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Stabilization of a Recombinant Ricin Toxin A Subunit Vaccine through Lyophilization

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Abstract

Lyophilization was used to prepare dry, glassy solid vaccine formulations of recombinant ricin toxin A-chain containing suspensions of colloidal aluminum hydroxide adjuvant. Four lyophilized formulations were prepared by using combinations of rapid or slow cooling during lyophilization and one of two buffers, histidine or ammonium acetate. Trehalose was used as the stabilizing excipient. Aggregation of the colloidal aluminum hydroxide suspension was reduced in formulations processed with a rapid cooling rate. Aluminum hydroxide particle size distributions, glass transition temperatures, water contents, and immunogenicities of lyophilized vaccines were independent of incubation time at 40°C for up to 15 weeks. Mice immunized with reconstituted ricin toxin subunit A (RTA) vaccines produced RTA-specific antibodies and toxin-neutralizing antibodies (TNA) regardless of the length of high temperature vaccine storage or the degree of aluminum adjuvant aggregation that occurred during lyophilization. In murine studies, lyophilized formulations of vaccines conferred protection against exposure to lethal doses of ricin, even after the lyophilized formulations had been stored at 40°C for 4 weeks. A corresponding liquid formulation of vaccine stored at 40°C elicited RTA-specific antibody titers but failed to confer immunity during a ricin challenge.

Keywords

Lyophilization; Freeze drying; Aluminum; Adjuvant; Stability; Biodefense; Aggregation; Ricin; Vaccine

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1. Introduction

Protein subunit vaccines, like therapeutic proteins [1,2,3], tend to be unstable and readily undergo physical and/or chemical degradation [4,5,6]. To slow this degradation, vaccines typically must be kept at low (e.g. subzero) temperatures for their entire shelf lives. The stringent cold-chain requirements of many vaccines thus provide a serious impediment to their use in developing countries or in emergency situations [7,8]. Excursions from the ideal cold-chain temperature are problematic [9]. For example, low-temperature excursions, which may cause accidental freezing, occur in 75-100% of liquid vaccine formulations during their distribution [9]. Freezing may result in loss of antigenicity [10].

The limitations imposed by cold-chain requirements are especially daunting for vaccines against bioterrorism threats. In contrast to vaccines against common diseases, it is not anticipated that bioterrorism vaccines would be administered routinely to patients. Instead, these vaccines would likely be administered only in the event of an imminent or actual bioterrorism attack. To meet the demands of such an emergency, large quantities of vaccines would need to rapidly be made available. In turn, this implies that stockpiles need to be created and maintained under conditions that preserve vaccine stability and efficacy. Thus, for typical vaccines requiring storage at 2-8°C or sub-zero temperatures, limits on available refrigerated storage capacity and refrigerated transport systems preclude their effective use.

Proteins are generally observed to be relatively weak antigens, and addition of microparticulate adjuvants to vaccine formulations typically is required for an appropriate immune response [11]. Currently, the only adjuvants that appear in vaccines approved for use in the United States are aluminum hydroxide, aluminum phosphate, and monophosphoryl lipid A adsorbed to aluminum hydroxide [12].

Lyophilization is used to stabilize therapeutic proteins [13] and potentially may extend the shelf life and thermostability of vaccines as well [14,15,16]. In the design of a lyophilized vaccine formulation, a primary objective is to use judiciously-chosen excipients [13] to embed the antigen in a glass whose high viscosity and low water content limit degradation reactions. In the first stage of a lyophilization process, temperature is reduced below the freezing point of a formulation, causing ice to crystallize and the remaining solute phase to become progressively more concentrated (approximately 30-100 fold), and viscous (approximately 10^{15}-fold). Eventually, the glass transition temperature at maximal freeze concentration (Tg') is reached, and the solute phase forms a glass, halting further crystallization of water. During the drying stages of lyophilization, the glass transition temperature of the formulation increases as water is removed. Ideally, at the end of the drying cycle the glass transition temperature is well above room temperature, allowing room-temperature storage while maintaining a low-mobility, glassy state. Commonly used glass-forming excipients include sugars such as sucrose and trehalose [13].

The formulation and lyophilization process must be optimized to confer stability not only to the antigen, but also to the adjuvant(s). Unfortunately, colloidal suspensions of aluminum adjuvant particles are unstable, and freezing-induced concentration of adjuvant suspensions causes aggregation during freeze-thawing [10,17,18,19,20]. Larger particles are less efficiently internalized by dendritic cells [21] and thus might be expected to produce a weaker immune response [22]. This expectation was consistent with results from a study of a recombinant hepatitis B vaccine formulated with aluminum hydroxide that demonstrated loss of immunogenicity when lyophilized, with larger adjuvant particle sizes correlating with lower immune responses [23]. In contrast, however, another study found that lyophilized vaccines with large (14-17μm) or small (1-2 μm) mean particle sizes were equally effective [24, 25]. The reason(s) for the different sensitivities of immune response to

particle size seen in the various studies remains unclear. During lyophilization, aggregation of colloidal aluminum hydroxide suspensions can be inhibited by reducing the extent of freeze-concentration with formulations that contain high concentrations of glass-forming excipients, and also by limiting the time over which the freeze-concentrated suspensions can aggregate by using rapid cooling procedures to accelerate glass formation [19].

Ricin toxin is a potential bioterrorism agent extracted from castor beans (*Ricinus communis)* [26]. The ricin heterodimer consists of two subunits, RTA and RTB [27,28]. RTA is an RNA N-glycosidase that selectively inactivates eukaryotic ribosomes, thereby inhibiting protein synthesis. RTB is a lectin that facilitates ricin attachment and entry into mammalian cells. In humans, ricin exposure via injection, inhalation and possibly ingestion can be lethal [26,29].

RiVax is a full-length derivative of RTA with attenuating point mutations at residues Y80 and V76 [30]. A liquid vaccine containing RiVax prepared without adjuvant produced RTA-specific neutralizing antibodies in mice [31,32], and lyophilized RiVax formulations that were reconstituted with a separate aluminum hydroxide adjuvant suspension protected mice against ricin exposure [33]. However, liquid RiVax vaccine formulations are unstable at elevated temperatures [34,35,36]. Previous studies of RiVax conformation in solution over a range of temperatures and pHs [35] and studies with RiVax adsorbed to alum [36] have both shown that the protein undergoes structural changes at a temperature around 40°C.

We hypothesized that the combination of a lyophilization process with controlled cooling rates and the addition of the glass-forming excipient trehalose to colloidal suspensions of aluminum hydroxide could be used to form ultra-stable lyophilized RiVax vaccine formulations. In addition, we tested the hypothesis that aggregation of aluminum hydroxide suspensions would reduce the potency of RiVax vaccines by manipulating cooling rates to induce different degrees of aluminum hydroxide aggregation. Both hypotheses were tested in a murine model.

2. Materials and Methods

2.1 Materials

High purity α,α-trehalose dihydrate and sulfuric acid were from Mallinckrodt Baker (Phillipsburg, NJ). L-Histidine, ammonium acetate, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). 2% Alhydrogel® (aluminum hydroxide adjuvant) was from Accurate Chemicals and Scientific Corp (Westbury, NY). 3 mL 13 mm glass lyophilization vials, caps and seals were from West Pharmaceutical Services (Lititz, PA). Concentrated 10X phosphate buffered saline (PBS) and Tween 20 were from Fischer Scientific (Fair Lawn, NJ). Peroxidase-conjugated affinipure donkey anti-mouse IgG (H+L) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 3,3′,5,5′-tetramentylbenzidine (TMB) was from Thermo Scientific (Rockford, IL).

2.2 Preparation of Vaccine Formulations

RiVax stock was received from the University of Kansas (Lawrence, KS) in 10% sucrose, 10 mM histidine 144 mM sodium chloride pH 6 solution. Stock RiVax was dialyzed overnight with three buffer exchanges into 10 mM histidine or ammonium acetate at pH 6, using a 10,000 MWCO SpectraPor7 Dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) and concentrated using a Millipore Amicon Ultra-15 MWCO 10,000 centrifugal filter unit. RiVax and placebo formulations were prepared with 0.85 or 1.0 mg Al/mL from Alhydrogel®, 0, 4, 8 or 12 w/v% trehalose and 0.2 or 0 mg/mL RiVax in 10 mM histidine or ammonium acetate buffer, pH 6. Vaccine formulations used for the stability study contained 0.85 mg Al/mL since this is the maximum allowable limit for aluminum in

vaccines in the US [37]. Placebo formulations used for measuring the particle size distribution of aluminum hydroxide with varying trehalose concentration used 1.0 mg Al/mL. Histidine buffer was chosen since it was shown previously to stabilize RiVax [35]. Ammonium acetate buffer was chosen because it is volatile and hence sublimes during the lyophilization process [38], reducing the tonicity of reconstituted formulations. In principle, higher concentrations of glass-forming excipients could thus be added to volatile buffer-containing formulations while still maintaining desired tonicity. Formulations were stirred at 2-8°C for 1 hour, after which time the amount of RiVax protein adsorbed to Alhydrogel® was determined by centrifuging samples containing 0.5 ml of vaccine formulation for 30 seconds at 14,500 × g in order to sediment Alhydrogel® particles with adsorbed RiVax protein. Protein remaining in the supernatant was measured by absorbance at 280 nm, and the protein adsorbed to Alhydrogel® was calculated by difference. In each of the formulations tested, the 1 hour mixing time was sufficient for approximately 50% of the RiVax to adsorb to the adjuvant.

2.3 Lyophilization

Lyophilization vials were filled with 1 mL of formulation. Vials were cooled at one of two rates. For rapid cooling, vials were placed on lyophilizer shelves pre-cooled to −10°C (FTS Systems Lyophilizer, Warminster, PA). Shelf temperatures were decreased at a rate of 0.5°C/minute to −40°C and then held at −40°C for 1 hour. For slow cooling, vials were placed on room temperature lyophilizer shelves, cooled to 0°C, held at 0°C for 1 hour, cooled to −40°C at a rate of 0.5°C/minute and then held at −40°C for 1 hour. To minimize radiation and edge vial effects, sample vials were surrounded with “dummy” vials. Primary and secondary drying was conducted as previously described [19]. After drying the chamber was backfilled with nitrogen until atmospheric pressure was achieved. Rubber stoppers were inserted under nitrogen atmosphere, and the vials were sealed with aluminum caps and stored at −80°C until use.

2.4 Stability Study

Lyophilized vaccine samples were used immediately after being removed from −80°C storage (denoted as “Time 0” samples) or placed in a 40°C incubator for accelerated degradation studies. Formulations subjected to accelerated degradation conditions were removed after incubation at 40°C for 1 week, 4 weeks, 8 weeks, or 15 weeks, and then stored at −80°C prior to administration to mice or further analysis.

2.5 Particle Sizing

Laser diffraction particle size analysis (LS 230, Beckman Coulter, Miami, FL) was performed on the initial liquid suspensions of aluminum hydroxide and lyophilized formulations reconstituted in 1 mL of 0.22 μm-filtered DI water. Previous studies showed no difference in particle size distributions between formulations with and without protein (data not shown) so no protein samples were used in the size analysis. The optical model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57 [39,40]. Approximately 6 mL of sample was required. For each run, laser diffraction intensities were recorded three times for 90-seconds each and averaged. Each formulation was run in triplicate.

Microflow image analysis (FlowCAM®, Fluid Imaging Technologies, Yarmouth, ME) was used for additional particle size characterization to visualize particles 2-2,000 μm. 0.1 mL of vaccine formulation was analyzed using a 100-μm flow cell with 10x objective and collimator. Dark and light settings of 17 and 20 were used, respectively.

2.6 Differential Scanning Calorimetry

Glass transition temperatures of lyophilized samples were determined using differential scanning calorimetry (Diamond DSC, Perkin Elmer, Waltham, MA). Triplicate samples were prepared inside an aluminum pan under dry nitrogen. Pans were cycled twice between 25°C to 150°C at a scan rate of 100°C/minute. The second heating scan was used to determine the onset glass transition temperature.

2.7 Moisture Content

Residual moisture in lyophilized vaccines was determined by Karl Fischer analysis (DL 37 coulometer, Mettler, Columbus, OH). Dimethylformamide with known moisture content was used to reconstitute the lyophilized vaccine. The total water present in the sample was determined in triplicate using pyridine-free vessel solution (PhotoVolt, Minneapolis, MN).

2.8 Murine Immunization Studies to Assess Immunogenicity of Vaccine Formulations

Murine studies were conducted under University of Colorado at Boulder Institutional Animal Care and Use Committee (IACUC) protocol #1103.07. Female Swiss Webster mice 5-6 weeks old were from Taconic (Hudson, NY) and allowed to acclimate for at least one week. Mice were housed 5 per cage and were allowed food and water ad libitum. Mice (10 per group) were injected subcutaneously on Days 0 and 21 with 50 μL of various vaccine formulations, each containing 0 or 10 μg RiVax. Blood was collected under isofluorane anesthesia on Days 0, 21 and 35 via the retro orbital cavity. Serum was separated by centrifugation at 10,000 rpm for 14 minutes at 4°C and stored at −80°C until use.

2.9 Total Antibody Enzyme Linked Immunosorbent Assay (ELISA)

Nunc MaxiSorb 96 well plates (Thermo Fischer Scientific, Rochester, NY) were coated with 50 μL/well of 1μg RiVax/mL diluted in PBS and incubated at 2-8°C overnight. Plates were washed 4 times with PBS containing 0.05% Tween 20. Plates were blocked with 300 μL/well of PBS with 1% BSA, incubated at room temperature for 2 hours, and washed again. Serum was initially diluted in PBS with 1% BSA, 0.05% Tween 20, either 800-fold for serum collected on days 0 and 21, or 10,000-fold for serum collected on Day 35. A series of in-plate 2.33-fold dilutions was made for each sample. Plates were incubated for 2 hours at room temperature and washed. 40 μL of HRP-conjugated donkey anti-mouse antibody diluted 10,000 times was added to each well and incubated for 2 hours at room temperature with shaking, followed by washing. 40 μL TMB was added to each well and incubated for 30 minutes, followed by quenching with 40 μL of 2N sulfuric acid. Plates were read at 450 nm on a Molecular Devices Kinetic Microplate Reader (Sunnyvale, CA).

To determine titers, average OD 450 values as a function of dilution were fit to a 4-parameter logistic equation using SigmaPlot software. The constraints $0 < \min < 0.15$ and $\max < 3.3$ were used. The cutoff value used was 0.25, which was at least 2.5 times the highest concentration of mouse serum dilution of each day 0 group average.

2.10 Vero cell cytotoxicity assay

Vero cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and were maintained in a humidified incubator (37°C, 5% CO_2). For cytotoxicity assays, the cells were trypsinized, adjusted to approximately $0.5\text{-}1.0 \times 10^5$ cells/mL, seeded (100 μl/well) onto white 96-well plates (Corning Life Sciences, Tewksbury, MA), and allowed to adhere overnight. Vero cells were then treated with either ricin at 10 ng/mL, ricin-serum antibody mixtures or medium alone for 2 hours at 37°C. The cells were washed and then incubated for 40 hours. Cell viability was assessed using CellTiter-Glo reagent (Promega, Madison, WI) according to the manufacturer's instructions, except that

the reagent was diluted 1:5 in PBS prior to use. Luminescence was measured with a SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA). All treatments were performed in triplicate, and 100% viability was defined as the average value obtained from wells where cells were treated with medium only. The neutralizing titer is defined as the dilution of mouse serum that inhibited ricin cytotoxicity in 50% of ricin treated cells (IC_{50}).

2.11 Ricin Challenge Study

Ricin challenge studies were conducted at the Wadsworth Center (Albany, NY) under Wadsworth Center's IACUC guidelines and protocol 10-384. Mice were vaccinated as described above and on day 49 mice were injected intraperitoneally with 100 ng/g of ricin diluted in PBS. Thereafter, the animals were allowed food and water ad libitum. Blood (<5 μl) was collected from the tail veins of the animals at 24 hour intervals to measure blood glucose levels with an Accu-Chek Aviva handheld blood glucose meter (Roche, Indianapolis, IN). Mice were euthanized when they became overtly moribund and/or when blood glucose levels fell below 25 mg/dL. For statistical purposes, readings at or below the meter's limit of detection of ~20 mg/dL were set to that value.

3. Results and Discussion

3.1 Aggregation of Aluminum Hydroxide during Lyophilization

Initial experiments were used to examine the effects of various concentrations of the glass-forming excipient trehalose on the aggregation of aluminum hydroxide suspensions during lyophilization. Prior to lyophilization, surface area-weighted particle size distributions (SA-PSD) for aluminum hydroxide suspensions in formulations without added antigen showed two surface-area weighted populations, one with a size of about 100 nm, and a secondary population with a size of about at 1-2 μm (Figure 1), similar to observations from earlier studies [19]. According to previous literature, Alhydrogel® consists of primary needle-like particles with diameters of about 2 nm [41]. These particles form stable aggregates with diameters of 1–5 μm in suspension in the as-received Alhydrogel® suspensions [41]. After lyophilization and reconstitution, aggregation of the aluminum hydroxide suspensions was evident in SA-PSDs for samples containing 0, 4, or 8% trehalose that had been cooled slowly. These SA-PSDs were shifted to larger sizes, with the main peak at roughly 10 μm. In contrast, minimal SA-PSD shifts were seen in slowly cooled samples containing 12% trehalose. Most likely, formulations with lower concentrations of the glass-forming excipient trehalose experience more concentration during freezing, which contributed to the observed increases in aggregation of aluminum hydroxide particles in these samples.

In samples that were cooled rapidly, shifts of SA-PSDs to larger sizes occurred only in samples containing the lowest levels of trehalose (0 or 4%). Thus, aggregation of aluminum hydroxide suspensions could be inhibited either by increasing the concentration of the glass-forming excipient trehalose, or by increasing the rate of glass formation by cooling rapidly [19]. In all cases, little difference was observed between placebo formulations buffered with histidine or volatile ammonium acetate (Figure 1). In liquid formulations prior to lyophilization, and in reconstituted formulations that had been lyophilized using rapid cooling, 90% of the total adjuvant surface area came from particles of size less than 1.5 μm equivalent spherical diameter. In contrast, larger particles contribute much more to the total surface area in formulations lyophilized with slow cooling, with 90% of the surface area associated with particles less than 18.5 μm in equivalent spherical diameter.

For subsequent studies, formulations were prepared using 8% trehalose, and adjuvant particles were sized, counted and microscopically imaged using the FlowCAM instrument. This allowed samples with two different particle size distributions to be prepared based on

the cooling rate applied during lyophilization. In FlowCAM analysis of samples prior to lyophilization, very few particles >2μm could be detected (Figure 2). After reconstitution, samples that had been lyophilized with rapid cooling showed large numbers (approximately 2×10^6/ml) of particles with an average equivalent spherical diameter of approximately 4 μm. Slowly-cooled samples showed even larger numbers (approximately 4-9 × 10^6/ml) of even larger particles with an average equivalent spherical diameter of approximately 6 μm.

3.2 Physical Stability of Lyophilized Formulations at Elevated Temperatures

For storage stability of lyophilized formulations, it is important that the glass transition temperature is well above the storage temperature. Water is a potent plasticizer, and even minute amounts of water may dramatically lower glass transition temperature and cause cake collapse and vaccine degradation. The physical stability of the placebo lyophilized cakes was assessed by visual appearance, glass transition temperature and water content. Even after 15 weeks of incubation at 40°C, there were no visual signs of cake collapse. The onset glass transition temperature was approximately 110°C and remained constant over the storage time (Table 1), suggesting that the lyophilized vaccines were stored in a glassy state and no water entered the cake over time (e.g. from the stopper) [42]. The onset glass transition temperatures were very similar to that of pure trehalose (110-120°C) [43]. The initial water content of the vaccines was less than 1% wt/wt and remained below this value throughout the storage period (Table 1). Laser diffraction and FlowCAM analysis of particle size in lyophilized samples reconstituted after up to 15 weeks of incubation at 40°C showed no change compared to samples analyzed immediately after lyophilization (data not shown).

3.3 Immunogenicity of Lyophilized RiVax Vaccines after High-Temperature Storage

Immunization of mice with liquid vaccine formulations of RiVax produced RTA-specific antibody titers of approximately 3×10^4 three weeks after the first injection, and 9×10^5 two weeks after a booster dose. Groups of mice injected with liquid RiVax or lyophilized RiVax vaccines had response rates of 80-100% and 100% after one and two injections, respectively (Figure 3A). No significant differences in endpoint RTA-specific antibody titers were detected between mice immunized and boosted with any of the vaccines (liquid or lyophilized) that had not been subjected to high temperature storage, based on a one way ANOVA on ranks test ($p=0.112$). The reciprocal endpoint antibody titer responses to any of the four lyophilized vaccines that had been stored for 15 weeks at 40°C were not significantly different based on Mann-Whitney Rank Sum Test ($p>0.05$) from the response to the un-stored lyophilized vaccine of the same group. No differences in response were detected between groups immunized with lyophilized vaccines containing histidine or ammonium acetate buffers based on Mann-Whitney Rank Sum Test ($p=0.182$). When lyophilized vaccines were compared by buffer group over incubation time, significant differences were not detected between the two lyophilization processing methods which produce “small” and “large” particles for the rapid and slow cooling methods, respectively, based on the Mann-Whitney Rank Sum Test or t-test for normally distributed groups, except for the histidine-containing lyophilized vaccines after 15 weeks of incubation ($p=0.022$) and ammonium acetate-containing lyophilized vaccines after 4 weeks of 40°C incubation ($p=0.002$). In both of these cases, the lyophilized vaccines that exhibited less aggregation of aluminum hydroxide particles (those made with rapid cooling) were slightly more immunogenic. An explanation for the minimal dependency of immune response on particle size is that, even when aggregation occurred, the majority of the aluminum hydroxide particles were still smaller than the upper size limits (ca. 10 μm) for phagocytosis by macrophage cells [44] and dendritic cells [21]. A similar lack of dependency of immune response on adjuvant particle size was reported previously for lyophilized lysozyme and alkaline phosphatase vaccines containing aluminum hydroxide or aluminum phosphate particles with average sizes ranging from 1 to 17 μm [24,25].

Neutralizing antibody titers were measured in serum two weeks after administration of a booster dose. All liquid and lyophilized vaccine groups, regardless of formulation buffer or particle size, produced neutralizing antibody titers that were not significantly different between groups based on a one way ANOVA on ranks test ($p=0.310$). Neutralizing titer responses to lyophilized vaccines did not decrease over incubation time at 40°C based on Mann-Whitney Rank Sum Test ($p>0.05$) (Figure 3B).

To determine whether the lyophilized vaccines were able to elicit protective immunity, mice were subjected to a prime-boost regimen as described above and then 28 days later challenged with an intraperitoneal injection of ricin. Hypoglycemia was used as a quantitative measure of ricin intoxication [45]. Before the ricin challenge, blood glucose levels were similar in all groups of mice based on one way ANOVA ($p=0.502$) (Table 2). Following the ricin challenge, naive mice experienced a rapid drop in blood glucose levels and expired within 24 hours (Figure 4). In contrast, mice immunized with a freshly prepared liquid version of the vaccine experienced a slight reduction in blood glucose levels but survived the ricin challenge. There was no statistically significant difference ($p>0.05$) based on a one way ANOVA in the blood glucose levels over time for mice immunized with liquid vaccine that was not subjected to high temperature storage, lyophilized vaccine formed by slow cooling in ammonium acetate buffer or lyophilized vaccine formed by rapid cooling in histidine buffer. There were statistically significant differences in the blood glucose levels between time 0 and 24 hours for mice that received lyophilized vaccine that had been formed by slow cooling in histidine buffer ($p=0.029$), lyophilized vaccine formed by rapid cooling in ammonium acetate buffer ($p<0.05$) and liquid vaccine that had been stored at 40°C ($p<0.001$). Mice immunized with the lyophilized vaccines that had been stored for 4 weeks at 40°C were protected (80-100%) against the ricin challenge, although the animals experienced a transient reduction in blood glucose levels. On the other hand, only 30% of mice immunized with the stored liquid vaccine survived the ricin challenge. This was consistent with results for titers where mice immunized with the liquid vaccine stored at 40°C decreased by 47% and 11% for antibody and neutralizing titers respectively from mice administered with the original liquid vaccine. When bound to aluminum hydroxide, RiVax undergoes changes in tertiary structure at 40°C as seen by a red shift in fluorescence peak position [36]. Likewise, protein aggregation, secondary and tertiary structural changes are observed in aqueous solutions of RiVax at 40°C [35]. These conformational changes in the protein structure most likely result in loss of epitopes critical to inducing rRTA-specific and neutralizing antibodies as well as a protective immune response. Since both the liquid and lyophilized heat-stressed vaccines were identical formulations with respect to excipients, antigen, and adjuvant content, we conclude from these studies that the lyophilized vaccine is more stable than the liquid formulation when stored at high temperatures.

Contrary to our initial expectations, the ability of lyophilized RiVax to protect against ricin challenge was not affected by the degree of aggregation of colloidal aluminum hydroxide. Even after storage for 4 weeks at 40°C, mice immunized with lyophilized vaccines containing aluminum hydroxide with larger particle sizes (slow-cooled lyophilization process) and smaller particle sizes (rapidly-cooled lyophilization process) showed equivalent blood glucose profiles and ricin challenge survival rates. All four lyophilized formulations were equally effective in terms of generation of rRTA-specific antibodies, neutralizing antibodies, and a protective response against challenge by ricin toxin.

4. Conclusions

Lyophilization-induced aggregation of colloidal aluminum hydroxide can be controlled by changing the concentration of the glass forming excipient trehalose, or cooling rate during the process. RTA-specific antibodies and neutralizing antibodies were elicited in immunized

mice regardless of whether the aluminum hydroxide aggregated. Antibody responses were not affected by high temperature storage. However, in ricin challenge studies, mice immunized with lyophilized vaccine that had been stored at high temperature were significantly better protected than mice immunized with liquid vaccine that had been incubated at high temperature.

The instability of colloidal suspension of aluminum hydroxide during freezing has discouraged the development of lyophilized, adjuvanted vaccine formulations, despite the advantages of superior thermal stability and reduced cold-chain requirements that such formulations might offer. Currently 67 vaccines are approved by the US Food and Drug Administration. Of these vaccines, 36% contain an aluminum salt adjuvant and 30% are lyophilized, but there are not currently lyophilized vaccines that contain an aluminum adjuvant [46]. The current work demonstrates that, through judicious choice of processing and formulation conditions, the instability of colloidal aluminum hydroxide suspensions can be mitigated, offering the potential for creation of thermally stable lyophilized formulations of vaccines containing aluminum hydroxide adjuvants.

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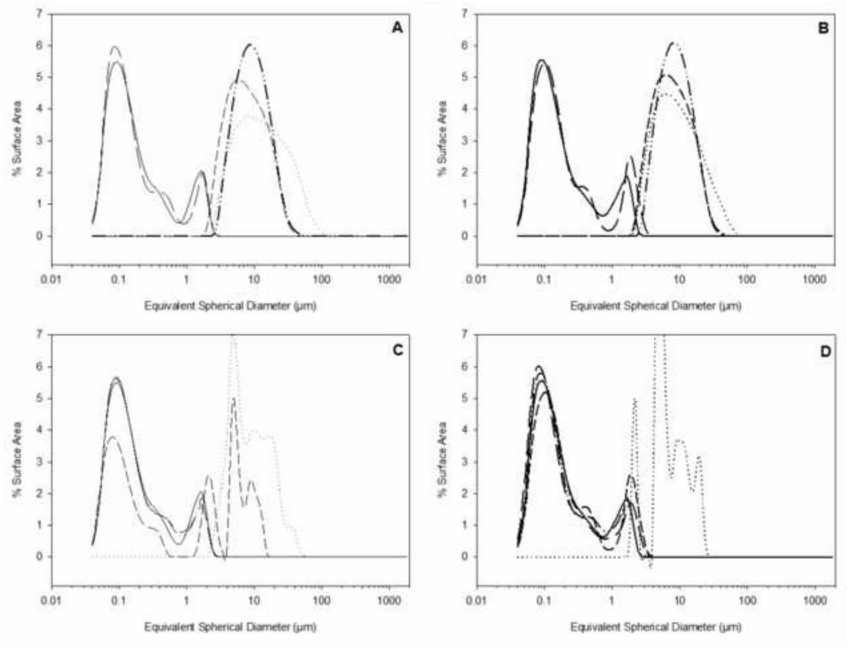

Figure 1.

Aluminum hydroxide particle size distributions. Lyophilization with rapid cooling and higher trehalose concentrations produce aluminum hydroxide particle size distributions measured after lyophilization and reconstitution that are more similar to the initial liquid particle size distribution (—). Formulations consist of 1 mg Al/mL, 0 (……) 4 (- - -) 8 (–..–..) or 12 (— —) w/v% trehalose in 10 mM histidine or ammonium acetate buffer at pH 6. A.) Lyophilization with slow cooling in histidine buffer; B.) Lyophilization with slow cooling in ammonium acetate buffer; C.) Lyophilization with rapid cooling in histidine buffer; D.) Lyophilization with rapid cooling in ammonium acetate buffer

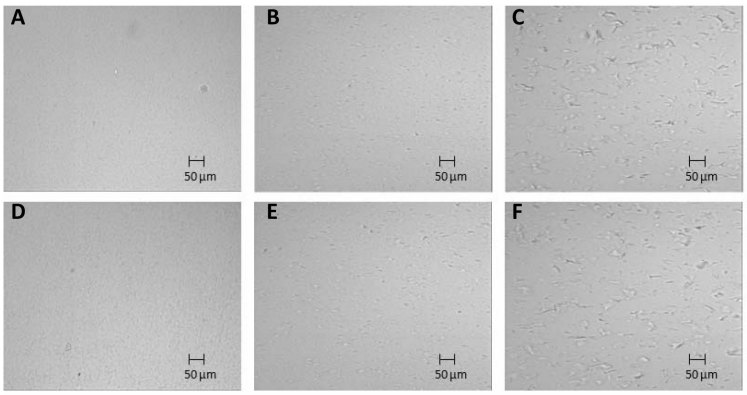

Figure 2.

Aluminum hydroxide particle images. FlowCAM images of undiluted placebo formulation before lyophilization in histidine buffer (A) and ammonium acetate buffer (D), after reconstitution of placebo vaccine lyophilized with rapid cooling in histidine buffer (B) and ammonium acetate buffer (E), after reconstitution of placebo vaccine lyophilized with slow cooling in histidine buffer (C) and in ammonium acetate buffer (F). All formulations contain 0.85 mg Al/mL with 8w/v% trehalose in 10 mM buffer pH 6.

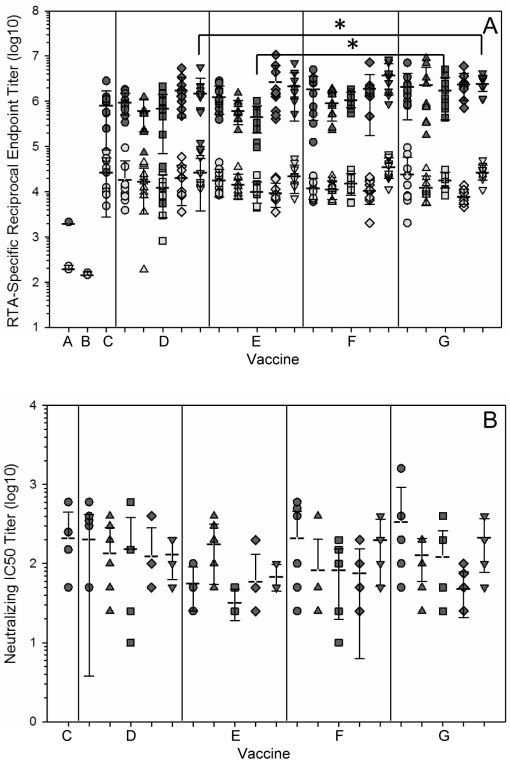

Figure 3.

RTA-specific and neutralizing antibody titers. A.) RTA-specific antibody titers after one injection (light gray) and after two injections (dark gray) for each vaccine after no high temperature storage (o), 1 week (△), 4 weeks (□), 8 weeks (◇) and 15 weeks (▽) of incubation at 40°C. Average titers are shown as the average of only the mice that responded with the standard deviation of those mice. Significant differences ($p<0.05$) between groups when comparing the lyophilization method with the same storage condition and buffer is shown by *. B.) Toxin neutralizing titer after 2 injections for vaccines with no high temperature storage (o) and vaccines stored at 40°C for 1 week (△), 4 weeks (□), 8 weeks (◇) and 15 weeks (▽). Vaccine groups: Negative control – vaccine without antigen lyophilized with slow cooling in histidine buffer (A), Negative control – vaccine without antigen lyophilized with slow cooling in ammonium acetate buffer (B), Positive control – freshly prepared liquid RiVax vaccine in histidine buffer (C), RiVax vaccine lyophilized with slow cooling in histidine buffer (D), RiVax vaccine lyophilized with slow cooling ammonium acetate buffer (E), RiVax vaccine lyophilized with rapid cooling in histidine buffer (F) RiVax vaccine lyophilized with rapid cooling in ammonium acetate buffer (G).

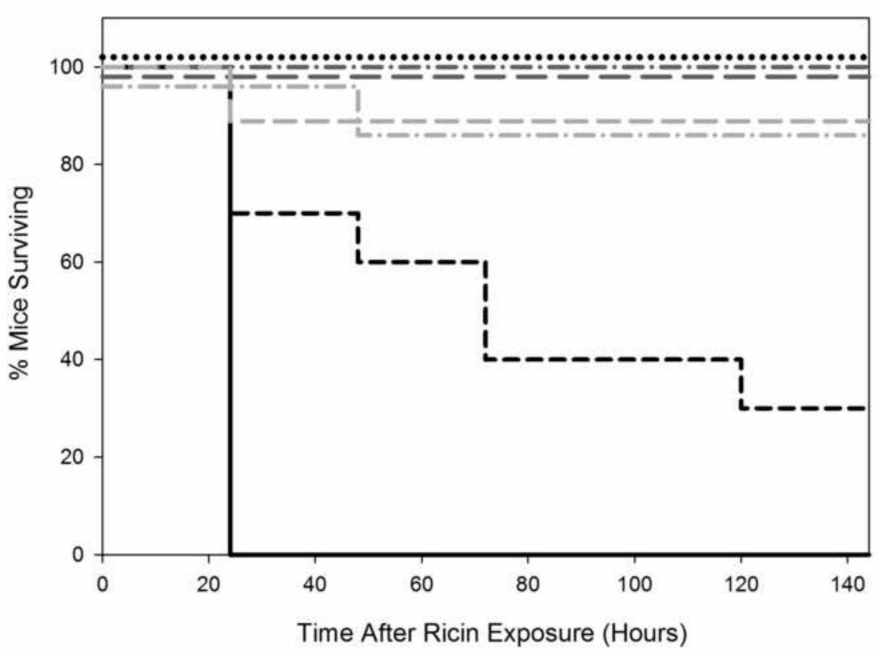

Figure 4.

Ricin challenge study. Mice immunized with lyophilized or liquid vaccine not exposed to high temperature storage had the highest survival rate during a ricin challenge. Vaccine formulations: No immunization ( ), liquid vaccine with no high temperature storage ( ), liquid vaccine with 3.5 weeks of high temperature storage at 40°C ( ), RiVax vaccine lyophilized with slow cooling in histidine buffer with high temperature storage for 4 weeks at 40°C ( ), RiVax vaccine lyophilized with slow cooling in ammonium acetate buffer with high temperature storage for 4 weeks at 40°C ( ), RiVax vaccine lyophilized with rapid cooling in histidine buffer with high temperature storage for 4 weeks at 40°C ( ) and RiVax vaccine lyophilized with rapid cooling in ammonium acetate buffer with high temperature storage for 4 weeks at 40°C ( ).

Table 1

Onset glass transition temperature and water content of placebo vaccines lyophilized with slow cooling in histidine buffer after storage at 40°C for various periods of time

Time Stored at 40°C	Onset Glass Transition Temperature (°C)	Water Content (%)
No Storage Time	107.7 ± 1.7	0.29 ± 0.02
1 Week	112.4 ± 1.5	0.45 ± 0.02
4 Weeks	111.9 ± 1.6	0.67 ± 0.25
8 Weeks	113.0 ± 4.7	0.63 ± 0.05
15 Weeks	110.1 ± 3.2	0.64 ± 0.02

Table 2

Blood glucose (BG) levels of mice before and after exposure to lethal doses of ricin. Average values and the range of values for each vaccine group are shown.

Vaccine		Time= 0hrs	Time = 24hrs	Time= 48hrs	Time = 72hrs
Lyophilized with Slow Cooling in Histidine buffer (4 weeks at 40°C)	Average BG (mg/DL)	130.7	108.1	129.7	122.9
	Range of BG Values	94-151	88-137	95-158	105-139
Lyophilized with Rapid Cooling in Histidine buffer (4 weeks at 40°C)	Average BG (mg/DL)	125.1	100.7	115.7	116.7
	Range of BG Values	93-152	80-121	33-136	71-147
Lyophilized with Slow Cooling in Ammonium Acetate buffer (4 weeks at 40°C)	Average BG (mg/DL)	118.4	100.0	114.7	114.6
	Range of BG Values	104-135	76-122	96-139	98-138
Lyophilized with Rapid Cooling in Ammonium Acetate buffer (4 weeks at 40°C)	Average BG (mg/DL)	135.8	111.6	122.6	124.9
	Range of BG Values	115-160	33-126	105-148	115-138
Liquid in Histidine buffer (No high temperature storage)	Average BG (mg/DL)	132.4	113.2	129.2	133.1
	Range of BG Values	103-185	89-139	112-149	111-162
liquid in Histidine buffer (3.5 weeks at 40°C)	Average BG (mg/DL)	124.4	75.0	71.7	86.0
	Range of BG Values	98-149	16-97	52-117	50-124
Sham Immunized with PBS	Average BG (mg/DL)	132.0			
	Range of BG Values	111-159			