuvant, to remove any potential concerns regarding the effect of adjuvant on interactions between PA and sCMG2 (which may or may not affect the stability of PA or the formation or stability of complexes of PA with sCMG2). Sera from mice immunized with either 10 $\mu$g (Fig. 3A) or 100 $\mu$g (Fig. 3B) preparations of PA were analyzed by ELISA for antibodies against PA. The data were expressed as the geometric mean titer (GMT) obtained from groups of mice at different times during the immunization process. All mice immunized with either 10 or 100 $\mu$g PA alone (without adjuvant) developed antibodies to PA 1 week after three immunizations (week 5), which increased through week 11. GMTs were approximately 5- to 10-fold higher in the 100 $\mu$g dose group, compared with the 10 $\mu$g dose group, from week 5 through 11. In contrast, mice immunized with 10 $\mu$g PA complexed with sCMG2 developed significantly diminished antibody titers, in comparison to PA vaccine alone, at each sampling point at weeks 5, 7, 9, and 11 after the primary immunization. For example, the GMT for the 10 $\mu$g dose group (GMT of 24,300) 2 weeks after the last immunization (week 7) was 140-fold higher than that for the
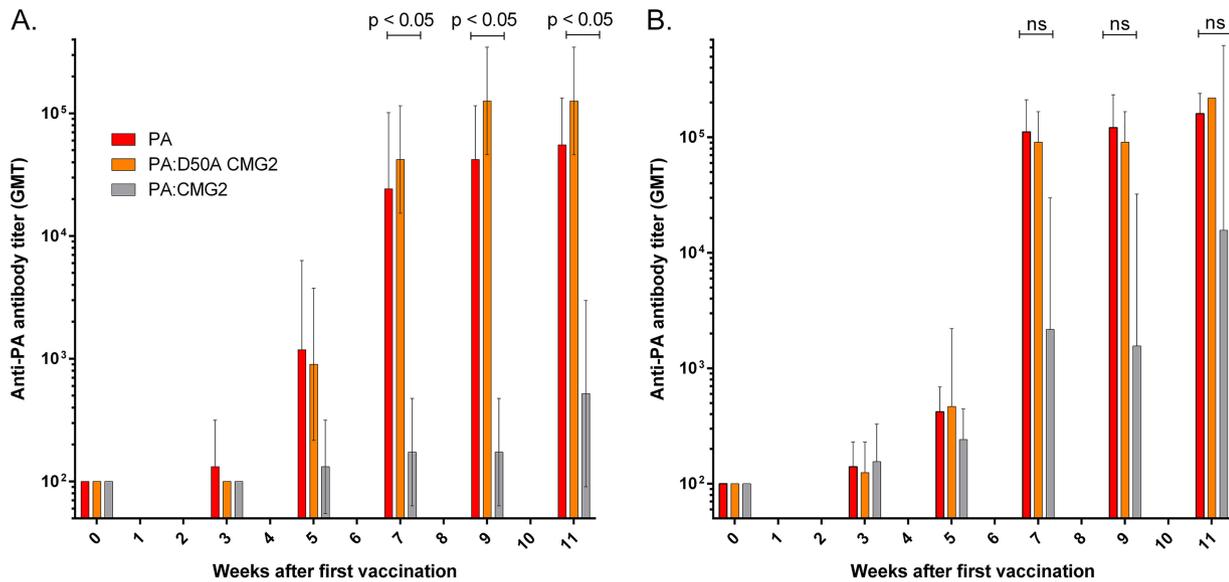
**FIG 3** Anti-PA IgG in mice immunized with PA, PA complexed with sCMG2, or PA in combination with sD50ACMG2. Groups of five mice were immunized three times, at 2-week intervals, with PA (red bars), PA complexed with sCMG2 (gray bars), or PA in combination with the PA-binding-deficient mutant sD50ACMG2 (orange bars). Serum samples were analyzed by ELISA (as described in Materials and Methods) for total antibodies binding to PA prior to immunization, 1 week after each immunization, and at 2-week intervals thereafter until week 11. (A) Mice immunized with PA or complexes containing 10 μg doses of PA immunogen. The Mann-Whitney test was performed with GraphPad Prism to determine the significance between PA-sCMG2 and PA or PA-sD50ACMG2 at each serum sampling point. (B) Mice immunized with PA or complexes containing 100 μg of PA. ns, not significant ($P > 0.1$). Bars represent the geometric means as the reciprocal endpoint titers, and error bars show the 95% confidence intervals.

group immunized with 10 μg PA complexed with sCMG2 (GMT of 173; $P = 0.02$); the statistical difference was also observable through week 11. Immunization with 100 μg PA plus sCMG2 also resulted in diminished PA antibody titers that were approximately 10-fold lower than those for PA alone at the week 7, 9, and 11 sampling points, although those differences were not statistically significant ($P = 0.12$), in comparison to the 100 μg PA dose. One mouse in each of the PA plus sCMG2 dose groups (100 and 10 μg) failed to respond. When mice were immunized with PA plus sD50ACMG2, in which PA does not form a stable complex, all mice treated with either 10 or 100 μg doses responded with titers against PA that were indistinguishable from those for PA alone at the same doses. This finding indicates that formation of the stable sCMG2-PA complex accounted for the significant differences in the responsiveness to PA, especially at the lower dose.

In the same study, we also analyzed sera for development of antibodies to sCMG2 (Fig. 4). Mice immunized with PA complexed with sCMG2 or PA plus sD50ACMG2 at the 10 μg PA dose exhibited little to no response to sCMG2. In contrast, mice immunized with 100 μg PA doses containing 150 μg of complexed sCMG2 or uncomplexed sD50ACMG2 developed immune responses to sCMG2 (Fig. 4). At the higher dose, however, the GMTs of anti-sCMG2 antibodies were statistically the same 2 weeks after the third immunization in the groups immunized with PA complexed with sCMG2 or with the PA vaccine in which sCMG2 was not complexed (PA plus sD50ACMG2). In marked contrast, antibodies to PA were drastically reduced by complexation with sCMG2 (Fig. 4, left). Thus, although the similarity in sequence between human CMG2 and mouse CMG2 is fairly high (84% identity), human sCMG2 is immunogenic in mice depending on dosage and complexation with PA does not affect that immune response.

**Toxin-neutralizing antibodies.** We also tested sera for the presence of TNAs, focusing on the peak titer at week 7. As can be seen in Fig. 5A, TNA titers were similar for both PA and PA plus sD50ACMG2 at either 10 or 100 μg, while sera from mice treated with PA plus sCMG2 exhibited significantly lower levels of TNAs. This finding
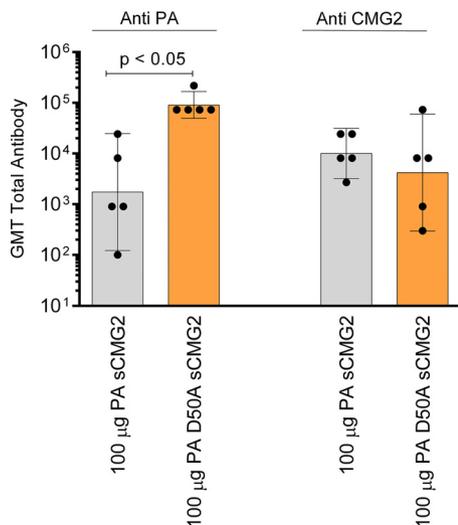
**FIG 4** Antibody titers for PA and CMG2. Serum samples obtained 3 weeks after the third immunization (week 7) from individual mice immunized with 100 $\mu$g of PA complexed with sCMG2 (gray bars) or PA in combination with sD50ACMG2 (orange bars) were analyzed by ELISA for total Ig to PA or sCMG2. Symbols are the results from individual mice. Reciprocal endpoint titers for either PA (left) or sCMG2 (right) were plotted as the geometric means, with error bars representing the 95% confidence intervals. The Mann-Whitney test was performed with GraphPad Prism to determine the significance between PA-sCMG2 and PA-sD50ACMG2.

correlated with total anti-PA IgG titers at week 7, which we show for comparison (Fig. 5B). While sCMG2 binds primarily to domain 4 and partially to domain 2, other toxin-neutralizing epitopes (for instance, domain 1) are presumably not affected. Thus, as we discuss below, if antigen processing and presentation were affected, this could decrease the Ig response, including the development of TNAs.
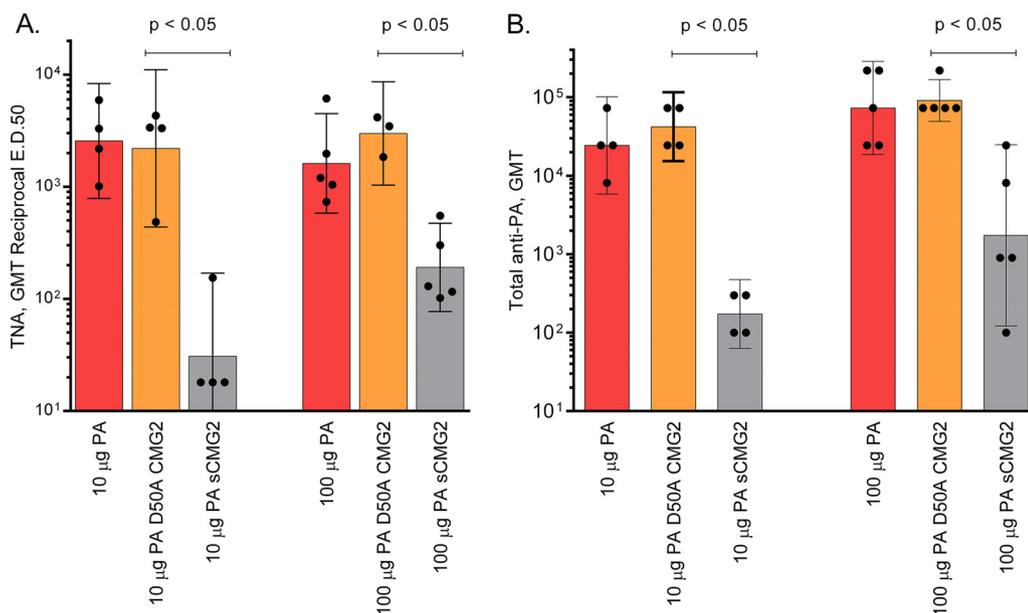


**FIG 5** Comparison of anthrax toxin-neutralizing ability and total Ig. Sera obtained from mice 3 weeks after the third immunization (week 7) were analyzed for TNAs (A) or for total Ig by ELISA (B) for both the 10 $\mu$g and 100 $\mu$g PA doses for PA (red bars), PA in combination with sD50ACMG2 (orange bars), and PA complexed with sCMG2 (gray bars). TNA titers were plotted as the geometric mean of the $ED_{50}$, as described in Materials and Methods. Significance was determined by the Mann-Whitney test for PA plus sCMG2 in comparison with PA plus sD50ACMG2, and the $P$ values are presented for both TNA and ELISA results.
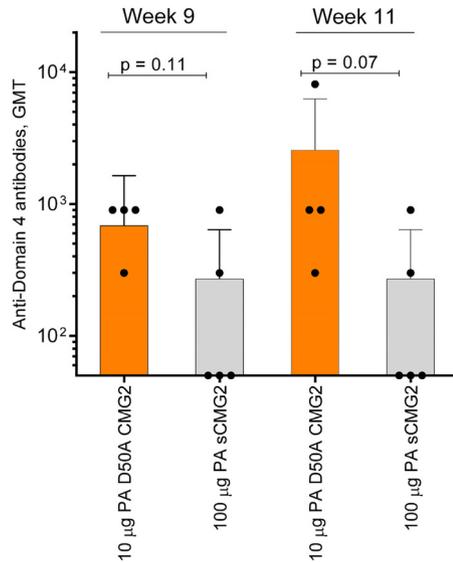
**FIG 6** Specificity of anti-PA antibodies toward domain 4. Sera were obtained 5 weeks (week 9) (left) or 7 weeks (week 11) (right) after the third immunization, from mice immunized with 10 μg PA in combination with sD50ACMG2 (orange bars) or 100 μg PA complexed with sCMG2 (gray bars). Anti-domain 4 antibodies were determined by ELISA, as described in Materials and Methods. Data are expressed as the geometric mean, with error bars representing the 95% confidence interval. Three of 5 mice in the PA plus sCMG2 group did not respond. Significance was determined by the Mann-Whitney test.

**Immunogenicity toward domain 4.** CMG2 binds primarily to domain 4 of PA but includes a small loop from domain 2. Isolated glutathione S-transferase (GST)-domain 4 has been shown to be protective in mice, in comparison to similar constructs encompassing domains 1, 2, and 3 (31), suggesting immunodominance of domain 4 with respect to protection against anthrax in the mouse model. However, other studies showed that levels of neutralizing antibodies were highest in mice immunized with either full-length PA or domain 1 and that dominant neutralizing antibodies could be mapped to the furin cleavage site within domain 1 (2, 3). We tested whether sera isolated from mice treated with either PA plus sD50ACMG2 (10 μg) or PA complexed with sCMG2 (100 μg) were reactive against isolated domain 4. We compared sera from animals immunized with the low dose of the uncomplexed PA plus sD50ACMG2 or the higher dose of complexed PA with sCMG2, since the higher dose of the PA plus sCMG2 complex was required to induce significant anti-PA antibodies and TNAs. Our expectation in this experiment was that, if sCMG2 were masking an epitope in domain 4, then we would detect antibodies directed at domain 4 in the PA plus sD50ACMG2 group but not in the PA plus sCMG2 group. The domain 4 we used was purified as a GST-domain 4 fusion and subsequently cleaved with thrombin to give the isolated domain 4, which we have shown is virtually identical in structure to domain 4 in the context of the full-length protein (32). Figure 6 shows that antibodies generated against domain 4 could be detected in every mouse immunized with the low dose of PA plus sD50ACMG2, while 3 of the 5 mice immunized with the high dose of PA plus sCMG2 were categorized as nonresponders. Furthermore, GMTs of ELISA antibodies against domain 4 were 5- to 7-fold lower in the sera of mice immunized with the high dose of PA plus sCMG2 at 5 weeks and 7 weeks after the third immunization, in comparison with the low dose of PA plus sD50ACMG2, although the results were not statistically significant ($P = 0.11$). In agreement with results showing decreased total antibodies to PA and lower TNA levels after immunization with the PA plus sCMG2 complex, antibodies to domain 4 were similarly affected. This result supports the notion that addition of sCMG2 could be blocking a conformational epitope located in domain 4, also resulting in a reduction in total antibodies against PA.

## DISCUSSION

Our findings indicate that complexation of sCMG2 with PA increases the thermal stability of PA, as measured by CD, while complexation to sD50ACMG2 has little effect on PA stability. These results are consistent with previous studies showing that sCMG2 significantly increases the stability of PA. However, we observed decreased generation of antibodies directed against PA, including TNAs and antibodies specific for domain 4, in mice immunized with PA plus sCMG2 but not in mice immunized with PA plus sD50ACMG2. These results indicate that a strategy to add sCMG2 to a PA vaccine to enhance the conformational stability of PA is likely to decrease the efficacy of any anthrax vaccine containing PA. The reasons for this inhibition of antibody formation when sCMG2 is complexed to PA could be multifold. One of the reasons for determining whether sCMG2 might be helpful in a vaccine formulation is the fact that the PA protein is more stable when bound to sCMG2 and this does not perturb the native structure. Why might a more stable protein be helpful in a vaccine formulation? Studies have shown that PA is rapidly cleaved to 20- and 63-kDa fragments after intraperitoneal or intravenous injection of PA (25) or to 20- and ~50-kDa fragments in human serum (26), which may negatively affect the immune response. Incubation of PA with thermolysin has been shown to result in the formation of 20- and ~50-kDa fragments (13, 33), a cleavage that occurs between residues 315 and 316 of PA. The C-terminal portion of this cleavage is further processed into smaller fragments upon prolonged incubation with thermolysin, but not if sCMG2 is bound (13), indicating that sCMG2 partially protects PA from proteolytic degradation.

The immune response to protein-based vaccines or therapeutic agents has been shown to be a two-part problem, one that is partly dependent on conformational determinants of the three-dimensional structure of the antigen (for binding to naive B cells, for instance) and another that is dependent on the uptake of the antigen and its subsequent proteolytic degradation. In the latter case, degradation is required for presentation of peptides from the antigen on MHC-II, which are then presented on the surface of professional APCs (B cells, macrophages, or dendritic cells), allowing binding of helper T cells and leading to the activation and differentiation/maturation of B and T cells (20, 34–36). Thus, to elicit an effective immune response, a native three-dimensional structure is needed to present conformational epitopes.

For instance, efforts to improve the ricin vaccine (37, 38), respiratory syncytial virus vaccine (24), or HIV-1 vaccine (39, 40) have focused in part on improving the conformational stability to enhance the presentation of conformational epitopes (B-cell response) that have been shown to provide protective immunity. Immunogenicity in those cases is very likely tied to the flexibility of certain regions of the respective immunogenic proteins, with reduction in flexibility inducing stronger antibody binding (40). Does sCMG2 binding to PA alter initial B-cell receptor binding? Perhaps sCMG2 is blocking a key epitope or epitopes that are required for initial B-cell receptor recognition? In accord with this notion, the evidence we present suggests that mice immunized with PA complexed with sCMG2 exhibit not only a diminished antibody response to PA but also a reduced TNA response. As mentioned in the introduction, the most effective monoclonal TNAs for anthrax generated to date, and now licensed by the FDA for postexposure treatment, are directed at domain 4. Thus, it is possible that, by prebinding sCMG2 to PA for immunization, we block a key conformational epitope in this domain (or domain 2) that is required for an effective immune response. In addition, we provide evidence that mice immunized with PA plus sCMG2 exhibited a weaker immune response to isolated domain 4, but further studies are required. For instance, to what extent do mice immunized with full-length PA generate conformational domain 4-specific antibodies, and are the majority of these domain 4 antibodies toxin neutralizing?

Another possibility is that the PA plus sCMG2 complex is not efficiently phagocytosed by APCs or is more weakly taken up by APCs, compared to PA alone (41). Indeed, Liu and coworkers have shown that myeloid cells (macrophages and neutrophils)

contain CMG2 on their surfaces and mice lacking CMG2 ($CMG2^{-/-}$) are resistant to lethal toxin exposure and killing (28). Thus, to establish infection, PA targets myeloid cells specifically for early destruction by targeting CMG2 (28). Addition of sCMG2 to the PA vaccine might have resulted in a decreased immune response due to the prevention of myeloid cells taking up and processing PA. Furthermore, there may be a more direct role for CMG2 as a receptor for PA on APCs for the induction of anti-PA antibodies. Our results are similar to results reported by Verma et al., who demonstrated decreased induction of anti-PA antibodies and TNAs when mice were immunized with a variant of PA with mutations in domain 4 that failed to bind to CMG2 on macrophages (42). In that case, the mutations in PA were shown not to affect the depletion of neutralizing antibodies from sera of mice immunized with PA, suggesting that the domain 4 mutants contained a full complement of structurally intact domain 4 epitopes. These results may also suggest a role for receptors on APCs in the presentation of intact surface-localized PA to B cells. With regard to stability, deamidation of asparagine residues in PA was associated with decreased potency, reduced levels of TNAs, and impaired binding of PA to cells (43).

Alternatively, it may be that uptake by APCs is not limiting but the intracellular processing and presentation of the PA plus sCMG2 complex is significantly slowed. Processing and presentation of epitopes through the class II MHC pathway are known to depend on the structure of the antigen, not only for proteolytic processing by cathepsins but also for initial recognition of the antigen by MHC-II (44–46). Our studies cannot discount that binding of sCMG2 impedes MHC-II recognition and that MHC-II primarily recognizes the same site as sCMG2, such that the occurrence of processing and presentation when sCMG2 is bound to PA necessitates binding to MHC-II through a weaker epitope. Alternatively, it may be that antigen binding to MHC-II is not limiting but the processing of the antigen by cathepsins is the limiting issue.

Several studies have focused on antigen processing, with the question being largely as follows: "what effect does the thermodynamic and proteolytic stability of the protein antigen have on the elicitation of an effective immune response?" For instance, studies by Delamarre and coworkers showed, using different variants of RNase A or horseradish peroxidase, that the uptake and presentation of peptides as MHC-II complexes in dendritic cells correlated with protein stability (as measured by susceptibility to proteolysis); the more stable variants gave rise to greater amounts of peptide-loaded MHC-II (47, 48). Proteolytic susceptibility in general correlates with a protein's thermal stability and thus serves as an excellent method for analyzing protein structure (49). If the protein is too stable, however, this can lead to incomplete processing, inadequate MHC-II presentation, and a decreased ability to activate T cells (21, 50, 51). Based on our stability studies, another explanation is that the increased stability of PA when bound to sCMG2 alters the rate of proteolytic degradation within endosomes and decreases the rate of presentation in the context of MHC-II molecules and the generation of T cells needed for production of antibodies (44).

While we cannot discount any of these possibilities, the studies presented here show rather unequivocally that immunization with PA complexed with sCMG2 leads to diminished induction of antibodies to PA, as well as TNAs and antibodies to domain 4. If indeed we have blocked key epitopes within domain 4 (or the area of domain 2 that sCMG2 binds that can also elicit TNAs [52, 53]), this would suggest that development of an effective immune response to PA necessitates the development of antibodies that can target these domains.

## MATERIALS AND METHODS

**Protein purification.** PA was purified as described previously, using periplasmic expression followed by anion exchange and gel filtration (54). Domain 4, sCMG2, and sD50ACMG2 were purified as GST-fusion proteins, as described previously (32). For vaccination studies, purified PA was diafiltered from a 20 mM Tris-HCl-150 mM NaCl buffer (pH 8.0) into a sucrose-mannitol lyophilization buffer containing 34 mg/ml mannitol, 10 mg/ml sucrose, and 0.73 mg/ml sodium phosphate dibasic (55), using a 15-ml Sartorius Vivaspin filter (molecular weight cutoff value of 10,000). Briefly, sucrose-mannitol buffer was added to the protein solution (1 ml) to 15 ml in the Vivaspin filter and centrifuged to 1 ml; this process was repeated another two times to give the final protein solution ready for lyophilization. In addition, prior to binding

of PA to sCMG2, sCMG2 and sD50ACMG2 were diafiltered into the same sucrose-mannitol buffer solution, and concentrations were determined using the extinction coefficients for PA ($78,400 \text{ M}^{-1} \text{cm}^{-1}$) and sCMG2 ($12,505 \text{ M}^{-1} \text{cm}^{-1}$). Protein purity was verified by SDS-PAGE.

**CD spectroscopy.** CD data were collected using a JASCO J810 spectropolarimeter. Thermal stability experiments were carried out in phosphate-buffered saline (PBS) (pH 7.4) with 10 mM MgCl$_2$. In these experiments, the final concentrations were 10 $\mu$M PA and 15 $\mu$M sCMG2. For sCMG2/sD50ACMG2, concentrations were kept at 15 $\mu$M. Samples were incubated for 1 h at room temperature after sCMG2 was added to PA. Variable temperature data were recorded at 222 nm using a 0.5 mm path length cell, with a 16 s time constant. Measurements were started at 20°C, and the temperature was increased 1°C/min using a Julabo F25-ME circulating water bath. Calculations of the $T_m$ were performed by fitting data for PA, PA plus sCMG2, sCMG2 alone, and sD50ACMG2 alone according to a two-state transition, using equations 5 through 8 in reference 56 and correcting for pre- and posttransition slopes, as described previously (56). Data were fit using Kaleidagraph v3.6 (Synergy Software).

**Vaccine preparation.** Two Eppendorf tubes, each containing PA (120 $\mu$M [10 mg/ml]), PA plus sCMG2 (120 and 180 $\mu$M [1:1.5]), or PA plus sD50ACMG2 (120 and 180 $\mu$M), were complexed for 2 h at room temperature in a sucrose-mannitol phosphate buffer (34 mg/ml mannitol, 10 mg/ml sucrose, and 0.73 mg/ml Na$_2$HPO$_4$), with an additional 130 $\mu$M magnesium chloride added to stabilize the complex. The protein complexes (final volume, 100 $\mu$l) were cooled gradually to −10°C using an ice-salt water bath and then were quick-frozen in liquid nitrogen. Solutions were lyophilized overnight in Eppendorf tubes under high vacuum using a Labconco lyophilizer, capped, and then shipped to Johns Hopkins University (JHU) or stored at −80°C. Lyophilized material was reconstituted in water (100 $\mu$l) immediately prior to vaccination and then was subsequently diluted to 500 $\mu$l with PBS (pH 7.4), giving a 2.0-mg/ml solution of PA. For 100 $\mu$g PA vaccinations, 50 $\mu$l of this solution was used; for 10 $\mu$g injections, 50 $\mu$l of a 0.2 mg/ml solution (a 1:10 dilution) was used.

**Animals.** Thirty-five female BALB/c mice (6 weeks of age) were used to perform the immunizations in the Animal Facility of the Bloomberg School of Public Health, JHU, where all of the care was given to the animals, according to ethics guidelines described below.

**Vaccination of animals.** Six groups of five female mice each were separated for different vaccination conditions; a control group was unvaccinated. Each animal received a 50 $\mu$l subcutaneous injection, in the back neck, containing 100 $\mu$g or 10 $\mu$g of PA, PA plus 150 or 15 $\mu$g sCMG2, or PA plus 150 or 15 $\mu$g sD50ACMG2, without adjuvant, every 2 weeks, resulting in three immunizations. Animals underwent retro-orbital bleeding, using a heparin-containing capillary, every 2 weeks; only blood necessary to check the antibody titer was collected, the eye was always changed from the last intervention, and isoflurane was used to alleviate pain. When vaccination was performed on the same day, the bleeding was always done prior to vaccination. The endpoint was determined as 2 weeks after the last immunization, when the animals were euthanized according to ethics guidelines.

**ELISAs.** Antibody titers were determined as described previously (2) by ELISAs using serum samples obtained from mice by retro-orbital bleeding. The PA and CMG2 antigens were analyzed by SDS gel electrophoresis to check for integrity, diluted to a 10 $\mu$g/ml concentration in 50 $\mu$l for each well in a 96-well plate. Bovine serum albumin (1%) was used as a blocking solution (200 $\mu$l) for 1 h at 37°C. Serum dilutions (50 $\mu$l) were incubated for 1 h at 37°C. Secondary antibodies were a mixture of goat anti-mouse IgG, IgA, IgM, and IgE labeled with alkaline phosphatase and diluted 1:1,000 (50 $\mu$l) and were incubated for 1 h at 37°C. To obtain the final result, plates were treated with 50 $\mu$l of an alkaline phosphatase substrate (*p*-nitrophenyl phosphate; Sigma), and the absorption was measured in a spectrophotometer at 405 nm.

**Toxin neutralization assays.** Anthrax lethal factor TNA assays were performed as described previously (57). Briefly, serially diluted sera were mixed with a fixed concentration of anthrax lethal toxin (50 ng/ml PA and 40 ng/ml lethal factor) and incubated for 30 min at 37°C. The mixture of sera and lethal toxin was transferred to a 96-well cell culture plate with $3 \times 10^4$ J774A cells/well. Cells were intoxicated for 6 h, and then cell survival was measured using the metabolic reporter dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). The reportable value is the reciprocal serum dilution at which 50% of the toxicity is neutralized (50% effective dose [ED$_{50}$]). Results were calculated using SAS v9.4 (SAS Institute Inc., Cary, NC). The lower limit of quantification (LLOQ) for the assay was 36, and values below the LLOQ were reported as 18.

**Statistical analysis.** Statistical differences between treatment groups were determined by representing group reciprocal endpoint titers as geometric means with 95% confidence intervals. *P* values for significance were calculated by the Mann-Whitney test using GraphPad Prism v6.0 (GraphPad Software, San Diego, CA), For calculation of geometric means, individual titers of nonresponding mice were assigned a value of 18, the LLOQ of the TNA assay, or 50 for ELISA, one-half the lowest dilution used in ELISAs.

**Storage.** The vaccines used and the sera obtained at the immunizations were stored at 4°C and were not subjected to thermal stress.

**Native PAGE.** Lyophilized proteins were resuspended in water (100 $\mu$l) as described above and were diluted further to a final concentration of 1 $\mu$M PA using 20 mM Tris (pH 8.0)-150 mM NaCl (pH 8.0) containing 5% glycerol. Protein samples were loaded (20 $\mu$l) onto a 4 to 20% native gel (Bio-Rad) and run at a constant voltage of 40 V for 17 h at 4°C. The gel was stained with GelCode blue safe protein stain (Invitrogen) and imaged using a Kodak Gel Logic 100 imaging system.

**Ethics.** All animals in this study were handled in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (58). All

experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Bloomberg School of Public Health, Johns Hopkins University (protocol MO15H134).

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