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Improved Formulation of a Recombinant Ricin A-Chain Vaccine Iincreases its Stability and Effective Antigenicity

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Improved formulation of a recombinant ricin A-chain vaccine increases its stability and effective antigenicity

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Abstract

Ricin is a potent toxin associated with bioterrorism for which no vaccine or specific countermeasures are currently available. A stable, non-toxic and immunogenic recombinant ricin A-chain vaccine (RTA 1-33/44-198) has been developed by protein engineering. We identified optimal formulation conditions for this vaccine under which it remained stable and potent in storage for up to 18 months, and resisted multiple rounds of freeze–thawing without stabilizing co-solvents. Reformulation from phosphate buffer to succinate buffer increased adherence of the protein to aluminum hydroxide adjuvant from 15 to 91%, with a concomitant increase of nearly threefold in effective antigenicity in a mouse model. Using Fourier-transform infrared spectroscopy, we examined the secondary structure of the protein while it was adhered to aluminum hydroxide. Adjuvant adsorption produced only a small apparent change in secondary structure, while significantly stabilizing the protein to thermal denaturation. The vaccine therefore may be safely stored in the presence of adjuvant. Our results suggest that optimization of adherence of a protein antigen to aluminum adjuvant can be a useful route to increasing both stability and effectiveness, and support a role for a "depot effect" of adjuvant.
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Keywords: Ricin; Adjuvant adsorption; Toxin

1. Introduction

Ricin, a highly potent toxin derived from the castor bean, kills human cells by depurinating a specific nucleoside of the 28S ribosomal RNA, thereby stopping translation and inducing apoptosis [1–3]. The ricin holotoxin contains two chains, A and B, linked by a disulphide bond (Fig. 1). The A-chain has *N*-glycosidase enzymatic activity against ribosomal RNA, while the B-chain is a lectin that binds to galactosyl moieties on the cell surface. Antibodies elicited against either the ricin A (RTA) or B-chain can neutralize the toxin, although antibodies to the A-chain are superior in this respect [4,5]. The major human B-cell epitope for RTA has been identified by Castelletti et al. [6] from cancer patients treated with a ricin-conjugate immunotoxin, and lies within residues 161–175. Some innate immunity to oral exposure to ricin is provided by oligosaccharide chains of sIgA molecules lining the gut [7].

Generation of non-toxic derivatives of RTA for use as vaccines has been attempted by several groups using formalin treatment, chemical deglycosylation, or mutagenesis by substitution or insertion [8]. Most of these efforts resulted in unstable protein products that aggregated in solution, had residual toxicity, or expressed poorly in recombinant form. At USAMRIID, an RTA derivative containing deletions of the C-terminal subdomain and an exposed surface loop (Fig. 1) was found to have high relative stability to thermal denaturation, no detectable cytotoxicity, and the ability to generate

Abbreviations: RTA, ricin A-chain; FTIR, Fourier-transform infrared spectroscopy; CD, circular dichroism; DLS, dynamic light scattering; MTD, mean time-to-death

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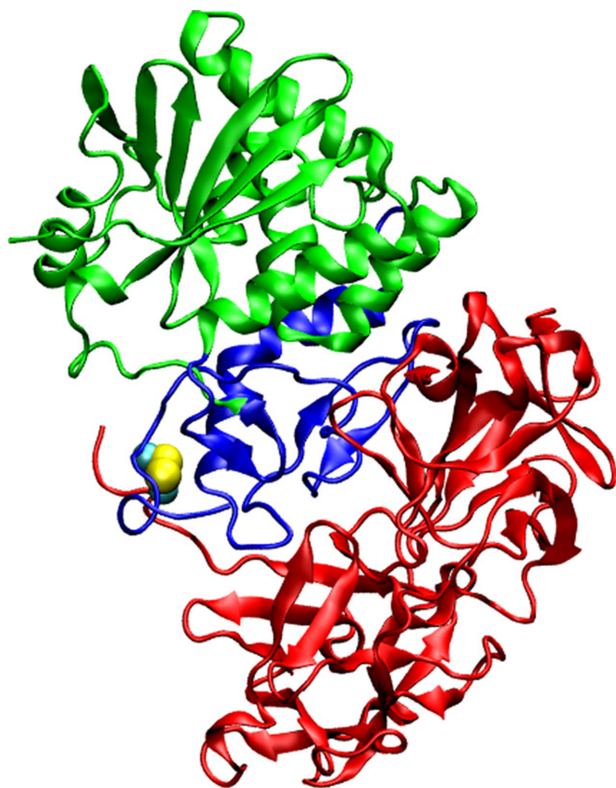

Fig. 1. The structure of ricin, showing the portion of the A-chain remaining in RTA 1-33/44-198 in green, the A-chain segments deleted in blue, the B-chain in red, and the cystine linking the two chains in VDW mode. Figure made using VMD and POV-Ray with protein coordinates 2AAI from Rutenber et al. [36].

neutralizing antibodies when used as an immunogen [9,10]. The RTA 1-33/44-198 vaccine completely protected 10 out of 10 mice against aerosol exposure to ricin. However, initial testing of a formulation with aluminum adjuvant in PBS buffer on non-human primates (NHP) provided only partial protection against aerosol challenge (Wannemacher et al., manuscript in preparation), suggesting that improvement of the vaccine formulation should be pursued to increase its effectiveness.

Herein we report the identification of a simple formulation recipe that increased the adherence of the RTA 1-33/44-198 protein to adjuvant and resulted in increased antigenicity in a mouse model. The high stability of this vaccine to long-term storage in bulk solution or while adhered to adjuvant demonstrated that it represents a substantial step forward in development of a viable anti-ricin vaccine.

2. Materials and methods

2.1. Vaccine protein and reagents

Ricin holotoxin (RCA_{60}) was obtained from Vector Laboratories at 25 mg/mL in PBS buffer. In BALB/c mice, one ricin toxin $IPLD_{50}$ was 20 μg/kg. RTA 1-33/44-198 was expressed as a recombinant protein in *Escherichia coli* and purified at the University of Nebraska-Lincoln Biological Process Development Facility (Meagher et al., manuscript in preparation). The protein preparation was >98% pure and contained 0.176 endotoxin units/mL by Limulus amoebocyte lysate assay, an acceptable level for human use. The MW_r of RTA 1-33/44-198 calculated from its amino acid sequence without an initiating methionine is 21,316. Protein concentrations were determined by UV spectroscopy with an A_{280} value of 0.721 for a 1 mg/mL solution. PBS buffer was 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (Sigma). 2% Alhydrogel® was from E.M. Sergeant Pulp and Chemical Co.

2.2. Spectroscopic methods

Circular dichroism was measured by using a Jasco J-810 spectropolarimeter as described [10]. A Peltier-device controlled cell holder was used with a 0.1 cm rectangular quartz cuvette and a protein concentration of 0.1 mg/mL. Thermal melting was monitored by CD at 205 nm at a heating rate of 1 K/min and was irreversible. Dynamic light scattering was measured at a 90° angle and 25 °C with a DynaPro instrument on protein at 0.65 mg/mL, after passage through a 0.1 μM Anodisk filter to remove dust particles. At least 20 measurements were taken and averaged to yield the hydrodynamic radius using the Dynamics software standard curve for globular proteins.

Fourier-transform infrared spectroscopy was done using a Jasco FT/IR-430 instrument and a 45° angle ZnSe horizontal attenuated total reflectance (HATR) flow-cell crystal of 25 reflections with a water jacket (Pike Technologies). Cell temperature was controlled using a water bath and monitored with a platinum RTD on the cell. One hundred microlitres of vaccine protein at 0.7–0.9 mg/mL, with or without 0.2% aluminum hydroxide adjuvant, was dried on the crystal surface with nitrogen gas. Deuteration of the protein sample was then performed with a flow of D_2O-saturated nitrogen gas over the crystal [11]. Two hundred and fifty scans were taken at 2 cm^{-1} resolution after stopping the gas flow. Within 10 min of deuteration, the absorbance was stable and the contribution of liquid water to the Amide I′ peak was eliminated. A weighted blank spectrum was subtracted to remove the residual contribution of water vapor in the instrument. All absorbances were normalized to zero at 1800 cm^{-1}. Data were not smoothed.

2.3. Assays of protein solubility and adherence to adjuvant

To measure protein adherence to adjuvant in different buffers, RTA 1-33/44-198 protein at 0.16 mg/mL was incubated with 0.2% Alhydrogel® for 1 h at 25 °C. The degree of adsorption was calculated from the concentration of protein remaining soluble after centrifugation, as determined by the Pierce Micro-BCA Protein Assay. To assay desorption from adjuvant, vaccine protein at 0.16 mg/mL was adhered

to 0.2% adjuvant in succinate buffer, pelleted by centrifugation, and resuspended in an equal volume of 2 M NaCl or PBS buffer. The concentration of free protein after varying times of incubation at 25 °C was measured by UV spectroscopy.

In experiments testing the effects of pretreatment of adjuvant with PBS, 0.2% Alhydrogel® was incubated with PBS buffer at 25 °C, followed by centrifugation to pellet the adjuvant, and resuspension in succinate buffer with vaccine protein. For assays of resistance to repeated freeze/thawing, the protein sample at 1.0 mg/mL in succinate buffer was frozen at −20 °C, before thawing at 37 °C. After removal of insoluble material by centrifugation, protein concentration measurements on the soluble fraction were compared to control samples kept at 4 °C to determine the percent protein remaining in solution.

2.4. Mouse vaccination and challenge

Female BALB/c mice, weighing 16–18 g, were obtained from the Frederick Cancer Research Facility. They were randomly divided into cages of 10 mice each and acclimated for 10–14 days. During acclimation, the mice had microchip transponders implanted and were mapped for animal identification codes. After acclimation, 30 mice for each vaccine group and buffer controls were injected intramuscularly (IM) at 0 and 3 weeks with the appropriate test or control article. Six days after the last IM injection, all the mice were bled from the periorbital sinus and the resulting sera were analyzed for ricin-specific IgG and neutralizing antibody concentrations. One day after bleeding, mice in each test and control group were injected intraperitoneally (IP) with 2.5, 5, 10, or 20 mouse $IPLD_{50}$ (20 μg/kg) of ricin toxin. The mice were observed for the next 7 days for signs of morbidity and mortality. All surviving mice were euthanized at 7 days post-exposure with IP ricin. During development of the mouse bioassay, mice were observed for 14 days but no deaths were noted after day 7 even at low dose challenge with IP ricin. Therefore, a 7-day post-challenge observation period was selected for this assay.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.5. ELISA

ELISAs were done as described in Lindsey et al. [12]. One hundred microlitre per well of ricin capture antigen at 5 μg/mL in PBS were dispensed into PVC microtiter plates (Dynex Technologies, Chantilly, VA) and allowed to adhere at 4 °C overnight. The plates were washed and then blocked with 5% skim milk in wash buffer and incubated at 37 ± 1 °C for 60 ± 5 min. After washing three times, unknown samples, standards, and controls were added to the plates (100 μL/well). The plates were incubated at 37 ± 1 °C for 60 ± 5 min and washed again. The secondary antibody was affinity-purified peroxidase-conjugated goat anti-mouse IgG (H + L) at 0.1 μg/mL. After developing with ABTS peroxidase substrate, the optical density (OD) was measured using an ELx808 Automated Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 405 nm within 30 min of addition of stop solution. The mean IgG concentration was calculated from the corresponding calibration curve run on each plate, using a four-parameter logistic (4PL) equation: $y = (a - d)/(1 + (x/c)^b) + d$.

2.6. Toxin-neutralization assay

The neutralizing anti-ricin IgG concentration was determined with an in vitro cytotoxicity neutralization assay. Anti-ricin IgG standards, unknown serum samples, and controls were diluted with leucine-free RPMI 1640 medium and added to 96-well, U-bottom microtiter plates (50 μL/well). Ricin toxin solution at 7.5 ng/mL (50 μL/well) was then added. After 30 min incubation at 37 ± 1 °C, 50 μL of EL4 cell suspension was added to each well and the plates were incubated at 37 ± 1 °C overnight. Fifty microlitres of ^{3}[H]Leucine medium was added to each well of the plates. After 4 h incubation at 37 ± 1 °C, the cells were collected onto filter mats by a cell harvester. The filter mats containing samples were dried in an oven (80–90 °C) for 10–20 min. Each filter mat was placed into a plastic bag and wetted with 9.5 mL of scintillation fluid. The sealed bag was placed inside a scintillation cartridge, which was counted with a Wallac Betaplate Scintillation Counter (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA). The corrected counts per minute for isotope (CCPMs) of each plate were collected. The concentration of neutralizing anti-ricin IgG in each unknown sample was calculated from a corresponding standard curve run on each plate using a four-parameter logistic (4PL) equation.

3. Results

3.1. Optimization of formulation

As an initial step to find optimal formulation conditions for the RTA 1-33/44-198 vaccine, we assayed the pH dependence of its thermal stability over the range of pH 5.5–8.0 using circular dichroism (Fig. 2). Within the range close to neutral, pH did not have a significant effect on the protein's stability. The denaturational mid-point for the vaccine under all the tested pH conditions was close to 58 °C. Fig. 2 also shows a melting scan for wild-type recombinant RTA, demonstrating the increased relative thermal stability of the vaccine protein [9,10].

Subsequent tests of the buffer and pH dependence of adherence of the protein to the aluminum hydroxide adjuvant

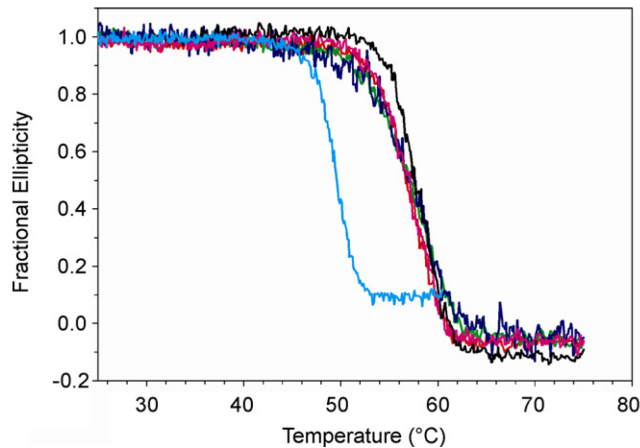

Fig. 2. The pH dependence of denaturation of RTA 1-33/44-198 protein was probed by circular dichroism as a function of increasing temperature. Buffers used were 20 mM NaBicarbonate, 100 mM NaCl, pH 7.3 (red), 20 mM NaAcetate, 100 mM NaCl, pH 5.5 (green), 20 mM NaSuccinate, 100 mM NaCl, pH 6.5 (blue), 20 mM NaBorate, 100 mM NaCl, pH 8.0 (pink), PBS, pH 7.4 (black). A melting scan of wild-type recombinant RTA protein in PBS buffer is shown in cyan.

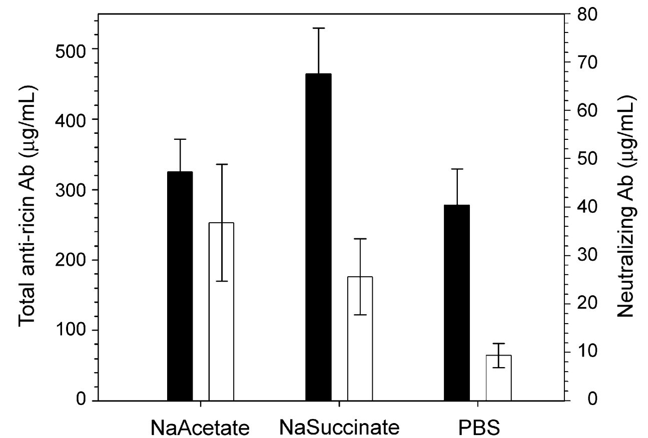

Fig. 3. Total anti-ricin IgG (black, left axis) and neutralizing antibody concentrations (white, right axis) from the sera of vaccinated mice.

Alhydrogel® revealed that several buffers gave adherence in the range of 88–96% of total protein (Table 1), with the exception of PBS, which had been used in our initial formulation [9]. In PBS, adherence was poor, averaging at 15%. The amount of adsorbed protein reached a stable value within 10 min of mixture of components. While pH within the tested range was not a significant factor influencing adherence, the identity of the buffer ion was critical. Adding PBS buffer to the protein–adjuvant complex released 75–80% of the protein within 5–15 min of addition, while addition of 2 M NaCl released only 10–15%. This result supports a specific role for phosphate ions rather than electrostatic effects in determining the degree of adherence. The level of adherence to aluminum phosphate adjuvant (Adju-Phos®) was only 7%. Pretreating aluminum hydroxide with PBS buffer for 2 h followed by assay of adherence in succinate buffer yielded 67% adsorption, indicating that modification of the adjuvant itself by ligand exchange of hydroxide for phosphate may have contributed to the lower adherence found in PBS, but was not the major factor reducing adsorption.

The efficacy of the vaccine formulated in various buffers was tested by challenging vaccinated mice by IP injection with 2.5 mouse $IPLD_{50}$ of ricin toxin (Table 1). Without vaccination, 0 of 10 mice survived challenge. All of the tested vaccine formulations in two doses protected 90–100% of the animals. PBS buffer yielded the lowest survival rate (18/20), although this figure is statistically not significantly different from the others.

The potency of the vaccine formulations was further tested using a two-dose assay in mice (Fig. 3). Total anti-ricin IgG in sera and the concentration of antibody able to neutralize ricin toxicity in a cell-based assay were determined (Table 2). To conserve animals, only sodium acetate, sodium succinate, and PBS buffers were tested. During the 3-week time course of this two-dose vaccination schedule, the amount of protein adhered to adjuvant did not significantly change in the prepared vaccine samples. Total anti-ricin IgG levels were highest with the succinate buffer formulation, and lowest with

Table 1
Adherence of vaccine protein to adjuvant and summary of survival after exposure to ricin toxin[a]

Buffer	Protein bound[b] (%)	Survival (alive/total)	Mean time-to-death[h] (days)
NaAcetate[c]	92 ± 4.6	19/20 (95%)	6.8 ± 0.2
NaBorate[d]	96 ± 0.6	20/20 (100%)	7
NaBicarbonate[e]	88 ± 7.5	20/20 (100%)	7
NaSuccinate[f]	91 ± 2.2	20/20 (100%)	7
PBS[g]	15 ± 4.0	18/20 (90%)	6.7 ± 0.24
No vaccine		0/10 (0%)	1.9 ± 0.09

[a] BALB/c mice were vaccinated as described in Section 2 and challenged by intraperitoneal exposure to 2.5 mouse $IPLD_{50}$ of ricin toxin. The experiment was terminated 7 days after ricin exposure. As described in Section 2, in earlier study no additional deaths were observed between days 7 and 14.
[b] Adherence of vaccine protein to Alhydrogel® in different buffers. Errors are the standard error of the mean ($n = 4$).
[c] 10 mM NaAcetate, 140 mM NaCl, pH 5.5.
[d] 10 mM NaBorate, 140 mM NaCl, pH 8.0.
[e] 10 mM NaBicarbonate, 140 mM NaCl, pH 6.5.
[f] 10 mM NaSuccinate, 140 mM NaCl, pH 6.5.
[g] Phosphate-buffered saline, pH 7.4.
[h] All mice euthanized at 7 days post-challenge with IP ricin.

Table 2
Antibody concentrations in sera of mice vaccinated with vaccine protein plus adjuvant in different buffer formulations[a]

Buffer	Binding to adjuvant (%)		Serum anti-ricin response	
	Day 0	Day 21	ELISA[b]	Neutralization[c]
NaAcetate[d]	99.9	99.9	325.2 ± 46.6	50.6 ± 16.6
NaSuccinate[e]	94.2	95.0	464.0 ± 65.5	35.2 ± 10.8
PBS	19.4	21.9	278.0 ± 51.2	12.9 ± 3.4

[a] BALB/c mice were vaccinated with two doses (0 and 3 weeks) of RTA 1-33/44-198 with 0.2% Alhydrogel® in different buffers. Each mouse received 20 μg of vaccine protein from a stock solution of 200 μg/mL.
[b] Total anti-ricin IgG measured by ELISA (μg/mL). Values are expressed as an average of 30 mice ± standard error of mean (S.E.M.).
[c] Neutralizing antibody assay (μg/mL).
[d] 10 mM NaAcetate, 140 mM NaCl, pH 5.5.
[e] 10 mM NaSuccinate, 140 mM NaCl, pH 6.5.

PBS. In the assay of toxin-neutralizing antibodies, acetate buffer gave the highest value, but the result for succinate buffer was the same within error. PBS formulation yielded an approximately threefold lower concentration of neutralizing antibodies. In the absence of any adjuvant, the antigenicity of the protein was much less. In mice challenged with 5 $IPLD_{50}$, 100% survived when vaccinated with the RTA vaccine plus adjuvant, compared to 50% in the absence of adjuvant. The anti-ricin ELISA titers were also reduced twofold without adjuvant.

The greater antigenicity, of the acetate and succinate buffer formulations versus PBS, was correlated with a high degree of adherence to adjuvant. Given these results, we decided that sodium succinate buffer at pH 6.5 would be used in further vaccine testing. A pH of 6.5 was chosen to remain within one unit of the relevant pK_a of succinate and as close as possible to neutral, to avoid damage to tissue at the site of injection. The toxicities of all the tested buffers are acceptable for use in a vaccine. Succinate is on the FDA's Everything Added to Food in the U.S. list, is a natural metabolite, and is used as a counterion in various drugs. Succinate was also preferred over acetate because of the greater volatility of the latter compound, which can result in an unstable pH.

3.2. Conformation of vaccine adsorbed to adjuvant

In order to determine the effects on protein conformation and stability of adjuvant adherence, we examined the adsorbed protein's secondary structure using horizontal attenuated total reflectance Fourier-transform infrared spectroscopy. Unlike most spectroscopic methods, infrared spectroscopy is not affected by the optical turbidity of aluminum adjuvant suspensions. Samples of protein alone and mixed with adjuvant were deuterated after application to the IR crystal to remove the very large contribution of liquid water to absorbance in the range of 1600–1700 cm^{-1} [11]. The IR spectrum in this region predominantly represents vibrations of the peptide backbone, and in a deuterated sample is known as the Amide I′. The shape of the spectrum is affected by protein secondary structure, with alpha helices yielding a peak between 1648 and 1660 cm^{-1} and beta-sheets one between 1625 and 1640 cm^{-1} [13]. Deuteration is expected to shift these values to 5–10 cm^{-1} lower wavenumber [14]. Protein denaturation and aggregation are associated with the appearance of an intense new band centered between 1610 and 1628 cm^{-1} [13,15].

To yield adequate signal, the method we employed required higher protein concentrations than were present in the formulation for vaccination (0.7–0.9 mg/mL versus 0.2 mg/mL), but much less than is typically used in transmission FTIR (>10 mg/mL), or HATR on protein/adjuvant mixtures without deuteration (≥2 mg/mL) [16]. We used protein concentrations as low as possible in order to prevent the appearance of artifacts due to self-association at high concentration.

Fig. 4a shows spectra of deuterated RTA 1-33/44-198 protein in sodium succinate buffer at temperatures from 25 to 65 °C. A lower concentration of succinate buffer (2 mM) was used in this experiment to minimize interference from a buffer-derived peak centered on 1560 cm^{-1}. Fig. 4b shows spectra of protein in succinate buffer adsorbed to Alhydrogel®, while Fig. 4c shows the same measurements done using PBS buffer, in which the degree of adherence was low. The shape of the Amide I′ spectra at 25 °C was slightly different between the adjuvant adsorbed and non-adsorbed samples. The adsorbed sample in succinate buffer showed a small relative increase in absorbance at 1635 cm^{-1} compared to the protein in PBS with Alhydrogel®, or protein alone in succinate buffer. At 25 °C, the peak maxima identified by first derivative analysis were 1644 cm^{-1} for protein alone in succinate, 1643 cm^{-1} in PBS with Alhydrogel®, and 1640 cm^{-1} adsorbed in succinate buffer.

On heating further to 65 °C (Fig. 4a), the protein alone in succinate buffer and in the presence of Alhydrogel® with PBS (Fig. 4c) developed a pronounced shoulder in the region of 1620–1630 cm^{-1}. The appearance of a new absorption peak is clear in the difference between the spectra at 65 and 45 °C, shown as a dashed line. This type of spectral change is believed to reflect the appearance of denatured protein aggregates containing strongly hydrogen-bonded beta-sheets [13,15]. Similar behavior was seen for protein heated in the absence of adjuvant. In contrast, the 1620–1630 cm^{-1} shoulder associated with denaturation was not present for the adjuvant-adsorbed protein in succinate (Fig. 4b). The

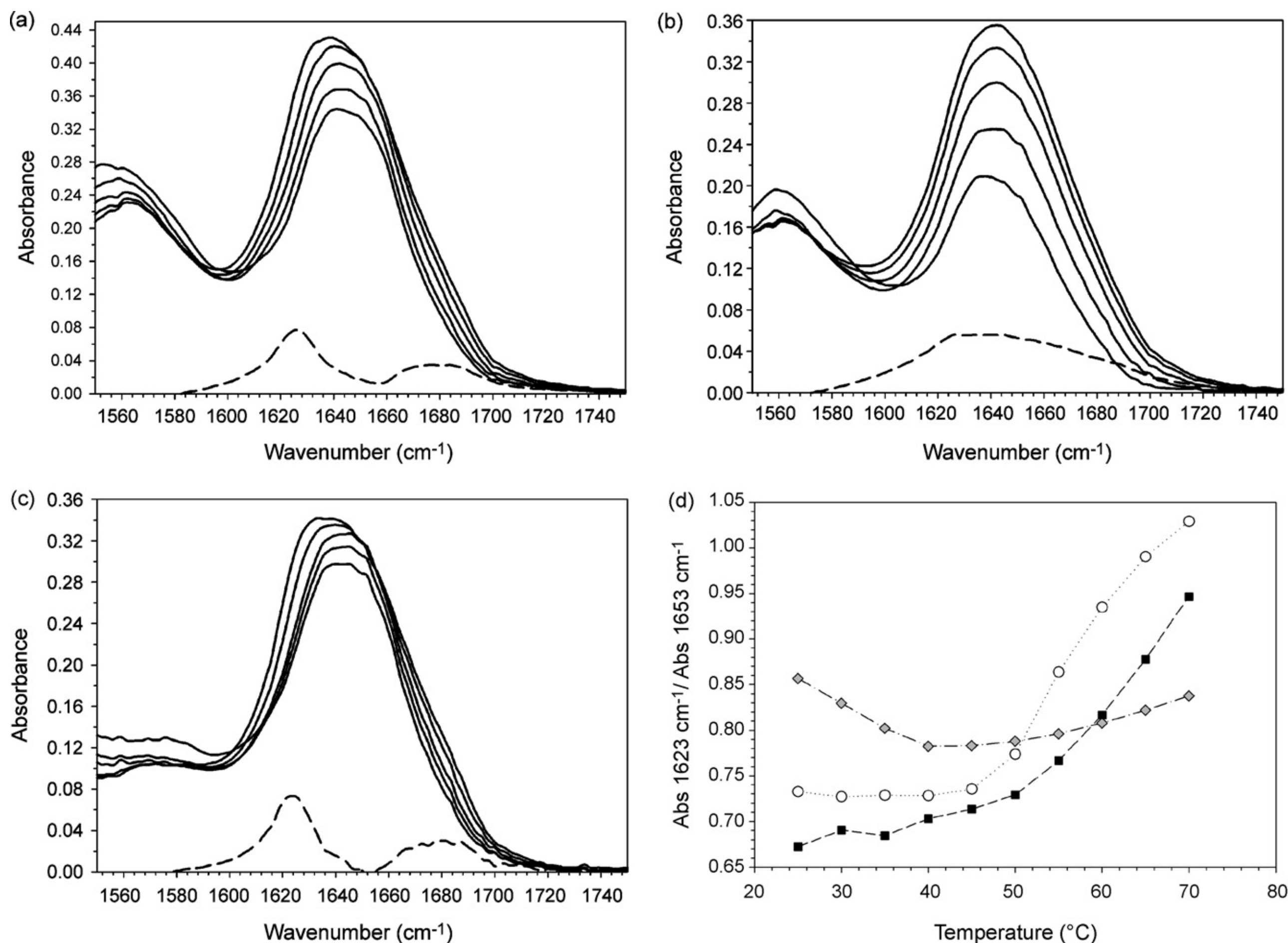

Fig. 4. FTIR data on RTA 1-33/44-198 protein. (a) Protein in 2 mM NaSuccinate, 100 mM NaCl, pH 6.5. Corresponding temperatures for ascending curves are 25, 35, 45, 55, and 65 °C. The difference between the curves at 65 and 45 °C is plotted as a dashed line. (b) Protein with 0.2% Alhydrogel® in 2 mM NaSuccinate, 100 mM NaCl, pH 6.5. (c) Protein with 0.2% Alhydrogel® in PBS buffer. (d) Temperature-dependent conformational changes monitored by the ratio of absorbances at 1623 and 1653 cm^{-1}. Circles: Protein plus Alhydrogel® in PBS; squares: protein alone in 2 mM NaSuccinate, 100 mM NaCl, pH 6.5; diamonds: protein with Alhydrogel® in 2 mM NaSuccinate, 100 mM NaCl, pH 6.5.

absence of this shoulder for the adjuvant-bound protein, compared to the pronounced difference seen with non-adsorbed protein, indicates that adsorption to adjuvant significantly stabilized the protein to denaturation. The general increase in absorbance intensity with temperature in Fig. 4b was not consistent in magnitude and may have been due only to changes in thickness of the layer of sample on the crystal, therefore alterations in shape of the spectra were more reliable indicators of conformational change.

The shifts in IR spectra of the protein samples as a functions of temperature are plotted as a ratio of absorbances at 1623 and 1653 cm^{-1} in Fig. 4d. For non-adsorbed protein, the denaturation process began near 50 °C and was similar for protein alone in succinate buffer, or in the presence of adjuvant with PBS buffer. For protein bound to Alhydrogel® in succinate buffer, the absorbance ratio decreased slightly with heating to 40 °C, and then increased gradually with further heating, but to a much lesser extent than for non-adsorbed protein. Solution tests of adherence in succinate buffer showed that the amount of adsorbed protein did not decrease with increase of temperature from 25 to 45 °C. The small difference in peak maxima of adsorbed and non-adsorbed protein at 25 °C may reflect a slight difference in protein conformation, or simply the effect of hydrogen-bonding to the adjuvant on peptide bond vibrations.

3.3. Retention of vaccine structure and potency during long-term storage

A study of the long-term stability of the vaccine was conducted to determine the best conditions for storage. Samples in solution in succinate buffer at 4 or −20 °C, or adhered to 0.2% Alhydrogel® at 4 °C, were tested for alterations in structure or potency over a period of 18 months. At time points of 0, 3, 6, 12, and 18 months of storage, the vaccine was examined by electrophoresis, circular dichroism, UV spectroscopy, mass spectrometry, dynamic light scattering, and a test of potency in a mouse model.

SDS-polyacrylamide gel electrophoresis on protein samples stored in solution at 4 or −20 °C for 18 months showed no

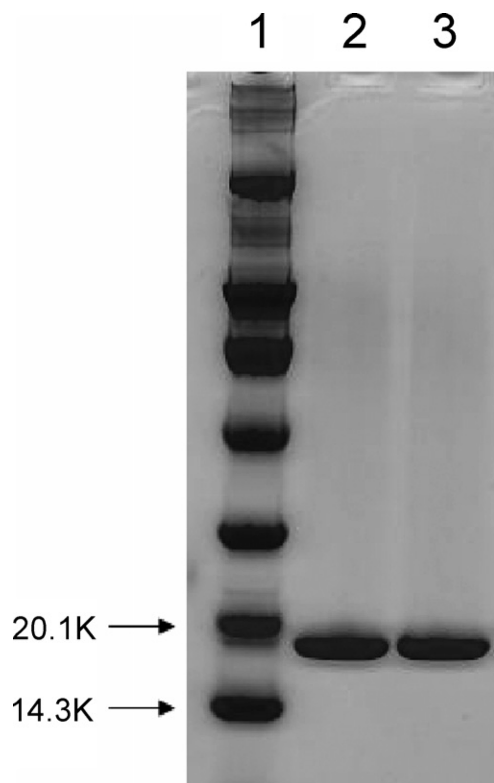

Fig. 5. SDS-polyacrylamide gel electrophoresis of vaccine protein samples stored for 18 months at −20 °C (lane 2) or 4 °C (lane 3) in 20 mM NaSuccinate, 100 mM NaCl, pH 6.5.

proteolytic degradation of the protein under either condition (Fig. 5). Electrospray ionization mass spectrometry (Harry Hines and Ernest Brueggemann) indicated that the mass of the protein sample did not change with storage. Two major peaks were detected at 21,447 and 21,316 Da. These values differed by the mass of a methionine residue and presumably reflected heterogeneity in the population of molecules retaining the initiating amino acid. UV spectroscopy was used to test for an increase in turbidity caused by protein aggregation. While a small number of individual vials of protein stored in solution at 4 °C showed visible aggregation after 1 year, no significant aggregation was detected in the samples stored at −20 °C even after 18 months.

Dynamic light scattering was measured to assay more sensitively for protein self-association. The hydrodynamic radius of the stored protein did not change over time or depend on the temperature of storage. A hydrodynamic radius of 2.2 ± 0.1 nm was found, corresponding to a globular protein of 21–23 kDa mass. Circular dichroism spectra (Fig. 6) and thermal melting scans (not shown) on the stored proteins also did not reveal a change in secondary structure content or thermostability.

The sensitivity of the protein to repeated cycles of freezing and thawing in the presence or absence of stabilizing sugars was tested to determine if addition of an excipient would be necessary for bulk storage at −20 °C. After one round of freeze/thawing, there was only a 2–3% loss of protein under any condition tested (Fig. 7). After five rounds, the protein in buffer alone had decreased in concentration to 94% of the original value. The excipients tested at 10% concentration had no significant stabilizing effect. Given the relatively high resistance of the vaccine protein to inactivation by freeze/thawing, bulk preparations may be stored frozen without an excipient at −20 °C.

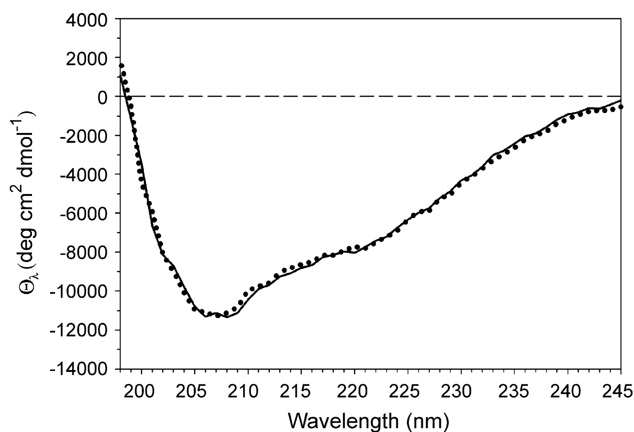

Fig. 6. Far-UV CD spectra on vaccine protein samples stored for 18 months at 4 °C (solid line) or −20 °C (dotted line) in 20 mM NaSuccinate, 100 mM NaCl, pH 6.5.

Spectroscopic measurements on the protein samples stored bound to adjuvant were not feasible due to their high turbidity and relatively low protein concentration. The integrity of the adsorbed vaccine over time was instead tested using a two-dose mouse potency assay. The degree of conferred protection was tested with increasing toxin challenge doses delivered IP. In developing the potency assay, the vaccine was adsorbed to Alhydrogel® in PBS buffer. Under these conditions, 80–100% of the mice survived a 2.5 $IPLD_{50}$ challenge, while only 40–50% of the mice survived at 5 $IPLD_{50}$ ricin challenge. When the stability study was started, a switch

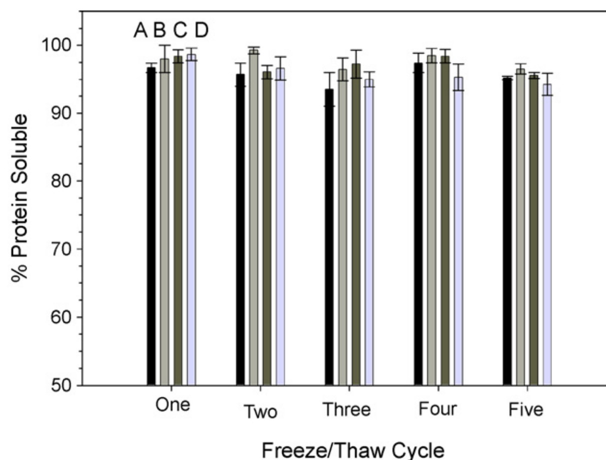

Fig. 7. The solubility of RTA 1-33/44-198 vaccine protein after repeated freeze–thaw cycles in the presence of different stabilizing excipients. Error bars are the standard error of the mean ($n = 3$). (A) 10% sucrose, (B) 10% glucose, (C) 10% sorbitol, (D) succinate buffer alone.

Table 3
Vaccine potency over long-term storage measured with a mouse bioassay

Storage (months)	Storage temperature (°C)	Storage with adjuvant[h]	Challenge dose ($IPLD_{50}$)	Survival[a]		MTD[c] (days)
				Alive/total	Percent	
0	4	+	2.5	9/10[b]	90	6.9 ± 0.10
0	4	+	5	8/10[b]	80	6.5 ± 0.40
0	−20	−	2.5	10/10	100	7[c]
0	−20	−	5	10/10	100	7[c]
3	4	+	2.5	10/10	100	7[c]
3	4	+	5	10/10	100	7[c]
3	−20	−	2.5	10/10	100	7[c]
3	−20	−	5	10/10	100	7[c]
6–7	4	+	2.5	9/10[b]	90	6.90 ± 1.0
6–7	4	+	5	10/10	100	7[c]
6–7	−20	−	2.5	10/10	100	7[c]
6–7	−20	−	5	10/10	100	7[c]
9	4	+	2.5	10/10	100	7[c]
9	4	+	5	9/10[b]	90	6.60 ± 0.40
9	4	+	10	10/10	100	7[c]
9	−20	−	2.5	10/10	100	7[c]
9	−20	−	5	9/10[b]	90	6.70 ± 0.30
9	−20	−	10	9/10[b]	90	6.80 ± 0.20
12	4	+	5	10/10	100	7[c]
12	4	+	10	9/10[b]	90	6.95 ± 0.05
12	4	+	20	8/10[d]	80	6.23 ± 0.56
12	−20	−	5	10/10	100	7[c]
12	−20	−	10	10/10	100	7[c]
12	−20	−	20	5/10[e]	50	5.03 ± 0.69[f]
18	4	+	5	9/10[b]	90	6.50 ± 0.50
18	4	+	10	6/10[b]	60	5.15 ± 0.77
18	4	+	20	6/10[b]	60	5.28 ± 0.80
18	4	−	5	9/10[b]	90	6.70 ± 0.30
18	4	−	10	6/10[b]	60	5.42 ± 0.75
18	4	−	20	5/10[e]	50	4.45 ± 0.86[g]
18	−20	−	5	9/10[b]	90	6.48 ± 0.52
18	−20	−	10	8/10[b]	80	6.77 ± 0.16
18	−20	−	20	7/9[b]	78	5.96 ± 0.66

[a] All buffer only controls died in 1–1.83 days at 2.5 LD_{50}, 0.67–1.83 days at 5 $IPLD_{50}$, and 0.67–0.83 days at 10 and 20 $IPLD_{50}$.
[b] Not significantly different from 10/10 by Fisher's Exact Test.
[c] All surviving mice euthanized at 7 days post-challenge with IP ricin.
[d] Not significantly different from 10/10 by Fisher's Exact Test.
[e] Significantly different from 10/10 but not significantly different from 8/10 or 7/9 by Fisher's Exact Test.
[f] Not significantly different from Mean time-to-death (MTD) of mice vaccinated with 4 °C stored product and challenged with 20 $IPLD_{50}$ ricin toxin.
[g] Not significantly different from MTD of mice vaccinated with 4 °C + 0.2% Alhydrogel® stored product and challenged with 20 $IPLD_{50}$ ricin toxin.
[h] For all groups with (−), adjuvant was added to vaccine 1 h prior to vaccination.

was made to adsorb the vaccine to Alhydrogel® in succinate buffer. In this buffer system, 90–100% of the mice survived the 2.5 and 5 $IPLD_{50}$ challenge. Therefore, after the 9-month stability the 2.5 $IPLD_{50}$ challenge was dropped to allow for the 20 $IPLD_{50}$ challenge group.

At a challenge dose of 5 mouse $IPLD_{50}$, there was no loss in potency of the ricin vaccine stored at either 4 °C with or without 0.2% Alhydrogel®, or at −20 °C in solution with succinate buffer (Table 3). At a 10 mouse $IPLD_{50}$ challenge, there was a non-significant trend for decreased potency between 9 and 18 months of storage at either temperature. The 20 $IPLD_{50}$ challenge gave an approximately 50% break-through in vaccine protection, with no significant difference between storage conditions and 12–18 months duration. No loss of adherence to adjuvant was found over 18 months storage at 4 °C. Because freezing of Alhydrogel® is not recommended, protein stored at −20 °C was tested by mixing with adjuvant 1 h before use.

4. Discussion

Vaccine stability can be greatly affected by formulation and is a critical characteristic of an effective pharmaceutical [17]. The degree of adjuvant adsorption is also an important quality control parameter for the life of the vaccine preparation [18]. Based on the reported findings, recommended formulation conditions for the RTA 1-33/44-198 vaccine would be adsorbed to 0.2% Alhydrogel® in succinate buffer, with a shelf-life of at least 18 months at 4 °C. Storage of the

vaccine bulk preparation was best done frozen in solution with succinate buffer. Because of the high intrinsic stability of the vaccine protein, adding stabilizing sugar excipients did not appear to be necessary. In comparison, an alternative ricin vaccine known as RiVax [19–22], containing substitution mutations at the active site, has a pronounced tendency towards aggregation. A study of required excipients by Peek et al. [23] recommended formulation of that protein with 50% glycerol.

Reformulation from PBS buffer to succinate strengthened the RTA 1-33/44-198 vaccine's effective antigenicity in a mouse model nearly threefold, while adherence increased from 15 to 91% (Tables 1 and 2). The adsorption of proteins to aluminum hydroxide and aluminum phosphate adjuvants is a complex phenomenon, depending on the surface charge of the protein and adjuvant as well as other factors [24–27]. In this case, charge did not appear to be the major factor driving adherence, as greater than 90% adherence could be achieved at pH 5.5–6.5, under which conditions both the protein and adjuvant had net positive charge. The measured p*I* of RTA 1-33/44-198 is close to 7.3 (Meagher, personnel communication), while the point of zero charge of aluminum hydroxide is 11.5.

The lower adherence found with phosphate buffer may have been due at least in part to the natural affinity of RTA for phosphate groups in its rRNA substrate [28]. Association of phosphate ions with the protein could alter its surface properties, affecting interactions with the adjuvant. Exchange of phosphate into the adjuvant gel itself also could affect adherence [27]. However, we found that pretreating aluminum hydroxide with PBS buffer resulted in a relatively small decrease in adherence. Conversely, Depaz et al. [29] found that the presence of phosphate in mixtures of Alhydrogel® with recombinant botulinum toxin heavy chain vaccines resulted in greater adsorption of protein.

The origin of the increased antigenicity of the RTA 1-33/44-198 vaccine in formulations of higher adjuvant adherence may be ascribed to the prolonged presence of antigen at the site of injection, known as the "depot effect" [30]. In theory, conformational changes may also occur when a vaccine is adsorbed to the adjuvant that affect its antigenicity. Jones et al. [16] found significant changes in the shape of ATR-FTIR spectra for bovine serum albumin, lysozyme and ovalbumin upon adsorption to aluminum adjuvants. Those authors concluded that a disruption of antigen conformation caused by adsorption destabilized the proteins' structures, and proposed that this phenomenon may itself contribute to the adjuvant effect. In contrast, our FTIR data showed only a very small change in spectral shape of the RTA vaccine on adherence to Alhydrogel®, that diminished on heating to physiological temperature.

Adjuvant adsorption greatly stabilized the vaccine protein to thermal denaturation, as judged from comparison of the spectra obtained in high-adherence (succinate) versus low adherence (PBS) buffers. From first principles, it should be expected that tight adherence of the native state of a protein to an adjuvant or any other ligand would stabilize that conformation. Along with potency studies, the high thermal stability of the adsorbed RTA 1-33/44-198 protein supports the conclusion that the vaccine may be safely stored with adjuvant for at least 18 months.

Other studies have reached varying conclusions on the significance for immunogenicity of vaccine adherence to aluminum adjuvant. Chang et al. [31] reported that the antigenicity of lysozyme in rabbits was independent of its adherence to aluminum adjuvant during preparation, while Romero Mendez et al. [32] proposed that trapping of a non-adsorbed protein in a porous matrix of aluminum phosphate adjuvant was adequate to stimulate the immune response. On the other hand, Capelle et al. [33] suggested that stronger adherence of an immunoglobulin antigen to adjuvant results in the formation of more condensed particles of greater immunogenicity. Berthold et al. [34] concluded that adsorption of recombinant anthrax protective antigen to aluminum phosphate enhances the immunogenicity of lower doses of antigen. Levesque et al. [35] also concluded that increased adsorption to adjuvant correlates with greater antigenicity of the proteins studied.

Our results suggest that optimization of adherence of a protein antigen to aluminum adjuvant should be pursued as a means to increase both antigenicity and product stability. Studies of the greater efficacy of the improved RTA 1-33/44-198 vaccine formulation in non-human primates will be reported elsewhere (Wannemacher et al., manuscript in preparation). At 6 months after the last vaccination of the NHP with the improved RTA 1-33/44-198 formulation, 100% survived a challenge of 10 LD_{50}s of aerosolized ricin.

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