Increasing the Potency of an Alhydrogel-Formulated Anthrax Vaccine by Minimizing Antigen-Adjuvant Interactions

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Aluminum salts are the most widely used vaccine adjuvants, and phosphate is known to modulate antigen–adjuvant interactions. Here we report an unexpected role for phosphate buffer in an anthrax vaccine (SparVax) containing recombinant protective antigen (rPA) and aluminum oxyhydroxide (AlOH) adjuvant (Alhydrogel). Phosphate ions bind to AlOH to produce an aluminum phosphate surface with a reduced rPA adsorption coefficient and binding capacity. However, these effects continued to increase as the free phosphate concentration increased, and the binding of rPA changed from endothermic to exothermic. Crucially, phosphate restored the thermostability of bound rPA so that it resembled the soluble form, even though it remained tightly bound to the surface. Batches of vaccine with either 0.25 mM (subsaturated) or 4 mM (saturated) phosphate were tested in a disease model at batch release, which showed that the latter was significantly more potent. Both formulations retained their potency for 3 years. The strongest aluminum adjuvant effects are thus likely to be via weakly attached or easily released native-state antigen proteins.

To combat infectious diseases, subunit vaccines, which consist of a recombinant antigen and an immune-stimulating adjuvant, are increasingly important. These vaccines provide a safer alternative to using live attenuated/inactivated microorganisms or partially purified microbial extracts, while still promoting protective immunity in individuals (1). Currently under development is an anthrax subunit vaccine (SparVax) for anthrax pre- and post-exposure prophylactic treatment. It uses a recombinant protein component, i.e., recombinant protective antigen (rPA), of the anthrax tripartite toxin (2) as the target antigen for toxin-specific neutralizing antibody production. rPA is a relatively poor immunogen by itself, and it needs to be formulated with an adjuvant to provide protection against anthrax infection. In line with several recombinant protein subunit vaccines (3), an aluminum-based adjuvant was chosen, since these mineral adjuvants have been shown to be highly effective and, having been administered to millions of people, have an extensive safety record. The selected adjuvant was Alhydrogel, which is essentially aluminum oxyhydroxide (AlOH) and, with a net positive surface charge, is known to bind to acidic proteins such as rPA (pI 5.6) (4).

The mechanism by which aluminum salts act as adjuvants for vaccine antigens has recently been intensively investigated at the cellular level. Originally they were considered to act simply as a depot maintaining local antigen concentrations (5), but now there are many observations that suggest that more-subtle effects lead to increased protection (6). These effects include NLRP3 inflammasome activation, progestandin production, release of endogenous danger signals such as uric acid or DNA following cell death, binding to membrane lipids, and B cell priming (6, 7). In a series of papers, Hansen and colleagues showed that the strength of antigen adsorption to an aluminum-containing adjuvant is inversely related to the immune response (8–10). They also showed that antigens do not need to be bound to the aluminum salt in order to benefit from the adjuvant effect (11) and that interstitial fluid can contribute to dissociation of the antigen–adjuvant complex in newly formulated vaccines but less so in older samples (12). Recently, it was demonstrated that aluminum-adjuvanted antigen dissociates readily from the adjuvant and removal of the injection site and associated alum depot 2 h after injection does not impair the immune response, which raises questions regarding the role of Alhydrogel in forming a stable antigen depot and of the physical interactions between antigen and adjuvant (13). Therefore, we wished to determine if the physical behavior of different formulations of a clinically relevant antigen–Alhydrogel complex had any correlation with short- or long-term potency.

Structurally, Alhydrogel consists of fine crystalline particles made of corrugated layers of aluminum oxyhydroxide (14). Each aluminum atom is coordinated by four oxygen atoms and two hydroxyl groups (15), the layers are held together by hydrogen bonds, and in aqueous solutions, the particles form aggregates ranging from 1 to 10 μm in diameter (16). Nonmodified Alhydrogel particles have a point of charge, i.e., the pH at which the charge on the colloidal particle is 0, of approximately 11. Therefore, Alhydrogel is positively charged at physiological pH and spontaneously adsorbs acidic proteins by an electrostatic attraction mechanism (17). Mixing the rPA protein with the Alhydrogel

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adjuvant at the appropriate concentrations readily, and very rapidly, yields the rPA-Alhydrogel (rPA-AIOH) complex (18).

Structural studies have shown that Alhydrogel-bound proteins, including rPA, preserve their secondary (19, 20), tertiary (18), and quaternary (21, 22) structures but exhibit decreased thermal stability, compared to their free counterparts in solution (23, 24). At the surfaces of adjuvant particles, bound proteins form a monolayer in which individual biomolecules are packed closely together, with no apparent preference for any particular surface orientation. Moreover, the size of protein–adjuvant particles appears to be similar to that of adjuvant particles alone (22, 25) and thus particle aggregation is not a feature of the process.

In addition to the rPA-AIOH complex, the formulation contained two excipients, i.e., (i) saline at physiological levels was included in the formulation to ensure that the anthrax vaccine was isotonic, for recipient comfort upon injection, and (ii) phosphate ions were included with the initial intention of providing buffering capacity. However, it is well established that phosphate groups ligand exchange with aluminum oxyhydroxide, resulting in modification of the surface properties of the colloidal particles (26).

For this subunit vaccine formulation, the immediate concern is that the ligand-exchange reaction modifies the point of zero charge to such an extent that the acidic recombiant protein becomes desorbed from the aluminum oxyhydroxide surface, resulting in high levels of unbound antigen with potential effects on the subsequent immune response. Furthermore, removing a phosphate ion from solution and replacing it with a hydroxyl group has the potential to modify the pH of the formulation. Indeed, it has also been shown that the pH microenvironment adjacent to the aluminum oxyhydroxide particle surface is approximately 2 pH units higher than that of the surrounding solution (27). Since antigen binding occurs in a monolayer at the surface of the aluminum oxyhydroxide particle (22), the antigen will reside in this microenvironment and be subject to conditions different from those of the bulk solution.

As discussed above, Wittayanukuluk and coworkers (27) showed phosphate to be a modifying agent for aluminum oxyhydroxide, due to the effects of the ligand-exchange reaction, in addition to its role as a buffer. Furthermore, by modifying the physical nature of the aluminum oxyhydroxide, phosphate may also affect the potency of any vaccine when it is used as an adjuvant.

Since phosphate constitutes the physiological buffering agent of the anthrax vaccine, we have studied the effects of phosphate ions on the rPA-AIOH formulation. The aims of these studies were to understand the effects of phosphate on the rPA-AIOH colloidal particles, the structure of the protein antigen, the stability of the formulation, and most importantly the potency of the rPA-AIOH vaccine. The data demonstrate that both surface-bound and free phosphate ions have subtle effects on the firmly bound rPA, causing it to behave like a soluble protein. This in turn results in an enhanced immune response, which helps to explain why the adsorption coefficient of rPA-AIOH binding is inversely related to potency (10).

**MATERIALS AND METHODS**

**rPA-Alhydrogel formulation.** All chemicals and reagents were purchased from either Sigma-Aldrich (United Kingdom) or Melford Laboratories (United Kingdom), unless otherwise stated. Recombinant protective antigen (rPA) was manufactured by current good manufacturing practices by Avecia Biologies (Billingham, United Kingdom). rPA was expressed as inclusion bodies using *Escherichia coli* strain UT5600(DE3)/pET29a. After solubilization with urea, the protein was refolded by dilution and then purified using anion-exchange and hydrophobic interaction chromatography. The highly purified rPA was then buffer exchanged into phosphate-buffered saline by diafiltration (28), and the concentration was adjusted to give ∼1.5 mg/ml. Aliquots of protein were stored at −80°C until required. Aluminum hydroxide gel adjuvant (Alhydrogel) was purchased from either Brenntag Biosector (Denmark) or Sigma-Aldrich (United Kingdom).

rPA was adsorbed to Alhydrogel adjuvant by adding the protein solution to the adjuvant suspension at ambient temperature. Unless otherwise stated, the rPA-AIOH formulations contained 200 μg/ml rPA, 2.6 mg/ml Alhydrogel, 0.9% NaCl, and 0.04% Tween 20 (pH 7.0), with differing concentrations of phosphate. rPA concentrations were measured as absorbance at 280 nm ($A_{280}$), using an extinction coefficient of 1.176 AU per mg/ml to give the concentration of rPA in solution in mg/ml by using quartz cuvettes with 1-cm path lengths (Hellma, Germany) in a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan).

**Alhydrogel phosphate titration.** Phosphate buffer (0 to 5 μmol) was added to 3 mg of Alhydrogel in 1 ml water and vortex mixed. The samples were incubated for 1 h with agitation at ambient temperature and then were centrifuged for 1 min at 20,000 × g. The supernatant was then analyzed for phosphate using a colorimetric assay (51); 400 μL of sample was added to 1,200 μL of reagent mixture (100 mM zinc acetate, 15 mM ammonium molybdate [pH 5.0]), vortex mixed, and allowed to react for 1 min before absorbance was measured at 350 nm in a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan), using quartz cuvettes with 1-cm path lengths (Hellma, Germany). Values were calibrated against a phosphate standard curve of NaH₂PO₄/Na₂HPO₄ (pH 7.0) containing 0 to 700 μM phosphate.

**rPA Langmuir adsorption isotherms.** rPA was combined with Alhydrogel in phosphate buffer (0 to 50 mM), and the samples were incubated for 1 h at ambient temperature, with gentle agitation. Subsequently, all samples were centrifuged for 5 min at 14,600 × g using a benchtop centrifuge, and rPA concentrations in the supernatant were measured using $A_{280}$. The amount of rPA adsorbed to Alhydrogel was calculated by subtracting the rPA remaining in solution from the total added. The adsorption coefficient ($K$) and the binding capacity ($Γ_{max}$) were obtained by linearizing the Langmuir equation (10, 29) and were determined using 1/y-axis intercept and 1/slope, respectively.

**Zeta potential measurements.** The zeta potential was determined using a Zetasizer (Malvern, United Kingdom). One-milliliter samples of rPA-AIOH were introduced into the DTS1060 capillary cells and pre-equilibrated at 20°C for 2 min prior to electrophoretic analysis, according to the manufacturer’s instructions. The zeta potential was automatically determined by the Zetasizer software, using the Smoluchowski equation. Samples were tested in triplicate, with an average of 6 readings per sample.

**Circular dichroism.** Conventional circular dichroism (CD) becomes inaccurate with particulates that settle and with highly scattering solutions (30). To avoid these problems, we used a solid-state CD technique reported previously, which reduces the effects of light scattering and protein aggregation by the use of a specialized rotating sample cell holder (31). The raw data were corrected for protein concentration and converted into differential extinction coefficient ($Δε$) units (M⁻¹ cm⁻¹) by using the molar concentration of amino acid residues for far-UV CD data and the molar concentration of rPA for near-UV data. The spectra were measured in triplicate from freshly prepared independent samples.

**Intrinsic tryptophan fluorescence.** Tryptophan fluorescence was measured at ambient temperature using a Cary Eclipse spectrophotometer (Agilent, United Kingdom) with an excitation wavelength of 280 nm; the emission spectra were measured between 300 and 400 nm. rPA-Alhydrogel samples were measured directly in quartz cuvettes with 1-cm path lengths (Hellma, United Kingdom). To prevent sedimentation and to maintain the homogeneity of the suspended rPA-Alhydrogel particles, samples were stirred using a small magnetic stirring bar placed inside the
cuvette. Data were comparable to those obtained from front-face illumination of a triangular cuvette, but stirring was more reliable in the standard square-footprint design.

In order to provide more-accurate evaluation of the emission spectra, the barycentric mean (the center of an integrated emission curve) was determined using the equation \( \lambda_{\text{bary}} = (\Sigma F_x \times \lambda) / \Sigma F_x \), where \( \lambda_{\text{bary}} \) is the barycentric mean, \( \lambda \) is the wavelength, and \( F_x \) is the point fluorescence at wavelength \( \lambda \) (32). Thermal denaturation was monitored via tryptophan fluorescence using a Cary Eclipse spectrophotometer equipped with a temperature controller and quartz cuvettes with 1-cm path lengths (Hellma, United Kingdom) with plastic lids. A small magnetic stirring bar inside the cuvette stirred the sample while the temperature was increased from 25°C to 65°C at a rate of 1°C min\(^{-1}\). The variation of fluorescence (\( F \)) with increasing temperature (\( T \)) was measured using wavelengths of 280 nm for excitation and 340 nm for emission. The transition temperature was determined as the peak value of the first-order derivative \( df/dT \).

**Calorimetry.** Differential scanning calorimetry measurements were made using a VP-DSC microcalorimeter (MicroCal, United Kingdom). Prior to analysis, all samples were degassed in a Thermostar unit (MicroCal, United Kingdom). Samples of rPA-Alhydrogel with 0.3 mg/ml rPA and 3 mg/ml phosphate-saturated Alhydrogel in either 1, 5, 10, 20, or 50 mM free phosphate were analyzed. Samples were scanned between 25°C and 60°C at the rate of 1°C min\(^{-1}\), and Alhydrogel diluted in the formulation buffer was used as a reference. Scans were not completely reversible, and thus the quantitative thermodynamic interpretation is limited. The data were simply processed using MicroCal Origin software to obtain the \( \Delta H \) and thus the quantitative thermodynamic interpretation is limited. The reference buffer was used as a reference. Scans were not completely reversible, and thus the quantitative thermodynamic interpretation is limited. The variation of fluorescence (\( F \)) with increasing temperature (\( T \)) was measured using wavelengths of 280 nm for excitation and 340 nm for emission. The transition temperature was determined as the peak value of the first-order derivative \( df/dT \).

**RESULTS**

**Phosphate titration of Alhydrogel.** The saturation curve of phosphate binding to Alhydrogel was determined in the absence of rPA. All added phosphate bound to the Alhydrogel until the saturation point was reached, when residual free phosphate appeared in the supernatant. It was evident from the binding curve that, for 1.5 mg of Alhydrogel in 1 ml, saturation was reached when 0.65 μmol of phosphate had bound (phosphate concentration of around 1 mM in this experiment). This gives a saturation level of 0.43 μmol of phosphate per mg of Alhydrogel (Fig. 1).

**Langmuir adsorption isotherms.** The ligand-exchange reaction introduces negative charge to the surface of Alhydrogel; to characterize the effect on rPA-Alhydrogel interactions, we determined the strength of binding (adsorption coefficient) and the binding capacity by fitting data to a Langmuir adsorption isotherm (10, 29). This analysis was performed with both nonmodified and phosphate-saturated Alhydrogel. When it was observed that rPA still bound to phosphate-saturated Alhydrogel, we extended the analysis to examine the effects of free phosphate on the protein-adsorbed interactions. The concentrations of free phosphate tested were between 0 and 50 mM. In Langmuir analysis, the strength of binding is given by the adsorption coefficient, with higher adsorption coefficient values indicating stronger interactions. For nonmodified Alhydrogel, rPA had an adsorption coefficient of 215 ml/mg. This value substantially decreased in the presence of phosphate (Fig. 2A). Similarly, there was a sharp reduction in the binding capacity (Fig. 2B). Both the adsorption coefficient and the binding capacity were further reduced by free phosphate, before reaching a steady state at around 5 to 10 mM phosphate.

To evaluate directly the effects of phosphate on the normal
vaccine formulation, rPA at 200 μg/ml was formulated with 2.6 mg/ml Alhydrogel at increasing concentrations of phosphate, and the level of unbound rPA was determined using UV absorbance (Fig. 2C). As expected, when the phosphate levels were increased, the levels of unbound rPA correspondingly increased. Whereas the unbound rPA content remained below 5% with 5 mM phosphate, the content increased to around 12% with 10 mM phosphate.

Zeta potential measurements. Changes in the Alhydrogel surface charge (zeta potential) as a function of phosphate concentration were measured using a Zetasizer Nano ZS system (Malvern). The analysis was performed with Alhydrogel (2.6 mg/ml) alone and with 200 μg/ml rPA bound to 2.6 mg/ml Alhydrogel, with increasing concentrations of phosphate. As expected from previous studies (33), the zeta potential of Alhydrogel was positive at physiological pH. With increasing phosphate saturation, the potential rapidly declined and eventually become negative (Fig. 3), with a point of 0 charge at 2.5 mM phosphate. Alhydrogel with adsorbed rPA exhibited similar changes; however, the initial zeta potential of Alhydrogel with bound rPA was lower than that of Alhydrogel alone and the point of 0 charge was at 2.3 mM phosphate. This initial difference was attributed to the negative charge on the adsorbed protein. The zeta potential values for Alhydrogel and rPA-AlOH converged around 3.5 mM.

Isothermal titration calorimetry of rPA binding to Alhydrogel. The modified adjuvant was prepared by treating Alhydrogel with phosphate to produce saturation levels of 10, 50, and 100%, according to the phosphate saturation curve in Fig. 1. Titration of

![Fig 1](image1.png)
**Fig 1** Saturation curve for binding of phosphate to Alhydrogel (ALH). These data were fitted to the Boltzmann sigmoid equation using nonlinear regression analysis in Origin software (version 7.5; OriginLab Corp.). (Inset) Raw absorbance data obtained in the spectrophotometric phosphate assay.

![Fig 2](image2.png)
**Fig 2** Results from linear regression fit to the Langmuir adsorption isotherm, showing the effects of phosphate on the adsorption coefficient (A), the binding capacity of Alhydrogel for rPA (B), and the fraction of rPA binding to Alhydrogel (C) in samples of 200 μg/ml rPA and 2.6 mg/ml Alhydrogel.
nonmodified Alhydrogel with rPA gave a series of endothermic peaks (Fig. 4A), whose magnitude decreased gradually through the titration due to a decrease of free binding sites on Alhydrogel. In stark contrast, the binding of rPA to phosphate-saturated Alhydrogel was exothermic, and the adjuvant was more rapidly saturated with the protein, implying that fewer binding sites were available (Fig. 4B). Similarly, titration of 50% phosphate-saturated Alhydrogel was exothermic but the magnitude was less than that of phosphate-saturated Alhydrogel (Fig. 4C). A complex result was observed with 10% phosphate-saturated Alhydrogel, which presumably presents mixed binding surfaces to the rPA. The interaction was endothermic at the beginning of titration but gradually changed to exothermic (Fig. 4D), suggesting that the initial entropy-driven interaction with AlOH was preferred over the subsequent enthalpy-driven binding to phosphate-modified surfaces.

**Solid-state circular dichroism.** The solid-state (31,34) far-UV CD spectra of rPA-AlOH closely resembled those of soluble rPA (35) (Fig. 5A), indicating minimal secondary structure perturbation upon binding to Alhydrogel. Increasing phosphate concentrations did not significantly affect the spectra. The near-UV solid-state CD spectra of rPA-AlOH at 0.25 mM phosphate, 4 mM phosphate, and 10 mM phosphate were also similar, with each showing the two prominent positive CD bands for tryptophan at 284 nm and 291 nm. These two bands, which are characteristic of rPA (31,35), were weaker than for soluble rPA in solution (Fig. 5B); in the 10 mM phosphate sample, two bands (272 nm and 276 nm) that were not present for soluble rPA were observed. In the

![FIG 4](image1.png)  
**FIG 4** Titration of Alhydrogel with rPA, showing thermograms for nonmodified Alhydrogel (A), 100% phosphate-saturated Alhydrogel (B), 50% phosphate-saturated Alhydrogel (C), and 10% phosphate-saturated Alhydrogel (D). (Insets) Diagrams showing the likely surface chemistry of the variously saturated adjuvants.

![FIG 5](image2.png)  
**FIG 5** Effects of phosphate (phos) on solid-state circular dichroism of 200 μg/ml rPA with 2.6 mg/ml Alhydrogel (Alh). (A) Far-UV CD spectra. (B) Near-UV CD spectra.
phenylalanine region, a broad positive band from 250 to 260 nm was seen for soluble rPA, which sharpened when rPA was bound to Alhydrogel, irrespective of the phosphate concentration. Thus, the phosphate modification of the Alhydrogel interaction has clear but subtle effects on the tertiary structure of rPA.

**Differential scanning calorimetry.** The transition temperature ($T_m$) and calorimetric enthalpy ($\Delta H_{cal}$) of rPA are strongly affected by binding to Alhydrogel (18, 23). Surprisingly, the protein had no measurable melting transition when it was adsorbed to nonmodified or phosphate-saturated Alhydrogel. However, as the free phosphate concentration increased, there appeared to be a distinct thermal transition that eventually resembled the behavior of free rPA (Fig. 6A). Between 1 and 50 mM phosphate, the $T_m$ rose from 43.3°C to 48.2°C and the $\Delta H_{cal}$ increased from 31 kcal mol$^{-1}$ to 139 kcal mol$^{-1}$ (Fig. 6B). The $T_m$ and $\Delta H_{cal}$ of nonadjuvanted rPA protein were 49.2°C and 219 kcal mol$^{-1}$, respectively. Plotting $T_m$ and $\Delta H_{cal}$ values against the fraction of bound protein revealed that these changes in thermodynamic parameters took place when $>90\%$ of rPA was still bound to Alhydrogel (Fig. 6C). Conversely, above 10 mM phosphate, when protein binding was less than 90\% (Fig. 2C), there were only minor changes in $T_m$ and $\Delta H_{cal}$. The phosphate concentration range in which major thermodynamic changes occurred was 1 to 10 mM, at which the majority of the rPA was in the bound form.

**Tryptophan fluorescence.** The tryptophan emission spectrum of rPA on Alhydrogel was similar to that of free rPA, irrespective of phosphate concentration (see the supplemental material). All spectra exhibited maxima near 330 nm and had the same barycentric mean values of 340.6 ± 0.1 nm (18). The tryptophan emission spectrum of rPA on Alhydrogel had weaker intensity than the emission spectrum of rPA without adjuvant, likely due to inhomogeneous distribution of fluorophores in solution, the colloidal nature of the sample, and light scattering. Overall, these data demonstrate no measurable changes in the protein tertiary structure due to phosphate (see the supplemental material).

Protein unfolding was monitored by tryptophan fluorescence while the temperature was increased from 25°C to 65°C at the rate of 1°C min$^{-1}$ (18). In samples containing nonmodified and 50\% and 100\% phosphate-saturated Alhydrogel, the protein had no distinct unfolding transition and exhibited only small changes in fluorescence, most of which were due to the quenching effects of higher temperatures (Fig. 7A). However, with free phosphate (i.e., around 2.6 mM), rPA had a well-defined transition region between 40°C and 50°C (Fig. 7B). It should be noted that increasing phosphate concentrations shifted the transition midpoint from 43.5°C to 48.0°C, compared to the 48.5°C transition temperature of nonadjuvanted rPA. It should also be noted that, in 10 mM phosphate, nearly 90\% of rPA was bound to Alhydrogel.

**Measurements of potency and stability.** For practical and ethical reasons, the study of the effects of phosphate ions on the potency of the rPA-AlOH formulation used only two formulations, namely, 0.25 mM and 4 mM phosphate rPA-AlOH. The 0.25 mM phosphate rPA-AlOH represented an unsaturated formulation in which the Alhydrogel retained surface hydroxyl groups, whereas the 4 mM phosphate rPA-AlOH represented phosphate-saturated Alhydrogel plus free/excess phosphate. The average pH values of the bulk solutions were 6.0 ± 0.3 ($n = 4$) and 7.1 ± 0.1 ($n = 7$) for the 0.25 mM and 4 mM phosphate rPA-AlOH formulations, respectively.

Potency was determined using the mouse anthrax challenge assay, evaluating several batches of these two formulation prototypes immediately following manufacture. For each potency analysis, a 4-fold dilution curve was determined. The ED$_{50}$ values were estimated by fitting a survival analysis model to the results of the potency assay, using a model that assumes that the survival times have a log-normal distribution and that there is a linear relation-
ship between log dose and log survival time (Table 1) (36). It
should be noted that, for three of the 4 mM phosphate rPA-AlOH
batches, more than one measurement of ED$_{50}$ was taken at release
and these data have been incorporated into the statistical analysis.
The weighted mean ED$_{50}$ for the 0.25 mM phosphate rPA-AlOH
batches was 0.21 µg/0.1 ml (back-transformed from the mean
log$_{10}$ED$_{50}$ value of −0.679), whereas the value for 4 mM phos-
phate rPA-AlOH was 0.04 µg/0.1 ml (mean log$_{10}$ED$_{50}$ value of
−1.398). The weighted mean was used (weighted by the inverse of
the variance of the log$_{10}$ED$_{50}$) to down-weight less-precise
log$_{10}$ED$_{50}$ estimates. Statistical analysis using analysis of variance
(ANOVA) indicated that, at release, 4 mM phosphate rPA-AlOH
was significantly more potent than 0.25 mM phosphate rPA-
AlOH, as shown by a difference in logED$_{50}$ values (P = 0.006).
The ANOVA took batch and operator into account. Comparison of
the mean ED$_{50}$ values indicated that the 4 mM phosphate rPA-
AlOH formulation was 5.25-fold more potent than the 0.25 mM
phosphate formulation.

In addition, long-term, real-time stability studies were per-
formed with a batch of 0.25 mM phosphate rPA-AlOH and a
batch of 4 mM phosphate rPA-AlOH. In accordance with Inter-
national Conference on Harmonisation guideline Q1A(R2) (37),
the materials were stored for at least 3 years at 2 to 8°C under
controlled conditions, which were monitored to ensure tempera-
ture compliance. During the 3 years of storage, samples were
analyzed for potency using the mouse anthrax challenge assay.
Trending analysis was performed after 3 years to determine the
overall stability of the two vaccine formulations, and the data are
presented in Fig. 8.

Linear regression analysis of data for the 0.25 mM phosphate
rPA-AlOH formulation after 39 months of storage at 2 to 8°C
revealed no evidence of an increase in logED$_{50}$ values after man-
ufacture (intercept = −0.414 [ED$_{50}$ = 0.385]; slope = 0.007; P = 
0.480). Similarly, the 4 mM phosphate rPA-AlOH formulation
exhibited no evidence of a significant increase in logED$_{50}$ after 36
months of storage at 2 to 8°C (intercept = −1.277 [ED$_{50}$ = 0.0528];
slope = 0.007; P = 0.673). Trending analysis was also
performed with three batches of the 4 mM phosphate rPA-AlOH
formulation, to provide supporting data on stability. Using rela-
tive potency versus a freshly formulated rPA-AlOH standard, the
rPA-AlOH formulation was shown to be stable for at least 37
months (see the supplemental material).

**DISCUSSION**

Aluminum-based adjuvants are used extensively in the formul-
lation of a range of vaccine types, including subunit vaccines, being
both effective in boosting immune responses and safe for use in
humans (14). Although mainly used against infectious diseases,
they are also effective in anticancer vaccines (38). Aluminum oxy-
hydroxide formulations, such as Alhydrogel, are the most com-
monly used since they are positively charged at physiological pH
and bind most antigens, which tend to be acidic. A significant
feature of aluminum oxyhydroxide chemistry is the ligand-ex-
change reaction, whereby surface hydroxyl groups are readily ex-
changed for phosphate ions (26). Consequently, the surface layer
of the aluminum oxyhydroxide is converted to aluminum phos-
phate, which radically changes the surface charge properties of the
Alhydrogel particles. Consequently, we performed a series of studies
to explore how phosphate modification of Alhydrogel (27)
affects the properties of the vaccine. Upon mixing, phosphate ions
rapidly bound to Alhydrogel particles until a saturation point was

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**TABLE 1** Summary of potency data at release for 0.25 mM phosphate
and 4 mM phosphate in final rPA-AlOH drug product

| Material type   | Batch no. | n  | LogED$_{50}$ | SD  | ED$_{50}$ (µg/0.1 ml) |
|-----------------|-----------|----|--------------|-----|----------------------|
| 0.25 mM phosphate FDP$^a$ | 1 | 1   | −0.130 | 0.285 |
|                 | 2 | 1   | −1.681 | 0.514 |
|                 | 3 | 1   | −0.484 | 0.193 |
|                 | 4 | 1   | −1.338 | 0.288 |
| Weighted mean   |           |    |            |     | 0.21                 |
| 4 mM phosphate FDP | 5 | 1   | −2.202 | 0.789 |
|                 | 6 | 4   | −1.133 | 0.154 |
|                 | 7 | 4   | −1.420 | 0.129 |
|                 | 8 | 1   | 0.348  | 0.594 |
|                 | 9 | 2   | −0.505 | 0.241 |
|                 | 10| 1   | −1.175 | 0.235 |
|                 | 11| 1   | −1.397 | 0.265 |
| Weighted mean   |           |    |            |     | 0.04                 |

$^a$FDP, final drug product.

$^b$An n value of 1 means 1 test item comprising a 4-point dilution series with 15 mice/
dose and compared to the reference standard. On some occasions, the analysis was
repeated up to 4 times to test reproducibility.
reached, with 0.43 μmol phosphate saturating 12.8 μmol (1 mg) of Alhydrogel. Since there are 2 mol of hydroxyl groups per 1 mol of Alhydrogel, the theoretical maximum for phosphate saturation requires 12.8 × 2 (i.e., 25.6) μmol phosphate. Thus, we assume that only 1.7% of potential reactive groups were on the surface and available for phosphate binding. This confirms that the majority of the hydroxyl groups are not exposed on the surface but are contained within the colloidal particle itself (21).

Despite the reversal in surface charge (Fig. 3), it was surprising that, even with supersaturating concentrations of phosphate, the acidic rPA protein at the formulation density of 200 μg per 2.6 mg of AlOH was still predominantly bound (>90%) to the Alhydrogel particles (Fig. 3). One possible explanation might be the variety of charged groups on rPA. Although acidic, with a pI of 5.6, rPA contains 85 cationic (Arg plus Lys) versus 96 anionic (Asp plus Glu) side chains, and the former may be sufficient to bind to the negatively charged phosphate groups in a more selective manner. The effects of increasing phosphate concentrations extended beyond the point of surface saturation, which suggests that the surface charge of the Alhydrogel is not determined purely by the extent of ligand exchange. There is thus also a role for free phosphate in modulating the interactions between the rPA and the Alhydrogel surface.

On native Alhydrogel, the interaction of rPA with the hydroxyl groups was endothermic, meaning that it was driven by an increase in entropy, an effect often attributed to dehydration effects. This would imply that there is a significant reduction of the water-accessible surface upon rPA binding (39, 40). In contrast, above the point of phosphate saturation, the reaction became exothermic and was no longer driven solely by entropy. This enthalpy-favored interaction could be associated with the formation of direct noncovalent bonds at the particle surface. At subsaturating phosphate concentrations, the thermodynamics are complex; at 10% saturation, there is evidence for a

FIG 8 Stability of logED_{50} for rPA-AlOH with 0.25 mM phosphate (A) and rPA-AlOH with 4 mM phosphate (B), both stored at 2 to 8°C. Error bars, standard deviation; solid line, trending line; dashed line, one-sided 95% confidence interval (CI).
genuinely mixed surface offering high-affinity (endothelial) and low-affinity (exothelial) sites.

On phosphate-saturated Alhydrogel, cooperative thermal unfolding (18, 23) was more evident and became increasingly pronounced with increased free phosphate, although the protein was still bound to the Alhydrogel. With increasing phosphate concentrations, surface-bound proteins thus appear to increasingly behave as though in solution. Thus, enhanced hydration (41) could explain both the ITC data and the increased protein thermostability.

Of paramount importance are the role of phosphate in the potency of the rPA subunit vaccine and its stability when stored under refrigeration. The phosphate-subsaturated 0.25 mM phosphate rPA-AlOH formulation was significantly less potent than the 4 mM phosphate rPA-AlOH formulation, and one contrasting feature of the two formulations was the pH of the bulk solution, with the 0.25 mM phosphate rPA-AlOH formulation having a pH of 5.9 and the 4 mM phosphate rPA-AlOH formulation a value of around 7.1. Consequently, the differing bulk solution pH values might explain the differences in ED50 values, particularly since rPA is acid labile (35, 42). However, the biophysical analysis of the protein structure of rPA-AlOH under low-phosphate conditions does not support any major acid-induced transformation of the protein structure (31, 35). Furthermore, with 0.25 mM phosphate rPA-AlOH, the Alhydrogel surface and bound antigen would have a local pH approximately 2 units higher, due to the positive surface charge attracting hydroxyl anions (27). In contrast, the negatively charged phosphate-saturated Alhydrogel would be expected to attract protons and hence have a more acidic microenvironment than the bulk solution.

Hansen and coworkers have hypothesized that the immunogenic response to an aluminum-adjuvanted protein antigen is inversely related to the adsorption coefficient (10). They and others have also shown that antigens can be rapidly released from the Alhydrogel depot, suggesting that the directly interacting adjuvant and antigen do not stimulate the immune response (11, 13, 43). Consequently, the ability of phosphate to modulate the adsorption coefficient of an acidic protein antigen and aluminum oxyhydroxide adjuvants might appear to be irrelevant. However, enhanced immunogenicity was seen when a recombinant Candida antigen (rAlk3p-N) was diluted with phosphate-buffered saline versus saline alone (44). Similarly, phosphate modulation of aluminum oxyhydroxide in both hepatitis B surface antigen and HIV-1 (SFI62D2gpl40) subunit vaccines demonstrated enhancement of immunogenicity (8, 9).

There have been several mechanisms of action for aluminum-containing adjuvants, including acting as a depot in tissues to produce prolonged exposure (45), enhanced delivery of antigen to antigen-presenting cells (46), induction of uric acid for activation of inflammatory dendritic cells (47), and enhanced proteolytic processing by the immune system due to destabilization of the antigen structure (23). However, compelling evidence has recently been produced to demonstrate that aluminum-containing adjuvants have direct effects on dendritic cells (6, 7, 48). Certainly a weakly bound antigen, as found in phosphate-saturated Alhydrogel, would be more likely to be internalized and processed by dendritic cells. It should be noted that the physiological level of phosphate is strictly regulated at 0.8 to 1.4 mM (49).

Upon injection into this phosphate concentration, exposed surface hydroxyl groups are likely to be replaced, and if it is not saturated with phosphate already, the aluminum oxyhydroxide will be saturated following injection. Surprisingly, our data show that the difference between the 0.25 mM phosphate rPA-AlOH and 4 mM phosphate rPA-AlOH formulations persists. It is possible that binding of the rPA to the Alhydrogel under subsaturating conditions occludes hydroxyl groups and prevents subsequent phosphate exchange. Under such circumstances, the stronger binding conditions may prevail even with later exposure to physiological phosphate concentrations. Although Iyer et al. showed that aluminum adjuvant interactions with a basic protein (lysozyme) could be reversed in interstitial fluid, this was true only for freshly formulated mixtures of acidic ovalbumin, and older samples did not release antigen (50). Thus, if both rPA (pI 5.6) and ovalbumin (pI 4.7) rearrange slowly on the adjuvant surface, then the effect of phosphate we observe may be indefinite maintenance of the loose interaction state.

Overall, the results are consistent with previous data showing that phosphate is a beneficial modulating agent for rPA binding to Alhydrogel. This study goes much further, however, in clarifying the role of phosphate, which could be explained by weaker interactions allowing a more complete water layer to be formed between the protein and the adjuvant. Thus, the hypothesis of Hansen and coworkers that the immunogenic response to an aluminum-adjuvanted protein antigen is inversely related to the adsorption coefficient (10) is strongly confirmed but also is extended to include the physical effects on the protein and the effects on potency over a long period. Combined with recent data indicating that some adjuvant-protein interactions rapidly dissociate upon injection (13), it appears that the strongest aluminum adjuvant effects are likely to be via weakly attached or easily released native-state antigen proteins.

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REFERENCES

1. Liljeqvist S, Stahl S. 1999. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. J. Biotechnol. 73:1–33.
2. Petosa C, Collier JR, Klimpel KR, Leppla SH, Liddington RC. 1997. Crystal structure of the anthrax toxin protective antigen. Nature 385:833–838.
3. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. 2008. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. Int. J. Pharm. 364:272–280.
4. Rinella JV, White JL, Hem SL. 1996. Treatment of aluminium hydroxide adjuvant to optimize the adsorption of basic proteins. Vaccine 14:298–306.
5. Glenny AT, Pope CG, Waddington H, Wallace U. 1926. Immunology notes. XXIII. The antigenic value of toxoid precipitated by potassium alum. J. Pathol. Bacteriol. 29:31–40.
6. Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, Seamone ME, Vilaysane A, Musci AD, Fong Y, Prenner E, Ling CC, Tschopp J, Muruve DA, Amrein MW, Shi Y. 2011. Alum interaction with dendritic
cell membrane lipids is essential for its adjuvanticity. Nat. Med. 17:479–487.
7. Levitz SM, Golenbock DT. 2012. Beyond empiricism: informing vaccine development through innate immunity research. Cell 148:1284–1292.
8. Hansen B, Mahalga P, Singh M, Sun Y, Srivastava I, Hogenesch H, Hem SL. 2011. Effect on the strength of adsorption of HIV 1 SF162/22gp 1-80 to aluminum-containing adjuvants on the immune response. J. Pharm. Sci. 100:3245–3250.
9. Hansen B, Belfast M, Soung G, Song I, Egan PM, Capen R, HogenEsch H, Mancinelli R, Hem SL. 2009. Effect of the strength of adsorption of hepatitis B surface antigen to aluminum hydroxide adjuvant on the immune response. Vaccine 27:8888–892.
10. Hansen B, Sokolovska A, HogenEsch H, Hem SL. 2007. Relationship between the strength of antigen adsorption to an aluminum-containing adjuvant and the immune response. Vaccine 25:6616–6624.
11. Romero Mendez IZ, Shi Y, HogenEsch H, Hem SL. 2007. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. Vaccine 25:825–833.
12. Jiang DP, Morefield GL, HogenEsch H, Hem SL. 2006. Relationship of adsorption mechanism of antigens by aluminum-containing adjuvants to in vitro elution in interstitial fluid. Vaccine 24:1665–1669.
13. Hutchison S, Benson RA, Gibson VB, Pollock AH, Garside P, Brewer JM. 2012. Antigen depot is not required for alum adjuvanticity. FASEB J. 26:1272–1279.
14. Shirodkar S, Hutchison RL, Perry DL, White JL, Hem SL. 1990. Aluminium compounds used as adjuvants in vaccines. Pharm. Res. 7:1288–1292.
15. Bruhne S, Gottlieb S, Assmus W, Alig E, Schmidt MU. 2008. Atomic structure analysis of nanocrystalline boehmite AIO(OH). Crystal Growth Design 8:489–493.
16. Yau PK, Schulze DG, Johnston CT, Hem SL. 2006. Aluminum hydroxide adjuvant produced under constant reaction concentration. J. Pharm. Sci. 95:1822–1833.
17. Seebet S, White JH, Hem SL. 1991. Predicting the adsorption of proteins by aluminium-containing adjuvants. Vaccine 9:201–203.
18. Soliakov A, Kelly IF, Lakey JH, Waterston A. 2012. Anthrax sub-unit vaccine: the structural consequences of binding rPA83 to Alhydrogel®. Eur. J. Pharm. Biopharm. 80:25–32.
19. Agopian A, Ronzon F, Sauzeat E, Sodoyer R, El Habib R, Buchet R, Chevalier M. 2007. Secondary structure analysis of HIV-1 gp41 in solution and adsorbed to aluminium hydroxide by Fourier transform infrared spectroscopy. Biochim. Biophys. Acta 1774:351–358.
20. Dong A, Jones LS, Kerwin BA, Krishnan S, Carpenter JE. 2006. Secondary structures of proteins adsorbed onto aluminium hydroxide: infrared spectroscopic analysis of proteins from low solution concentrations. Anal. Biochem. 351:282–289.
21. Harris JR, Soliakov A, Lewis RJ, Depoez F, Waterston A, Lakey JH. 2012. Alhydrogel® adjuvant, ultrasonic dispersion and protein binding: a TEM and analytical study. Micron 43:192–200.
22. Soliakov A, Harris JR, Waterston A, Lakey JH. 2010. The structure of Yersinia pestis PstG1 polymer in free and adjuvant bound states. Vaccine 28:5746–5754.
23. Jones LS, Peak LJ, Power J, Markham A, Yazzie B, Middaugh CR. 2005. Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. J. Biol. Chem. 280:13406–13414.
24. Peak LJ, Martin TT, Nation CE, Pegram SA, Middaugh CR. 2007. Effects of stabilizers on the destabilization of proteins upon adsorption to aluminum salt adjuvants. J. Pharm. Sci. 96:547–557.
25. Morefield GL, HogenEsch H, Robinson JP, Hem SL. 2004. Distribution of adsorbed antigen in mono-valent and combination vaccines. Vaccine 22:1973–1984.
26. Hem SL, HogenEsch H. 2007. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiating activity. Expert Rev. Vaccines 6:685–698.
27. Wittayanuskuluk A, Jiang DP, Regnier FE, Hem SL. 2004. Effect of microenvironment pH of aluminum hydroxide HIIVJ adjuvant on the chemical stability of adsorbed antigen. Vaccine 22:1172–1176.
28. Williamson ED, Hodgson I, Walker NJ, Topping AW, Duchsars MG, Mott JM, Estep J, LeBatt C, Flick-Smith HC, Jones HE, Li H, Quinn CP. 2005. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect. Immun. 73:5978–5987.
29. Langmuir J, 1916. The constitution and fundamental properties of solids and liquids: part 1: solids. J. Am. Chem. Soc. 38:2221–2295.
30. Bustamante C, Maestre MF. 1988. Statistical effects in the absorption and optical activity of particulate suspensions. Proc. Natl. Acad. Sci. U. S. A. 85:4882–4886.
31. Ganesan A, Waterston A, Moore BD. 2012. Biophysical characterisation of thermal-induced precipitates of recombinant anthrax protective antigen: evidence for kinetically trapped unfolding domains in solid-state. Eur. J. Pharm. Biopharm. 82:475–484.
32. Lakey JH, Magetedana R, Ptak M. 1989. The lipopeptide antibiotic A21978C has a specific interaction with DMPC only in the presence of calcium-ions. Biochim. Biophys. Acta 985:60–66.
33. Lindblad EB. 2004. Aluminum compounds for use in vaccines. Immuno. Cell Biol. 82:497–505.
34. Ganesan A, Lyle C, Halling PJ, Kelly S, Price N, Moore BD. 2006. Assessing the structure of immobilised proteins and antigens using circular dichroism. J. Pharm. Pharmacol. 58:9–10.
35. Chalton DA, Kelly IF, McGregor A, Ridley H, Waterston A, Miller J, Lakey JH. 2007. Unfolding transitions of Bacillus anthracis protective antigen. Arch. Biochem. Biophys. 465:1–10.
36. Collett D. 2003. Modelling survival data in medical research, 2nd ed. CRC Press, Boca Raton, FL.
37. Food and Drug Administration. 2004. International Conference on Harmonisation: evaluation of stability data: availability. J. Pharm. Sci. 93:215–230.
38. Cooper A, Johnson CM, Lakey JH, Nollmann M. 2001. Heat does not come in different colours: entropy-enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions. Biochim. Biophys. Chem. 3:215–230.
39. Jelnesarov I, Bosshard HR. 1999. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. J. Mol. Recognit. 12:3–18.
40. Svergun DI, Richard S, Koch MHJ, Sayers Z, Kuprin S, Zaccar G. 1998. Protein hydration in solution: experimental observation by x-ray and neutron scattering. Proc. Natl. Acad. Sci. U. S. A. 95:2267–2272.
41. Young JAT, Collier RJ. 2007. Anthrax toxin: receptor binding, internalization, pore formation, and translocation. Annu. Rev. Biochem. 76:243–265.
42. Noe SM, Green MA, HogenEsch H, Hem SL. 2010. Mechanism of immunopotentiation by aluminium-containing adjuvants elucidated by the relay between antigen retention at the inoculation site and the immune response. Vaccine 28:3588–3594.
43. Lin I, Ibrahim AS, Avasians E, Edwards V, Fu Y, Baquir B, Taub R, Spellberg B. 2008. Considerable differences in vaccine immunogenicities and efficacies related to the diluted used for aluminum hydroxide adjuvant. Clin. Vaccine Immunol. 15:582–584.
44. Hunter RL. 2002. Overview of vaccine adjuvants: present and future. Vaccine 20(Suppl 3):S7–S12.
45. Brewer JM, Pollock KGJ, Tetsky L, Russell DG. 2004. Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. J. Immunol. 173:6143–6150.
46. Kool M, Souliffe T, van Nimwegen M, Willart AM, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205:869–882.
47. Kuroda E, Ishii KJ, Uematsu S, Ohata K, Coban C, Akira S, Aritake K, Urade Y, Morimoto Y. 2011. Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. Immunity 34:514–526.
48. Keele C, Neil E, Joels N. 1984. Samson Wright's applied physiology, 13th ed. Oxford Medical Publications, Oxford, England.
49. Iyer S, HogenEsch H, Hem SL. 2003. Relationship between the degree of antigen adsorption to aluminum hydroxide adjuvant in interstitial fluid and antibody production. Vaccine 21:1219–1223.
50. Bencini DA, Wild JR, O'Donovan GA. 1983. Linear one-step assay for the determination of orthophosphate. Anal. Biochem. 132:254–258.