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Research paper

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 (TNAs) 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.

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1. Introduction

Protein subunit vaccines, like therapeutic proteins [1–3], tend to be unstable and readily undergo physical and/or chemical degradation [4–6]. 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 subzero 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 [14–16]. In the design of a lyophilized vaccine formu-

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lation, 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 (T'_g) 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 [10,17–20]. 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 (14–17 μm) 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 [34–36]. 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 10× 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 h, 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 s at 14,500g 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 h 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/min to −40 °C and then held at −40 °C for 1 h. For slow cooling, vials were placed on room temperature lyophilizer shelves, cooled to 0 °C, held at 0 °C for 1 h, cooled to −40 °C at a rate of 0.5 °C/min and then held at −40 °C for 1 h. 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

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