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Vaccines with Aluminum-Containing Adjuvants: Optimizing Vaccine Efficacy and Thermal Stability

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Abstract

Aluminum-containing adjuvants have been used to enhance the immune response against killed, inactivated and subunit antigens for over seven decades. Nevertheless, we are only beginning to gain important insight as to what may be some very fundamental parameters for optimizing their use. For example, there is evidence that the conventional approach of maximizing antigen binding (amount and/or strength) may not result in an optimal immune response. Adsorption of antigen onto the adjuvant has recently been suggested to decrease the thermal stability of some antigens; however, whether adsorption-induced alterations to the structure and/or stability of the antigen have consequences for the elicited immune response is unclear. Finally, the thermal stability of vaccines with aluminum-containing adjuvants is not robust. Optimizing the stability of these vaccines requires an understanding of the freeze sensitivity of the adjuvant, freeze and heat sensitivity of the antigen in the presence of the adjuvant, and perhaps most importantly, how (or whether) various approaches to formulation can be used to address these instabilities. This review attempts to summarize recent findings regarding issues that may dictate the success of vaccines with aluminum-containing adjuvants.

INTRODUCTION

Aluminum-containing adjuvants have been included in vaccines to boost the immune response to antigens for over seven decades.^{1–3} Despite their long record of success at improving vaccine immunogenicity, the process of formulating vaccines with these adjuvants is largely based on trial and error. In the past decade, our understanding of the important factors in the design of vaccines with aluminum-containing adjuvants as well as approaches to address challenges of vaccine stability has been greatly improved. The intensive research in the area of vaccines with aluminum-containing adjuvants in recent years can be divided broadly into three categories: (1) those primarily directed towards understanding the inherent effects these adjuvants have on the immune system at the cellular and molecular levels; (2) those focused on antigen – adjuvant interactions; and (3) those focused on improving vaccine stability. An in-depth discussion on the effects of adjuvants alone (without antigen) on the immune system is beyond the scope of this review. For this, the reader is directed to the reviews and original works that have been published primarily within the last 2 years on this subject.^{3–16} The following review will attempt, however, to put into context the research focusing on the important parameters and consequences of the antigen/adjuvant interaction as well as ways to improve the thermal stability of vaccines.

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In the sections that follow, we will discuss research that we believe may suggest a need for paradigm shift in how vaccine formulation is approached. We will revisit the importance of binding and strength of the binding interaction in light of studies explicitly designed to investigate these parameters with regard to the immune response as well as the effects adsorption has on antigen structure and stability. The inadequate thermal stability, especially under freezing temperatures, of vaccines with the aluminum containing-adjuvants is a challenge to ensuring the delivery of efficacious vaccines. In the past few years; however, approaches to improve the stability of such vaccines have been pursued, and research in this area will also be highlighted.

THE IMPORTANCE OF ANTIGEN ADSORPTION TO ALUMINUM-CONTAINING ADJUVANTS

Conventional wisdom dictates that in vaccines necessitating the use of aluminum-containing adjuvants, antigen adsorption should be maximized.¹⁷ This concept dates back to the first vaccines containing these adjuvants – alum-precipitated diphtheria and tetanus toxoid vaccines. The World Health Organization (WHO) requires that at least 80% of the antigens in these vaccines be adsorbed.¹⁸ The necessity of adsorption and the importance of the strength of adsorption have recently become topics of consideration with respect to the efficacy of the final vaccine product. Studies addressing both factors are discussed below.

Adsorptive capacity and immunogenicity

For vaccines, the adsorptive capacity provides information regarding the maximum amount of a specific antigen that can be adsorbed onto the adjuvant under given conditions.¹⁷ Monolayer coverage is assumed, and the Langmuir equation is used to describe the relationship. Electrostatic interactions and phosphate exchange are two of the most important mechanisms for the adsorption of antigens onto aluminum-containing adjuvants. Accordingly, the determined adsorptive capacity is antigen, adjuvant, and formulation dependent. This is evident from the ranges of adsorptive capacities listed in Table 1. Values for a given antigen and adjuvant combination that are divergent reflect the differences in the formulations (i.e., buffer salts, pH, salt concentrations, etc.).

As suggested with the WHO recommendations for diphtheria and tetanus toxoid vaccines, the percentage of the antigen that is bound in the vaccine vial, and not the calculated adsorptive capacity, is more often the practical concern for the final product. In an actual vaccine with an aluminum-containing adjuvant, the relative amount of antigen to adjuvant is usually far less than the calculated adsorptive capacity. For example, the reported adsorptive capacities of HBsAg to the various aluminum-containing adjuvants in Table 1 range from 0.58 to 1.7 mg antigen/mg aluminum. The FDA-approved monovalent HBsAg vaccine with the highest mass ratio of HBsAg to aluminum, Engerix-B®, contains only 10 µg HBsAg per 250 µg aluminum from aluminum hydroxide (amounts obtained from packaging insert).¹⁹ This ratio, 0.04 mg HBsAg/mg aluminum, is likely far in excess of the adsorptive capacity of the adjuvant for this antigen in the vaccine, ensuring complete adsorption. Research regarding the importance of the extent to which the antigen is actually adsorbed, however, is calling to question whether formulations with a higher extent of antigen bound are necessarily better. Findings from some recent studies are summarized in Table 2 and discussed below.

For some vaccines, studies suggest that the fraction of the antigen that is adsorbed to the adjuvant in the vaccine vial does not correlate with the elicited antibody titer. For example, Chang et al demonstrated that the antibody response elicited in rabbits against hen egg white lysozyme (HEL) was irrespective of the fraction of antigen adsorbed onto the adjuvant.²⁰

Vaccines with adjuvants having a range of adsorption percentiles (3% to 90% lysozyme adsorbed) were created using a commercial aluminum hydroxide adjuvant (3%), phosphate-treated aluminum hydroxide adjuvant (10%, 85%, and 90% -- note: the adjuvants with 85% and 90% antigen adsorption were vaccines of identical formulas prepared on different days), or a commercial aluminum phosphate adjuvant (35%). All vaccines elicited antibody titers approximately four times higher than that obtained using only soluble protein or co-administration of soluble protein and an aluminum-containing adjuvant at different sites. Moreover, there was no significant difference in the anti-HEL antibody titers of the vaccines containing the HEL and an aluminum-containing adjuvant. Similar results regarding the relationship between the extent of adsorption in the vial and anti-HEL antibodies produced in mice were obtained by Clausi et al with mice immunized with vaccines of HEL with varying degrees of adsorption onto aluminum hydroxide and aluminum phosphate adjuvants as a function of processing (freezing and freeze-drying).²¹ This series of vaccines was produced using a single starting adjuvant and varying the manufacturing procedure. The resultant vaccines had differing fractions (amounts) of antigen adsorbed following reconstitution or thawing. No correlation was found between the percentages of HEL adsorbed (10% to 90%) and the anti-HEL antibody production. It should be noted that the vaccine dosages were not titrated to evaluate the dependence on the lack of the effect of adsorption on the amount of antigen administered. It is possible, therefore, that the absence of a dependence on antigen concentration was because even at lowest amount of HEL adsorbed, the antigen dosages used in these studies were sufficient to maximize the response due to the presence of adjuvant, even if some population of the vaccine had changed. At lower dosages of antigen, however, the outcome may differ.

A 2005 study considered the importance of degree of anthrax antigen (recombinant protective antigen – rPA) adsorption (0, 80% or 100%), adjuvant choice and total antigen content.²² Broadly described, the vaccines consisted of aluminum hydroxide adjuvant in saline with 100% rPA adsorbed (reminiscent of the only licensed anthrax vaccine approved for use in humans), aluminum phosphate adjuvant in saline with $\geq 80\%$ rPA adsorbed, and aluminum phosphate adjuvant in sodium phosphate buffer with no rPA adsorbed, only in solution. In the case of this antigen, binding of the protein to adjuvant was not essential for the aluminum-containing adjuvants to boost the anti-rPA response in CD1 mice, but instead the mere presence of adjuvant was capable of enhancing anti-PA antibody response, relative to antigen alone in solution. There were striking differences, however, in the dose-response behavior of the two vaccines containing aluminum phosphate adjuvant. The vaccine with the adsorbed antigen was insensitive to antigen dose, but the vaccine with the soluble antigen yielded a trend of decreasing response with the decreasing antigen concentration. This suggests that at least for rPA, MW ~83 kDa, some degree of adsorption is important in maximizing antibody production when antigen is limited. Interestingly, the only vaccines that elicited neutralizing antibody titers above those elicited by the adjuvant-free rPA solution were those vaccines containing the aluminum phosphate adjuvant, which contained both soluble and adsorbed antigen. The vaccine with 100% of the antigen adsorbed, i.e., that with the aluminum hydroxide adjuvant, did not have a significant affect on the production of neutralizing antibodies and was similar to the soluble control. It is unclear whether the lack of production of neutralizing antibodies is because 100% of the antigen was adsorbed or whether some other factor (i.e., structural perturbations of the adsorbed antigen, differences in the mechanisms of actions of the adjuvants, particle size, etc.) played a role.

The generality of the importance of any adsorption at all in the vaccine vial with respect to the antigen-specific antibody titers was addressed in a more recent study using three model antigens (HEL, dephosphorylated α -casein, and ovalbumin).²³ The study assessed antigen-specific antibody titers elicited by mice immunized with vaccines consisting of solutions of antigen, suspensions of adjuvant in an antigen solution (no adsorption of antigen onto

adjuvant), or adsorbed antigens. Mice receiving the vaccines with adjuvant, even in the absence of adsorption, produced significantly greater antibody titers compared to the group only receiving the specified antigen in solution alone. The authors of the study provided a possible explanation of how the vaccine is capable of enhancing an appropriate immune response when the antigen is not adsorbed to the adjuvant: the antigen is trapped in the interstitial spaces of the adjuvant and can be retained at the site of injection for presentation. They acknowledge, however, that due to the limited range of molecular weights of the antigens in the study, further investigation is necessary to determine if there is a potential for antigen size limitation in regards to accessing the void spaces of the adjuvant. The results suggest that for vaccines with antigens of relatively small molecular sizes, adsorption may lack any real importance in formulation for immunopotentiality; however, whether some degree of adsorption is necessary for protective immunization was not addressed.

Evidence that adsorption may be important for some antigens within the size range of the model proteins from the above-cited study (14.3–43 kDa) comes from a recent study of *Streptococcus pneumoniae* vaccines.²⁴ The vaccine antigens in this study (Sp1 – 69.9 kDa, Sp2 – 30.1 kDa, and Sp3, a Sp1/Sp2 fusion protein, -- 99.5 kDa) did not adsorb to aluminum phosphate adjuvant but adsorbed to aluminum hydroxide adjuvant. It was reported that all vaccines with aluminum hydroxide adjuvant elicited significantly higher antibody responses than the vaccines with aluminum phosphate adjuvant. The geometric mean antibody titers were also higher for the vaccines with the aluminum hydroxide in comparison to the corresponding adjuvant-free vaccine as well. Interestingly, the difference between the antibody responses of an adjuvant formulation and its corresponding adjuvant-free formulation was only determined to be significant for Sp2, with the aluminum hydroxide formulation yielding the higher response. This suggests that antigen adsorption is particularly important for some vaccines, although the size dependence remains unclear. Unfortunately, percentages of adsorption were not reported for this study. The single study for which antigen (Sp3) adsorption was reported, compared freshly prepared versus aged vaccines with a constant aluminum content and varying ratios of aluminum hydroxide:aluminum phosphate adjuvants to potentiate adsorption. Thus, it is impossible to make any definitive statements regarding the importance of the extent of adsorption for immunopotentiality or simply differences due to the properties of the adjuvants.

Collectively, this literature suggests that although adsorption may be important for some vaccines (i.e., diphtheria and tetanus toxoid vaccines, and more recently *Streptococcus pneumoniae*), for other vaccines, antigen adsorption may not be required for immunogenicity. Evidence of enhanced immunogenicity when the antigen and adjuvant are delivered at distinct sites, however, still remains elusive. Therefore, adsorption may not be required, but it appears that proximity of the antigen and adjuvant is important. Adsorbed or not, the evidence still suggests for most vaccines antigen and adjuvant must be formulated and administered together to achieve an enhancement in the immune response.

Effect of adsorptive coefficient

Antigen adsorption onto adjuvant is not merely defined by the adsorptive capacity: a second factor, the adsorptive coefficient, which is a measure of the strength of the adsorption, must also be considered.¹⁷ Initial studies regarding the strength of adsorption dealt primarily with the fate of antigen in the presence of interstitial fluid as a measure of strength of adsorption. In the above mentioned Chang et al study regarding extent of HEL adsorption and anti-HEL antibody production, the extent of HEL that remained adsorbed following exposure to interstitial fluid was ~ 40%, regardless of the adsorption in the vial.²⁰ This led the group to hypothesize that antibody production may be driven by the extent of adsorption after administration, i.e., the amount adsorbed in the environment of the interstitial fluid. Subsequent studies examined the immune response of several model vaccines that

experienced antigen desorption in *in vitro* studies using model interstitial fluid.^{20,23,25,26} The results suggest that the antigen-specific antibody production can be boosted through the use of aluminum-containing adjuvants, regardless of whether the antigen remains adsorbed post-administration. The ability of the antigen to readily desorb upon exposure to interstitial fluid was also found to be unnecessary for potentiation of the immune response.²⁵ Microscopy studies reveal that *in vitro*, dendritic cells are capable of taking up both soluble antigen as well as antigen that remains adsorbed onto adjuvant.^{23,27}

As one might expect, it is difficult to vary separately adsorptive coefficient and the percentage of antigen adsorbed. The importance of the adsorptive coefficient, or strength of adsorption, has been studied in vaccines consisting of the model antigens ovalbumin, α -casein and dephosphorylated α -casein, formulated with aluminum hydroxide and phosphate-treated aluminum hydroxide adjuvants¹⁷ as well as vaccines with more pharmaceutically relevant antigens such as HBsAg^{28,29} and candidiasis antigen.³⁰ In the model protein antigen studies, the strength of adsorption was altered via modifying the interaction between the antigen and adjuvant: adjuvant phosphate modification and/or dephosphorylation of the antigen. These methods were able to produce a range of formulations where the adsorption coefficient and fraction of antigen adsorbed were decreased in comparison to the unmodified antigen adsorbed to aluminum hydroxide salt. When the immune response was examined as a function of adsorptive strength within a series of formulations containing the same antigen (e.g., only α -casein based antigens), a trend of increasing immune response in mice as a function of decreasing adsorptive strength and fraction of antigen population that was adsorbed was observed.¹⁷

The adsorptive strength was not explicitly the focus of the investigation of a subunit vaccine candidate against candidiasis, nevertheless this study also points to an enhancement in the immune response when the adsorption strength is weaker.³⁰ Specifically, it was observed that there was a slightly significant ($p = 0.04$) reduction in the adsorptive capacity of Alhydrogel when the adjuvant was diluted with PBS versus saline. Although they did not measure the binding strength, the authors do suggest that the binding strength is likely less in the PBS formulation than the saline formulation based on the well-known phosphate exchange reaction that occurs when aluminum hydroxide adjuvant is formulated in phosphate buffers. When the two formulations were compared, there was not a significant difference in the antibody titers in mice using the two vaccines. Nevertheless, the vaccine formulated with PBS resulted in significantly higher Th1 and Th2 cell proliferation in spleenocytes and increased survival following a challenge.³⁰

Finally, two groups have investigated the effect of tightness of binding of HBsAg to aluminum hydroxide on anti-HBsAg antibodies produced by immunized mice.^{28,29} The outcome of both studies was that the vaccine formulations with the tightest adsorption of the HBsAg onto the adjuvant yielded the lowest antibody response. Moreover, the methodology of the Hem and colleagues study allowed evaluation of the effect of adsorptive strength in the absence of differences in the amount adsorbed. They observed that even when the antigen was essentially completely adsorbed in the vaccine formulations (aluminum hydroxide adjuvant: 100% adsorption, phosphate-treated aluminum hydroxide adjuvant: 99% adsorption), there was a significantly higher antibody response (GMT (95% CI): 813 (321, 2057) vs. 2605 (1044, 6499)) for the vaccine in which the strength of adsorption was weaker (Adsorptive coefficient, ml/mg: 3660 vs. 1550). Interestingly, Hem et al found no statistical difference between the antibody titers for the four vaccines using the phosphate-treated aluminum hydroxide adjuvants with various adsorptive coefficients (1550 to 250).²⁹

The only definitive conclusions that can be made from the literature discussed here are as follows: The strength of the association between the antigen and adjuvant may have

important implications for immunopotential. Tighter adsorption of the antigen to the adjuvant in the formulated vaccine is not necessarily better.

EFFECTS OF ADSORPTION ON THE ANTIGEN

It is intuitively attractive that adsorption should have effects on protein structure. Although aluminum-containing adjuvants have been in use in vaccine formulations for nearly a century and it has been assumed during much of that period that adsorption of antigens to those adjuvants is important, investigations into the direct effects of antigen adsorption on antigen conformation and stability have only recently begun. The work of Hem and colleagues demonstrated that the environment of the protein adsorbed to a mineral salt adjuvant can be significantly different from the bulk environment in which the protein stability is normally studied.³¹ The pH difference, which could be several units, suggests that caution should be taken when applying solution studies at bulk pH to predict the stability or conformation of the adsorbed antigen. Finally, it is also valid to assume that there may be differences in polarity and charge density on the adjuvant surface, which have implications for protein binding.^{31,32}

Recent studies report contradicting evidence regarding the extent of structural perturbation following adsorption. In one of the earliest such studies published, Tleugabulova et al used SDS-PAGE, denaturing size exclusion chromatography (SEC) and microscopy to investigate the structural perturbations of HBsAg following adsorption.³³ Under reducing conditions it was observed that, following adsorption, only half of the HBsAg was recoverable. It was further reported that increasing the time that the antigen was exposed to the reducing conditions (SDS sample buffer with dithiothreitol and 2-mercaptoethanol at 100°C, boiling) only increased the amount of the degradation peaks in the antigen exposed to adjuvant. They stated that this may suggest that the “structure of VLPs may be compromised by the adsorption on adjuvant.”³³ It is important to note however that both the chromatography and the SDS-PAGE were performed on antigen that had been intentionally reduced and denatured. Further evidence for structural modifications upon adsorption was detected via microscopy techniques.

Later works investigating the potential structural perturbation upon adsorption using more direct, spectroscopic techniques not requiring antigen reducing or boiling prior to analysis have mostly focused on model antigens. In a study of three model vaccines using lysozyme (adsorbed to aluminum phosphate), ovalbumin (adsorbed to aluminum hydroxide) and bovine serum albumin, BSA, (adsorbed to aluminum hydroxide) potential structural perturbations upon adsorption and as a consequence of thermal treatment were assessed via fluorescence spectroscopy, attenuated total reflectance (ATR) FTIR spectroscopy, and differential scanning calorimetry.³⁴ At relatively low temperatures (10–25°C), the spectral techniques suggested structural changes when the proteins were adsorbed. In another study, Zheng et al used ATR-FTIR to compare the secondary structure of soluble and aluminum hydroxide adjuvant adsorbed BSA and β -lactoglobulin in dried films and also concluded that the structures of the model antigens are altered following adsorption.³⁵ Moreover, Zheng et al, found that the structural perturbations were dependent on the amount of protein adsorbed, with lower amounts of adsorbed protein yielding greater spectral alterations following adsorption.

Not all studies investigating the effect of adsorption on antigen structure detected changes. Dong et al did not detect adsorption-induced changes in the secondary structure of six model protein antigens, including ovalbumin.³⁶ Unlike the other studies to detect secondary structural changes, Dong et al used transmission FTIR, which avoids potential artifacts associated with ATR-FTIR. The differences in the findings with ovalbumin might also be

attributable to the differences in the buffer, Dong et al used phosphate buffers, which as stated above is known to attenuate the binding interaction between ovalbumin and aluminum hydroxide adjuvant. Agopian et al used ATR-FTIR and concluded that the secondary structure of their antigen of interest, gp41, was not altered following adsorption onto aluminum hydroxide adjuvant.³⁷ They measured the spectra in 5 mM sodium formate buffer pH 5.2 or acetonitrile /water/detergent mixtures, and found α -helix and β -sheet contents similar, though not identical, to that of the soluble protein in 50 mM sodium formate buffer or ACN/water/detergent mixtures. They stated that 50 mM sodium formate or acetonitrile/water/no detergent prevent adsorption. Moreover, adjuvant dissolution was observed in 50 mM sodium formate buffer. This would suggest that the antigen is not tightly bound, but the strength of the binding interaction is not discussed. Nevertheless, both studies indicate that the secondary structures of antigens are not significantly altered under conditions that do not maximize the binding interaction. Collectively, studies investigating adsorption-induced structural perturbations of antigens suggest that all factors affecting antigen adsorption – including the antigen, buffer, and physiochemical properties of the aluminum-containing adjuvant – will dictate to what extent the antigen is perturbed. Antigen structures must be monitored in the formulation of interest, as extrapolations based on other formulations may prove unreliable.

Potential effects of adsorption and structural perturbations on the inherent thermal stability of antigens in the presence of adjuvant have been investigated as well. In the Jones et al. study using lysozyme, ovalbumin and BSA, marked changes in thermal behavior, including a decreased unfolding temperature and alterations of the number of thermally-induced transitions, were observed when these model antigens were bound to adjuvants.³⁴ Similar studies by others revealed that several potential vaccine antigens, including malaria,³⁸ ricin toxin,³⁸ and botulinum toxin³⁹ experience similar thermal destabilization when adsorbed to adjuvants when monitored by DSC, fluorescence, and second derivative UV spectroscopy. These studies suggest that it is inappropriate to use solution state stability data alone to predict the stability of the antigen in the presence of an aluminum-containing adjuvant. Instead, ensuring optimization of the vaccine requires that the antigen stability be studied in the presence of the adjuvant.

The thermal stability of cytochrome c, α -chymotrypsinogen A and ovalbumin as a function of adsorption onto aluminum hydroxide was determined by Bai and Dong using transmission FTIR spectroscopy.⁴⁰ They determined that the changes in the secondary structures of the proteins as a function of temperature were, at most, marginally affected by adsorption. Although they observed little to no differences in the relative intensity of the predominant second derivative peaks for the proteins in the adsorbed and solution states, they did determine that the strength of intermolecular β -sheet aggregations formed as a function of heating the samples was weaker in the adsorbed samples than the solution state proteins. Finally, ovalbumin exhibited the biggest spectral differences as a function of adsorption when heated, with the adsorbed sample having a band for random coil that was absent in the solution state. It was stated that this band represented an incomplete transition of the adsorbed protein from an unfolded state to aggregate.

In contradiction to the work by Jones et al, Zheng et al. suggest that the secondary structures of BSA and BLG in the adsorbed state had increased thermal stability in comparison to the solution state proteins.⁴¹ ATR-FTIR data monitoring the amide I and amide II regions of the antigens showed that BSA in solution lost α -helix structure and gained intermolecular β -sheet structure at high temperature, but no significant spectral changes were observed for BSA adsorbed to aluminum. Similar results were obtained for BLG although it was stated that “the thermal effect on stability of the adsorbed BSA and BLG is not the same.”⁴¹ Although the authors conclude that adsorption stabilizes the structures of the proteins, it is

important to keep in mind that the spectra of each protein in the adsorbed and solution states prior to heating were not alike. Thus, the “stabilized state” of the adjuvant formulation was already perturbed from that of the solution state. It was not the conformational state of the protein observed in the adjuvant-free formulation. Zheng et al. suggested that there were multiple reasons for the differences of this work in comparison to Jones et al including measurement temperature (Jones et al measured samples at elevated temperatures and Zheng et al measured previously heated samples at room temperature). They also point out differences in sample handling: Jones et al analyzed a slurry following centrifugation while Zheng et al. analyzed evaporated films. Therefore, although it would be advantageous to compare these studies it may be impossible.^{34,35,41}

While the immediate effects of binding to adjuvant are somewhat ambiguous or small, longer-term studies indicate greater effects of structural perturbation. Studies on a potential trivalent botulinum toxin vaccine showed greater changes in pH, DSC profile, fluorescence maximum, and 2D-UV peak position for antigen bound to adjuvant over 9 weeks, at 4°C and 37°C, than controls stored in similar conditions without adjuvant.³⁹ In addition, adsorbed antigens also showed a sharply decreased ability to desorb from the adjuvant. Similarly treated botulinum toxin vaccines were also examined by MALDI-TOF mass spectrometry after protease digestion in an attempt to localize sites of chemically modified residues (through oxidation or deamidation) for each of the proteins involved.³² It appears that binding to adjuvants causes chemical modifications to appear sooner, within two weeks of storage rather than 9 weeks, though in some cases it also decreases the number of chemical modifications present.³² These differences could indicate that the minor structural perturbations detected in most studies exposed different sections of the protein to conditions under which they are reactive, including the increased microenvironment pH surrounding adjuvants.³¹ There is some evidence that addition of excipients can improve stability of adjuvant-bound proteins, as will be discussed later. A significant consequence of chemical and physical alterations is the inability to adequately desorb proteins from the adjuvant, which as alluded to early in this review may negatively impact the immunogenicity of the vaccine.^{17,25,29} although it must be noted that this has not been investigated.^{32,39}

APPROACHES TO IMPROVE THE THERMAL STABILITY OF VACCINES WITH ALUMINUM-CONTAINING ADJUVANTS

Most vaccines are currently manufactured and transported as refrigerated liquid suspensions, and do not tolerate either increased temperatures or freezing well. The consequence is that a robust cold chain is necessary to ensure that efficacious vaccines reach the patients. Recently, several different approaches to achieve vaccines with improved thermal stability, at subzero and elevated temperatures, have been examined. These are highlighted below.

Improving the stability of suspensions at elevated temperatures

One approach to improving the thermal stability of vaccines with aluminum-containing adjuvants is to use a combination of buffers to alter the surface chemistry of the adjuvant and control the ionization state of the antigen's amino acid side chains. As stated in the previous sections, adsorption can compromise the thermal stability of some antigens. Jezek et al found that this was indeed the case for HBsAg, and although HBsAg antigenic activity dependence on bulk pH was only marginal the adsorbed antigen in a phosphate-free buffer, the thermal stability of soluble antigen was strongly pH-dependent.⁴² The addition of phosphate (20–100 mM) significantly improved the thermal stability of the adsorbed HBsAg, across the bulk pH range examined. This effect was attributed to a change in the pH of the microenvironment near the adjuvant surface due to the phosphate exchange that occurred with the aluminum hydroxide adjuvant. Moreover, the thermal stability was

improved by also including a second buffering species, especially histidine or lactate. Interestingly, the best formulations included histidine or lactate at pH 5.2, with considerably less buffering capacity than the other excipients examined, malate and succinate. The mechanism of the stabilizing effects of histidine and lactate was not elucidated. It was, however, hypothesized that the effect was due to differing abilities of the two groups of excipients to exchange protons with HBsAg side chains on the basis of the pKa's of the ionic excipients. Whether this approach of using phosphate buffer to modify the pH at the adjuvant surface and supplementing the formulation with an additional "buffer" species with all pKa's ≥ 1 pH unit from the bulk pH has a broader applicability for improving other vaccines with aluminum-containing adjuvants remains to be determined.

Middaugh et al have approached improving the thermal stability of vaccines through high throughput screening and empirical "phase diagrams" to define formulation conditions that will maintain the desired structural properties.^{38,43-49} The goal is that the actual formulation will result in a product well within the phase boundaries (e.g., temperature, pH) of interest. The strategy starts by using spectroscopic techniques (UV spectroscopy, fluorescence spectroscopy, circular dichroism, etc.) to create an empirical, quantitative "phase diagram" for the antigen based on a unique vectoral basis set that allows optimization of general formulation conditions (pH, temperature) for a particular antigen.⁵⁰ The construction of this phase diagram is followed by another set of high-throughput studies to screen excipients for increased stabilization under stress conditions, including thermal stress, denaturing solvent conditions, or physical agitation. This approach to rational vaccine stabilization for vaccines with aluminum-containing adjuvants relies on two assumptions: (1) that biophysical measures can provide information about the immunological integrity of antigens, and (2) that the conditions and excipients that stabilize antigens in solution will also stabilize them when adsorbed to adjuvants.³⁸

In recent studies for antigens that require an adjuvant for efficacy, the excipients that provided the most stabilization were subjected to a final series of tests with antigens adsorbed to adjuvants.³⁸ Some high throughput screening and phase diagram studies focused on enhancing the stability of the antigens in solution and evaluating the ability of the antigen to be adsorbed onto an aluminum-containing adjuvant in the stabilizing formulations. These studies included the use of the following antigens: region II of the non-glycosylated erythrocyte-binding antigen (EBA-175 RII-NG) – a vaccine candidate against malaria infections⁴⁹ and a recombinant ricin toxin A-chain V76M/Y80A (rRTA).⁴⁶ Other studies have not only used this technique to identify potential stabilizers but have also included studies on how the stabilizers affect the stability of the adsorbed antigens. EBA-175 RII-NG, rRTA, Clostridium difficile toxoids A and B, as well as the model antigens lysozyme, ovalbumin, and BSA have been the subject of these studies.^{38,44} From the results of these studies, Middaugh et al concluded that formulating with excipients identified as stabilizers of the antigen in the solution state indeed resulted in formulations with improved thermal stability of the antigen in the adsorbed state.

Improving the stability of suspensions at sub-zero temperatures

Although heat instability is better appreciated, as evidenced by the near-universal use of refrigeration in vaccine transport and storage, protection against damage from freezing temperatures has become an equally important consideration. In both developed and developing countries, significant amounts of vaccine are accidentally subjected to freezing temperatures.⁵¹ Exposure to freezing temperatures has been shown to decrease vaccine efficacy.^{51,52} Unlike heat degradation, which appears to be mostly attributable to the instability of the antigen, addressing instability at subzero temperatures may require consideration of both the antigen and the adjuvant.

For aluminum-containing adjuvants, freezing of adjuvant suspensions results in adjuvant particle aggregation.^{21,52–57} Several mechanisms have been proposed for adjuvant aggregation. These include overcoming the repulsive forces between particles of like-charge via mechanical forces created by growing ice crystals⁵⁷ and alteration of the particles' surface chemistry via freeze-concentration and ion-exchange of buffer salts on adjuvant particles.⁵⁶ A combination of these mechanisms is also possible. It has been observed that particle surface area is a determinant of IgE and IgG2 reactions to injected polystyrene particles⁵⁸ and that smaller particles are better internalized by dendritic cells,²⁷ though some studies show no relation between particle size and immunogenicity for particles ~ 2 to 10 μm — see below.²¹ It has been suggested that a loss of adjuvant activity after freezing could be attributed to adjuvant particle aggregation.^{52,53,55}

One possible measure that could be taken to prevent freeze-damage to vaccines is to simply prevent freezing. Freezing point depression can be accomplished through the addition of many different excipients due to colligative properties. Propylene glycol, polyethylene glycol 300 (PEG 300), and glycerol have all been used to prevent freezing, and thus prevent vaccine aggregation.^{54,55} This approach has been successful for preventing agglomeration of hepatitis B monovalent and DTaP (diphtheria, tetanus, acellular pertussis) trivalent vaccines. Moreover, propylene glycol and PEG 300 inhibited the red-shift of HBsAg's intrinsic fluorescence emission peak in the stabilized vaccines. Interestingly, glycerol (50% v/v) caused a blue shift in the emission spectrum without apparent detriment to vaccine efficacy. *In vitro* antigenicity and anti-hepatitis B antibody titers in mice were similar to properly stored control vaccine when using vaccines containing PEG 300, propylene glycol, or glycerol at a concentration that prevented freezing and that were subjected to multiple exposures to temperatures down to -20°C .

Polyethylene glycol and propylene glycol have also been shown to prevent the agglomeration of vaccine particles (monovalent HBsAg vaccines and multivalent vaccines) at concentrations too low to inhibit freezing.^{54,55} Temperatures as low as -80°C were explored. It is thought that the excipients may be preventing agglomeration even in the event of freezing through steric hindrance. As in the studies using sufficient concentrations of excipient to prevent freezing, the *in vitro* antigenicity and *in vivo* immunogenicity of these freeze-thaw stressed vaccines also indicated that the efficacy of the HBsAg component, the only component examined, was preserved.⁵⁵ A vaccine formulation including propylene glycol with the above-mentioned phosphate-histidine buffer for hepatitis B vaccines has achieved enhancement of both heat- and freeze-stability in a monovalent hepatitis B vaccine.⁵⁴

Studies to evaluate the effect of excipients on the freezing stress of lyophilization processes have also recently revealed a variety of excipients that may suppress freeze-thaw-induced agglomeration of vaccine particles. One group examined varying concentrations of trehalose and sodium succinate buffer for their abilities to minimize agglomeration of Alhydrogel[®] when frozen on a lyophilizer shelf while cooling to -40°C at a rate of $0.5^{\circ}\text{C}/\text{min}$.⁵⁶ Trehalose, at a concentration of 15% w/v, yielded a particle size distribution similar to the untreated, unaggregated control (mean particle diameter $2.25 \pm 0.37 \mu\text{m}$). At concentrations between 5% and 10%, a significant population of aggregates was observed. Spray-freezing and increasing the cooling rate reduced the amount of trehalose necessary to maintain the original particle size distribution (PSD), with spray-frozen samples requiring no trehalose. Only a small succinate dependence on the mean particle diameter was observed for shelf freeze-thaw (F/T)-treated samples containing 7.5% w/v trehalose and 0–250 mM succinate – increasing slightly with increasing succinate content. Antigen binding and enhancement of the immune response of the F/T treated adjuvant was not measured in this study; however, a subsequent study of model vaccines with lysozyme as the antigen revealed an increase in the

amount of lysozyme bound to aluminum hydroxide adjuvant in a sodium succinate buffer formulation following either freezing on a lyophilizer shelf or spray-freezing.²¹ Whether up to 7.5% trehalose had a significant affect on the increase in antigen binding was not discussed.²¹

Another group examined several formulations of 10 mg/ml aluminum hydroxide adjuvant (Alhydrogel®) under dry-ice freezing conditions, again with the intentions of examining what occurred during the freezing step for lyophilized samples.⁵⁹ The formulations that resulted in 24-hr sedimentation volumes following a single dry ice/RT (3h/1h) freeze-thaw cycle similar to non-frozen control all contained 0.1% polysorbate 80 and one additional excipient. The specific excipients conferring protection against agglomeration were saccharose, sorbitol, glycine, mannitol, polyvidone (PVP) K12, and PVP K25, all at 10% w/v, and trehalose (20%). The effect of the excipients on antigen binding capacity of the adjuvant was not discussed. Additionally, neither the PSD (before or after a freeze-thaw treatment) nor immunogenicity of model vaccines formulated with these excipients was examined.

Dry powder approaches to enhanced stability

Another strategy that can address both heat- and freeze-stability issues is to formulate vaccines containing aluminum adjuvant as dry powder. Dry powder formulations generally show slower kinetics of degradation, even at elevated temperatures. Moreover, a solid, low-water formulation is also less susceptible to the stresses following additional exposure to subzero temperatures after attaining the dried state. Finally powder formulations allow different routes of administration, including inhalation and trans-dermal modes. There are, however, several obstacles to the use of dried formulations. Most methods of drying require either heating or freezing, both of which can be problematic for vaccine components – see above. In addition, dried vaccine formulations often require an additional reconstitution step prior to administration; this can add an additional source of error and another stress condition to vaccine preparations. Nevertheless, advances have been made regarding the creation of dry powder vaccines with aluminum-containing adjuvants.

Conventional freeze-drying, even in the presence of excipients that offer protection against freeze-thaw damage, results in agglomerated particles following rehydration.^{21,56} Spray freeze-drying, however, has been used to successfully produce dry powder vaccines with aluminum-containing adjuvants with little to no particle agglomeration following reconstitution.^{21,56} It has been suggested that the lack of agglomeration in the spray freeze-dried vaccines is attributable, at least in part, to the minimal agglomeration afforded by the faster cooling rate of spray freezing prior to drying.^{53,56} According to conventional wisdom of what is known regarding particle size and the immune response, it would be hypothesized that a conventionally lyophilized vaccine will yield reduced anti-antigen antibody titers and spray-freeze dried samples could yield similar antibody titers as the liquid control. Interestingly, Randolph and colleagues found no correlation between particle size and anti-lysozyme antibody titers in mice immunized with control vaccine, vaccine subjected to conventional lyophilization and spray freeze-dried vaccine.²¹ The only formulation that resulted in antibody titers significantly different from the control vaccine suspension was the spray freeze-dried formulation – which yielded a larger mean anti-lysozyme antibody response. The mean particle sizes of the vaccines ranged from 2 to 14 μm , dependent on the formulation and treatment. It was suggested that perhaps the lack of correlation between particle size and anti-lysozyme antibody titers was because the group with the largest particles (i.e., excipient-free vaccine dried via conventional lyophilization then reconstituted) had a mean particle size that was similar to the 10 μm threshold suggested for optimal uptake by macrophages. Given the strong correlation between particle size and anti-HBsAg antibody titers that we previously observed for samples subjected to freeze-thaw

stress, the lack of correlation between particle size and antibody titers in dried samples is not likely to be universal.

Another method for producing dried biological formulations involves use of supercritical fluids. At the critical point, the boundary between the liquid and gas phases ceases to exist. Carbon dioxide (CO₂) is one of the most common compounds for supercritical applications. This is due to its very accessible critical points (31.3°C and 7.38 MPa), low toxicity, and low environmental impact.⁶⁰

Carbon dioxide Assisted Nebulization with a Bubble-Dryer® (CAN-BD) is a super-critical CO₂ process that has been successfully used to produce dried small molecules, proteins, and vaccines.^{60–62} Supercritical fluids aid in making an aerosol of the pharmaceutical formulation. In the case of vaccines, two streams, one of aqueous antigen (and adjuvant, if necessary) with stabilizers and one of supercritical CO₂, are mixed at high pressure and sprayed into a drying chamber at atmospheric pressure. A rapid expansion of both mixed supercritical CO₂ and CO₂ dissolved in water produce micron-scale droplets, which are rapidly dried by heated nitrogen and collected on a filter.^{61,63} This process has been used to produce a dry aluminum-hydroxide-adjuvanted HBsAg vaccine formulation with little detectable loss in potency or immunogenicity in mice, following reconstitution.⁶² The mean volume-weight aerodynamic diameter of the resulting particles was 1.6 µm, with the majority sized between 1 and 10 µm. Moreover, Sievers et al reported that the powder formulation was stable following storage at 66°C (up to 10 days) and –20°C (up to 43 days). The method has also been used to produce an inhalable powder formulation for a live-attenuated measles vaccine.^{60,62,64} Similar to the freeze-drying methods mentioned earlier, most of these formulations require one or more stabilizers, usually surfactants or saccharides.

It is apparent from the original works reviewed here that thermal stability of some vaccines containing the aluminum-containing adjuvants has been successfully addressed through formulation (buffers and surfactants). Limited evidence exists that suggest that dry powder approaches (spray-freeze drying, supercritical fluid methodologies, etc.) to improved stability may also result in stabilized vaccines. It is unclear whether these strategies will be universally applicable to all vaccines, as each method discussed has limitations that must be considered in vaccine development. Regardless of whether the inherent lack of thermal stability is improved through formulation or in combination with a drying method the (1) excipient effects must be monitored and (2) implications on overall immunogenicity of the end-product must be validated for efficacy.

CONCLUSIONS

With their long history of use, aluminum-containing adjuvants will likely continue to be in vaccines for decades to come. The results of recent research however suggest that the approach to creating and formulating vaccines with these adjuvants may and should be changing. The conventional wisdom regarding these vaccines has been challenged. First, with respect to the design of the antigen/adjuvant complex, it is no longer a given that strong binding of the antigen to the adjuvant will result in the most robust immune response. This observation combined with the potential inherent immune stimulating differences of the aluminum-containing adjuvants with differing chemistries will likely have an impact on the balance of free versus absorbed antigen in candidate vaccine formulations. Although large conformational changes do not necessarily immediately result when antigens are adsorbed, antigens that are tightly bound to adjuvant do appear to have subsequent decreases in thermal stability. Moreover, as the vaccine ages, the extent of conformational changes can

also change. The question remains however whether some degree of destabilization is beneficial or detrimental to the immunogenicity of properly stored vaccine.

New approaches to vaccine stability have also been presented. We now know that insight into how to stabilize the antigen in the solution state can provide guidance into minimizing the extent of adsorption induced thermal destabilization of the antigen. Moreover, recent success with the improved heat stability of a hepatitis B vaccine highlights the importance of recognizing that changes to the adjuvant's surface chemistry and choice of buffer salts can have profound effects on the stability of the antigen. This same vaccine has served as a useful model in approaches to achieving enhanced stability in the subzero domain, in both the liquid and dried state. Moreover, the lyophilization studies with model proteins are beginning to suggest that some degree of agglomeration may even be acceptable in a reconstituted lyophilized product. Although the dry powder approaches that retain particle size of the liquid vaccines may be farther on the horizon, the approaches to enhancing the stability of these vaccines in the liquid state are relatively low cost, and by utilizing excipients with a good safety track record for injectable pharmaceuticals, should come to fruition in the not too distant future. The universality of the approaches to enhancing the thermal stability of vaccines with aluminum-containing adjuvants will only become clear as these approaches are attempted with additional vaccine candidates.

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Table 1

Examples of adsorptive capacities and adsorptive coefficients

Antigen	Adjuvant ¹	Mean adsorptive capacity (mg/mg Aluminum)	Adsorptive coefficient (ml/mg)	Ref.
Lysozyme	AH	0.772	6	65
Lysozyme	AH	~0.3 ²	Not reported	66
Lysozyme	AP	1.49	1.8	67
Lysozyme	AP, 0% EG ³ AP, 20% EG AP, 40% EG	0.66 0.57 0.47	Not reported	68
Lysozyme	AP	1.4	0.47	34
BSA ⁴	AH AH	1.77 2.59	35 12	67
BSA	AH	2.67	197	65
BSA	AH (1) AH (2) AH (3)	1.71 1.61 1.43	Not reported	68
BSA	AH, 20% EG AH, 40% EG	1.70 1.68	Not reported	68
BSA	AH	2.2	3.1	34
α -casein	AH	2.1	Not reported	27
α -casein	AH	2.5	2000	69
α -casein	AH	2.4	2409	17
α -casein	PT-AH (A) PT-AH (B)	1.5 1.4	Not reported	27
α -casein	PT-AH	0.9	419	17
α -casein	PT-AH (E) PT-AH (F)	1.7 1.4	150 130	69
α -casein	AP	0.4	Not reported	27
DP- α -casein	AH	2.5	75	69
DP- α -casein	AH	0.4	59	17
DP- α -casein	PT-AH	0.03	0.8	17
DP- α -casein	PT-AH (E) PT-AH (F)	0.6 0.1	65 65	69
Ovalbumin	AH	2.7	86	69
Ovalbumin	AH	1.6	1.6	34
Ovalbumin	PT-AH (A) PT-AH (B) PT-AH (C) PT-AH (D) PT-AH (E)	2.4 2.0 1.7 1.3 0.8	62 43 19 11 7	69
HBsAg	AH	1.7	6000	70
HBsAg	AH	0.91	3660	29

Antigen	Adjuvant ¹	Mean adsorptive capacity (mg/mg Aluminum)	Adsorptive coefficient (ml/mg)	Ref.
HBsAg	PT-AH (A)	0.81	1550	29
	PT-AH (B)	0.79	635	
	PT-AH (C)	0.62	320	
	PT-AH (D)	0.58	250	
rPa	AH	0.73	100	71
rPa	PT-AH	0.50	4.6	71
Myoglobin	AH	1.14	2	65
α -lactalbumin	AH	1.85	1806	65
Fibrinogen	AP	0.753	2215	65

¹ **AH**: Aluminum Hydroxide Adjuvant; **AH (#)**: Aluminum Hydroxide Adjuvant in NaCl (going in increasing amount of NaCl); **PT-AH**: Phosphate-Treated Aluminum Hydroxide Adjuvant; **PT-AH (A-Z)**: Phosphate-Treated Aluminum Hydroxide adjuvant treated with phosphate (going in increasing order of phosphate content). The letter designation is ONLY consistent within a referenced manuscript; **AP**: Aluminum Phosphate Adjuvant

² Value is approximated from a graph in the original manuscript.

³ **EG**: ethylene glycol. All samples with this designation also contain 0.06M NaCl

⁴ For this entry, the two AH differ in crystalline size (i.e. surface area).

Table 2
Summary of recent studies investigating the importance of antigen adsorption on immunological outcome

Antigens	Adjuvants ¹	Percentage of the antigen that is adsorbed	Immunological Outcome	Amount bound important?	Ref
Lysozyme	AH PT-AH AP	3% 10%, 85–90% 35%	1 All vaccines with antigen pre-bound to adjuvant resulted in immunological titers 4 times that of soluble protein or administration of soluble protein and adjuvant and different sites.	1 No	20
			2 Titers of vaccines with antigen and adjuvant formulated together, regardless of the percentage of the antigen that is adsorbed, were not statistically different.	2 No	
Lysozyme	AH	~10–80% (varied w/ processing and trehalose content)	No correlation between the amount of antigen bound and IgG1 titer in mice.	No	21
Lysozyme	AP	~15–90% (varied w/ processing and trehalose content)	No correlation between the amount of antigen bound and IgG1 titer in mice	No	21
rPA	AP in phosphate buffer, AP in saline AH in saline	0% ≥80% 100%	1 All enhanced the immunological response relative to soluble antigen with no adjuvant present.	1 No	22
			2 For the AP formulations, adsorption of the antigen rendered the immunological response insensitive to the antigen dose in the range examined.	2 Yes	
			3 The only vaccines that elicited neutralizing antibodies at levels above adjuvant-free vaccine were those with AP adjuvant.	3 Maybe	
Lysozyme	F-AP AP	0% 38%	All vaccines containing adjuvant, even if almost none of the antigen was adsorbed, yielded higher antibody titers in mice than soluble antigen without adjuvant present.	No	23
DP-α-casein	AP AP in phosphate buffer	100% 2%	All vaccines containing adjuvant, even if almost none of the antigen was adsorbed, yielded higher antibody titers in mice than soluble antigen without adjuvant present.	No	23
OVA DP-OVA	AP	38% 0%	All vaccines containing adjuvant, even if almost none of the antigen was adsorbed, yielded higher antibody titers in mice than soluble antigen without adjuvant present.	No	23
Sp1 Sp2 Sp3	AP AH	Not adsorbed Adsorbed	Antigens adsorbed to AH produced significantly higher titers than those not adsorbed, in solution alone (Sp-2 only) or with AP.	Yes	24

¹ AP: aluminum phosphate adjuvant; AH: aluminum hydroxide adjuvant; PT-AH: phosphate treated-aluminum hydroxide adjuvant; F-AP: fibrinogen-treated aluminum phosphate adjuvant