

Freeze-thaw stress of Alhydrogel[®] alone is sufficient to reduce the immunogenicity of a recombinant hepatitis B vaccine containing native antigen



Tanya Clapp^a, Michael W. Munks^b, Ruchit Trivedi^a, Uday B. Kompella^a,
LaToya Jones Braun^{a,*}

^a Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Denver, 12850 E. Montview Boulevard, C238, Aurora, CO 80045, United States

^b Integrated Department of Immunology, National Jewish Health, 1400 Jackson Street, Denver, CO 80206, United States

ARTICLE INFO

Article history:

Received 18 December 2013

Received in revised form 18 April 2014

Accepted 7 May 2014

Available online 20 May 2014

Keywords:

Alhydrogel[®]

Aluminum-containing adjuvant

Adjuvant

Aluminum hydroxide adjuvant

Freeze–thaw (FT)

Recombinant hepatitis B surface antigen (rHBsAg)

ABSTRACT

Preventing losses in vaccine potency due to accidental freezing has recently become a topic of interest for improving vaccines. All vaccines with aluminum-containing adjuvants are susceptible to such potency losses. Recent studies have described excipients that protect the antigen from freeze-induced inactivation, prevent adjuvant agglomeration and retain potency. Although these strategies have demonstrated success, they do not provide a mechanistic understanding of freeze–thaw (FT) induced potency losses. In the current study, we investigated how adjuvant frozen in the absence of antigen affects vaccine immunogenicity and whether preventing damage to the freeze-sensitive recombinant hepatitis B surface antigen (rHBsAg) was sufficient for maintaining vaccine potency. The final vaccine formulation or Alhydrogel[®] alone was subjected to three FT-cycles. The vaccines were characterized for antigen adsorption, rHBsAg tertiary structure, particle size and charge, adjuvant elemental content and in-vivo potency. Particle agglomeration of either vaccine particles or adjuvant was observed following FT-stress. In vivo studies demonstrated no statistical differences in IgG responses between vaccines with FT-stressed adjuvant and no adjuvant. Adsorption of rHBsAg was achieved; regardless of adjuvant treatment, suggesting that the similar responses were not due to soluble antigen in the frozen adjuvant-containing formulations. All vaccines with adjuvant, including the non-frozen controls, yielded similar, blue-shifted fluorescence emission spectra. Immune response differences could not be traced to differences in the tertiary structure of the antigen in the formulations. Zeta potential measurements and elemental content analyses suggest that FT-stress resulted in a significant chemical alteration of the adjuvant surface. This data provides evidence that protecting a freeze-labile antigen from subzero exposure is insufficient to maintain vaccine potency. Future studies should focus on adjuvant protection. To our knowledge, this is the first study to systematically investigate how FT-stress to adjuvant alone affects immunogenicity. It provides definitive evidence that this damage is sufficient to reduce vaccine potency.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Antigens have been combined with aluminum-containing adjuvants since 1926, when Glenny and colleagues originally

precipitated diphtheria toxoids with alum and observed an improved immune response over a soluble antigen inoculation [1]. Today, the majority of the recommended vaccines for children are formulations with the antigen adsorbed onto an aluminum-containing adjuvant. An important formulation and stability concern for vaccines with aluminum-containing adjuvants is their reduced efficacy following freeze–thaw (FT) stress. Exposure to FT stress results in agglomeration of the vaccine particles and vaccine potency loss.

For vaccines with aluminum-containing adjuvants, there is a narrow range of temperatures at which the vaccines are stable during storage: 2 °C to 8 °C. The cold chain vulnerability has

* Corresponding author. Present address: Department of Pharmaceutical Sciences, School of Pharmacy, Regis University, School of Pharmacy, 3333 Regis Blvd., H-28, Denver, CO 80221, United States. Tel.: +1 303 964 6698.

E-mail addresses: tanyamclapp@gmail.com (T. Clapp),

MunksM@NJHealth.org (M.W. Munks), ruchit.trivedi@ucdenver.edu (R. Trivedi), uday.kompella@ucdenver.edu (U.B. Kompella), lbraun001@regis.edu (L.J. Braun).

been documented in the developing and developed world during both transport and storage [2–5]. In 2012, the DHHS audited 45 providers for vaccine management practices, and 89% did not meet qualifications for adherence to the CDC's temperature monitoring requirements. Furthermore, 42% were found to have refrigerators at less than 2 °C for five or more hours during a two week time period [5].

Vaccines that have been damaged following accidental freezing should not be used, but discarded. It is not always ideal, however, to discard a vaccine if accidental freezing is only suspected, especially when the result is an unimmunized population and usable vaccines in the trash. Nevertheless, it is also important to not provide a false hope of protection by immunizing with damaged vaccines. One way to minimize the risk of administering a previously frozen vaccine at the point-of-use is to use the shake test. This qualitative test relies on correctly evaluating relative rates of vaccine particle sedimentation. It has been widely reported that following freeze-thaw stress, FT-stressed vaccines have large agglomerates with faster sedimentation rates [6–8]. A validation study by the WHO determined that, when performed correctly, the shake test could identify previously frozen vaccines with aluminum-containing adjuvants with 100% accuracy [6]. There are two major problems with the shake test. First, the shake test is not always performed. In addition, if the personnel are improperly trained in conducting this assay, accurate assessment of vaccine quality is not guaranteed [9].

The characteristic size range of the particles in vaccines with aluminum-containing adjuvants has been reported to be on the order of 1–10 μm , with the average size less than 5 μm [10–12]. A cut-off size for adjuvants to retain their immune-stimulating properties has been suggested to be less than 10 μm [12]. Particles larger than 10 μm in size have been reported to yield decreased antigen uptake by antigen presenting cells (APC) [13]. It has also been observed that the particle surface area affects the resulting IgE and IgG₂ reactions. Specifically, the large surface area of small particles has been suggested to promote cell contact and cellular responses [14]. Most recently, the freezing of aluminum-containing vaccines was reported to result in disruption of the antigen-adjuvant lattice. The formation of aluminum clusters, based on microscopy and increases in the analyzed aluminum content of the particles, was attributed to the lattice disruption [15]. Although particle agglomeration is a likely reason for reduced immune response, it may not be the only cause. In our previous publications, we have reported losses on the order of only 10% of the particles that were under 10 μm in size (sizes evaluated 1.5 to 9.0 μm) but significant losses of potency [16,17]. We hypothesize that some of the loss in antibody response when using vaccines subjected to FT stress may be due to antigen FT instability.

To test our hypothesis, this study compares the total anti-rHBsAg IgG response in mice following administration of a non-stressed rHBsAg vaccine, a vaccine containing rHBsAg formulated with FT-treated Alhydrogel[®], or soluble rHBsAg. We further investigated FT-induced physicochemical alterations to the vaccine based on extent of antigen adsorption, tertiary structure of the adsorbed antigen, adjuvant zeta potential, vaccine particle size distribution and adjuvant microscopic structural/elemental analyses. To the best of our knowledge, this is the first study to evaluate the effect of damaging the adjuvant alone on vaccine potency.

2. Material and methods

2.1. Vaccine preparation

A *Saccharomyces cerevisiae*-derived recombinant hepatitis B surface antigen (rHBsAg), subtype adw (purity >98%) was purchased from Advanced Immunochemical Services, Inc. (Long Beach, CA).

Aluminum hydroxide adjuvant (Alhydrogel[®] 2%) was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Sodium phosphate monobasic anhydrous (USP), dibasic anhydrous (USP) and sodium chloride (ACS) were purchased from Fisher Scientific. Distilled de-ionized water was used to prepare the buffers. Buffers were filtered with 0.22 μm Stericup filter units (Millipore, Billerica, MA) prior to use. Stock protein solutions of 0.3 mg/ml rHBsAg were prepared in 1.5-ml lo-bind microcentrifuge tubes (Eppendorf, Hamburg, GERMANY). Stock Alhydrogel[®] suspensions (1.8 mg Al/ml) were prepared in 15-ml conical tubes (Fisher Scientific, Pittsburgh, PA). The formulation buffer for both was 9 mg/ml NaCl, 0.78 mg/ml Na₂HPO₄, 0.54 mg/ml NaH₂PO₄ buffer, referred to hereon as phosphate buffer. Borosilicate vials with butyl rubber stoppers and aluminum seals were purchased from Wheaton (Millville, NJ).

Samples were prepared in 2-ml borosilicate vials aseptically in a laminar-flow hood. Samples designated for FT treatment were settled at room temperature for 1 h prior to placement at –20 °C. Twenty-two hours later, the frozen samples were removed from the freezer to thaw at room temperature for 2 h. This was repeated two additional times, such that all FT-stressed samples underwent three FT-cycles. Three rHBsAg vaccine formulations (see Table 1) were prepared at a final concentration of 50 $\mu\text{g/ml}$ HBsAg with 1.5 mg/ml Alhydrogel[®]. A fourth formulation was 50 $\mu\text{g/ml}$ rHBsAg in phosphate buffer. All formulations were prepared in triplicate. In accordance with the designated timing of the final vaccine preparation (see Table 1), vaccines containing adjuvant were rotated on a bench-top rotisserie for 15 min at room temperature to facilitate antigen adsorption. For the formulation in which only the adjuvant was frozen (i.e., Alhydrogel[®] 3FT@ –20 °C), antigen adsorption was conducted at the conclusion of the final 2-h thawing time. For vaccine formulation characterizations, samples were diluted to a final concentration of 20 $\mu\text{g/ml}$ rHBsAg with or without 600 $\mu\text{g/ml}$ Alhydrogel[®].

2.2. rHBsAg vaccine formulation characterization

2.2.1. Particle sizing

Vaccine formulation particle sizing and counting was conducted using microflow imaging with a digital particle analyzer (DPA) model DPA 4100 (Protein Simple, Santa Clara, CA). For all formulations, the instrument was in the “set point 3” configuration (100 μm flow cell, low magnification and open diaphragm). This mode allows for a minimum particle size detection of 1 μm [18,19]. The formulation buffer was used as the diluent, and measurements of diluent were analyzed for background. For analysis, sample measurements were grouped in the following size intervals: 1.0–3.0 μm , 3.0–6.0 μm , 6.0–9.0 μm , 9.0–12.0 μm , 12.0–15.0 μm and 15.0–30.0 μm .

Particle sizing and counting of adjuvant samples were conducted using an AccuSizer 780 SIS (Particle Sizing Systems, Port Richey, FL). This technique combines light scattering and light obscuration detectors to cover a size range of 0.5–500 μm . The adjuvant sample measurements were grouped in the following size intervals: 0.5–1.0 μm , 1.0–3.0 μm , 3.0–6.0 μm , 6.0–9.0 μm , 9.0–12.0 μm , 12.0–15.0 μm and 15.0–30.0 μm . Analysis of this data was truncated at the 15.0–30.0 μm bin to correlate with the MFI data. No particles above 60 μm were detected, and the excluded data represents less than an additional 1% of the total particle count.

2.2.2. Determination of anti-rHBsAg IgG by ELISA

Immulon-1B high-binding polystyrene plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated by incubating each well with 100 μl of 5 $\mu\text{g/ml}$ goat anti-rHBsAg (ad/ay) (Meridian Life Science, Inc., Memphis, TN) in sodium bicarbonate/carbonate buffer

Table 1
Administered vaccine formulations.

		Abbreviation	Contains Alhydrogel®?	FT-treated?	rHBsAg adsorbed prior to FT stress?
1	Positive Control vaccine (4 °C)	AH/HB	Yes	No	N/A
2	FT-treated conventional vaccine (3FT @ -20 °C)	AH _{FT} /HB _{FT}	Yes	Yes	Yes
3	FT-treated Alhydrogel® vaccine (3FT @ -20 °C)	AH _{FT} /HB	Yes	Yes	No
4	Soluble rHBsAg (no adjuvant): antigen alone baseline	HB	No	No	N/A

(100 mM and pH 9.3) overnight at 4 °C. Plates were washed three times (3×) with 8 mM Na₂HPO₄, 150 mM NaCl, 2 mM KH₂PO₄, 3 mM KCl, 0.05% Tween® 20, pH 7.4 (1× PBS-T) using a plate washer (ELx405 Auto Plate Washer, Bio-Tek Instruments, Inc., Winooski, VT). The plates were then blocked with a solution of 50 mM Tris, 100 mM NaCl, 0.1% NaN₃, and 1% BSA (TNN) for 30 min. Afterwards, the plates were rinsed 3× with 1× PBS-T, and then 100 μl of 1.67 μg/ml rHBsAg in TNN was added to each well. Uncaptured rHBsAg was removed by rinsing 3× with 1× PBS-T. Appropriate dilutions of sera and/or positive control (mouse monoclonal HBsAg IgG₁ (MyBioSource, San Diego, CA)) in TNN were added. After washing (3×) with 1× PBS-T, 1:1000 horseradish peroxidase (HRP) goat anti-mouse IgG, Fcγ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) with 20 mM Tris, 150 mM NaCl and 1% BSA (TBS-T) was added to each well. Plates were washed 5× with PBS-T, and the HRP substrate TMB (3,3',5,5'-tetramethylbenzidine) (1-step Ultra TMB-Substrate, Thermo Fisher Scientific Inc. (Waltham, MA) was added to all wells. The reaction was allowed to proceed for up to 20 min. The reaction was stopped by addition of 2 M H₂SO₄, and the optical density measured at 450 nm (ELx808 Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT).

2.2.3. Antigen adsorption

The amount of rHBsAg adsorbed to Alhydrogel® was measured by analyzing the supernatant using a microBCA kit (Pierce, Rockford, IL). An rHBsAg standard curve was generated and used to interpolate the value in the supernatant. The amount adsorbed was calculated by subtracting the interpolated value from the amount in the control.

2.2.4. Intrinsic tryptophan fluorescence spectroscopy

The tertiary structure of rHBsAg was analyzed using a QuantaMaster spectrofluorometer (Photon Technologies International, Lawrenceville, NJ). An excitation wavelength of 280 nm was used and emission was monitored from 295 to 400 nm. The data were analyzed starting at 310 nm to minimize scattering effects. Front face fluorescence sampling geometry was attempted for all samples. Samples were settled overnight in the cuvettes, and the resulting cakes were analyzed. Front face fluorescence geometry was not useful for the conventional vaccine formulation that was frozen (i.e., AH_{FT}/HB_{FT}) because the vaccine particle layer was too compact; therefore, this sample was analyzed suspended in a square cuvette. The emission peak positions were determined by processing the spectra using Origin version 7.0 software. Initially, a 7-point Savitsky–Golay smoothing function was applied to all spectra. The peak positions were then determined using first derivative analysis.

2.2.5. Particle zeta potential

The zeta potential of each sample (with and without rHBsAg) was measured with a Zetasizer Nano Series ZS (Malvern, Westborough, MA). Samples were diluted and resuspended in the formulation buffer. Three readings of each sample were averaged as one of the triplicate sample values for zeta potential measurement.

2.2.6. Scanning electron microscopy and energy dispersive spectroscopy

Alhydrogel® samples at 600 μg Al³⁺ with no rHBsAg were examined by scanning electron microscopy. Samples were washed with sterile water in triplicate prior to analysis. A 5 μl volume of suspended adjuvant was mounted onto a silicon wafer. The samples were dried overnight at room temperature and micrographed at 300× and 4000× using the scanning microscope JEOL 6480LV (JEOL, Peabody, Massachusetts) with an electron beam energy of 5 kV. Elemental content was determined using a NORAN System SIX Energy Dispersive X-ray Spectrometer (EDS) (Thermo Scientific, Waltham, MA).

2.3. In-vivo immunogenicity

All animal experiments were approved and conducted according to the University of Colorado Institutional Animal Care and Use Committee. For antibody studies, five 5-week old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) per formulation were used. Mice were injected on day 0 with a total volume of 100 μl/mouse (50 μl intramuscularly in each hind-leg). Serum samples were collected every two weeks through day 28.

3. Results and discussion

3.1. Particle size and count distribution following freeze-thaw (FT) stress

The particle size distribution in terms of particle counts were evaluated for all vaccines formulations containing Alhydrogel®. The majority of particles in all formulations are in the 1.0–3.0 μm range (Fig. 1). Vaccines subjected to three 24-h FT cycles at -20 °C resulted in agglomerated particles and yielded an altered particle size distributions when compared to the Positive Control (AH/HB) (Fig. 1). This was consistent with results previously reported by

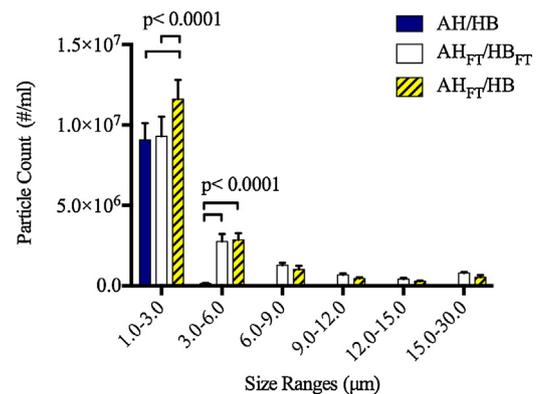


Fig. 1. The effect of three 24-h FT cycles at -20 °C on the agglomeration of vaccine formulations containing Alhydrogel®, (AH_{FT}/HB_{FT}) and FT-treated Alhydrogel® (AH_{FT}/HB) vaccines compared to Positive Control (AH/HB) vaccine was measured using MFI. Total particle count data was evaluated by binning the data in narrow size bins between 1.0 μm and 30.0 μm. (results of two-way ANOVA analyses with Tukey's post-test to compare the control mean values to the FT-treated Alhydrogel® (AH_{FT}/HB) vaccine; error bars the standard deviation of three independent replicates.).

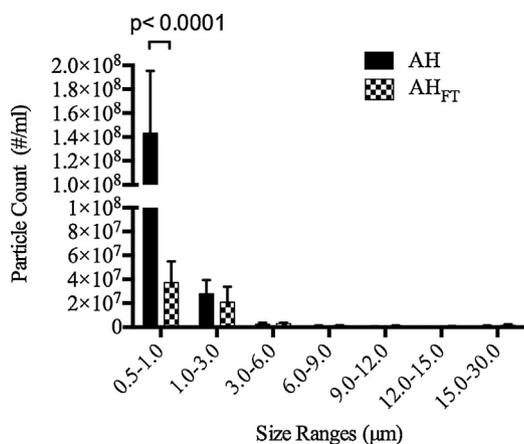


Fig. 2. The number of nanoparticles in Alhydrogel® (AH) samples (no rHBsAg) down to 0.5 µm were measured using an Accusizer particle sizer. (Results of two-way ANOVA analyses with Tukey's post-test: error bars the standard deviation of three independent replicates.).

our own group and others [20–22]. There were no statistical differences in the total particle counts in the less than 3.0 µm across the conventional vaccine formulations (i.e., AH/HB and AH_{FT}/HB_{FT}), regardless of thermal treatment (Fig. 1). In contrast, the FT-treated Alhydrogel® vaccine formulation had significantly more particles in this size range than either conventional vaccine formulation. Both frozen formulations had significantly more particles than the Positive Control vaccine in the 3.0–6.0 µm range. These results suggest that the 1.0–3.0 µm range is being repopulated during the FT-treatment.

Aluminum hydroxide adjuvant has a primary particle size on the order of 10 nm [11]. The 1.0–10.0 µm particles that are often described as being the adjuvant particle is in fact a porous agglomerate of these primary nanoparticles. The increases in the numbers of particles in the 1.0–10.0 µm range in the FT-treated formulations relative to the Positive Control vaccine suggest that (1) large agglomerates were broken into smaller particles due to shear during the MFI measurement and/or (2) populations of nanoparticles agglomerated to yield micron-sized particles after the FT-stress. These phenomena would also explain the similar particle counts for the 1.0–3.0 µm range of the Positive Control and the AH_{FT}/HB_{FT} vaccines despite a larger particle count in the 3.0–6.0 µm range for the AH_{FT}/HB_{FT} vaccine (Fig. 1).

A limitation of MFI is that it cannot measure particles below 1.0 µm. To explore the possibility that nanoparticles in the formulation agglomerate, a particle sizer based on light scattering and light obscuration was used. Unlike MFI, the light scattering detector can detect nanoparticles. The Accusizer is reported to measure particles down to 0.5 µm [23]. To investigate the possibility that nanoparticles repopulated the 1.0–3.0 µm range, a separate study comparing non-stressed and FT-stressed Alhydrogel® preparations lacking antigen was conducted. The results, presented in Fig. 2, indicate that not only did nanoparticles exist in the non-FT-stressed Alhydrogel® but they accounted for a majority of particles measured by the Accusizer. Moreover, this technique yielded a statistically significant decrease in the number of particles in the 0.5–1.0 µm range following FT-stress that was concurrent with a static particle count in the 1.0–3.0 µm range (Fig. 2).

3.2. Antigen adsorption and tertiary structure of adsorbed rHBsAg

The current convention for formulations with aluminum-containing adjuvants is that the antigen should be adsorbed [24]. The World Health Organization requires that in diphtheria and tetanus toxoid vaccines at least 80% of those antigens be adsorbed

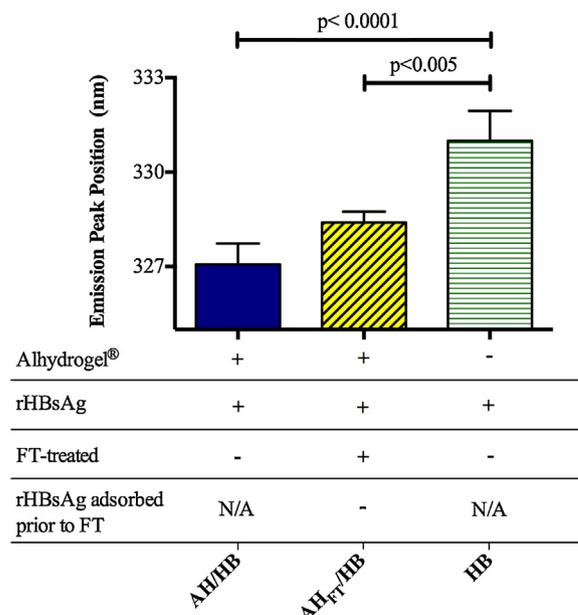


Fig. 3. Intrinsic fluorescence emission peak position: Positive Control (AH/HB), FT-treated Alhydrogel® (AH_{FT}/HB) and soluble rHBsAg (HB) vaccines. (Results of one-way ANOVA analyses with Tukey's post-test to compare the mean values to rHBsAg solution state: error bars the standard deviation of three independent replicates).

to aluminum [25]. In all formulations with Alhydrogel®, the amount of rHBsAg in solution following adsorption was below the limit of detection of the microBCA assay (i.e., less than 2 mg/ml; data not shown). Thus, the microBCA assay indicated that all vaccine formulations with adjuvant met the conventional formulation criteria.

The tertiary structures of adsorbed and solution-state rHBsAg were evaluated using intrinsic fluorescence spectroscopy, a technique that is very sensitive to changes in the local environment of tryptophan (Trp). This type of measurement provides insight into the polarity of the Trp environment. Using excitation wavelengths of 280 nm or 295 nm, the emission spectra peak maxima range for proteins is typically ~308 nm to ~350 nm, correlating to Trp in non-polar or polar environments, respectively. A decrease in the average polarity of the Trp environment results in a blue shift in the spectral peak. Exposure to a more polar environment results in a red shift of the emission peak [26].

The emission peak maximum for rHBsAg in solution was at 331.0 ± 0.9 nm, indicating the Trp residues are in a relatively apolar environment. When rHBsAg was adsorbed to Alhydrogel® in the Positive Control (AH/HB) vaccine or FT-treated Alhydrogel® (AH_{FT}/HB) vaccine, there was a statistically significant blue shift compared to solution state (Fig. 3). It was not possible to analyze the AH_{FT}/HB_{FT} vaccine using front face fluorescence geometry because the vaccine particle layer was too compact; therefore, these samples were analyzed suspended in a square cuvette (data not shown). The blue shift for rHBsAg adsorbed in the Positive Control vaccine or in the FT-treated Alhydrogel® vaccine suggests that the tertiary structure was perturbed upon adsorption to Alhydrogel®. There are conflicting reports in the literature regarding structural perturbation of rHBsAg and other proteins when adsorbed to an aluminum-containing adjuvant [20,27,28]. The data reported here is in agreement with reports that rHBsAg's structure is likely perturbed upon adsorption. However, it is important to state that antigen source, formulation, and vaccine age prior to analysis may affect the observed result. Interestingly, the data in Fig. 3 suggests that the structure is less perturbed when the antigen is adsorbed onto FT-treated Alhydrogel® compared to the Positive Control vaccine.

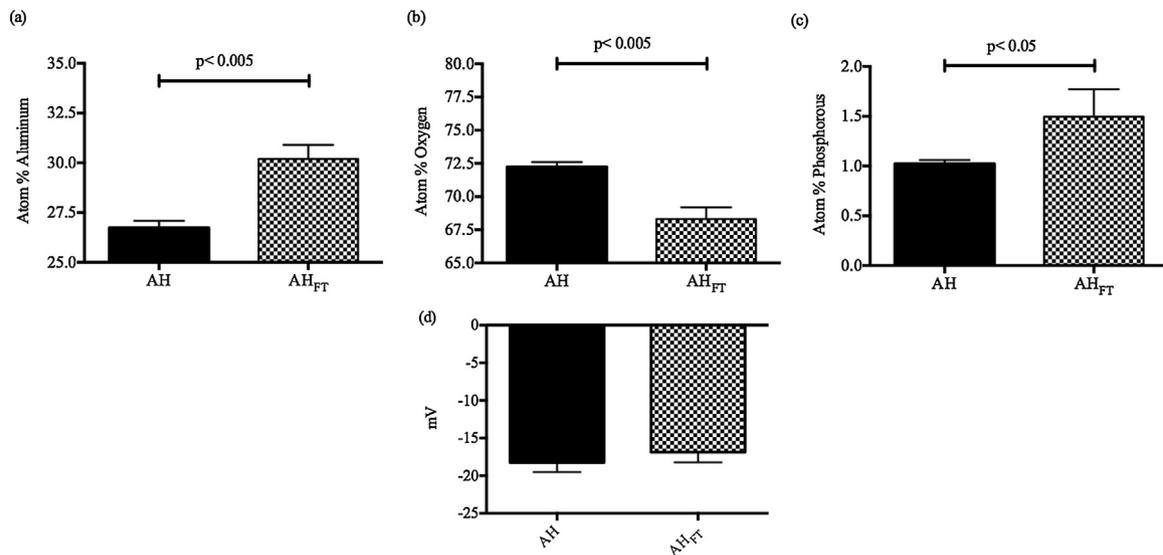


Fig. 4. Atom % of (a) aluminum (b) oxygen (c) phosphorous of Alhydrogel® (AH) in the absence of rHBsAg following three 24-h FT cycles at -20°C measured by SEM-EDS. (d) Effect of FT on the Alhydrogel®'s zeta potential. (Results of unpaired *T*-test. Error bars for EDS results are the standard deviation of three independent sections on one silicon wafer. For zeta potential, three readings of each sample were averaged as one of the triplicate sample values for zeta potential measurement; error bars are indicative of the standard deviation of the triplicate measurements.)

3.3. Elemental content of Alhydrogel following FT stress

Another possible interpretation of shifts in the fluorescence emission peak position of an adsorbed protein is that simply the chemistry (and hence polarity) of the local environment of Trp residues has changed due to a chemical change of the surface, without necessarily a change in the conformation of the protein. A recent study of structural damage of FT-stressed adsorbed vaccines demonstrated that the lattice between the antigen and adjuvant was broken following FT-stress, and it was suggested that it was accompanied by a change in the elemental composition of the surface of the particles [15]. Kurzatkowski et al. did not investigate adjuvant alone samples (i.e., absent of antigen); therefore, structural/elemental changes could not be solely attributed to alterations in the adjuvant elemental properties. In our study, Alhydrogel® was formulated in buffer and stored for 72-h at 4°C or subjected to three FT-cycles and then analyzed by zeta potential and SEM in the absence of antigen. Thus, the observed physico-chemical and elemental content differences would be solely due to changes to the adjuvant.

EDS was utilized to investigate the elemental content of the adjuvant precipitates. The detected elements were aluminum, oxygen and phosphorous (Fig. 4a–c). The contribution of phosphorous was due to ligand exchange with buffer for hydroxyl groups on the adjuvant surface, further verified by the negative zeta potential (Fig. 4d). In both adjuvant formulations, weight and atom percent (wt% and at%) content was highest in oxygen and lowest in phosphorous (Fig. 4b and c, respectively). The oxygen content in FT-stressed adjuvant slightly, but significantly, decreased (Fig. 4b). The aluminum and phosphorous contents in FT-stressed adjuvant slightly, but significantly, increased, (Fig. 4a and c). In the previous report, the loss of oxygen and high aluminum content was suggested to be due to the damaged structure of lattice between antigen and adjuvant [15]. We speculate this loss may be due to dehydration of the adjuvant during freezing or a potential change in the alumina phase, which has been reported to occur with high heat stress [29,30].

Previously, autoclaving of the adjuvant was reported to result in deprotonation/dehydration reactions resulting in a decreased surface area and increased crystallinity [29]. In recent literature,

high heat treatment of boehmite (the mineral form of Alhydrogel®) results in dehydration, an elimination of hydroxyl groups, and formation of various transition aluminas. Furthermore, transition aluminas have much lower surface areas than boehmite [30]. Further investigation of whether the altered elemental content also coincides with an alumina phase transition and/or reduced surface area is therefore warranted.

We also speculate that the increase in the at% of aluminum and phosphorous and decrease in the at% of oxygen is due to fewer exchangeable hydroxyl groups following FT treatment. Less exchangeable hydroxyl groups would reduce phosphate ligand exchange of rHBsAg and result in weaker adsorption strength following FT. In fact, it has previously been reported by others that decreasing exchangeable hydroxyl groups by increasing the phosphorous content in the buffer results in weaker adsorption of rHBsAg onto aluminum hydroxide adjuvant [31].

3.4. In-vivo anti-rHBsAg concentrations

With the exception of the AH_{FT}/HB_{FT} vaccine, the rHBsAg in the formulations was kept at 4°C until use and had not been subjected to FT-stress. The Positive Control vaccine, which contained non-FT stressed Alhydrogel®, was immediately mixed with rHBsAg and injected into mice within 1-h of mixing. All vaccine formulations containing Alhydrogel® were compared to an antigen alone formulation. Inoculating some mice with an adjuvant-free formulation established whether the adjuvant in the remaining formulations had provided a boost in the native anti-rHBsAg antibody response. As seen in Fig. 5, rHBsAg administered without Alhydrogel® elicited a weak immune response at day 28 post-injection (average antibody concentration: $12.33 \pm 0.82 \mu\text{g/ml}$ anti-rHBsAg IgG, refer to Table 1 for formulation abbreviations) in comparison to the Positive Control vaccine (average antibody concentration: $96.93 \pm 25.97 \mu\text{g/ml}$ anti-rHBsAg IgG). This result suggested that we should be able to detect loss of the adjuvant effect in the FT-stressed formulations.

The AH_{FT}/HB_{FT} vaccine was a traditionally formulated vaccine with rHBsAg adsorbed to Alhydrogel® prior to exposure to FT-stress. In addition to the FT induced agglomeration of the vaccine particles, FT labile rHBsAg had the potential to be damaged or

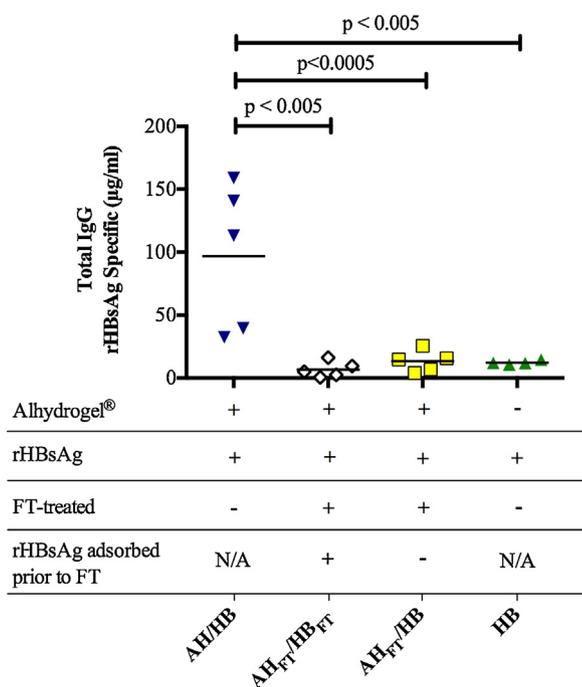


Fig. 5. In vivo anti-rHBsAg ($\mu\text{g/ml}$) at day 28 following intramuscular injection with $5 \mu\text{g}$ rHBsAg alone or adsorbed to $150 \mu\text{g}$ of Alhydrogel®: Positive Control (AH/HB), Negative Control (AH_{FT}/HB_{FT}), FT-treated Alhydrogel® (AH_{FT}/HB) and soluble rHBsAg (HB) vaccines. (Statistical significance was determined by a one-way ANOVA test, followed by a Tukey's multiple comparison test; the line is indicative of the average anti-rHBsAg response.)

obscured by particle agglomeration following the FT-treatments. Thus, stress to the rHBsAg was also anticipated to negatively impact the production of IgG antibodies against native rHBsAg compared to the Positive Control (AH/HB) vaccine and the FT-stressed Alhydrogel® (AH_{FT}/HB) vaccine. As expected, the AH_{FT}/HB_{FT} vaccine elicited the lowest antibody concentration ($6.83 \pm 2.79 \mu\text{g/ml}$). Interestingly, the response was not statistically different than the test formulation where Alhydrogel® was frozen in the absence of rHBsAg (FT-treated Alhydrogel® vaccine) or the Alhydrogel®-free, Soluble rHBsAg formulation. In fact, both formulations exposed to FT-stress elicited anti-rHBsAg titers similar to the Soluble rHBsAg formulation and were statistically less than the Positive Control vaccine.

Studies investigating structural perturbations and chemical modifications following antigen adsorption have reported chemical modification within two weeks of adsorption [32,33]. Furthermore, these studies have correlated these modifications and time following adsorption with a decreased ability to desorb the antigen from the aluminum-containing adjuvant [33]. Previous reports for hepatitis B surface antigen and model antigen vaccines found an inverse relationship between tightness of binding and the elicited immune response [24,31]. The tightness of the interaction between the antigen and adjuvant is expected to increase with time as the ability of desorption decreases with time. In this study the rHBsAg in the AH_{FT}/HB_{FT} vaccine was in the presence of Alhydrogel® during the three 24-h FT cycles; therefore, we evaluated the function of rHBsAg incubation time (three-24 h cycles at 4°C) with non-FT-stressed and FT-stress on the elicited anti-rHBsAg concentration. Interestingly, we did not observe a statistically different immune response; Supplementary Fig. S1. This suggests that an incubation time of 72-h in which rHBsAg is in the presence of Alhydrogel® does not alter the immune response compared to samples administered within 1 h of rHBsAg adsorption. Furthermore, the effect of an extended incubation time on the tertiary structure of

rHBsAg when adsorbed to non-FT-stress Alhydrogel® was investigated and found to not result in a statistically significant shift in the emission peak maximum when compared to the Positive Control vaccine (Supplementary Fig. S2).

It has been previously established that FT-stress of vaccines with adjuvants such as Alhydrogel® can lead to reduced vaccine efficacy [16,20,34–36]. The prevailing thought is that agglomeration of the vaccine particles is largely to blame; however, the emphasis has been primarily on the increase in vaccine particles that are larger than $10.0 \mu\text{m}$. Our results suggest, however, that losses in the immune response are not correlated to losses in the number of particles between $1.0 \mu\text{m}$ and $10.0 \mu\text{m}$ and increases in particles $10.0 \mu\text{m}$ and larger. Instead, the results of a particle size measurements of antigen-free formulations suggest that the reduced total anti-rHBsAg IgG response observed in mice administered FT-stressed formulations with rHBsAg may be correlated to losses in the measured nanoparticle population. Interestingly, recent studies comparing the immune stimulating properties of nanoparticles versus microparticles of other potential adjuvants suggest that nanoparticles may have a more potent effect on the immune response than micron-sized particles [14,37]. Moreover, our adjuvant only investigations indicated that the surface chemistries of non-FT-stressed Alhydrogel® and FT-stressed Alhydrogel® differed. Collectively, these findings suggest that the specific properties that have traditionally been the focus of studies to better understand the FT-inactivation of aluminum-containing adjuvants have not necessarily probed the parameters that are most important to the immune response.

4. Conclusions

AH_{FT}/HB_{FT} and FT-treated Alhydrogel® (i.e., AH_{FT}/HB) vaccines were used to investigate whether losses in antibody concentration following FT-stress were due to adjuvant agglomeration/size, antigen FT instability or a combination of mechanisms. This result suggests that FT damage to Alhydrogel® alone was sufficient to result in a significant reduction in total IgG antibody concentration against rHBsAg. Since no FT-stressed formulation responded statistically better than antigen alone, instability of the antigen does not appear to be a major contributor to the loss of activity in this system. This result suggests that vaccine stabilization strategies should be aimed toward understanding and characterizing the physical properties of the adjuvant, which are necessary to prevent FT-induced efficacy loss.

In all adsorbed formulations, rHBsAg was completely adsorbed and experienced a blue shift in the tertiary structure compared to solution state antigen. All formulations exposed to FT-stress elicited anti-rHBsAg concentrations similar to antigen alone and statistically less than the Positive Control vaccine. Interestingly, the immunological outcome did not correlate to a loss in the number of particles between $1.0 \mu\text{m}$ and $10.0 \mu\text{m}$. Instead, initial evidence collected for Alhydrogel® alone samples (i.e., no rHBsAg) suggests that the immunological outcome may correspond to a distinct loss of particles in the nanoparticle range instead of a loss of micron-sized particles. Furthermore, investigation into the adjuvant's physicochemical properties suggested altered elemental content and surface properties. It still has not been clearly determined what other adjuvant properties are altered following FT-stress. This is the first study to provide definitive evidence, for an rHBsAg vaccine at least, that damage to the adjuvant alone is sufficient to reduce vaccine potency. We suggest that future formulation strategies should be focused on protecting the adjuvant from FT damage to retain vaccine potency. This work highlights a need for a better fundamental understanding of additional physicochemical properties of the adjuvant that are

altered and to more rationally determine how these adjuvant properties can best be stabilized against the adverse effects of FT-stress.

Conflicts of interest statement

The authors declare no conflict of interest.

Acknowledgments

We are thankful to Dr. Philippa Marrack and the Marrack lab for their guidance and technical assistance in the early stages of this study. We are also grateful to Natalia Varaksa and the Nanomaterials Characterization Facility (NCF) at CU-Boulder for assistance with the microscopy studies. Tanya Clapp was supported through a Pre-doctoral Fellowship in Pharmaceutics.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.05.037>.

References

- [1] Glenny APC, Waddington H, Wallace U. The antigenic value of toxoid precipitated by potassium alum. *J Pathol Bacteriol* 1926;29:38–45.
- [2] Matthias DM, Robertson J, Garrison MM, Newland S, Nelson C. Freezing temperatures in the vaccine cold chain: a systematic literature review. *Vaccine* 2007;25:3980–6.
- [3] Immunization News and Information. The VacScene Newsletter. Seattle, WA: Public Health Seattle & King County; 2012.
- [4] McColloster P, Vallbona C. Graphic-output temperature data loggers for monitoring vaccine refrigeration: implications for pertussis. *Am J Public Health* 2011;101:46–7.
- [5] Levinson DR. Vaccines for children program: vulnerabilities in vaccine management. In: OEI-04-10-00430; 2012.
- [6] Kartoglu U, Ozguler NK, Wolfson LJ, Kurzatkowski W. Validation of the shake test for detecting freeze damage to adsorbed vaccines. *Bull World Health Organ* 2010;88:624–31.
- [7] Zapata MI, Feldkamp JR, Peck GE, White JL, Hem SL. Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels. *J Pharm Sci* 1984;73:3–8.
- [8] Dimayuga R, Scheifele D, Bell A. Effects of freezing on DPT and DPT-IPV vaccines, adsorbed. *Canada communicable disease report = Relevé des maladies transmissibles au Canada*, 21; 1995. p. 101–3.
- [9] Grover A, Singh A. Validity of the shake test to identify the frozen damaged vaccine vials. *Indian Pediatr* 2005;42:724–5.
- [10] Johnston CT, Wang SL, Hem SL. Measuring the surface area of aluminum hydroxide adjuvant. *J Pharm Sci* 2002;91:1702–6.
- [11] Hem SL, HogenEsch H. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotential. *Expert Rev Vaccines* 2007;6:685–98.
- [12] Gupta RK. Aluminum compounds as vaccine adjuvants. *Adv Drug Delivery Rev* 1998;32:155–72.
- [13] Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. *Vaccine* 2005;23:1588–95.
- [14] Nygaard UC, Samuelsen M, Aase A, Lovik M. The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. *Toxicol Sci: Off J Soc Toxicol* 2004;82:515–24.
- [15] Kurzatkowski W, Kartoglu U, Staniszweska M, Gorska P, Krause A, Wysocki MJ. Structural damages in adsorbed vaccines affected by freezing. *Biologicals: J Int Assoc Biol Stand* 2013;41:71–6.
- [16] Braun LJ, Tyagi A, Perkins S, Carpenter J, Sylvester D, Guy M, et al. Development of a freeze-stable formulation for vaccines containing aluminum salt adjuvants. *Vaccine* 2009;27:72–9.
- [17] Braun LJ, Jezek J, Peterson S, Tyagi A, Perkins S, Sylvester D, et al. Characterization of a thermostable hepatitis B vaccine formulation. *Vaccine* 2009;27:4609–14.
- [18] Barnard JG, Rhyner MN, Carpenter JF. Critical evaluation and guidance for using the Coulter method for counting subvisible particles in protein solutions. *J Pharm Sci* 2012;101:140–53.
- [19] Barnard JG, Babcock K, Carpenter JF. Characterization and quantitation of aggregates and particles in interferon-beta products: potential links between product quality attributes and immunogenicity. *J Pharm Sci* 2013;102:915–28.
- [20] Chen D, Tyagi A, Carpenter J, Perkins S, Sylvester D, Guy M, et al. Characterization of the freeze sensitivity of a hepatitis B vaccine. *Hum Vaccin* 2009;5:26–32.
- [21] Clausi AL, Merkley SA, Carpenter JF, Randolph TW. Inhibition of aggregation of aluminum hydroxide adjuvant during freezing and drying. *J Pharm Sci* 2008;97:2049–61.
- [22] Salnikova MS, Davis H, Mensch C, Celano L, Thiriot DS. Influence of formulation pH and suspension state on freezing-induced agglomeration of aluminum adjuvants. *J Pharm Sci* 2012;101:1050–62.
- [23] Particle Sizing Systems, Accusizer 780 Syringe Injection Sampler 21 CFR Operations Manual, 2006, Particle Sizing Systems; Santa Barbara, CA.
- [24] Hansen B, Sokolovska A, HogenEsch H, Hem SL. Relationship between the strength of antigen adsorption to an aluminum-containing adjuvant and the immune response. *Vaccine* 2007;25:6618–24.
- [25] World Health Organization. Expanded Programme on Immunization, Manual for production and control of vaccine—tetanus toxoid. Geneva: World Health Organization; 1977.
- [26] Duy C, Fitter J. How aggregation and conformational scrambling of unfolded states govern fluorescence emission spectra. *Biophys J* 2006;90:3704–11.
- [27] Jones LS, Peek LJ, Power J, Markham A, Yazzie B, Middaugh CR. Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. *J Biol Chem* 2005;280:13406–14.
- [28] Tleugabulova D, Falcon V, Penton E. Evidence for the denaturation of recombinant hepatitis B surface antigen on aluminium hydroxide gel. *J Chromatogr B, Biomed Sci Appl* 1998;720:153–63.
- [29] Burrell LS, Lindblad EB, White JL, Hem SL. Stability of aluminium-containing adjuvants to autoclaving. *Vaccine* 1999;17:2599–603.
- [30] Boumaza A, Favaro L, Lédion J, Sattonnay G, Brubach JB, Berthet P, et al. Transition alumina phases induced by heat treatment of boehmite: an X-ray diffraction and infrared spectroscopy study. *J Solid State Chem* 2009;182:1171–6.
- [31] Hansen B, Belfast M, Soung G, Song L, Egan PM, Capen R, et al. Effect of the strength of adsorption of hepatitis B surface antigen to aluminum hydroxide adjuvant on the immune response. *Vaccine* 2009;27:888–92.
- [32] Estey T, Vessely C, Randolph TW, Henderson I, Braun LJ, Nayar R, et al. Evaluation of chemical degradation of a trivalent recombinant protein vaccine against botulinum neurotoxin by LysC peptide mapping and MALDI-TOF mass spectrometry. *J Pharm Sci* 2009;98:2994–3012.
- [33] Vessely C, Estey T, Randolph TW, Henderson I, Cooper J, Nayar R, et al. Stability of a trivalent recombinant protein vaccine formulation against botulinum neurotoxin during storage in aqueous solution. *J Pharm Sci* 2009;98:2970–93.
- [34] PATH. Effects of freezing on vaccine potency: literature review. PATH; 2003.
- [35] The effects of freezing on the appearance, potency and toxicity of adsorbed and unadsorbed DPT vaccines. *Wkly Epidemiol Rec* 1980;55:385–392.
- [36] Boros CA, Hanlon M, Gold MS, Robertson DM. Storage at -3°C for 24 h alters the immunogenicity of pertussis vaccines. *Vaccine* 2001;19:3537–42.
- [37] Li X, Aldayel AM, Cui Z. Aluminum hydroxide nanoparticles show a stronger vaccine adjuvant activity than traditional aluminum hydroxide microparticles. *J Controlled Release* 2014;173:148–57.