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Thermal Stability and Epitope Integrity of a Lyophilized Ricin Toxin Subunit Vaccine

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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.

Keywords

vaccine; toxin; B cell epitope; monoclonal antibodies; biodefense

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Author Contributions

JW, JY, GVS and DE conducted the experiments; JW, JY, GVS, DE, TM and OD analyzed the results; JW, JY, GVS and NJM wrote the manuscript.

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JW, JY, GVS, DE and NJM declare no competing interests. RS, TM and OD are employees of Soligenix, Inc. which holds the NIH contract and license for RiVax[®].

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 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 RVEc, 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]. RVEc-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 $3 \times \text{LD}_{50}$ (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]

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 $5 \times \text{LD}_{50}$ (50 $\mu\text{g/kg}$) 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 20% of pre-challenge weight, and/or became hypoglycemic ($<25 \text{ mg/dL}$).

2.3 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 $\mu\text{g/well}$ 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 & Perry Labs, Gaithersburg, MD) and analyzed using a SpectroMax 250 spectrophotometer equipped with Softmax Pro 5.4.5 software (Molecular Devices, Sunnyvale, CA). The endpoint titer was defined as the minimal dilution whose absorbance (450 nm) was > 3 times background, with background being defined as the average absorbance produced by wells with buffer alone. Seroconversion was defined as the endpoint titer of 1:50. Geometric mean titers (GMTs) were calculated from the endpoint titers. Mice that had not seroconverted, as determined by ELISA, were assigned a GMT of 1 for the purposes of statistical analysis. Vero cell cytotoxicity assays were performed as previously described [29, 30].

2.4 RiVax competition ELISA (RiCoE).

Nunc 96-well plates were coated overnight at 4 °C with ricin or native RTA (1 µg/well), washed with PBS-T and then blocked for 2 h with 2% goat serum in PBS-T. Samples were prepared in a separate 96 well PVC plate. Two-fold serial dilutions of RTA or RiVax were mixed with indicated mAbs at concentrations equivalent to their relative 90% maximal effective concentration (EC₉₀). The antigen-mAb mixtures were incubated for 1 h at room temperature and then applied to microtiter plates coated with RTA or ricin, in duplicate, and incubated for 1 h. Control wells contained only mAb at concentrations required to achieve EC₉₀. Plates were then washed with PBS with Tween-20 and probed with HRP-conjugated goat anti-mouse IgG for 1 h. The plates were washed again and developed with SureBlue TMB, as noted above. Relative half maximal effective concentration (EC₅₀) values were generated following the guidelines provided by Seabaugh [31]. Results were plotted to represent the percent of mAb able to bind RTA on the plate when compared to wells that contained mAb alone without competitor. EC₅₀ values determined with a 4-PL sigmoidal curve fit using GraphPad Prism v. 7.01 (GraphPad Software, San Diego, CA).

2.5 EPICC analysis.

Epitope profiling immunocapture competition (EPICC) assays were conducted as described in a separate manuscript (G. Van Slyke, D. Ehrbar, D. Vance, N. Mantis, *manuscript in preparation*). Briefly, Immulon 4HBX 96 well plates (ThermoFisher Scientific, Pittsburgh, PA) were coated overnight with a capture anti-RTA mAb (1 µg/ml). The following day the plates were blocked with 2% goat serum, washed, and then overlaid with a mixture of biotin-tagged ricin and polyclonal antibody (pAb) mouse serum (1:25). The amount of biotin-ricin used in the competition ELISA was equivalent to the EC₉₀ for each capture mAb (range: 100–150 ng/ml). After 1 h incubation, plates were washed and developed with streptavidin-HRP (1:1000; ThermoFisher Scientific, Pittsburgh, PA) and TMB, as described above for ELISAs. The percent (%) inhibition of ricin binding to the capture mAb in the presence of pAb serum was calculated from the optical density (OD) values as follows: $1 - \frac{\text{value OD}_{450}(\text{biotin-ricin} + \text{competitor mAb})}{\text{value OD}_{450}(\text{biotin-ricin without competitor mAb})} \times 100$.

2.6 Statistical analysis.

Statistical analysis was performed with GraphPad Prism v. 7.01. Endpoint titers were log-transformed prior to statistical analysis. Endpoint titers were compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Survival data were tested using the log rank Mantel-Cox test. In all cases, the significance threshold was set at $P < 0.05$.

3. Results

3.1 Thermostability of lyophilized RiVax adsorbed.

We wished to determine the long-term stability profile of the lyophilized formulation of RiVax adsorbed to Alhydrogel[®] (0.85 mg/ml). Previous studies indicated that the lyophilized vaccine was stable for at least 30 days at 40 °C, whereas the liquid vaccine

adsorbed to Alhydrogel® failed within that same time frame [32]. Here we sought to extend the period of analysis to one year.

Towards this end, we first established baseline mouse potency values of the lyophilized and liquid RiVax formulations. The lyophilized and liquid RiVax formulations were administered to groups of mice by SC injection at low (0.3 µg), medium (1 µg) and high (3 µg) doses on days 0 and day 21. Analysis of serum samples collected on day 30 indicated that both vaccine formulations elicited ricin toxin IgG antibody titers in a dose-dependent manner (Figure 1A, B). On day 35, vaccinated and control mice were challenged with $5 \times \text{LD}_{50}$ ricin and then monitored for 7 days. The lyophilized vaccine elicited dose-dependent immunity to ricin, with all 12 animals that received the high dose vaccine (3 µg) surviving toxin exposure and only one animal in the low dose (0.3 µg) vaccine group surviving (Figure 1C). The liquid vaccine formulation performed identically to the lyophilized vaccine at the low and medium doses (0.3 and 1.0 µg) but differed slightly at the high dose (3 µg), where 9 of 12 mice survived toxin challenge (Figure 1D). Following challenge, the vaccinated mice experienced transient weight loss and hypoglycemia, but in each case those values rebounded by 72 h (Figure 1E, F).

Next, to examine the impact of thermal stress on RiVax potency, the liquid and lyophilized vaccine formulations were stored at 2–8°C (“unstressed”) or 40 °C (“stressed”) for 12 months. Aliquots were removed at 3, 6 and 12-months for potency analysis and the stressed and unstressed RiVax formulations at 3 µg doses were administered to groups of mice in a prime (day 0) and boost (day 21) regimen, as described above and shown in Figure 2A. Analysis of serum samples collected on day 30 indicated that the ricin endpoint titers elicited by the lyophilized and liquid vaccines stored at 4 °C were identical across the three-time points examined (*i.e.*, 3, 6 and 12 months) (Figure 2 B, C). The vaccinated and control groups of mice were next challenged with $5 \times \text{LD}_{50}$ ricin on day 35. Mice that received vaccines stored for 3 months at 4 °C were immune to ricin toxin challenge, as evidenced by complete survival in both cohorts (Figure 2 D, E). The potency of the liquid vaccine declined significantly after storage for 3 months at 40 °C, as evidenced by a reduction in ricin-toxin GMT, as well as a significant decline in protective immunity (Figure 2C, E). The liquid vaccine formulation stored for 6 months at 40 °C was completely ineffective at stimulating immunity to ricin, as none of the vaccinated mice survived ricin challenge (Figure 2G). Because the liquid RiVax formulation had effectively failed by 6 months, we chose not to vaccinate additional mice with liquid vaccine that had been stored at 40 °C for 12 months. In contrast, the lyophilized vaccine incubated at 40 °C for up to 12 months did not experience a decline in potency. Mice vaccinated with the “stressed” lyophilized vaccine had ricin-specific serum IgG titers equivalent to those found in mice vaccinated with the “unstressed” vaccine (Figure 2F). Thus, we conclude that the thermostabilized, lyophilized RiVax formulation retains *in vivo* potency for at least one year at high temperature.

3.2 Interrogating the conformational integrity of RiVax using toxin-neutralizing mAbs.

We have generated a near comprehensive B cell epitope map of RTA using a collection of camelid-derived single domain antibodies alongside more than a dozen different toxin-neutralizing mAbs (G. Van Slyke, D. Vance, Y. Rong and N. Mantis, manuscript submitted)

[33, 34]. We postulated that a subset of these mAbs might have utility as probes of RiVax conformational integrity that could be used as a tool in product profiling alongside more conventional potency assays. Three mAbs, namely PB10, WECB2 and SyH7, encompass the two most immunodominant regions on RiVax. PB10 and WECB2 recognize overlapping epitopes in so-called cluster I that encompass RTA's α -helix B (residues 94–107) (Figure S1). It should be noted that PB10's epitope is linear at its core, even though it is sensitive to local conformational changes [33, 35]. SyH7 recognizes a discontinuous epitope that involves RTA's α -helix A (residues 14–24) and α -helices F-G (residues 184–207) within so-called epitope cluster II.

To determine whether PB10, WECB2 and SyH7 are sensitive to antigen conformation, native RTA and RiVax protein were tested for the ability to competitively inhibit each of the three mAbs from binding to plate bound ricin in a direct ELISA format. Specifically, PB10, WECB2 and SyH7 were incubated with a range of concentrations of native RTA or RiVax in solution and then applied to microtiter plates coated with ricin. Relative EC₅₀ values for both RTA and RiVax were established from the inhibition profiles, as shown in Figure 3 and Figure S2. To be clear, EC₅₀ values for each of the three mAbs, PB10, WECB2 and SyH7, were defined as the amount of soluble RTA or RiVax required to reduce maximal binding by 50% in the ricin ELISA.

As a rule, the EC₅₀ values for RTA and RiVax were essentially identical for each of the three mAbs tested (Figure 3). When the competition ELISAs were repeated with thermally denatured antigens (RiVax and RTA), the EC₅₀ values were markedly increased (red symbols, Figure 3), indicating that the denatured antigens in solution were less effective at competitively inhibiting PB10, WECB2 and SyH7 from binding to plate bound ricin. For example, the EC₅₀s for thermally denatured RTA and RiVax protein probed with PB10 increased ~100-fold, consistent with PB10 recognizing a linear epitope that is sensitive to local changes in antigen conformation. By comparison, thermal denaturation of RTA and RiVax protein effectively destroyed WECB2 and SyH7's epitopes, as neither antigen was able to competitively inhibit WECB2 and SyH7 from recognizing plate-bound ricin (Figure 3). For simplicity, we refer to the RiVax competition ELISA as RiCoE.

To gauge the reproducibility of the RiVax competition ELISA (or RiCoE) and its applicability of RiVax adsorbed to aluminum salts, a single reference vaccine lot was probed with PB10, WECB2 and SyH7 at defined intervals over a 12-month period. We found that the EC₅₀ values were consistent for each of the three mAbs across the one year time frame with coefficients of variation (CV) less than 20% (Table 1; Figure S3). Thus, we conclude that PB10, WECB2 and SyH7 may have utility as reporters of RiVax conformational integrity, even when RiVax is adsorbed to aluminum.

We next subjected the liquid and lyophilized RiVax adsorbed vaccine formulations that had been placed at 4 °C or 40 °C for 12 months to the RiCoE assay. At time zero, the liquid and lyophilized RiVax adsorbed formulations had comparable EC₅₀ values (Figure 4). At 3, 6 and 12 months, the liquid and lyophilized RiVax formulations stored at 4 °C were stable, as evidenced by no gross change in EC₅₀ values over the 12 month period (left column, Figure 5). Similarly, the EC₅₀ values of the lyophilized vaccine stored at 40 °C in the RiCoE assay

were unaltered after storage for 12 months, suggesting that the integrity of lyophilized RiVax is preserved despite exposure to prolonged thermal stress. In contrast, the liquid RiVax stored at 40 °C for 3 months had completely lost its capacity to competitively inhibit SyH7 and WECB2 from binding to RTA in the RiCoE (Figure 5), while the same vaccine was ~7-fold less effective at inhibiting PB10. The RiCoE values obtained at 6- and 12-month time points were essentially identical to the 3-month time point (data not shown), indicating that most degradation occurs within the first 12 weeks. These results are consistent with a decline in the conformational integrity of the liquid vaccine upon incubation at 40 °C, but not the lyophilized vaccine.

3.3 Epitope-specific responses elicited by lyophilized and liquid RiVax formulations

As further validation of the integrity of the lyophilized RiVax formulations, we subjected sera from vaccinated mice to epitope profiling using a competition ELISA referred to as EPICC, described in full in a separate manuscript (G. Van Slyke, D. Ehrbar, J. Westfall, J. Yates, and N. Mantis, *manuscript in preparation*). Briefly, EPICC is a modified capture ELISA in which antisera is tested for the ability to compete with toxin-neutralizing mAbs to capture biotinylated ricin. The degree of competition (*i.e.*, % inhibition of ricin capture) of a given serum sample is an estimate of the relative number of antibodies against the particular epitope recognized by the capture mAb. The EPICC assay has utility as a surrogate marker of immunity to ricin. With respect to the current study, we found that mice vaccinated with the lyophilized vaccine stored at 40 °C had significantly higher serum antibody titers against the PB10 epitope, as compared to mice vaccinated with the stressed liquid RiVax preparation (median = 19.37 % inhibition for the lyophilized preparation vs. 3.5% inhibition for the liquid preparation, $U = 18$, $p = 0.0011$, as determined by a two-tailed Mann Whitney U test). The same relationship held true for SyH7 (median = 52.11% inhibition for the lyophilized preparation vs. 2.96 % inhibition for the liquid preparation, $U = 0$, $p < 0.0001$). This result demonstrates, in principle, that the PB10 and SyH7 epitopes were intact in the lyophilized RiVax formulation, while also suggesting that the liquid formulation had undergone conformational changes prior to being administered to mice (Figure 6).

4. Discussion

Biodefense vaccines are manufactured with the expectation that they may be stockpiled for use only in the event of public health emergency. In previous studies, it was shown that a lyophilized, dry, glassy solid vaccine formulations of RiVax containing suspensions of colloidal aluminum hydroxide adjuvant were stable for at least 4 weeks at 40 °C, while the potency of a comparable liquid vaccine under the same conditions declined notably [32]. [32]. In the current report we subjected lyophilized RiVax adsorbed formulations to prolonged (12 month) thermal stress. We found that the lyophilized vaccine demonstrated no significant decline in potency, as evidenced by the onset of ricin toxin-specific antibodies and survival in a lethal dose ricin challenge model in vaccinated mice.

In this report, we also investigated the use of a mAb-based competition ELISA (“RiCoE”) to assess the conformational integrity of RiVax over time. The underlying concept behind the RiCoE assay is that specific mAbs known to be directed against immunodominant,

discontinuous neutralizing epitopes can serve as tools to probe the conformation and structural integrity of a vaccine antigen. RiVax was particularly amenable to this type of analysis, considering we have recently generated, using a large collection of single domain camelid antibodies (V_HHs) [34] and conventional murine mAbs [33], a near saturating B cell epitope map of the four primary neutralizing “hotspots” on the surface of RiVax. In this specific study, we employed three murine mAbs: PB10, WECB2 and SyH7. PB10 and WECB2 have overlapping footprints encompassing the area around α -helix B (residues 94–107) on RTA, while SyH7’s epitope is focused on α -helices F and G (residues 184–207). In the RiCoE assay, PB10, WECB2 and SyH7 were used to probe liquid and lyophilized RiVax formulations that had been incubated for 12 months at 4 °C or 40 °C. Previous studies had demonstrated that the liquid vaccine “fails” at 40 °C within a matter of weeks, whereas the lyophilized preparation retains potency for at least one month [32]. We have now demonstrated that the lyophilized formulation was stable for up to one year at 40 °C, while the confirmed that the liquid vaccine’s potency declined rapidly. The failure of liquid RiVax formulation in the RiCoE assay (*i.e.*, inability to competitively inhibit WECB2 or SyH7 binding to plate-bound ricin) correlated with a decline in potency in the mouse model. Moreover, the decline in potency also correlated with reduced serum antibody competition with SyH7 or PB10 for ricin binding in the so-called EPICC assay – potentially linking vaccine conformation and perturbation thereof with generation of antibody responses *in vivo*.

At this point in time, the RiCoE assay serves strictly as a qualitative, rather than a quantitative measure of RiVax integrity, since we have not demonstrated directly that the failure of mAb recognition is due to local conformational changes in specific epitopes and not occlusion of specific epitopes due to protein-protein aggregation. Nonetheless, it is interesting that WECB2 and SyH7 gave binary outputs (*i.e.*, competition versus no competition) in the RiCoE assay, whereas PB10’s output was continuous (*i.e.*, change in EC₅₀ values across a range of values). This obvious difference in readout is consistent with changes in epitope conformation and not occlusion. WECB2 and SyH7’s epitopes are discontinuous in nature and easily perturbed when RiVax is subject to stress or denaturing agents, while PB10 recognizes a linear epitope at its core but is context sensitive [35, 36]. We speculate that PB10 may be the most discriminating among the three mAbs with respect to probing RiVax’s tertiary conformation and therefore have the most utility going forward.

We propose that a RiCoE-like assays may be a useful supplement to current biochemical and biophysical methods being used to assess integrity of RiVax [23, 32, 37, 38]. While the sensitivity of RiCoE remains to be established, one advantage of the assay is that it affords a means to interrogate RiVax in solution, as well as RiVax adsorbed to aluminum salt adjuvant. Moreover, the assay can be completed within a matter of hours using conventional microtiter-based ELISA methodologies and a spectrophotometer. While the idea of using mAbs to probe vaccine integrity is not novel, it has yet to gain widespread application. Zhu and colleagues developed a RiCoE-like assay for hepatitis B surface antigen vaccine and made a strong case for its application in manufacturing and predicting potency [39]. A similar approach was reported for hepatitis E [40], although in neither case were the *in vitro* assays validated with parallel *in vivo* potency studies like we have done here with RiVax. The best example of how we envision RiCoE being used for ricin vaccine development is the

work by Coombes et al., in which they used mAbs to interrogate tetanus toxoid and diphtheria toxoid vaccines and linked *in vitro* assays to *in vivo* potency across a range of vaccine formulations and batches [41, 42].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Baseline mouse potency studies of lyophilized and liquid RiVax formulations. Groups of BALB/c mice ($n = 12$ per experimental group and 6 for control group) were immunized by the SC route on days 0 and 21 with low-, medium-, or high-dose lyophilized (A and C) or liquid (B and D) vaccine formulations. Sera were collected on day 30 and analyzed for ricin-specific IgG by ELISA (A and B). Mice were challenged with ricin on day 35 (C and D). Body weight (E) and blood glucose levels (F) were monitored following ricin challenge with those from mice immunized with 3 μ g being shown here. Symbols: squares, lyophilized RiVax; circles, liquid RiVax; triangles, control mice. Colors: pink, 0.3 μ g; green, 1 μ g; blue, 3 μ g.

Figure 2: Prolonged thermal stress leads to a decline in potency of liquid RiVax

RiVax potency was evaluated after 3, 6, and 12 months of storage at 40°C by immunizing BALB/c mice (n = 12 per experimental group, 6 for control group) by the SC route on days 0 and 21 with 3 µg of stressed or unstressed lyophilized (left panels) or liquid (right panels) RiVax formulations. Serum was collected on day 30 and analyzed for ricin-specific IgG by ELISA (B and C). A 5 × LD₅₀ ricin challenge was administered on day 35. (D and E) Survival curves from mice that received either lyophilized (D) or liquid (E) RiVax at the 3 month time point. (F and G). Survival over the course of the 12 month study for groups receiving lyophilized (F) or liquid (G) RiVax. Colors: blue, unstressed sample; red, stressed sample; black, control.

Figure 3: Inhibition of mAb binding is altered upon thermal denaturation of RTA and RiVax. Native (black) and denatured (red) RTA and RiVax protein were competed with the ricin-specific mAbs PB10 (A), WECEB2 (B), and SyH7 (C), to assess sensitivity to antigen conformation. Symbols: circles, RiVax protein; squares, RTA.

Figure 4: Epitope integrity of liquid and lyophilized RiVax formulations, as determined by RiCoE.

Lyophilized and liquid RiVax formulations were challenged with PB10 (A), WECEB2 (B), or SyH7 (C) in the RiCoE to establish baseline EC_{50} values for each mAb. Symbols: squares, lyophilized RiVax; circles, liquid RiVax.

Figure 5: Conformational change evident in liquid RiVax following thermal stress

Lyophilized (**open squares**) and liquid (**open circles**) RiVax formulations were assessed by RiCoE after storage for 3 months at 4°C (left panels) or 40°C (right panels). The vaccine formulations were tested against the mAbs PB10 (A and B), WECB2 (C and D), and SyH7 (E and F) in the RiCoE.

Figure 6: Mice vaccinated with thermally-stressed liquid RiVax had reduced serum antibody response to SyH7 and PB10 epitopes.

Cohorts of BALB/c mice ($n = 12$ per experimental group) were vaccinated with $3 \mu\text{g}/\text{mouse}$ of liquid or lyophilized preparations of RiVax that had been either thermally-stressed (stored at 40°C) or left unstressed (stored at 4°C) for 3 months. Serum samples were collected on day 30 and profiled via a modified capture ELISA known as EPICC as a means to assess relative epitope reactivity within a given serum sample. ELISA plates were coated with SyH7 (A) or PB10 (B) and then probed with biotin-labeled ricin in the absence or presence of antisera from RiVax-vaccinated animals, as indicated above. A reduction (% inhibition) is inferred as being competition for epitope access. Two-tailed Mann-Whitney U tests were performed to compare median inhibition values between liquid and lyophilized groups for both the stressed and unstressed preparations. ** $p < 0.01$, **** $p < 0.0001$.

Table 1.

RiCoE analysis of lyophilized RiVax reference vaccine over 1 year

mAb	Time (months)				Ave	±SD	%CV
	0	3	6	12			
PB10	0.086 ^a	0.075	0.098	0.077	0.084	0.0092	10.94
SyH7	2.27	2.79	3.59	2.94	2.896	0.471	16.29
WECB2	2.69	2.66	3.86	2.78	2.99	0.501	16.75

^aEC₅₀ values (µg/mL) derived from RiCoE assays provided in Figure S2.