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Improving the stability of recombinant anthrax protective antigen vaccine

Anita Verma *, Drusilla L. Burns

Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD, USA

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ABSTRACT

Development of recombinant protective antigen (rPA)-based anthrax vaccines has been hindered by a lack of stability of the vaccines associated with spontaneous deamidation of asparagine (Asn) residues of the rPA antigen during storage. In this study, we explored the role that two deamidation-prone Asn residues located directly adjacent to the receptor binding site of PA, Asn^{713} and Asn^{719}, play in the stability of rPA-based anthrax vaccines. We modified these residues to glutamine (Gln) and generated rPA (N713Q/N719Q), since Gln would not be expected to deamidate on a time scale relevant to vaccine storage. While wild-type rPA vaccine formulated with aluminum hydroxide lost immunogenicity upon storage, as measured by induction of toxin-neutralizing antibodies in mice, the rPA(N713Q/N719Q) vaccine did not exhibit a significant loss in immunogenicity. This finding suggests that modification of Asn^{713} and Asn^{719} of rPA to deamidation-resistant amino acids may improve the stability of rPA-based anthrax vaccines.

1. Introduction

Anthrax, a disease caused by *Bacillus anthracis*, is a serious biothreat because of its lethal nature and the potential for *B. anthracis* spores to be dispersed over a large area. Since anthrax vaccines are not administered routinely to the general population, stockpiling of anthrax vaccines ensures their availability in an emergency. Therefore, a long shelf life is a desirable characteristic of an anthrax vaccine.

Most anthrax vaccines target anthrax toxin, a virulence factor that is essential for disease progression, since animal studies have shown that toxin-neutralizing antibodies correlate with protection against anthrax disease [1–3]. Anthrax toxin is composed of a receptor binding-component known as protective antigen (PA) and two enzymatically active components, lethal factor (LF) and edema factor (EF) [4]. Because of the pivotal role of PA in anthrax toxin action, efforts have been underway to develop new anthrax vaccines composed of a purified recombinant form of PA (rPA) adsorbed to aluminum adjuvant capable of inducing a robust toxin-neutralizing response. However, attempts to develop such vaccines have been hampered by a lack of stability of the vaccine upon storage [5].

Spontaneous modification of amino acids including deamidation, oxidation, isomerization and hydrolysis can contribute to the instability of recombinant protein antigens. Of these, deamidation of asparagine (Asn) residues resulting in its conversion to aspartate (Asp) or isoAsp (occurring in a ratio of approximately 1:3) is the most common post-translational modification that takes place during storage of proteins [6,7]. The 68 Asn residues of PA have been predicted to deamidate with half-lives ranging from days to thousands of years [8]. Several of these Asn residues have been demonstrated to undergo deamidation in time frames that are relevant to vaccine dating periods [8,9]. We and others have shown that the spontaneous deamidation of Asn residues of rPA results in loss of immunogenicity [10,11].

Recently, we demonstrated that binding of PA to its receptors on the surface of antigen presenting cells leads to an enhanced toxin-neutralizing antibody response [12]. Deamidated PA has been shown to exhibit reduced receptor binding [8], suggesting that some of the loss of immunogenicity of PA upon deamidation may be due to impaired receptor-binding. Two of the deamidation-prone residues of PA, Asn^{713} and Asn^{719}, are located directly adjacent to residues that are part of the receptor binding site of PA [13], thus deamidation of these residues might be expected to perturb receptor binding either through steric interference or by altering the conformation of the receptor-binding site. Of note, D'Souza et al. [11] demonstrated that the rates of deamidation of Asn^{713} and Asn^{719} increased significantly when PA was

* Corresponding author at: CBER, FDA HFM-434, Building 52/72, Room 3324, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA.
E-mail address: anita.verma@fda.hhs.gov (A. Verma).

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adsorbed onto aluminum hydroxide adjuvant, an adjuvant that is commonly used in vaccines, further implicating these residues as ones that might be at least partially responsible for loss of immunogenicity of adjuvanted rPA vaccines upon storage.

To explore the possibility that deamidation of Asn^{713} and Asn^{719} might contribute to loss of immunogenicity of rPA vaccines upon storage, we created altered forms of rPA that would not be expected to undergo deamidation in a time frame relevant to vaccine dating periods. We then compared the immunogenicity and stability of freshly prepared and stored vaccines formulated with either wild-type rPA or with the altered forms of rPA.

2. Methods

2.1. Ethics statement

Animal procedures were performed at the facilities of Cocalico Biologicals, Inc., Stevens, PA and the U.S. Food and Drug Administration animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animals at both facilities were handled in strict accordance with the recommendations outlined in the Guide for the Care and Use of The Laboratory Animals of the National Institutes of Health and National Research Council. The animal protocols were approved by the Institutional Animal Care and Use Committee of either Cocalico Biologicals, Inc. (Animal Welfare Assurance Number D16-00398 or A3669-01) or of the U.S. Food and Drug Administration, depending on where the study was conducted.

2.2. Mutation of the pagA gene, gene expression and protein purification

Mutated *pagA* genes of *B. anthracis* encoding PA with codons substitution at positions 713 [AAT (Asn) → CAA (Gln) or TCA (Ser)] and 719 [AAC (Asn) → CAG (Gln) or TCA (Ser)] were generated using QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene, CA). The mutated *pagA* genes express PA protein with Gln or Ser residues at positions 713 and 719 in place of Asn residues. These PA proteins are referred to as rPA(N713Q/N719Q) and rPA(N713S/N719S). The mutated *pagA* genes were cloned in pET-22b(+) plasmid carrying an N-terminal *pelB* signal sequence. Wild-type and mutant *pagA* genes were expressed in *E. coli* BL21 (DE3). Wild-type rPA, rPA(N713Q/N719Q) and rPA(N713S/N719S) were purified essentially as previously described [14]. Purified rPA proteins were approximately 95–99% pure as determined by SDS-PAGE.

2.3. Immunization studies

Purified wild-type rPA, rPA(N713Q/N719Q) and rPA(N713S/N719S) were used to prepare vaccine formulations by mixing 50 μg/ml rPA protein with 1.5 mg/ml of aluminum adjuvant in normal saline solution (0.9% NaCl, wt/vol) as described previously [14]. The mixture was gently vortexed and allowed to stand for 1 h at 25 °C for adsorption. For the accelerated stability studies, adsorbed vaccine preparations were stored at 25 °C for 4 weeks as described previously [10]. Groups of 10 mice (six-week-old female CD-1 mice) were immunized once intraperitoneally with either freshly prepared or stored rPA-aluminum hydroxide vaccine formulations. A control group of 10 mice was simultaneously immunized with aluminum hydroxide adjuvant in normal saline solution. Immunizations and serum collections were carried out by Cocalico Biologicals, Inc., Stevens, PA or at the U.S. Food and Drug Administration animal facility. Mice were bled 28 days post-immunization.

2.4. Receptor binding assay

In vitro receptor binding assays with wild-type rPA and rPA (N713Q/N719Q) were performed at 4 °C using a DC 2.4 murine dendritic cell line. DC 2.4 cells were grown in six-well plates to 90% confluence. Cells were washed with 1× PBS, prechilled and incubated with rPA protein for 3 h at 4 °C. After incubation, cells were washed several times with 1× PBS to remove unbound rPA protein. Cells were lysed with mammalian protein extraction reagent (MPER) lysis buffer (Pierce) containing 1× Halt protease inhibitor cocktail and 10 U of DNase/ml. The cell lysates (30 μg of total protein) were subjected to SDS-PAGE analysis. Proteins were then transferred to nitrocellulose membranes, followed by Western blotting using anti-PA mouse monoclonal antibody (QED, Biosciences, Inc.) to visualize the bound rPA protein.

2.5. Cytotoxic activity assay

Biological (cytotoxic) activity of wild-type rPA and rPA(N713Q/N719Q) was measured using the macrophage lysis assay. The assay was performed using J774A.1 cells essentially as described previously [15]. Briefly, cytotoxicity was measured using 2-fold dilutions of wild-type rPA or rPA(N713Q/N719Q) mixed with a fixed concentration of LF (40 ng/ml). Percent viability of the cells was expressed as the viability of the toxin treated cells divided by the viability of untreated control cells X 100. Percent viability was plotted against rPA protein concentration and fitted using a four-parameter logistic regression model. The inflection point, which indicates 50% survival, is reported as the half maximal effective concentration (EC_{50}).

2.6. Toxin-neutralizing antibody (TNA) assays

Serum samples collected from groups of immunized mice were analyzed individually using the TNA assay, performed essentially as described previously [14]. Neutralization of lethal toxin cytotoxicity was measured by assessing J774A.1 cell viability with two-fold serial dilutions of the test serum or a reference rabbit polyclonal serum (NR-3839) mixed with a set concentration of lethal toxin as described earlier [14]. A four-parameter logistic regression model was used to fit the data points generated when the absorbance was plotted versus reciprocal of the serum dilution. The inflection point, which indicates 50% neutralization is defined as the reciprocal of the serum dilution at 50% inhibition and is reported as the ED_{50} [16].

2.7. Statistical analyses

Statistical analyses were conducted using GraphPad Prism software (version 6, GraphPad Software, La Jolla, CA). For the cytotoxic activity curves of PA proteins, a four-parameter logistic regression model was used to fit the data. For TNA assays, an unpaired *t*-test was performed to calculate statistical significance and the conventional $P = 0.05$ was used as the level of significance.

3. Results and discussion

To investigate whether deamidation of Asn^{713} and Asn^{719} of PA contributes to the loss of immunogenicity of rPA vaccines during storage, we generated rPA(N713Q/N719Q) in which Asn^{713} and Asn^{719} were replaced with Gln. We chose this substitution since Gln residues would not be expected to deamidate on a time scale relevant to vaccine storage [17,18]. Moreover, since Asn and Gln differ only by a single methylene group, substitution of Gln for Asn would represent a conservative

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