Chapter 6
Formulation Approaches and Strategies for Vaccines and Adjuvants

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Abstract In order to create safe and efficacious vaccines, formulations that confer stability must generally be developed. In this chapter, formulation considerations consisting of solution conditions, particles, delivery route, endotoxin level, and preservatives will be covered along with the addition of adjuvants currently approved for use in vaccines and adjuvants currently being researched. Methods to increase vaccine stability and analytical techniques used to monitor vaccines will be discussed.

6.1 Introduction

Currently, there are 30 diseases that are preventable by vaccination (WHO et al. 2009), and numerous new vaccines currently are under development. Since vaccines prevent disease at a low cost, they have become the most cost-effective healthcare intervention (WHO et al. 2009) and offer the hope for combating a number of challenging diseases, including malaria, tuberculosis, human immunodeficiency virus, and cancer. For the full promise of vaccines to be realized, formulations must be developed that allow optimal immune responses while at the same time providing for retention of activity during storage, transportation, and delivery to patients. This chapter will discuss topics in vaccine formulation such as types of vaccines,

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current and future adjuvants, particulate formulations, route of delivery, endotoxin levels, preservatives, stability, and challenges associated with analytical techniques needed for vaccines.

6.2 Vaccine Versus Protein Formulations

There is now a significant literature dealing with strategies for developing formulations that are appropriate for therapeutic proteins (Volkin et al. 2002; Wang et al. 2007; Carpenter et al. 1997; Frokjaer and Otzen 2005; Stolnik and Shakesheff 2009; Kamerzell et al. 2011; Chang and Hershenson 2002; Akers et al. 2002). Vaccine formulations have much in common with these formulations, but differ in a critical aspect: the desirability of an immune response. A strong immune response to a vaccine is a requirement, whereas an immune response to a therapeutic protein formulation could be very detrimental to the patient and disease treatment (Nechansky and Kircheis 2010).

To help stimulate a suitable immune response to an administered antigen, adjuvants are frequently added to vaccine formulations. These adjuvants are typically used as suspensions of nano- or microparticles. Although the addition of such particles lowers the required amount of antigen needed to create an appropriate immune response, formulation design is also complicated because the physical and chemical stability of adjuvants as well as antigens must be considered.

Vaccines are able to create strong immune responses with relatively low concentrations of protein (10–100 μg/mL) (FDA 2012a) due to the high native immunogenicity of the antigen being used or the presence of an adjuvant in the formulation. Therapeutic protein formulations require much higher protein concentrations to be an effective treatment of a disease such as antibody formulations which often require as much as 100 mg/mL of protein (Shire et al. 2004).

Although the mechanism of action for protein therapeutics and vaccines is very different, both types of formulations need to be stabilized. Excipients used to stabilize protein therapeutics are often used to also stabilize vaccines. Methods to monitor stability and increase formulation stability will be discussed in later sections.

6.3 Types of Vaccines

Depending on the characteristics of the pathogen of interest and target population to be vaccinated, different types of vaccines can be formulated. There are three main types of vaccines: live attenuated, killed/inactivated, and subunit vaccines. Live attenuated vaccines consist of a weakened version of the pathogen. Since live attenuated vaccines are normally immunogenic enough on their own, they rarely require

an adjuvant (Pulendran and Ahmed 2011). Live attenuated vaccines can be problematic if they revert back to a stronger form of the pathogen, which could potentially cause harm in non-vaccinated or immunocompromised people (Singh et al. 2006). To avoid a pathogen from being able to revert to a stronger form, killed, also known as inactivated vaccines are created using whole pathogens that have been either heat or chemical treated. The safest type of vaccine is the subunit vaccine where only a portion of the pathogen is used (Pulendran and Ahmed 2011). Although the subunit vaccines have less risk in terms of the potential for the pathogen causing the disease, they are also less immunogenic because they are highly purified. The low immunogenicity often requires the vaccine to contain an adjuvant or be given in multiple doses (Pulendran and Ahmed 2011).

Since subunit vaccines only contain a portion of the actual pathogen, they can come in many forms depending on which portion of the pathogen they include. Examples of specific types of subunit vaccines are toxoid vaccines, conjugate vaccines, and DNA vaccines (Pulendran and Ahmed 2011). Toxoid vaccines are used when an invading pathogen secretes a toxic material to the body. Toxoid vaccines contain an inactivated version of the toxic material, so that in the event of exposure to the actual toxic material the body would be protected. A conjugate vaccine takes advantage of the immune system being able to recognize bacteria coated in polysaccharides by linking the antigen of interest to polysaccharides. A DNA vaccine carries genetic material, DNA, which the body can then use to produce the desire antigen and create an immune response.

The main focus of this chapter will be on subunit vaccines. The main components of subunit vaccines typically are the antigen, adjuvant, stabilizer, buffer, and preservative when necessary.

6.4 General Formulation Considerations

Intuitively, one might expect based on physiological conditions that buffer pH values near 7 might be optimal for a vaccine formulation. However, a broader range of pH (e.g., 5–8) may be explored for vaccine formulations. Practical limitations on formulation conditions include the relatively rapid rate of deamidation reactions observed at alkaline pH, and acid-catalyzed degradation reactions that can be accelerated at acidic pH values. Stability for many proteins is optimal in solutions formulated at pH 5–6. Pain on injection may be dependent on formulation pH, tonicity, osmolarity, solution temperature, drug concentration, and injection volume (Brazeau et al. 1998), but can sometimes be mitigated by using formulations with reduced buffer capacity. The buffer solution should also be adjusted so that the overall vaccine formulation is isotonic. Isotonicity of the vaccine will reduce tissue damage and pain of injection. Preservatives can be added to vaccines in cases where potential contamination is a concern, such as in multidose vaccine formulations.

6.5 Adjuvants

Adjuvants are materials that are used along with the antigen in a formulation with the primary goal of eliciting a stronger and more efficacious immune response compared to the antigen alone. In addition, an ideal adjuvant should possess the following properties:

1. By eliciting a strong immune response, an adjuvant should be capable of lowering the required antigen dose (Vogel 2000; O'Hagan and De Gregorio 2009), hence reducing or eliminating any antigen-induced toxicity effects, and reducing the per-dose cost for expensive antigens.
2. The adjuvant should induce both cellular and humoral immune response to the antigen (O'Hagan and De Gregorio 2009).
3. Adjuvanted formulations should be capable of producing a rapid onset and prolonged immune response (O'Hagan and De Gregorio 2009).
4. Adjuvants should aid in creating an immune response in populations not able to originally create an immune response such as elderly, young children, and immunocompromised people (Vogel 2000; O'Hagan and De Gregorio 2009).
5. Any interactions between the adjuvant and the antigen should not result in a loss of structural or chemical integrity of the antigen (O'Hagan and De Gregorio 2009).
6. The adjuvant should be safe and easy to formulate (O'Hagan and De Gregorio 2009).

Currently in the United States, the Food and Drug Administration (FDA) has approved two aluminum-based adjuvants. The first approved adjuvant is alum which is most commonly present as the mineral salts aluminum phosphate or aluminum hydroxide. The second approved adjuvant is AS04. AS04 is an adjuvant system containing monophosphoryl lipid A (MPL) adsorbed to aluminum. In addition to alum and AS04, the European Union (EU) has approved three other adjuvants for use in vaccines. The oil-in-water emulsions MF59 and AS03 have been approved along with virosomes (Rappuoli et al. 2011).

6.5.1 Aluminum Salt Adjuvants

Aluminum salt adjuvants have been used safely in vaccines for over 70 years. The two main aluminum salt adjuvants used are aluminum hydroxide and aluminum phosphate. Aluminum hydroxide is also known as Alhydrogel®, and aluminum phosphate is also known as AdjuPhos®. The type of aluminum salt chosen for the vaccine formulations is based on the mechanism of antigen adsorption to the adjuvant. The antigen can adsorb to the adjuvant surface through van der Waals forces, hydrogen bonding, electrostatic forces, and ligand exchange. Since van der Waals forces and hydrogen bonding provide much weaker binding of antigen to adjuvant,

we will focus on only the other two stronger mechanisms of adsorption. The World Health Organization (WHO) recommends that over 80 % of antigen be adsorbed to adjuvant based on a tetanus vaccine (WHO 1977). However, studies with aluminum salt-adjuvanted vaccines based on recombinant protective antigen (Berthold et al. 2005), lysozyme (Clausi et al. 2008a; Chang et al. 2001; Romero Mendez et al. 2007), dephosphorylated α-casein (Romero Mendez et al. 2007), and ovalbumin (Romero Mendez et al. 2007) have shown that antigen need not be fully adsorbed to adjuvant to be effective (Clapp et al. 2011).

To maximize attractive electrostatic interactions and encourage adsorption of antigen to adjuvant, the antigen and adjuvant should have opposite charges (Seeber et al. 1991). Critical parameters for design of adjuvanted formulations thus include the isoelectric point (p*I*) of the antigen and the point of zero charge (PZC) of the adjuvant. At these two pHs, the protein and adjuvant, respectively, will exhibit net charges of zero. For aluminum hydroxide to PZC is approximately 11, and for aluminum phosphate the PZC is between 4 and 5.5 (Peek et al. 2007). Based on the pH of the vaccine formulation, the charge of the antigen and adjuvant will change; stronger binding is generally seen at solution pH values where the antigen and the adjuvant are oppositely charged (Seeber et al. 1991). To optimize the PZC for aluminum salt adjuvants, aluminum hydroxide can be treated with phosphate ions. In the presence of phosphate, aluminum hydroxide surfaces are converted to the more thermodynamically favored aluminum phosphate, thus lowering the PZC (Hem et al. 2010). Due to the relatively high ionic strength found under physiological conditions, antigens that are adsorbed via electrostatic interactions can often desorb from aluminum salt adjuvants once injected into the body (Hem et al. 2010).

Ligand exchange is another means by which antigen can be attached to adjuvant surfaces. Phosphate groups on antigens may exchange with adjuvant hydroxyl groups (Hem et al. 2010). To reduce the amount of ligand exchange between the antigen and aluminum salt adjuvant, the aluminum hydroxide adjuvant can be treated with phosphate ions, thus reducing the number of site for potential ligand exchange (Hem et al. 2010). Adsorption strength was varied by pretreating aluminum hydroxide adjuvant with phosphate ions, and it was found that the strength of adsorption was inversely proportional to the immune response for HIV gp140 antigen (Hansen et al. 2011), In-labeled alpha casein (Noe et al. 2010), and hepatitis B surface antigen (Egan et al. 2009). Since ligand exchange is a stronger mechanism of adsorption than electrostatics, antigen will not readily elute from the antigen-adjuvant complex once it is injected into the body and comes into contact with fluid (Hem et al. 2010).

The US Code of Federal Regulations recommends that vaccine formulations contain less than 0.85 mg Al^{3+} per dose when assayed and less than 1.14 mg Al^{3+} when calculated, whereas the WHO and European standards recommend less than 1.25 mg Al^{3+} per dose (Vecchi et al. 2012). The toxic levels of aluminum were evaluated to be around 36.42 mg of Al^{3+}, in an acute toxicity study in rats (Titkov and Oganesian 1995), which is 43 times greater than the FDA recommended dose. The regulatory agencies have presumably recommended a low dose of aluminum to avoid possibilities of chronic toxicity.

Many vaccines can protect people against a disease through a humoral response wherein antibodies are produced once a pathogen invades the body. The antibodies can help the immune system clear the invading pathogen from the body. Some pathogens, however, require the body to initiate a cellular response in order for the pathogen to be cleared. A cellular immune response is important in vaccines protecting against intracellular pathogens (Mbow et al. 2010). In particular, malaria and tuberculosis vaccines require a cellular immune response to be effective (Wilson-Welder et al. 2009). Since aluminum salt adjuvants create a humoral immune response which is not ideal for vaccines protecting against all pathogens, other vaccine adjuvants need to be investigated (Garcon et al. 2007).

6.5.2 MF59

MF59 was the second approved vaccine adjuvant after alum (Rappuoli et al. 2011). MF59 is an oil-in-water emulsion. In the emulsion, squalene oil nanodroplets approximately 160 nm in diameter are surrounded by the nonionic detergents polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85) (Schultze et al. 2008). When stored at temperatures between 2 and 8 °C, MF59 is able to retain a constant particle size for up to 3 years (Schultze et al. 2008). MF59 is commonly used as an adjuvant in influenza vaccines (O'Hagan et al. 2011). In one study, the antigens diphtheria toxoid, tetanus toxoid, group C meningococcal conjugate, hepatitis B surface antigen, and recombinant MB1 were formulated with both aluminum adjuvant and MF59 adjuvant. For all antigens except diphtheria toxoid, formulations containing MF59 adjuvant were able to create a stronger immune response than corresponding formulations containing aluminum, as shown by geometric mean IgG titers after two doses of the vaccine (Singh et al. 2006).

6.5.3 AS04

AS04 is an adjuvant system created by GlaxoSmithKline Biologicals that contains 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) adsorbed to an aluminum salt. Lipopolysaccharide (LPS) is known to stimulate Toll-like receptor (TLR) 4, helping create a cellular immune response. MPL comes from the portion of LPS found in the cell walls of gram-negative bacteria (Casella and Mitchell 2008). Since LPS is too toxic to be used directly as an adjuvant, MPL is derived from LPS to have a similar effect on TLR 4 without the unwanted toxicity (Baldridge et al. 2004). The AS04 adjuvant can help create both humoral and cellular immune responses (Garcon et al. 2007).

AS04 is currently included in the FDA-approved human papillomavirus (HPV) vaccine Cervarix (Descamps et al. 2009). The AS04 adjuvant present in a hepatitis B vaccine was tested in comparison to a hepatitis vaccine without AS04.

It was found that after one dose of vaccine containing AS04 adjuvant, the patient seropositivity rate was 77 %, whereas patients receiving vaccine without AS04 had only a 37 % seropositivity rate. After injections at 0 and 6 months, the AS04 group had 98 % seroprotected, and after injections at 0, 1, and 6 months, the group without AS04 had 96 % seroprotected, showing that the vaccine formulated with AS04 was equally effective as the vaccine without AS04 but required fewer doses (Boland et al. 2004).

6.5.4 AS03

AS03 is an oil-in-water emulsion adjuvant system created by GlaxoSmithKline Biologicals. This adjuvant contains squalene and α-tocopherol, a form of vitamin E. Hepatitis B surface antigen (HBsAg) formulated with AS03 had a 10 times higher geometric mean titer than antigen alone formulated with alum after two intramuscular doses (Morel et al. 2011). A significantly higher antibody titer was also seen when an H5N1 influenza vaccine was formulated with AS03 in comparison to vaccine without an adjuvant (Morel et al. 2011). In addition to producing higher antibody titers with HBsAg, the AS03-adjuvanted influenza formulations were able to produced both Th1 and Th2 cytokines in greater amounts than alum (Morel et al. 2011). To be most effective AS03 should be injected in the same location and at the same time as the antigen (Morel et al. 2011).

6.5.5 Virosomes

Virosomes are viruslike particles containing portions of virus envelope without genetic material of the virus. When virosomes are used as an adjuvant, they can create both a humoral and cellular immune response (Reed et al. 2009). Virosomes are approximately 100–200 nm in diameter (Bachmann and Jennings 2010). Viruslike particles can be found in hepatitis A and B, human papillomavirus, and influenza vaccines licensed in Europe (Moser et al. 2011).

6.6 Future Adjuvants

Adjuvants are an integral part of an effective subunit and inactivated microorganism vaccine formulations, and scientists have consistently directed their efforts to discover new adjuvant molecules that are safer and more effective than alum. However, new adjuvant research involves thorough in-depth understanding of the mechanism of action, stability pattern, toxicity profile across various doses and populations, as well as compatibility with the vaccine candidate in the desired formulation.

6.6.1 OM-174

In a review by Corradin and Giudice, several adjuvant candidates were discussed (Corradin and Giudice Giuseppe 2005). They classified the adjuvants based on solubility (aqueous or oil soluble) (Corradin and Giudice Giuseppe 2005). The water-soluble adjuvants included OM-174, which is a derivative of MPL. The authors reported "excellent safety" of this adjuvant when used with a malaria synthetic subunit vaccine. Also, it was mentioned that this adjuvant can be either administered alone in a formulation or as a co-adjuvant with alum.

6.6.2 QS-21

QS-21 is another water-soluble adjuvant. Chemically, it is an acylated 3.28-o-bisdesmodic triterpenoid saponin derived from the bark of the *Quillaja saponaria* tree (Kensil et al. 1991). This adjuvant has been tested in several clinical trials for vaccines against infectious diseases such as HIV-1 (Evans et al. 2001), influenza (Mbawuike et al. 2007), and malaria (Stoute et al. 1997), as well as in cancerous patients with melanoma (Helling et al. 1995), breast cancer, or prostate cancer (Kensil and Kammer 1998). QS-21 has been extensively used with MPL in the malaria vaccine with satisfactory results. However, being a natural product, QS-21 exhibits variability in composition depending on the source, and also can be expensive to extract and purify (Kamstrup et al. 2000). Also, dose-dependent immune responses for QS-21 pose a challenge in cancer patients, who develop local erythema and flu-like symptoms at doses greater than 150 μg (Adams et al. 2010). Additionally, QS-21 degrades during long-term storage in aqueous solutions (Cleland et al. 1996). Synthetic saponins have been investigated to overcome these problems (Adams et al. 2010).

6.6.3 Immunostimulating Complexes

Another adjuvant that contains a saponin is immunostimulating complexes (ISCOM). ISCOM contains cholesterol, phospholipids, saponin, and protein. ISCOMATRIX is similar to ISCOM except it does not contain protein (Pearse and Drane 2005). When the ISCOMATRIX components combine, they form approximately 40 nm, cage-like structures (Pearse and Drane 2005). The ISCOMATRIX has been shown to be stable when refrigerated for 2 year, stored at 40 °C for a few months, after freeze-thaw cycles and during freeze-drying (Pearse and Drane 2005). Both humoral and cellular immune responses can be generated with this adjuvant (Sun et al. 2009). An increased amount of local reactions to the ISCOMATRIX in a clinical trial for human papillomavirus (HPV) vaccine was seen in comparison to

the group containing no adjuvant (McKenzie et al. 2010). In vaccine trials for HPV, hepatitis C virus, and influenza, ISCOMATRIX was found to be safe (McKenzie et al. 2010).

6.6.4 Montanide ISA

Montanide ISA 720 is a squalene-based adjuvant designed for human use that consists of mannide monooleate emulsifier and forms stable water-in-oil droplets with the idea of promoting sustained release of antigen at the injection site (Aucouturier et al. 2002). In clinical studies involving a malaria vaccine (*P. falciparum* CSP C-terminal fragment 282–283) formulated with ISA 720 and alum, high antibody titers were obtained along with good lymphocyte proliferation and production of IFN-γ that is critical for the elimination of malaria parasite (Roestenberg et al. 2008; Lopez et al. 2001). Another compound in this category is Montanide ISA 51, which is based on mineral oil that can be metabolized has also been shown to be safe for human use (Aucouturier et al. 2006).

6.6.5 Microorganism Compounds

Components derived from microorganisms such as bacteria hold promise as "immunopotentiating" adjuvants. For example, specific mutants (produced by site-directed mutagenesis) of heat-labile enterotoxin derived from *Vibrio cholerae* or *Escherichia coli* have been investigated as candidates for mucosal adjuvants that provoked increased serum IgG levels in mice and pigs when administered nasally in a microsphere delivery system (Vajdy et al. 2004). However, toxicity of such molecules has limited their use in humans (Vajdy et al. 2004). Another example in this category is a fusion gene (CTA1 gene from cholera toxin fused with a synthetic analogue of *S. aureus* protein A encoding gene) that exhibited less toxicity compared to wild-type cholera toxin (Lycke 2004).

6.6.6 Cytokines

Cytokines can also be potential adjuvant candidates. However, a variety of interleukins (IL-1, IL-2, IL-12) evaluated for this purpose exhibited in vivo stability and toxicity issues (Vajdy et al. 2004). Another example is IRX-2 which contains a natural mixture of Th1 cytokines (IL-1, IL-2, and IFN-γ) that enhances the antigen-processing capacity of lymph nodes by stimulating the Th1 pathway (Naylor et al. 2010).

6.6.7 Toll-Like Receptors

TLRs are pattern-recognition receptors produced by cells of the innate immune system. The TLRs bind to a variety of infectious agents and stimulate pathways that finally protect the host cells from the pathogen. Therefore, synthetic or purified TLRs have been the interest for adjuvant purposes (Steinhagen et al. 2011). One such example of TLR agonist is a repeating sequence of CpG dinucleotides, which has been found to be immunostimulatory and has been tested as an adjuvant in hepatitis B vaccine (Cooper et al. 2005). Imiquimod and resiquimod are small molecule TLR-7/TLR-8 agonist molecules, which are being studied as a topical adjuvant for skin disease (Gnjatic et al. 2010).

6.6.8 Polymer Particles

Micro- and nanoparticle formulations can also be employed for vaccine delivery resulting in sustained-release vaccine formulations. Such formulations involve the use of biodegradable polymers such as polylactic acid (PLA), poly-lactic-*co*-glycolic acid (PLGA), polyethylene glycol (PEG), and polyphosphazene to formulate the micro- or nanoparticles (Oyewumi et al. 2010). Researchers have tried to correlate immune response and particle size. In general, smaller particles can cross biological barriers via tight junctions or via endocytosis and get to systemic circulation, which might be expected to result in better efficacy. However, smaller particle sizes do not always correlate with enhanced immune response. For example, HBsAg entrapped in PLA particles of diameter 2,000–8,000 nm produced greater anti-HBsAg antibody response than HBsAg entrapped in 200–600 nm PLA particles (Kanchan and Panda 2007). Various formulation parameters such as formulation materials, dose, antigen loading method, uniformity of particle size, and various routes of administration can be held responsible for such contrasting observation (Oyewumi et al. 2010).

6.7 Vaccine Particles

The size, shape, and surface molecular organization of antigens have been found to affect the immune response (Bachmann and Jennings 2010). By using adjuvants of controlled sizes, vaccine particles can be made to be of sizes similar to those of the target pathogen (Bachmann and Jennings 2010; O'Hagan et al. 1997). Viruslike particles and immunostimulating complexes can be on the same order of magnitude of viruses. Emulsions, liposomes, and virosomes can be on the same order of magnitude of size as larger viruses, bacteria, fungi, and protozoa. Microparticles and mineral salts can be on the same order of magnitude size as bacteria, fungi, and protozoans (Bachmann and Jennings 2010). In addition to adjuvant particles being

a similar size to potential pathogens, it is also important for adjuvants to be taken up by antigen presenting cells.

Freeze-drying parameters can be varied to create vaccines containing a range of aluminum particle sizes (Clausi et al. 2008b). In a study conducted with a model freeze-dried lysozyme vaccine, formulations containing aluminum particles ranging in average size from 2 to 14 μm all produced similar anti-lysozyme IgG1 titers after two doses (Clausi et al. 2008a).

6.8 Route of Delivery

An ideal vaccine should be effective, safe, and administered in a minimally invasive manner. The route of vaccination is a very important consideration as some infectious disease pathogens invade the host cells on mucosal surface; in such cases, the ideal vaccine needs to induce systemic immunity as well as mucosal immunity (Devriendt et al. 2012). Oral administration of vaccine is one of the routes of administration that yield the highest patient compliance and does not require syringes or trained personnel. However, a vaccine delivered via the oral route must be robust enough to survive the acidic pH in the stomach and proteolytic enzymes and should be suitably transported across the gastrointestinal tract in order to reach the systemic circulation. Approaches to modulate delivery across the gastrointestinal tract includes altering physicochemical properties of the vaccine for enhanced uptake or formulating the vaccine in micro- or nanoparticles that protect the antigen from acid degradation in stomach. However, particle-based formulations face a major hurdle in crossing the intestinal barrier and therefore generally offer very poor protection at the mucosal site. Several ligand-based delivery systems have been recently explored to identify gastrointestinal surface receptors as vehicles of delivery of antigen via endocytosis to elicit a strong immune response. Such ligands include lectin-based targeting, bacterial adhesins, bacterial toxins, and antibody-mediated targeting (Russell-Jones 2000). Live attenuated vaccines are administered orally as the antigen needs to have an inherent ability to attach to mucosal cells. Presently, the vaccines that have been approved for oral administration include cholera, influenza, polio virus, rotavirus, and *Salmonella typhimurium* (Holmgren and Czerkinsky 2005).

The nasal route of administration can also produce mucosal and systemic immune responses. It is an attractive alternative to oral vaccines as the antigen is not subjected to acid degradation. Also, this route of administration is easily accessible, highly vascularized, and ideal for mass immunization. However, the vaccine still needs to overcome the nasal mucosal barriers to produce systemic effects. Solution, dry powder, or suspension formulations can be delivered via this route. Nasal vaccination possibly demonstrates a more rapid onset compared to oral vaccines (Davis 2001). Flumist® is an example of nasal delivery system consisting of temperature-sensitive attenuated influenza virus.

The most common route of vaccine administration is via intramuscular or subcutaneous injection. Intramuscular injection optimizes the immunogenicity of the

vaccine and greatly reduces any adverse reaction at the site of administration. Transcutaneous vaccination has also become a topic of interest for vaccine delivery. The skin is the largest organ in the human body and is the first natural barrier against harmful pathogens. However, the transport of antigens across the stratum corneum represents a significant barrier to this route of vaccine delivery. It is expected that adjuvants such as alum, MPL, and bacterial endotoxins will have limited penetration across the skin due to their large size. However, preclinical transcutaneous studies indicate that cholera toxin (CT) and heat-labile *E. coli* toxin (LT) can be used as adjuvants as they stimulate immune response against other antigens. The most successful delivery via transcutaneous route consisted of physically disrupting the skin barrier with the help of microneedles followed by delivery of the formulation (Bal et al. 2010).

6.9 Endotoxin Levels

Endotoxin comes from LPS found in the cell membranes of gram-negative bacteria (Magalhaes et al. 2007). LPS commonly contains distinct regions of an O-antigen region, core oligosaccharide, and hydrophobic lipid (lipid A), with the lipid A region being responsible for toxicity (Magalhaes et al. 2007). Endotoxin can be introduced into formulations when components of the vaccines are produced in gram-negative bacteria, such as recombinant proteins produced in *E. coli* (Magalhaes et al. 2007). When the body is exposed to large dose of endotoxin or small doses of endotoxin systematically, an inflammatory reaction occurs which can cause shock, tissue damage, or death (Magalhaes et al. 2007). To avoid damage caused by endotoxin, endotoxin levels should be kept low in formulations. The threshold pyrogenic dose of endotoxin in humans is 5 EU/kg (Malyala and Singh 2008), making it desirable to keep endotoxin levels below this amount. Although specific endotoxin limits have not been set by United States Pharmacopeia (USP), it is recommended to keep endotoxin levels low. Brito and Singh suggested upper endotoxin limits for different types of vaccines based on DTwP and cholera vaccines as follows: genetic vectors 10 EU/mL, recombinant subunit 20 EU/mL, polysaccharide 20 EU/mL, live attenuated 200 EU/mL, inactivated 500 EU/mL, and toxoid 200,000 EU/mL (Brito and Singh 2011).

Endotoxin present in formulation is most commonly measured by the gel clotting in the Limulus Amebocyte Lysate (LAL) test. If levels of endotoxin are too high, endotoxin can be removed throughout steps in the vaccine manufacturing process. Since endotoxin is stable at high temperature, heat sterilization will not inactivate endotoxin unless temperatures exceeding 250 °C for 30 min and 180 °C for 3 h are used (Magalhaes et al. 2007; Gorbet and Sefton 2005). Concentrations of acids and alkalis above 0.1 M are capable of inactivating endotoxin. Endotoxin present in protein solutions can be removed by LPS affinity resins, two-phase extractions, hydrophobic interaction chromatography, ion exchange chromatography, gel filtration chromatography, sucrose

gradient centrifugation, and membrane adsorbers. If protein is not present in the desired solution for endotoxin removal ultrafiltration can be used (Magalhaes et al. 2007).

6.10 Preservatives

Although preservatives are not normally used in single-use vials, preservatives are normally added to multidose vials to prevent growth of microorganisms as recommended by the United States Code of Federal Regulations for vaccines not containing live attenuated viruses. Preservative that have been used in US FDA-approved vaccines include thimerosal, phenol, benzethonium chloride, and 2-phenoxyethanol (Geier et al. 2010). At an acidic pH thimerosal is able to kill bacteria and at an alkaline or neutral pH thimerosal prevents bacteria and fungus from replicating (Rowe et al. 2009). Thimerosal is not compatible with aluminum and should therefore not be used with an aluminum salt adjuvant (Rowe et al. 2009). Vaccines recommended for children under 6 years old, except for influenza vaccines, have had the thimerosal reduced to trace levels or lower (FDA 2012b). Thimerosal is currently used in tetanus toxoid vaccine, influenza vaccines, and multidose Menomune-A/C/Y/W-135. Phenol is able to be used against both gram-negative and gram-positive bacteria, mycobacteria, some fungi, and viruses (Rowe et al. 2009). Phenol is currently included in Pneumovax 23. Benzethonium chloride has an optimal antimicrobial activity from pH 4–10 and is not compatible with anionic surfactants (Rowe et al. 2009). Benzethonium is currently included in BioThrax. 2-Phenoxyethanol is able to protect against gram-negative organisms but has reduced activity when nonionic surfactants are present (Rowe et al. 2009). 2-Phenoxyethanol is currently included in inactivated poliovirus vaccine (IPOL).

6.11 Stability

In order for vaccines to be economically feasible and able to be delivered to patients, they generally should have a shelf life of 2 years or longer. To determine the stability of a given formulation, both real-time stability studies and accelerated stability studies can be conducted. In accelerated stability testing, a stress such as elevated temperature, elevated humidity, light exposure, agitation, freeze-thawing, extremes of pH, or redox conditions (Chang and Hershenson 2002; Brandau et al. 2003) is applied to the formulation, and the rates at which the formulation degrades is monitored. Extrapolation of degradation rate data as a function of stress level allows an estimate of shelf life in the absence of stress to be obtained.

Many parameters such as pH, ionic strength, osmolarity, and the type and concentration of excipients present may play a role in vaccine stability. pH affects vaccine stability by changing the rate at which hydrolysis and deamidation reactions

occur. pH also changes the charge of molecules in solution which can then cause changes in protein structure or changes in adsorption of protein to adjuvant or other surfaces (Brandau et al. 2003). Lower ionic strength can increase the solubility of biomolecules, and the solution ionic strength can change how molecules assemble (Brandau et al. 2003). Excipients can also be added to formulations for stability (Brandau et al. 2003).

Excipients are commonly added to formulations to increase the formulation stability, maintain pH, modify tonicity, or help increase antigen solubility. Excipients commonly added to increase stability consist of surfactants, sugars, salts, and antioxidants (Chang and Hershenson 2002). Surfactants are commonly used to prevent unwanted protein adsorption to surfaces. Proteins often denature when adsorbed to surface. Sugars in solution are able to protect proteins from denaturing by preferential hydration and excluding sugar molecules from the protein surface. Sugars protect lyophilized formulations by slowing molecular motions in the dried solid state, and by providing hydrogen bonds with protein in the place of water. Salts can be added to formulations to increase the formulation ionic strength and can be added to help maintain a particular pH. Antioxidants are used to protect against oxidation.

To predict the formulation conditions and excipients that maximize the vaccine formulation stability from complex data sets, empirical phase diagrams can be used to better interpret the data (Maddux et al. 2011). Empirical phase diagrams take mathematical data collected from a variety of spectroscopic techniques and convert it into colors. Similar colors represent similar stabilities. Techniques commonly used in collecting the spectroscopic data for phase diagrams consist of circular dichroism, near-UV absorbance, extrinsic fluorescence, dynamic light scattering, OD 350, and intrinsic UV fluorescence (Maddux et al. 2011). To determine regions of stability, controlled formulation parameters (e.g., temperature, pH, excipient concentration, protein history, or other relevant conditions) need to be varied.

Since vaccines have potential to experience both hot and cold temperatures before being delivered to patients, the vaccine stability should be tested with several cycles of freezing and thawing. Loss of or decreased potency has been observed for vaccines containing an adjuvant (e.g., Alhydrogel) due to freeze-thawing (Braun et al. 2009). Several studies in the literature have implicated freezing-induced agglomeration of Alhydrogel for loss of potency (Diminsky et al. 1999). A study by Jones et al. subjected hepatitis B and DTaP vaccine formulations to controlled freeze-thaw cycles; they also evaluated the freezing-induced protection effects provided by additives such as glycol, PEG 300, and glycerin (Braun et al. 2009).

To increase stability and allow for higher storage temperatures, vaccines can be dried. In the dried solid state, degradation reactions occur at a much slower rate and much less water is present allowing for less degradation. Methods of drying that have been used consist of lyophilization (Carpenter et al. 1997; Clausi et al. 2008a, 2009; Amorij et al. 2008), Xerovac (a dehydration process not involving freezing) (Worrall et al. 2001), spray drying (Amorij et al. 2008; Bowey and Neufeld 2010; Sou et al. 2011), spray freeze-drying (Amorij et al. 2008), and carbon dioxide-assisted nebulization with a Bubble Dryer® (CAN-BD) (Amorij et al. 2008; Burger et al. 2008) (a drying process used to produce an inhalable powder).

Although vaccines can be created with antigen and adjuvant produced separately and then mixed together before administration in the clinic, it is recommended to have antigen and adjuvant formulated together. If the antigen and adjuvant will be stored separately, both components of the vaccine will need to undergo stability studies separately and then throughout the stability study antigen and adjuvant will need to be combine to test the whole vaccine. Variations in the vaccine such as adsorption of antigen to adjuvant caused by amount of time combine and mixing conditions will be created when the antigen and adjuvant are combined before use by different people. Slightly variations in the mixing procedure used could cause potential changes in the vaccine. These variations in the vaccine could potentially cause a loss in efficacy or safety.

6.12 Challenges of Analytical Techniques

When developing antigens to include in vaccine formulations, high-resolution techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM) should be used (Maddux et al. 2011). When vaccine formulations are monitored over time, lower-resolution, faster techniques are more appropriate (Maddux et al. 2011) and will be focused on for the rest of this section. Changes in the vaccine formulations could be an indication of instability leading to a loss of safety and efficacy. Since vaccines frequently contain adjuvants which can scatter light as well as low protein concentrations, analytical techniques can often become difficult.

Primary structure can be looked at by breaking apart the antigen of interest through proteolysis and then analyzing the fragments with mass spectroscopy for areas of degradation. The amino acids, glutamine, and asparagine are more prone to deamidation and should be monitored through a loss of carboxylic acid group. The glutamine and asparagine residues should be especially monitored for deamidation when surrounded by a glycine residue allowing for greater flexibility for the deamidation reaction (Manning et al. 1989). Oxidation is more common in the aromatic residues tyrosine and tryptophan and along with cysteine and methionine.

Secondary structure has been examined by infrared spectroscopy. A study conducted with the six model proteins, cytochrome c, ovalbumin, α-chymotrypsinogen A, recombinant human IL-1ra, IgG1, and sTNF-R1 compared the standard solution infrared spectrum at protein concentrations of 15 mg/mL to lower protein concentrations of 1.0 and 0.5 mg/mL with protein adsorbed to Alhydrogel adjuvant and found that the spectra were very similar (Dong et al. 2006). The technique developed of looking at the secondary structure through infrared spectra of adjuvant-protein pellet would be applicable to vaccines formulated with aluminum adjuvants containing low concentrations of antigen. The secondary structure of proteins adsorbed to aluminum hydroxide, glass, and cellulose was able to be examined by a similar method (Bee et al. 2009; Fradkin et al. 2011).

Tertiary structure has been examined by tryptophan fluorescence quenching for protein adsorbed to glass, cellulose, silica, and alum (Bee et al. 2009; Fradkin et al. 2011). Since proteins contain the amino acid tryptophan which gives off a fluorescent emission depending on how buried the tryptophan residues are in the protein, the amount of unfolding can be monitored by measuring how easily the fluorescence from these residues can be quenched. The Stern-Volmer constant can be used to help determine the amount of quenching taking place. The Stern-Volmer equation uses the ratio of fluorescence intensity without quencher present, F_o, to fluorescence intensity with quencher present, F, equaling one plus the Stern-Volmer constant, K_{SV}, multiplied by the quencher concentration $[Q]$. The Stern-Volmer equation is as follows (Bee et al. 2009):

$$\frac{F_o}{F} = 1 + K_{SV}[Q]$$

Aggregation of vaccine antigen and particles present in vaccine formulations can be examined by many different techniques based on the size of particles present in the formulation and the desired information (particle count, particle size distribution, particle images). Imaging particle size techniques using instruments such as micro-flow imaging (MFI) or FlowCAM can count, size, and image particle if particles are greater than 2 μm. Nanosight instruments are capable of sizing particles in the nanometer range. If only the particle size distribution is required, laser diffraction can be used for formulations when particles are much smaller in the range of 0.04–2,000 μm. For small particles on the order of nanometers, dynamic and static light scattering can be used.

To monitor the thermal stability of vaccines, differential scanning calorimetry (DSC) can be used to find to melting temperature (T_m). A higher melting temperature would be more desirable for a formulation. Studies have been conducted to compare melting temperatures of formulations with different excipients with and without adjuvant to determine the formulation with the best thermal stability (Peek et al. 2007). In addition, enthalpy of unfolding can also be determined for proteins in which the heat-induced conformational change is reversible (i.e., no aggregation) (Vessely et al. 2009). Peek et al. employed DSC as a method of looking at thermal transitions of proteins adsorbed to Alhydrogel in the absence and presence of stabilizers. The overall increasing T_m of protein-Alhydrogel samples in presence of stabilizers (e.g., sorbitol, caprylate) indicate that proteins adsorbed to adjuvant are stabilized (Peek et al. 2007). In another example, measles vaccine powder was analyzed using DSC where the various energy-related (endotherms and exotherms) transformations were seen such as glass transition (T_g), T_m, and recrystallization (LiCalsi et al. 2001). However, a powder form may be quite complex consisting of various additives and excipients, and in such cases it becomes challenging to assign peaks to particular components or events.

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