Structural and Immunological Analysis of Anthrax Recombinant Protective Antigen Adsorbed to Aluminum Hydroxide Adjuvant

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New anthrax vaccines currently under development are based on recombinant protective antigen (rPA) and formulated with aluminum adjuvant. Because long-term stability is a desired characteristic of these vaccines, an understanding of the effects of adsorption to aluminum adjuvants on the structure of rPA is important. Using both biophysical and immunological techniques, we compared the structure and immunogenicity of freshly prepared rPA-Alhydrogel formulations to that of formulations stored for 3 weeks at either room temperature or 37°C in order to assess the changes in rPA structure that might occur upon long-term storage on aluminum adjuvant. Intrinsic fluorescence emission spectra of tryptophan residues indicated that some tertiary structure alterations of rPA occurred during storage on Alhydrogel. Using anti-PA monoclonal antibodies to probe specific regions of the adsorbed rPA molecule, we found that two monoclonal antibodies that recognize epitopes located in domain 1 of PA exhibited greater reactivity to the stored formulations than to freshly prepared formulations. Immunogenicity of rPA-Alhydrogel formulations in mice was assessed by measuring the induction of toxin-neutralizing antibodies, as well as antibodies reactive to 12-mer peptides spanning the length of PA. Mice immunized with freshly prepared formulations developed significantly higher toxin-neutralizing antibody titers than mice immunized with the stored preparations. In contrast, sera from mice immunized with stored preparations exhibited increased reactivity to nine 12-mer peptides corresponding to sequences located throughout the rPA molecule. These results demonstrate that storage of rPA-Alhydrogel formulations can lead to structural alteration of the protein and loss of the ability to elicit toxin-neutralizing antibodies.

Bacillus anthracis is a Gram-positive, aerobic, spore-forming bacterium that secretes a tripartite toxin composed of a binding component known as protective antigen (PA) and two catalytically active components known as lethal factor (LF) and edema factor (EF). Manifestations of anthrax disease are believed to be caused primarily by the effects of lethal toxin (PA plus LF) and edema toxin (PA plus EF). Following its binding to cell surface receptors, PA is cleaved by furin (13) into a 20-kDa amino-terminal fragment and a 63-kDa polypeptide which, in turn, heptamerizes, binds to LF and/or EF, and mediates their translocation into the cell cytosol. LF is a zinc metalloprotease which inactivates mitogen-activated protein kinase kinase signaling, whereas EF is an adenylyl cyclase that increases the cellular concentration of cyclic AMP (7). Functional studies (9, 24), as well as the crystal structure (28), of PA have demonstrated that the protein is folded into four distinct domains, each of which plays a role in toxin function. Domain 1 (residues 1 to 258) contains the furin recognition site, which is cleaved to release 167 amino acids at the N-terminal end of the protein (domain 1a). The remaining portion of domain 1 (domain 1b) forms the LF/EF binding site. Domains 2 (residues 259 to 487) and 3 (residues 488 to 595) are involved in heptamerization and are responsible for the formation of the pore through which LF and EF travel to enter the cytosol. Domain 4 (residues 596 to 735), along with domain 2, forms the receptor binding pocket of the protein (17, 23).

Animal studies have shown that protective immunity to anthrax disease correlates with induction of neutralizing anti-PA antibodies (11, 20, 29). Therefore, in recent years, efforts have been made to develop anthrax vaccines composed of purified recombinant PA (rPA). Vaccines based on recombinant protein antigens often require an adjuvant to induce a suitable immune response to achieve protection from disease. With regard to rPA vaccines, adjuvants have been shown to increase rPA immunogenicity in animal models (3, 21). The most commonly used adjuvants are aluminum salts, usually aluminum hydroxide or aluminum phosphate. Although aluminum-containing adjuvants have been used in vaccine formulations for almost a century, the effects of adjuvant adsorption on antigen structure, conformation, and stability have only recently begun to be investigated (6). The effects of adsorption to aluminum adjuvants on the structure of different protein antigens, including hepatitis B surface antigen, gp41, and model antigens such as lysozyme, ovalbumin, and BSA have been investigated by using a variety of biophysical techniques (1, 8, 16, 26, 27, 34, 36). Those studies yielded various results regarding the extent to which structural alterations occurred following adsorption of the protein antigen to aluminum adjuvants. A major use of rPA vaccines would be in an emergency situation which cannot be predicted; thus, these vaccines would likely be stockpiled. Therefore, the long-term stability of these vaccines will be a primary consideration in their development. Initial ef-
forts to develop an rPA vaccine were stalled because of vaccine stability issues (2); however, the molecular basis of the lack of stability has yet to be elucidated. A recent study examined the structure of rPA immediately after adsorption to aluminum adjuvant (33). The authors of that study concluded that the interactions of rPA and Alhydrogel (aluminum hydroxide) have little effect on the structure of the protein. Although the immediate effects of the adsorption of the rPA antigen to Alhydrogel were demonstrated to be small, the study did not examine the stability of rPA when adsorbed to Alhydrogel for longer periods of time. Currently, little information is available concerning structural alterations of rPA that may occur after long-term adsorption to aluminum adjuvants. In order to begin to address this issue, we have performed accelerated stability studies in which we stored adsorbed rPA at either room temperature (RT) or 37°C for 3 weeks. The structure and immunogenicity of the stored preparations were compared to those of freshly adsorbed material by using both biophysical and immunological techniques in order to provide an assessment of the changes in rPA that might occur upon prolonged storage of an aluminum-adjuvanted rPA vaccine.

MATERIALS AND METHODS

Materials. B. anthracis recombinant PA83 (NR-140 and NR-164), recombinant LF (NR-142), anti-rPA rabbit reference polyclonal sera (NR-3839), and murine macrophage-like J774A.1 cells (NR-28) were from the National Institutes of Health (NIH) Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases (NIAID), NIH (Bethesda, MD). Aluminum hydroxide adjuvant, Alhydrogel was obtained from Brenntag Biossector, Frederikssund, Denmark. The anti-PA monoclonal antibodies (MAbs) used in this study (13B3, I3G, 3F9, and 14B7) have been described previously (10, 22, 23). Wistar-derived mouse macrophage-like cell line J774A.1 was grown in Dulbecco’s modified Eagle medium (containing a high glucose concentration and sodium pyruvate) supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (25 U/ml), streptomycin sulfate (25 µg/ml), and 10 mM HEPES. Briefly, cells were plated in 96-well flat-bottom plates (40,000/well) and incubated for 17 to 19 h at 37°C in a 5% CO₂ incubator. Neutralization of LT cytotoxicity was measured by assessing cell viability with 2-fold serial dilutions of the rabbit polyclonal serum (NR-3839) as the reference serum sample. Test serum samples were prepared in a separate 96-well microtiter plate by using an appropriate starting dilution, followed by 2-fold dilutions for a total of seven dilutions per sample. The samples were then incubated with a constant concentration of LT (PA at 50 ng/ml plus LF at 40 ng/ml) for 30 min prior to addition to the cells. This concentration of LT kills approximately 95% of the cells in the absence of any neutralizing serum sample. The serum-toxin mixtures were transferred to the 96-well cell plate, and cells were incubated for 4 h at 37°C. Following incubation, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide was added to plates. After 2 h of incubation, cells were lysed by the addition of solubilization buffer (90% isopropanol, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 38 mM HCl). The plates were read for optical density using a microplate reader at A570. A four-parameter logistic regression model was used to fit the data points generated when the optical density was plotted versus the reciprocal of the serum dilution. The inflection point, which indicates 50% neutralization, was reported as the mean effective dilution (ED₅₀).

Preparation of rPA-Alhydrogel formulations. All of the rPA-Alhydrogel formulations used for mouse immunization studies were prepared by using rPA at a concentration of 50 µg/ml and 1.5 mg/ml of aluminum in normal saline solution (0.9% NaCl). The mixture was gently vortexed and allowed to stand for 1 h at RT for adsorption. The degree of adsorption of rPA to Alhydrogel was determined after centrifugation to pellet the adjuvant and collection of the supernatant, which was examined for protein content using the Pierce BCA protein assay (Pierce, Rockford, IL) and for rPA content using a macrophage lysis assay that has been described previously (35). The adsorption of rPA to the adjuvant was estimated by these methods to be ≥99%.

Freshly prepared rPA-Alhydrogel formulations are referred to as “0-week” preparations throughout this report. Preparations were subsequently stored at either RT/25°C or 37°C for 3 weeks. In this work, three independent immunization studies of mice were performed. In the first immunization study, an rPA-Alhydrogel formulation stored at RT (approximately 22 to 25°C) was used, whereas in two subsequent studies, rPA-Alhydrogel formulations stored at a controlled temperature of 25°C for 3 weeks were used for immunizations. For the purpose of consistency, RT incubation is referred to throughout this report for all three immunization studies. For the direct Alhydrogel formulation immunoassay and intrinsic fluorescence experiments, rPA was used at 30 µg/ml with 0.9 mg/ml of aluminum and at 200 µg/ml with 1.5 mg/ml of aluminum, respectively. All formulations were stored at 25°C or 37°C for 0 and 3 weeks.

Immunization of mice. All mouse experiments (immunizations and serum collections) were carried out by Cocalico Biologicals, Inc. (Reamstown, PA), in compliance with the guidelines of its Institutional Animal Care and Use Committee. Mice (six-week-old female CD-1 mice) were immunized once intraperitoneally with 200 µl of vaccine (equivalent to 10 µg of rPA/mouse). Groups of 20 mice were immunized with either freshly prepared rPA-Alhydrogel formulations or formulations stored at RT or 37°C for 3 weeks. An Alhydrogel control group of 20 mice was simultaneously immunized with only Alhydrogel in normal saline solution. At 28 days postimmunization, mice were bled. Serum samples collected from different groups of mice were analyzed individually or as pools, as indicated. Sample pools for each treatment group were prepared by combining equal volumes of serum from individual mice.

Cell culture and TNA assay. Sera obtained from mouse immunization studies were analyzed by the toxin neutralization antibody (TNA) assay using J774A.1 cells essentially as described previously (30). The murine macrophage-like cell line J774A.1 was grown in Dulbecco’s modified Eagle medium (containing a high glucose concentration and sodium pyruvate) supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (25 U/ml), streptomycin sulfate (25 µg/ml), and 10 mM HEPES. Briefly, cells were plated in 96-well flat-bottom plates (40,000/well) and incubated for 17 to 19 h at 37°C in a 5% CO₂ incubator.

Neutralization of LT cytotoxicity was measured by assessing cell viability with 2-fold serial dilutions of the rabbit polyclonal serum (NR-3839) as the reference serum sample. Test serum samples were prepared in a separate 96-well microtiter plate by using an appropriate starting dilution, followed by 2-fold dilutions for a total of seven dilutions per sample. The samples were then incubated with a constant concentration of LT (PA at 50 ng/ml plus LF at 40 ng/ml) for 30 min prior to addition to the cells. This concentration of LT kills approximately 95% of the cells in the absence of any neutralizing serum sample. The serum-toxin mixtures were transferred to the 96-well cell plate, and cells were incubated for 4 h at 37°C. Following incubation, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide was added to plates. After 2 h of incubation, cells were lysed by the addition of solubilization buffer (90% isopropanol, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 38 mM HCl). The plates were read for optical density using a microplate reader at A570. A four-parameter logistic regression model was used to fit the data points generated when the optical density was plotted versus the reciprocal of the serum dilution. The inflection point, which indicates 50% neutralization, was reported as the mean effective dilution (ED₅₀).

Intrinsic fluorescence. Intrinsic fluorescence measurements of rPA-Alhydrogel formulations were carried out using a quartz cuvette with a 5-mm path length in a Jasco J-815 spectrophotometer (Jasco Inc.) at 20°C. The emission spectrum between 290 and 400 nm at an excitation wavelength of 280 nm was recorded. Intrinsic fluorescence spectra were measured for rPA alone in normal saline solution, as well as for rPA-Alhydrogel formulations prepared at 200 µg/ml of rPA and stored for 0 and 3 weeks at 25°C and 37°C. Fluorescence intensity in arbitrary units was measured for rPA alone in normal saline solution, as well as for rPA-Alhydrogel formulations prepared at 200 µg/ml of rPA and stored for 0 and 3 weeks at 25°C and 37°C. Fluorescence intensity in arbitrary units was plotted against the wavelength in nanometers by using Microsoft Office Excel. Three independent experiments were performed to measure fluorescence intensities.

Mapping of MAb binding to rPA. The individual protein domains of rPA, composed of amino acids 1 to 258 (domain 1), 259 to 487 (domain 2), 488 to 595 (domain 3), and 596 to 735 (domain 4), were cloned into Escherichia coli strain BL21, expressed, and purified as described previously (4). Each recombinant domain was resolved from different groups of mice were analyzed individually or as pools, as indicated. Sample pools from each treatment group were prepared by combining equal volumes of serum from individual mice.
binding to rPA was evaluated by dot blot assay. For these experiments, 1 μg of purified rPA domain was spotted directly onto nitrocellulose. Blotting then proceeded as for the immunoblot assays described above.

**Direct Alhydrogel formulation immunoassay.** A direct Alhydrogel formulation immunoassay, previously described by Zhu et al. (39), was performed with freshly prepared or stored rPA-Alhydrogel formulations, with slight modifications, in an ELISA format. Briefly, the different rPA-Alhydrogel formulations (100 μl/well) were added to black, opaque, 96-well U-bottom plates (Corning Inc., Corning, NY). rPA-Alhydrogel pellets were washed three times with phosphate-buffered saline (PBS, pH 7.4) by resuspension-centrifugation (1,000 × g for 5 min at RT) cycles. rPA-Alhydrogel pellets were then blocked with 200 μl of 3% bovine serum albumin (BSA) in PBS for 1 h at RT with shaking, followed by 3 washes with PBS. In a separate 96-well plate, different MAbs were serially diluted in 1% BSA–PBS and 100 μl of each dilution was transferred to respective wells in the blocked rPA-Alhydrogel plate as the primary antibody reagent. Plates were incubated for 1 h at RT with shaking, followed by three washes with PBS. Goat anti-mouse IgG (H+L) conjugated to fluorescein was added then proceeded as for the immunoblot assays described above.

**Results**

**Analysis of rPA-Alhydrogel formulations by intrinsic fluorescence.** To determine if structural alterations of rPA can occur when bound to Alhydrogel, we first examined the intrinsic fluorescence of tryptophan/tyrosine residues in adsorbed rPA formulations that had been freshly prepared or stored for 3 weeks. Because fluorescence emission is dominated by tryptophan rather than tyrosine, the intrinsic fluorescence of rPA, which has seven tryptophan residues, would be due mainly to that of tryptophan residues. Tryptophan residues which are exposed on the surface of a protein generally have spectra which are shifted by 10 to 20 nm toward a longer wavelength (340 to 350 nm) than tryptophans buried in the hydrophobic core of the protein (~320 to 330 nm) (18). The spectrum of the freshly prepared rPA-Alhydrogel material (Fig. 1) has an emission maximum at 330 nm, which was the same as the emission maximum of rPA in solution (data not shown). These results suggest that when rPA is initially adsorbed onto the surface of Alhydrogel, the tryptophans of the protein are generally buried and in an environment similar to that of those of the solution structure of the protein, as has been reported recently (33). However, spectra of rPA-Alhydrogel formulations stored for 3 weeks at 25°C or 37°C exhibited emission maxima that were shifted toward a longer wavelength, with maxima at ~340 nm (Fig. 1), thus indicating that the local environment around the tryptophan residues of rPA adsorbed to Alhydrogel changed upon storage.

**Structural characterization of rPA adsorbed to Alhydrogel using a direct Alhydrogel formulation immunoassay.** To further explore structural changes/alterations of a specific region(s) or domains of rPA molecules adsorbed to Alhydrogel upon storage, we used a direct Alhydrogel formulation immunoassay (39). In this assay, an anti-rPA polyclonal antibody serum and several MAbs that recognize epitopes in different domains of rPA were used to directly probe the structure of rPA on the aluminum adjuvant. Figure 2 shows the reactivity profiles that were obtained for the different antibody preparations by using this assay. Anti-PA polyclonal sera (Fig. 2A) exhibited no difference in binding to rPA-Alhydrogel formulations stored for 0 or 3 weeks at 25°C. Mab 13B3 and Mab 1G3 exhibited stronger binding to the stored rPA-Alhydrogel formulation than did the freshly prepared formulation (Fig. 2B and C). We mapped Mab 13B3 binding to rPA to domain 1 (data not shown) by immunoblot analysis of recombinant fragments corresponding to the domains of rPA as described in Materials and Methods. Mab 1G3, is known to specifically recognize an epitope located between residues Ser168 and Phe314 (23). We further localized the binding site for this Mab by immunoblot analysis and found that the Mab bound to domain 1 of rPA but not domain 2 (data not shown). Anti-PA MAb BA16-0104 and MAb 2P9, which we found bound to domains 2 and 3.
respectively (data not shown), bound in similar manners to the freshly prepared and stored rPA-Alhydrogel formulations (Fig. 2D and E). Similar results were obtained with MAb 14B7, which recognizes a conformational epitope in PA domain 4 (31) (Fig. 2F). rPA-Alhydrogel formulations stored at 37°C exhibited reactivity profiles that were similar to those of the formulations stored at 25°C (data not shown).

Assessing the immunological response of rPA-Alhydrogel formulations using the TNA assay. In order to explore the immunological stability of rPA on the aluminum hydroxide adjuvant Alhydrogel, we performed TNA assays of serum samples from mice that had been immunized with freshly prepared rPA-Alhydrogel formulations (0 weeks) or preparations stored for 3 weeks at either RT or 37°C. Three independent immunization studies were performed, and representative data from one of these studies are shown in Fig. 3. Sera from groups of mice immunized with the freshly prepared rPA-Alhydrogel formulation exhibited significantly higher (>0.05) lethal toxin-neutralizing antibody titers than groups of mice immunized with rPA-Alhydrogel formulations stored at either RT or 37°C for 3 weeks. No significant difference in toxin-neutralizing antibody titers was seen between the groups of mice immunized with the freshly prepared rPA-Alhydrogel formulation and those immunized with rPA-Alhydrogel formulations stored at RT or 37°C for 3 weeks. As shown in Fig. 3, a substantially greater number of nonresponder mice was evident in groups immunized with rPA-Alhydrogel formulations stored at either RT or 37°C than in the group immunized with the freshly prepared formulation. The decrease in the TNA titers and the increase in the number of mice that did not mount a TNA response indicate a decreased ability of our rPA-Alhydrogel formulations to elicit neutralizing antibodies upon storage at either RT or 37°C.

Peptide epitope mapping of the immune response to rPA adsorbed to Alhydrogel. The structure and stability of rPA adsorbed to Alhydrogel were further characterized by examining the ability of freshly prepared or stored formulations to induce antibodies to specific linear epitopes. Serum samples, generated by immunization of mice with either freshly prepared or stored preparations of rPA-Alhydrogel, from the three independent immunization studies described above, were tested against a peptide array spanning the length of the mature rPA protein. The array consisted of 182 peptide 12-mers, with an overlap of 8 amino acids each. The antibody response profile of pooled sera from one of the studies is shown in Fig. 4. Measurable responses directed to peptides corresponding to regions within all four domains of the rPA protein were observed.

We further analyzed the data to determine whether responses to specific peptides differed depending on the immunizing material used (freshly prepared versus stored formulations). Analyzing data from all three immunization studies, we identified 13 peptides (Table 1) that, in at least two of the three studies, exhibited significant reactivity (optical density, >0.3) to pooled sera from mice immunized with at least one of the three preparations, that is, the freshly prepared formulation, the preparation stored at RT, or the preparation stored at 37°C. Of note, if adjacent peptides met the criteria described above, we listed only the peptide which had the greater reactivity since responses to adjacent peptides were likely due to recognition of overlapping amino acids and not likely the result of responses to distinctly different amino acid sequences.

For each of the 13 peptides listed in Table 1, we examined the reactivity of individual serum samples from 60 mice (20 mice/immunization group for each of three immunization studies) in the peptide ELISA. We determined the number of mice from each immunization group responding to each peptide. A mouse was considered a responder to a peptide if its serum sample reacted in the peptide ELISA to give an optical density reading of at least 0.3. Results for the 13 antigenic peptide epitopes are shown in Table 1. The proportion of responders in the group of mice immunized with formulations stored for 3 weeks at RT was significantly different from that for the group immunized with the freshly prepared material for 6 of the 13 peptides. The proportion of responding mice in the group immunized with the formulation...
stored for 3 weeks at 37°C was significantly different from that of the group immunized with freshly prepared material for 8 of the 13 peptides. No significant differences were observed between the groups immunized with formulations stored at RT and 37°C (Table 1). Of note, in all cases except one, more responders were seen in the groups immunized with formulations stored at the elevated temperatures than in those immunized with the freshly prepared material. The only exception was peptide 501, a peptide for which the number of responders in the group immunized with the preparation stored at 37°C was equal to that in the group immunized with the fresh preparation.

DISCUSSION

Previous studies have identified structural changes in proteins occurring upon adsorption to aluminum adjuvants. In a study by Jones et al. (16), decreases in the thermal stability of lysozyme, ovalbumin, and bovine serum albumin were observed by using differential scanning calorimetry and spectroscopic techniques. Thermal destabilization of the malarial antigen EBA-175 RII-NG and of a mutant form of ricin toxin A chain upon adsorption to aluminum adjuvants has also been reported (27). Of note, others have reported that the secondary structures of six model proteins were not significantly affected upon adsorption to aluminum adjuvants as measured by Fourier transform infrared spectroscopy (8). Less is known about longer-term changes in the structure of proteins that might occur after adsorption to aluminum adju-
In this regard, Vessely et al. reported that fluorescence and UV spectroscopy suggested changes in the structure of botulinum neurotoxin protein-derived vaccine antigens during storage on aluminum adjuvant (36).

In this study, we examined the structure and immunogenicity of rPA-Alhydrogel formulations to assess changes in rPA that might occur upon prolonged storage on the aluminum hydroxide adjuvant Alhydrogel. Using both biophysical and immunological techniques, we have found evidence that structural alterations of rPA occurred upon storage on Alhydrogel.

Intrinsic fluorescence measurements demonstrated that the environment around the tryptophans present in rPA was different in the freshly prepared rPA-Alhydrogel formulations from that in the stored preparations.

FIG 4 Reactivity of sera from mice immunized with freshly prepared or stored rPA-Alhydrogel formulations to rPA peptides. Mice were immunized with a freshly prepared (0 week) rPA-Alhydrogel formulation (white bars) or a formulation stored for 3 weeks at RT (black bars) or 37°C (hatched bars). Four weeks after immunization, sera from the mice were collected and, for each group, equal volumes of sera were pooled. The reactivity of the pooled sera (indicated on the y axis) to 12-mer peptides spanning the length of rPA was assessed as described in Materials and Methods. Each value on the x axis corresponds to the residue number of the first amino acid of a 12-mer peptide within the mature PA protein sequence.
TABLE 1 Reactivities of peptides with sera from mice immunized with rPA-Alhydrogel formulations

| PA domain(s) | Amino acids | 12-mer peptide amino acid sequence | No. of responders<sup>a</sup> | Statistical significance<sup>b</sup> |
|--------------|-------------|-----------------------------------|-----------------------------|----------------------------------|
|              |             | 0 wk at RT 3 wk at 37°C             |                             | 0 wk vs 3 wk at RT 37°C          |
| 1            | 197–208     | KNKRTFLSPWIS                        | 8                           | No                               |
| 1            | 245–256     | KVSPEAHPLV                          | 1                           | No                               |
| 2            | 433–444     | TMNYNQELEK                          | 15                          | No                               |
| 2            | 473–484     | TGSNSWEVLPIQ                        | 6                           | No                               |
| 3            | 489–500     | ARIIFNGKDLNL                        | 3                           | No                               |
| 3            | 501–512     | VERRIAAVNPS                        | 7                           | No                               |
| 3            | 521–532     | MTIKEALKIAFG                        | 6                           | Yes                              |
| 3            | 545–556     | KDITEFDFFDQ                         | 8                           | Yes                              |
| 3, 4         | 585–596     | AKMNILIRDKRF                        | 4                           | Yes                              |
| 4            | 609–620     | ESVVEKAEHREVI                       | 21                          | No                               |
| 4            | 633–644     | KDIRKILSGYIV                       | 0                           | Yes                              |
| 4            | 673–684     | KTFRFDCYYNDK                       | 5                           | Yes                              |
| 4            | 721–732     | IKKILFSSKKGY                       | 1                           | No                               |

<sup>a</sup> There were 60 mice in each treatment group.
<sup>b</sup> Significance (P < 0.05) was determined by using the Marascuilo test as described in Materials and Methods.

PA contains seven tryptophan residues, five in domain 1 and two in domain 2 (37). A shift in the emission maximum toward a longer wavelength (from 330 to 340 nm) was seen when spectra of stored formulations were compared to those of freshly prepared formulations (Fig. 1). This red shift in the emission maximum is indicative of exposure of tryptophan residues to a more polar environment (18). These results suggest that, upon storage on Alhydrogel, certain portions of domain 1 and/or domain 2 of rPA were rearranged such that previously buried regions became more surface exposed.

The results of our analysis using a direct Alhydrogel formulation immunoassay also suggest that structural alterations in domain 1 of rPA can occur upon storage on Alhydrogel. MAbs mapping to domains 1a and 1b exhibited stronger reactivity to rPA-Alhydrogel formulations that had been stored than to freshly prepared formulations (Fig. 2). In contrast, polyclonal antibody sera and three MAbs mapping to domains 2, 3, and 4 showed very little or no difference in reactivity. At least one of these MAbs, 14B7, which maps to domain 4, recognizes a conformational epitope (23,31). These results suggest that portions of domains 2, 3, and 4 remain structurally unchanged after storage. However, on the basis of these results, we cannot exclude the possibility that some structural changes in domains 2, 3, and/or 4 might have occurred upon storage that were not detectable with this particular set of antibodies.

In addition to results from direct analysis of the structure of rPA on Alhydrogel, analysis of the immunogenicity of the freshly prepared and stored formulations were consistent with changes in rPA occurring upon storage. We examined the ability of fresh and stored formulations to elicit toxin-neutralizing antibodies, as well as antibodies directed to specific peptide epitopes. Toxin-neutralizing epitopes on rPA might either be composed of contiguous amino acids or be conformational in nature (i.e., composed of amino acids that are spatially adjacent in the three-dimensional structure of the protein but which are not contiguous on the polypeptide chain). We found that stored formulations exhibited less of an ability to induce toxin-neutralizing antibodies than did freshly prepared formulations (Fig. 3). In striking contrast, we found that the stored formulations elicited antibody responses to peptide (contiguous) epitopes that were either similar to or greater than the responses elicited by the fresh formulations. The peptide epitopes that exhibited increased reactivity to sera generated by the stored preparations were located throughout rPA in all four domains of the protein (Table 1 and Fig. 5). Examination of the three-dimensional structure of the protein (28) (Fig. 5) indicates that peptide epitopes 245, 473, 521, 545, 585, 633, and 673 correspond to regions located at domain interfaces, suggesting that a “loosening” of interdomain interactions may occur upon storage of rPA-Alhydrogel formulations such that regions located at domain interfaces become more accessible to antibody induction. Peptide epitopes 197, 585, and 633 are located in primarily buried regions of the molecule, which would indicate that certain buried regions of the protein may become more accessible to the immune system upon storage of rPA-Alhydrogel formulations. When the TNA assay results and peptide ELISA results are con-

FIG 5 Locations of antigenic peptide epitopes within the crystal structure (28) of PA. Shown in purple are the nine peptide epitopes that exhibited significantly and reproducibly stronger reactivity to sera from mice immunized with rPA-Alhydrogel formulations stored for 3 weeks at either RT or 37°C than to sera from mice immunized with freshly prepared formulations. Peptides are numbered with the residue number of the first amino acid of the 12-mer peptide sequence. PA domains 1 (orange), 2 (blue), 3 (green), and 4 (yellow) are indicated.
sidered together, it is noteworthy that a loss of neutralizing epitopes upon storage of the rPA-Alhydrogel formulations appears to be associated with a gain in the immunogenicity of certain contiguous epitopes. This observation could be explained if a general destabilization of the protein takes place upon storage that would disrupt conformational neutralizing epitopes but increase the exposure of certain contiguous (linear) epitopes such that they are more readily accessible for antibody induction.

Recently, Soliakov et al. (33) have shown minimal effects of Alhydrogel adsorption on the structure of rPA during a very brief period of interaction/storage; however, those results do not address the long-term stability of rPA on Alhydrogel. Our data extend those results to demonstrate that structural destabilization of the rPA molecule, likely involving all four domains, can occur upon storage on Alhydrogel. The molecular events that lead to such destabilization remain to be elucidated. Among possible causes for the observed destabilization are direct interaction of previously buried or inaccessible regions of rPA with Alhydrogel, accelerated deamidation of asparagine residues important for structural integrity of the protein due to the Alhydrogel microenvironment (38), and loss of one or both of the calcium ions that are important in the stabilization of PA structure in solution (5, 12, 14, 15, 28). Another possibility is that minute quantities of contaminating proteases copurifying with rPA could lead to proteolysis of the adsorbed protein upon prolonged storage; although SDS-PAGE analysis of rPA stored in solution for up to 3 weeks at 25°C did not show significant proteolysis (data not shown).

In conclusion, the data presented here indicate that dynamic changes in the structure of rPA can occur while it is adsorbed to Alhydrogel. In our work, the immunological properties of rPA were affected upon storage on Alhydrogel, including its ability to elicit toxin-neutralizing antibodies. Several animal studies (11, 20, 29) have shown that protective immunity to anthrax disease correlates with the induction of functional neutralizing anti-PA antibodies. Thus, a loss of the ability of rPA to induce neutralizing antibodies could reflect a loss of vaccine efficacy. These findings, describing the types of changes in rPA structure that can occur upon storage on Alhydrogel, are a first step toward understanding the biochemical basis of rPA vaccine instability. These findings also emphasize that considerable efforts should be made toward the stabilization of rPA on aluminum adjuvants to increase vaccine shelf life.

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REFERENCES

1. Agopian A, et al. 2007. Secondary structure analysis of HIV-1-gp41 in solution and adsorbed to aluminum hydroxide by Fourier transform infrared spectroscopy. Biochim. Biophys. Acta 1774:351–358.
2. Baillie LW. 2009. Is new always better than old? The development of human vaccines for anthrax. Hum. Vaccin. 5:806–816.
3. Berthold I, Pommo ML, Wagner L, Arciniega JL. 2005. Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine 23:1993–1999.
4. Brady RA, Verma A, Meade BD, Burns DL. 2010. Analysis of antibody responses to protective antigen-based anthrax vaccines through use of competitive assays. Clin. Vaccine Immunol. 17:1390–1397.
5. Chaffton DA, et al. 2007. Unfolding transitions of Bacillus anthracis protective antigen. Arch. Biochem. Biophys. 465:1–10.
6. Clapp T, Siebert P, Chen D, Jones Braun L. 2011. Vaccines with aluminum-containing adjuvants: optimizing vaccine efficacy and thermal stability. J. Pharm. Sci. 100:388–401.
7. Collier RJ, Young JA. 2003. Anthrax toxin. Annu. Rev. Cell Dev. Biol. 19:45–70.
8. Colonna A, Jones LS, Kerwin BA, Krishnan S, Carpenter JF. 2006. Secondary structures of proteins adsorbed onto aluminum hydroxide: infrared spectroscopic analysis of proteins from low solution concentrations. Anal. Biochem. 351:282–289.
9. Elliott JL, Mogridge J, Collier RJ. 2000. A quantitative study of the interactions of Bacillus anthracis edema factor and lethal factor with activated protective antigen. Biochemistry 39:6706–6713.
10. Furcha JW, Ribot WJ, Jendrek S, Little SF. 1998. Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of Bacillus anthracis. Appl. Environ. Microbiol. 64:982–991.
11. Fellows PF, et al. 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by Bacillus anthracis isolates of diverse geographical origin. Vaccine 19:3241–3247.
12. Gao-Sheridan S, Zhang S, Collier RJ. 2003. Exchange characteristics of calcium ions bound to anthrax protective antigen. Biochem. Biophys. Res. Commun. 306:61–64.
13. Gordon VM, Klimpel KR, Arora N, Henderson MA, Leppa SH. 1995. Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. Infect. Immun. 63:82–87.
14. Gupta PK, et al. 2003. Conformational fluctuations in anthrax protective antigen: a possible role of calcium in the folding pathway of the protein. FEBS Lett. 554:505–510.
15. Gupta PK, et al. 2003. Acid induced unfolding of anthrax protective antigen. Biochem. Biophys. Res. Commun. 311:229–232.
16. Jones LS, et al. 2005. Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. J. Biol. Chem. 280:13406–13414.
17. Lacy DB, Wigelsworth DJ, Melynka RA, Harrison SC, Collier RJ. 2004. Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. Proc. Natl. Acad. Sci. U. S. A. 101:13147–13151.
18. Lakowicz JR (ed). 1983. Principles of fluorescence spectroscopy. Springer, New York, NY.
19. Li H, et al. 2008. Standardized, mathematical model-based and validated in vitro analysis of anthrax lethal toxin neutralization. J. Immunol. Methods 333:89–106.
20. Little SF, Ivins BE, Fellows PF, Friedlander AM. 1997. Passive protection by polyclonal antibodies against Bacillus anthracis infection in guinea pigs. Infect. Immun. 65:5171–5175.
21. Little SF, Ivins BE, Webster WM, Norris SL, Andrews GP. 2007. Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine. Vaccine 25:2771–2777.
22. Little SF, Leppla SH, Cora E. 1988. Production and characterization of monoclonal antibodies to the protective antigen component of Bacillus anthracis toxin. Infect. Immun. 56:1807–1813.
23. Little SF, et al. 1996. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of Bacillus anthracis using monoclonal antibodies. Microbiology 142(Pt 3):707–715.
24. Mogridge J, Cunningham K, Lacy DB, Mourez M, Collier RJ. 2002. The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. Proc. Natl. Acad. Sci. U. S. A. 99:7045–7048.
25. Ngundi MM, Meade BD, Lin TL, Tang WJ, Burns DL. 2010. Comparison of three anthrax toxin neutralization assays. Clin. Vaccine Immunol. 17:895–903.
26. Peck LJ, Brandau DT, Jones LS, Joshi SB, Middaugh CR. 2006. A systematic approach to stabilizing EBA-175 RII-NG for use as a malaria vaccine. Vaccine 24:5839–5851.
27. Peck LJ, Martin TT, ELK Nation C, Pegram SA, Middaugh CR. 2007.
Effects of stabilizers on the destabilization of proteins upon adsorption to aluminum salt adjuvants. J. Pharm. Sci. 96:547–557.
28. Petosa C, Collier RJ, Klimpel KR, Leplla SH, Liddington RC. 1997. Crystal structure of the anthrax toxin protective antigen. Nature 385:833–838.
29. Pitt ML, et al. 2001. In vitro correlate of immunity in a rabbit model of inhalational anthrax. Vaccine 19:4768–4773.
30. Quinn CP, et al. 2004. Immune responses to Bacillus anthracis protective antigen in patients with bioterrorism-related cutaneous or inhalation anthrax. J. Infect. Dis. 190:1228–1236.
31. Rosovitz MJ, et al. 2003. Alanine-scanning mutations in domain 4 of anthrax toxin protective antigen reveal residues important for binding to the cellular receptor and to a neutralizing monoclonal antibody. J. Biol. Chem. 278:30936–30944.
32. Savolainen L, et al. 2008. Pilot study of diagnostic potential of the Mycobacterium tuberculosis recombinant HBHA protein in a vaccinated population in Finland. PLoS One 3:e3272. doi:10.1371/journal.pone.0003272.
33. Solakov A, Kelly IF, Lakey JH, Watkinson A. 2012. Anthrax sub-unit vaccine: the structural consequences of binding rPA83 to Alhydrogel. Eur. J. Pharm. Biopharm. 80:25–32.
34. Tleugabulova D, Falcon V, Penton E. 1998. Evidence for the denaturation of recombinant hepatitis B surface antigen on aluminium hydroxide gel. J. Chromatogr. B Biomed. Sci. Appl. 720:153–163.
35. Verma A, et al. 2008. Role of the N-terminal amino acid of Bacillus anthracis lethal factor in lethal toxin cytotoxicity and its effect on the lethal toxin neutralization assay. Clin. Vaccine Immunol. 15:1737–1741.
36. Vessely C, et al. 2009. Stability of a trivalent recombinant protein vaccine formulation against botulinum neurotoxin during storage in aqueous solution. J. Pharm. Sci. 98:2970–2993.
37. Welkos SL, et al. 1988. Sequence and analysis of the DNA encoding protective antigen of Bacillus anthracis. Gene 69:287–300.
38. Wittayanukulluk A, Jiang D, Regnier FE, Hem SL. 2004. Effect of microenvironment pH of aluminium hydroxide adjuvant on the chemical stability of adsorbed antigen. Vaccine 22:1172–1176.
39. Zhu D, et al. 2009. Development of a direct alhydrogel formulation immunoassay (DAFIA). J. Immunol. Methods 344:73–78.