**Temperature-mediated recombinant anthrax protective antigen aggregate
development: implications for toxin formation and immunogenicity**

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Running title: Immunogenicity of recombinant anthrax protective antigen aggregates
formed by heating

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Abbreviations: Protective Antigen (PA); recombinant PA (rPA); Lethal Toxin (LeTx);
Macrophage Lysis Assay (MLA); Toxicity Neutralization Assay (TNA); Lethal Factor
(LF); Edema Factor (EF); Room Temperature (RT)

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ABSTRACT

Anthrax vaccines containing recombinant PA (rPA) as the only antigen face a stability issue: rPA forms aggregates in solution after exposure to temperatures $\geq 40^{\circ}\text{C}$, thus losing its ability to form Lethal Toxin (LeTx) with Lethal Factor. To study rPA aggregation's impact on immune response, we subjected rPA to several time and temperature combinations. rPA treated at 50°C for 30 min formed high mass aggregates when analyzed by gel electrophoresis and failed to form LeTx as measured by a macrophage lysis assay (MLA). Aggregated rPA-formed LeTx was about 30 times less active than LeTx containing native rPA. Mice immunized with heat-treated rPA combined with $\text{Al}(\text{OH})_3$ developed antibody titers about 49 times lower than mice immunized with native rPA, as measured by a Toxicity Neutralization Assay (TNA). Enzyme Linked Immunosorbent Assay (ELISA) of the same immune sera showed anti-rPA titers only 2—7 times lower than titers elicited by native rPA. Thus, rPA's ability to form LeTx correlates with its production of neutralizing antibodies, and aggregation significantly impairs the protein's antibody response. However, while these findings suggest MLA has some value as an in-process quality test for rPA in new anthrax vaccines, they also confirm the superiority of TNA for use in vaccine potency.

Keywords: Anthrax; Vaccine; Stability; Protective Antigen; Aggregation

INTRODUCTION

Anthrax is a bacterial infection caused by the entry of endospores of *Bacillus anthracis* into the host through abrasions in the skin, by inhalation or ingestion [1]. Depending of the route of infection, anthrax can occur as one of three syndromes: cutaneous, gastrointestinal, or inhalational [2]. Notwithstanding that anthrax represents mainly an occupational risk for those handling ruminants or their products, and therefore has a low incidence in humans, the disease can be serious and even fatal, especially as a pulmonary infection. Among 235 cases of human anthrax reported in the period 1955-2000, there were 11 inhalational. On the other hand, the 2001 bioterrorism incident in the United States produced 22 cases, 11 of the inhalational type, resulting in five fatalities [3]. The high virulence, environmental persistence and ease of transmission of anthrax spores, as well as the high rates of morbidity and mortality that result from spore exposure [4], and the simplicity of growth of the bacterium in laboratory conditions, facilitates its potential use as a bioterrorism weapon [5].

The pathogenesis of *B. anthracis* is mainly explained by two virulence factors: a poly- γ -D glutamic acid capsule and a tripartite exotoxin. The virulence plasmid pXO2 contains the genes for the synthesis of the capsule [6,7], which prevents bacterial phagocytosis and lysis by cationic serum proteins [8]. Meanwhile, plasmid pXO1 contains genes for the exotoxin components: protective antigen (PA), lethal factor (LF), and edema factor (EF) [9]. PA's key contribution to the pathology mediated by the anthrax toxins is its role in binding and internalization of the other exotoxin components [10,11]. Two types of receptors on target cells can bind PA: epithelial cells of the skin, lung, and intestine

express tumor endothelial marker 8 (TEM8 or ANT XR1) [10,12], while capillary morphogenesis protein 2 (CMG2 or ANT XR2) is widely expressed by other types of human tissue [13]. Upon binding to the cellular receptor, the 83 kDa PA is cleaved by a furin-like protease [14], to yield a 63 kDa fragment (PA₆₃) and the N-terminal 20 kDa fragment (PA₂₀) [15]. PA₆₃ forms a heptamer that can bind up to three molecules of either LF or EF [16] to constitute lethal toxin (LeTx) or edema toxin, respectively. The toxins are then endocytosed by the cell, and after acidification of the endosome, LF and EF are translocated into the cytosol [17]. LF is a zinc-dependent metalloprotease that cleaves most isoforms of the mitogen-activated protein kinase kinases (MAPKKs or MEKs), and EF is a calmodulin-dependent adenylate cyclase that affects ion homeostasis and cell signaling, increasing the cytosolic cAMP concentration [18,19].

Antibodies to PA protect against lethal infection in animal models, which is consistent with the disruption of the intoxication mechanism [17]. Although present in variable amounts and along with lower quantities of LF, EF and other anthrax secreted proteins [20,21], PA is the predominant antigen present in Anthrax Vaccine Adsorbed (AVA), the vaccine currently licensed in the United States. LeTx activity and antibody-mediated neutralization can be evaluated *in vitro* using a macrophage lysis assay (MLA) [22] and a toxin neutralization assay (TNA) [23], respectively. Protection afforded by AVA or similar vaccines has been attributed to PA antigen content, because protective immunity in animals correlates with serum levels of PA-neutralizing antibodies [24–27].

Novel anthrax vaccines, containing purified recombinant PA (rPA), can induce protective immunity in animals and humans comparable to AVA [24,28,29]. On the other hand, rPA vaccines do not exhibit the local side effects that AVA does [4], which may hinder continued or expanded use of the latter. In contrast, rPA vaccines are less stable than AVA, which is a subject of concern [30]. It has been reported that rPA loses its biological activity at temperatures as low as 37°C for a period of 48 h [31,32], behavior which may forecast stability problems at lower temperatures for longer periods. In general, purified proteins such as rPA are particularly susceptible to degradation through non-enzymatic modifications such as deamidation, oxidation and aggregation [33–35]. PA is thermally unstable, showing not only a loss of toxin-forming activity upon exposure at relatively mild temperature for short periods, but also aggregation upon exposure for few minutes to temperatures above 40°C [32]. Additionally, our group has reported a decrease in immunogenicity in mice of experimental rPA vaccines exposed to 45°C for 2 min, as measured by TNA [36]. Although it is unlikely that vaccines will be exposed at these temperatures during storage or transportation, the study of degraded forms of rPA produced under these conditions constitutes a useful strategy to assess the stability-indicating characteristics of different laboratory methods [37].

In this research we evaluated the effect of the exposure of rPA in solution to temperatures outside refrigeration, including two above its melting point (between 40 and 46°C) [32], on the formation of aggregates and the relation of this aggregation to the ability of the protein to form active LeTx and to elicit neutralizing antibody response in mice.

MATERIAL AND METHODS

Antigens

rPA (NR-140, recombinant, produced using a plasmid introduced into a non-sporulating, avirulent strain of *B. anthracis* lacking both pX01 and pX02 and NR-2629, recombinant, produced in *Escherichia coli*) and recombinant LF (rLF; NR-142) were provided by NIH-NIAID, Biodefense and Emerging Infections Research Resources Repository (Bethesda, MD). Freeze dried rPA NR-140 and rLF NR-142 were reconstituted according to the supplier directions to a concentration of 1 mg/mL, while rPA NR-2629 (frozen) was thawed and diluted to the same concentration. Samples were stored at -80°C until use. Although both rPA preparations are essentially identical, rPA NR-2629 was used for the experiments that required larger amounts of material, because stocks of rPA NR-140 are limited, and extensive experience has been gained with the latter used in serological testing.

Reference sera

In-house freeze-dried reference serum MR02-1 was used to estimate total antibody contents in test sera in U/mL. MR02-1 was obtained by immunizing mice with up to three doses of an experimental rPA vaccine [38]. Immunoglobulin from pooled serum was purified by ammonium sulfate precipitation. Ammonium sulfate was removed by dialysis against PBS, then diluted with PBS containing 10% normal mouse serum (NMS), and lyophilized. Antibody content (400 U/mL) was assigned to MR02-1 by ELISA, in relative terms to a pool of sera of mice immunized twice with AVA, to which 800 U/mL

was arbitrarily assigned [39]. MR02-2 reference serum (720 U/mL vs. MR02-1 at 400 U/mL in the TNA) was obtained by immunizing 500 CD-1 mice intraperitoneally with an experimental vaccine [38] containing rPA and aluminum hydroxide on days 0, 21 (6 µg), and 42 (3 µg), and bleeding them on day 63. Immunoglobulin from pooled serum was purified by ammonium sulfate precipitation. Ammonium sulfate was removed by dialysis against PBS; the preparation was then diluted with PBS containing 10% normal mouse serum (NMS), and lyophilized [36].

Polyacrylamide gel electrophoresis and Western blot

Temperature-treated and control rPA samples were mixed with Native Sample Buffer (Bio-Rad, Hercules, CA), or Laemmli Sample Buffer (Bio-Rad, Hercules, CA) to which mercaptoethanol (5%) was added (for reductive denaturing conditions). Samples in Laemmli buffer were heated at 95°C for 5 min. Aliquots containing 5 µg of protein were separated on a 4-20% polyacrylamide gradient gel (Mini protean gel, Bio-Rad, Hercules, CA). Proteins were visualized by staining with Bio-Safe Coomassie G-250 Stain (Bio-Rad, Hercules, CA). To perform the Western blotting, proteins were electrophoretically transferred to Trans-Blot nitrocellulose membranes, 0.45 µm pore size (Bio-Rad, Hercules, CA). Membranes were blocked with incubation buffer (IB: 0.05% Tween 20 in phosphate buffered saline [PBS-T], plus 5% skim milk), and then incubated during 2 h at room temperature (RT) with MR02-2 or NMS diluted with IB. After washing with PBS-T, the membranes were incubated at RT for 1 h with alkaline phosphatase-labeled goat anti mouse IgG (H+L: Heavy and Light chains) antibody (KPL, Inc. Gaithersburg, MD)

diluted in IB. BCIP/NBT Western Blot Substrate (KPL, Inc. Gaithersburg, MD) was used to reveal phosphatase activity according with manufacturer instructions.

rPA thermal treatment and vaccine formulation

rPA NR-2629 (at 1 mg/mL) was incubated during 30 min, one, four or 24 h at 22 (RT), 50 or 75°C. Some treated samples (Table 1) and the control rPA were formulated into a vaccine as described previously [40]. rPA vaccine freshly thawed and then formulated at RT was used as a negative control. In brief, aluminum-containing adjuvant (Alhydrogel® Brenntag Biosector, Denmark; 1.42mg/mL Al) was combined with 30 µg/mL rPA (control or treated). After vigorous agitation using a Vortex mixer, the suspensions were incubated at RT for 1h.

Immunization and sera collection

With the approval of CBER's Animal Care and Use Committee, a total of three hundred female CD-1 mice, four to six week old, were distributed in groups of twenty and then immunized once intraperitoneally with 6 µg of control or treated rPA, one group per treatment. Twenty-eight days post-immunization, mice were bled from the tail veins and sera separated. Samples were stored at -20°C until use. A serum pool from non-immunized mice was used as negative control.

Anti-rPA ELISA

ELISA was performed as described previously [39], with some modifications. In brief, ELISA plates were coated overnight with a 1µg/mL solution of rPA (NR-140). Samples,

negative control or reference serum diluted in IB were added to the coated plates and then incubated at RT for 2 h. After incubation, plates were washed three times with 0.8% NaCl containing 0.05% Tween 20, and alkaline phosphatase-labeled goat anti mouse IgG (H+L) antibody diluted in PBS-T was added. After another cycle of incubation/wash, the substrate p-nitrophenyl phosphate (Sigma Chemicals, St. Louis, MO) was used to develop color. Optical density was measured at 450 and 505 nm after 30 min in a microplate reader and units were calculated relative to reference MR02-1 using a reference-line algorithm (Unitcalc v1.3; BioSys Inova HB, Stockholm, Sweden). The limit of quantification (LOQ) of the assay was established as 37 U/mL [39]. Values >37 U/mL were interpreted as positive responses.

Macrophage Lysis Assay (MLA)

A published method [41] with some modifications was used to measure the ability of heat-treated rPA to form LeTx. In brief, RAW 264.7 cells (ATCC, Manassas, VA) with no more than twenty-four passages, were cultured in 75 cm² flasks in complete DMEM medium. Cells were harvested and suspended in complete DMEM at a density of 4.5×10^6 viable cells/mL, assessed via trypan blue exclusion assay using an automated cell counter (Countess; Invitrogen, Waltham, MA); 100 μ L aliquots of this suspension were deposited in a polystyrene 96-well round-bottom tissue culture plate (BD, Franklin, NJ) to a final density of 4.5×10^5 cells/well, and then incubated for 17-19 h at 37°C under a 5% CO₂ atmosphere. Before incubation with rPA and rLF, the culture medium was discarded. One hundred μ L of toxin mixture were added to culture plates: heat-treated or untreated rPA plus rLF, to a final concentration of 50 ng/mL of rPA and 160 ng/mL of rLF per well.

Cell plates were then incubated at 37°C/4 h. Cell viability was estimated by the addition of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] 5 mg/ml in phosphate-buffered saline; Sigma). After 2 hours of incubation, cells were lysed by the addition of solubilization buffer (90% isopropanol, 0.5% [wt/vol] SDS, 38 mM HCl) [42] and optical density was read using a microplate reader at 570 nm with a 690 nm reference filter. The percentage of surviving cells was calculated as follows: $100\% \times (\text{OD}_{\text{rPA-samples}} / \text{OD}_{\text{non-rPA}})$, where rPA-samples corresponds to reading of wells containing cells and toxin mixture (treated or non-treated rPA, plus rLF) in a complete DMEM, whereas non-rPA represents the reading of wells containing cells and complete DMEM only.

Toxin Neutralizing Assays (TNA)

We used a published method [23], slightly modified, to measure neutralizing activity in mouse sera. Briefly, RAW 264.7 cells (ATCC, Manassas, VA) were cultured and seeded in 96-well round-bottomed plates as described for the MLA. Test and reference sera were pre-diluted and then serially diluted (1:2) in a microtiter plate containing complete DMEM. Afterward, plates were incubated with LeTx at a constant concentration (50 ng/mL of rPA; 160 ng/mL of rLF) for 30 min at 37°C in a 5% CO₂. Toxin-serum mixtures were then added to cell plates and then incubated (37°C in 5% CO₂) for 4 h. Cell viability was estimated as describe for MLA, by the addition of MTT. After 2 hours of incubation, cells were lysed by the addition of solubilization buffer and optical density was read. Neutralizing activity (neutralizing units per mL; U/mL) was calculated by multiplying the Neutralization Factor 50 ($\text{NF}_{50} = \text{ED}_{50} \text{ sample} / \text{ED}_{50} \text{ reference}$, where ED_{50} represents the reciprocal of the dilution corresponding to 50% neutralization read

from each fitted curve [sample and reference serum]) by the activity of the reference serum (MR02-2). ED_{50} was derived from a four-parameter logistic regression model (GraphPad Software, Inc., San Diego, CA) applied to a transformation of the change of optical density at 570 and 690 nm for each titration curve [36].

Statistical analysis

JMP 9.0.0 software (SAS Institute, Cary, NC) was used for the statistical analysis. The nonparametric Dunn test was performed to compare medians of the test groups to that of the control.

RESULTS

Gel electrophoresis of rPA subjected to 50°C showed the presence of aggregate formation following exposure to this temperature for as little as 30 min, while exposure of rPA at RT for up to 24 h did not cause any apparent change in its MW (Figure 1). The absence of bands at 50 and 75°C in the native gels suggests either complete degradation of rPA or the formation of high mass aggregates that cannot penetrate the polyacrylamide mesh. Additional analysis by SDS-PAGE and WB indicated a time-dependent loss of rPA (83 kDa) with the concomitant appearance of higher molecular weight material including some bands exceeding 250 kDa. These latter bands suggest the formation of aggregate structures (Figure 2). The absence of lower mass staining by SDS-PAGE and Western blotting suggests proteolysis is not occurring under the conditions tested.

We also performed a measurement of the size of aggregates using a single particle optical sizing system (AccuSizer FX Nano, Particle Sizing Systems, Port Richey, FL). Particles larger than 0.2 microns were determined from samples of rPA heated at 22 and 50°C, finding that the sample heated at 50°C had almost four times the concentration of particles >0.2 microns than the sample that remained at 22°C (data not shown). This formation of detergent-resistant high molecular weight protein bands correlated with a loss of rPA's ability to form LeTx: as temperature and time of exposure increased, the proportion of macrophages that survived in MLA, in the presence of a mixture of treated rPA and fresh LF, also increased (Table 1). Without heating, rPA was able to form LeTx with undiminished killing ability over 24 h. In contrast, heating for as little as 1 h led to almost total loss of toxicity.

Serum collected from mice immunized with several rPA preparations indicate that exposure of rPA to the high temperatures moderately impaired the antibody response when measured by ELISA, but significantly eliminated the ability of the protein to induce neutralizing antibodies, where approximately 86% of the mice failed to develop measurable protective immunity (Fig 3B). This reduced immunogenicity of rPA matches its inability to form LeTx (Table 1 and Figure 3).

DISCUSSION

The potential use of *B. anthracis* in warfare and terrorism has stimulated the manufacturing and stockpiling of licensed anthrax vaccines, as well as the development

of new vaccines. Stability-indicating tests are important for assessing vaccine quality over time; development of some of these tests includes exposure of the products to different temperatures, even outside their recommended storage conditions [43]. PA is a thermolabile molecule [31,44], with a denaturation temperature around 50°C [45]. We selected three temperatures to characterize the stability-indicating properties of three testing procedures, one below the denaturation temperature of PA (22°C, as control), one around its denaturation temperature (50°C) and one above its denaturation temperature (75°C). We hypothesized that any detectable loss in rPA activity due to temperature-dependent structural changes would occur faster at higher temperatures. The formation of aggregates of rPA that we detected, following its exposure to 50°C for a period as short as 30 minutes, corresponds to the findings of Belton and Miller, who analyzed the mechanics of the aggregation of rPA, induced by its heating [46]. These authors found that the aggregation rate increased substantially after 43°C, and also that the particle diameter of the aggregated rPA increased when the temperature was raised from 43°C to 49°C.

Anthrax vaccines must have reliably long shelf-lives, since all lots that enter the National Strategic Stockpile must remain potent throughout protracted storage periods. Vaccine stability requires the development of suitable manufacturing conditions to preserve antigen integrity for the product's lifetime. For example, as part of recent efforts aimed to produce a stable single-antigen anthrax vaccine, the use of a freeze-dried dominant negative inhibitor (DNI) homolog of rPA has been proposed [47,48]. A thorough biophysical and immunological characterization of DNI exposed to temperatures outside

of the range recommended for vaccine storage has been performed, as compared to rPA. The immunogenicity of a liquid formulation of DNI adsorbed to aluminum adjuvant, measured as induction of neutralizing antibodies in mice, decreased after its storage at 40°C for 8 weeks [47]. On the contrary, in a study similar to ours, the immunogenicity of freeze-dried DNI subjected to thermal stress (40°C and 70°C for up to 4 weeks) and formulated into a vaccine with aluminum adjuvant was not affected [48].

Potency testing is a quality control check for vaccines used throughout the dating period of the product, being performed immediately prior to release for clinical use and throughout stability testing [37,49]. Because antibodies directed to PA alone can protect animals from anthrax infection [50,51], we have previously suggested quantifying immunogenicity in mice by either ELISA or TNA to measure anthrax vaccine potency [36,52]. On the other hand, the MLA has been proposed to evaluate rPA quality prior to formulation into a final vaccine bulk. MLA quantifies the ability of rPA to form LeTx *in vitro* when mixed with LF. Although separate potency testing is conducted on the final vaccine product, the use of routine animal testing to predict effectiveness in clinical use is becoming undesirable [53]. The MLA might be a suitable replacement of an animal-based potency test for anthrax vaccines if its outcome reflected not only rPA integrity, but if it were also a reliable predictor of the ability of the antigen to elicit neutralizing antibodies. A main concern is that the MLA may quantify loss of rPA biochemical function, while the protein retains its ability to elicit protective immunity. In such a scenario, MLA results without accompanying animal potency data will be misleading. However, we found out that rPA's ability to form LeTx correlates with its production of

neutralizing antibodies, and aggregation also significantly impairs the antibody response to the protein; but a challenge to the use of the MLA as a potency test, at least in terms of its stability-indicating characteristics, is the impact that the binding of rPA to the adjuvant may have on its structure. It has been proposed that the interaction of rPA with the aluminum hydroxide gel used as adjuvant is destabilizing, and the direct cause of a reduction in the capacity of vaccines formulated with this material to induce neutralizing antibodies [54,55]. Regarding the mechanism responsible for the loss of immunogenicity upon storage, we have previously reported that spontaneous deamidation predicted to occur over an extended period would adversely affect vaccine immunogenicity [35]. However, D'Souza et al. showed rapid deamidation of asparagines in the positions 713 and 719 of rPA, when the protein was adsorbed onto aluminum hydroxide gel, but not when it was kept non-adsorbed at the same temperature (in the cold) [54]. On the other hand, Hassett et al. [47] reported changes in DNI structure and increased deamidation at elevated temperatures, which ran parallel to the loss of neutralizing immunogenicity, after only one week of storage of their liquid adsorbed and non-adsorbed DNI vaccine formulations at 40 °C [47]. Moreover, Verma et al. recently reported that the loss of immunogenicity of rPA vaccine due to deamidation was independent of the presence of adjuvant [56].

The relation between aggregation and deamidation in our results is unclear: it remains to be studied if deamidation of rPA in solution occurs in short periods at the temperatures we used, and if deamidation contributes to aggregation or vice versa. On the one hand, as the temperature nears its denaturation temperature, proteins partially unfold, exposing

hydrophobic regions, which induce aggregation [57]. On the other, the rate of deamidation of asparagine residues can be influenced by the flexibility of the peptide backbone [58]. At the same time, the resulting changes in charge from the amino residues of asparagines to the carboxylic residues of aspartic acid and/or iso-aspartic acid may also alter the way the proteins will be refolded [59]. The triggering of protein aggregation by deamidation has been reported elsewhere [60], while the rate of deamidation of a model peptide has been reported to follow Arrhenius behavior between 5 and 65°C [61]. In fact, D'Souza et al. have proposed that the deamidation of rPA that they detected is mostly driven by the adsorption-mediated secondary structural conformation, which promotes the formation of the succinimide intermediate [54].

An explanation on the impact of rPA's tertiary structure on the induction of neutralizing antibodies is also lacking. Recently Verma et al. have suggested that the differences in the tertiary structures of wild-type rPA and a six-Asp mutant rPA appear to be relatively minor, and reported that mutation of these six deamidation-prone Asn residues to Asp residues did not result in obvious alteration of epitopes critical for the induction of toxin-neutralizing antibodies [56]. However, it is interesting to highlight that while the structure of domains 3 and 4 [62] of the protein is destabilized by aggregation at 45 °C, the structure of domains 1 and 2 is somewhat preserved at this temperature [63], findings which may suggest that neutralizing epitopes on rPA tend to cluster on domains 3 and 4: in our hands, while the induction of neutralizing antibodies by aggregates produced at 50°C essentially disappeared, non-neutralizing antibodies were still induced by these aggregates.

In summary, the results presented in this study indicate that rPA aggregates generated by its exposure to 50°C for as short as 1 h, form some amount of LeTx measurable in the MLA and elicit reduced or little detectable antibody, as measured by both ELISA and TNA, respectively, suggesting that these methods of quantification can distinguish rPA of good vs. bad immunogenic quality. MLA is unsuitable as alternative to immunogenicity for use as potency test, not only because the binding of rPA to the adjuvant may affect its structure, but because desorption may also affect the characteristics of the protein. Nevertheless, the results of the current study allow us to ascertain the stability-indicating properties of the three methods. While the MLA demonstrates that rPA has a 50% loss of toxin-forming activity when treated at 50°C for 1 h before formulation into a final vaccine lot, TNA data show that antibodies elicited by this heat-treated antigen formulated into a vaccine are reduced by over a hundred fold, compared to those elicited by unheated antigen. ELISA data, in contrast to TNA data, indicate only a relatively minor reduction in antibody titer elicited by heat-treated rPA. These findings suggest that although the MLA may not be used to replace immunogenicity as a potency test for anthrax vaccines, and it is somewhat limited in its stability-indicating characteristics, it is still an important in-process test that contributes to the use of rPA of good immunogenic quality in the manufacturing of new anthrax vaccines. Our results also confirm the superiority of the TNA over ELISA for use in the immunogenicity test [36,52], in terms of its sensitivity to detect changes on rPA potentially relevant for protection of the population for which an rPA-only anthrax vaccine may be prescribed.

ICH recommends that studies be conducted on vaccines under accelerated and stress conditions [64]; in particular, stress testing includes the effect of temperatures above those used for accelerated testing, which helps identifying the likely degradation products. In turn, results of stress testing can help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used [65]. ICH also recommends the careful selection of stress conditions on a case-by-case basis [64]. Further knowledge of the mechanism or mechanisms responsible for the loss of rPA protective immunogenicity would help inform the selection of the combination of time and temperature exposure that will best help validate the tests described in this research.

CONCLUSIONS

This study shows, by exposing rPA to elevated temperatures over time, that the formation of supramolecular structures (aggregates) of the protein correlates with the degradation of one of its functions and with its ability to induce antibodies in mice. Although particles larger than 0.2 μm were detected in preparations of rPA held at RT, the number of such particles increased four-fold upon incubation at 50°C. Aggregated protein was deficient in its ability to form LeTx and also lost its ability to induce an antibody response as measured by both ELISA and TNA. The contrasting results obtained in this study, among the methods investigated, represent a contribution towards the selection of quality control procedures for anthrax vaccines with suitable stability-indicating characteristics. Further work is needed to understand how changes occur on rPA, both before and after its adsorption to the adjuvant.

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Treatment		MLA % survival	ELISA Median (U/mL)	TNA Median (U/mL)
Temperature (°C)	Time (h)			
22	0	4.2	405.5	144.4
	1	6.8	503.5	168.5
	24	3.0	455.5	128.8
50	1	52.2	110.0*	3.0*
	4	98.4	151.8*	3.0*
	24	101.6†	173.8*	3.0*
75	1	98.3	83.5*	3.0*
	4	98.7	61.8*	3.0*
	24	103.4†	38.1*	3.0*

Table 1. Correlation between the ability to form LeTx of rPA and its immunogenicity.

rPA was subjected to different temperature-time combinations. The ability of rPA to form LeTx was determined by macrophage lysis assay (MLA). Immunogenicity was measured by ELISA (U/mL, which represent total serum anti-rPA antibodies) and a toxin neutralization assay (TNA; U/mL, which corresponds to serum LeTx-neutralizing antibodies). †Percent survivals above 100% were due to fluctuation of the OD readings of the wells containing cells and medium only. *Values for ELISA and TNA significantly different than the control (22°C/0 h; p=0.05). For calculation purposes, an arbitrary value of 3.0 was assigned to samples for which antibodies were not detected. Temperatures above 50 °C impair the ability of rPA to produce neutralizing antibodies (TNA), and match a decrement of LeTx formation; meanwhile there are some total antibodies production at 50 and 75 °C. The treatment at 22 °C shows no significative changes in the production of antibodies.

Figure 1. Native PAGE for rPA treated at the temperatures shown, for the indicated periods. Freshly prepared rPA (83 KDa) corresponds to time 0 at 22°C (control). M = molecular weight markers. rPa incubated at 22°C by the periods indicated shows no degradation, relative to the control. Meanwhile, no native rPA is present in any lane when samples are heated. In some lanes corresponding to samples heated at 50°C and 75°C, bands barely show up at the bottom of the loading wells, suggesting the formation of molecular structures so big that they are not able to enter the polyacrylamide mesh.

Figure 2. SDS-PAGE and Western Blot (WB) patterns of heat treated rPA at 22°C, 50°C, and 75°C for 0.5 and 24 h. The control preparation consisted of freshly thawed and diluted rPA. Strips of representative gels and the corresponding WB were put side-by-side for comparison. It was expected that the higher sensitivity of WB could propitiate detection of additional protein bands. The rPA monomer band present in the product incubated at 22°C appears to be of similar intensity to the band corresponding to the control, and contains similar degradation products below 83 KDa. A few bands above rPA in the WB for 0.5 and 24 h suggest the formation of supramolecular structures. The reduction of the intensity of the band corresponding to the rPA monomer becomes evident at 50°C, but is especially obvious at 75°C. WB points to some degradation of the rPA monomer, but also to the formation of more structures of molecular mass larger than 83 KDa, especially at 75°C. Monomeric rPA, degradation products and detergent-resistant aggregates were detected in the WB using polyclonal anti-rPA antibodies.

Figure 3. Antibody response of mice immunized with rPA that had received different time-temperature treatments (showed in the bottom rows), formulated into a vaccine. Control vaccine, prepared at room temperature using fresh rPA, is represented by 0 h at 22°C. Twenty mice per group are shown in a box plot format. A) U/mL represents the total serum antibody response measured by ELISA. Antibody response to rPA decreased following its thermal treatment, but was still detectable, even after its exposure to 75°C for 24 hours. B) U/mL corresponds to the serum-neutralizing antibody response measured by TNA. The induction of neutralizing antibodies was seriously compromised by incubation of rPA at 50°C and 75°C.