Journal of Controlled Release 177 (2014) 20–26

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Elimination of the cold-chain dependence of a nanoemulsion adjuvanted vaccine against tuberculosis by lyophilization

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ARTICLE INFO

Article history:
Received 16 October 2013
Accepted 22 December 2013
Available online 29 December 2013

Keywords:
Nanoemulsion
Lyophilization
Vaccine
Adjuvant

ABSTRACT

Next-generation rationally-designed vaccine adjuvants represent a significant breakthrough to enable development of vaccines against challenging diseases including tuberculosis, HIV, and malaria. New vaccine candidates often require maintenance of a cold-chain process to ensure long-term stability and separate vials to enable bedside mixing of antigen and adjuvant. This presents a significant financial and technological barrier to worldwide implementation of such vaccines. Herein we describe the development and characterization of a tuberculosis vaccine comprised of both antigen and adjuvant components that are stable in a single vial at sustained elevated temperatures. Further this vaccine retains the ability to elicit both antibody and T_H1 responses against the vaccine antigen and protect against experimental challenge with *Mycobacterium tuberculosis*. These results represent a significant breakthrough in the development of vaccine candidates that can be implemented throughout the world without being hampered by the necessity of a continuous cold chain or separate adjuvant and antigen vials.

1. Introduction

Although lyophilization of protein, live-attenuated or inactivated virus or bacteria-containing vaccines is a routine practice, to date there have been no reports of successful lyophilization and thermostability characterization of an adjuvanted licensed vaccine [1]. Thermostable vaccines can greatly increase effective distribution of vaccines in the developing world and eliminate a prime contributor to high vaccine wastage rates [2,3]. Development of vaccines that do not require cold-chain maintenance would significantly reduce the cost and technological hurdles of implementation of new vaccines worldwide, especially in low resource settings. Additionally cold-chain maintenance cannot be ensured during natural disasters when power supplies may be compromised. Lyophilization of protein-containing pharmaceuticals such as vaccines is a commonly employed method to prolong shelf-life and increase resistance to thermal stress [4,5], and multiple marketed vaccines are distributed as lyophilized products [1]. New vaccines under development for induction of cell-mediated immunity against diseases such as malaria or tuberculosis may require adjuvant components in order to enhance and shape immune responses effectively [6]. However, the addition of adjuvant(s) to a vaccine antigen results in a more complex formulation with the potential for multiple interactions among components. Thus, maintaining long-term stability in adjuvanted vaccines can present a significant challenge to vaccine developers; for this reason, some adjuvanted vaccines are administered following bedside-mixing with a separate adjuvant vial [7]. Moreover, none of the existing marketed lyophilized vaccines contain adjuvant in the lyophilized formulation [1]. Indeed, adjuvant formulations already used in approved human vaccines such as aluminum salts or oil-in-water nanoemulsions may be particularly challenging to lyophilize [8,9]. The complex nature of approved vaccine adjuvants (e.g. alum, oil-in-water nanoemulsions and/or monophosphoryl lipid A (MPLA)) presents a substantial hurdle to developing lyophilized adjuvanted vaccines.

Approximately 1.5 million people die of tuberculosis (TB) each year worldwide, with an estimated 2 billion people currently infected, 10% of whom will develop active disease at some point in their lives [10]. The only approved vaccine for TB, Bacillus Calmette-Guérin (BCG), was first used in humans in 1921 and has been effective in reducing the incidence of disseminated TB in children. However, BCG has proven ineffective at preventing pulmonary TB in adolescents and adults [11–13]. Mathematical modeling of the impact of implementing a hypothetical new vaccine against TB with 60% efficacy predicts an 80% drop in incidence by 2050 [14]. Thus there is an urgent need for a new TB vaccine to either boost immunity primed by BCG or replace BCG. Protective immunity against *Mycobacterium tuberculosis* (*Mtb*) requires both TNF and IFN-γ production by CD4 T cells [15,16]. We have developed a recombinant fusion protein antigen consisting of four *Mtb* proteins, designated ID93, that when paired with an adjuvant such as the synthetic TLR4 agonist glucopyranosyl lipid adjuvant formulated in a squalene-in-water stable nanoemulsion (GLA-SE), induces robust T_H1 responses and is protective against *Mtb* challenge [17–19]. In the absence of the

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http://dx.doi.org/10.1016/j.jconrel.2013.12.025

TLR agonist, immunization with ID93 generates a modest T_H2 response that is not protective against *Mtb* challenge [20]. ID93 + GLA-SE is currently undergoing Phase I safety testing in human volunteers. An effective, thermostable tuberculosis vaccine formulation could have a dramatic impact on global health, with easier worldwide distribution and reduced vaccine wastage. Herein, we describe the lyophilization, thermostability characterization, and biological efficacy of a nanoemulsion-adjuvanted tuberculosis vaccine candidate, ID93 + GLA-SE.

2. Material and methods

2.1. Sample preparation and lyophilization

The construction, expression, and purification of the ID93 tandem fusion protein containing the *Mtb* genes Rv3619, Rv1813, Rv3620, and Rv2608 have been described previously [17]. Briefly the ID93 fusion protein was expressed in *E. coli*, purified under denaturing conditions by chromatography on DEAE and Q Sepharose columns, and analyzed by SDS-PAGE on a 4–20% Tris glycine gel (Invitrogen). GLA (also known as PHAD) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). GLA-SE containing 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was formulated according to the previously described methods [19,21]. Briefly, GLA-SE was produced by mixing a buffered aqueous phase (poloxamer 188 and glycerol in ammonium phosphate buffer pH 5.7) and oil phase (DMPC and GLA dispersed into squalene by sonication at 70 °C) and then microfluidizing the mixture using the Microfluidics M110P (Newton, MA) for 12 passes at 30,000 psi. Component concentrations in the emulsions consisted of 10% v/v squalene, 1.9% w/v DMPC, 0.1% w/v poloxamer 188, 2.3% w/v glycerol, and 25 mM ammonium phosphate buffer. GLA-SE was diluted to the specified concentrations for use.

Liquid and lyophilized samples were prepared with 1.5 mL fill volumes in 3 mL glass vials. Covialed samples containing ID93 (5 μg/mL) + GLA-SE (50 μg/mL, 2% total oil) were prepared in 20 mM tromethamine (Tris) at pH 8.0 [22]. Separately vialed ID93 or GLA-SE were prepared at twice the concentration of covialed samples (10 μg/mL ID93 or 100 μg/mL GLA, 4% total oil GLA-SE) and mixed 1:1 prior to injection. Samples for SDS-PAGE were prepared at 100 μg/mL ID93 to facilitate analysis. Lyophilized samples also contained 5% (w/v) D-trehalose dehydrate as a stabilizer and were lyophilized using a VirTis (Gardiner, NY) AdVantage 2.0 EL-85 benchtop freeze dryer. The lyophilization recipe utilized a thermal treatment schedule including a 10-h freezing step from 4 to −40 °C, and an annealing step at −15 °C. The primary drying phase (at 100 mTorr) lasted 18 h from −40 °C to 25 °C. Finally, a secondary drying phase at 50 mTorr was employed at 25 °C for 9 h. All samples were stoppered in atmospheric gas at 500 mTorr, sealed using aluminum caps, and stored at 4 °C until use. The lyophilization process utilized is a non-optimized developmental process designed to be compatible with a variety of excipient systems. Trehalose was selected as an excipient based on previous observations with other emulsion systems (data not shown). Heat stressed samples were incubated at 50 °C for 30 days and unstressed samples were stored at 4 °C prior to injection.

2.2. Reducing SDS-PAGE

Reducing SDS-PAGE was performed using Life Technologies (Grand Island, NY) NuPAGE LDS sample buffer, with 1.25% β-mercaptoethanol added, and incubated at 90 °C for 15 min. Samples were run at 180 V for 65 min using 1 μg of ID93 per lane in Life Technologies Novex 4–20% acrylamide tris-glycine precast gel cassettes. Gels were stained overnight using Life Technologies SimplyBlue SafeStain before destaining, drying, and imaging. Band intensities were compared using ImageJ software (NIH) [23].

2.3. Particle analysis

Particle size, polydispersity, and zeta potential measurements were made as described previously [24] using a Malvern (Worcestershire, UK) Nano-ZS after 100 times dilution into ultrapure water filtered througha 20 nm Whatman (Maidstone, Kent, UK) Anotop plus filter. Nanoparticle tracking analysis was performed with a NanoSight LM10 (Amesbury, UK) with a 405 nm laser and a Hamamatsu Orca Flash 2.8 CMOS camera (Hamamatsu, JP). Samples were diluted 1:10^5 in 20-nm filtered ultrapure water in three steps. Each sample was diluted and analyzed four times, independently, to account for dilution error. Ninety seconds of video was recorded for each sample with optimized shutter and gain settings. The camera histogram gating was adjusted to maximize sensitivity. Data analysis was performed using NanoSight NTA 2.3 software (Wiltshire, UK) in standard mode.

2.4. Chemical integrity of GLA-SE

Concentrations of squalene, DMPC, and GLA were monitored using reversed-phase HPLC (RP-HPLC) as described previously [24]. An Agilent 1200 (Santa Clara, CA) and an ESA Biosciences Corona Charged Aerosol Detector (CAD; Chelmsford, MA) were used with a Waters (Milford, MA) Atlantis C18 5 μm column (4.6 mm × 250 mm). Mobile phase A contained 75:15:10 (v/v/v) methanol, chloroform, and water with 20 mM ammonium acetate and 1% acetic acid. Mobile phase B contained 50:50 (v/v) methanol and chloroform, 20 mM ammonium acetate, and 1% acetic acid. Samples were prepared by dilution (1:20) into mobile phase B, 9 μL was injected onto a 30 °C column, and elution with a gradient of 100% to 10% mobile phase A over 45 min was used. Standard curves were fit with a second order polynomial, as recommended by the detector manufacturer, and sample concentrations determined by interpolation.

2.5. Animals and immunizations

6–8 week old female C57BL/6 mice were purchased from Charles River and maintained in Specific Pathogen Free conditions. After infection animals were maintained in ABSL3 containment according to the regulations and guidelines of the IDRI Institutional Animal Care and Use Committee. Mice were immunized three times three weeks apart by intramuscular injection of 100 μL of the indicated vaccine preparation. For BCG immunization 5 × 10^4 CFU (Pasteur strain, Sanofi Pasteur) were injected intradermally once at the time of the first subunit immunization.

2.6. Blood cell counts

Peripheral blood was collected from mice ($N = 5$/group) eighteen hours after immunization. Whole blood was stained for CD90.2 (clone 53–2.1) and CD19 (clone 6D5). Sphero AccuCount Rainbow Particles (Spherotech.com) were added according to the manufacturer's instructions. Cells were washed and resuspended in PBS. Up to 10^6 events were collected on a four laser LSRFortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo. Absolute numbers of $CD19^+$ B cells and $CD90.2^+$ T cells per microliter of blood were calculated according to the manufacturer's instructions.

2.7. Antibody responses

Mouse sera ($N = 5$/group) were prepared 21 days after immunization by collection of retro-orbital blood into microtainer serum collection tubes (VWR International, West Chester, PA), followed by centrifugation. Each serum sample was then analyzed by antibody capture ELISA. Briefly, ELISA plates (Nunc, Rochester, NY) were coated with 1 μg/mL recombinant antigen in 0.1 M bicarbonate buffer and blocked with 1% BSA-PBS. Then, in consecutive order and following