

Mechanistic Analysis of the Effect of Deamidation on the Immunogenicity of Anthrax Protective Antigen

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The spontaneous modification of proteins, such as deamidation of asparagine residues, can significantly affect the immunogenicity of protein-based vaccines. Using a “genetically deamidated” form of recombinant protective antigen (rPA), we have previously shown that deamidation can decrease the immunogenicity of rPA, the primary component of new-generation anthrax vaccines. In this study, we investigated the biochemical and immunological mechanisms by which deamidation of rPA might decrease the immunogenicity of the protein. We found that loss of the immunogenicity of rPA vaccine was independent of the presence of adjuvant. We assessed the effect of deamidation on the immunodominant neutralizing B-cell epitopes of rPA and found that these epitopes were not significantly affected by deamidation. In order to assess the effect of deamidation on T-cell help for antibody production elicited by rPA vaccine, we examined the ability of the wild-type and genetically deamidated forms of rPA to serve as hapten carriers. We found that when wild-type and genetically deamidated rPA were modified to similar extents with 2,4-dinitrophenyl hapten (DNP) and then used to immunize mice, higher levels of anti-DNP antibodies were elicited by wild-type DNP-rPA than those elicited by the genetically deamidated DNP-rPA, indicating that wild-type rPA elicits more T-cell help than the genetically deamidated form of the protein. These results suggest that a decrease in the ability of deamidated rPA to elicit T-cell help for antibody production is a possible contributor to its lower immunogenicity.

Inhalation anthrax is a serious, often fatal, disease caused by *Bacillus anthracis*. Because of the fatal nature of the disease and the ease of dispersion of *B. anthracis* spores, *B. anthracis* is one of the most feared of all bioterror weapons. Due to the low incidence of natural anthrax disease in humans, anthrax vaccines are not given routinely. However, the potential for the use of *B. anthracis* as a bioterror agent has prompted stockpiling of anthrax vaccines by national governments. Long-term stability is a highly desired characteristic of a stockpiled vaccine since a long shelf life significantly decreases the cost of the stockpile.

Many anthrax vaccines are based on protective antigen (PA), which is a nontoxic component of anthrax toxin. Anthrax toxin is a critical virulence factor of *B. anthracis* and is essential for disease symptomatology and progression (1, 2). The toxin is composed of PA, lethal factor (LF), and edema factor (EF). PA binds to cell receptors, heptamerizes, and then binds LF or EF to form lethal toxin (LT) and edema toxin (ET), respectively (3). Internalization of LT and ET leads to introduction of the enzymatically active effector proteins LF and EF into the cell cytosol where they exert their cytotoxicity (2). Vaccines based on PA or a recombinant form of PA (rPA) elicit antibodies, in particular functional toxin-neutralizing antibodies (TNAs), that have been correlated with protection against the disease (4–6).

Unfortunately, progress to develop new-generation rPA-based anthrax vaccines has been hampered by vaccine instability (7), which is believed to be due, at least in part, to spontaneous deamidation of the protein upon long-term storage (8, 9). Deamidation results in posttranslational conversion of asparagine (Asn) to aspartate (Asp) or isoaspartic acid (isoAsp). Deamidation of Asn occurs *in vivo* on a time scale ranging from a few seconds to centuries, depending on the local environment (10). In theory, deamidation might adversely affect the interaction of rPA with the aluminum adjuvant of the vaccine, might alter the epitope structure of the antigen, and/or might affect the ability of the protein to

elicit T-cell help for antibody production which, in turn, might affect the vaccine immunogenicity (11–15).

Earlier studies have demonstrated that certain Asn residues of rPA deamidate during the purification process and upon storage (8, 16, 17). Previously, we generated a mutant form of rPA in which the six Asn residues that are the most prone to deamidation were changed to Asp residues (six-Asp mutant rPA). The residues that were changed were Asn⁴⁰⁸, Asn⁴⁶⁶, Asn⁵³⁷, Asn⁶⁰¹, Asn⁷¹³, and Asn⁷¹⁹. We have used this “genetically deamidated” form of the protein as a model for the naturally deamidated form of the protein that would be expected to result upon prolonged vaccine storage (8). In that previous study, we demonstrated that six-Asp mutant rPA possesses lower immunogenicity than wild-type rPA. Recently, D’Souza et al. (9) confirmed that rPA adsorbed to Alhydrogel rapidly deamidates and further confirmed that this deamidation resulted in the loss of immunogenicity. Taken together, these results provide substantial support for the idea that the loss of stability of rPA vaccines is due, at least in part, to deamidation. However, little is known about the mechanism(s) responsible for this loss of immunogenicity. In this study, we exploited our genetically deamidated six-Asp mutant rPA in order to better understand the immunological mechanism(s) responsible for the low immunogenicity of deamidated rPA.

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MATERIALS AND METHODS

Materials. *B. anthracis* recombinant PA83 (NR-140), recombinant LF (NR-142), anti-rPA rabbit reference polyclonal serum (NR-3839), and murine macrophage-like J774A.1 cells (NR-28) were from the NIH Bio-defense and Emerging Infections Research Resources Repository, NIAID, NIH (Bethesda, MD). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). The aluminum hydroxide adjuvant, Alhydrogel, was obtained from Brenntag Biosector (Denmark). 2,4-Dinitrobenzenesulfonic acid hydrate (DNBS), 2,4-dinitrophenyl (DNP), and Sypro Orange dye were obtained from Sigma-Aldrich (St. Louis, MO). DNP conjugated to bovine serum albumin (DNP-BSA), used for coating the enzyme-linked immunosorbent assay (ELISA) plates, was from Santa Cruz Biotechnology (Dallas, TX). The anion-exchange column (HiPrep Q HP 16/10) and the gel-filtration chromatography column (Superdex 200 10/300GL) were from GE Healthcare (Sweden).

Cloning and expression of wild-type and six-Asp mutant rPA genes. Genes encoding wild-type and six-Asp mutant rPA were cloned in pET-22b(+) plasmid carrying an N-terminal *pelB* signal sequence for periplasmic localization and expressed and purified in *Escherichia coli* BL21(DE3) plyS essentially as previously described (18). Briefly, *E. coli* BL21(DE3) plyS cultures containing recombinant plasmid constructs were grown at 37°C in LB broth containing 100 µg/ml of ampicillin. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C for 3 to 4 h. The bacterial cells were harvested and resuspended in 20 mM Tris-HCl, and 1 mM EDTA (pH 8.0) containing 20% sucrose. The suspension was stirred gently at 4°C for 10 min and centrifuged. After centrifugation, cell pellets were resuspended in 20 mM Tris-HCl (pH 8.0) containing 5 mM MgSO₄ to release the periplasmic content. The suspension was again stirred gently at 4°C for 10 min, and the rPA proteins in the periplasmic content were collected after centrifugation. Wild-type and six-Asp mutant rPA proteins were then further purified using a HiPrep Q HP 16/10 anion-exchange column with a 0 to 1 M NaCl gradient. Finally, the rPA preparations were purified by size exclusion chromatography on a Superdex 200 10/300GL column. The purified rPA proteins obtained using this procedure were approximately 95 to 99% pure as determined by SDS-PAGE. We have previously shown that wild-type rPA exhibits only minor amounts of deamidation (8).

Assessment of melting temperatures. A real-time PCR instrument (CFX96 RT-PCR; Bio-Rad, Hercules, CA) was used to monitor the protein unfolding (melting temperature) of the rPA proteins as previously described (19). Briefly, the protein unfolding was detected by binding of the dye Sypro Orange to rPA using the fluorescence resonance energy transfer (FRET) channel, which has an excitation wavelength range of 450 to 490 nm and an emission range of 560 to 580 nm. The purified protein preparations of wild-type and six-Asp mutant rPA at 0.2 mg/ml protein in 10 mM sodium phosphate buffer (pH 7.2) containing 25 mM NaCl were mixed with Sypro Orange dye at 5× concentration. A sample volume of 30 µl/well was loaded to a 48-well clear PCR plate (Bio-Rad, Hercules, CA), and the plate was sealed using optically clear PCR sealer. Samples were analyzed by holding the temperature at 25°C for 5 min followed by a temperature increase to 85°C in 1°C increments. Fluorescence measurements were collected every 1°C after the temperature was held for 1 min, and the resulting data were recorded as a plot of relative fluorescence units (RFUs) versus temperature.

Conjugation of DNP hapten to wild-type and six-Asp mutant rPA proteins. DNP was conjugated to purified wild-type and six-Asp mutant rPA proteins as previously described (20). Briefly, 1 mg of each of the purified rPA proteins was incubated with 10 mg of DNBS in 1 ml of 0.14 M K₂CO₃ (pH 8.0) overnight at 25°C. After incubation, the conjugate complexes were dialyzed extensively against 10 mM sodium phosphate buffer (pH 7.2). The conjugation ratios were estimated based on the absorbance of the conjugates at 360 nm using a molar extinction coefficient of 17,500 for DNP. The DNP-conjugated wild-type (wild-type rPA-DNP) and six-Asp mutant rPA (six-Asp mutant rPA-DNP) proteins with similar conjugation ratios were used for the mouse immunization studies.

Preparation of rPA-Alhydrogel formulations and immunization studies. All of the rPA-Alhydrogel formulations used for mouse immunization studies were prepared using either unconjugated or DNP-conjugated wild-type and six-Asp mutant rPA purified proteins at a 50 µg/ml concentration with Alhydrogel equivalent to 1.5 mg/ml of aluminum in normal saline solution (0.9% NaCl [wt/vol]). The formulation mixture was gently vortexed and allowed to stand for 1 h at room temperature (RT) for adsorption. All mouse 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 were immunized once intraperitoneally with rPA-Alhydrogel formulations. Groups of 10 or 20 mice (6-week-old female CD-1 mice) were immunized with freshly prepared rPA-Alhydrogel formulations (equivalent to 10 µg of rPA/mouse). Control groups of 10 mice were simultaneously immunized with either Alhydrogel or DNP-Alhydrogel (2 µg of DNP in Alhydrogel equivalent to 1.5 mg/ml of aluminum in normal saline solution). This concentration of DNP with Alhydrogel provided 2-fold more DNP for immunization than the total DNP molecules in the DNP-labeled rPA preparations used for immunization. Mice were bled 28 days postimmunization.

Mouse immunization studies for wild-type and six-Asp mutant rPA proteins without Alhydrogel were also performed in a fashion similar to that described above. In these studies, groups of 20 mice were immunized with 15 µg of wild-type and six-Asp mutant rPA purified proteins in normal saline without any Alhydrogel. The serum samples collected from the different groups of mice were analyzed individually using the toxin-neutralizing antibody (TNA) assay or DNP ELISA, as appropriate.

TNA assays. Sera from mouse immunization studies were analyzed by the TNA assay using J774A.1 cells essentially as described previously (21). Neutralization of LT cytotoxicity was measured by assessing the cell viability with 2-fold serial dilutions of the test serum and a reference rabbit polyclonal serum (NR-3839) as described earlier (22). 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 50% effective dose (ED₅₀) (23).

For the homologous TNA and heterologous TNA assays, serum samples obtained from a group of mice immunized with wild-type rPA protein were tested in a TNA assay that utilized LT prepared either using wild-type rPA plus LF (homologous TNA) or six-Asp mutant rPA plus LF (heterologous TNA). Similarly, the serum samples obtained from a group of mice immunized with six-Asp mutant rPA were tested in a TNA assay that utilized LT prepared either using six-Asp mutant rPA plus LF (homologous TNA) or wild-type rPA plus LF (heterologous TNA).

DNP ELISA. The presence of antibodies to DNP in mouse sera was measured by ELISA essentially as described previously (22). Briefly, microtiter plates were coated with DNP-BSA (100 ng/well) in phosphate-buffered saline (PBS) (pH 7.4) overnight at 4°C. Plates were washed three times with 300 µl of wash buffer (PBS [pH 7.4] with 0.05% Tween 20). Individual serum samples from different groups of immunized mice were initially diluted to 1:12.5 in 5% skim milk in PBS with 0.1% Tween 20 and then serially diluted 4-fold in a separate plate. The diluted serum samples (100 µl) were transferred to the ELISA plate and incubated for 1 h at 37°C. After incubation, the plates were washed, and 100 µl of horseradish peroxidase-labeled anti-mouse IgG(H+L) (KPL, Gaithersburg, MD) was added to each well at a 1:4,000 dilution. After 1 h of incubation at 37°C, the plates were washed, and 100 µl of SureBlue Reserve TMB (KPL, Gaithersburg, MD) substrate was added to each well and incubated for 30 min at 37°C. Color development was stopped by the addition of TMB stop solution. The absorbance at 450 nm was measured. 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 of the curve, which indicates 50% response, was reported as the antibody titer.

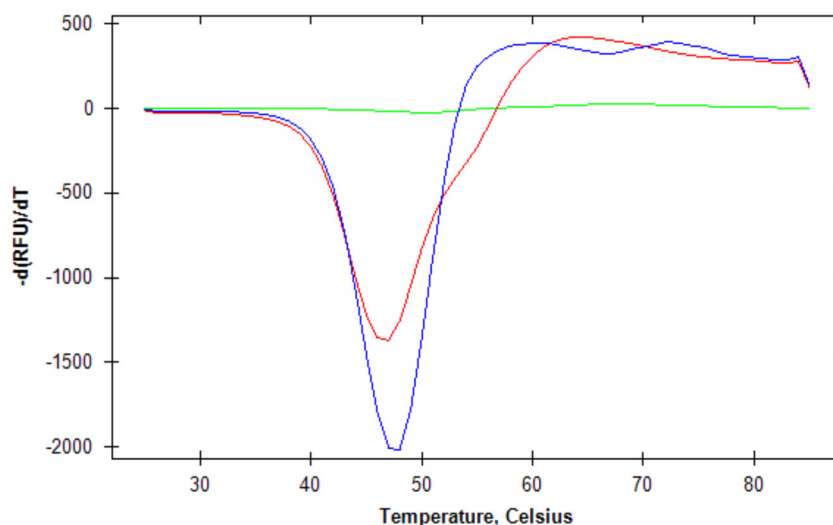


FIG 2 Melting temperatures for wild-type rPA (blue), six-Asp mutant rPA (red), and dye only (green). The melting temperatures were determined as described in Materials and Methods. The graph shows the negative first derivative of the relative fluorescence units (RFUs) as a function of temperature. The minimum of each curve represents the melting temperature.

from mice immunized with either the wild-type rPA-Alhydrogel or six-Asp mutant rPA-Alhydrogel formulations were tested for their abilities to neutralize both the homologous and the heterologous toxins. The results are shown in Fig. 3B. Sera from mice immunized with the wild-type rPA vaccine were able to neutralize both the homologous and the heterologous toxins equally well. Similarly, sera from mice immunized with the six-Asp mutant rPA vaccine were able to neutralize the homologous and heterologous toxins equally well. Taken together, these results suggest that alterations of deamidation-prone Asn residues to Asp residues did not result in alteration of immunodominant B-cell epitopes that are critical for the induction of toxin-neutralizing antibodies.

Does deamidation affect the ability of rPA to induce T-cell help for antibody production? The results described above suggest that immunodominant B-cell epitopes are not significantly altered upon rPA deamidation. Another possible contributor to the lower immunogenicity observed with six-Asp rPA mutant than with wild-type rPA might be differences in the T-cell response to the antigen, possibly due to alterations in antigen processing and/or antigen presentation to T cells. Altered processing and/or presentation of T-cell epitope(s) might result in less effective T-cell help for the B-cell antibody response.

To explore this possibility, we conjugated the hapten DNP to both the wild-type and six-Asp mutant rPA proteins to assess and compare the capabilities of these rPA proteins to act as carrier proteins that provide T-cell help for the generation of anti-DNP antibodies. Haptens are small chemical molecules and cannot stimulate antibody responses by themselves. However, when coupled to a carrier protein, a hapten-specific antibody response can be achieved since the carrier protein-hapten conjugate will activate primary T cells through peptides derived from the carrier protein, thus providing the T-cell help that is needed for generation of the antibody response (24, 25). To perform these experiments, we conjugated the hapten DNP to both the wild-type and six-Asp mutant rPA proteins (wild-type rPA-DNP and six-Asp mutant rPA-DNP) such that each protein was modified to the same extent (9.5 DNP molecules/wild-type rPA molecule and 10.0

DNP molecules/six-Asp mutant rPA molecule, respectively). Groups of mice were then immunized with each of these haptenated rPA proteins that had been adsorbed to Alhydrogel. Sera from the immunized mice were then tested by ELISA for anti-DNP antibodies. As shown in Fig. 4, sera from mice immunized with wild-type rPA-DNP exhibited significantly higher anti-DNP antibody titers ($P = 0.017$; unpaired t test) than mice immunized with six-Asp mutant rPA-DNP. The reduced anti-DNP antibody response observed with six-Asp mutant rPA-DNP suggests that the six-Asp mutant rPA carrier protein is less effective in eliciting a primary T-cell response than the wild-type rPA carrier protein. Sera from mice immunized with DNP plus Alhydrogel did not exhibit detectable levels of anti-DNP antibodies (data not shown), as expected since the hapten alone would not be expected to elicit an antibody response.

DISCUSSION

Over the past decade, considerable amounts of effort and resources have gone into developing new-generation anthrax vaccines and in understanding the mechanism of protection of these vaccines. Data from a number of studies suggest that PA-based anthrax vaccines can protect against disease through an antibody-mediated mechanism and that anti-PA antibodies, especially toxin-neutralizing antibodies, correlate with protection (4, 26, 27). For that reason, the antibody response generated by a PA-based vaccine is critical, and the vaccine should be able to elicit the same antibody response at the end of its dating period as that generated immediately after its manufacture.

Our previous findings (8) as well as those of others (9) suggest that deamidation of rPA upon storage of a vaccine decreases the anti-PA antibody response elicited by that vaccine and thus would be expected to decrease the protective capacity of the vaccine. In this study, we sought to better understand the mechanisms underlying this decrease in antibody response since such knowledge would inform development of strategies that will prevent or circumvent this problem. We examined three possible mechanisms by which deamidation might decrease the immunogenicity of rPA

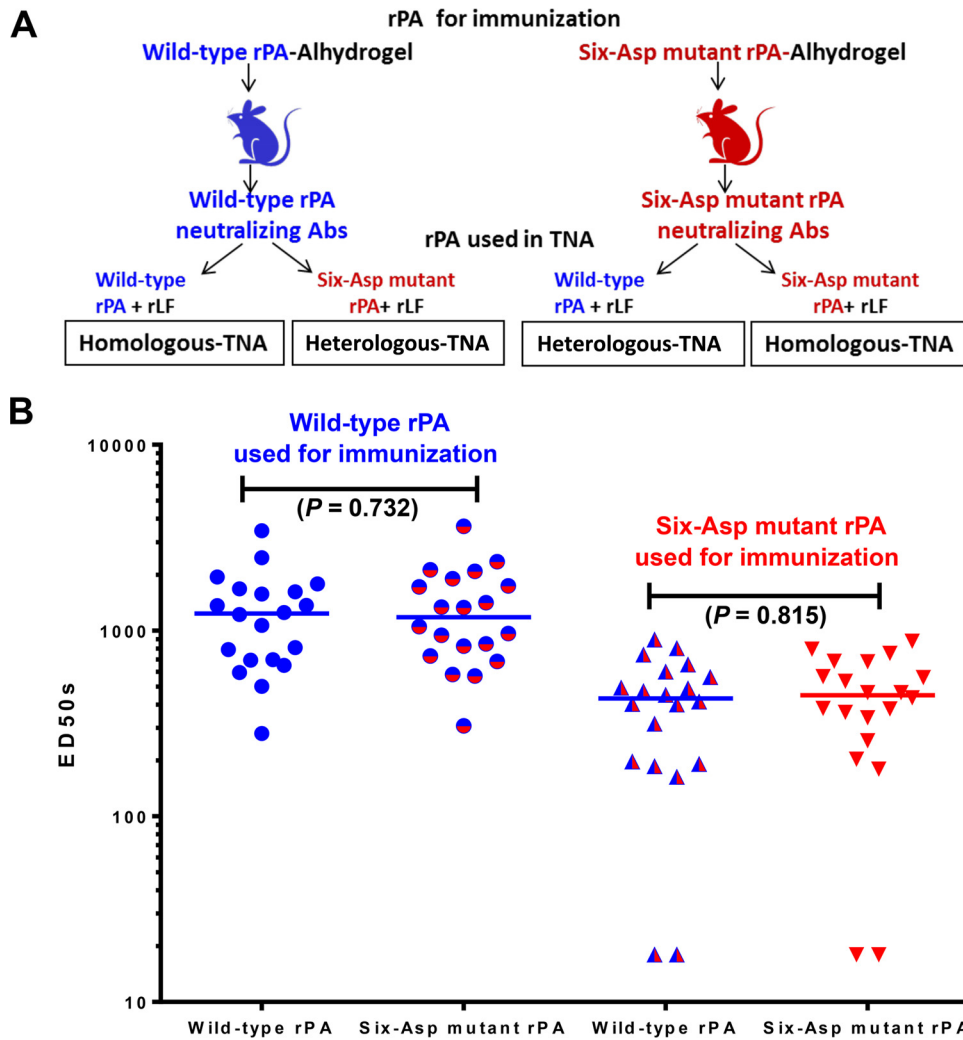


FIG 3 Ability of sera from mice immunized with either wild-type rPA vaccine or six-Asp mutant rPA vaccine to neutralize the homologous and heterologous toxins. (A) A schematic diagram of the experimental design is shown. (B) Two groups of 20 mice each were immunized with either freshly prepared wild-type or six-Asp mutant rPA Alhydrogel formulations (10 μ g of rPA/mouse). At 28 days postimmunization, mice were bled, and serum samples were analyzed in a TNA assay which utilized either the homologous or the heterologous toxin as described in Materials and Methods. The graph shows the TNA titers (expressed as ED_{50} s) for each mouse. The x axis indicates the type of rPA used to prepare the LT used in the TNA assay. The horizontal lines represent the medians for the groups. A nonparametric Mann-Whitney test was performed to calculate the statistical significance using GraphPad Prism software. The *P* values are indicated. This figure is representative of three independent immunization studies. Abs, antibodies; rLF, recombinant lethal factor.

vaccines: (i) changes in the interaction of the rPA protein with the vaccine adjuvant, (ii) changes in immunodominant B-cell epitopes, and (iii) changes in the ability of the rPA to induce T-cell help to elicit an antibody response.

We first investigated whether differences in interactions of the antigen with the adjuvant could account for the lower immunogenicity of the genetically deamidated rPA protein. Previously, others have reported an inverse correlation between the strength of the interaction of an antigen with an aluminum adjuvant and its ability to elicit an antibody response (15). PA has been determined to bind to an aluminum hydroxide adjuvant primarily through electrostatic interactions (28). Deamidation of rPA increases the net negative charge of the protein and, as such, lowers the pI. Thus, the deamidated form of the protein would be expected to bind to the positively charged Alhydrogel more tightly than wild-type rPA, which might decrease its immunogenicity. In order to

determine whether changes in the interactions of the antigen with the adjuvant might be the cause of the lower immunogenicity we observed with the genetically deamidated mutant, we examined the immunogenicity of wild-type and six-Asp mutant rPA that had not been adsorbed to the aluminum adjuvant. While the immunogenicity of both proteins was lower than that observed with the adjuvant, lower immunogenicity of six-Asp mutant rPA than of wild-type rPA was still observed. Thus, a difference in the adsorption of the two protein forms to the adjuvant or the strength of that interaction was not the cause of the decreased immunogenicity of the six-Asp mutant rPA.

We also investigated whether changes in B-cell epitopes that are capable of eliciting a strong neutralizing antibody response might be the cause of the lower immunogenicity of the genetically deamidated mutant. In our routine TNA assay, the abilities of antibodies elicited by rPA or rPA variants to neutralize wild-type

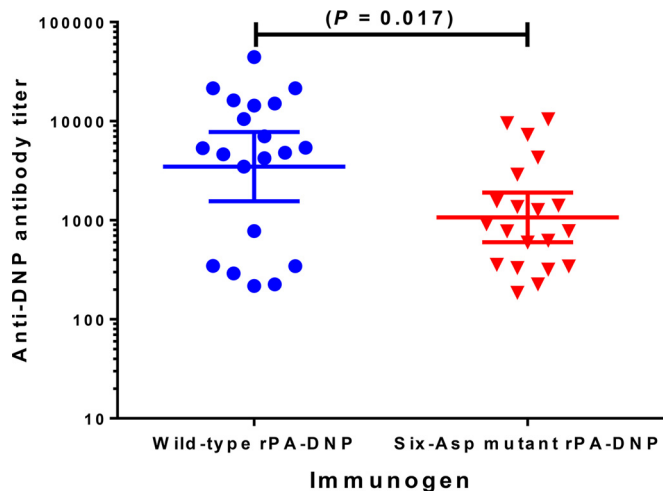


FIG 4 DNP ELISA titers of mice immunized with wild-type and six-Asp mutant rPA conjugated to DNP. Two groups of 20 mice each were immunized with freshly prepared wild-type rPA-DNP (9.5 DNP molecules/rPA molecule) or six-Asp mutant rPA-DNP (10.0 DNP molecules/rPA molecule) Alhydrogel formulations (10 μ g of rPA conjugated to DNP/mouse). At 28 days postimmunization, mice were bled, and serum samples were analyzed by ELISA for anti-DNP antibody titers as described in Materials and Methods. The graph shows the anti-DNP antibody titer for each mouse. The horizontal lines represent the geometric mean titer (GMT) with 95% confidence interval (CI) for each group. The GMT for the wild-type rPA-DNP group was 3,475 (95% CI, 1,552 to 7,780); the GMT for the six-Asp mutant rPA-DNP group was 1,069 (95% CI, 601 to 1,896). An unpaired *t* test was performed to calculate the statistical significance using GraphPad Prism software. The *P* value is indicated. This figure is a representative of two independent immunization studies.

toxin are measured. If critical B-cell epitopes were altered by the deamidation of rPA, the antibody repertoire elicited by the deamidated rPA would be expected to differ. If the epitopes that were altered were immunodominant, then the resulting antibody repertoire would likely show diminished neutralization of the wild-type toxin since the epitopes that elicited the antibody response would have different primary structures than those of the toxin that is to be neutralized. However, we found that sera from mice immunized with the wild-type rPA vaccine neutralized both the homologous (wild-type rPA plus LF) and heterologous (six-Asp mutant rPA plus LF) toxins equally well and that sera from mice immunized with the six-Asp mutant rPA vaccine neutralized its homologous (six-Asp mutant rPA plus LF) and heterologous (wild-type rPA plus LF) toxins equally well. If the immunodominant epitopes were different in the two proteins, then one would expect that the antibodies would bind to epitopes of the homologous toxin better than the heterologous toxin and therefore be more efficient in neutralizing the homologous toxin. Thus, our results suggest that deamidation does not alter B-cell epitopes to the extent that the neutralizing antibody repertoire that is elicited by the deamidated protein is substantially changed.

In contrast to our findings concerning the lack of a role of the adjuvant or the lack of effect of deamidation on the immunodominant epitopes of rPA, we did find that deamidation diminished the ability of rPA to elicit T-cell help for antibody production since six-Asp mutant rPA when coupled to DNP was less able to elicit antibodies to the hapten than was wild-type rPA when coupled to an equal amount of DNP. In the case of the rPA vaccine, T-cell

help for antibody production would be elicited when the rPA is taken up by antigen-presenting cells and processed, and peptides from the processed rPA are presented to cognate T cells by major histocompatibility complex (MHC) class II molecules. Changes in the primary structure of rPA due to deamidation might affect antigen processing and presentation by several different mechanisms. First, a change from Asn to Asp or isoAsp due to deamidation, if such an amino acid were located within a critical T-cell epitope, might alter the ability of the epitope to interact with and activate T cells. Alternatively, changes in the amino acid sequence of rPA due to deamidation might affect antigen processing by altering critical target sites for the proteases involved in antigen processing. Such a phenomenon has been described for tetanus toxin fragment C (12). Those authors showed that deamidation of Asn residues interfered with processing of the protein, which affected generation of a T-cell response as assessed using an *in vitro* assay. Another possible mechanism for diminished T-cell help due to deamidation might be changes in the efficiency of antigen uptake by antigen-presenting cells.

The results described here provide insight into possible mechanisms by which deamidation of rPA diminishes immunogenicity of the protein. Our results suggest that while the overall structure of the protein is not substantially affected by deamidation, local changes in amino acid sequence (i.e., Asn→Asp or isoAsp) appear to affect the ability of rPA to generate T-cell help for antibody production. This knowledge should be useful in the development of strategies to minimize or circumvent the loss of immunogenicity of rPA due to spontaneous deamidation.

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