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Increasing the Potency of an Alhydrogel Formulated Vaccine by Minimising Antigen–Adjuvant Interactions.

Allan Watkinsona§, Andrei Soliakovb§, Ashok Ganesanc, Karie Hirstd, Chris LeButte, Kelly Fleetwoodf, Peter C. Fuscod, Thomas R. Fuerstd and Jeremy H. Lakeyb#.

aPharmAthene UK Ltd, Billingham, TS23 1XN, UK: currently GSB Pharma, 3 Edston Drive, Guisborough, TS14 6GG, UK

bInstitute for Cell and Molecular Biosciences, Faculty of Medical Sciences, University of Newcastle, Newcastle- upon-Tyne, NE2 4HH, UK.

cXstalBio Limited, University Avenue, Glasgow, G12 8QQ, UK

dPharmAthene Inc, One Park Place, Suite 450, Annapolis, MD 21401 USA

eDefence Science and Technology Laboratory, Porton Down, Salisbury,SP4 0JQ, UK.

fQuantics, Hudson House, 8 Albany Street, Edinburgh, EH1 3QB, U.K.

§ Both authors should be considered as equal lead author with this manuscript.

#Corresponding author Jeremy H Lakey jeremy.lakey@ncl.ac.uk (T=+441912228865)

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Running Title. Phosphate enhances Anthrax vaccine potency
Abstract

Aluminium salts are the most widely used vaccine adjuvants and phosphate is known to modulate the antigen-adjuvant interaction. Here we report an unexpected role of phosphate buffer in an anthrax vaccine (SparVax™) comprising recombinant Protective Antigen (rPA) and aluminium oxyhydroxide (AlOH) adjuvant (Alhydrogel™). Phosphate ions bind to AlOH to produce an aluminium phosphate surface with a reduced rPA adsorption coefficient and binding capacity. However, these effects continued to increase as free [phosphate] 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 (sub-saturated) and 4 mM (saturated) phosphate, tested in a disease model at batch release, showed that the latter was significantly more potent. Both formulations retained their potency for three years. The strongest aluminium adjuvant effects are thus likely to be via weakly attached or easily released, native state antigen proteins.

Abbreviations

rPA; recombinant protective antigen from *Bacillus anthracis* expressed in and purified from *Escherichia coli*. 
Introduction

To combat infectious diseases subunit vaccines, comprising a recombinant antigen and an immune stimulating adjuvant, are increasingly important. These vaccines provide a safer alternative to using live attenuated/inactivated micro-organisms or partially purified microbial extracts, while still promoting protective immunity in an individual (1). Currently under development is an anthrax subunit vaccine (SparVax™) for anthrax pre- and post-exposure prophylaxis treatment. It uses a recombinant protein component, Protective Antigen (rPA), of the anthrax tripartite toxin, (2) as the target antigen for toxin neutralizing antibody production. By itself, rPA is a relatively poor immunogen and, to provide protection against anthrax infection, needs to be formulated with an adjuvant. In line with several recombinant protein sub-unit vaccines (3), an aluminium-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. More specifically the selected adjuvant was Alhydrogel®, which is essentially aluminium oxyhydroxide and, with a net positive surface charge, is known to bind to acidic proteins like rPA (pI= 5.6) (4).

The mechanism by which aluminium salts act as adjuvants for vaccine antigens has recently been intensively investigated at the cellular level. Originally they were considered to simply act as a depot maintaining local antigen concentration (5) but there are now many observations which suggest that more subtle effects lead to increased protection(6). These include NLRP3 inflammasome activation, prostaglandin 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, Hem and colleagues showed that the strength of antigen adsorption to an aluminium-containing adjuvant is inversely related to the immune response(8-10). They also showed that antigens do not need to be bound to the aluminium salt in order to benefit from the adjuvant effect (11) and furthermore 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 aluminium adjuvanted antigen dissociates readily from the adjuvant and the removal of the injection site and associated alum depot 2 h after injection does not impair the immune response, thus questioning the role of Alhydrogel
in forming a stable depot of antigen(13). These results fundamentally question the role of the physical interaction between antigen and adjuvant. We therefore wished to determine if the physical behaviour of different formulations of a clinically relevant antigen-Alhydrogel complex has any correlation with its short or long term potency.

Structurally, Alhydrogel® comprises fine crystalline particles made of corrugated layers of aluminium oxyhydroxide (14). Each aluminium atom is coordinated by four oxygen atoms and two hydroxyl groups (15), the layers are held together by hydrogen bonding and, in aqueous solutions, the particles form aggregates ranging from 1 to 10 µm in diameter (16). Unmodified Alhydrogel particles have a point of zero charge of approximately 11, i.e., the pH at which the charge on the colloidal particle is zero. 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 adjuvant, at the appropriate concentrations readily, and very rapidly, forms the rPA-Alhydrogel (rPA-AlOH) complex (18).

Structural studies have shown that Alhydrogel-bound proteins, including rPA, preserve their secondary (19, 20), tertiary (18), and quaternary structure (21, 22) but exhibit a decreased thermal stability compared to their free counterparts in solution (23, 24). At the surface of adjuvant particles, bound proteins form a monolayer in which individual biomolecules are closely packed 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) so particle aggregation is not a feature of the process.

In addition to the rPA-AlOH complex, the formulation comprised two excipients: (1) saline at physiological levels was included in the formulation to ensure that the anthrax vaccine was isotonic for recipient comfort upon injection and (2) phosphate ions were included with the initial intention of providing buffering capacity. However, it is well established that phosphate groups will ligand exchange with aluminium oxyhydroxide resulting in a modification of the surface properties of the colloidal particles (26). For this sub-unit vaccine formulation, the immediate concern is that the ligand exchange reaction modifies the point of zero charge to such an extent that the acidic recombinant
protein becomes desorbed from the aluminium 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 aluminium oxyhydroxide particle surface is approximately two pH units higher than that of the surrounding solution (27). Since antigen binding occurs as a monolayer at the surface of the aluminium oxyhydroxide particle (22), it will reside in this microenvironment and be subject to conditions different from those of the bulk solution.

As discussed above, Hem and co-workers (27) have shown phosphate to be a modifying agent for aluminium oxyhydroxide, due to the effects of the ligand exchange reaction and in addition to its role as a buffer. Furthermore, by modifying the physical nature of the aluminium oxyhydroxide, phosphate may also affect the potency of any vaccine where it is used as an adjuvant.

Since phosphate comprises the physiological buffering agent of the anthrax vaccine, we have studied the effect of phosphate ions on the rPA-AlOHH formulation. The aim of these studies was to understand the effect of phosphate on the rPA-AlOHH colloidal particles, the structure of the protein antigen, the stability of the formulation, and most importantly the potency of the rPA-AlOHH 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 resulted in an enhanced immune response and helped to explain why the adsorption coefficient of rPA-AlOHH binding is inversely related to potency (10).

**Materials and Methods**

**rPA-Alhydrogel formulation**

All chemicals and reagents were purchased from either Sigma-Aldrich Company Ltd (UK) or Melford Laboratories (UK), unless otherwise stated.

Recombinant protective antigen (rPA) was manufactured to cGMP by Avecia Biologics, Billingham, U.K. rPA was expressed as inclusion bodies using Escherichia coli strain UT5600 (DE3)/pET29a.
After solubilisation 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 adjusted to give ~ 1.5 mg/ml. Aliquots of protein were stored at -80°C until required. Aluminium hydroxide gel adjuvant (Alhydrogel™) was purchased from either Brenntag Biosector (Denmark) or Sigma-Aldrich Company Ltd (UK).

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

**Alhydrogel Phosphate Titration**

Phosphate buffer (0 - 5 μmol) was added to 3 mg of Alhydrogel in 1 ml water and vortexed. The samples were incubated for 1 hour with agitation at ambient temperature and then centrifuged at 20000 x g for 1 minute. The supernatant was then analysed for phosphate using a colorimetric assay (Bencini et al., 1983). 400 μl of sample was added to 1200 μl of reagent mixture (100 mM zinc acetate, 15 mM ammonium molybdate, pH 5.0), vortexed and allowed to react for 1 minute before measuring absorbance at 350 nm in a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan) using 1 cm path length quartz cuvette (Hellma, GmbH & Co., Germany). Values were calibrated against a phosphate standard curve of NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0 containing 0 - 700 μM phosphate.

**rPA Langmuir adsorption isotherms**

rPA was combined with Alhydrogel in phosphate buffer (0 – 50 mM) and the samples were incubated for 1 hour with gentle agitation at an ambient temperature. Subsequently, all samples were centrifuged for 5 min at 14600 x g using a bench top centrifuge and supernatant [rPA] was assessed by $A_{280}$. The
amount of rPA adsorbed to Alhydrogel was calculated by subtracting the rPA remaining in solution from the total added. Adsorption coefficient (K) and binding capacity (Γ_{max}) were obtained by linearizing the Langmuir equation (10, 29) and determined using 1/y intercept and 1/slope, respectively.

**Zeta Potential Measurements**

Zeta potential was determined using a Zetasizer (Malvern, U.K.). 1 ml samples of rPA-AlOH were introduced into the DTS1060 capillary cells and pre-equilibrated at 20°C for 2 min prior to electrokinetic analysis, as per 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 becomes inaccurate when using particulates that settle and in 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 specialised rotating sample cell holder(31). The raw data was corrected for protein concentration and converted into differential extinction coefficient (∆ε) units (M⁻¹ cm⁻¹), using the molar concentration of amino acid residues for far UV CD and for near-UV the molar concentration of rPA. 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 spectrofluorometer (Agilent, UK) with an excitation wavelength of 280 nm and the emission spectra were measured between 300 and 400 nm. rPA-Alhydrogel® samples were directly measured in a 1 cm path length quartz cuvette (Hellma, UK). 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 with that obtained from front face
illumination of a triangular cuvette but stirring was more reliable in the standard square footprint design.

In order to provide a more accurate evaluation of the emission spectra, the barycentric mean (the centre of an integrated emission curve) was determined using the equation:

\[
\lambda_m = \frac{\sum F_\lambda \times \lambda}{\sum F_\lambda}
\]

where, \(\lambda_m\) is the barycentric mean, \(\lambda\) is the wavelength, \(F_\lambda\) is the point fluorescence at wavelength \(\lambda\) (32). Thermal denaturation was monitored via tryptophan fluorescence using a Cary Eclipse equipped with a temperature controller and a 1 cm path length quartz cuvette (Hellma, UK) with a plastic lid. A small magnetic stirring bar inside the cuvette stirred the samples while the temperature was raised 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 VP-DSC micro-calorimeter (MicroCal, UK). Prior to analysis, all samples were de-gassed in a ThermoVac unit (MicroCal, UK). Samples of rPA-Alhydrogel 0.3 mg/ml PA; 3 mg/ml phosphate saturated Alhydrogel; 0, 1, 5, 10, 20, and 50 mM free phosphate were analysed. 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 protein melting temperature (\(T_m\)) and, from the area under the peak, enthalpy (\(\Delta H\)). Isothermal titration calorimetry was conducted in a VP-ITC titration micro-calorimeter (MicroCal, UK). All samples were equilibrated by dialysis against the same buffer (150 mM NaCl, pH 7.0) and de-gassed as above. The Alhydrogel suspension was placed in the stirred chamber (300 rpm) and rPA solution in the syringe. Titration was performed with injection volumes of 10 µl for 20 s and injection spacing of 200 s, a cell temperature of 25°C and
reference power of 15 µCal/sec. The ITC data were processed, including subtracting reference scans (rPA into buffer) from sample scans, in Origin software.

Mouse Anthrax Challenge Assay

The mouse anthrax challenge assay, which emulates the human immunological response to anthrax infection, was used for all release and stability testing of the rPA vaccine. All investigations involving animals were carried out according to the requirements of the UK Home Office legislation and the Animal (Scientific Procedures) Act 1986. Potency was determined using a mouse challenge assay using a single immunization followed by subsequent challenge with anthrax STI spores. Female A/J mice (Harlan Laboratories, U.K.), between 7 and 12 weeks of age, were injected with either test rPA-AlOH vaccine (either 0.25mM phosphate or 4mM phosphate) or with a freshly formulated reference standard comprising rPA-AlOH. During immunization, each dose was administered in a total volume of 0.1 ml, administered in two 0.05 ml aliquots intra-muscularly from the same syringe into each hind-limb. Both the reference and test items were administered as two parallel 4-point dilution series ranging between 0.2 and 0.004 ug/ml doses, with 15 mice/dose. The mice were transferred to the ACDP Level III isolator for spore challenge. At day 21 following the single immunization, each mouse was challenged with $2 \times 10^6$ Bacillus anthracis STI spores intraperitoneally (range of 0.9 to 5 x $10^6$ spores) which is equivalent to a $2 \times 10^3$ median lethal dose. All immunizations and spore challenges were performed using a single operator. Following spore challenge, all animals were closely monitored for up to 8 days and the time to death was recorded. Humane end points were strictly observed for animals showing signs of disease.

Potency was determined using a survival model, which describes the relationship between vaccine concentration and mouse survival and ED50 values were determined. In this study, the ED50 is the estimated concentration at which 50% of the mice survive to 8 days. For a single material the predictor is the $\log_{10}$ vaccine concentration and the response is the number of days survived by each mouse. For mice that survive until day 8 the survival time is censored, i.e. the model takes into account that they are alive on the final day of the test. With this model the survival time is assumed to
follow a lognormal distribution, which means that the log of survival time follows a normal
distribution.

For a single material the following parameters are defined for each concentration $i (i = 1, 2, 3, 4)$:

- $C_i$ the vaccine concentration for group $i$,
- $N_i$ the number of mice challenged for group $i$. $N_i$ is usually 15.
- $S_{ij}$ the number of days the $jth$ mouse from group $i$ survived, where $j = 1, 2, \ldots, N_i$. $S_{ij}$ is censored at 8 days.

The survival model for a given material can be expressed as:

$$E(\log_e(S)) = \alpha + \beta \times \log_{10}(C).$$

The equation describes a straight line with slope $\beta$ and intercept $\alpha$.

Once $\alpha$ and $\beta$ have been estimated it is possible to calculate the ED50 of the material. For the
lognormal distribution this is equivalent to the concentration at which the mean log survival time is
$\log_e(8)$ days. At the ED50 we have:

$$\log_e(8) = \frac{\log_e(8) - \alpha}{\beta} \times 10$$

$$ED_{50} = \frac{\log_e(8) - \alpha}{\beta}$$

The variance of the $\log_{10}(ED_{50})$ can be approximated using the Taylor series method:

$$\text{var}(\log_{10}(ED_{50})) = \
\frac{1}{\beta^2} \left[ \text{var}(\alpha) + 2. \text{cov}(\alpha, \beta) \cdot \log_{10}(ED_{50}) + \right]$$

Long term Stability determination

The stability of each of the materials was investigated using weighted linear regression. Each estimate
of log ED50 was weighted by the inverse of the variance of the estimate, such that precise estimates
were given more weight than imprecise estimates. The weighted linear regression produces a line of
best fit to the data, such that the intercept provides an estimate of the log ED50 at manufacture and the slope provides an estimate of the change in log ED50 per month. A p-value was calculated for the slope, indicating whether the log ED50 changes over time. All analysis was conducted using the statistical software R (version 2.12.1 for Windows).

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 supernatent. It was evident from the binding curve that, for 1.5 mg of Alhydrogel in 1ml, saturation was reached when 0.65 mol of phosphate had bound (around 1 mM [phosphate] in this experiment). This gives a saturation level of 0.43 mol of phosphate per mg of Alhydrogel (Figure 1).

Langmuir Adsorption Isotherms

The ligand exchange reaction introduces negative charge to the surface of Alhydrogel and to characterise 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 on both non-modified and phosphate saturated Alhydrogel. When it was observed that rPA still bound to phosphate saturated Alhydrogel, we extended the analysis to examine the effect of free phosphate on the protein-adjuvant interaction. 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, the higher the value of adsorption coefficient the stronger the interaction. For non-modified Alhydrogel, rPA had an adsorption coefficient of 215 ml/mg. This value substantially decreased in the presence of phosphate (Figure 2A). Likewise, there was a sharp reduction in the binding capacity (Figure 2B). Both adsorption coefficient and binding capacity were further reduced by free phosphate, before reaching a steady state around 5 to 10mM phosphate.
To evaluate directly the effect of phosphate upon 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 (Figure 2C). As expected, when the phosphate levels were increased, the levels of unbound rPA correspondingly increased. Whereas at 5 mM the unbound rPA content remained below 5%, this had increased to around 12% with 10 mM phosphate.

**Zeta potential measurements**

Changes in Alhydrogel surface charge (zeta potential) as a function of phosphate concentration, were measured using a Zetasizer Nano ZS (Malvern). The analysis was performed on 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 would be expected from previous studies (33), the zeta potential of Alhydrogel was positive at physiological pH. With increasing phosphate saturation, it rapidly declined and eventually become negative (Figure 3) with a point of zero charge at 2.5 mM phosphate. Alhydrogel with adsorbed rPA exhibited similar changes; however, the initial zeta potential of Alhydrogel with bound rPA had a lower value compared to that of Alhydrogel alone and the point of zero charge was 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 Figure 1. Titration of non-modified Alhydrogel with rPA gave a series of endothermic peaks (Figure 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 (Figure 4B). Similarly, titration of 50% phosphate-saturated Alhydrogel was exothermic but the magnitude was less compared to that of phosphate-saturated Alhydrogel (Figure 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 (Figure 4D), suggesting that the initial entropy driven interaction with AlOH was preferred over subsequent enthalpy driven binding to phosphate modified surfaces.

**Solid State Circular Dichroism**

Solid state (31, 34) far UV CD spectra of rPA-AlOH (Fig 5a) closely resembled that of soluble rPA (Chalton et al, 2007), indicating minimal secondary structure perturbation upon binding to Alhydrogel. Increasing phosphate concentrations did not significantly affect the spectrum. Near UV solid state CD spectra of rPA-AlOH at 0.25 mM phosphate, 4 mM phosphate and 10mM phosphate were also similar, with each showing the two prominent positive tryptophan CD bands at 284 nm and 291 nm. These two bands, characteristic of rPA (31, 35), were weaker than in soluble rPA in solution (Figure 5B) and in the 10 mM phosphate sample two bands (272nm and 276nm) are observed that were not present with soluble rPA. In the phenylalanine region a broad positive band from 250-260 nm was seen in 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 rPA tertiary structure.

**Differential Scanning Calorimetry (DSC)**

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 non-modified or phosphate saturated Alhydrogel. However, as the free phosphate concentration increased there appeared a distinct thermal transition that eventually resembled the behaviour of free rPA (Figure 6A). Between 1 and 50 mM phosphate, the $T_m$ rose from 43.3 °C to 48.2 °C and $\Delta H_{cal}$ increased from 31 kcal mol$^{-1}$ to 139 kcal mol$^{-1}$ (Figure 6B). The $T_m$ and $\Delta H_{cal}$ of non-adjuvanted 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 (Figure 6C). Conversely, above 10 mM phosphate, when protein binding was less than 90 % (Figure 2C), there were only minor changes in
The phosphate concentration range when major thermodynamic changes occurred was 1-10 mM, when 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 (supplementary data). All spectra exhibited their maximum 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 compared to the emission spectrum of rPA without adjuvant, likely due to an inhomogeneous distribution of fluorophores in solution, the colloidal nature of the sample, and light scattering. Overall, these data demonstrate no measurable change in the protein tertiary structure due to phosphate (Supplementary data).

Protein unfolding was monitored by tryptophan fluorescence while increasing the temperature from 25 °C to 65 °C at the rate of 1 °C min⁻¹ (18). In samples containing non-modified, 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 effect of higher temperatures (Figure 7 A). However, with free phosphate, i.e. around 2.6 mM, rPA had a well-defined transition region between 40 °C and 50 °C (Figure 7 B). Note that increasing phosphate concentration shifted the transition midpoint from 43.5 °C to 48.0 °C, compared to the 48.5 °C transition temperature of non-adjuvanted rPA. Also, it should 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 the effect 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 where the Alhydrogel retained surface hydroxyl groups, whereas, in contrast, the 4 mM phosphate rPA-AlOH represented a 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, respectively.

Potency was determined using the anthrax mouse-challenge assay, evaluating several batches of these two formulation prototypes immediately following manufacture. With each potency analysis a four-fold dilution curve was performed. The ED50 values were estimated by fitting a survival analysis model to the results of the potency assay, using a model that assumes the survival times have a log normal distribution and that there is a linear relationship between log dose and log survival time (Table 1) (36). Note that for three of the 4 mM phosphate rPA-AlOH, batches more than one measurement of ED50 was taken at release and this data has been incorporated into the statistical analysis. The weighted mean ED50 for the 0.25 mM phosphate rPA-AlOH batches was 0.21 μg/0.1mL (back-transformed from the log10 ED50 mean value of -0.679), whereas for the 4 mM phosphate rPA-AlOH the value was 0.04 μg/0.1mL (log10 ED50 mean value of -1.398). The weighted mean was used (weighted by the inverse of the variance of the log10 ED50) to down weight less precise log10 ED50 estimates. Statistical analysis using ANOVA indicated that at release 4 mM phosphate rPA-AlOH was significantly more potent than 0.25mM phosphate rPA-AlOH, as shown by a difference in log ED50 (p=0.006). The ANOVA took batch and operator into account. Comparison of the mean ED50 values indicated that the 4 mM phosphate rPA-AlOH was 5.25 fold more potent than the 0.25mM phosphate formulation.

In addition long term, real time stability studies were performed on a batch of 0.25 mM phosphate rPA-AlOH and one of 4 mM phosphate rPA-AlOH. In accordance with ICH Harmonised Tripartite Guidelines Q1A(R2) (Stability Testing of New Drug Substances and Products) (37), the materials were stored for at least three years at 2-8°C under controlled conditions, which were monitored to ensure temperature compliance. During the 3 years storage, samples were analysed for potency using the mouse anthrax challenge assay. Trending analysis was performed after three years to determine the overall stability of the two vaccine formulations and the data are presented in Figure 8. Linear regression analysis of the 0.25mM phosphate rPA-AlOH after 39 months storage at 2-8°C revealed no evidence of an increase in log ED50 post manufacture (intercept = -0.414 (ED50 of 0.385); slope = 0.007; p = 0.480). Likewise, the 4mM phosphate rPA-AlOH exhibited no evidence of
a significant increase in logED50 after 36 months storage at 2-8°C (intercept = -1.277 (ED50 of
0.0528); slope = 0.007; p = 0.673). Trending analysis was also performed on three batches of the 4
mM phosphate rPA-AlOH formulation to provide further supporting data for stability. Using relative
potency (RP) against a freshly formulated rPA-AlOH standard, the rPA-AlOH was shown to be stable
for at least 37 months (Supplementary Data).

Discussion
Aluminium based adjuvants are extensively used in the formulation of a range of vaccine types,
including sub-unit vaccines, being both effective in boosting immune responses and safe for use in
man (14). Although mainly used against infectious diseases they are also effective in anti-cancer
vaccines (38). Aluminium oxyhydroxide formulations, such as Alhydrogel, are the most commonly
used since they are positively charged at physiological pH and bind most antigens, which tend to be
acidic. A significant feature of aluminium oxyhydroxide chemistry is the ligand exchange reaction,
whereby surface hydroxyl groups are readily exchanged for phosphate ions (26). Consequently, the
surface layer of the aluminium oxyhydroxide is converted to aluminium phosphate, radically changing
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 reached, with 0.43 µmol phosphate saturating 12.8 µmol (1 mg) of Alhydrogel. Since there are 2
moles of hydroxyl groups per 1 mole of Alhydrogel, the theoretical maximum phosphate saturation
requires 12.8 x 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 surface exposed, but contained within the colloidal particle itself (21).
However, despite the reversal in surface charge (Figure 3), it was surprising that even with supra-
saturating 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 (Figure 3). One
possible explanation might be due to the variety of charged groups on rPA. Although acidic, with a pI
of 5.6, rPA still contains 85 cationic (Arg + Lys) versus 96 anionic (Asp + 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 concentration extended beyond the point of surface saturation and suggest that the surface charge of the Alhydrogel is not purely determined by the extent of ligand exchange. There is thus also a role for free phosphate in modulating the interaction between the rPA and the Alhydrogel surface.

On native Alhydrogel the interaction of rPA with the hydroxyl groups was endothermic meaning that it is driven by an increase in entropy, an effect often attributed to dehydration effects. This would imply that there is a significant reduction of 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 favoured interaction could be associated with the formation of direct non-covalent bonds at the particle surface. At sub-saturating phosphate concentrations, the thermodynamics are complex and at 10% saturation there is evidence for a genuinely mixed surface offering high (endothermic) and low (exothermic) affinity 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. Increasing phosphate concentration thus appears to surface bound proteins to behave as though in solution. Thus enhanced hydration (41) could explain both the ITC data and the increased protein thermostability.

Of paramount importance is role of phosphate in the potency of the rPA sub-unit vaccine and its stability when stored under refrigeration. The phosphate sub-saturated 0.25 mM phosphate rPA-AlOH was significantly less potent than that of 4 mM phosphate rPA-AlOH and one contrasting feature of the two formulations was the pH of the bulk solution, the 0.25 mM phosphate rPA-AlOH having a pH of 5.9 and the 4 mM phosphate rPA-AlOH around 7.1. Consequently, the differing bulk solution pH 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 in 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).
contrast, the negatively charged phosphate saturated Alhydrogel would be expected to attract protons, and hence have a more acidic microenvironment compared to the bulk solution.

Hem and co-workers have hypothesized that the immunogenic response to an aluminium adjuvantized 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 implying 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 aluminium oxyhydroxide adjuvants might appear to be irrelevant. However, enhanced immunogenicity was seen when a recombinant *Candida* antigen (rAls3p-N) was diluted with phosphate buffered saline as opposed to saline alone (44). Similarly, phosphate modulation of aluminium oxyhydroxide in both hepatitis B surface antigen and HIV 1 SF162dV2gp140 subunit vaccines demonstrated an enhancement of immunogenicity (8, 9).

There have been several proposed mechanisms of action for aluminium-containing adjuvants, including: a depot in tissues to produce prolonged exposure (45), an enhanced delivery of antigen to antigen presenting cells (46), an induction of uric acid for activating inflammatory dendritic cells (47), and an enhanced proteolytic processing by the immune system due to destabilisation of the antigen structure (23). However, compelling evidence has recently been produced to demonstrate that aluminium-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 the 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 not phosphate saturated beforehand, the aluminium oxyhydroxide will saturate 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 in sub-saturating conditions occludes hydroxyl groups and prevents subsequent phosphate exchange. Under such circumstances the stronger binding conditions may prevail even when later exposed to physiological phosphate concentrations.

Although Iyer *et al* showed that aluminium adjuvant interactions with a basic protein (lysozyme)
could be reversed in interstitial fluid, with acidic ovalbumin this was only true of freshly formulated mixtures 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, the effect of phosphate we observe may be the 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 however goes much further 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 adjuvant. Thus the hypothesis of Hem and co-workers that the immunogenic response to an aluminium adjuvantized protein antigen is inversely related to the adsorption coefficient (Hansen et al, 2007) is strongly confirmed but also extended to include the physical effects on the protein and the effect on potency over a long period. Combined with recent data that some adjuvant protein interactions rapidly dissociate upon injection (13) it appears that the strongest aluminium adjuvant effects are likely to be via weakly attached or easily released, native state antigen proteins.

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Figure legends

**Figure 1:** Saturation curve for binding of phosphate to Alhydrogel. These data were fitted to the Boltzmann Sigmoid equation using non-linear regression analysis in the Origin software (version 7.5, OriginLab Corporation). The insert shows raw absorbance data obtained in the spectrophotometric phosphate assay.

**Figure 2:** Results from linear regression fits to the Langmuir adsorption isotherm showing the effect of phosphate on (A) adsorption coefficient; (B) binding capacity of Alhydrogel for rPA and (C) Fraction of rPA binding to Alhydrogel in samples of 200ug/ml rPA and 2.6mg/ml Alhydrogel.

**Figure 3:** Zeta potential: curves exhibit the effect of phosphate on zeta potential of Alhydrogel with and without rPA.

**Figure 4:** Titration of Alhydrogel with rPA showing thermograms for: A) Non-modified Alhydrogel; B) 100 % phosphate saturated Alhydrogel; C) 50 % phosphate saturated Alhydrogel; and D) 10 % phosphate saturated Alhydrogel. Inset diagrams show the likely surface chemistry of the variously saturated adjuvants.

**Figure 5:** The effect of phosphate on Solid state Circular Dichroism of 200ug/ml rPA with 2.6mg/ml Alhydrogel: (A) far UV CD spectra and (B) near UV CD

**Figure 6:** Phosphate effect on the structure and stability of adjuvanted rPA: A) An overlay showing DSC endotherms of rPA on Alhydrogel in 0, 2, 5, 10, 20, 45 mM phosphate and rPA without adjuvant respectively; B) T_m and ΔH_cal values as function of phosphate concentration; C) T_m and ΔH_cal change as fraction of bound rPA. Samples contained 0.3 mg/ml rPA and 3 mg/ml phosphate saturated Alhydrogel.

**Figure 7:** Tryptophan fluorescence: thermal denaturation of rPA on Alhydrogel: A) rPA adsorbed to non-modified, 50 % and 100 % phosphate-saturated Alhydrogel; B) rPA on Alhydrogel in 1-10 mM free phosphate. The thermal transition of non-adjuvanted rPA (solid line) occurred at 48.5 °C. All curves are normalised to values of 1 at 25°C and 0 at 65°C.
Figure 8: logED50 Stability data for (A) 0.25 mM Phosphate rPA-AlOH and (B) 4 mM Phosphate rPA-AlOH, both stored at 2-8°C. The error bars represent ± SD; the solid line is the trending line and the broken line is the one-sided 95% confidence interval.
Table 1: Summary of potency data at release for 0.25 mM phosphate and 4 mM phosphate rPA-AlOH in Final Drug Product (FDP).

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