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Review Article

Theme: Emerging Concepts for Vaccine Development and Vaccination
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A Rational, Systematic Approach for the Development of Vaccine Formulations

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Abstract. With the continuous emergence of new infectious diseases and new strains of current diseases, such as the novel H1N1 influenza in 2009, in combination with expanding competition in the vaccine marketplace, the pressure to develop vaccine formulations right the first time is increasing. As vaccines are complex, costly, and have high risk associated with their development, it is necessary to maximize the potential for development of a successful formulation quickly. To accomplish this goal, the historical empirical approach to formulation development needs to be updated with a rational, systematic approach allowing for more rapid development of safe, efficacious, and stable vaccine formulations. The main components to this approach are biophysical characterization of the antigen, evaluation of stabilizers, investigation of antigen interactions with adjuvants, evaluation of product contact materials, and monitoring stability both in real time and under accelerated conditions. An overview of investigations performed for each of these components of formulation development is discussed. The information gained in these studies is valuable in forming the base of knowledge for the design of a robust formulation. With the use of continually advancing technology in combination with maintaining a rational, systematic approach to formulation development, there is a great increase in the probability of successfully developing a safe, effective, and stable vaccine formulation.

KEY WORDS: adjuvant; development; formulation; stability; vaccine.

INTRODUCTION

Development of vaccines is a complex and costly undertaking with high risk associated as the majority of vaccine candidates fail in preclinical and phase I development (1). Because of the complexity of manufacturing vaccine products, it is important to have a good understanding of what factors can impact the safety, efficacy, and stability of the formulation all along the development path. Failure to understand factors that can adversely impact the vaccine formulation can result in selection of sub-optimal conditions leading to failures of safety, efficacy, or stability causing project delays or cancelation. In addition, with the continuous emergence of new infectious diseases and new strains of current diseases, such as with the novel H1N1 influenza in 2009, along with expanding competition in the vaccine marketplace the pressure to develop vaccine formulations quickly is increasing.

An outline of a rational, systematic approach for the development of robust vaccine formulations is presented in Fig. 1. The main components of this approach are: biophysical characterization of the antigen, evaluation of stabilizers, investigation of antigen interactions with adjuvants, evaluation of product contact materials such as sterile filter membranes, and monitoring stability both in real time and under accelerated conditions. In this approach, development is initiated with biophysical characterization of the antigen with various analytical techniques with the goal of determining appropriate pH, buffer species, and ionic strength to prevent antigen aggregation and maintain the antigen in an appropriate folded state for early preclinical studies. These studies are followed up with an evaluation of stabilizers to enhance the physical and chemical stability of the antigen moving towards the typical goal for vaccines of a 3-year shelf life. Next, investigations of what the most appropriate adjuvant is to obtain the desired immune response and how the antigen and adjuvant interact with one another should be performed. As development moves towards a formulation for phase I clinical trials initial evaluations of how the formulation is impacted by product contact materials such as sterile filter membranes should be initiated. All of these investigations need to be supported by real-time and accelerated stability studies to verify that changes to the formulation during the development process maintain the antigen in a chemically and physically stable state. Each of these components occurs over multiple development phases and are complimentary with one another. An overview of each of these steps in the development process will be discussed. By replacement of historical empirical approach to development of vaccines with a rational and systematic approach quality can be built into the formulation allowing for more rapid development of safe and reliable vaccines.

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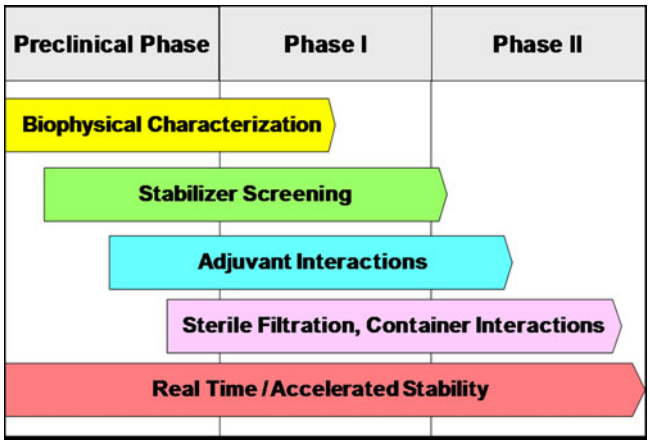

Fig. 1. Components of a rational and systematic approach to the development of vaccine formulations are biophysical characterization, stabilizer screening, adjuvant interactions, sterile filtration and container interactions, and stability studies. Each of these components occurs over multiple phases of development and are complimentary to one another

Biophysical Characterization

In the early phases of vaccine development, it is important to understand the physical characteristics of the antigen. It is critical to understand how parameters such as pH, buffer species, and ionic strength impact the folded state of the antigen as well as the propensity of the antigen to aggregate. Knowing how characteristics of the formulation will impact physical stability of the antigen will aid selection of appropriate excipients during the development process. The laboratory of C Russell Middaugh at the University of Kansas has performed extensive research in the area of biophysical characterization of vaccine antigens and therapeutic proteins through the use of spectroscopic techniques (2). Studies performed by Peek *et al.* (3,4) used a systematic three-step approach towards development of ricin toxin A-chain and erythrocyte binding antigen vaccines. In this systematic approach, the stability of the antigen is first evaluated as a function of pH and temperature, then a library of compounds is screened for their potential to stabilize the antigen, and finally the adsorption characteristics of the antigen to aluminum-containing adjuvants is investigated. In the evaluation of antigen stability as a function of pH, they utilized empirical phase diagrams which are a valuable tool to combine data from various analytical techniques to obtain a broad view of antigen stability.

Empirical Phase Diagrams

When initiating preformulation studies for the systematic development of a vaccine formulation, a logical place to start is understanding how the physical stability of the antigen is impacted by changes in pH and temperature. The pH of the formulation can impact both the physical stability of the antigen, such as whether the antigen maintains the appropriate folding and if the antigen will aggregate, as well as the chemical stability of the antigen. The pH can impact the chemical degradation rate of many mechanisms of degradation such as hydrolysis, oxidation, and deamidation (5). The empirical phase diagram offers a convenient way to display how the physical stability of an antigen is impacted with changes in pH and temperature. Generally in this approach, characterization data are taken from various spectroscopic techniques such as second derivative UV/Vis (6), intrinsic fluorescence, extrinsic fluorescence (7,8), and circular dichroism (9,10) are combined and transformed into data vectors to construct the empirical phase diagram (11). An example of data typically transformed into vectors is from second derivative UV/Vis spectroscopy and is shown for an antigen of *Clostridium difficile* (Fig. 2). In this technique, shifts in the wavelength of absorbance peak minima of phenylalanine, tyrosine, and tryptophan residues are associated with changes in the protein structure. A shift in the blue direction is associated with unfolding as the amino acid residues are exposed to a more polar environment (Fig. 2a). A shift in the red direction is associated with aggregation as the amino acid residues are exposed to a less polar environment (Fig. 2b). So depending on the environment the antigen was exposed to either unfolding or aggregation occurred. These data can be combined with other characterization data into vectors which are assigned a color based on the magnitude and an empirical

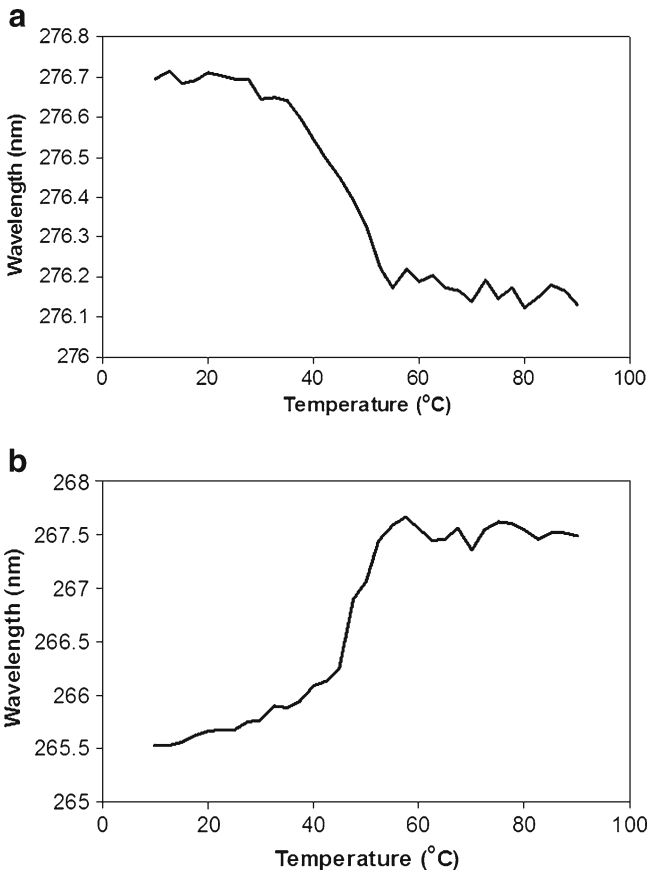

Fig. 2. Second derivative UV/Vis spectroscopy is a useful technique for antigen characterization. In this technique, shifts in wavelength of absorbance peak minima for phenylalanine, tyrosine, and tryptophan residues reveal if the protein changes states with increasing temperature. Under various formulation conditions an antigen for *Clostridium difficile* can be observed in different physical states of unfolding (**a**) and aggregation (**b**). Unfolding of the antigen is indicated by a blue shift in the wavelength of the absorbance peak minima while aggregation is indicated with a red shift in the peak minima

phase diagram of pH versus temperature can be constructed from these color vectors (Fig. 3). In empirical phase diagrams, regions of similar color indicate conditions where the antigen is in a similar state. Different color regions indicate that the antigen is in a different state under those formulation conditions. Depending on what information is known about the antigen, regions of the phase diagram can be labeled for example as: native structure, unfolded, and aggregated. If information on the stability of the various forms of the antigen is know the regions can be identified as stable or unstable states. The empirical phase diagram approach has been used in development of viral (12), bacterial (13), as well as sub-unit (14,15) vaccine antigens and is a valuable tool for selection of appropriate pH conditions early in development.

In addition to pH evaluation, the empirical phase diagram approach can be utilized to determine the impact of other variables on antigen stability like buffer type and concentration, ionic strength, and impact of product contact materials. For instance, it was investigated what the effect on physical stability would be of increasing levels of phosphate buffer for the *C. difficile* antigen discussed previously (Fig. 4). At the lowest concentration of phosphate buffer investigated a transition in the physical state of the antigen was observed at approximately 43°C. This is shown on the empirical phase diagram by the shift in color from brown to green. This transition was associated with an unfolding event of the antigen. There were also transitions seen at the same temperature for the higher buffer concentrations. However, those transitions resulted in a different physical state of the antigen than was observed at the low buffer concentration indicated by the purple and blue colors on the empirical phase diagram. The transitions at the 100 and 150 mM phosphate buffer concentrations were associated with antigen aggregation and reduced stability. The data suggest that high concentrations of phosphate should be avoided to maintain optimal physical stability of the antigen. The empirical phase diagram approach is a convenient method to have a broad overview of the impact of various formulation parameters on the physical stability of an antigen. This allows for a more rational selection of formulation conditions moving forward through the development phases.

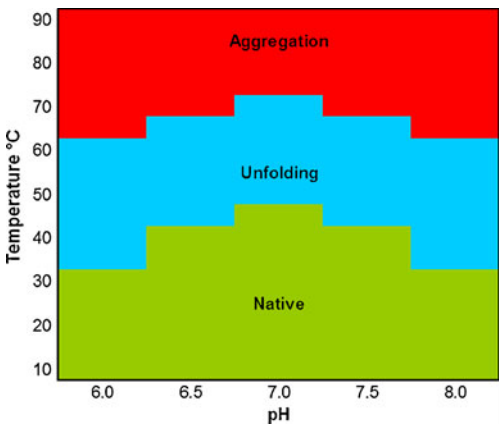

Fig. 3. When evaluating antigen stability by the empirical phase diagram approach regions of like color indicate under those conditions the antigen is in a similar state. A change in color suggests that an event such as unfolding or aggregation has occurred. In the empirical phase diagram presented here for a theoretical antigen as the color changes from *green* to *blue* to *red*, the antigen goes from the native conformation to an unfolded state and finally to an aggregated state. The diagram also indicates that the antigen is most stable at pH 7.0 as the transition temperatures are highest at that pH

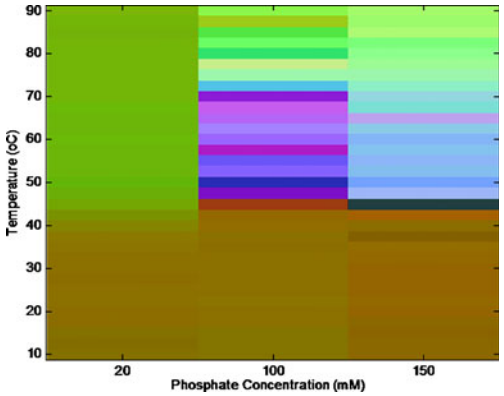

Fig. 4. An empirical phase diagram for a *Clostridium difficile* antigen exhibits how buffer concentration can impact the physical state of an antigen. There is a transition in the physical state of the antigen at all levels of phosphate buffer at approximately 43°C. The transition observed at the low level of phosphate was associated with protein unfolding while the transition at the higher buffer levels was associated with aggregation of the antigen

Evaluation of Stabilizers

Optimization of formulation parameters such as pH, ionic strength, and buffer species may not prove to be enough to stabilize an antigen for the typically desired 3-year shelf life of vaccine products. In this case stabilizing excipients need to be investigated for incorporation into the vaccine formulation. Evaluation of antigen stabilizers typically begins with investigation of generally regarded as safe (GRAS) excipients. By utilizing GRAS excipients, development may proceed more rapidly as regulatory concerns regarding safety of the formulation excipients will be lower. Since at the early stage of development the primary mechanism of antigen degradation may not be known it is important to evaluate excipients from various classes of stabilizers (Table I). For stabilizer investigation, information on the physical stability of the antigen obtained from the empirical phase diagram approach can be used to select appropriate formulation conditions in which to perform the studies. It is useful to select formulation conditions in which the antigen exhibits some instability in order to be able to detect stabilization

Table I. Classes of Excipients and Examples of GRAS Compounds in Each Class that can be Evaluated as Stabilizers in the Development of a Vaccine Formulation

Excipient class	Examples
Amino acids	Arginine, aspatate, glycine, glutamate, lysine, and proline
Antioxidants	Ascorbic acid, EDTA, and malic acid
Cyclodextrins	α-Cyclodextrine, β or γ 2-hydroxypropylcyclodextrine
Proteins	Albumim and gelatin
Sugars/Sugar Alcohols	Sucrose, trehalose, lactose, dextrose, glycerol, sorbitol, and mannitol
Surfactants	Brij, pluronic, and Tween

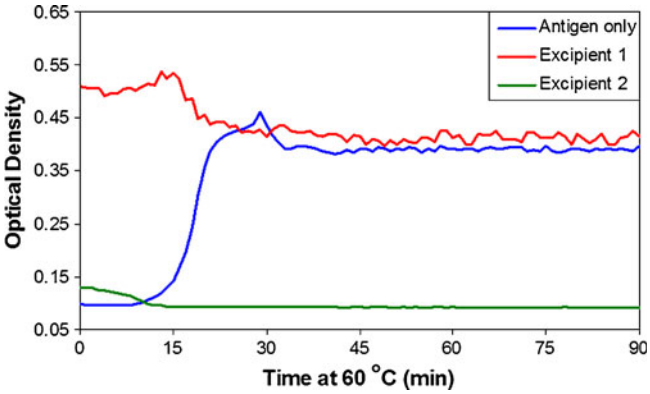

Fig. 5. One method to assess the ability of excipients to stabilize an antigen is to monitor optical density of each formulation over time. Two excipients were evaluated for their ability to stabilize a meningococcal serotype B recombinant protein antigen. The optical density of the formulations was monitored over time at 60°C to evaluate the onset of aggregation

effects of excipients more easily. Using the empirical phase diagram for the theoretical antigen in Fig. 2, a low pH of 6.0 or a high pH of 8.0 would be selected to evaluate stabilizers for that antigen since, at those pHs, the transition temperatures of the antigen is lower. Once the formulation conditions are selected the impact of each stabilizer on the onset of aggregation are determined for each excipient or combination of excipients. The excipient(s) that exhibit the greatest increase in the transition temperature, or the time until the transition occurs at a given temperature, should be considered for inclusion in the final formulation. The ability of two excipients to stabilize a meningococcal serotype B recombinant protein antigen against aggregation was evaluated (Fig. 5). In this experiment, optical density of each formulation was monitored at 60°C and the time until the onset of aggregation was determined. The formulation with no added excipient exhibited aggregation after approximately 13 min. Excipient 1 actually destabilized the antigen and was aggregated immediately upon monitoring the formulation indicated by the high optical density. Excipient 2 was a good stabilizer of the antigen as it prevented aggregation through the duration of the experiment. Therefore, excipient 2 was selected to perform further development studies to optimize the formulation.

Excipient screening such as monitoring of optical density or extrinsic fluorescence can be performed in a 96 well format to allow high-throughput screening of many excipients and excipient combinations at one time. This is a powerful approach to obtain a large quantity of stability data quickly and has been used in the development of *C. difficile* (16,17), *Bacillus anthracis* (14), measles (12), hepatitis (18), as well as other vaccines. Once the pH, buffer, and stabilizer(s) have been determined, focus of development can shift towards understanding how environmental stresses such as low or high temperatures as well as incorporation of adjuvants to enhance immunogenicity may impact antigen stability.

Correlation of Real-time and Accelerated Stability

It is important to understand how extreme environmental conditions such as high temperatures can impact the stability of the formulation. Correlation of accelerated stability with real-time data is valuable to support activities such as expiration dating and assessment of the impact of temperature excursions during shipment and storage of the vaccine for clinical trials. When initiating stability studies it is important to understand what potential mechanism the antigen can degrade by. In general, physical instability is associated with loss of protein structure and aggregation while common forms of chemical degradation are oxidation and deamidation (19). One method to evaluate the stability of the antigen against these types of degradation is Arrhenius kinetics. The temperature dependence of the rate constants for elementary chemical reactions can be described simply by the Arrhenius equation (20):

$$k = Ae^{-E_a/RT}$$

In the equation, k is the rate constant, A is the pre-exponential factor, E_a is the activation energy, R is the gas constant, and T is the temperature. If a plot of Ln (k) versus $1/T$ is linear the data obtained from the accelerated conditions can be extrapolated to real-time storage conditions such as refrigerated at 4°C. An underlying assumption of the Arrhenius equation is that the reaction mechanism does not change in the temperature range of interest. Generally, a change in the mechanism of degradation results in a non-linear Arrhenius plot. An example of an antigen whose degradation follows Arrhenius kinetics is meningococcal serotype A polysaccharide conjugated to tetanus toxoid (Fig. 6). This does not necessarily mean there is a single degradation mechanism for the conjugate that is an elementary chemical reaction, but that the rate limiting reaction follows Arrhenius kinetics or possibly the average of all the potential degradation mechanisms approximates Arrhenius type behavior. From this type of data, predictions can be made regarding whether the antigen will remain stable for the desired shelf life of the product. This allows decisions to be made earlier on in the development process regarding the necessity of further development work to reach stability goals. The accelerated data should always be supported with real-time studies to verify the predicted relationship between antigen degradation at high and low temperatures.

Obtaining data on how extreme temperatures impacts the vaccine formulation is also important for supporting clinical studies. Temperature excursions, where the vaccine formulation is exposed to temperatures outside of the recommended range such as freezing or ambient temperature

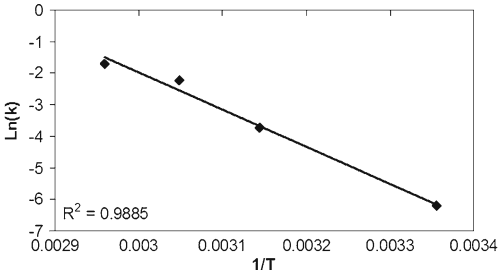

Fig. 6. The degradation of meningococcal serotype A conjugate can be described by Arrhenius kinetics. By extrapolation of data from accelerated conditions, predictions of the kinetics in real time can be made

for refrigerated products, can occur during the shipment and storage of clinical trial materials. The data obtained in accelerated stability studies are useful for determination whether a vaccine remains acceptable for administration to a patient following exposure to a temperature higher than the recommended storage temperature. For example, if a vaccine containing the meningococcal conjugate discussed above, in which refrigeration is the recommended storage condition, was left overnight at ambient temperature the equation for the best fit line of the Arrhenius plot above could be used to predict if the vaccine remains within the potency specification and can still be administered to patients.

In addition to high temperature excursions it is useful to determine the impact of other factors on formulation stability. Environmental stresses such as exposure to cycles of freezing and thawing, extended exposure to light as well as contact with various storage container materials. As more knowledge is obtained on how environmental stresses impact the formulation this information can be used to develop a more robust and stable vaccine. These early characterization and stability investigations also give indications regarding whether a lyophilized formulation will be required to meet the shelf life goals of the product. While this review focuses on development for liquid presentations there are reviews in the literature regarding the considerations needed when developing a lyophilized vaccine formulation (21–23).

Adjuvantation

A side effect of vaccine antigens becoming more pure as purification technology has advanced is a reduction in the immunogenicity of the antigen. The first vaccines were killed or inactivated bacteria or viruses (24). These formulations retained intrinsic molecules such as Toll-like receptor agonists and exotoxins that could activate the immune system. However, the potential reactogenicity of these whole cell or virus formulations presents a safety issue versus a highly purified antigen. To retain antigen immunogenicity with more highly purified antigens, adjuvants can be incorporated into the vaccine formulation. Adjuvants interact with the immune system through various mechanisms thereby enhancing the immune response. Currently, the most utilized adjuvants in licensed products are aluminum salts and squalene-based oil-in-water emulsions. Use of these adjuvants will be the focus of this section, however there are many other types of adjuvants in development for use in vaccines such as Toll-like receptor agonists, examples include LPS mimics, flagellin, and CpG motif DNA, saponins such as QS-21, and cytokines including type I Interferons and IL-12 (25–32).

Aluminum Adjuvants

Aluminum-containing adjuvants are currently the most widely used in marketed vaccines. These adjuvants have a long history of use and an excellent safety profile. The adjuvant effect of aluminum salts was first discovered by Glenny *et al*. in 1926. He observed an increase in the immune response of diphtheria toxoid when precipitated with potassium alum versus a solution of the toxoid (33). Since that time much has been learned about the properties and mechanism of action of aluminum-containing adjuvants, particularly from Stanley Hem's laboratory at Purdue University. This section described what aluminum-containing adjuvants are, how they interact with vaccine antigens, and current hypotheses on the mode of action of these adjuvants.

Characterization of Antigen Interactions with Aluminum-Containing Adjuvants

When formulating a vaccine with aluminum-containing adjuvants, it is important to understand both the nature of the surface of the adjuvant and how the antigen interacts with that surface. When considering the nature of the adjuvant surface it is convenient to imagine a continuum of surfaces with slightly different properties, with aluminum hydroxide adjuvant on one end of the continuum, and aluminum phosphate adjuvant on the other. Aluminum hydroxide adjuvant is chemically crystalline aluminum oxyhydroxide AlO(OH) (34). The surface of aluminum hydroxide adjuvant is composed of hydroxyls that are able to accept and donate protons which allow the surface to have an electrical charge (35). Because the adjuvant surface can have a positive or negative charge depending on the pH the adjuvant has a point of zero charge (PZC). It is important to understand what the PZC of the adjuvant is as this gives an indication of whether the surface charge at a given pH will be positive or negative which in turn impacts antigen adsorption. The PZC of aluminum hydroxide adjuvant is 11.4 so at physiological pH the surface has a positive charge. The PZC of aluminum hydroxide adjuvant can be decreased to an acidic value by treatment of the adjuvant with phosphate anions (36). The decrease in PZC is proportional to the level of phosphate exposure as the greater amount of phosphate exposure the lower the PZC value.

Aluminum phosphate adjuvant is chemically amorphous aluminum hydroxyphosphate, $Al(OH)_x(PO_4)_y$, and is not a stoichiometric compound. The PZC of aluminum phosphate adjuvant decreases with increasing phosphate incorporation and commercially available material generally has a PZC between 5.0 and 5.5 (37). Therefore, at physiological pH aluminum phosphate adjuvant generally has a negative surface charge.

Once the physical properties of the adjuvant are known, how a given antigen will interact with the adjuvant can be determined. There are many mechanisms by which an antigen can adsorb to an aluminum-containing adjuvant such as electrostatic attractive forces, hydrophobic interactions, ligand exchange, hydrogen bonding and van der Waals forces (38). Of these types of interaction forces, electrostatic attractive forces, ligand exchange, and hydrophobic interactions are typically the predominate adsorption mechanisms observed in vaccine formulations.

Electrostatic attractive forces act in formulations where the antigen and adjuvant have opposite electrical charges. At physiological pH, aluminum hydroxide adjuvant has a positive charge and will have electrostatic attraction with antigens having an acidic isoelectric point (pI). Aluminum phosphate adjuvant has a negative charge at physiological pH and will have electrostatic attraction with antigens that have a basic pI. This has been demonstrated with model antigens, as albumin which has an acidic pI and adsorbs preferentially to aluminum hydroxide adjuvant and lysozyme which has a basic

pI adsorbs preferentially to aluminum phosphate adjuvant (39). A method to determine the extent to which electrostatic attractive forces are involved in adsorption of an antigen is to treat the formulation with increasing concentrations of sodium chloride (40). The ions from the salt shield the charges on the adjuvant surface and antigen, therefore as the ionic strength increases the adsorption level of the antigen will decrease if electrostatic attractive forces are the predominate adsorption mechanism.

Ligand exchange is the strongest adsorption force and occurs when a phosphate group on an antigen exchanges with a hydroxyl on the surface of an aluminum-containing adjuvant. Ligand exchange can occur even in the presence of electrostatic repulsive forces. This was demonstrated by preparing ovalbumin with varying amounts of phosphorylation and determining the level of adsorption with aluminum phosphate adjuvant (41). All of the different ovalbumin samples had electrostatic repulsive forces present in the system. Ovalbumin with low phosphate content exhibited no adsorption to the adjuvant as electrostatic repulsion was the dominant force. Hyperphosphorylated ovalbumin could overcome the electrostatic repulsive force with ligand exchange binding and was 99% adsorbed to aluminum phosphate adjuvant. However, this high strength of binding can have a deleterious impact on antigen immunogenicity. It was shown with hepatitis B surface antigen as well as with alpha-casein, a model antigen that as the strength of binding increased the resulting geometric mean antibody titer in mice decreased (42,43). It was determined that the reduction in immunogenicity, seen in systems exhibiting very tight binding of antigen to the adjuvant, resulted from interference in antigen processing and presentation as no activated T cells were found in the spleens of mice vaccinated with the tightly bound antigen formulations. These data demonstrate that antigen adsorption strength must be considered, in addition to the amount of adsorption when developing a stable, immunogenic vaccine formulation with aluminum-containing adjuvants.

Historically, it was hypothesized that the vaccine antigen must be adsorbed to the surface of the adjuvant to observe an enhancement of immunogenicity. However, recently it was shown that antigen adsorption is not required for potentiation of the immune response with aluminum-containing adjuvants (44). The necessity of adsorption is antigen dependent. Some antigens do require a high level of adsorption to maintain optimal immunogenicity, while for others equivalent immunogenicity is achieved for adsorbed and non-adsorbed formulations, and others exhibit optimal immunogenicity in non-adsorbed formulations. It is important to understand early in formulation development the importance of adsorption if aluminum-containing adjuvants are utilized. This can generally be achieved by evaluating the immunogenicity of an antigen in adsorbed and non-adsorbed formulations in an appropriate animal model. Formulations in which the antigen is adsorbed and not adsorbed to the adjuvant can typically be obtained by manipulation of the surface charge of the adjuvant through addition of phosphate anion (Fig. 7). In this example, meningococcal serotype A conjugate was combined with a series of aluminum-containing adjuvants and the adsorption level was determined. As the conjugate has an acidic pI, it is not surprising that it is nearly all adsorbed to the adjuvant surfaces that have a positive surface charge due to electrostatic attractive forces. As the surface charge of the adjuvant becomes more negative, the

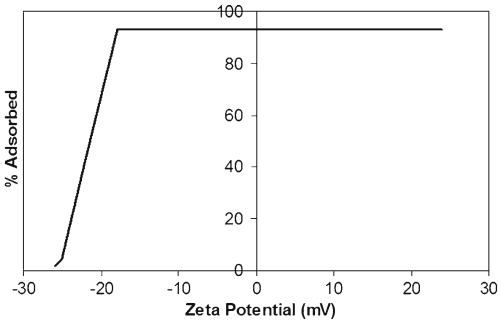

Fig. 7. Adsorption profile of meningococcal serotype A conjugate on a series of aluminum-containing adjuvants with varying surface charges

conjugate remains adsorbed even in the presence of electrostatic repulsive forces. This is due to the ability of serotype A conjugate to adsorb by the ligand exchange mechanism. However, once all of the ligand exchange binding sites are blocked electrostatic repulsive forces become dominant and the adsorption level quickly drops. For this antigen, the adjuvant selected for the adsorbed formulation would likely have a zeta-potential near −10 mV. An adjuvant having a more positive surface charge would generally not be selected as the binding strength would be too great due to ligand exchange binding and issues with stability would result over time. For the non-adsorbed system, the adjuvant selected would have a zeta-potential around −35 mV. Adjuvants with surface charges on the steep slope of the curve should be used with caution as it may be difficult to consistently prepare a formulation having an intermediate level of adsorption due to the inherent variability of adjuvant and formulation preparation. Once the importance of adsorption is determined the focus of subsequent development efforts can be targeted towards optimizing the stability of the adsorbed or non-adsorbed formulation.

Impact of the Micro-environment pH

If adsorption is critical for antigen immunogenicity, then the impact of the micro-environment pH must be evaluated. As vaccine formulations with aluminum-containing adjuvants are suspensions of charged particles in an aqueous solution, the charged adjuvant surface attracts ions of opposite charge from the surrounding water. This includes attraction of protons by a negatively charged adjuvant and attraction of hydroxyls by a positively charged adjuvant. The result of attracting protons or hydroxlys to the surface of the adjuvant produces a micro-environment pH which can be up to pH 2 units different than what can be measured in the bulk of the formulation (45). The difference in pH between the bulk and micro-environment can have a dramatic impact on the stability of adsorbed antigens (46). The stability of most antigens exhibits a dependence on pH. Figure 8 shows the pH dependence for the degradation of the meningococcal serotype A polysaccharide conjugate discussed previously. The data indicate that the conjugate exhibits greater stability at a pH lower than physiological pH. The adjuvant used to prepare an adsorbed formulation of the serotype A conjugate would have a zeta-potential of −10 mV. Since the adjuvant surface has a negative charge the micro-environment pH will be lower than the bulk pH. Therefore, a formulation having a pH near physiological could still be utilized as the pH

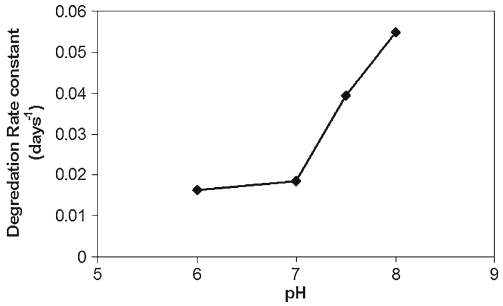

Fig. 8. The pH dependence of the degradation of meningococcal polysaccharide conjugate serotype A at 45°C

experienced by the conjugate will be lower and in the range of enhanced stability.

In contrast, if the only factor used to select the adjuvant for adsorption of the conjugate was to optimize electrostatic attractive forces an adjuvant having a positive surface would likely be selected. In this case the micro-environment pH would be greater than that seen in the bulk due to the attraction of hydroxyls to the adjuvant surface. This would result in destabilization of the conjugate if the formulation was prepared at physiological pH. A much lower formulation pH would need to be utilized to maintain stability of the conjugate in this situation. However, care must be taken when considering formulating at a pH away from physiological pH as the potential of pain associated with administration of the vaccine increases the further away from physiological pH the formulation gets. Therefore, when formulating vaccines with antigens adsorbed to aluminum-containing adjuvants the optimal formulation is often found with conditions that balance adsorption level, adsorption strength, and the micro-environment pH.

Mechanism of Action of Aluminum-Containing Adjuvants

Aluminum-containing adjuvants play many roles in the potentiation of the immune response to an antigen from recruitment of antigen-presenting cells (APCs), enhancing the uptake of antigen, and influencing the differentiation of APCs and T cells. Following administration of a vaccine with an aluminum-containing adjuvant, an inflammatory response is induced that recruits immune cells such as monocytes, neutrophils, and eosiniophils to the administration site (47–50). The adjuvant then facilitates uptake of the antigen by resident and infiltrating APCs. This is likely a result of the adjuvant making the antigen particulate in nature so it can be internalized by APCs through the efficient phagocytosis mechanism (51). Finally, it is thought that aluminum-containing adjuvants impact the activation and differentiation of dendritic cells through the Nalp3 inflammasome and up-regulation of cytokines, though this is still under investigation (52–56). Historically, a T_h2 type response with a high level of IgG1 antibodies is obtained with the use of aluminum-containing adjuvants (26).

Emulsions

An emulsion is a dispersion of at least two immiscible liquid phases stabilized with an emulsifying agent (57). The most typical emulsion types utilized in formulation of vaccines are water-in-oil and oil-in-water emulsions, though nonaqueous emulsions can be prepared. Emulsions were first evaluated in vaccine formulations by Freund in the 1940s (58–60). He found that enhanced immunogenicity could be obtained by formulating antigens in a water-in-paraffin oil emulsion either with *Mycobacterium tuberculosis*, complete Freund's adjuvant, or without the mycobacteria, incomplete Freund's adjuvant (IFA). Salk and colleagues evaluated IFA with whole virus influenza vaccine in humans and nonhuman primates and found that formulation with the adjuvant induced a faster and longer lasting immune response than formulation with saline alone (61–63). The mechanism of action of water-in-oil emulsions was thought to be the formation of an antigen depot in the continuous oil phase at the site of injection (64). The success of water-in-oil emulsion vaccine formulations in clinical studies led to the licensure of a seasonal influenza vaccine with IFA in the UK (65). In the initial years of licensure, around one million doses of the emulsion adjuvanted influenza vaccine were administered. However, water-in-oil emulsion adjuvanted vaccines exhibited high reactogenicity and their use was discontinued over time (66).

Currently, the primary focus of vaccine formulation development is on squalene-based oil-in-water emulsions. Squalene is a biodegradable oil which is a precursor of cholesterol. The primary source of squalene is shark liver oil; however, methods for obtaining squalene from renewable sources such as olives are being developed (67). Typically, nonionic surfactant emulsifiers such as Tween 80 and Span 85 are used to prepare stable emulsions of squalene. To manufacture the emulsion generally the two phases are prepared separately then mixed together. The squalene is mixed with the low hydrophilic-lipophilic balance (HLB) emulsifier as a low HLB indicates a preference for the oil phase, and the high HLB emulsifier is mixed with the aqueous phase. The oil phase is then dispersed into the water phase. Finally, a homogeneous preparation having a sub-micron particle size can be obtained through processes such as microfluidization or temperature induced phase inversion. This provides a preparation that can be sterilized by terminal filtration and stored ready to use. Squalene emulsions are generally stored refrigerated to stabilize the oil from degradation due to chemical oxidation (68).

Squalene-based oil-in-water emulsions act on the immune system in multiple ways to provide their adjuvant effect (69). The emulsion stimulates the release of chemokines which attract monocytes and granulocytes to the site of injection. The adjuvant induces maturation of the recruited monocytes into dendritic cells and enhance antigen uptake by those cells by stimulating endocytosis. Finally, migration of mature dendritic cells to the draining lymph node for antigen presentation to T cells is enhanced through induction of chemokine receptors. In addition to these direct effects on cells of the immune system it has also been shown that squalene emulsions can interact with skeletal muscle to promote the immune response (70). All of these mechanisms of action contribute to the ability of squalene emulsions to induce robust antibody responses.

Squalene-based emulsion adjuvants currently in development include MF59 (Novartis Vaccines and Diagnostics), AS03 (GlaxoSmithKline), and AF03 (Sanofi Pasteur). The primary target for use of these emulsion adjuvants has been pandemic and seasonal inactivated influenza vaccines. All three of these squalene emulsion adjuvants have been found to boost the immune response to both seasonal and pandemic strains of influenza (71–76). While the frequency of reactions at the injection site for vaccines containing squalene emulsion adjuvants is generally higher than non-adjuvanted vaccines these reactions tend to mild and of short duration (77,78). Because of the ability to induce T_h1 responses, the dose sparing potential and relative safety demonstrated by the squalene-based emulsion adjuvants, they will continue to play an important role in the future for seasonal and pandemic influenza vaccines as well as vaccines for other emerging infectious diseases.

Sterile Filtration

As vaccines are administered to infants, children, and adults who are generally healthy at the time of injection there is a high level of safety that must be ensured when manufacturing the product. Prevention of microbial contamination of vaccines is an important part of producing a safe vaccine formulation. Typically, this can be achieved through aseptic processing and sterile filtration of the vaccine formulation. However, formulations with aluminum-containing adjuvants cannot be sterilized by filtration due to the particle size of the adjuvant being greater than 0.2 μm. Materials used to prepare vaccines with aluminum-containing adjuvants must be sterilized prior to formulation and handled aseptically during the formulation and filling process.

Sterile filter membranes are produced with various materials. Typical membranes used in vaccine production are cellulose acetate (CA), polyethersulfone (PES), and polyvinylidene fluoride (PVDF) (79–81). Components of vaccine formulations can interact with the filter membrane material and it must be determined if that interaction may be detrimental to the vaccine. Investigating the amount of antigen lost during filtration, the amount of material than can be filtered prior to clogging, and the ability of the filter to retain microbial organisms following exposure to the vaccine formulation are important for determining the optimal membrane material for sterile filtration. These parameters were used to investigate the most appropriate membrane for an influenza formulation (Fig. 9). In pressure monitoring experiments, it was observed that CA and PVDF membrane materials clogged more rapidly than PES. The amount of influenza antigen lost during filtration was analyzed by serial radial immunodiffusion and these results correlated with the pressure data as the PES membrane had the least loss of material. These data suggest that PES should be used as the membrane for sterile filtration. However, before moving forward the PES membrane was evaluated in the bacterial challenge test to confirm that interaction with the formulation did not diminish the ability of the membrane to retain microbial organisms. In this case the membrane did maintain its ability to retain microbial contaminants following exposure to the influenza formulation. Therefore, a PES membrane was selected for use in sterilization of the influenza formulations for future development studies. These investigations are important for both ensuring the safety of the vaccine by removing potential contamination from the formulation and avoiding loss of product during production.

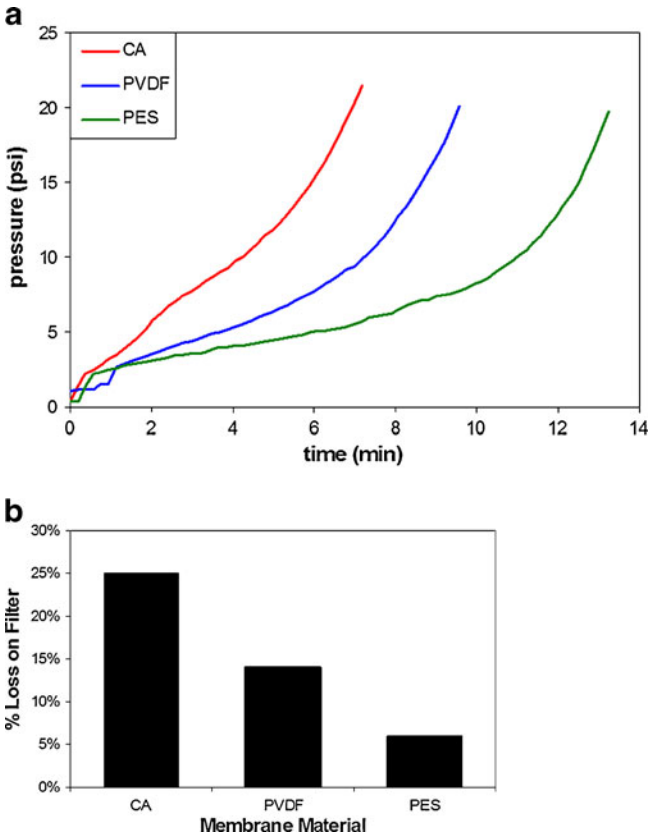

Fig. 9. It is important to understand how the formulation interacts with the membrane during sterile filtration. Pressure (**a**) and antigen loss (**b**) were monitored to investigate the optimal sterile filtration membrane material for an influenza formulation

CONCLUSIONS

With the continuous emergence of new infectious diseases and new strains of current diseases, such as with the novel H1N1 influenza in 2009, in combination with expanding competition in the vaccine marketplace the pressure to quickly develop robust vaccine formulations is increasing. Utilizing a rational, systematic approach allows for more rapid development of safe, efficacious, and stable vaccine formulations. The main components to this approach are biophysical characterization of the antigen, evaluation of stabilizers, investigation of antigen interactions with adjuvants, evaluation of product contact materials, and monitoring stability both in real time and under accelerated conditions. While this is not a wholly comprehensive list of the investigations that are needed to complete development of a vaccine formulation the information gained in these studies should form the base of knowledge for a robust formulation. Building this broad package of knowledge on factors that impact the formulation is valuable in supporting decisions for moving through the various phases of development projects. In addition, information gained in these studies can aid in determining the impact of unplanned events such

as deviations in manufacturing or temperature excursions during storage allowing decisions regarding the suitability of use of the product to be made with greater confidence. With the use of continually advancing technology and maintaining a rational, systematic approach to formulation development there is increasing probability of success in developing a safe, effective, and stable formulation.

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REFERENCES

1. Douglas RG, Sadoff J, Samant V. The vaccine industry. In: Plotkin S, Orenstien W, Offit P, editors. Vaccines. Amsterdam: Elsevier; 2008. p. 37–44.
2. Brandau DT, Jones LS, Wiethoff CM, Rexroad J, Middaugh CR. Thermal stability of vaccines. J Pharm Sci. 2003;92:218–31.
3. Peek LJ, Brey RN, Middaugh CR. A rapid, three-step process for the preformulation of a recombinant ricin toxin A-chain vaccine. J Pharm Sci. 2007;96:44–60.
4. Peek LJ, Brandau DT, Jones LS, Joshi SB, Middaugh CR. A systematic approach to stabilizing EBA-175 RII-NG for use as a malaria vaccine. Vaccine. 2006;24:5839–51.
5. Volkin DB, Mach H, Middaugh C. Degradative covalent reactions important to protein stability. Meth Mol Biol. 1995;40:35–63.
6. Mach H, Middaugh CR. Simultaneous monitoring of the environment of tryptophan, tyrosine, and phenylalanine residues in proteins by near-ultraviolet second-derivative spectroscopy. Anal Biochem. 1994;222:323–31.
7. Matulis D, Baumann CG, Bloomfield VA, Lovrien RE. 1-Anilino-8-naphthalene sulfonate as a protein conformational tightening agent. Biopolymers. 1999;49:451–8.
8. Ali V, Prakash K, Kulkarni S, Ahmad A, Madhusudan KP. Bhakuni V.1-Anilino-8-naphthalene sulfonic acid (ANS) induces folding of acid unfolded cytochrome c to molten globule state as a result of electrostatic interactions. Biochemistry. 1999;38:13635–42.
9. Yamada T, Iwabuki H, Kanno T, Tanaka H, Kawai T, Fukuda H, *et al*. Physicochemical and immunological characterization of hepatitis B virus envelope particles exclusively consistent of the entire L (pre-S1þpre-S2þS) protein. Vaccine. 2001;19:3154–63.
10. Constantino HR, Schwendeman SP, Griebenow K, Kilbanov AM, Langer R. The secondary structure and aggregation of lyophilized tetanus toxoid. J Pharm Sci. 1996;85:1290–3.
11. Kueltzo LA, Ersoy B, Ralston JP, Middaugh CR. Derivative absorbance spectroscopy and protein phase diagrams as tools for comprehensive protein characterization: a bGCSF case study. J Pharm Sci. 2003;92:1805–20.
12. Kissmann J, Ausar SF, Rudolph A, Braun C, Cape SP, Sievers RE, *et al*. Stabilization of measles virus for vaccine formulation. Hum Vaccin. 2008;4:350–9.
13. Zeng Y, Fan H, Chiueh G, Pham B, Martin R, Lechuga-Ballesteros D, *et al.* Towards development of stable formulations of a live attenuated bacterial vaccine: a preformulation study facilitated by a biophysical approach. Hum Vaccin. 2009;5:322–31.
14. Jiang G, Joshi SB, Peek LJ, Brandau DT, Huang J, Ferriter MS, *et al*. Anthrax vaccine powder formulations for nasal mucosal delivery. J Pharm Sci. 2006;95:80–96.
15. Cai S, He F, Samra HS, de la Maza LM, Bottazzi ME, Joshi SB, *et al*. Biophysical and stabilization studies of the Chlamydia trachomatis mouse pneumonitis major outer membrane protein. Mol Pharm. 2009;6:1553–61.
16. Salnikova MS, Joshi SB, Rytting JH, Warny M, Middaugh CR. Physical characterization of *Clostridium difficile* toxins and toxoids: effect of the formaldehyde crosslinking on thermal stability. J Pharm Sci. 2008;97:3735–52.
17. Salnikova MS, Joshi SB, Rytting JH, Warny M, Middaugh CR. Preformulation studies of *Clostridium difficile* toxoids A and B. J Pharm Sci. 2008;97:4194–207.
18. He F, Joshi SB, Bosman F, Verhaeghe M, Middaugh CR. Structural stability of hepatitis C virus envelope glycoprotein E1: effect of pH and dissociative detergents. J Pharm Sci. 2009;98:3340–57.
19. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res. 1989;6:903–18.
20. Martin A. Physical Pharmacy. 4th ed. Philadelphia: Lea & Febiger; 1993.
21. Chang LL, Pikal MJ. Mechanisms of protein stabilization in the solid state. J Pharm Sci. 2009;98(9):2886–908.
22. Abdul-Fattah AM, Truong-Le V, Yee L, Pan E, Ao Y, Kalonia DS, *et al*. Drying-induced variations in physico-chemical properties of amorphous pharmaceuticals and their impact on Stability II: stability of a vaccine. Pharm Res. 2007;24(4):715–27.
23. Clausi AL, Merkley SA, Carpenter JF, Randolph TW. Inhibition of aggregation of aluminum hydroxide adjuvant during freezing and drying. J Pharm Sci. 2008;97(6):2049–61.
24. Vogel FR, Hem SL. Immunological adjuvants. In: Plotkin S, Orenstien W, Offit P, editors. Vaccines. Amsterdam: Elsevier; 2008. p. 59–71.
25. Lahiri A, Das P, Chakravortty D. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine. 2008;26:6777–83.
26. Morefield GL, Hawkins LD, Ishizaka ST, Kissner TL, Ulrich RG. Synthetic Toll-like receptor 4 agonist enhances vaccine efficacy in an experimental model of toxic shock syndrome. Clin Vaccine Immunol. 2007;14(11):1499–504.
27. Garçon N. Preclinical development of AS04. Meth Mol Biol. 2010;626:15–27.
28. Mizel SB, Bates JT. Flagellin as an adjuvant: cellular mechanisms and potential. J Immunol. 2010;185(10):5677–82.
29. McCluskie MJ, Krieg AM. Enhancement of infectious disease vaccines through TLR9-dependent recognition of CpG DNA. Curr Top Microbiol Immunol. 2006;311:155–78.
30. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. Vaccine. 2009;27:1787–96.
31. Prchal M, Pilz A, Simma O, Lingnau K, von Gabain A, Strobl B, *et al*. Type I interferons as mediators of immune adjuvants for T- and B cell-dependent acquired immunity. Vaccine. 2009;27 Suppl 6:G17–20.
32. Wright AK, Briles DE, Metzger DW, Gordon SB. Prospects for use of interleukin-12 as a mucosal adjuvant for vaccination of humans to protect against respiratory pneumococcal infection. Vaccine. 2008;26:4893–903.
33. Glenny AT, Pope CG, Waddington H, Wallace U. The antigenic value of toxoid precipitated by potassium alum. J Pathol Bacteriol. 1926;29:31–40.
34. Shirodkar S, Hutchinson RL, Perry DL, White JL, Hem SL. Aluminum compounds used as adjuvants in vaccines. Pharm Res. 1990;7:1282–8.
35. Strumm W. Chemistry of the Solid-Water Interface: Process at the Mineral-Water and Particle-Water Interface in Natural Systems. New York: Wiley; 1992.
36. Iyer S, HogenEsch H, Hem SL. Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. Pharm Dev Technol. 2003;8:81–6.
37. Jiang D, Johnston CT, Hem SL. Using rate of acid neutralization to characterize aluminum phosphate adjuvant. Pharm Dev Technol. 2003;8:349–56.
38. Peek LJ, Matin TT, Nation CE, Pegram SA, Middaugh CR. Effects of stabilizers on the destabilization of proteins upon adsorption to aluminum salt adjuvants. J Pharm Sci. 2007;96:547–57.
39. Seeber SJ, White JL, Hem SL. Predicting the adsorption of proteins by aluminium-containing adjuvants. Vaccine. 1991;9:201–3.
40. Al-Shakhshir RH, Regnier FE, White JL, Hem SL. Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminum-containing adjuvants. Vaccine. 1995;13:41–4.
41. Morefield GL, Jiang D, Romero-Mendez IZ, Geahlen RL, Hogenesch H, Hem SL. Effect of phosphorylation of ovalbumin

on adsorption by aluminum-containing adjuvants and elution upon exposure to interstitial fluid. Vaccine. 2005;23:1502–6.
42. Hansen B, Belfast M, Soung G, Song L, Egan PM, Capen R, *et al*. Effect of the strength of adsorption of hepatitis B surface antigen to aluminum hydroxide adjuvant on the immune response. Vaccine. 2009;27:888–92.
43. Hansen B, Sokolovska A, HogenEsch H, Hem SL. Relationship between the strength of antigen adsorption to an aluminum-containing adjuvant and the immune response. Vaccine. 2007;25:6618–24.
44. Romero Méndez IZ, Shi Y, HogenEsch H, Hem SL. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. Vaccine. 2007;25:825–33.
45. Wittayanukulluk A, Jiang D, Regnier FE, Hem SL. Effect of microenvironment pH of aluminum hydroxide adjuvant on the chemical stability of adsorbed antigen. Vaccine. 2004;22:1172–6.
46. Estey T, Vessely C, Randolph TW, Henderson I, Braun LJ, Nayar R, *et al*. Evaluation of chemical degradation of a trivalent recombinant protein vaccine against botulinum neurotoxin by LysC peptide mapping and MALDI-TOF mass spectrometry. J Pharm Sci. 2009;98(9):2994–3012.
47. Goto N, Akama K. Histopathological studies of reactions in mice injected with aluminum-adsorbed tetanus toxoid. Microbiol Immunol. 1982;26:1121–32.
48. Goto N, Kato H, Maeyama J, *et al*. Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminum hydroxide with different physical properties. Vaccine. 1997;15:1364–71.
49. Walls RS. Eosinophil response to alum adjuvants: involvement of T-cells in not-antigen-dependent mechanisms. Proc Soc Exp Biol Med. 1977;156:431–5.
50. Verdier F, Burnett R, Michelet-Habachi C, Moretto P, Fievet-Groyne F, Sauzeat E. Aluminum assay and evaluation of the local reaction at several time points after intramuscular administration of aluminum containing vaccines in the cynomolgus monkey. Vaccine. 2005;23:1359–67.
51. Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. Vaccine. 2005;23:1588–95.
52. Sokolovska A, Hem SL, HogenEsch H. Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants. Vaccine. 2007;25:4575–85.
53. Kool M, Soullié T, van Nimwegen M, Willart MA, Muskens F, Jung S, *et al*. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med. 2008;205:869–82.
54. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature. 2008;453:1122–6.
55. Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol. 2009;21:23–9.
56. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 2009;9:287–93.
57. Nairn JG. Solutions, emulsions, suspensions, and extracts. In: Gennaro A, editor. Remington: the Science and Practice of Pharmacy. Baltimore: Lippincott Williams & Wilkins; 2000. p. 721–52.
58. Freund J, Sommer HE, Walter AW. Immunization against malaria: vaccination of ducks with killed parasites incorporated with adjuvants. Science. 1945;102:200–2.
59. Freund J, Thomson KJ, Sommer HE, Walter AW, Schenkein EL. Immunization of Rhesus monkeys against malarial infection (P.Knowles) with killed parasites and adjuvants. Science. 1945;102:202–4.
60. Freund J. The mode of action of immunological adjuvants. Adv Tuberc Res. 1956;7:130–48.
61. Salk JE, Laurent AM. The use of adjuvants in studies on influenza immunization. I. Measurements in monkeys of the dimensions of antigenicity of virus-mineral oil emulsions. J Exp Med. 1952;95:429–47.
62. Salk JE, Bailey ML, Laurent AM. The use of adjuvants in studies on influenza immunization. II. Increased antibody formation in human subjects inoculated with influenza virus vaccine in a water in-oil emulsion. Am J Hyg. 1952;55:439–56.
63. Salk JE, Contakos M, Laurent AM, Sorensen M, Rapalski AJ, Simmons IH, *et al*. Use of adjuvants in studies on influenza immunization. III. Degree of persistence of antibody in human subjects two years after vaccination. J Am Med Assoc. 1952;151:1169–75.
64. Vogel FR, Caillet C, Kusters IC, Haensler J. Emulsion-based adjuvants for influenza vaccines. Expert Rev Vaccines. 2009;8:483–92.
65. Perkins FT. Experience in the United Kingdom with oil adjuvant influenza vaccines. Ann Allergy. 1972;30:288–91.
66. Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants—a balance between toxicity and adjuvanticity. Vaccine. 1993;11:293–306.
67. Fox CB. Squalene emulsions for parenteral vaccine and drug delivery. Molecules. 2009;14:3286–312.
68. Bulland JL, Hughes H. The primary thermal oxidation product of squalene. J Chem Soc. 1949;26:492–7.
69. Seubert A, Monaci E, Pizza M, O'Hagen DT, Wack A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J Immunol. 2008;180:5402–12.
70. Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, *et al*. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci USA. 2008;105:10501–6.
71. Gasparini R, Pozzi T, Montomoli E, Fragapane E, Senatore F, Minutello M, *et al*. Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects. Eur J Epidemiol. 2001;17:135–40.
72. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, *et al*. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet. 2001;357:1937–43.
73. Leroux-Roels I, Borkowski A, Vanwolleghem T, Dramé M, Clement F, Hons E, *et al*. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet. 2007;370:580–9.
74. Baras B, Bouveret N, Devaster JM, Fries L, Gillard P, Sänger R, *et al*. A vaccine manufacturer's approach to address medical needs related to seasonal and pandemic influenza viruses. Influenza Other Respi Viruses. 2008;2:251–60.
75. Levie K, Leroux-Roels I, Hoppenbrouwers K, Kervyn AD, Vandermeulen C, Forgus S, *et al*. An adjuvanted, low-dose, pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. J Infect Dis. 2008;198:642–9.
76. Caillet C, Piras F, Bernard MC, de Montfort A, Boudet F, Vogel FR, *et al*. AF03-adjuvanted and non-adjuvanted pandemic influenza A (H1N1) 2009 vaccines induce strong antibody responses in seasonal influenza vaccine-primed and unprimed mice. Vaccine. 2010;28:3076–9.
77. O'Hagan DT, Wack A, Podda A. MF59 is a safe and potent vaccine adjuvant for flu vaccines in humans: what did we learn during its development? Clin Pharmacol Ther. 2007;82:740–4.
78. Leroux-Roels G. Prepandemic H5N1 influenza vaccine adjuvanted with AS03: a review of the pre-clinical and clinical data. Expert Opin Biol Ther. 2009;9:1057–71.
79. Przybylowski M, Bartido S, Borquez-Ojeda O, Sadelain M, Rivière I. Production of clinical-grade plasmid DNA for human Phase I clinical trials and large animal clinical studies. Vaccine. 2007;25:5013–24.
80. Dunleavy M. Polyethersulfone membranes facilitate sterile filtration of biological materials. BioScience Technol. 2003;44:46.
81. Sundaram S, Auriemma M, Howard Jr G, Brandwein H, Leo F. Application of membrane filtration for removal of diminutive bioburden organisms in pharmaceutical products and processes. PDA J Pharm Sci Technol. 1999;53:186–201.