Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Thermal stability and epitope integrity of a lyophilized ricin toxin subunit vaccine

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ARTICLE INFO

Article history:
Received 20 February 2018
Received in revised form 18 August 2018
Accepted 23 August 2018
Available online xxxx

Keywords:
Vaccine
Toxin
B cell epitope
Monoclonal antibodies
Biodefense

ABSTRACT

Biodefense vaccine are destined to be stockpiled for periods of time and deployed in the event of a public health emergency. In this report, we compared the potency of liquid and lyophilized (thermostabilized) formulations of a candidate ricin toxin subunit vaccine, RiVax, adsorbed to aluminum salts adjuvant, over a 12-month period. The liquid and lyophilized formulations were stored at stressed (40 °C) and unstressed (4 °C) conditions and evaluated at 3, 6 and 12-month time points for potency in a mouse model of lethal dose ricin challenge. At the same time points, the vaccine formulations were interrogated *in vitro* by competition ELISA for conformational integrity using a panel of three monoclonal antibodies (mAbs), PB10, WECB2, and SyH7, directed against known immunodominant toxin-neutralizing epitopes on RiVax. We found that the liquid vaccine under stress conditions declined precipitously within the first three months, as evidenced by a reduction in *in vivo* potency and concomitant loss of mAb recognition *in vitro*. In contrast, the lyophilized RiVax vaccine retained *in vivo* potency and conformational integrity for up to one year at 4 °C and 40 °C. We discuss the utility of monitoring the integrity of one or more toxin-neutralizing epitopes on RiVax as a possible supplement to animal studies to assess vaccine potency.

1. Introduction

The development and stockpiling of countermeasures against biothreat agents remains a high priority in many countries, including the United States [1,2]. There are, however, formidable challenges associated with the testing, manufacturing, licensure, and, ultimately, the stockpiling of vaccines and therapeutics for use in the biodefense realm [3]. First, the Centers for Disease Control and Prevention's (CDC's) list of Select Agents and Toxins consists of more than two dozen toxins, viruses, and highly pathogenic bacteria that require special containment facilities for handling and testing. Second, evaluating the efficacy of candidate vaccines and therapeutics must rely on well-established animal models, often non-human primates, to serve as human surrogates, since the incidence of human exposure to most select agents and toxins is too low to afford a meaningful clinical study population. Finally, because medical countermeasures will be stockpiled and used only in the rare event of a public health emergency, it is essential that therapeutics and vaccines have long shelf lives, as well as means to readily monitor overall stability.

Ricin is classified by the Centers for Disease Control and Prevention (CDC) as a putative biothreat agent, alongside other protein toxins like botulinum, abrin, and *Staphylococcus* enterotoxin (SEB). Ricin is a natural byproduct of the castor bean plant (*Ricinus communis*), which is grown worldwide for its oils extensively used in industrial and cosmetic applications. The toxin itself is a ~65 kDa glycoprotein that makes up five percent of the dry weight of a castor bean. In its mature form, ricin consists of two subunits, ricin toxin A-chain (RTA) and ricin toxin B-chain (RTB), joined by a single disulfide bond [4]. RTA is an RNA N-glycosidase (EC 3.2.2.22) that cleaves the sarcin-ricin loop (SRL) of 28S rRNA, thereby stalling ribosome translocation [5,6]. RTB is a galactose/N-acetyl galactosamine (Gal/GalNAc)-specific lectin that facilitates toxin entry into and transport within mammalian cells. Exposure to ricin, particularly via inhalation, results in severe inflammation and localized cell death by apoptosis [7,8]. At the present time, medical intervention following ricin exposure is strictly supportive [9].

Considerable efforts have been invested in the development of a safe and effective ricin toxin subunit vaccine [10–12]. RiVax is one

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https://doi.org/10.1016/j.vaccine.2018.08.059
0264-410X/

of two candidate ricin vaccines that have been evaluated in Phase I clinical trials [12]. RiVax is a full-length variant (267 amino acids) of RTA with two point mutations: one at position Y80 that disrupts RTA's RNA N-glycosidase activity and one at position V76 that eliminates RTA's propensity to induce vascular leak syndrome (VLS) [13–15]. The other subunit vaccine is RV*Ec*, a truncated version of RTA that lacks residues 199–267, as well as a small hydrophobic loop in the N-terminus (residues 34–43) [16–18]. Pilot phase I clinical trials have indicated that both RiVax and RiVax-adsorbed to Alhydrogel® are safe and immunogenic in healthy adults [19,20]. RV*Ec*-adsorbed has also been shown to be safe and immunogenic in humans [21].

Long term stability is a challenge associated with all vaccines but is particularly critical within the context of ricin. When RTA is not complexed with its binding partner (RTB), it has a propensity to unfold at temperatures above 37 °C [22]. Not surprisingly, RiVax is similarly unstable, a fact that has raised concerns about its useful shelf life [18,23] and its reliance on cold chain storage and distribution. To overcome these concerns, Smallshaw and colleagues lyophilized RiVax and showed that it retained full potency in mice when reconstituted and freshly adsorbed to Alhydrogel® before injection [24]. Hassett and colleagues successfully developed a dry, glassy solid vaccine formulation containing colloidal aluminum hydroxide adjuvant and trehalose as a stabilizing excipient. In a mouse model, the adjuvanted lyophilized vaccine formulations retained full immunogenicity and potency after incubation for 4 weeks at 40 °C (the longest time previously examined), while the comparable liquid vaccines failed. The lyophilized aluminum-adjuvanted RiVax formulation ("lyophilized RiVax") was subsequently tested (unstressed) in an intramuscular vaccination protocol in Rhesus macaques and was shown to elicit ricin-specific serum antibodies of sufficient quantity and quality to protect the animals against $3 \times LD_{50}$ (LD_{50} = median lethal dose) aerosol challenge [25]. Antibody quantity was defined as ricin-specific serum IgG endpoint titers, while antibody "quality" was defined as serum IgG reactivity against different immunodominant toxin-neutralizing epitopes on RiVax, as demonstrated by competition ELISA with a panel of monoclonal antibodies (mAbs).

In the present study, we sought to more fully evaluate lyophilized RiVax for stability and conformational integrity. We subjected liquid and lyophilized formulations of RiVax adsorbed to a 12-month accelerated decay study at 40 °C. At 3, 6 and 12-month time points, the different vaccine formulations were interrogated for conformational integrity using a competition ELISA with three different mAbs (PB10, WECB2, and SyH7) directed against immunodominant toxin-neutralizing epitopes on RiVax and RTA. We report that lyophilized RiVax retained conformational integrity and potency in a mouse model for up to one year at 40 °C. In the mouse model, the degree of serum antibody reactivity with SyH7 and PB10 epitopes correlated with survival against ricin challenge, thereby linking epitope integrity to the actual antibody response elicited following immunization. Based on these results, we conclude that the lyophilized RiVax adsorbed formulation is stable for extended periods at elevated temperatures. We discuss the utility of monitoring the integrity of one or more toxin-neutralizing epitopes on RiVax as a possible substitute to animal studies to assess vaccine potency.

2. Materials and methods

2.1. Ricin toxin and RiVax vaccine formulations

Ricin (*Ricinus communis* agglutinin II) and RTA were purchased from Vector Laboratories (Burlingame, CA). Ricin was purchased without azide and sterility of the preparation was confirmed prior to use in animal studies. RiVax protein was expressed in *Escherichia coli* and purified by affinity purification and stored in 50% w/w glycerol, 10 mM histidine, and 140 mM NaCl at pH 6.0, as described [23,25]. A proprietary ThermoVax® thermostabilization technology was employed to generate a lyophilized aluminum salt-adsorbed vaccine formulation, preparations of which are reconstituted with water for injection (WFI) immediately prior to use [26]. For this particular study, "liquid RiVax" (10 mM histidine, 150 mM NaCl, 200 μg/ml RiVax®, 0.85 mg/ml aluminum, pH 6.5) and thermostabilized "lyophilized RiVax" (10 mM histidine, 8% (w/v) trehalose, 200 μg/ml RiVax®, 0.85 mg/ml aluminum, pH 6.5) were generated from the same batch of recombinant RiVax protein. Each formulation was stored at 2–8 °C. Thermal stress was defined as 40 °C with 75% relative humidity. Liquid and lyophilized adsorbed RiVax formulations were manufactured, stored at the designated temperatures and then provided to the Wadsworth Center by Soligenix, Inc. (Princeton, NJ). Analytical studies of the four different RiVax formulations over the course of the 12 month incubations periods are provided as Supplementary Tables 1–4. The reference lot used in this study was described previously by Roy and colleagues [25].

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.08.059.

2.2. Mouse vaccination studies

Mouse studies were conducted under strict compliance with the Wadsworth Center's Institutional Animal Care and Use Committee (IACUC). Female BALB/c mice aged 6–8 weeks at the start of the experiments were obtained from Taconic Biosciences (Germantown, NY) and housed under conventional, specific-pathogen-free conditions. Mice (n = 12 per experimental group; n = 6–8 for control groups) were vaccinated with liquid or lyophilized RiVax formulations (3 μg, 1 μg, and 0.3 μg). Lyophilized RiVax was reconstituted with sterile water (1 mL) immediately prior to use and diluted in PBS as needed to achieve final desired dose. The vaccines were administered to mice on days 0 and 21 in 50 μl final volumes via the subcutaneous (SC) route. Blood was collected from mice via the submandibular vein on days 14 and 30.

2.3. Ricin challenge

Mice were challenged by intraperitoneal (IP) injection with the equivalent of 5 x LD_{50} (50 μg/kg) ricin, as established in our laboratory for this specific batch of ricin toxin. Survival was monitored over a 7-day period. Body weight and blood glucose levels were measured daily. Mice were euthanized when they became overtly moribund, experienced weight loss $\geq$ 20% of pre-challenge weight, and/or became hypoglycemic (<25 mg/dl).

2.4. Direct ELISAs and toxin-neutralizing assays

ELISAs using ricin were done essentially as previously described [27,28]. Nunc Maxisorb F96 microtiter plates (ThermoFisher Scientific, Pittsburgh, PA) were coated overnight at 4 °C with ricin (1 μg/well in PBS), washed with PBS with 0.05% (v/v) Tween-20 (PBS-T), and then blocked for 2 h with PBS-Tween (PBST) containing 2% (v/v) goat serum (Gibco, MD, USA). Three-fold serial dilutions of serum (starting at 1:50) were then applied to plate for 1 h at ambient temperature, washed and detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL). The ELISA plates were developed using SureBlue 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard &