Manmohan Singh *Editor*

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Preface

Recent advances in our understanding of innate immunity have fueled the field of drug discovery with emphasis on developing better immune potentiators that can boost the host innate immune system. Targeting the host innate compartment has the unique advantage of triggering a rapid mobilization of key effector mechanisms to engender a strong immune response to a vaccine antigen. Several new class of molecules that target the innate immune system have been recently tested as potential vaccine adjuvants. These novel adjuvants also require rationally designed vaccine formulations and delivery systems that can provide maximum potency with acceptable safety in a prophylactic setting. These delivery systems contribute greatly on modifying and controlling the level of systemic exposure, avoiding the potential production of proinflammatory cytokines, improving safety and/or tolerability of the novel adjuvant.

One of the fascinating aspects of vaccine delivery for several decades now has been fully exploring the benefits and limitations of mucosal delivery. Exciting new advents have been made in this regard using gene-based and attenuated oral vaccines. Immunization through the skin as the route of delivery of antigens holds great promise in making vaccines more patient compliant and needle free. Since most vaccines are complex compositions, new medium and high throughput tools have now emerged that help screen rapidly for excipients and stabilizers prior to running expensive preclinical animal studies. Several biophysical tools and assays are now being adapted to better characterize vaccine formulations.

In this book we have made an effort to cover new vaccine delivery technologies and discuss some of the next generation immune potentiators that could potentially be part of licensed products in the future. Detailed description of all leading vaccine technologies with their limitations should be of great help to researchers and students to enhance their understanding of these novel concepts. The book also has chapters on clinical and non-clinical safety evaluation of vaccine formulations which should be of great value in moving vaccines from research to clinic.

Manmohan Singh

Contents

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Section I
Novel Immune Potentiators and Delivery Systems for Enhancing Vaccine Potency

Chapter 1
TLR7/8 Agonists as Vaccine Adjuvants

Mark A. Tomai and John P. Vasilakos

1.1 Introduction

Small molecule TLR7/8 agonists have demonstrated great potential as vaccine adjuvants, since they quantitatively and qualitatively enhance both humoral and cellular immune responses. However, most small molecule TLR agonists evaluated thus far as vaccine adjuvants are highly soluble and have a propensity to rapidly disperse away from the vaccination site, resulting in decreased efficacy and increased systemic adverse effects. Intense effort and progress has been made to increase their ability to maintain close proximity to antigen at the administration site. Here, we will discuss three vaccine approaches utilizing small molecule TLR7/8 agonists as vaccine adjuvants. These approaches are designed to improve the adjuvanticity and to reduce the potential for systemic adverse events associated with these small molecule TLR7/8 agonists when used as vaccine adjuvants. One approach utilizes the TLR7/8 agonist resiquimod gel as a topically applied adjuvant at the vaccination site. The other two approaches utilize novel TLR7/8 agonists in a conventional vaccine format where the adjuvant and antigen are administered together. These novel TLR7/8 agonists are lipid modified or chemically modified for conjugation to antigen—all three approaches are designed to promote retention of the TLR7/8 agonists at the administration site in order to maintain their spatial and temporal proximity to the antigen, resulting in enhanced immune responses and reduced systemic adverse effects.

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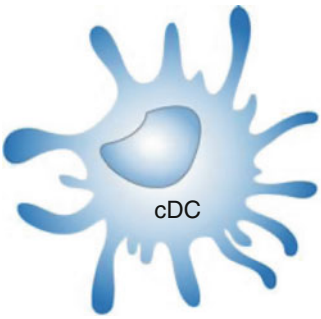
1.2 The Need for New Vaccine Adjuvants

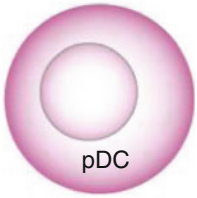
Despite the development of numerous successful vaccines, vaccines do not exist for many pathogens or cancers. Currently, inactivated pathogens, recombinant proteins, purified peptides, and DNA vaccines are being explored in order to address adverse events associated with live vaccines, and in some cases, to address the fact that live infectious agents do not confer protection. The major problem with most protein, peptide, and DNA vaccines is that they are poorly immunogenic or elicit an inappropriate immune response, and don't provide protection against the infectious agent or cancers. The question isn't whether an adjuvant is required, but what type of adjuvant or adjuvant combination will work best with a specific antigen or antigens for a specific disease indication. In addition, each antigen and adjuvant combination must be formulated to provide adequate stability and to ensure that the vaccine maximally stimulates the appropriate immune response in an acceptably safe and tolerable manner. Hence, the three key components of a vaccine are the antigen, adjuvant, and formulation. This chapter will focus on adjuvants, specifically small molecule TLR7/8 agonists.

Currently, there are very few vaccine adjuvants approved for human use. Aluminum salts (i.e., ALUM) are one of few US Food and Drug Administration approved adjuvants and is the most widely used adjuvant [1]. Additionally, MF59, an oil-in-water squalene emulsion, has been approved in some countries [2]. More recently, AS03 adjuvant (DL-a-tocopherol, squalene, polysorbate 80) and AS04 adjuvant (ALUM and monophosphoryl lipid A) have also been approved in some countries [3, 4]. These adjuvants are safe, but they do not uniformly or sufficiently enhance cell-mediated immune responses that are required for elimination of many intracellular organisms and cancers. Hence, adjuvants that drive cellular immunity, both CD4 and CD8 responses, are being investigated.

1.3 Toll-Like Receptors and Toll-Like Receptor Agonists

In order to understand how vaccines induce adaptive immune responses, we first must begin with how the innate immune system recognizes microorganisms. Several recognition strategies have been developed by the innate immune system to deal with the problem of detecting a broad range of heterogeneous and rapidly evolving pathogens. Specifically, the innate immune system possesses receptors, broadly classified as pattern recognition receptors (PRR), which specifically recognize conserved microbial molecular patterns [5, 6]. PRRs are predominantly expressed on or in phagocytic cells, such as dendrtitic cells, macrophages, neutrophils, monocytes, and to a lesser extent on other cell types. PRRs are germ-line encoded receptors, and unlike T-cell or B-cell receptors, don't undergo somatic mutation and clonal distribution. As such, PRRs are "hard-wired" to recognize conserved microbial molecular patterns known as pathogen-associated molecular patterns (PAMPs). PAMPs are classically characterized as a limited set of conserved molecular patterns unique to the microbial world and invariant among entire classes of pathogens [7]. Engagement of PRRs with PAMPs results in antimicrobial and inflammatory responses, including the production of





TLR	TLR Ligand
3	Poly (I:C)
4	MPL
5	Flagellin
8	Resiquimod

TLR	TLR Ligand
7	Resiquimod
9	CpG

IL-12

IFN-α

Fig. 1.1 Conventional DC express TLR3, 4, 5, and 8, and when ligated with specific agonists, produce IL-12. Plasmacytoid DC express TLR7 and TLR9, and when ligated with their specific agonists, produce IFN-α. Adapted from Coffman et al. [3]

cytokines and chemokines that affect innate and adaptive immune responses. Indeed, it has become clear that innate immune cells and their PRRs are usually critical for the induction, magnitude, and quality of adaptive immune responses.

Toll-like receptors (TLRs) are one type of PRRs utilized by the innate immune system to recognize microbial pathogens. There are ten human TLRs; they are transmembrane-signaling proteins expressed on surface of the plasma membrane or endosomes. TLR1, 2, 4, 5, 6, and 10 are cell surface expressed, and TLR3, 7, 8, and 9 are expressed in endosome/lysosome membranes. Although innate immune cells express TLRs, all innate immune cells do not express the same TLRs. As an example, the majority of human dendritic cells (DC) can be classified as conventional DC (cDC) or plasmacytoid DC (pDC). Conventional DC express TLR3, 4, 5, and pDC express TLR7 and 9. Ligation of cDC TLRs results in the production of numerous cytokines including IL-12. In contrast, ligation of pDC TLRs results in the production of interferon alpha. Therefore cell-specific TLR expression results in differences in cytokine responses induced by various TLR agonists (Fig. 1.1).

Although the schematic in Fig. 1.1 is an oversimplification of the complexities of TLR expression patterns and cytokine responses resulting from TLR ligand

stimulation, Fig. 1.1 highlights a critical aspect of differential TLR expression patterns on human innate immune cells and the TLR ligands used to activate them: TLR agonists influence the cytokine repertoire of the innate immune system, which in turn influences adaptive immunity. Additionally, most TLR agonists stimulate either cDC or pDC, except for the small molecule TLR7/8 agonists, such as resiquimod.

1.4 TLR Agonists as Vaccine Adjuvants

Improved understanding of how innate immune responses can be initiated, specifically through PRRs, has allowed us to understand how innate immunity affects adaptive B- and T-cell responses. It has been recognized for almost two decades now that innate immunity impacts adaptive immunity [8]. The discovery of *toll* in drosophila and its link to immunity, and the discovery of TLRs in mammals, along with the elucidation of mammalian TLR expression patterns and signaling pathways has led us to understand the history of vaccinology and has provided a guide to rationally develop vaccine adjuvants [8–12].

We have come to recognize that in the 1890s, Coley's toxin, a mixture of bacterial cell lysate that exhibited immunostimulatory properties, was able to ameliorate progression of some cancers [13]. Springing forward to the 1980s, we determined that bacterial DNA was a critical component within Coley's toxin that elicited immune responses, and then later determined that those responses were mediated through TLR9. Today, numerous papers have demonstrated the vaccine adjuvant ability of TLR9 agonists [14]. Additionally, recent evaluation of some commonly used preventative vaccines, BCG Vaccine "SSI" (live attenuated vaccine, Statens Serum Institut), Influvac® (inactivated subunit vaccine, Abbott Laboratories), and Typhim Vi® (subunit vaccine, Sanofi Pasteur SA), has demonstrated that they contain TLR agonists which optimally induce DC maturation for induction of Th1 adaptive immunity [15]. Finally, several studies have demonstrated that one of the most effective vaccines available, the live attenuated yellow fever vaccine 17D (YF-17D), is known to activate multiple DC subsets (i.e., cDC and pDC) via TLR2, TLR7, TLR8, and TLR9 to elicit immune modulatory cytokines such as IL-12p40, IL-6, and IFN-α [16, 17]. The resulting adaptive immune responses are characterized by a mixed Th1/Th2 response and antigen-specific $CD8^+$ T cells. Hence we have empirically learned that many vaccines owe their effectiveness, in part, to TLR agonists.

1.5 Small Molecule TLR7, TLR8, and TLR7/8 Agonists as Vaccine Adjuvants

Currently, we are purposefully adding defined TLR agonists to vaccine formulations with the intent of improving the effectiveness of the vaccine. The approval of the toll-like receptor (TLR) 4 agonist monophosphoryl lipid A (MPL), as a vaccine adjuvant lends credibility to using TLR agonists as vaccine adjuvants,

from the perspective that TLR agonists can be safe, effective, and manufactured to commercial scale for human vaccine use. MPL has been approved for human use with human papilloma virus vaccine (Cervarix™, GlaxoSmithKline, London, UK); this is the first globally approved vaccine containing a TLR agonist. MPL is also approved with hepatitis B virus vaccine (FENDrix®, GlaxoSmithKline, London, UK); this was the first approved vaccine containing a TLR agonist. MPL is also approved with a pollen vaccine (Pollinex®-R Quattro, Allergy Therapeutics Ltd., London, UK).

As indicated above, TLR7 and TLR8 are expressed in the major human dendritic cell (DC) subsets, and this is an important reason why TLR7/8 agonists are of considerable interest as vaccine adjuvants. TLR7 and TLR8 can be activated by certain synthetic TLR7/8 agonists, such as the imidazoquinolines imiquimod (R-837, TLR7 agonist) and resiquimod (R-848, TLR7/8 agonist) [18–21]. Resiquimod and imiquimod are prototypical imidazoquinoline molecules, typically 250–500 Da. Initially identified as antiviral molecules, it was later discovered that these molecules directly activate the innate immune system, inducing production of various cytokines and maturation of dendritic cells, and therefore have vaccine adjuvant potential. Aldara® Imiquimod 5% cream was the first small molecule TLR agonist approved for clinical use. It is approved for HPV-mediated external genital warts, superficial basal cell carcinoma, and actinic keratosis. Both resiquimod and imiquimod have been evaluated in numerous clinical studies for chronic viral infection and cancer because of their immune modulatory activity.

Stimulation of TLR7 and TLR8 with these molecules enhances DC activation as well as cell-mediated immunity [20, 22–26]. Much of what is known about the vaccine adjuvant potential of TLR7 and TLR8 agonists has been discovered using the imidazoquinoline molecules. These molecules directly activate antigen presenting cells resulting in the induction of co-stimulatory molecules and numerous cytokines that modulate adaptive immunity. In short, these molecules have the fingerprint of vaccine adjuvants. As might be expected, there currently are various organizations developing similar TLR7 and TLR8 agonists as vaccine adjuvants. To list a few, Novartis (Basal, Switzerland) has a platform of TLR7 and TLR8 agonists in early stage development. Telormedix (Bioggio, Switzerland) is evaluating a TLR7 agonist as part of a malaria vaccine, and VentiRx (San Diego, CA) is evaluating a TLR8 agonist VTX-744 as a vaccine adjuvant.

1.6 Resiquimod as a Vaccine Adjuvant

A number of small molecule TLR7/8 agonists have been evaluated as vaccine adjuvants. Since resiquimod is the most well-studied TLR7/8 agonist, we will focus on its use as a vaccine adjuvant. First, a number of in vitro studies using resiquimod have shown that this molecule enhances human dendritic cell maturation, and cellular and humoral adaptive immunity [22, 27–30]. These in vitro

studies clearly demonstrate the critical link between innate and adaptive immunity. The predominant evidence that resiquimod and analogs, such as imiquimod, may be effective vaccine adjuvants has come from studies demonstrating the ability of these molecules to activate antigen presenting cells, induce immune modulatory cytokines, and activate adaptive immune responses in numerous species, specifically in mice, rats, guinea pigs, and monkeys. These studies utilized conventional s.c., i.m., or i.n. vaccination and incorporated model antigens, clinically relevant antigens, and DNA vaccines [31–41]. Resiquimod injected with antigen in an aqueous buffer, ALUM, or Montanide enhanced Th1-like immunity, humoral immunity, and concomitantly inhibited Th2-like immunity. Consistent with these findings, resiquimod has been shown in numerous allergic models to inhibit Th2-like allergen-specific adaptive immune responses [42–44]. Although resiquimod and analogs have been effective vaccine adjuvants in many systems, a number of studies did not appear to demonstrate that these TLR7/8 agonists were strong adjuvants [40, 45–48]. Interestingly, a number of studies showed that small molecule TLR7 or 8 agonists were not very effective adjuvants when simply mixed with antigens, but the adjuvanticity of these small molecules could be substantially improved by appropriate formulation with or conjugation to the antigen [39, 49–51]. These findings imply that formulation of these small molecule TLR agonists is likely a critically important aspect regarding the use of these molecules and that a close spatial relationship between the small molecule TLR7/8 agonist and the antigen is critical for effective induction of adaptive immunity.

Why do all studies not show resiquimod as a strong vaccine adjuvant? One reason may be the water soluble nature of resiquimod (molecular weight 314 Da) which, upon injection, may disperse away from the injection site throughout the body rather than staying at the site of injection, which is presumably where the antigen resides (unpublished pharmacokinetic data). The short half-life of resiquimod at the administration site is likely not optimal for local (at the injection site) activation of DC, which are critically important for initiating adaptive immune responses. Also, in standard vaccine formulations like oil-in-water emulsions or alum emulsions, resiquimod tends to enter into the aqueous phase rather than the oil phase, and as indicated above, resiquimod may disperse away from the vaccination site, dissociating itself from the antigen. Additionally, in some systems it appears that TLR activation by itself is not sufficient to drive certain types of adaptive responses, such as CD8 T-cells, and therefore, TLR7/8 agonists may need to be coupled with other stimuli for optimal activity [52–54]. Indeed, accumulating evidence indicates that combinations of TLR agonists such as resiquimod and TLR3, TLR4, or TLR9 agonists, or resiquimod combined with cytokines can improve DC maturation and T-cell activation [34, 53–57]. Therefore, it is possible that for some vaccines, a more complex approach may be necessary, where multiple immune modulatory molecules will be needed to achieve the adaptive immune response required for disease resolution.

The ability to keep the antigen and TLR agonist in close physical proximity to each other at the vaccination site improves vaccine outcome as evidenced by

Table 1.1 Pros and cons of resiquimod gel as a vaccine adjuvant

Advantages	Disadvantages
Enhances localization of the adjuvant effects at administration site	Resiquimod is intrinsically soluble, and therefore some of the active drug can enter the systemic circulation, thereby increasing the chance for systemic adverse effects
	Better therapeutic window than injectable resiquimod but may not be sufficient in all cases
Compared to injectable resiquimod formulations, resiquimod gel induces less systemic adverse effects due to systemic cytokine production	Potential for local adverse effects at treatment site
Stable and cost-effective to reproducibly manufacture	More cumbersome to administer as a vaccine adjuvant
	Two steps are required: (1) vaccinate and (2) apply resiquimod gel to vaccination site
Validated adjuvant activity in nonclinical and clinical proof-of-concept studies	Greater opportunity for variability in results due to operator variation in topical administration

studies using topical (dermal) application of these small molecule TLR agonists, including cancer vaccine clinical studies [58]. An additional advantage to dermal delivery of TLR7/8 agonists is that these molecules can be dosed at effective levels for inducing local immune activation without increasing systemic cytokines that might lead to systemic side effects. Dermal application of imiquimod or resiquimod (imiquimod cream or resiquimod gel) in rodents and primates induced local cytokine and chemokine production, Langerhans cell migration to draining lymph nodes, and infiltration of various immune cells to the application site [59–67]. Furthermore, adaptive immune responses to infectious agents have been enhanced by topical administration of these TLR agonists using protein and DNA vaccines [25]. Recently, resiquimod gel was shown to be a potent adjuvant for locally administered subcutaneous vaccines, inducing antitumor CTL responses following a single application at the time of subcutaneous vaccination [59]. Indeed, resiquimod gel is currently being evaluated in FDA-approved clinical cancer vaccine trials in conjunction with cancer antigen vaccines (www.clinicaltrials.gov). In these studies, 0.06% or 0.2% resiquimod gel is or will be applied directly at the vaccination site either prior to or after immunization. The concept here is that resiquimod gel applied topically at the vaccination site induces activation of innate immune cells at the site of antigen localization, resulting in cytokine production, DC maturation, and enhanced DC migration to the draining lymph nodes. Topical studies with resiquimod gel or imiquimod cream have substantiated this concept. A summary highlighting the advantages and disadvantages of using topical formulations of resiquimod is shown in Table 1.1.

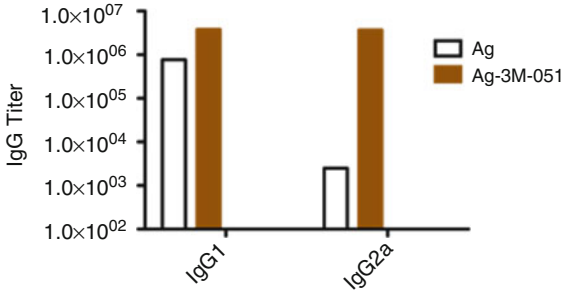


Fig. 1.2 3M-051 conjugated to a recombinant protein induces a balanced Th1/Th2 response. Balb/c mice (5/group) were immunized with (1) a recombinant protein that induces a weak cellular immune response when administered without adjuvants or with (2) a recombinant protein-TLR7/8 agonist conjugate (Ag-3M-051). Mice were immunized s.c. three times, once every 2 weeks, with the antigen alone or the conjugate at a TLR agonist:protein molar ratio of 10:1. Twenty-one days after the last immunization, the levels of Ag-specific serum IgG1 and IgG2a were determined by ELISA. The results are presented as geometric mean IgG titers

1.7 Novel Imidazoquinolines as Vaccine Adjuvants

Physical association between adjuvant and antigen correlate with optimal immune responses. Interestingly, resiquimod was found to physically associate with keyhole limpet hemocyanin (KLH) more effectively than to OVA or human serum albumin, and resiquimod enhanced KLH adaptive immunity more efficiently than the other two antigens (data not shown). Such results prompted further investigation into developing systematic strategies to physically associate small molecule TLR agonists with antigens. Recent studies have evaluated small molecule TLR agonist-antigen conjugates in mouse and primate systems. In mice, TLR7/8-OVA and TLR7/8-HIV-1 GAG protein conjugates enhanced antigen-specific Th1 and CTL responses more effectively than conventional aqueous resiquimod vaccine formulations. Similarly in monkeys, the TLR7/8-antigen conjugate more effectively enhanced antigen-specific CD4 and CD8 responses than the non-conjugate formulation [39, 40, 49].

Important lessons have been learned using prototype conjugatable small molecule TLR7/8 agonists; however, the conjugation method of these prototype small molecules to proteins or peptides was not optimized (i.e., less controlled conjugation). Hence, the recent development of newer conjugatable TLR7/8 agonists focused on improving the predictability and consistency of conjugation to many types of antigens and peptides. As such, a new class of molecules has been developed; an example of this novel class of conjugatable TLR7/8 agonist molecules is 3M-051. The results in Fig. 1.2 demonstrate the adjuvant activity of a 3M-051 conjugated to antigen X (Ag). These results show that a weakly immunogenic recombinant protein conjugated to 3M-051 can induce a robust

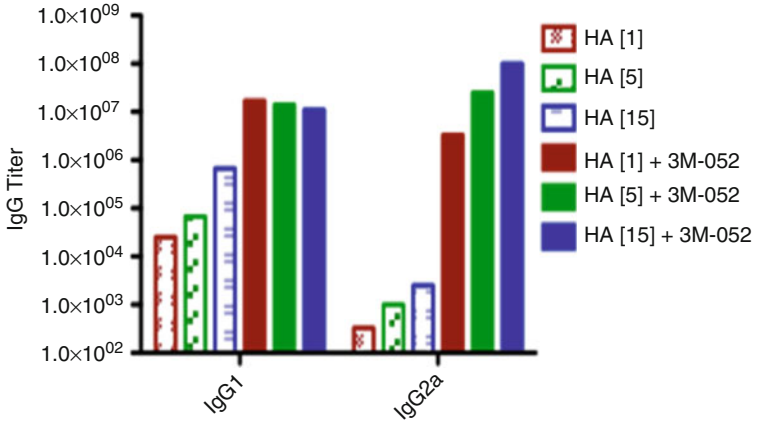
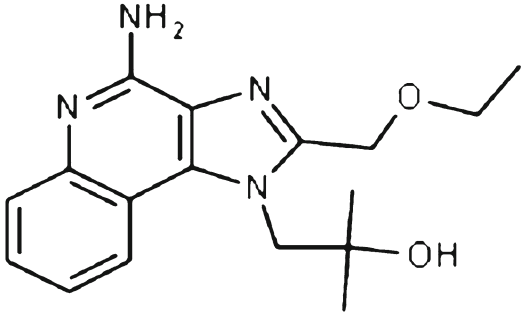
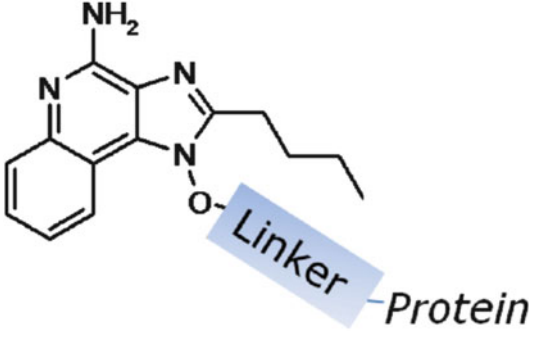


Fig. 1.3 Antigen-sparing effect of HA vaccine adjuvanted with 3M-052 TLR agonist. Balb/c mice (5/group) were immunized with influenza hemagglutinin (HA) with or without the TLR7/8 agonist 3M-052. Mice were immunized s.c. three times, once every 2 weeks, with 1, 5, or 15 μg/mouse with or without 0.1 mg/kg 3M-052. Twenty-one days after the last immunization, the levels of HA-specific serum IgG1 and IgG2a were determined by ELISA. The results are presented as geometric mean IgG titers

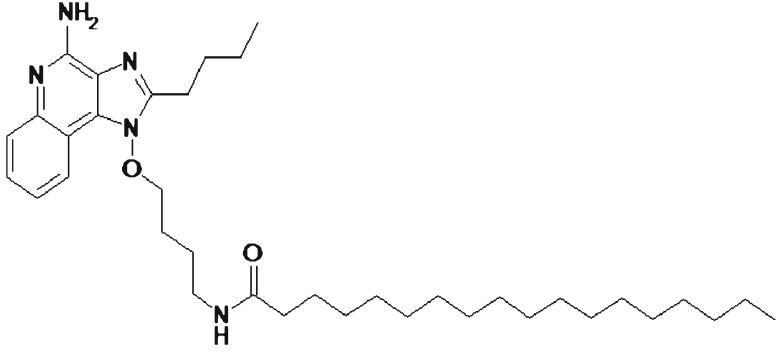
humoral immune responses—significantly improving the immunogenicity of this weak antigen.

In addition to conjugating an antigen to small molecule TLR agonists, another approach to maintaining close physical and temporal proximity of antigen and TLR agonist at the vaccination site is to develop TLR agonists with physical–chemical properties that inhibit their distribution away from the administration site. Note that resiquimod and other similar TLR7/8 agonists administered parenterally quickly distribute throughout the body post injection. TLR 7/8 agonists that induce local adjuvant effects without inducing systemic cytokines should be better vaccine adjuvants. Therefore, the novel TLR7/8 agonist 3M-052 was synthesized on the basis of its physical–chemical properties, which allow it to stay at the vaccination site when injected as part of typical vaccine formulations. Most recently, formulations of 3M-052 with hemagglutinin demonstrated enhanced Th1 immunity without induction of systemic cytokines [68]. Similar unpublished results were seen with other antigens formulated with 3M-052. Figure 1.3 demonstrates that, consistent with previously published results using resiquimod, vaccines formulated with 3M-052 can enhance IgG1 and IgG2a responses. Unlike resiquimod, 3M-052 did not induce systemic cytokines, even when dosed at 1 mg/kg, and 3M-052 demonstrated superior antigen-sparing activity. The structures of resiquimod, 3M-051, and 3M-052 are shown in Table 1.2 along with a brief description of their preparation as vaccine adjuvants.

Table 1.2 TLR7/8 vaccine adjuvants and their preparation for in vivo testing

Resiquimod		Resiquimod gel was prepared in a formulation containing polypropylene glycol, colloidal silicon dioxide, and glycerol triacetate (triacetin). The final percentage of resiquimod (gel) evaluated in clinical studies ranged from 0.01% to 0.25%. Dosing areas ranged from 10 to 50 cm^2 consisting of 0.02–2.5 mg of drug applied to the skin
3M-051		3M-051 was conjugated to proteins by a proprietary method. In the described study, various amounts of 3M-051 were conjugated to an antigen resulting in 10:1, 5:1, and 2:1 M ratios of 3M-051 to antigen. After the conjugation procedure was performed, the conjugate was purified by size exclusion chromatography, and the protein concentration and molar ratio of TLR agonist to protein was determined. The soluble conjugate was injected s.c.

3M-052



3M-052 was dissolved in $CHCl_3$. Dioleoylphosphatidylcholine (PC) was also dissolved separately in $CHCl_3$. Various proportions of the two solutions were mixed in order to give a 10:1 mass ratio of PC to 3M-052. $CHCl_3$ was removed under vacuum and sterile saline was added to the resulting 3M-052/PC residue. After vortexing, the suspension was sonicated, and mean particle diameter was 420 nm and single modal. This stable suspension was admixed with antigen in PBS and injected s.c.

1.8 Conclusion

Since small molecule TLR7/8 agonists can activate appropriate innate immune cells resulting in the modulation of humoral and cellular immunity, these agonists have been found to be excellent adjuvant candidates with various antigens, including recombinant proteins that are poorly immunogenic and tumor antigens. It is apparent that in order for these TLR7/8 agonists to be optimally effective and safe, immune activation at the application site without systemic activation is important. Thus far, most of the small molecule imidazoquinolines evaluated as vaccine adjuvants have demonstrated the propensity to rapidly disperse away from the vaccination site. Three approaches are being evaluated to solve this issue.

Topical application of resiquimod gel to the dermis along with conventional vaccination partially solves the issue of rapid systemic distribution of the TLR agonist. Such an approach promotes local immune stimulation at the vaccine site while diminishing systemic immune activation. Additionally, two new classes of TLR7/8 agonists have been developed that promote close association between TLR agonist and antigen and can be formulated and administered together as a single vaccine product. One class of molecules, i.e. 3M-051, is designed to be conjugated directly to antigens, thereby ensuring close association between TLR agonist and antigen. Another class of molecules, i.e. 3M-052, has been developed that promotes retention of the TLR agonist at the administration site with the antigen, due to the physical–chemical properties of this class of TLR agonists.

By promoting close association of TLR agonist and antigen, the probability of activating the same antigen presenting cells that process and present antigen is increased, which should more efficiently enhance adaptive immunity and limit systemic adverse effects.

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Chapter 2
Preclinical and Clinical Development of Synthetic *i*NKT-Cell Glycolipid Agonists as Vaccine Adjuvants

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2.1 Introduction

NKT cells are a separate lineage of T lymphocytes that co-express receptors for the T-cell and natural killer (NK) cell lineages. Most NKT cells express a semi-invariant T-cell receptor (TCR), Vα14-Jα18 paired with Vβ8.2, Vβ7 or Vβ2 in mice and Vα24-Jα18/Vβ11 in human [1–5]. These cells are referred to as *i*NKT cells type I NKT cells, or NKT cells, in contrast to type II NKT cells comprising the remaining NKT cells expressing non-invariant TCR [6]. These cells share phenotypic and functional characteristics of T and NK cells. The phenotype of NKT cells expresses a T-cell receptor αβ (TCRαβ), the CD4 or the CD8 co-receptor or neither of them [double-negative (DN) phenotype], the NK1.1 marker, and some Ly49 receptors [7–10]. Emerging evidence indicates that $CD4^+$ and $CD4^-$ *i*NKT cell subsets are functionally distinct [11–13]. The distribution of *i*NKT cells has been well studied in mice, and less well in human. Murine *i*NKT cells represent approximately 0.5% of the T-cell population in the blood and peripheral lymph nodes (LN), and up to 30% of T cells in the liver, and this population appears to be ten times less frequent in humans. However, high and low expressers are found in humans and mice [14–17].

NKT cells recognize glycosphingolipids presented by the non-classical major histocompatibility complex (MHC) molecule CD1d. CD1d proteins are expressed on the surface of a variety of antigen-presenting cells and many non-hematopoietic cells. They present cellular self-lipids and exogenous lipids with an α-anomerically linked sugar, to *i*NKT cells. In humans, the CD1 family consists of the group I

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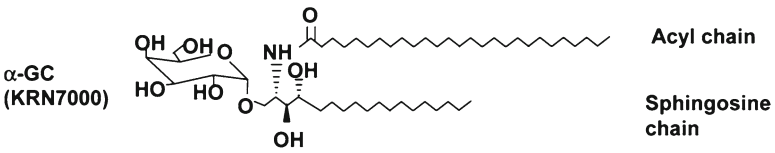
(CD1a, CD1b, CD1c and CD1e) and II proteins (CD1d). It has been suggested that the group I CD1 proteins are not universally present in all species, whereas group II are, and rodents such as mice and rats only display CD1d molecules.

The glycosphingolipid alpha-galactosyl ceramide (α-GalCer) was the first activator of *i*NKT cells to be discovered and has been employed extensively as an experimental tool to study *i*NKT cells. α-GalCer is a structurally well- characterized compound, containing a phytosphingosine moiety, an amide-linked acyl chain and an O-linked galactopyranosyl polar head, and its most distinguishing feature is that the galactose head group is attached to the sphingosine through an α-linkage at the anomeric carbon. Thus far, α-anomeric D-glycosyl ceramides have not been detected in mammals, as similar mammalian glycosyl ceramides contains a β linked galactopyranosyl, which changes the relative orientation of the carbohydrate moiety.

α-GalCer Structure



One of the featuring characteristics of both mouse and human *i*NKT cells is their vigorous response to α-GalCer stimulation, associated with a rapid and robust secretion of both T helper1 (Th1)-type (IFN-γ) and T helper2 (Th2)-type (IL-4) cytokines [18–21]. As a result, *i*NKT have the ability to either enhance or suppress Th1 antigen-specific immune responses.

Administration of high doses of soluble α-GalCer by the i.v. route in mice can result in the acquisition of an anergic phenotype on repeated exposure to α-GalCer [22, 23]. However, this is not observed at doses administered by the i.m. route that is effective as vaccine adjuvants [24].

The recently solved crystal structure of α-GalCer bound to human and mouse CD1d has allowed rational design of novel *i*NKT cell ligands. The crystal structures of both mouse and human CD1d have identified the antigen binding site consisting of two channels or pockets; the A′ channel that can accommodate an alkyl chain of up to 26 carbons long (the acyl chain of α-GalCer) and the F′ channel that can accommodate an alkyl chain of up to 18 carbons long (the sphingosine chain of α-GalCer). These studies have revealed that the lipid chains fit tightly into the CD1d binding groove, and α-GalCer protrudes from the binding groove with only the galactose head group for recognition by the *i*NKT TCR [25–27].

The affinity of TCR binding to the glycolipid/CD1d complex and stability of the bound glycolipid/CD1d complex are believed to largely influence the immune response. Indeed the results of structural, kinetic and functional studies have facilitated the process of rational optimization of *i*NKT agonists and have led to the

identification of a series of novel synthetic compounds, with modifications in the polar head and lipid tails, which can be used to modulate *i*NKT activity.

The successful induction of both innate and acquired immunity with co-administration of antigen plus glycolipid antigens such as α-GalCer has been shown in several prophylactic and therapeutic models. α-GalCer has been tested in conjunction with peptides, DNA, irradiated tumour cells or loaded onto a variety of cells including antigen-presenting cells (APC) such as dendritic cells (DC), and tumours. Here we review different strategies that have been used in preclinical models for the development of glycolipids as adjuvants for vaccines, as well as clinical data that have been obtained with these products.

2.2 Preclinical and Clinical Experience with Soluble α-GalCer as Vaccine Adjuvant

2.2.1 Preclinical Studies with Soluble α-GalCer

Preclinical studies have shown substantial promise for *i*NKT cell-based treatments of infections, cancer, autoimmune and inflammatory diseases, using free α-GalCer as adjuvant.

2.2.1.1 Antitumour Immunity

α-GalCer as Adjuvant with DNA Vaccines

α-GalCer displayed adjuvant effects with DNA vaccines against tumours, when used for the primary immunization. A DNA vaccine expressing human papillomavirus (HPV) type 16 E7 (pcDNA3-CRT/E7) was combined with α-GalCer at the prime phase, and generated a higher number of E7-specific $CD8^+$ T cells in vaccinated mice through stimulating maturation of DCs. In fact, priming with a DNA vaccine in the presence of α-GalCer and boosting with E7-pulsed DC vaccine led to a significant enhancement of E7-specific $CD8^+$ effector and memory T cells. The antitumour immunity significantly improved therapeutic and preventive effects against an E7-expressing tumour model (TC-1) in vaccinated mice suggesting that the potency of the DNA vaccine combined with α-GalCer could be further enhanced by boosting with an antigen expressing DC-based vaccine [28].

α-GalCer as Adjuvant with Irradiated Tumour Cells as Antigens

α-GalCer has been shown to represent an important adjuvant for improving the efficacy of tumour cell-based vaccines to treat ovarian cancer. Using a transplantable

mouse ovarian surface epithelial carcinoma (MOSEC) model as well as a murine Müllerian inhibiting substance type II receptor T antigen (TgMISIIR-TAg) transgenic mouse model that is capable of developing ovarian cancer spontaneously, it was shown that administration of irradiated MOSEC tumour cells with adjuvant α-GalCer generated significant protective and therapeutic antitumour effects against MOSEC tumours in vaccinated mice. α-GalCer treatment led to an increase in the IFN-γ serum levels in the presence or absence of irradiated MOSEC tumour cells, demonstrating activation of *i*NKT cells. In addition, i.p. vaccination with irradiated MOSEC tumour cells together with α-GalCer was capable of generating a significant number of cytotoxic T lymphocytes against MOSEC tumour cells compared to vaccination with either irradiated MOSEC tumour cells or α-GalCer alone. Furthermore, treatment of the TgMISIIR-TAg transgenic mice with ovarian tumour cell-based vaccines combined with adjuvant α-GalCer led to prolonged survival as well as increased numbers of tumour-specific $CD8^+$ T cells [29].

α-GalCer as Adjuvant Combined to Nontoxic B Subunit of Shiga Toxin-Based Vaccines

Another study reported synergy between α-GalCer and STxB (nontoxic B subunit of Shiga toxin, a nontoxic homopentameric protein responsible for toxin binding and internalization into target cells) based vaccine leading to potent $CD8^+$ T-cell response with the use of very low dose of antigen (50 ng) through enhanced cross-presentation mediated by a α-GalCer. When mice were immunized twice with the STxB-OVA conjugate (50 μg) alone, an induction of anti-$OVA_{257–264}$ $CD8^+$ T cells corresponding to 0.4% of $CD8^+$ T cells was demonstrated. After two immunizations with STxB-OVA and α-GalCer via the i.p. route, 4.6% of $CD8^+$ T cells stained positively with $OVA_{257–264}/K^b$ tetramer directly ex vivo without any in vitro restimulation step. In addition, in mice immunized twice with STxB coupled to a polypeptide derived from the HPV16-E7 protein (STxB-$E7_{43–57}$) at a low dose (1 μg), a marked induction of anti-E7 CTL was detected ex vivo by the $E7_{49–57}/D^b$ tetramer (1.12% of $CD8^+$ T cells) while only low levels of E7-specific CTL (0.12% of $CD8^+$ T cells) were detected after immunization with STxB-$E7_{43–57}$ alone. In addition, using a transgenic mouse model that expresses OVA on the surface of all cells, it was shown that α-GalCer could break tolerance against self-antigens. Vaccination of mice with STxB-OVA and α-GalCer conferred potent protection against recombinant vaccinia virus encoding OVA (rVV-OVA), with virus titres in the ovaries reduced by 5 log compared with those of mice treated with PBS, while mice immunized with OVA and α-GalCer exhibited a slight reduction of infectious virus titres corresponding to less than 1 log reduction of virus titres. This study demonstrates that STxB-based vaccines combined with α-GalCer resulted in improvement of the STxB Ag delivery system as assessed by the more powerful $CD8^+$ T-cell response observed even at very low doses of immunogen. This vaccine formulation was also efficient to break tolerance against a self-antigen and to induce viral immunity. The potential mechanisms underlying the synergy between STxB-OVA and α-GalCer were

enhanced cross-presentation of STxB-OVA by α-GalCer and α-GalCer-mediated increased STxB uptake by DCs [30].

2.2.1.2 Anti-infectious Immunity

α-GalCer as Adjuvant for HIV Vaccines

The first study to show that α-GalCer can enhance the immunogenicity of DNA vaccines investigated the adjuvant activity of α-GalCer on HIV-1 DNA vaccines.

To investigate whether the immunogenicity of DNA vaccination could be enhanced by α-GalCer, BALB/c mice were co-administered i.m. with 2 μg of α-GalCer with a DNA vaccine, encoding HIV-1 env and gag. Compared to mice vaccinated with DNA only, co-administration of α-GalCer with suboptimal doses of DNA vaccines greatly enhanced antigen-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses. The adjuvant effect of α-GalCer was dependent on CD1d and IFN-γ. Even at the lowest dose tested (0.25 μg), α-GalCer still displayed adjuvant activity on DNA vaccination. In contrast to other vaccines, α-GalCer was also able to enhance an HIV-specific antibody response tenfold; however, the adjuvant activity of α-GalCer was most profound when co-administered at the priming, but not at the boosting phase and concomitant delivery of α-GalCer with DNA vaccine provided optimal adjuvant activity. In order to explore the mechanisms underlying the adjuvant activity of α-GalCer displayed only during the priming phase, the levels of IFN-γ, IL-4 and IL-12 cytokines were tested, in mice receiving a single dose of α-GalCer versus a repeated dose of α-GalCer. The mice that received a single dose of α-GalCer produced significant levels of all three cytokines, whereas the levels of cytokine production were strongly diminished in a second group of mice receiving a repeated dose of the same amount of α-GalCer [31].

α-GalCer as Adjuvant for Malaria Vaccines

α-GalCer has been used as an adjuvant to modulate and/or augment protective immune responses elicited by malaria vaccines. BALB/c mice were immunized i.v. with irradiated *Plasmodium yoelii* sporozoites together with α-GalCer, and the levels of protective anti-malaria immunity was measured by determining the amount of parasite-specific rRNA in the liver following challenge with live *P. yoelii* sporozoites. It was shown that administration of α-GalCer significantly enhanced, in a dose-dependent manner, the level of protective immunity as the parasite load in the livers of immunized mice administered with 2 μg of α-GalCer with irradiated *P. yoelii* sporozoites was ten times smaller than that in the livers of mice immunized with irradiated sporozoites alone. However, α-GalCer treatment did not affect the antimalarial humoral response as the antibody titres were identical among the groups of immunized mice regardless of whether or not they received α-GalCer. Strikingly α-GalCer treatment increased the number of IFN-γ-secreting

circumsporozoite protein (CS)-specific $CD4^+$ T and $CD8^+$ T cells. Subcutaneous (s.c.) immunization of α-GalCer with recombinant adenovirus expressing the whole *P. yoelii* CS protein or recombinant sindbis virus expressing the $CD8^+$ T-cell epitope of the CS protein, significantly enhances the protective immune response induced by the two different recombinant viruses suggesting that the enhancement of the cellular immune response by treatment with α-GalCer is independent of the antigen delivery system (attenuated pathogen or recombinant virus) and the epitope. α-GalCer treatment failed to increase the number of CS-specific CD8+ T cells induced by irradiated *P. yoelii* sporozoites immunization in CD1d-deficient mice and the number of CS-specific IFN-γ-secreting CD8+ and CD4+ T cells in the irradiated sporozoite-immunized knockout mice lacking the IFN-γ receptor (IFN-γ R−/−) demonstrating that the adjuvant activity of α-GalCer was dependent on both CD1d molecules and on IFN-γ production [32].

α-GalCer as Adjuvant for *Bacillus anthracis* Vaccine

The potency of α-GalCer to augment the efficacy of the current *Bacillus anthracis* vaccine has been investigated. This vaccine consists largely of protective antigen (PA), the protein of anthrax toxin that mediates entry of edema factor (EF) or lethal factor (LF) into cells. PA interacts with LF and EF to form lethal toxin (LT) and edema toxin (ET), which together are referred to as anthrax toxin. PA induces protective antibody-mediated immunity against *B. anthracis* but has limited efficacy and duration.

C57BL/6, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice were immunized with PA or PA plus α-GalCer and later boosted with PA alone before determining anti-PA endpoint titres in serum. PA alone stimulated a strong antibody response in C57BL/6 mice that was further enhanced three to fourfold by the inclusion of α-GalCer during the primary immunization and consistent with the lack of CD1d and NKT cells, α-GalCer had no effect on the antibody titres in the knockout mice. Sera from the immunized mice were then tested for the ability to neutralize LT in vitro and it was shown that type I NKT activation with α-GalCer led to an enhanced neutralization capacity while sera from $CD1d^{-/-}$ mice immunized with PA plus α-GalCer had a poor neutralization capacity. While there was some resistance among PA-immunized C57BL/6 and $CD1d^{-/-}$ mice challenged with a single dose of LT, a second dose of LT revealed better protection in PA/α-GC-immunized C57BL/6 mice. Using multiple doses toxin challenge, PA-immunized mice succumbed rapidly to LT, while PA/α-GalCer-immunized mice were resistant over a period of several months, demonstrating that NKT activation led to a sustained protective antibody response. This may be particularly important for the current anthrax vaccine, for which multiple boosters are required to maintain protection [33].

α-GalCer as Adjuvant for Genital Herpes Vaccine

The efficacy of α-GalCer as a mucosal adjuvant for induction of protective immunity against genital herpes has been assessed. Intranasal immunization with HSV-2

glycoprotein D (gD) in combination with α-GalCer elicited strong systemic gD-specific IgG Ab responses as well as lymphoproliferative responses with a mixed Th1/Th2 cytokine profile in the spleen, mediastinal lymph nodes, and genital lymph nodes. Importantly, such an immunization scheme conferred complete protection against an otherwise lethal vaginal HSV-2 challenge. Similarly, intravaginal immunization with gD plus α-GalCer generated potent gD-specific lymphoproliferative and IFN-γ responses in the genital lymph nodes and spleen. Furthermore, the vaginally immunized mice developed a strong systemic and mucosal IgG antibody response and protection against vaginal HSV-2 challenge. To ascertain whether the adjuvant effect of α-GalCer was mediated via the CD1d molecule, C57BL/6 and $CD1d^{-/-}$ mice were immunized i.n. with gD plus α-GalCer three times. Contrary to the immunized $CD1d^{-/-}$ mice, the immunized C57BL/6 mice showed high gD-specific IgG Ab titres, had no or low viral replication and no or only mild symptoms of disease with 100% survival demonstrating that the adjuvant effect of α-GalCer in induction of antibody response and protection against genital herpes was dependent on the usage of the CD1d molecule [34].

α-GalCer as Adjuvant for Influenza Vaccines

α-GalCer as a Mucosal Adjuvant

The efficacy α-GalCer combined with peptides as a mucosal adjuvant was examined in several studies; the study by Young et al. showed for the first time that a single nasal immunization of inactivated virus and α-GalCer is a safe and effective means of preventing influenza infection. To examine the effect of a single co-administration with α-GalCer in the early phases of immune responses, BALB/c mice were immunized with inactivated PR8 alone (1, 10 or 50 μg) or together with 0.5 μg of α-GalCer via the intranasal route, and challenged with 20 LD50 of live PR8 virus 2 weeks later. Three days after infection, the virus titres in the lung washes from all mice immunized with inactivated PR8 alone were lower than those in mice immunized with the vehicle or α-GalCer alone, and the reductions were dose dependent. Mice immunized with a medium dose (10 μg) of inactivated PR8 and α-GalCer were completely protected against the infection, while those receiving inactivated PR8 alone were not. When inactivated PR8 was administered alone, a high dose (50 μg) was required to achieve complete clearance of the live virus. These results indicate that the single co-administration of α-GalCer as a nasal vaccine adjuvant can induce protective immune responses against live virus infection in mice even with reduced dose of inactivated PR8. To assess the PR8-specific humoral immune responses induced by α-GalCer, the mice were sacrificed 2 weeks after the immunization and serum and lung washes were collected. The levels of PR8-specific IgG antibodies in serum were significantly higher in the mice immunized with inactivated PR8 alone or together with α-GalCer than in those receiving the vehicle or α-GalCer alone, and they were increased in a dose-dependent manner. Co-administration of α-GalCer and 10 μg (but not 50 μg) of inactivated PR8 increased IgG levels significantly, and in a small dose (1 μg) inactivated PR8 regime, the small amount of antigen did not elicit a sufficient specific antibody response and

so the adjuvanticity of α-GalCer was not clearly observed. However, mice co-immunized with 10 μg or 50 μg of inactivated PR8 together with α-GalCer produced significantly higher levels of IgA Abs in lung washes than did those immunized with the same dose of inactivated PR8 alone. The levels of IgG1 were remarkably higher in the mice co-immunized with inactivated PR8 and α-GalCer than in those with 1 and 10 μg of inactivated PR8 alone indicating that a single intranasal immunization with inactivated PR8 and α-GalCer induced both mucosal and systemic Ab responses that were slightly biased toward Th2-type responses by some of the dose regimens. The levels of PR8-specific IgG Abs in serum were higher at 3 months post-immunization than at 2 weeks and mice co-immunized with α-GalCer had significantly higher levels of IgA antibodies in lung washes than did those immunized with inactivated PR8 alone 3 months after a single nasal administration. In addition, IL-4 and IL-5 cytokine productions by lymphocyte-derived spleen and cervical lymph were significantly higher in the group of mice co-immunized with α-GalCer than in those receiving inactivated PR8 alone for all doses, while, IFN-γ production was significantly lower in mice given nasal inactivated PR8 and α-GalCer than in mice given nasal inactivated PR8 alone. Consistent with the ratio of serum IgG subtypes, these cytokine profiles demonstrated that the immune responses against inactivated PR8 were biased toward Th2-type responses by the α-GalCer vaccine adjuvant. Interestingly, spleen cells from the mice immunized with inactivated PR8 and α-GalCer elicited distinct CTL responses against virally infected cells, while those receiving inactivated PR8 alone did not, indicating that the mice co-immunized with inactivated PR8 and α-GalCer induced cell-mediated responses against inactivated PR8. Altogether, this study demonstrated that α-GalCer is a safe and appropriate mucosal adjuvant that not only potentiated the immunogenicity of inactivated virus vaccine, but also had positive effects on the mortality of immunized mice against lethal viral infection [35].

In the study by Miller et al., α-GalCer was combined with peptides designed across the highly conserved influenza precursor haemagglutinin [HA(0)] cleavage loop, as a vaccine. Peptides designed across the HA(0) of influenza A/H3N2 viruses, delivered to mice via the intranasal route with α-GalCer as an adjuvant, provided 100% protection following H3N2 virus challenge. Similarly, i.n. inoculation of peptides across the HA(0) of influenza A/H5N1 with α-GalCer completely protected mice against heterotypic challenge with H3N2 virus. Results of these studies demonstrated that HA(0) peptides adjuvanted with α-GalCer have the potential to form the basis of a synthetic, i.n. influenza vaccine [36].

The efficacy of nasal vaccination with α-GalCer as a mucosal adjuvant for the induction of protective immunity against nontypeable Haemophilus influenzae (NTHi), a major pathogen of otitis media and other upper respiratory tract diseases was investigated in the study of Noda et al. Mice were immunized i.n. on days 0, 7, and 14 with the P6 outer membrane protein of NTHi, and 2 μg of α-GalCer. On day 21, the number of CD11c+ DCs and NKT cells was investigated; CD11c+ DCs and the number of α-GalCer-CD1d tetramer-positive NKT cells significantly increased in the nasal-associated lymphoid tissue (NALT) of the P6 + α-GalCer treated mice. The level of IgA antibodies in nasal washes and IgG antibodies in the

serum was also significantly elevated as well as P6-specific IgA-producing cells. IFN-γ-, IL-4-, and IL-6-producing cells were investigated, and the increase of these cytokine-producing cells was shown in nasal passages and spleens of the P6 + α-GalCer group. Following i.n. challenge of mice with live NTHi, enhanced NTHi clearance was observed in the groups of P6 and P6 α-GalCer treated mice, as indicated by reduced numbers of live NTHi in nasal washes, but the effect of nasal immunization was most potent in the P6 + α-GalCer group. This study demonstrated that α-GalCer was an effective mucosal adjuvant and that co-administration with 2 μg of α-GalCer might be an optimized dose for the induction of P6-specific protective immunity [37].

α-GalCer Adjuvant in Subcutaneous Immunization

The adjuvant effect of α-GalCer administered subcutaneously to enhance protective efficacy of inactivated (*i*) influenza A virus (IAV) has also been investigated. Mice were injected s.c. with *i*IAV together with 1 μg of α-GalCer or PBS and sampled 7 days later to measure virus-specific CTL responses directly ex vivo. The primary response to the nucleoprotein (NP) peptide DbNP366 was clearly dominant in both spleen and draining lymph nodes (LN). Surprisingly, mice that were vaccinated with *i*IAV and α-GalCer together displayed significantly reduced percentages of DbNP366 compared with those given *i*IAV. Repeated immunization of *i*IAV ± α-GalCer one to three times at 2-week intervals did not boost DbNP366-specific CD8+ T cell numbers 7 days after the last vaccination, and in fact, the CTL response was diminished by the α-GalCer treatment. However, in contrast to the early (day 7) time point, a significant increase in the proportion and number of DbNP366-specific memory CTLs was observed in mice given *i*IAV + α-GalCer 6 weeks following vaccination. Interestingly, the diminished effector magnitude at the acute time point correlated with an increased ratio of CD62Lhi to CD62Llo DbNP366-specific T cells, but there was no difference in this CD62Lhi to CD62Llo ratio at the memory time point. As such, α-GalCer + *i*IAV apparently favours the generation of the "central memory" cells (TCM) set early after vaccination, contributing to an increased pool of memory T cells. The extent of NKT cell activation following α-GalCer administration was also evaluated using α-GalCer-loaded CD1d tetramer, and a week after the α-GalCer treatment, both the percentage and absolute numbers of NKT cells were significantly increased. To determine if the α-GalCer effect was mediated via NKT cell activation, NKT-deficient CD1d−/− or wild-type CD1d+/+ mice were vaccinated with *i*IAV ± α-GalCer. The DbNP366-specific CTL response was then analyzed at acute (d7) and memory (d42) time points, and, as expected, there was a diminished CTL response in wild-type mice after *i*IAV + α-GalCer administration compared with *i*IAV alone, while effector D^bNP_{366}-specific CTL responses were significantly greater in the $CD1d^{-/-}$ mice irrespective of α-GalCer treatment, suggesting that α-GalCer-induced NKT cell activation impairs the development of acute, *i*IAV-induced CTL responses in normal mice. To investigate whether α-GalCer augmentation of CTL memory enhances recovery following

heterologous IAV infection, mice were primed with *i*IAV PR8 (H1N1) ± α-GalCer and challenged i.n. with H3N2 IAV at least 6 weeks later. Secondary i.n. challenge with A/H3N2 of iPR8 IAV-primed mice resulted in a large recall response 8 days after infection and the DbNP366-specific recall response was significantly greater in both the mediastinal lymph node (MLN) and the respiratory tract airways (isolated by bronchoalveolar lavage) of mice vaccinated with iPR8 + α-GalCer compared with mice primed with iPR8 alone. This result suggested that α-GalCer co-administration with *i*IAV augments the recall CTL response to heterologous IAV challenge, presumably as a consequence of the increased numbers of CTL memory precursors. In addition, mice that received iPR8 + α-GalCer showed evidence of significantly enhanced virus clearance on days 5 and 6 following i.n. challenge with the H3N2 IAV 6 weeks after vaccination. Mechanistically, it was shown that the decrease in the acute IAV-specific CTL responses in *i*IAV + α-GalCer-immunized mice was not reflecting impaired DC function as α-GalCer induced up-regulation of costimulatory molecules on DC of the immunized mice, but rather reflected increased Indoleamine 2,3-dioxygenase (IDO) expression. NKT cell-derived IFN-γ induced increased IDO expression, which in turn inhibited the full expansion and maturation of the acute CTL effector response after *i*IAV + α-GalCer priming. It was suggested that α-GalCer-induced inhibition of acute CTL effector generation and α-GalCer-increased memory CTL cells may reflect independent mechanisms. In fact, the α-GalCer treatment was associated with increased bcl-2 expression in CD44+CD8+ CTLs (memory phenotype) compared with the same population from mice that received *i*IAV alone. This α-GalCer-dependent increase in bcl-2 levels was observed in both the spleen and the draining LN supporting the notion that α-GalCer promotes CTL survival and development into long-lived memory by inducing the expression of survival genes [38].

2.2.2 Clinical Experience with Free α-GalCer as a Drug

Three studies have evaluated the efficacy of free α-GalCer over a wide variety of ranges as a monotherapy against cancer and infectious diseases, but not in the adjuvant setting. However, these studies give indications on the safety and *i*NKT activation following administration of free α-GalCer in the clinic.

2.2.2.1 Patients with Refractory Solid Tumours

A dose escalation of KRN7000 (α-GalCer) was studied in advanced cancer. Patients with refractory solid tumours received i.v. KRN7000 (50–4,800 μg/m^2) on days 1, 8, and 15 of a 4-weekly cycle. Patients with solid tumours were given one cycle and, in the absence of dose-limiting toxicity or progression, treatment was continued. The major end point of this study was to identify the maximum tolerated dose (MTD) and the optimal biologically active dose (OBAD) of KRN7000. No serious

drug-related adverse events occurred during this study. One patient on dose level 1 developed grade 3 fever (temperature up to 40.1 °C and prophylactic paracetamol was used thereafter). One patient on dose level 6 experienced transient flush immediately after injection, which occurred after each administration. On dose level 7, one patient experienced sneezing during each drug administration. Importantly, although KRN7000 can induce liver toxicity in mice, no signs of KRN7000-induced liver toxicity were found; however, the MTD could not be identified. It was shown that the number of circulating NKT cells in cancer patients was significantly lower than that of healthy volunteers. The NKT cells in the peripheral blood (PB) in the NKT-high group decreased to undetectable levels within 24 h after the first administration of KRN7000 at all dose levels, and recovery to the preadministration levels was not observed within a week. Here, only one patient (patient 3 on the first cohort), who had a relatively high pretreatment NKT cell count, showed a faint but detectable increase in serum of IFN-γ at 6 h after the first KRN7000 administration and an increase in serum IL-12 peaking 8 h after KRN7000 administration while these cytokines were never observed in the NKT-low group. In the NKT-high group, five of ten patients showed an increase in both GM-CSF and/or TNF-α levels, peaking 4–6 h after KRN7000 injection and amongst of these, patient 3, who experienced severe fever after the first administration of KRN7000, also developed the highest levels of GM-CSF and TNF-α. In contrast, in the NKT-low group, only slight fluctuations of GM-CSF and TNF-α serum levels were seen after KRN7000 administration. Two patients were not evaluable for the antitumour response: one received only one cycle, and the general conditions deteriorated, preventing treatment continuation and tumour evaluation; in the other patient, the tumour could not be properly assessed. No partial or complete responses were observed; 7 stabilizations for a median of 123 days (range, 83–216 days) and 15 tumour progressions were recorded. In conclusion, this phase I study of KRN7000 did not reach the MTD and did not record substantial toxicities across a broad range of doses. Moreover, no OBAD was defined, despite a very intensive immunological monitoring in all of the patients included in the study [39]. Results of this study strongly suggested other therapeutic strategies aiming at reconstitution of the deficient NKT cell population in cancer patients.

2.2.2.2 Patients with Chronic Hepatitis B Infection

In a dose-escalating phase I/II trial aiming at investigating the safety, tolerability and the antiviral effect of α-GalCer as a novel class of treatment of patients with chronic hepatitis B infection, patients were randomly assigned to 0.1 μg/kg ($n=8$), 1 μg/kg ($n=6$) or 10 μg/kg ($n=6$) α-GalCer or placebo ($n=7$) treatment. This phase I/II dose-escalation trial was performed in a randomized, double-blind and placebo-controlled manner. After completion of 8 weeks of treatment, with injections at 0, 4, and 8 weeks, patients were monitored without further therapy for an additional 16 weeks.

At baseline, the number of circulating NKT ($CD3^+$ $V\alpha24^+$ $V\beta11^+$ or α-GalCer CD1d-tetramer staining) did not significantly differ between the groups. The first administration of α-GalCer induced a rapid decrease in circulating NKTs in all dose levels, which was followed by a recovery of NKT numbers. Although less pronounced, this decrease in NKT numbers was also observed after the second and third administration of α-GalCer. Furthermore, albeit not significantly different, the number of NKTs was still decreased at the end of treatment (EOT, day 84) and approached baseline levels at the end of follow-up (EFU, day 168). The NKT numbers in patients receiving placebo did not significantly differ during the study. Of note, all patients exhibiting high baseline NKT levels who received ≥1 μg/kg α-GalCer developed fever and severe rigours 1 h to 2 days after drug administration. NKT subset analysis in these patients revealed that after the first administration of α-GalCer, the proportion of $CD4^+$ NKTs decreased and the proportion of $CD8^+$ NKTs increased. α-GalCer treatment significantly changed the number of NK cells 2 days post-injection; in patients receiving 0.1 or 1 μg/kg α-GalCer, NK cell numbers significantly decreased whereas the highest dosage induced an increase in NK cells. Activated NK cells, defined as $CD69^+$ cells, were observed in all treated patient groups, but the most pronounced increase in CD69-expressing NK cells was observed in patients with high NKT numbers at baseline. Significant differences in circulating T-cells and DCs were not observed. Cytokine levels remained undetectable in the patient group with low NKT numbers; however, in five of nine patients with high NKT levels, a transient increase in TNF-α was observed. The patient exhibiting the highest TNF-α level (35 pg/ml) experienced severe fever shortly after α-GalCer administration. In addition, the patients exhibiting a period of fever shortly after α-GalCer administration demonstrated an increase in IL-6 from 2 ± 3 pg/ml at baseline to 719 ± 906 pg/ml 4 h after drug administration that returned to baseline levels at day 2. No detectable levels of IFN-γ, IL-1β, IL-10, IL-5 and GM-CSF were observed in serum of those patients. No significant decreases in HBV DNA following the first administration of α-GalCer were observed in any of the three dosages groups. There were also no clear and significant differences in the alanine aminotransferase (ALT) values over time in the three different dose levels of α-GalCer-treated patients compared with placebo. Four α-GalCer-treated patients discontinued therapy early because of an episode of fever shortly after drug administration. All these episodes resolved spontaneously. These side effects limited further development of treatment with α-GalCer in chronic hepatitis B patients [40]. Results from this trial suggested that higher dosage of α-GalCer might be more effective, but will be probably limited by its side effects.

2.2.2.3 Patients with Chronic Hepatitis C Infection

The safety and the antiviral activity of α-GalCer as a novel class of treatment of chronic hepatitis C patients was investigated. Forty patients were randomly assigned to a dose of 0.1 μg/kg ($n = 9$), 1 μg/kg ($n = 9$), 10 μg/kg ($n = 11$) or to placebo ($n = 11$).

Small decreases in HCV RNA directly following the first administration of α-GalCer was frequently observed but not in the placebo group. At the end of treatment and at the end of follow-up no statistically significant changes in HCV RNA were observed in either group. Among patients with high baseline *i*NKT cell levels (*i*NKT cells >1,000 NKT cells/10^6 T cells) of whom six received α-GalCer no statistically significant changes in HCV RNA were found.

There were no significant changes in mean ALT levels among treated patients compared to the placebo group. At the end of follow-up, one patient in dose level 1 and one patient in the placebo group had normal ALT levels. There was no significant effect of α-GalCer on IFN-α- and IL-5 levels, and no statistically significant changes in serum levels of IFN-γ were observed in any of the treatment groups analyzed as a whole. Similarly, no statistically significant changes in serum levels of TNF-α were observed in dose levels 1, 2 and the placebo group. In dose level 3 there was a small overall increase in serum TNF-α levels after 4 h that returned to baseline levels at day 2. α-GalCer induced a reproducible increase in IFN-γ and TNF-α levels in several individual patients and the maximum increase in serum TNF-α occurred in the patient with the highest baseline *i*NKT cell count (6,885 *i*NKT cells/10^6 T-cells). This patient showed a marked decrease in HCV RNA compared to baseline after the first administration of α-GalCer that was accompanied by a rise in serum ALT levels, suggestive of an immune response to HCV-infected hepatocytes. However, although the second administration of α-GalCer also led to high serum levels of both TNF-α and IFN-γ in this patient, no reduction in HCV RNA load was observed after the second administration, and only a minor reduction in HCV RNA load was observed after the third administration.

Concerning *i*NKT cells, NK and T cells, the first administration of α-GalCer resulted in a rapid and significant decrease in circulating *i*NKT cells (staining with monoclonal antibodies against TCR Vα24 and Vβ11 chains or α-GalCer CD1d-tetramer) in all dose levels which was followed by a recovery of *i*NKT cell numbers, but not in the placebo group. While the proportion of $CD4^+$ *i*NKT cells was not significantly altered, the proportion of DN *i*NKT was significantly decreased and the proportion of $CD8^+$ *i*NKT cells significantly increased. The second and third administration of α-GalCer did not result in any significant changes in the contribution of each *i*NKT cell subset to the total *i*NKT cell pool. Both the first and second administration of α-GalCer, but not placebo, resulted in a significant decrease in the number of circulating T cells that was caused by a decrease in both $CD4^+$ and $CD8^+$ T cells and no statistically significant changes were found in the expression of CD69 on T cells.

All reported adverse events in this study were scored grade I. None of the patients discontinued treatment because of adverse events and no serious adverse events related to the study drug occurred. In addition, there were no significant changes in mean ALT levels among treated patients compared to the placebo group. In conclusion, α-GalCer used as monotherapy in doses of 0.1–10 μg/kg in this study was safe and it exerts moderate immunomodulatory effects [41].

2.3 Preclinical and Clinical Experience with Glycolipid-Loaded or Transduced Cells

2.3.1 Preclinical Experience with α-GalCer-Loaded and α-GalCer-Transduced Cells

2.3.1.1 α-GalCer-Loaded Tumour Cells

Some studies have indicated that tumour cells are capable of presenting α-GalCer on CD1d molecules and elicit combined NKT and NK responses. Even though the tumour cells lacked expression of CD40, CD80, and CD86 costimulatory molecules, the i.v. injection of tumour cells loaded with α-GalCer (tumour/Gal) resulted in IFN-γ secretion by NKT and NK cells that was comparable to or better than α-GalCer-loaded DCs (DC/Gal). Tumour cells that expressed low levels of endogenous CD1d, or were transduced to express higher levels of CD1d in a stable fashion when loaded with α-GalCer, failed to establish tumour upon i.v. injection, and the resistance against tumour cells was independent of $CD4^+$ and $CD8^+$ T cells but dependent upon NKT and NK cells [42]. However, mice injected s.c. with B16/Gal and with tumour cells that had been transfected to express high levels of CD1d and loaded with α-GalCer ($CD1d^{hi}$-B16/Gal) and mice given DC/Gal i.v. were not protected against B16 challenge s.c. Unexpectedly when mice were injected with transfected $CD1d^{hi}$-B16/Gal i.v. and then challenged s.c. 2 weeks later with B16 tumour cells, all the mice became resistant to B16 tumour, and several tumours induce resistance when exposed to α-GalCer and injected i.v. This resistance proved to be T-cell mediated as $CD4^{-/-}$ and $CD8^{-/-}$ mice did not develop resistance to a subsequent s.c. challenge with B16 tumour cells. In addition, vaccination with $CD1d^{hi}$-B16/Gal tumour cells i.v. induced $CD8^+$ T cells specific for defined melanoma differentiation antigens such as peptides from the gp100, tyrosinase-related, and dopachrome tautomerase (DCT)/tyrosinase-related protein 2 (TRP-2) antigens, in relatively low doses into mice. α-GalCer-loaded tumour cells were superior inducers of T-cell immunity than DCs, as DCs coated with peptide with or without α-GalCer were poorly immunogenic when given by the i.v. as well as the s.c. route, while EL4(OVA) tumour cells induced stronger immunity if the cells were loaded with α-GalCer and injected i.v. but not when given s.c. The response to tumour/Gal was entirely dependent on the presence of $V\alpha14^+$ NKT cells as indicated with the appropriate knockout mice. Mechanistically, it was demonstrated that tumour/Gal induced adaptive immunity through the capture and cross-presentation of glycolipid by DCs in vivo. The ensuing presentation of glycolipid by DCs to NKT cells induce DC differentiation or maturation, and the maturing DCs will then be able to trigger the adaptive T-cell immunity, resulting in long-term T-cell resistance to the tumour. [43]. Shimizu et al. also demonstrated that α-GalCer-loaded human leukemic cell lines and primary leukemic cells as well as human dendritic cells (DCs) loaded with α-GalCer (hDC/Gal) injected into C57BL/6 mice have the capacity to stimulate murine NKT cells in vivo [44].

2.3.1.2 α-GalCer-Loaded Myeloid-Derived Suppressor Cells

A study has investigated whether myeloid-derived suppressor cells (MDSCs) or even monocytes can enhance immunity with the help of activated NKT cells. Immature myeloid cells (also called MDSC), macrophages, granulocytes, immature dendritic cells, monocytes, and other myeloid cells in early differential stages (which are known to be accumulated in the blood, spleen, and bone marrow of tumour-bearing mice and cancer patients) were tested as APCs for a cellular vaccine. These cells have phenotypical similarity with inflammatory monocytes and may be differentiated from the same precursors as monocytes. Immunization of mice with α-GalCer-loaded monocytes presenting Her-2/$neu_{63–71}$ peptide (Mo/hP63/αGC), but not with α-GalCer-unloaded monocytes presenting Her-2/$neu_{63–71}$ peptide (designated Mo/hP63), induced significant levels of CTL responses against the Her-2/$neu_{63–71}$ peptide, as did bone marrow-derived DC (BmDC). To test the antitumour effect elicited by the manipulated monocyte vaccine, BALB/c mice were injected i.v. with 2×10^5 cells of Her-2/CT26 tumour on day 0, followed by vaccination on day 1. Although both Mo/αGC and Mo/hP63/αGC induced significant antitumour activity, Mo/hP63/αGC led to a significant extension in mean survival time of tumour-challenged mice as compared to Mo/αGC-treated mice. To assess whether a monocyte-based vaccine expressing whole tumour Ag instead of peptide could also induce antitumour immunity, mice were immunized with an adenovirus (AdHM) expressing Her-2/*neu* tumour antigen on the cell surface of monocytes. Mo/AdHM/αGC-immunized group showed a significant increase in survival over the group immunized with Mo/AdHM or Mo/αGC demonstrating that vaccination of mice with α-GalCer-loaded monocytes presenting a tumour Ag induced strong Ag-specific CTL responses and successful antitumour immunity against circulating metastatic tumour cells. In addition, MDSCs loaded with Ag peptide and αGalCer (MDSC/hP63/αGC) induced significantly higher hP63-specific cytolysis than that observed in MDSC/hP63-immunized mice, and increased protection against the development of Her-2/CT26 metastases. Vaccination with MDSC/hP63/αGC led to a significant extension in survival time, one that was comparable to the antitumour effect of bone marrow-derived DCs loaded with hP63 and α-GalCer (BmDC/hP63/αGC) and while BmDC/hP63 vaccination induced strong CTL activity in itself, MDSC-based vaccine required α-GalCer loading to generate successful CTL activity in vivo. Mice vaccinated with MDSC/AdHM/αGC showed significantly higher resistance to tumour challenge than those immunized with MDSC/AdHM, and depletion of Treg cells significantly increased the antitumour effects of the MDSC vaccine. It was shown that α-GalCer loading did not increase the CTL activity of the MDSC/OVA peptide$_{257–264}$ (designated MDSC/pep) vaccine in CD1d$^{-/-}$ mice as it had done in C57BL/6 wild-type mice suggesting that α-GalCer-loaded MDSCs induced NKT cell activation even in tumour-bearing mice and that activated NKT cells augmented Ag-specific CTL responses induced by MDSC immunization. Depletion experiments showed that both CD8$^+$ T cells and NK cells are necessary for the induction of antitumour effects by the α-GalCer-loaded MDSC vaccine and that NKT cell activation by α-GalCer in MDSC-based vaccine can compensate the

helper function of $CD4^+$ T cells for the generation of successful antitumour CTL activity in the absence of $CD4^+$ T cells. The characteristics of transformed MDSC after activation by NKT cells were checked by assessing the phenotypes and maturation of MDSC in vivo. α-GalCer-loaded MDSCs increased the expression of CD86, demonstrating the shift of MDSCs into activated APCs after stimulation by activated NKT cells. The influence of MDSC vaccine on CTL responses was investigated in an established tumour-suppressive environment and results demonstrated that MDSC/pep/αGC treatment significantly enhanced the Ag-specific IFN-γ secretion even in tumour-bearing mice. This study suggested that MDSC vaccines induced Ag-specific CTLs when loaded with α-GalCer rather than suppressing CTL function and that α-GalCer-loaded MDSC vaccine could be immunogenic for $CD4^+$ T cells rather than increasing regulatory T cells [45].

2.3.1.3 α-GalCer-Loaded B Cells

Although resting B cells are known for being poorly immunogenic and for inducing T-cell tolerance, studies have attempted to test the efficiency of α-GalCer-loaded, peptide-pulsed B cells in generating cytotoxic immunity and antitumour activity. In vitro, α-GalCer-loaded B cells (B/α-GalCer) efficiently stimulated NKT hybridoma (DN32.D3) cells to produce interleukin (IL)-2, equalling the rate of the DC group of IL-2 production when at higher ratios to the hybridoma. In vivo, i.v. injection of B/α-GalCer into syngenic mice stimulated NKT to produce IFN-γ whereas B/vehicle did not. The characteristics of B cells after injection were checked and high levels of CD86 but not CD80 expression were induced within 24 h on B/αGalCer. The B-cell-based vaccine approach induced long-lasting memory cytotoxic immunity as only the B/α-GalCer/peptide ($ovalbumin_{257–264}$)-treated group completely lysed peptide-pulsed targets even 5 weeks after a single vaccination and showed a significant increase in the number of IFN-γ-producing $CD8^+$ T cells against the peptide compared to C57BL/6 mice vaccinated with B alone, B/α-GalCer, or B/peptide. It was shown that B/α-GalCer/peptide was as efficient as DC/α-GalCer/peptide in generating cytotoxicity and that the CTL immunity required both CD8+ T and NKT cells but not CD4+ T or NK cells. This study also demonstrated that B cells act as real APCs rather than peptide reservoir, and the loading of α-GalCer and peptide on the same B cell was required for CTL generation, as i.v. vaccinated C57BL/6 mice with B/α-GalCer plus B/pep failed to generate in vivo cytotoxicity contrary to i.v. injected mice with B/α-GalCer/peptide. Vaccination with B/α-GalCer/peptide also generated antitumour immunity in both prophylactic and therapeutic settings. Indeed, following vaccination before s.c. transplantation of ovalbumin-transfected B16 melanoma (MO-5), a slightly delayed pattern of tumour growth was observed in mice vaccinated with B/α-GalCer, although all mice finally developed tumours while, no mice receiving B/α-GalCer/peptide, DC/peptide, or DC/α-GalCer/peptide developed tumour growth.

To examine whether these mice established long-term antitumour activity, the surviving mice were re-challenged s.c. with MO-5 tumours 70 days after the first tumour inoculation. Tumour growth was not observed in those mice, showing that

vaccination with B/α-GalCer/peptide established memory immunity against the tumour. In the therapeutic model, mice were vaccinated (a) 1 or (b) 9 days after s.c. transplant when tumours had become palpable. In the 1-day model, vaccination with DC/peptide, DC/αGalCer/peptide or B/αGalCer/peptide almost completely suppressed tumour growth. In the 9-day model, none of these vaccinations completely destroyed the growing tumour due to the aggressive nature of the B16 melanoma. However, in mice vaccinated with B/αGalCer/pep, tumour growth was less pronounced than in "B alone" group of mice and resembled that observed in the DC/pep-vaccinated or DC/αGalCer/pep-vaccinated group. This B-cell-based vaccine regimen was then applied to HER-2/*neu* tumour antigen. Again, a significant level of HER-2/*neu*-specific cytotoxicity in vivo was observed in mice given α-GalCer-loaded HER-2/$neu_{63–71}$-pulsed B cells. After i.v. or s.c. tumour inoculation with HER-2/*neu*-expressing colon carcinoma (CT26-HER-2/*neu*), all mice vaccinated with B/α-GalCer/peptide (HER-2/$neu_{63–71}$-pulsed B/α-GalCer) survived the duration of the experiment, showing that a B-cell-based vaccine regimen proved to be as effective as DC-based vaccines in generating both prophylactic and therapeutic antitumour immunity [46].

2.3.1.4 α-GalCer-Loaded Antigen-Transduced B Cells

To extend the B-cell vaccine approach to the whole antigen, and to overcome the MHC restriction, a non-replicating adenovirus was used to transduce B cells with an antigenic gene. Primary B cells transduced with an adenovirus-encoding truncated Her-2/neu (AdHM) efficiently expressed Her-2/neu. Compared with the moderate antitumour activity induced by vaccination with adenovirus-transduced B cells (B/AdHM), vaccination with α-GalCer-loaded B/AdHM (B/AdHM/α-GalCer) induced significantly stronger antitumour immunity, especially in the tumour-bearing mice. The depletion study showed that CD4+, CD8+, and NK cells were all necessary for the therapeutic immunity. Confirming the results of the depletion study, B/AdHM/α-GalCer vaccination induced cytotoxic NK cell responses but B/AdHM did not. Vaccination with B/AdHM/α-GalCer generated Her-2/neu-specific antibodies more efficiently than B/AdHM immunization, and B/AdHM/α-GalCer could prime Her-2/neu-specific cytotoxic T cells more efficiently and durably than B/AdHM. CD4+ T cells appeared to be necessary for the induction of antibody and CTL responses. This study demonstrated that with the help of NKT cells, antigen-transduced B cells efficiently induce innate immunity as well as a wide range of adaptive immune responses against the tumour, suggesting that they could be used to develop a novel cellular vaccine [47].

2.3.1.5 Allogeneic Fibroblasts Transfected with Antigen-Encoding mRNA, Loaded with α-GalCer

In this study, allogeneic fibroblasts transfected with mRNA encoding tumour antigen were used as a source of antigen, an approach that could be clinically useful in

situations where access to autologous tumour is limited or where response to a specific tumour antigen is desired. In these experiments NIH3T3 fibroblasts were selected as expression of OVA protein by OVA mRNA-transfected NIH3T3 was similar to that of the parental B16 transfectants. Parental B16 melanoma cells and NIH3T3 cells expressed lower levels of CD1d than bone marrow-derived DCs, and stable variants that had been transduced with a retrovirus expressing high levels of murine CD1d were established as previously (CD1d^{hi}-NIH3T3).

To monitor the in vivo antigen-presenting capacity of transfectant fibroblasts, OVA mRNA transfectants were loaded with or without α-GalCer and given to mice that had received an injection of OT-I cells. Mice given α-GalCer-loaded, OVA mRNA-transfected CD1d^{hi}-NIH3T3 (CD1d^{hi}-NIH3T3/Gal-OVA) showed greater OT-I cell proliferation than mice given OVA mRNA-transfected CD1d^{hi}-NIH3T3 (CD1d^{hi}-NIH3T3-OVA). Thus, CD1d^{hi}-NIH3T3/Gal-OVA were able to generate OT-I cellular proliferation in vivo, suggesting cross-presentation by endogenous DCs in the allogeneic hosts.

The capacity of allogeneic cells with or without α-GalCer to stimulate the innate immune system in vivo was measured. NK cell responses were analyzed by flow cytometry for the expression of CD69 and IFN-γ 16 h after immunization. NK cells up-regulated CD69 and secreted IFN-γ in mice given CD1dhi-NIH3T3/Gal and only a weak allogeneic response was seen in NK cells from mice injected with NIH3T3- or CD1dhi-NIH3T3. NKT-cell activation was analyzed in an IFN-γ ELISPOT assay following restimulation of spleen cells with or without α-GalCer. The number of IFN-γ-producing spots in NIH3T3/Gal- or CD1dhi-NIH3T3/Gal-injected mice was similar to B16/Gal or CD1dhi-B16/Gal indicating that CD1dhi-NIH3T3/Gal as well as CD1dhi-B16/Gal act as antigen-presenting cells for innate *i*NKT-cell and NK cell responses in vivo.

To assess antitumour effects, a B16 melanoma lung metastasis model was used in which resistance to the establishment of lung metastases mainly depends on NK and *i*NKT cells. Mice given allogeneic fibroblasts without α-GalCer 3 h following challenge readily developed lung metastases while this did not occur in mice given NIH3T3/Gal or CD1d^{hi}-NIH3T3/Gal. Jα18$^{-/-}$ mice, which do not have *i*NKT cells, did not demonstrate this resistance to tumour metastases, indicating that the activation of innate lymphocytes by NIH3T3/Gal or CD1d^{hi}-NIH3T3/Gal in vivo was sufficient to block the establishment of lung metastases.

DCs from mice immunized with NIH3T3/Gal or CD1d^{hi}-NIH3T3/Gal i.v. showed changes consistent with DC maturation, but not DCs from animals immunized with NIH3T3 cells or CD1d^{hi}-NIH3T3. The indications of DC maturation were ablated in Jα18-deficient mice indicating *i*NKT cells were necessary for DC maturation. These results suggested that DCs began to mature soon after injection of allogeneic fibroblasts loaded with α-GalCer. The glycolipid loaded on fibroblasts activated *i*NKT cells (directly and indirectly after capture by host DCs), which in turn matured the DCs.

To study the importance of *i*NKT-cell activation and CD1d-expressing fibroblasts in the induction of OVA-specific T-cell responses, mice were immunized with variations of parental or CD1d^{hi}-NIH3T3 cells transfected with OVA mRNA: NIH3T3-OVA,

NIH3T3/Gal-OVA, CD1d^{hi}-NIH3T3-OVA, and CD1d^{hi}-NIH3T3/Gal-OVA. The number of OVA-tetramer-positive cells increased in mice given NIH3T3/Gal-OVA or CD1d^{hi}-NIH3T3/Gal-OVA, but not in mice given NIH3T3-OVA or CD1d^{hi}-NIH3T3-OVA, and this did not occur when Jα18-deficient mice were used as recipients. Mice immunized with CD1d^{hi}-NIH3T3/Gal-OVA generated higher number of $OVA_{257–264}$ peptide-specific T cells than mice given NIH3T3/Gal-OVA. When comparing the magnitude of T-cell responses after priming with the NKT-cell ligand α-GalCer versus ligands of NK cells, α-GalCer-loaded, antigen-carrying fibroblasts led to a stronger immune response by linking innate and adaptive immunity in naive mice.

To evaluate whether the T-cell response in mice immunized with CD1d^{hi}-NIH3T3/Gal-OVA can lead to antitumour immunity, mice were challenged s.c. with EL4 thymoma or OVA-expressing EL4 (EG7) 2 weeks after i.v. immunization with NIH3T3-OVA, CD1d^{hi}-NIH3T3-OVA, NIH3T3/Gal-OVA, or CD1d^{hi}-NIH3T3/Gal-OVA. Protection against tumour development after s.c. inoculation requires CD4$^+$ and CD8$^+$ T-cell responses. Antitumour effects in mice given CD1d^{hi}-NIH3T3/Gal-OVA were shown against EG7, but not EL4, indicating tumour-specific immune response, and the vaccination failed to provide the protective effect in mice immunized with NIH3T3/Gal-OVA, NIH3T3, CD1d^{hi}-NIH3T3-OVA, or CD1d^{hi}-NIH3T3/Gal.

This concept was then applied to real tumour models by immunizing mice with CD1d^{hi}-NIH3T3/Gal cells transduced with mRNA encoding the melanocyte differentiation antigen, tyrosinase-relating protein 2 (trp2). Adaptive antitumour responses to injected trp2-encoding mRNA-transfected CD1d^{hi}-NIH3T3/Gal were assessed. When the mice were given s.c. challenge of B16 melanoma cells 2 weeks later to assess antitumour protection, growth of B16 tumour cells was inhibited in mice that received i.v. immunization of CD1d^{hi}-NIH3T3/Gal-trp2 but not CD1d^{hi}-NIH3T3-trp2 or CD1d^{hi}-NIH3T3/Gal, and none of the immunized mice demonstrated any antitumour immunity against EL4 thymoma cells. The effect of immunization with trp2-encoding mRNA-transfected CD1d^{hi}-NIH3T3/Gal on established s.c. B16 tumours was assessed. Mice were injected with B16 cells s.c. then i.v. with CD1dhi-NIH3T3/Gal-trp2 cells on days 5 and 12, and tumour size was evaluated. Inhibition of tumour growth was seen in immunized mice until day 20, although no mouse demonstrated complete rejection of the tumour. Thus, glycolipid-loaded, mRNA-transfected allogeneic fibroblasts act as cellular vectors to provide *i*NKT-cell activation, leading to DC maturation and T-cell immunity [48]

2.3.1.6 α-GalCer Transduced in Live Vectors

The adjuvant effect of α-GalCer transduced in live vectors has been also evaluated. An approach for stably incorporating α-GalCer and its analogue α-C-GalCer into live BCG organisms was developed, and the impact of this on the stimulation of T-cell responses and protective immunity evaluated. For the initial assessment of the biological activity of *i*NKT cell-activating glycolipids incorporated into live BCG, a standard *i*NKT cell hybridoma stimulation assay was used. The DCs infected with glycolipid-modified BCG preparations strongly stimulated IL-2 production by

*i*NKT cell hybridoma cells in comparison with DCs infected with unmodified BCG, indicating that the incorporated glycolipids could be presented by CD1d molecules. The activity of α-GalCer/BCG or α-C-GalCer/BCG for stimulation of a human *i*NKT cell clone in culture with infected monocyte-derived human DCs was also tested and results demonstrated that α-GalCer/BCG was strongly stimulatory toward human *i*NKT cells and activated their secretion of IFN-γ, TNF-α, and IL-13 at relative levels comparable to those obtained with addition of free α-GalCer. To determine the in vivo activity of the glycolipids incorporated stably into live BCG, serum cytokine levels were measured at various time points after i.p. injection of α-GalCer/BCG into C57BL/6 mice. α-GalCer/BCG was clearly active in vivo and induced low but detectable serum levels of IFN-γ, IL-12, and IL-4 within 6–12 h. Injection of unmodified BCG induced low levels of serum IL-12p70 and no detectable IFN-γ or IL-4 over a 48-h period, and a single injection of free α-GalCer rapidly induced all three cytokines. Since α-GalCer and α-C-GalCer have been reported to induce differentiation and maturation of DCs, the expression of MHC class II and costimulatory molecules on the CD11c+ cells in the spleens and livers of C57BL/6 mice that were injected i.p. with α-GalCer/BCG or α-C-GalCer/BCG was also assessed. In the spleen, neither unmodified nor glycolipid-modified BCG had a significant effect on the surface levels of MHC class II molecules. However, the costimulatory molecules CD80, CD86, and CD70, while only slightly increased on CD11c+ cells by unmodified BCG, showed pronounced induction with α-GalCer/BCG and also to a lesser extent with α-C-GalCer/BCG. In the liver, marked increases of MHC class II were observed with both α-GalCer/BCG and α-C-GalCer/BCG, as well as increases in CD80, CD86, and CD70. In all cases, these effects were greater than those observed with unmodified BCG. These effects depended on *i*NKT cell activation, since they did not occur in parallel experiments conducted in CD1D−/− knockout mice which lack *i*NKT cells, suggesting that the enhancement of DC maturation was an important consequence resulting from incorporation of *i*NKT cell agonist glycolipids into BCG.

In marked contrast to CD4+ T-cell differentiation that proceeded similarly in mice immunized with unmodified and glycolipid-modified BCG, evaluation of Ag-specific CD8+ T-cell priming and recall responses revealed a dramatic effect of the glycolipid incorporation. To analyze recall responses of endogenous CD8+ T cells, C57BL/6 mice were vaccinated with unmodified or glycolipid-modified BCG-OVA and analyzed after 3 or 8 weeks for SIINFEKL-responsive CD8+ T cells in the spleen by IFN-γ ELISPOT. This revealed significantly enhanced responses to the SIINFEKL peptide in mice that had received α-GalCer/BCG-OVA or α-C-GalCer/BCG-OVA compared with BCG-OVA without glycolipid incorporation. IFN-γ ELISPOT responses to an H-2Kd presented epitope (GYAGTLQSL) shared by the endogenous mycobacterial Ags TB10.3 and TB10.4 (TB10.3/10.4) confirmed this finding as responses to this peptide were also significantly enhanced in BALB/c mice vaccinated 2 weeks previously with αGalCer/BCG compared with unmodified BCG. Taken together, these results provided strong evidence that mycobacterial Ag-specific CD8+ T-cell responses were significantly accelerated and enhanced by incorporation of α-GalCer or α-C-GalCer into live BCG. Experiments conducted to address the

question of whether the effect of α-GalCer or α-C-GalCer on enhancing CD8+ T-cell responses required the physical association of the glycolipids with the immunizing bacteria revealed that only direct physical incorporation of the glycolipids into live BCG organisms was able to induce significantly improved CD8+ T-cell priming against an H-2Kb presented TB10.3/10.4 epitope (QIMYNPAM). Immunization and challenge studies were performed to determine whether the enhanced CD8+ T-cell priming associated with α-GalCer/BCG or α-C-GalCer/BCG could improve protective immunity induced by BCG vaccination. C57BL/6 mice that were either naive or immunized 2 months earlier by intradermal inoculation with live BCG, αGalCer/BCG, or α-C-GalCer/BCG were challenged by low-dose aerosol infection with virulent *Mycobacterium tuberculosis* H37Rv, and CFU counts in tissues were determined at 3 and 6 weeks after challenge. In naive mice, substantial growth in the lungs and dissemination to spleens were detected at 3 and 6 weeks after challenge. However, vaccination with BCG, α-GalCer/BCG, or α-C-GalCer/BCG considerably reduced *M. tuberculosis* bacterial loads in both lungs and spleens of aerosol-challenged mice compared with naive controls. Interestingly α-C-GalCer/BCG vaccination protected significantly better than BCG, at the 3 weeks time point, in both lungs and spleen. Immunization with α-C-GalCer/BCG also showed a more prolonged effect on control of *M. tuberculosis* infection compared with BCG immunization, with reductions in CFU in both organs at 6 weeks after challenge. Similar trends toward enhanced protection were observed with α-GalCer/BCG immunization, although this was clearly less pronounced than with α-C-GalCer/BCG and achieved statistical significance only at the 6-week time point in the lungs. These results provide the basis for a simple modification of BCG that could overcome the CD8+ T-cell priming defect inherent in this vaccine and potentially lead to a more effective vaccine for prevention and control of *M. tuberculosis* infections [49].

2.3.1.7 Glycolipid-Loaded DC

α-GalCer-Loaded Cells Derived from Mouse Embryonic Stem Cells

The capacity of α-GalCer-loaded dendritic cells derived from mouse embryonic stem cells (ES-DC) to stimulate NKT cells was evaluated both in vitro and in vivo, in comparison with that of bone marrow-derived dendritic cells (BM-DC). DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, were generated using a method based on in vitro differentiation of DC from mouse ES cells. ES-DC or BM-DC preincubated with α-GalCer similarly activated *i*NKT cells both in vitro and in vivo as demonstrated by a significant cytotoxity against YAC-1 cells in comparison with ES-DC loaded with vehicle alone. Treatment with i.p. injection of ES-DC loaded with α-GalCer did not show any therapeutic effect in mice following s.c. injection of MO4 tumour cells originating from NK-sensitive B16 melanoma cells, but elicited a significant but limited protective effect against the i.p. disseminated tumour cells. However, i.p. injection of ES-DC expressing OVA (ES-DC-OVA) loaded

with α-GalCer elicited a significant antitumour effect in mice following s.c. injection of MO4 cells expressing OVA, but the loading of α-GalCer to ES-DC-OVA did not improve the effect. In contrast, the treatment with ES-DC-OVA loaded with α-GalCer elicited a potent effect to prolong the survival time of the mice using the i.p. disseminated tumour cells indicating that the NKT cells activated by α-GalCer presented by ES-DC together with OVA and specific CTL primed by OVA antigen presented by the same ES-DC acted synergistically to protect the mice. In addition, α-GalCer-loaded ES-DC-OVA simultaneously expressing the chemokine SLC (ES-DC-OVA/SLC) exhibited a far more potent protective effect than α-GalCer ES-DC-OVA, and it was shown that the SLC produced by ES-DC dominantly enhanced the activation of antigen-specific CTL rather than NKT or NK cells [50].

On the basis of the previously established methods to generate DC from mouse embryonic stem cells (ES-DC), four kinds of genetically modified ES-DC, which expressed the melanoma-associated antigens, glypican-3, secreted protein acidic and rich in cysteine, tyrosinase-related protein-2, or gp100 were generated. Anticancer effects elicited by immunization with the ES-DC were assessed in preventive and also therapeutic settings in the models of peritoneal dissemination and spontaneous metastasis to lymph node and lung. The in vivo transfer of a mixture of three kinds of tumour associated antigens (TAA)-expressing ES-DC protected the recipient mice from melanoma cells more effectively than the transfer of ES-DC expressing single TAA, and loading ES-DC with α-GalCer further enhanced the anticancer effects, suggesting that excellent synergic effects of TAA-specific cytotoxic T lymphocytes and natural killer T cells against metastatic melanoma can be achieved by using genetically modified ES-DC [51].

α-GalCer-Loaded DC

When comparing the ability of α-GalCer-charged DCs and the free drug to manipulate NKT numbers and function systemically in mice, Fujii et al. have shown that DCs elicited NKT responses distinct from those seen with the drug alone. The response to DC α-GalCer, as assessed by the number of IFN-γ-secreting NKT cells after α-GalCer challenge, was much stronger and more prolonged. To evaluate protection against the development of B16 melanoma metastases that could be induced by free α-GalCer versus α-GalCer-charged DC, the tumour cells and α-GalCer were both administered via the i.v. route and metastases to the lungs were evaluated 2 weeks later. Immunization with DCs pulsed with glycolipid markedly reduced lung metastases and provided more effective resistance to B16 melanoma metastases, independent of NK cells. DCs from mice given free α-GalCer were fully capable of inducing prolonged NKT cell responses upon adoptive transfer to naïve animals, but not in the recipients given free drug showing that mice develop a stronger, more prolonged and effector type of NKT response, when α-GalCer is selectively targeted to DCs, but that this response can be blocked by the induction of anergy after presentation of α-GalCer on other cells [52].

Nagaraj et al. confirmed that an increase of IFN-γ-producing splenocytes cells is detectable after immunization of mice with α-GalCer-pulsed DCs as compared to mice immunized with free α-GalCer or unpulsed DCs. To assess the antitumour activity of α-GalCer, mice were pre-vaccinated s.c. on day 0 with PBS only or with α-GalCer-pulsed DCs, and challenged on day +7 by injecting 1×10^5 PancO2 cells, a murine pancreatic ductal adenocarcinoma, highly resistant to antitumour agent, which has been shown to produce rapidly growing local tumours following s.c. inoculation. On days +21 and +28, mice were post-vaccinated (PBS only or DCs pulsed with α-GalCer, respectively). Immunization of mice with DCs pulsed with α-GalCer prevented tumour growth until week 4 and strongly decreased tumour growth in comparison with the control group as demonstrated by a decrease in tumour volume and increase in the percentage of tumour-free mice. In addition, survival time was prolonged by the use of α-GalCer-pulsed DCs [53].

In another study, immunization with DCs pulsed with CTL epitope peptide together with α-GalCer at priming phase, but not at boosting phase elicited a specific CTL activity and protective immunity against infection of intracellular bacteria. The effect of immunization with dendritic cells (DCs) pulsed with α-GalCer and listeriolysin O (LLO) 91–99 peptide, a dominant cytotoxic T lymphocyte (CTL) epitope of Listeria monocytogenes was evaluated by observing the responses of specific CD8+ T cells and in vivo CTL activity. Immunization with DCs pulsed with α-GalCer and LLO91–99 at priming phase and with DCs pulsed with LLO91–99 alone at boosting phase induced stronger in vivo CTL activity, reduced the bacterial load in spleens of Listeria-challenged mice and augmented CD62L+ CD8+ central memory T cells compared with other immunization protocols. The blockade of IFN-γ at boosting phase reversed the induction of CD8+ central memory T cells and reduced the bacterial load in spleens of Listeria-challenged mice immunized with DCs pulsed with α-GalCer and LLO91–99 at both phases, suggesting that α-GalCer at boosting phase has deleterious effects through IFN-γ production [54].

Shimizu et al. compared α-GalCer-pulsed and non-pulsed DC to induce long-term NK- and NKT-cell activation at the single cell level and demonstrated that DC therapy in mice induces long-lasting innate NK- and NKT-cell activation through a pathway that requires host DC and $CD4^+$ T cells and that the continued generation of active NK cells resists the establishment of metastases in vivo. Mice immunized with DC and DC/α-GalCer were protected against an i.v. challenge of live B16 tumour cells and fewer metastases developed in mice given DC/α-GalCer versus DC only, but the protection induced by both lasted 12 months even though the mice had not been immunized with B16 melanoma. NK depletion and also the use of IFN-$\gamma^{-/-}$ mice ablated the protection that had been induced by vaccination with either DC or DC/Gal. Sixteen to 24 hours after B16 tumour challenge, CD69 markers of NK and NKT activation were optimally detected and DC, but not B16 melanoma itself, could induce this heightened responsiveness of NK cells to B16 challenge. In addition, NK and NKT cells in DC/Gal-immunized mice responded to syngeneic (B16, EL4 and YAC-1) and allogeneic J558 tumour cells, and produced IFN-γ 16 h later, demonstrating the heightened reactivity of these cells to several tumours in the spleens of DC-vaccinated mice. The authors also showed

that the priming of NKT and NK cells with DC/Gal was longer lasting than with DC, and that mice receiving DC or DC/α-GalCer several months earlier have heightened NKT and NK reactivity long term and systemically to challenge with various tumours. In contrast to DC, tumour cells and B blasts did not induce tumour-reactive NK cells, but cells that would produce IFN-γ after challenge with B16 melanoma. Using appropriate knockout mouse models and depletion experiments, it was shown that the function of both DC and $CD4^+$, but not $CD25^+$ or $CD8^+$ T cells in the recipient mice is required for heightened NKT and NK reactivity to develop. Finally, the long-term NK activation in DC-vaccinated mice does not reflect true memory but rather continuous reactivation for many months in mice that are immunized with DC or DC/α-GalCer [55].

2.3.2 Clinical Experience with Glycolipid-Loaded DC

2.3.2.1 α-GalCer-Pulsed Immature Monocyte-Derived DC

In a phase I dose escalation study, patients with advanced non-small cell lung cancer or recurrent lung cancer received i.v. injections of α-GalCer-pulsed DC (level 1: $5 \times 10^7/m^2$; level 2: $2.5 \times 10^8/m^2$; and level 3: $1 \times 10^9/m^2$) to test the safety, feasibility, and clinical response. Patients were given four i.v. injections on days 1 and 8 in a two-course treatment protocol. The phenotypes of APC containing DC prepared for each administration were analyzed by flow cytometry for each administration and in all preparations the DC-rich population showed an immature monocyte-derived DC phenotype expressing HLA-DR, CD11c, CD80, and CD86. The administered cells contained substantial numbers of $CD3^+$ cells in addition to $CD3^-$ cells. No major (above grade 2) toxicity or severe side effects were observed in any patient. The frequency of peripheral blood NKT cells in all patients was measured by FACS analysis, and one patient in the level 3 group showed dramatic increase in the circulating NKT cell number after the first and second α-GalCer-pulsed DC administration. In this patient, the absolute numbers of Vα24 NKT cells decreased transiently to a nadir around 1–2 days after the α-GalCer-pulsed DC injection, and subsequently increased >20-fold 3 days after second α-GalCer-pulsed DC injection. The increased levels were sustained for at least 1 week. This sharp fluctuation, however, could not be detected after the third and fourth α-GalCer-pulsed DC injection. The number of peripheral blood NKT cells from the other two patients in the level 3 group increased only after the first α-GalCer-pulsed DC administration, and in the remaining six cases in the level 1 and level 2 groups, no clear relationship was found between the number of circulating NKT cells and α-GalCer-pulsed DC administration. In addition, IFN-γ production in Vα24 NKT cells increased following the administration of α-GalCer-pulsed DC in the one case in which the number of circulating NKT cells changed strikingly, but after the third and fourth administration of aGalCer-pulsed DCs, no obvious elevation in the level of IFN-γ production was detected. There were no cases of complete response or partial response, five

cases of no change, and four cases of disease progression. Three patients receiving dose level 3 were followed up for 23–26 weeks after the clinical trial period and all were classified as no change. This clinical trial demonstrated α-GalCer-pulsed DC administration was well tolerated and was safe even in patients with advanced disease [56].

2.3.2.2 α-GalCer Loaded Monocyte-Derived Mature DC

Chang et al. have investigated i.v. injection of monocyte-derived mature DC that were loaded with α-GalCer in five patients who had advanced cancer. All patients received an initial i.v. injection of five million unpulsed monocyte-derived mature DC, followed at monthly intervals by two additional injections of mature DC that were pulsed with α-GalCer. None of the patients had detectable circulating NKT cells at baseline, probably due to extensive therapy of the underlying malignancy. The injection of unpulsed DC did not lead to an increase in NKT cells in any patient at any time point. In contrast, the injection of α-GalCer-pulsed DC led to >100-fold increase in circulating NKT cells in all patients. The numbers of NKT cells stayed above baseline for >84 days in all patients, and were elevated above baseline for >6 months in two patients with longer follow-up. In one patient who had myeloma, sustained expansion (for >3 months after vaccination) of invariant NKT cells was found in the marrow tumour bed before and after DC vaccination. Thus, injection of α-GalCer-loaded DC led to a sustained increase in NKT cells in blood and the tumour bed. To assess changes in antigen-specific T cells, viral antigen-specific T cells against influenza matrix protein and CMVpp65 were monitored by Elispot and MHC tetramers. An increase in CMVpp65-specific, but not influenza matrix peptide (Flu-MP)-specific IFN-γ producers in fresh peripheral blood mononuclear cells (PBMCs) was observed in three of four patients who were tested following the injection of α-GalCer-pulsed, but not unpulsed DC and was associated with a significant increase in CMV-specific memory T cells—but not Flu-MP-specific T cells—in all three individuals. One of the patients received an inactivated influenza vaccine as a part of routine care, shortly after the injection of α-GalCer-loaded DCs, and there was significant expansion of Flu-MP-specific, IFN-γ-producing, and memory T cells in this patient which was consistent with enhancement of vaccine-induced immune response with α-GalCer-pulsed DCs [57].

2.3.2.3 α-GalCer-Pulsed IL-2/GM-CSF-Cultured PBMCs Containing DCs

A phase I–II study of α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC administration in patients with advanced non-small cell lung cancer (NSCLC) was conducted to evaluate the safety, immunological responses, and clinical outcomes. A substantial number of $CD3^+$ T cells and $CD3^-$ cells in the IL-2/GM-CSF-cultured PBMCs expressed HLA-DR, CD11c, and CD86. For the 17 patients

who completed the study, the treatment was well tolerated; all abnormality of laboratory findings data were within the criteria of grade I, and no cases needed an additional treatment. Immunological assays were performed for 17 patients who completed the course of four i.v. aGalCer α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC injections. The frequency of peripheral blood $V\alpha24^+V\beta11^+$NKT cells and $CD3^+CD56^+$NK cells was measured by flow cytometry analysis and six patients showed a dramatic increase (twofold or greater) in the circulating NKT cell number after the first, second, and third α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC administration. In the remaining 11 cases, no obvious increase was found in the number of circulating NKT cells and high values of baseline NKT cell number did not always correlate with the augmentation of peripheral blood NKT cell numbers. The absolute number of Vα24 NKT cells increased at various ranges, up to 21.1-fold and in three cases that showed expanded NKT cell number, the proportion of $CD4^+$NKT cells appears to be reduced in vivo after stimulation with α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC while in one case the percentage of $CD8^+$NKT cells were increased. The number of IFN-γ producing cells in PBMC was assessed in vitro by an ELISPOT assay after restimulation with α-GalCer. In ten patients, the number of cells with IFN-γ production increased more than twofold after the administration of α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC (good responders) and in the remaining seven patients, a minimal alteration of IFN-γ producing capacity was observed (poor responders). IFN-γ production in the patient PBMC in the ELISPOT system was due to both $CD56^+CD3^-$ NK cells and $CD3^+CD56^+$NKT cells. After the injection of α-GalCer-pulsed IL-2/GM-CSF cultured PBMC, the estimated median survival time (MST) of the 17 cases was 18.6 months (range, 3.8–36.3 months). Ten patients who displayed increased IFN-γ producing cells (≥ twofold) showed prolonged MST (31.9 months; range, 14.5–36.3 months) as compared with poor-responder patients (n =7) MST (9.7 months; range, 3.8–25.0 months) [58].

2.3.2.4 α-GalCer-Pulsed Antigen-Presenting Cells

A phase I study evaluated the safety and feasibility α-GalCer-pulsed antigen-presenting cells (APC) treatment administered in the nasal submucosa of patients with head and neck cancer. Nine patients with unresectable or recurrent head and neck cancer received two treatments 1 week apart, of 1×10^8 of α-GalCer-pulsed autologous APC into the nasal submucosa. During the clinical study period, no serious adverse events (Common Terminology Criteria for Adverse Events version 3.0 greater than grade 3) were observed. After the first and the second administration of α-GalCer-pulsed APC, an increased number of NKT cells was observed in four patients and enhanced natural killer activity was detected in the peripheral blood of eight patients. The administration of α-GalCer-pulsed APC into the nasal submucosa was found to be safe and induce antitumour activity in some patients [59].

2.4 Combination Therapies with Glycolipids and Other Adjuvants

2.4.1 α-GalCer and TLR Ligands

Some studies have highlighted the potential for manipulating the interactions between TLR ligands and *i*NKT cell activation in the design of effective vaccine adjuvants.

When wild-type mice were administered OVA protein together with α-GalCer and MPL, this combination acted synergistically to induce expansion of $OVA_{257–264}$-specific $CD8^+$ T-cell responses measured in the blood using MHC class I/peptide tetramers. DC maturation was examined in the spleens of animals treated i.v. with the two compounds and correlated with significant increased expression of the costimulatory molecules CD80 and CD86, and MHC molecules showing that microbial signals and *i*NKT cell-mediated signals can be coordinated to modulate DC-induced T-cell immunity [60].

In another study of Hermans et al., the combination of α-GalCer and MPL examined had a synergistic effect on the induction of $CD8^+$ T-cell responses to OVA in wild-type animals, as measured in the blood using H-$2K^b$/$OVA_{257–264}$ tetramers. The OVA-specific $CD4^+$ T-cell responses and OVA-specific IgG were also strongest with the combination of α-GalCer and MPL. DC isolated from animals treated with the combination of α-GalCer and MPL provided the strongest stimulus to T cells. Antigen-specific $CD8^+$ T-cell responses induced in the presence of the MPL and α-GalCer showed faster proliferation kinetics and increased effector function than those induced with either ligand alone. Whereas the primary $CD8^+$ T-cell responses induced in the presence of α-GalCer and MPL was shorter lived than those induced with either ligand alone, the combination treatment did not impair the capacity of these responses to be restimulated. In addition, when human DC and human *i*NKT cells were combined in vitro, the levels of costimulatory molecules were dramatically enhanced in the presence of both α-GalCer and MPL compared with either stimulus alone, suggesting that cooperative action of TLR ligands and *i*NKT cells on DC function applies across species [61].

2.4.2 α-GalCer and Quil A

Using the HCV soluble E2 envelope glycoprotein (sE2), a major target for HCV neutralizing antibodies, it was shown that combinations of QuilA and α-GalCer adjuvants can act antagonistically. In BALB/c mice immunized with purified sE2 alone or in combination with various adjuvants that act through distinct molecular mechanisms, sE2 combined with MPL or CpG ODN elicited statistically higher levels of IgGs than the protein alone, whereas adjuvanting with α-GalCer did not significantly boost antibody titres. Immunization with sE2/QuilA + CpG ODN

significantly enhanced overall antibody titres compared to sE2/QuilA, but the combination of sE2, QuilA and α-GalCer resulted in a significant decrease of total and E2-specific IgG levels when compared to sE2/QuilA indicating that combinations of QuilA and α-GalCer adjuvants act antagonistically. In an experiment analyzing the ability of sera from mice immunized with sE2 and a combination of QuilA and CpG ODN, or QuilA and α-GalCer to neutralize HCV pseudoparticles (HCVpp) entry, the combination of E2, QuilA and CpG ODN elicited significantly higher mean half-maximal neutralizing titres (NT50) while no differences in neutralization ability were observed between sera derived from sE2/QuilA and sE2/QuilA + α-GalCer immunized mice, and the overall NT50 values were lower in this set of immunizations [62].

2.4.3 Combination of DC Immunization with α-GalCer

The combination of DC immunization with *i*NKT cell activation to enhance antitumour $CD8^+$ T-cell responses induced by immunization with antigen-loaded dendritic cells (DCs) was assessed in animals treated with anti-CD25 antibody to inactivate regulatory T cells (Treg) that might be triggered by cytokines released by *i*NKT cells. Combining DC immunization with *i*NKT cell activation was found to significantly enhance antitumour activity that was associated with a prolonged proliferative burst of responding $CD8^+$ T cells, suggesting that inactivating regulatory T cells and eliciting *i*NKT cell activation can improve antitumour immunization with antigen-loaded DCs [63].

2.5 α-GalCer Bound to Soluble CD1d

Stirneman et al. have reported a noninvasive strategy to induce a sustained activation of *i*NKT cells and to promote their activation at the tumour site using α-GalCer-loaded recombinant mouse soluble CD1d molecules (sCD1d) (α-GalCer/sCD1d) or fused to an antitumour antibody (anti-HER2) to confer tumour localization properties to the α-GalCer/sCD1d. The sCD1d–anti-HER2 fusion protein specifically bound to HER2-expressing tumour cell lines, such as the murine B16 melanoma cell line stably transfected with the human HER2 antigen and the naturally HER2-expressing human breast carcinoma cell line SK-BR-3. The model of experimental lung metastasis induced by the i.v. injection of the B16 melanoma cell line was used to test the antitumour activity of the recombinant CD1d complexes. As a first approach, B16 melanoma cells, wild type or stably transfected with the human HER2 antigen were preincubated with equimolar amounts of either α-GalCer alone or the α-GalCer/sCD1d–anti-HER2 fusion or the intact anti-HER2 mAb, before being injected i.v. into naive mice. Co-injection of

α-GalCer with the tumour cells completely inhibited tumour development, whether or not the tumour cells expressed the HER2 antigen. In contrast, the α-GalCer/sCD1d–anti-HER2 fusion inhibited tumour growth only when HER2 was expressed on the tumour cells and the intact anti-HER2 mAb did not inhibit lung metastases of B16-HER2 tumour cells, indicating that the antitumour effect of the bound fusion protein was *i*NKT cell mediated. In these preincubation settings, the antitumour activity of the sCD1d–anti-HER2 protein was not superior to the already optimal effect of free α-GalCer coinjected with tumour cells. However, the selective effect on HER2-expressing tumour cells provided evidence that the sCD1d–anti-HER2 fusion protein targeted on HER2-expressing cancer cells can redirect *i*NKT cells to the tumour site. In systemic treatments started at different time points after the injection of B16-HER2 melanoma cells, the mice were injected five times i.v. every 3–4 days. Free α-GalCer, if injected 2 or 6 days after tumour graft, had no significant antitumour effect on the development of lung metastasis while systemic treatment with equimolar amounts of the α-GalCer/sCD1d–anti-HER2 fusion protein, when started 2 days after tumour graft, had a potent antitumour effect against lung metastasis and established s.c. tumours even when treatment was delayed until 6 days after injection of the tumour cells. Importantly, the α-GalCer/sCD1d–anti-HER2 fusion protein was unable to block tumour growth in $CD1d^{-/-}$ mice, demonstrating the essential role of *i*NKT cells in mediating this antitumour effect.

2.6 Oral and Intranasal Administration of α-GalCer

The effectiveness of co-administering α-GalCer as an adjuvant with a CTL-inducing HIV envelope peptide, via either the oral or intranasal route, to prime antigen-specific immune responses in multiple systemic and mucosal compartments has been analyzed. Mice were immunized by the intranasal or oral route one to three times at 5-day intervals, with a combination of the CTL inducing R15K peptide at 100 μg/mouse/dose and the synthetic glycolipid α-GalCer at 2 μg/mouse/dose. Both intranasal and oral immunization of peptide antigens ad-mixed with α-GalCer induced antigen-specific CTL responses systemically and antigen-specific IFN-γ producing cells both systemically and in various mucosal compartments including the gut lymph nodes. Delivering a third dose of adjuvant–antigen mixture by the i.n. route induced strong peptide-specific CTL response in the spleen, at comparable levels to that seen after the two-dose immunization scheme. Additionally, peptide-specific IFN-γ producing cells were observed in the spleen as well as MLN demonstrating that administration of up to three doses of free α-GalCer via the i.n. or oral routes does not hinder the induction of antigen-specific T-cell responses, and multiple doses were in fact beneficiary to induce antigen-specific T-cell responses in the various mucosal tissues [64].

2.7 Analogues of α-GalCer with C Glycosidic Linkage

Candidate derivatives of α-GalCer with selectivity towards either Th1 or Th2 cytokines have been extensively explored. The C-glycoside analogue α-GalCer with a C-glycosidic bond between galactosyl moiety and ceramide (replacement of the glycosidic O with C) remained highly active.

A synthetic α-C-galactosylceramide (α-C-GalCer) has been shown to act as a natural killer T-cell ligand in vivo, and stimulated an enhanced Th1-type response in mice. In two disease models requiring Th1-type responses for control, namely malaria and melanoma metastases, α-C-GalCer exhibited a 1,000-fold more potent antimalarial activity and a 100-fold more potent antimetastatic activity than α-GalCer. Moreover, α-C-GalCer consistently stimulated prolonged production of the Th1 cytokines interferon-γ and interleukin (IL)-12, and decreased production of the Th2 cytokine IL-4 compared with α-GalCer [65, 66].

2.7.1 α-C-GalCer as Adjuvant for Malaria and Tumour Vaccines

In wild-type (WT) and IL-12-deficient mice treated with equal doses of glycolipid 3 days before challenge with sporozoites, α-C-GalCer suppressed liver stage development to a much greater degree than α-GalCer; in IL-12-deficient mice the antimalarial activity of both glycolipids was totally abolished showing that IL-12 is a key factor not only driving α-C-GalCer's superior antimalarial effect, but also mediating the antiplasmodial effect of both glycolipids. However, the ability of α-C-GalCer to better inhibit liver stages was the same in WT mice and in IL-4- and IL-10-deficient mice, indicating that α-C-GalCer's superior antimalarial activity does not involve IL-4 and IL-10 production.

To assess the role of NK cells in α-GalCer- and α-C-GalCer-mediated protection against malaria, mice were depleted of NK. In nondepleted control mice, α-C-GalCer exhibited better antimalarial activity than did α-GalCer, as expected, while α-C-GalCer's superior antimalarial activity in mice depleted of NK cells was abrogated. Hence, α-C-GalCer's enhances IL-12 production, which then triggers NK cells to produce more IFN-γ needed to suppress malarial liver stage development. Given the importance of DCs in the in vivo physiological response to *i*NKT ligands, the ability of α-GalCer and α-C-GalCer to induce maturation of DCs was investigated. The first marker to show up-regulation on CD11c+ DCs splenocytes after injection of either glycolipid was MHC class II. α-GalCer-treated mice showed increased expression of this marker 2 h after injection, while α-C-GalCer-treated mice showed increased expression 6 h after injection, and by 24 h post-treatment, α-GalCer and α-C-GalCer-treated mice expressed the highest MHC class II expression on CD11c+ DCs. In α-GalCer-treated mice, CD86 expression first increased at 6 h post-injection; whereas in α-C-GalCer-treated mice this marker did not show increased expression until 24 h post-injection. The highest expression of CD86 was found at 24 h after injection, and α-GalCer-treated mice expressed more marker

than α-C-GalCer-treated mice. In both α-GalCer- and α-C-GalCer-injected mice, increased expression of CD40 was only observed 24 h after treatment, at similar levels for both glycolipds, in contrast to MHC class II and CD86. Overall, the up-regulation data indicated that α-GalCer induces a faster maturation of CD11c+ DCs than does α-C-GalCer, which appears to induce a more prolonged maturation of this cell type.

When analyzing proliferation of NKT cells, Vα14i NKT cells from α-GalCer-treated mice rapidly downregulated their TCRs, becoming undetectable in both spleen and liver by 5 h, and remaining so at 24 h. At 48 h, Vα14i NKT cells from α-GalCer-treated mice become detectable again in both spleen and liver, and returned to levels comparable to that detected prior to injection until 168 h post-injection. In contrast, Vα14i NKT cells from mice treated with α-C-GalCer exhibited a slower and shorter TCR downregulation, with small percentages of cells still present at 5 h in both spleen and liver. At 24 h the cells were almost completely undetectable, but started reappearing at 48 h, although at lower levels than that detected prior to injection. α-C-GalCer stimulated a far greater expansion of Vα14i NKT cells in both spleen and liver than did α-GalCer and the difference was most striking at 72 h; in the spleen, the percentage of cells was approximately ten times higher than that observed at the start; similarly, in the liver the percentage was about three times higher. At 120 h, the levels of Vα14i NKT cells started to come down in both the spleen and liver, but still remained higher than that observed prior to injection. By 168 h, the percentages continued to come down, approaching baseline in the liver, but remaining high in the spleen. Overall, this data indicated that despite its poor ability to stimulate cytokine production by NKT cell, α-C-GalCer was a better in vivo stimulus for Vα14i NKT cell proliferation than α-GalCer, and the TCRβ usage did not affect in vivo Vα14i NKT cell activation stimulated by α-GalCer or α-C-GalCer [65, 66].

2.7.2 α-C-GalCer Loaded DCs for Antitumour Therapy

α-C-GalCer was shown to be a more active inducer of the innate production of cytokines than the prototype α-GalCer, and the innate response was qualitatively different. Graded doses of α-GalCer or α-C-GalCer were administered i.v. and the innate response was monitored in terms of elevations of serum cytokines. For induction of IFN-γ, the response to α-GalCer began to decrease at 20 ng per mouse, whereas for α-C-GalCer, 2 ng was the limiting dose. Another distinction was that α-C-GalCer induced more prolonged production of IFN-γ and higher levels of IL-12 but did not induce IL-4 or TNF-α. It was also shown that IL-12 and IFN-γ were produced by distinct CD11c+ cells in spleen, the former by costimulatory DCs and the latter by NK cells activated by the DC–NKT interaction.

In addition, α-C-GalCer proved to be more potent than α-GalCer, either as a free drug or pulsed onto DCs, as in vivo primed NKT cells with either free glycolipid or glycolipid-loaded DCs secreted more IFN-γ or IL-4 as measured 2 days following iv

administration. α-C-GalCer loading onto DCs required just 2 h contrary to α-GalCer which required 12 h, and expanded NKT cells much greater than α-GalCer as measured 5 days following i.v. administration into mice, indicating that binding of α-C-GalCer to DCs was more stable than binding of α-GalCer.

A standard assay in which the establishment of metastases of MHC-class I low B16 melanoma cells is retarded was used to test the in vivo efficacy of the innate response. Lung metastases were evaluated 2 weeks after i.v. co-administration of DCs loaded with either glycolipid together with B16 melanoma cells. The α-C-GalCer-loaded DCs were more effective than α-GalCer-loaded DCs in reducing the size and the number of lung metastases, and depletion experiments indicated that both NKT and NK cells were contributing to the resistance induced by α-C-GalCer-loaded DCs.

Furthermore, 20 ng of α-C-GalCer was more potent adjuvant than α-GalCer for the presentation of cell-associated antigens in mice and adaptive resistance to tumour cells. Mice that were given irradiated J558 tumour cells and either 20 ng of α-C-GalCer or 2 μg of α-GalCer, but not 20 ng of α-GalCer, developed resistance to challenge with MHC class I positive J558 tumour cells. Only α-C-GalCer up-regulates CD40L expression on NKT cells even though both glycolipids induced high levels of CD86 costimulatory molecules on DCs, demonstrating that improved adjuvant function correlated with CD40L up-regulation on the NKT cells and presentation of cell-associated antigens, but not on up-regulation of CD86 [67].

2.7.3 α-C-GalCer as Adjuvant for a Live Attenuated Influenza Virus

The adjuvant activity of α-C-GalCer was tested with a live attenuated influenza virus vaccine containing an NS1 protein truncation (rNS1 1–73 virus). To determine whether the adjuvant increases protection of the vaccine, BALB/c mice were vaccinated with either 10^2, 10^3, or 10^4 PFU of the rNS1 1–73 virus with 0, 1, 2, or 4 μg of the α-C-GalCer. While all of the mice vaccinated with 10^2 PFU of the rNS1 1–73 virus without adjuvant died after challenge, 80% of mice vaccinated with 10^2 PFU of the rNS1 1–73 virus with 1 μg adjuvant survived, indicating that the adjuvant can increase the protection of the vaccine and reduce mortality due to influenza virus challenge. Increasing the amount of adjuvant did not further increase the protection of the vaccine. Most of the mice vaccinated with 10^2 PFU of the rNS1 1–73 virus with adjuvant had more of both subtypes of IgG, IgG1 and IgG2a antibodies. The adjuvant increased the number of influenza virus-specific $CD8^+$ T cells that recognize influenza virus peptides in mice vaccinated with both the 10^2 and 10^3 PFU of the rNS1 1–73 viruses. In addition, $CD1d^{-/-}$ mice vaccinated with the virus and adjuvant did not have detectable levels of IFN-γ in their sera, consistent with the lack of NKT cells in these animals. Moreover survival after challenge was similar in $CD1d^{-/-}$ mice vaccinated with and without adjuvant indicating that the adjuvant increases immunogenicity and enhances protection of the live attenuated influenza virus vaccine only in wild-type mice in which NKT cells can be stimulated [68].

However, α-C-GalCer was found to be a rather weak ligand against human *i*NKT *cells* in vitro. Therefore, several C-glycoside analogues have been synthesized and some studies have identified α-C-GalCer analogues that display a strong stimulatory activity not only in mice but also in human *i*NKT cells, providing new therapeutic tools that can be used as vaccine adjuvants in humans [69]. The adjuvant activity of these α-C-GalCer analogues has yet to be evaluated in preclinical models.

2.8 Analogues of α-GalCer with Non Glycosidic Linkages

Some studies described chemical synthesis and immunological characterization of a number of nonglycosidic α-GalCer analogues in which galactose, the focal point of *i*NKT TCR recognition, is substituted with nonglycosidic variants. The synthetized compounds were functional and induced lower levels of cytokines than did α-GalCer, induced selective expansion and activation of *i*NKT cells, allowing the identification of analogues with clinically more desirable features than α-GalCer. The CD1d-binding, lipid-comprising ceramide, ether linked to sugar alcohols with four carbons (threitolceramide, referred to as ThrCer), activated both human and mouse *i*NKT cells as defined by the maturation of human and mouse DCs, the expansion of human *i*NKT cells in vitro, and in vivo secretion of IFN-γ and IL-4. Activation of human *i*NKT cells by ThrCer-pulsed DCs, while inducing DC maturation as defined by IL-12 production and IFN-γ secretion, ensured a greater proportion of live DCs as compared with the DC survival after the activation of human *i*NKT cells by α-GalCer. Immune responses in mice injected i.v. with 1 μg ThrCer and 400 μg OVA were comparable to those seen with α-GalCer, and OVA-specific T cells in mice injected with OVA and ThrCer rejected E.G7-OVA tumour cells compared to control groups. In contrast to *i*NKT cells stimulated in vivo with α-GalCer, *i*NKT cells stimulated with ThrCer in vivo were initially refractory to a subsequent challenge with α-GalCer in vitro, but recovered the ability to produce IFN-γ after 14 days. These results indicated that the unresponsiveness of *i*NKT cells after their in vivo stimulation with ThrCer was shorter lived than *i*NKT cell unresponsiveness caused by in vivo stimulation with α-GalCer, a property that may have advantages for the use of ThrCer in vaccination strategies. This compound is now in development for clinical evaluation as adjuvant in vaccines against cancer and infectious diseases [70, 71].

2.9 Analogues of α-GalCer with Phytosphingosine and Fatty Acid Chains of Varying Length and Saturation

2.9.1 α-GalCer Analogues with Branched Acyl Chains

Two α-GalCer analogues, KBC-007 and KBC-009, that have different branched chain lengths were prepared and evaluated for their efficacy as nasal influenza vaccine

adjuvants. These analogues displayed improved solubility over α-GalCer and potently stimulated NKT cells in both murine and in vitro human systems. Examination of serum cytokines in vivo revealed that these analogues elicited different cytokine release profiles compared to α-GalCer. KBC-009 induced both Th1/Th2 cytokines, whereas KBC-007 induced a more Th2-polarized cytokine response with diminished IFN-γ production. The adjuvant efficacy of these α-GalCer analogues were evaluated with a nasal influenza vaccine. BALB/c mice were immunized i.n. with inactivated A/PR8 (1 μg) alone or with 0.5 μg of either α-GalCer or α-GalCer analogues, then anti-PR8 IgG and anti-PR8 IgA titres were measured in the serum, lung and nasal washes after immunization. Co-immunization with α-GalCer or analogues resulted in increased IgG1/IgG2a ratios (>1) relative to immunization with inactivated PR8 alone (0.63), indicating induction of Th2-polarized antibody responses. To investigate antibody responses in the mucosal compartment, PR8-specific IgG and IgA were measured in lung and nasal washes. Immunization with KBC-009 induced significantly stronger PR8-specific IgA and IgG responses in lung washes and IgG responses in nasal washes than those observed in mice immunized with A/PR8 alone. The ability of KBC-009 to stimulate mucosal immune responses was comparable to that of α-GalCer. Analysis of cellular immune responses showed that mononuclear cells (MNC) from spleens and mediastinal (MdLNs) of all mice co-immunized with α-GalCer analogues exhibited much more pronounced proliferative responses to antigen restimulation compared to MNCs from mice immunized with PR8 alone. Consistent with the increased antigenicity, just as immunization with α-GalCer caused complete protection from live virus infection, adjuvantation with KBC-009 also completely protected mice from infection. In contrast, KBC-007 did not improve virus clearance over treatment with inactivated PR8 alone, indicating that co-administration of the α-GalCer analogue KBC-009 with intranasal vaccination can generate significant protective immune responses against live virus infection. These results indicated that α-GalCer analogues, especially KBC-009, significantly increase cell-mediated immunity as measured by Ag-specific lymphocyte proliferation, cytokine production and CTL activity against virus-infected cells. Taken together, the data showed that branched chain-containing α-GalCer analogues exhibit strong stimulatory activity on human *i*NKT cells and potential for clinical application [72].

2.9.2 α-GalCer Analogues with Modification of the Fatty Acyl or Phytosphingosine Chain

In studies by Li et al. [73, 74], a library of 25 synthetic analogues of α-GalCer was generated with analogues in group A having a modification of the fatty acyl chain, whereas analogues in group B have a terminal benzene ring on the phytosphingosine chain. These analogues were then screened to identify glycolipids that can potently activate *i*NKT cells and dendritic cells (DC) and thus display robust adjuvant activity. Based on the level of IFN-γ produced by the glycolipids, nine analogues C11, C18, C22, C23, C24, 6DW116C9, 7DW8-4, 7DW8-5, and 7DW8-6 were

selected as initial candidates for further assessment of their biological activities. Co-cultures of human *i*NKT cells and autologous DCs secreted IFN-γ, IL-4, GM-CSF, and IL-12 in the presence of all of the selected analogues. In particular, C18, C22, C23, 7DW8-5, and 7DW8-6 were shown to induce a significantly higher level of all of the Th1-related cytokines, IFN-γ, IL-12, and GM-CSF, than the parental compound, α-GalCer. Based on the results from this set of assays, five analogues were selected which include C18, C22, C23, 7DW8-5, and 7DW8-6 for further assessment of their biological activities. 7DW8-5 was identified as the lead compound for further adjuvant testing in HIV and malaria diseases.

After selecting 7DW8-5, its adjuvant effect was compared to that of α-GalCer against various HIV vaccine platforms, namely Ad5-p24 and DNA-p24. For this purpose, different doses of each adjuvant were co-administered intramuscularly with a suboptimal dose of either Ad5-p24 or DNA-p24. Two weeks after a single immunizing dose of Ad5-p24 co-administered with each glycolipid, 7DW8-5 was shown to enhance the most the level of p24-specific CD8+, as well as CD4+ T-cell responses that secrete IFN-γ, as determined by ELISpot assay. Similarly, when mice were primed with a DNA-p24 vaccine plus either 7DW8-5 or α-GalCer, and then boosted with the DNA-p24 vaccine alone, 7DW8-5 displayed a stronger adjuvant effect than α-GalCer, eliciting significantly higher p24-specific CD8+ T-cell and humoral responses. To confirm that the adjuvant effect of 7DW8-5 on HIV Ad5-p24 and DNA-p24 vaccines was mediated by CD1d molecule, CD1d-deficient mice were immunized with HIV vaccines co-administered with 7DW8-5 or α-GalCer, and as expected, 7DW8-5 failed to display any adjuvant effect in CD1d-deficient mice immunized with DNA-p24 or Ad-p24.

The adjuvant effect of 7DW8-5 on the efficacy of a malaria vaccine was investigated. For this purpose, mice were immunized with a suboptimal dose of a recombinant adenovirus expressing a *P. yoelii* CS protein, AdPyCS, together with 1 μg of glycolipid, then the level of PyCS-specific $CD8^+$ T-cell response, anti-PyCS antibody response as well as liver parasite burden following challenge with *P. yoelii* sporozoites were determined. 7DW8-5 enhanced the malaria-specific $CD8^+$ T-cell response significantly more than α-GalCer and also enhanced the malaria-specific humoral response equally, or slightly stronger than α-GalCer. Importantly, 7DW8-5 was able to display a significantly stronger adjuvant effect than α-GalCer in enhancing protective efficacy of AdPyCS after a single immunizing dose. In addition 7DW8-5 displayed a dramatic dose sparing effect since co-administration of 1 ng of 7DW8-5 elicited a similar level of PyCS-specific $CD8^+$ T-cell response compared with that induced by 100 ng of α-GalCer. The adjuvant effect of 7DW8-5 is to be evaluated in clinical trial with malaria vaccines [74].

2.9.3 Wittycell iNKT Agonists with Modification in the Polar Head and Acyl Chains

Wittycell (WTC) has developed screens for the identification of *i*NKT agonists and created a library of synthetic ligands, with modifications in the polar head and fatty

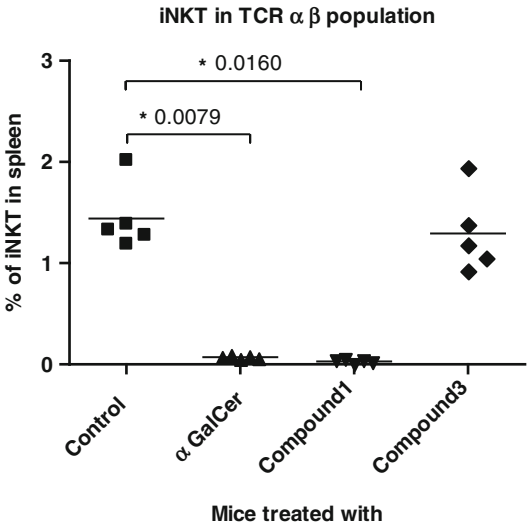


Fig. 2.1 Downregulation of *i*NKT cells in the spleen upon activation with Wittycell glycolipid *i*NKT agonists. Mice were injected with 1 μg WTC glycolipids or α-GalCer or PBS (controls) via the i.v. route. One day later, the spleens were removed and the cells stained with anti-TCRβ and WTC glycolipid/CD1d tetramer. *i*NKT percentages were calculated after flow cytometry analysis. Individual values are shown from a representative experiment with five animals per group

acyl. Compounds 1–4 were selected as initial candidates for further assessment of their biological activities and adjuvant capacities.

To test the capacity of WTC glycolipids to stimulate *i*NKT cells, C57BL/6 mice were injected via the i.v. route with 1 μg of the glycolipids, and the behaviour of *i*NKT cell was monitored in the spleen by CD1d WTC glycolipid-loaded-tetramer staining [75]. *i*NKT cells represented approximately 2% of the total spleen cells prior to glycolipid administration. *i*NKT cell percentage showed significant decrease in groups of mice treated with the prototype ligand αGalCer and WTC compounds. Mice from the group treated with compound 3 (Fig. 2.1) did not show any difference in *i*NKT percentages in spleen compared to vehicle-treated control animals, indicating that not all the glycolipids are potent stimulators of *i*NKT cells. In agreement with previous studies [76–79], *i*NKT cells virtually disappeared from the spleen within 24 h following compounds 1 and 2 administration, indicating that they vigorously expanded.

The ability of the compounds to induce cytokine release was also assessed in vivo. Examination of serum cytokines revealed that these compounds elicitated different cytokine release profile compared to α-GalCer. Compounds 2 and 4 induced both Th1 and Th2 cytokines, whereas compound 1 gave a more Th1 polarized cytokine response with increased IFN-γ production (Fig. 2.2a, b).

The effect of the glycolipids on DC maturation was investigated by analyzing the expression of CD40, CD80, and CD86 costimulatory molecules on the main subsets of myeloid $CD8\alpha^+$ or $CD8^-$ $CD11c^+$ splenic DC, 24 h following i.v. injection of 1 μg WTC glycolipids. A typical experiment showing DC maturation following glycolipid administration via the i.v. route is presented in Fig. 2.3a, b; this experiment shows that injection of 1 μg of compound induced up-regulation of CD40, CD80 and CD86 costimulatory molecules on DC subsets, demonstrating that WTC glycolipids activate *i*NKT cells that provide essential signals to APC to induce adaptive immunity.

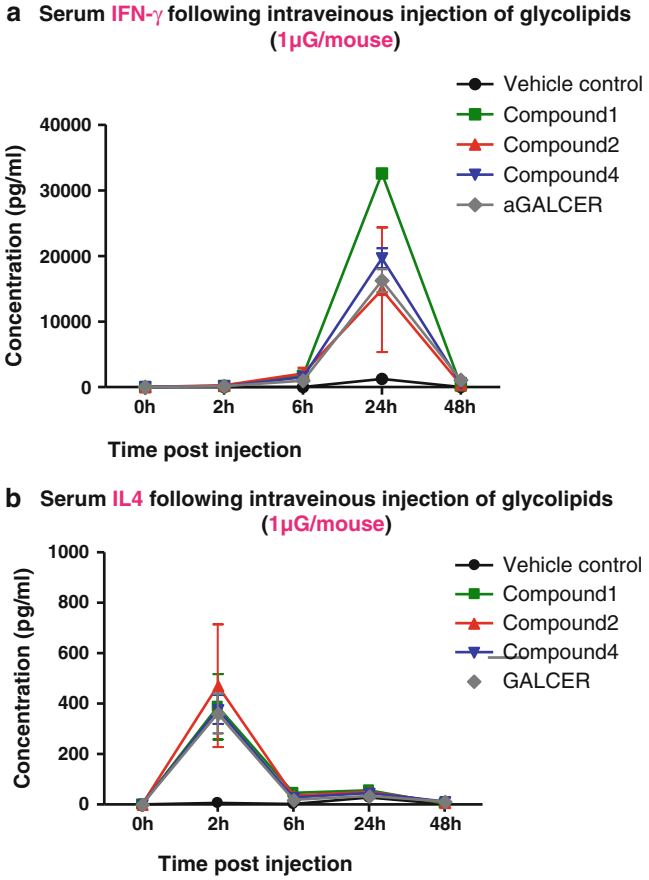


Fig. 2.2 Kinetics of cytokine release into serum following in vivo administration of Wittycell glycolipids *i*NKT agonists. C57BL/6 mice were injected i.v. with 1 μg of WTC glycolipids ($n=3$). Sera were collected at indicated times for analysis of IFN-γ (**a**) and interleukin-4 (IL-4) (**b**) by CBA

The adjuvant capacity of WTC glycolipids was assessed with OVA as a model antigen. MHC class I/peptide pentamers were used to determine the overall generation of SIINFEKL-specific CTL, following i.m. immunization of C57BL/6 mice with WTC compounds and OVA full-length protein. As indicated in Fig. 2.4, responses primed in the presence of WTC glycolipids were significantly higher, yielding an unprecedented up to 25% pentamer positive cells with compound 4 fourteen days following prime.

The adjuvant effect of WTC glycolipids on adaptive antitumour immunotherapy was investigated in mouse models of metastatic lung cancer induced by B16 and B16/OVA tumour cell lines in syngenic immunocompetent C57BL/6 mice. Figure 2.5a, b shows a representative tumour challenge experiment in a prophylactic setting, in which mice were vaccinated with OVA protein in the presence of compound 2 and challenged with 5×10^5 B16-OVA cells or B16 parental cells, 14 days

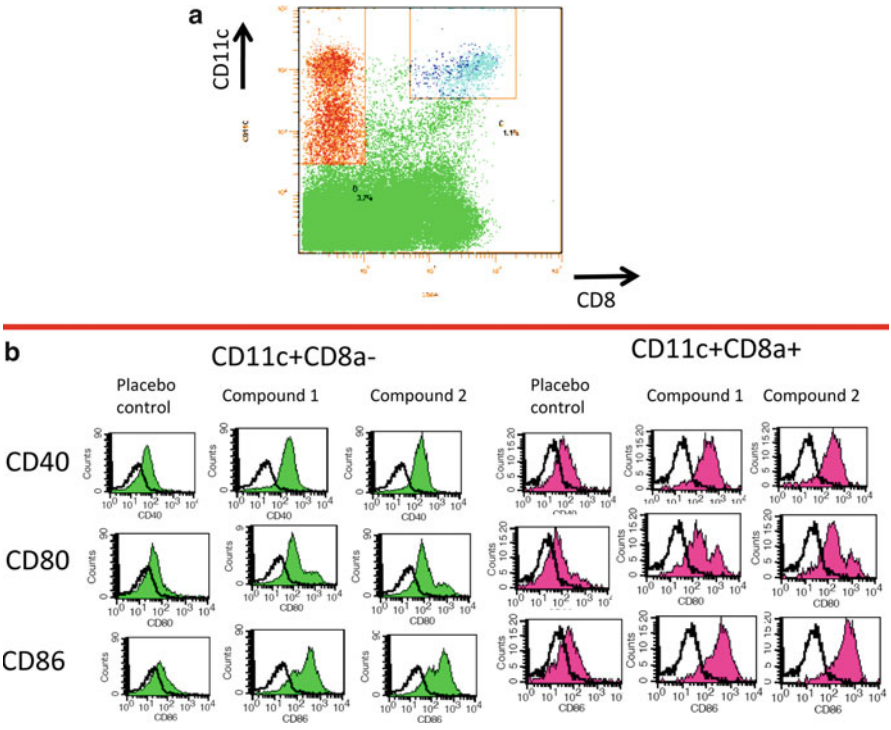


Fig. 2.3 Increased costimulatory molecule expression on CD11c high splenic dendritic cells maturing in response to Wittycell glycolipid *i*NKT agonists. C57BL/6 mice were injected i.v. with 1 μg of glycolipids. Spleens were removed 1 day later for antibody staining and flow cytometry analysis of the expression of CD40, CD80 and CD86 on CD11c dendritic cells. Representatives FACS profiles of CD11c high (**a**); CD40, CD80 and CD86 expression on CD11c high CD8α^{+} or CD11c high CD8α^{-} (**b**)

following i.m. priming. In this metastatic model, tumour metastases develop in the lungs between 20 and 26 days following challenge. Mice were monitored for clinical signs and body weight and were sacrificed when the weight loss was above 20% of the initial body mass, then their lungs were assessed for the presence of tumours. Mice vaccinated with OVA protein and compound 2 developed resistance to challenge with B16/OVA cell line, indicating that compound 2 was a potent adjuvant for specific adaptive antitumour responses. In the treatment setting of antitumour immunotherapy, mice were challenged on day 0 with B16/OVA tumours then injected with WTC glycolipids in combination with OVA protein 7 or 14 days following challenge. Figure 2.5c, d shows a representative experiment with prolongation of survival of mice i.m. treated with compounds 2 and 4 and OVA protein, when the treatment was given until 7 days post challenge. Significant prolongation of survival was also observed with compounds 2 and 4 when the treatment was administered until 14 days post challenge (not shown).

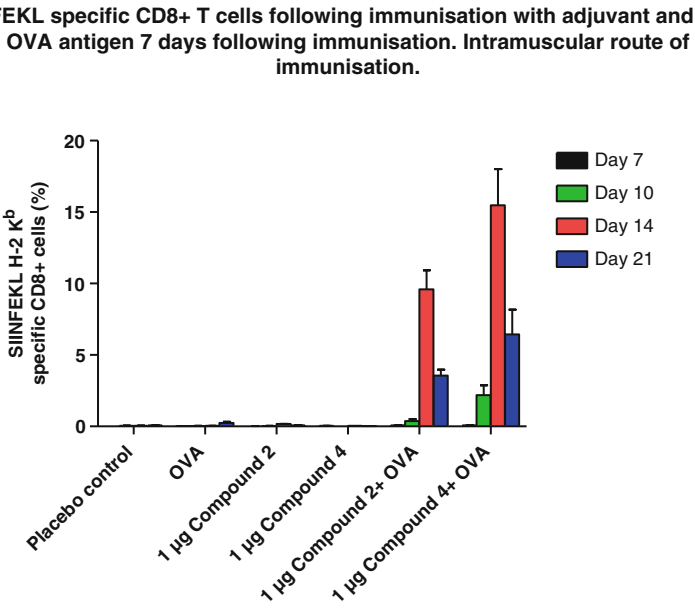


Fig. 2.4 Wittycell glycolipid *i*NKT agonists induce high levels of SIINFEKL/H2Kb pentamer-specific $CD8^+$ T cells following immunization with OVA antigen. Quantitative representation of percentages of SIINFEKL/H2Kb CD8+ specific cells 7, 14 and 21 days after i.m. vaccination with mixtures of 50 μg OVA and glycolipids. Values are the means of six mice ± SD. The experiments have been repeated at least twice, yielding similar results. Negative control animals were treated with placebo (PBS) and yielded pentamers below 0.05% at all times

The adjuvant effect of the WTC compounds were tested in different models and indications such as influenza, genital herpes, hepatitis B, showing enhanced cellular and humoral responses (unpublished data). Wittycell is preparing a phase I clinical trial of its lead compound, compound 4, to evaluate the adjuvant effect in combination with HBsAg, with the primary objective to analyze the safety in humans. GLP toxicity studies demonstrated that the Wittycell lead compound is non-mutagenic and nontoxic following repeated intramuscular administrations in mice and monkeys and there were no histological indications of any systemic effect, with or without antigen.

2.10 Other Synthetic Glycolipids

2.10.1 α-L-Fucosylceramides

Both D- and L-fucose (6-deoxy galactose) are widely found in nature. Of interest, L-fucose is predominantly found in the α-configuration in the lipopolysaccharides

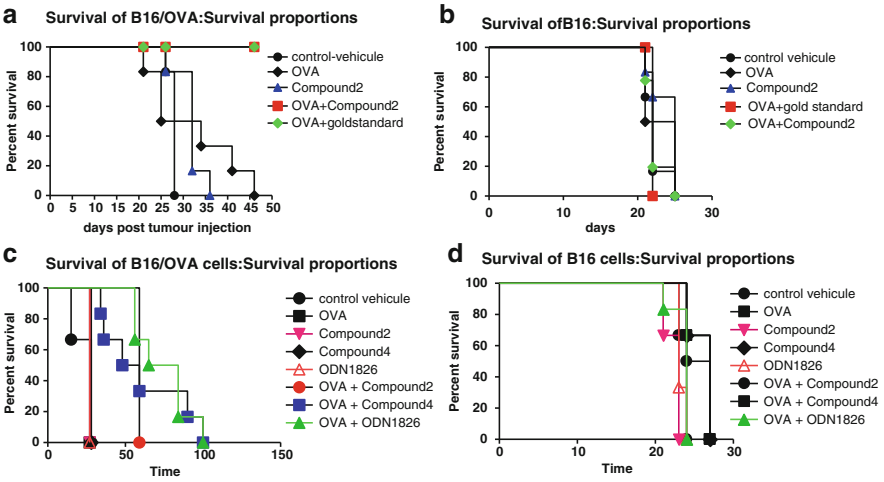


Fig. 2.5 WTC adjuvants induce antitumour immunity following immunization with OVA proteins. Groups of six female C57BL/6 mice were immunized i.m. on day 0 with a mixture of 50 μg OVA protein and 1 μg of the WTC adjuvants or 50 μg CPG as a reference adjuvant. The mice were challenged 2 weeks after vaccination by tail-vein injection of 5×10^5 B16 expressing OVA (B16/OVA) cells or the parental cell line B16. Animals were sacrificed when they began to show signs of disease (typically 24–28 days following tumour challenge) and when the weight loss was above 20% of the initial body mass (**a**, **b**). Groups of six female C57BL/6 mice were challenged on day 0 by tail-vein injection of 5×10^5 B16 expressing OVA (B16/OVA) cells or the parental cell line B16. The mice were immunized 7 days later with a mixture of 50 μg OVA protein and 1 μg of the WTC adjuvants. Animals were sacrificed when they began to show signs of disease (typically 24–28 days following tumour challenge) and when the weight loss was above 20% of the initial body mass (**c**, **d**)

(LPS) of Gram-negative bacteria and animal glycosphingolipids. With the exception of the monohexylceramide α-L-fucosylceramide, most fucosphingolipids are usually ceramide oligosaccharides. Veerapen et al. have developed an efficient method for the synthesis of a series of α-L-fucosylceramides and compared these to α-GalCer (KRN7000), in particular in their ability to induce the expansion of *i*NKT cells in samples of human PBMC during an eight-day in vitro culture. The results showed that both the percentages and absolute numbers of *i*NKT cells in cultures were increased by stimulation with α-L-fucosylceramides with C26:0 > C18:0 (OH) > C24:0. The α-L-fucosylceramide containing a C26:0 fatty acid was the most active of the fucosyl series, and stimulated *i*NKT cell expansions in some donors that approached those seen with the prototype *i*NKT cell agonist KRN7000. In contrast, the α-L-fucosylceramide containing the C20:2 fatty acid was found to lack detectable *i*NKT cell stimulating activity in any of the donors tested [80]. The adjuvant activity of these compounds is yet to be fully explored.

2.10.2 Beta-Linked Glycosylceramides

Thus far, alpha-anomeric D-glycosylceramides have not been detected in mammals. In view of the lack of α-structured GSLs in mammals and the lack of a profound effect of alpha-anomeric compounds when tested in humans with cancers, recent studies have suggested that endogenous β-structured glycosphingolipids may be the potential endogenous ligands for NKT cells [81]. Far fewer studies have characterized beta-linked glycosylceramides. In order to examine the ability of these glycolipids to induce antitumour immunity, BALB/c mice were injected i.v. with syngeneic colon carcinoma CT26 cells, followed by glycolipid administration. A strong protection was induced by β mannosylceramide (β-ManCer) at a low dose of 50 pmol which was similar to protection after treatment with α-GalCer. β-ManCer was shown to be 100-fold more potent than α-mannosylceramide (α-ManCer) and α-GalCer, and all protection was lost in *Jα18−/−* mice, confirming that *i*NKT cells are necessary for β-ManCer-induced protection.

The lack of detectable cytokine production after in vitro stimulation with β-ManCer was also confirmed in vivo (β-ManCer induces a low level of cytokines in vitro with only TNF-α detected at concentrations of less than 30 nM), as there was no substantial increase in IFN-γ, IL-4, IL-13, or TNF-α levels after treatment with 50 pmol β-ManCer and only a modest increase in IL-12, which was still lower than that observed with α-GalCer. While β-ManCer was consistently less potent than α-GalCer, results showed that β-ManCer stimulates NKT cells with a similar Vβ repertoire as α-GalCer. In addition, simultaneous treatment with β-ManCer and α-GalCer resulted in a significant 79% reduction of the median number of tumour nodules, suggesting that α-GalCer and β-ManCer work synergistically to eliminate/prevent CT26 lung metastases [82]. The adjuvant activity of these compounds is yet to be fully explored.

2.10.3 6′-Derivatized α-GalCer Analogues

Aspeslagh et al. generated a series of analogues with aromatic groups connected via different linkages to the C6′ of the galactose group aiming at generating extra hydrophobic interactions. Addition of an aromatic moiety at the 6′-position of the galactose moiety leads to a marked functional Th1 polarization in vivo. Interestingly, the IFN-γ response to these glycolipid-pulsed BMDC was markedly higher compared to α-GalCer, leading to a sustained Th1 bias in vivo. When administered directly at high doses, all of the tested glycolipids prevented tumour growth and were significantly more potent in preventing tumour growth when loaded onto BMDC and adoptively transferred. Importantly, *i*NKT cell recognition by these glycolipids ultimately results in a stronger *i*NKT cell proliferation in mice and human [83]. The adjuvant activity of these compounds is yet to be fully explored.

2.11 Conclusion

The ability of glycolipids to act as vaccine adjuvants has been well established in animal models. α-GalCer has been shown to have a strong adjuvant effect in models of infectious diseases and cancer with various types of vaccines: DNA-based vaccines, peptide-based vaccines, cell-based vaccines or live vectors. In addition glycolipids are effective adjuvants via both systemic and mucosal delivery. A large number of analogues of α-GalCer have been screened by different groups, in an attempt to improve on its biological and physico-chemical properties. Amongst these, compounds have been identified with decreased or increased efficacy, and with modified activity or formulation characteristics. Different analogues have been shown to bias responses towards either Th1 or Th2 responses in animal models. Also, combinatorial adjuvant approaches such as α-GalCer and TLR ligands can provide stronger stimulus to T cells, although not all adjuvant combinations have proved useful, at least under the conditions tested.

α-GalCer has been tested in several clinical trials, principally in a non-vaccine setting. Clinical administration of the free drug α-GalCer or α-GalCer-pulsed APC was well tolerated. Evidence for *i*NKT cell activation and innate immune responses has been obtained in these trials. α-GalCer induced more potent *i*NKT cell responses when loaded on mature DCs compared to immature DCs or when used as a free drug in the clinic.

Analogues of α-GalCer have not yet been tested in combination with vaccine antigens in the clinic. Further clinical trials are in preparation to assess the safety and efficacy of vaccination strategies targeting *i*NKT with glycolipids in humans.

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Chapter 3
Mucosal Vaccination: Opportunities and Challenges

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3.1 Introduction

Mucosal vaccination has been the common generic name attributed to the oral, intranasal, pulmonary, rectal, and vaginal routes of vaccine administration. Mucosal surfaces, with a combined surface area of about 400 m^2 [1], are undoubtedly the major site of entry for most pathogens. Therefore, these vulnerable surfaces are associated with a large and highly specialized innate and adaptive mucosal immune system that protects the surfaces and the body against potential destructive agents and harmless substances from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immune cells [2]. These immune cells accumulate in a particular mucosa or circulate between various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system [1]. In theory, mucosal surfaces seem to be the more accessible lymphoid organ for the induction of an immune response such as that required for immunization. Nevertheless, one of the more important reasons for the development of mucosal vaccines is the increasing evidence that local mucosal immune responses are important for protection against disease, principally for diseases which start on mucosal surfaces such as the respiratory, gastrointestinal, or urogenital mucosae. On the other hand, mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, while injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces. However, even

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with the many attractive features of mucosal vaccination described, it has often proven difficult in practice to stimulate strong sIgA immune responses and protection by mucosal antigen administration [2]. As a consequence, no more than half a dozen mucosal vaccines are currently approved for human use and no subunit vaccines are listed among those approved.

3.2 Anatomophysiology of the Mucosal Immune System

MALT includes the gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), the mammary and salivary glands, and the urogenital organs. The common mucosal immune system (CMIS) acts as an integrated pathway that establishes communication between the organized MALT (inductive sites) and the diffuse mucosal tissues (effector sites). However, there is some evidence supporting the theory that this CMIS is compartmentalized. For instance, stimulation at one mucosal site in MALT can induce an immune response at remote mucosal effector sites [3, 4]. However, the extent of the immune response at the effector sites depends on where the induction occurred. Holmgrenn and Czerkinsky recently summarized this phenomenon in this way: "Oral immunization may induce substantial antibody responses in the small intestine (strongest in the proximal segment), ascending colon and mammary and salivary glands and it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestines, tonsils or female genital tract mucosa. Conversely, intranasal immunization in humans results in antibody responses in the upper airway and cervicovaginal mucosa, and regional secretions (saliva, nasal secretions) without inducing an immune response in the gut" [2]. Important evidences that may explain, at least in part, the dependence of the mucosal site where the IgA is generated on the route of antigen administration were recently summarized by Kiyono [5].

3.2.1 Gut-Associated Lymphoid Tissue

The GALT described elsewhere [1] lines the digestive system and has two organizational levels to its structure: one with little organization, characterized by loose clusters of lymphoid cells in the lamina propria of the intestinal villi, and the other with a high level of organization called Peyer's patches.

The so-called intraepithelial lymphocytes (IELs) can be found in the outer mucosal epithelial layer, and the majority of these cells are CD8+ T-lymphocytes. Due to its localization, it is thought that this population of T cells may function to encounter antigens that enter through the intestinal mucous epithelium. Under the epithelial layer is the lamina propria, which contains large numbers of B cells, plasma cells, activated T_H cells, and macrophages in loose clusters. It is interesting to note that in healthy children, histological sections of the lamina propria have revealed more than 15,000 lymphoid follicles in total (described in [1]).

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain between 30 and 40 lymphoid follicles organized as macroscopic nodules or aggregates. In a similar way to what happens with lymphoid follicles in other sites, those from mature Peyer's patches can develop into secondary follicles with germinal centers, supported or connected by follicular dendritic cells.

Parafollicular T-lymphocyte zones located between the large B-cell follicles present a large number of high endothelium venules, allowing cellular migration and lymphocytes recirculation.

Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the subepithelial dome (SED).

The FAE is the name given to the mucous membrane overlying the organized lymphoid follicles. The FAE is a small region characterized by the presence of specialized flattened epithelial cells called M-cells. Together, the FAE, lymphoid follicles, and associated structures form the antigen sampling and inductive sites of the mucosal immune system [6].

The function and structural characteristics of microfold epithelial cells (M cells) have been described in several recent reviews [1, 6]. It has been widely accepted that M cells are probably playing a key role in mucosal infection and immunity. It is thought that the main role of M-cells is the sampling of antigens to transport them across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. In addition, M-cells are a common route for complex antigens and pathogen invasion, for example, several invasive *Salmonella* species, *Vibrio cholerae*, *Yersinia* species, *Escherichia coli* and the polio virus [6].

M-cells have been identified in the epithelia of a variety of mucosal tissues and within the FAE of a wide variety of animal species, including laboratory animals (mice, rats, rabbits), domestic pets, and man. In mice and men, M-cells reside in about 10% of the FAE in contrast with 50% in the rabbit. In the gut, M-cells are easily recognized by the lack of surface microvilli and the normal thick layer of mucus that characterizes the rest of the epithelial cells. Additionally, M-cells contain a deep invagination similar to a pocket in the basolateral cytoplasmic membrane that contains one or more lymphocytes and occasional macrophages [6].

3.2.2 Nasopharynx-Associated Lymphoid Tissue

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered to be analogous to Waldeyer's ring in humans (pharyngeal lymphoid tissue that includes adenoid, tubal tonsil, palatine tonsil, lingual tonsil) [7]. In the rat, lymphoid aggregates are situated at the nasal entrance to the pharyngeal duct [8]. Detailed reviews of NALT and nasal vaccination can be found elsewhere [8–10]. NALT is a well-organized structure consisting of B- and T-cell-enriched areas which are covered by an epithelial layer containing

M-cells, the so-called FAE. The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches [5]. Although NALT and Peyer's patches share certain similarities, they two differ markedly in morphology, lymphoid migration patterns, and the binding properties of the [high] endothelial venules [7]. Additionally, IELs and antigen-presenting cells including dendritic cells (DCs) and macrophages can also be found in NALT [11]. Therefore, according to Kiyono [5], NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune response to antigens delivered to the nasal cavity.

3.3 Immune Responses Initiated by MALT

MALT plays an important role in antigen sampling and generation of lymphocytes, including specific IgA effector B cells, memory B cells and T cells. This involves active lymphocyte proliferative activity, local production of cytokines, and continuous cellular trafficking [12]. Antigens from the lumen can be internalized by antigen-processing dendritic cells which move into the epithelium and then migrate back to local or distant organized tissues. In the intestinal and airway epithelia, mucosal epithelial cells are sealed by tight junctions; therefore, most of antigen (predominantly particulates) transport is carried out by the M cells. Luminal antigens are endocytosed into vesicles that are transported from the luminal membrane to the underlying M-cell pocket membrane. Vesicles and the pocket membrane experience fusion, and the antigens are delivered to the clusters of lymphocytes present within the pocket. It is not known whether M cells participate in antigen processing and presentation nor if they express MHC class II molecules [12, 13]. Simultaneously, it is believed that the intact antigens are processed by professional antigen-presenting cells (APCs) such as macrophages and dendritic cells, either in the epithelium or in the underlying dome region immediately below M-cells which is thus ideally located to sample transported antigens. Moreover, chemokines secreted by the FAE result in an additional attraction of DCs to the FAE, resulting in a high density of phagocytic cells at sites of entry of foreign antigens and pathogens [14]. Phenotypically immature DCs are subsequently moved to the T-cell areas, where they upregulate the expression of maturation markers and MHC molecules [14].

In the follicle, B cells undergo immunoglobulin class switching from expression of IgM to IgA under the influence of several local factors, including transforming growth factor (TGF-β), IL-10 and cellular signals delivered by dendritic cells and T cells [13]. Furthermore, it is thought that because dendritic cells are migratory cells, they can transport microbes to the mesenteric lymph nodes and to the spleen for the induction of systemic responses [15]. Therefore, these cells also possibly transport antigens, especially those sampled directly from the luminal contents.

The lymphocytes primed in the Peyer's patches move through the draining lymphatics to the mesenteric lymph nodes (MLN) where they can reside for an undefined period for further differentiation before they migrate again to the mucosa.

Peyer's patches contain all the cellular and microarchitectural environments (e.g., a B-cell follicle including germinal centers, a dendritic cell network, and an interfollicular T-cell area) needed for the generation of IgA-committed B cells [16]. Therefore, B cells primed in the Peyer's patches or in NALT and transported to the MLN migrate again to the diffuse mucosal effector tissues such as the lamina propria of the upper respiratory and intestinal tract where full maturation is achieved under the influence of IgA-enhancing cytokines IL-5, IL-6, and IL-10 and are transformed into immunoglobulin-secreting active plasma or blast cells [5, 16].

How the lymphocytes know where to return is an interesting and important aspect of the mucosal immune response. It seems to be well established already that following activation in organized mucosal lymphoid tissues, B- and T-cells are able to upregulate the expression of tissue-specific adhesion molecules and chemokine receptors that function as "homing receptors" to guide the lymphocytes back to the mucosa through the recognition of endothelial counter-receptors in the mucosal vasculature [14, 17, 18].

Although IgA constitutes only 10–15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears, and mucus of the bronchial, genitourinary, and digestive tracts [1]. In humans, more IgA is produced than all other immunoglobulin isotypes combined, and IgA is concentrated over 1 mg/mL in secretions associated with the mucosal surfaces [14].

The secretory immunoglobulin A has several functions in mucosal defense as described elsewhere [2, 5, 14]. So-called immune exclusion is a mechanism that consists of the entrapment of antigens or microorganisms by the sIgA in mucus, preventing direct contact of the antigen with the mucosal surface [14, 19]. Additionally, specific sIgA might block or sterically hinder the microbial surface molecules that mediate epithelial attachment [20].

IgA on the mucosal surface and within the lamina propria is able to complex with food or environmental antigens. The resulting immune complexes may be destroyed locally or excreted through the overlying epithelium, thus preventing potentially antigenic materials from reaching the circulation where they may be able to induce IgE antibodies with subsequent development of food allergy. Therefore, IgA also serves as an immunological barrier to environment antigens.

3.4 Challenges in Oral and Nasal Vaccine Design

Vaccines administered through one of the mucosal surfaces encounter the same host mechanisms as harmless antigens, such as food proteins and commensal bacteria or the same defense barriers as do microbial pathogens and other foreign macromolecules. Therefore, after mucosal administration, vaccines can be diluted in mucosal secretions, detained in mucus gels, attacked by proteases and nucleases and barred by epithelial barriers. Therefore, it is estimated that large doses of antigen would be required. Moreover, soluble non-adherent antigens are taken up at low levels if at

all, and in the intestine, such antigens can induce immune tolerance [21] or simply be ignored be the mucosal immune system [22].

3.4.1 Active Components in Gastrointestinal Luminal Fluids

Besides the barrier function of the mucus covering all mucosal surfaces, the gastrointestinal system has additional important specificities that constitute a barrier to vaccine (attenuated or killed bacteria, antigen proteins, peptides) administration, the gastric and intestinal fluids. The mucosal layer of stomach is an epithelium covered with tiny gastric pits that are entrances to millions of gastric glands. These glands contain cells that secrete some of the products needed to digest food. The secretion of the gastric juice is stimulated by signals from the stretch receptors that are activated by food entrance into stomach. The most important components of gastric juice are pepsinogen, the precursor for the digestive enzyme pepsin, hydrochloric acid (HCl), and lubricanting mucus. It is long known that pepsinogen is converted to the digestive enzyme pepsin by the highly acid conditions of the stomach. HCl causes proteins in the digestive contents to unfold, exposing their peptide linkages to hydrolysis by pepsin. The HCl also kills most of the bacteria that reach the stomach and stops the action of the salivary amylase. In duodenum, chime or other foreign compounds (vaccines, proteins, bacteria, virus, etc.) contact with a fluid that result from the contribution of gastric, pancreatic and liver secretions. Therefore, the luminal fluids of the first segment of the intestine have high concentrations of pancreatic enzymes, which include proteases (active forms are trypsin, carboxypeptidase), an amylase, nucleases, lipases, and bicarbonate ions ($H_2CO_3^-$). The liver contributes also with $H_2CO_3^-$ and bile (bile salts, cholesterol, and bilirubin) which are important for the emulsion of the food fats.

Brush-border epithelial cells on the villi of small intestine secrete water and mucus into the intestinal contents. These cells also produce enzymes (some examples are: disaccharidases such as lactase, aminopeptidase, nucleases, nucleotidases, nucleosidases) that complete the digestion of carbohydrates (disaccharides), proteins (large peptides, dipeptides) and nucleic acids (nucleotides). A large amount of other proteases not mentioned in this text are secreted into luminal fluids. All constitute an enzymatic barrier for peptide and protein antigens GI delivery (reviewed in [23]).

3.4.2 Physical Epithelial Barriers

Mucosal epithelial cells are tightly linked via intracellular junctions that form a continuous barrier which is resistant to microbial passage, the epithelial tight junctions.

The other barrier to infection is the cell surface mucin barrier and the glycocalyx.

3.4.2.1 Tight Junctions

Tight junctions are a form of cell–cell adhesion in epithelial and endothelial cellular sheets. They are responsible for intercellular sealing. Therefore they act as a primary barrier or "gate" to the diffusion of solutes or larger particles, including pathogens, through the intercellular space. But many physiological situations require that various materials are selectively transported across cellular sheets, and this occurs either by transcellular transport through the cell or by paracellular flux through tight junctions. So, tight junctions are not simply impermeable barriers: they show ion as well as size selectivity, and vary in tightness depending on the cell type. In addition to the "barrier function," tight junctions are thought to function as a "fence" [24] to prevent diffusion or intermixing of plasma membrane components between the apical and basolateral domains. It has been demonstrated that some human pathogens are able to invade the body through epithelial cells. It was demonstrated that in some cases they interfere with epithelial polarity to enhance binding to the apical surface, enter into cells, and/or cross the mucosal barrier [25]. On other cases it was demonstrated that dendritic cells (DCs) open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample bacteria. In addition, because DCs express tight-junction proteins such as occludin, claudin 1, and zonula occludens 1, the integrity of the epithelial barrier is preserved [26]. On the other hand, tight junctions can be opened using diverse absorption enhancers. Among those compounds, chitosan has been intensely studied [27].

3.4.2.2 Extracellular Mucus Barrier

Epithelial layers in the body are protected from pathogens and similar stresses by mucus. However, successful enteric pathogens have created strategies to circumvent these barriers. Early investigations of diffusion through mucus gels demonstrated that small molecules can readily diffuse through mucus whereas mucus is an impermeable elastic barrier to bacterium-sized particles. This appears rational, since the end products of digestion, such as monosaccharides and disaccharides, or small peptides, could penetrate the mucous layers to reach the enterocytes and to undergo subsequent absorption. More recent work clearly demonstrates that virus-sized particles can readily diffuse through mucus gels [28]. Therefore, detailed knowledge of mucin dynamics is required to understand the interaction of the mucosal barrier with particulates (bacterial, virus, artificial particulates) and macromolecules. The topic has been reviewed by several authors (see [29–31]).

The essential, protective role of mucus is perhaps most evident in the physiology of the lung, which is continuously exposed to airborne pathogens, toxins, and contaminants. Many of these foreign particles become trapped in the sticky gel of mucus lining the lumen of the bronchoalveolar epithelium and are expelled from the lungs via coughing or cilial motion. In the gastrointestinal tract, the thickness of mucus ranging from 700 μm in the stomach and large intestine to a diameter between 150 and 300 μm in the small intestine [32]. The secreted mucus forms two layers,

a thinner inner layer that is sterile and difficult to dislodge and an outer layer that is not sterile and is more easily removed. Normally, anaerobic commensal microorganisms live in outer mucus layer, leaving the inner mucus layer effectively sterile.

The major components of these barriers are mucin glycoproteins that are produced by mucus cells (goblet cells). Secreted mucin were described by McGuckinin as "a secreted glycoprotein with a central domain containing a dense array of *O*-linked oligosaccharides and amino- and carboxy-terminal cystein-rich domains that oligomerize the mucins into a large macromolecular complex, giving mucus its viscous properties." However, mucus is also formed by other molecules involved in host defense against infection like antimicrobial molecules (cationic and amphipathic peptides or lectins) produced by Paneth cells, or secretory antibodies, IgA and IgG, which are produced by B cells in lamina propria of the intestine and are secreted into the mucus by epithelial cells. All mucus components were exhaustively described by McGuckinin (see [29]).

Evidently, pathogens have evolved many ways of evading the mucosal barrier. Among these mechanisms, some allow efficient penetration of the mucus (presence of flagella), production of enzymes that degrade the mucus, modulation of pathways that allow evasion of the barrier (inflammatory and apoptotic), and disruption of the cells that produce the barrier. Finally, a large number of enteric pathogens have evolved strategies to infect the host via the normal physiological sampling of bacteria and particulates that are carried out by M cells that reside in the dome ephithelium. Goblet cells are not present at dome epithelium, so is not covered by thick mucus layer, leaving holes in the mucus barrier. This anatomophysiological particularity has been appointed as an opportunity to the development of mucosal vaccines.

3.4.3 Immunological Tolerance

Epithelial cells are dynamic participants in the mucosal defense. They have been described as working as sensors detecting danger signals like microbial components through pattern recognition receptors such as Toll-like receptors (TLRs) [14]. The epithelial cells respond to the danger signals by producing cytokine and chemokine signals to underlying mucosal cells, such as dendritic cells (DCs) and macrophages, to trigger innate, nonspecific defenses and promote adaptive immune responses [14, 33].

In the intestine, the environment is extraordinarily rich in food antigens and microorganisms that constitute the normal flora. For this reason, there are mechanisms that reduce and modulate the cytokine and chemokine signals to avoid undesirable responses (reviewed in [34–36]) such as mucosal inflammation.

The mucosal surfaces are in a permanent state of alert, but they "adapt" to the presence of foreign microorganisms. As a consequence, vaccines that produce a strong immune response if injected in sterile tissues such as muscle could be ignored when administered through mucosal surfaces [14]. This state of unresponsiveness or so-called immunological tolerance is dependent on the route of administration of the vaccine and has been appointed as one of the biggest challenges for mucosal

vaccine development. Therefore, intended mucosal vaccination strategies should overcome mucosal tolerance mechanisms, and will require a more detailed understanding of the underlying mechanisms behind the phenomenon. Although the phenomenon of oral tolerance has been known for almost a century, the mechanistic basis is still not fully understood. For instance, the molecular mechanism by which the innate immune system distinguishes commensal from pathogenic bacteria is a topic of great interest which is so far not fully understood. Answers to this and others questions will provide vital information for the development of effective oral vaccines. Some review articles about the state of the art of this knowledge have been published recently [13, 21, 22, 37].

Increasing evidence has shown that the induction of mucosal tolerance is related to the pathway for antigen internalization. One important pathway for tolerance might involve passing through intestinal epithelial cells, escaping capture by lamina-propria phagocytes and transport through blood capillaries to the liver [21]. Another important pathway for the entrance of the antigens from the lumen is via dendritic cells, which can intercalate between epithelial cells and sample antigens directly from the intestinal lumen [26]. It was recently demonstrated that the expansion of dendritic-cell populations mediates the enhancement of oral tolerance [38]. Moreover, these unprocessed antigens are carried through the lymphatics to the mesenteric lymph nodes, which have been implicated in oral tolerance [21, 39]. On the contrary, as demonstrated in more recent studies, Peyer's patches appear not to have an important role in the induction of tolerance [40–42], while the uptake of antigens via Peyer's patches is essential for the induction of an immune response and determines the profile of the induced immune response when using particles as oral antigen carriers [43].

Another important observation is the induction of immunological tolerance that can be induced following the administration of a single high dose of the antigen or a repeated exposure to lower doses. These two forms so-called high- and low-dose tolerance are mediated by distinct mechanisms described recently [21]. It is thought that T-cells are the major cell type involved in the induction of mucosal tolerance. It is generally agreed that the status of oral tolerance can be explained by clonal anergy, clonal deletion of T-cells or by active suppression by regulatory T-cells through the secretion of inhibitory cytokines. The most controversial issue is how and where the antigen-specific T-cells in the MLNs first encounter antigen, and Mowat [13] has reviewed several studies addressing this question. According to the same author, however, it seems more probable that presentation of the antigen to naïve T-cells occurs in the MLNs themselves due to unprocessed antigen brought there by APCs that traffic to the MLNs after being loaded with antigen in the mucosa or Peyer's patches [13].

3.5 Mucosal Adjuvants

To circumvent or minimize these barriers, vaccine formulations and delivery strategies have to be carefully designed in order to efficiently stimulate the innate and adaptive immune response appropriate for the target pathogen [14, 44]. Following this idea,

delivery strategies are likely to be most promising when they mimic pathogens. Therefore, particulate delivery systems that adhere to mucosal surfaces or even better that would be able to selectively target M-cells are likely to be the most effective [14]. Moreover, to be distinguished from commensal microorganisms, the vaccine formulations should also carry substances that activate innate signaling pathways in the epithelial cells and/or in the underlying antigen-presenting cells. These substances which are included in vaccine formulations with the aim of enhancing its immunogenicity are termed adjuvants (*adjuvare*; latin, to help). Presently, there is no optimal adjuvant classification. Although the complete working mechanism of many adjuvants is not entirely known at the moment, classification based on their mode of action has been suggested [45, 46]. Increasing evidence has demonstrated that most non-particulate mucosal adjuvants act by binding to specific receptors, and this adjuvant-class is frequently named immunopotentiators. Particulate adjuvants mainly function to concentrate vaccine components and to target vaccines towards APCs or carry out a depot action.

3.5.1 Micro- and Nanoparticles as Polymeric Vaccine Delivery Systems

The category of particulate carriers includes different particles which have been widely reviewed in the recent scientific literature, including microemulsions (such as MF59) [46, 47], iscoms [48, 49], liposomes [48], virosomes [50], virus-like particles, and polymeric microparticles [46, 51–55]. These particles have a common feature, which is that their size should be similar to the size of a pathogen in order to be taken up by APCs [56, 57] and subsequently deliver the associated antigen into these cells. Therefore, the main role of the delivery systems is to concentrate the antigen in the lymphoid tissues responsible for the induction of the immune response. However, the potency of these delivery systems can be significantly improved by the association of an immunopotentiator. This aspect is of particular importance for recombinant vaccines and other weak antigens. Regarding oral and nasal vaccination, the entrapment of vaccine antigens in delivery systems has two main purposes. The first goal is to protect the antigen against degradation on mucosal surfaces, and the other is the enhancement of their uptake in MALT. The most successful work in achieving these two goals has been done with nano- and microparticles. The interaction between particulates and the GALT has been a subject of several reviews [58–61] since a deep understanding of this interaction would be key in the design of successful nanoparticles. The uptake of inert particles has been shown to take place transcellularly through normal enterocytes and specialized M-cells or to a lesser extent across paracellular pathways through the tight junctions between cells [59]. Although transport by the paracellular route has been shown, for example, with polyalkylcyanoacrylate nanocapsules in the jejunal mucosa of the rat [62], the probability of its incidence does not seem to be high since the opening diameter of the gap junctions between the cells is between 7 and 20 nm in diameter [59].

Regarding the transcellular transport, its occurrence via M-cells appears to be a very natural mechanism since M-cells are specialized for endocytosis and subsequently transport the particulates to the adjacent lymphoid tissue (Peyer's patches in the gut). Therefore, after the particle binds to the M-cell apical membranes, the particulates are rapidly internalized and offered to the continuous lymphoid tissue. Depending on their size, the particles can be retained within the lymphoid tissue (>3 μm) [58], or they can be internalized by phagocytic cells and subsequently transported to another lymphoid tissue through the lymphatic vessels that innervate the PP dome area. There is a broad consensus that M-cells, associated with Peyer's patches are the main target for vaccination purposes. However, several questions have arisen regarding this issue. One issue is related to the number of Peyer's patches in the gut and therefore the total area covered with M-cells. Mice and rats have between 6 and 10 discrete Peyer's patches, while a human being has many hundreds [63]. In this respect, the differences between mice and men mean that one must take extreme caution when extrapolating from animal models to humans. On the other hand, these uptake studies have been performed in a small target area in the animal models. Another question is related to the factors that may influence the particle uptake across the gastrointestinal tract epithelium. Some examples reviewed in references [58, 64] are the particle size, ideally it should be smaller than 10 μm for being take up by M-cells of Peyer's patches in intestine and hydrophobicity, increasing the surface hydrophobicity of particles, permeability through mucin also increase whilst decreasing translocation across the cell interior, which has a more hydrophilic environment. Particle surface charge seems to be also an important factor; theoretically, positively charged particles are better positioned to interact with the negatively charged mucin. Additionally, other factors that may influence uptake studies are particle dose, administration vehicle, animal species and age, feeding state of the animals, use of penetration enhancers and use of targeting agents.

3.5.2 Immunopotentiators

Nonmicrobial particles, macromolecules, and protein-subunit antigens generally induce weak or undetectable adaptive immune responses when applied mucosally. The encapsulation of the antigen in a particulate delivery system can direct the antigen to the inductive site, ideally to the Peyer's patches, but may not be sufficient to evoke an appropriate immune response, because it may not be recognized as a harmful particulate. To be distinguished from harmless substances and nutrients, mucosal vaccines should raise alarms in the mucosa by including substances in the formulations that activate innate signaling pathways [14].

The best-known mucosal immunopotentiators are the secreted enterotoxins of *V. cholerae* and *E. coli*, cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT). Both CT and LT are exceptionally potent oral-mucosal immunogens (their mechanisms are reviewed in [65]). However, this kind of adjuvants has been shown to be toxic

for humans. Therefore, several genetically modified forms have been engineered to reduce or eliminate the toxicity associated with the enzymatic A subunits of these toxins [66, 67]. In spite of this, some concerns have recently been raised about the use of CT- or LT-derived adjuvants for use in intranasal vaccines. This was based on reports from studies in mice that were intranasally administered CT and LT. These compounds could be localized in the olfactory bulb of the brain, apparently as a result of retrograde transport via the olfactory nerve [68].

Furthermore, many live attenuated mucosal vaccine vectors, including poliovirus, adenovirus, and enteric bacteria are currently under development and have been extensively reviewed [69, 70]. A practical advantage of these live antigen delivery systems is that it avoids the effort and cost associated with antigen purification. Although the superiority of these live attenuated pathogens as mucosal vaccine vectors is due in part to their ability to target the antigen to the appropriate tissue, enhance its uptake to yield a more robust immune response and activate multiple innate immune responses, some safety (virulence reversion) and ethic issues associated with genetic manipulation will delay their use in humans. Therefore, the same safety concerns observed for the live attenuated vaccines already in the market for more than forty years.

Meanwhile, with the recent progress in this area, a number of immunopotentiators have become available for inclusion in vaccines, which have been extensively reviewed elsewhere [46, 71, 72]. Moreover, in more recent years, new information about the functions of immunomodulatory cytokines and the discovery of TLRs has provided promising new alternatives. It has also been demonstrated that the vertebrate innate immune system uses pattern recognition receptors, including TLRs, specifically to detect pathogen-associated molecular patterns (PAMPs) present in infectious agents [73]. To date, at least ten different human TLRs have been identified, as well as a number of naturally occurring TLR ligands. For example, various TLR ligands including CpG-containing oligonucleotides [73], flagellin [74], and bacterial porins [75] have shown adjuvant activity when administered mucosally together with antigens. Synthetic TLR ligands have also been identified, including imidazoquinoline compounds such as imiquimod and resiquimod (R-848), which activate human TLR7 and TLR8 [73] as well.

3.6 Final Remarks

Most pathogens gain access to their hosts through mucosal surfaces. The induction of helpful specific antigen mucosal antibodies is feasible only when the antigen is administered by one of the mucosal routes. On the other hand, a number of obstacles must be overcome in order to efficiently stimulate innate immune responses and evoke adaptive immune responses without disturbing mucosal homeostasis or inducing tolerance. Tolerance mechanism is maybe the most important obstacle. Pathogenic bacteria and virus normally surpass this barrier and therefore theoretically attenuated virus or bacteria are the ideal antigen producers and vectors. Inspired by these vectors, polymeric carriers can be designed in order to have similar sizes as the pathogens, and may be loaded with antigens and immunopotentiators

molecules that will activate innate immune response. Therefore, the investigation of novel nontoxic adjuvants, like delivery systems and immunopotentiators, which should be efficacious on mucosal surfaces is urgently required and is as important as the investigation of new antigens for the development of new vaccines.

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Chapter 4
Oral Vaccination: Attenuated and Gene-Based

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4.1 Introduction

The ability to deliver vaccines by a pill, capsule, chewable candy, or even as a liquid slurry represents a delivery improvement over injected vaccines. Besides the pain of watching our young children return from the pediatrician with multiple band-aids on their legs and tears in the eyes, vaccines that can be administered in the absence of needles have several advantages. Distribution and manufacturing are greatly simplified. A pill can be handed out by anyone, not necessarily by qualified medical support. No sterile filling of syringes or vials is necessary because the stomach and intestinal track handle non-sterile food all the time. Unwanted needle sticks and sharps disposal are avoided. From a performance improvement standpoint, delivering a vaccine mucosally could improve the immune responses mucosally since 90% of pathogens invade by this route and parenteral delivery is not particularly adept at inducing immunity at a mucosal surface. Several approved oral vaccines have been developed, and several oral platform approaches are under investigation that might expand the available pool of vaccines. This chapter reviews the history of oral vaccines, both approved vaccines and those in early stages of development.

4.2 Attenuated Pathogens Given Orally to Prevent Infection

This section describes the use of classical oral vaccines and the infections they prevent. All of these vaccines (Polio, Rotavirus, Typhoid, and Cholera) rely on attenuating pathogens that use the oral route as their natural route of infection.

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All of these vaccines are efficacious and were in widespread use, although the polio vaccine has been generally replaced with an injected form because of the issue of vaccine shedding and reversion to virulent forms.

4.2.1 Poliomyelitis and Polio Vaccines

Poliovirus is an extremely infectious enteric virus caused by three polio serotypes (types 1–3) [1]. It is mainly spread via the fecal–oral route although oral–oral transmission can also occur. It enters the body through the mouth and replicates in the intestine and shed in the feces. Before the introduction of vaccines, virtually all children under the age of 5 were infected with polio [2]. The vast majority of infections do not cause serious disease, but in about 1% of cases the virus does enter the central nervous system damaging motor neurons and causing paralysis that is sometimes permanent [2, 3]. Of those people that are paralyzed, a fatality rate of 2–20% exists but is higher if bulbar polio develops [2].

In the early 1950s before vaccination began there were 20,000 cases of paralytic poliomyelitis in the USA per year (CDC, reported morbidity and mortality in the USA 1981). Vaccination began in 1955 using an inactivated vaccine (IPV) administered intramuscularly (i.m.) and developed by Jonas Salk. By 1960 the number of cases had fallen to 3,000. Oral polio vaccination (OPV) developed by Albert Sabin was introduced in 1961, and by 1979 there were only ten reported cases of poliomyelitis per year (CDC; Polio Vaccine Information Statement). OPV soon became the vaccine of choice because of a number of factors, including its ease of administration, its lower cost, and better mucosal immunity that prevent person-to-person transmission.

Initially poliomyelitis was thought to be a disease of the developed world but it was later recognized as a scourge of the developing world as well. The Global Polio Eradication Initiative (TGPEI) was launched in 1988. Its goal was to eradicate polio virus worldwide using OPV by the year 2000. While that goal has not been entirely met, in 2005 the annual number of cases worldwide had dropped by 99% [3]. Wild-type virus still circulates in four countries (Afghanistan, Pakistan, Nigeria, and India) [4]. In the case of Afghanistan, Pakistan and Nigeria a simple cause of inadequate vaccination is to blame for the continued circulation of wild-type virus [5]. But in India it appears to be vaccine failure that has prevented eradication. The problems lie in just two Indian regions, Uttar Pradesh and Bihar. Although reasons for vaccine failure are not fully understood, the regions are swamped with infectious enteric diseases, and it is possible that the competition in the gut flora leads to inadequate vaccine take [6].

While OPV has been responsible for great gains made in polio eradication, it is not without its risks. In some cases the vaccine can cause vaccine-associated paralytic poliomyelitis (VAPP) when the vaccine strains revert to more neurovirulent, wild-type like strains. In addition the vaccine strains can become circulating

vaccine-derived polioviruses (cVDPV), which are biologically equivalent to wild-type strains [7]. Because of these issues, vaccination with OPV eventually needs to be halted and replaced by the enhanced-potency IPV (eIPV was developed in 1978 to be more antigenic than the original IPV. It was licensed in 1984 and is also given by i. m. injection). To this end the USA stopped immunization with OPV in 2000 and replaced it with eIPV. The switch to eIPV is the ultimate goal of TGPEI.

To highlight the eventual need to halt global OPV vaccination and switch to eIPV is a case that occurred in 2005 in Minnesota. A 5-month-old Amish baby was hospitalized for vomiting, persistent fever, and bloody stools. Type 1 vaccine-derived polio virus (VDPV) was isolated from her stool. Fortunately paralysis did not occur but subsequently VDVP of the same serotype was isolated from stools of eight other Amish children in the community (who were not sick). It was estimated that the VDPV had been circulating in the community for 2 months prior to the baby becoming sick. It was later found that the baby suffered from severe combined immune deficiency. VDPV are shed by OPV vaccines, and since the USA had ceased vaccination with OPV in 2000 the original source was thought to be an OPV receiver in the developing world [8]. This example highlights the risks from VDPV in under-vaccinated communities and underscores the some of the issues with the OPV, and why switching to eIPV has been carried out in the USA.

Today there are four types of OPV all produced by either Sanofi Pasteur or Panacea in India; OPV is a trivalent vaccine that confers protection to all three polio strains (PV1, PV2 and PV3). It was developed in 1961 by Sabin and consists of a mixture of three live attenuated viruses. Because there is competition by each of the three strains to induce immunity, OPV confers the best protection to PV2. Therefore monovalent vaccines consisting of live attenuated PV1 (mOPV1) or PV3 (mOPV3) and a bivalent vaccine (bOPV) consisting of PV1 and PV3 have been developed. These monovalent and bivalent vaccines are being used tactically in the last polio endemic countries to match the circulating strains within each country [6].

4.2.2 Rotavirus and Rotavirus Vaccines

Rotavirus is a dsRNA virus that has 11 genes. Several species of Rotavirus have been identified (A–E), although humans are most commonly infected with Rotavirus species A. Of species A, several serotypes have been identified and classified based upon two surface structural proteins, the glycoprotein VP7 which defines G-types and the protease-sensitive protein VP4 which defines P-types. Strains are usually designated by their G serotypes, for example G1–G4 and G9, and the P-type is indicated by a number followed by a letter and a number in square brackets for the P-genotype. A study examining the global distribution of Rotavirus serotypes found that four common G serotypes G1–G4 in conjunction with P[8] or P[6] represented over 88% of worldwide strains [9].

Worldwide Rotaviruses are the leading cause of severe diarrheal disease and dehydration in infants and children under 5 years old [10]. In 2004 an estimated 527,000 children died from Rotavirus infection with 85% of those deaths occurring in South Asia and sub-Saharan Africa [11]. In addition in 2009 the Global Surveillance Network reported that 36% of children under 5 who were hospitalized with diarrhea tested positive for Rotavirus (data was collected from 43 countries) [12].

In 2009 after rigorous clinical trial testing for safety and efficacy in the America's, Europe, Asia, and Africa, the World Health Organizations recommended the use of Rotavirus vaccines in all national immunization programs [13]. The effectiveness of Rotavirus vaccines is being closely watched and already positive trends have started to emerge. For example in El Salvador that introduced routine vaccination in 2006 there has been a 69–81% decline in Rotavirus-specific hospitalizations during 2008–2009 compared to years without vaccination [14]. In the USA from 2006 to 2009 there was a 58–86% reduction in rotavirus hospitalizations [15]. In Mexico from 2007 to 2009 there was a 40% decline in diarrhea-related hospitalizations after a rotavirus vaccination program was introduced [16]. And in Queensland Australia from 2007 to 2009 there was an 89–94% reduction in rotavirus-related hospitalizations in children under 5 years old [17].

The first Rotavirus vaccine licensed in the USA was a tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV). It was initially developed at the NIH and was licensed in 1998 as "Rotashield" by Wyeth-Lederle. It was made by combining the rhesus parent strain RRV (serotype G3) with three human-rhesus reassortant strains of G serotypes 1, 2, and 4. However despite extensive testing that showed 48–68% protection against RV disease and 64–91% protection against severe disease [18–21] it was pulled from the market in 1999 due to an association with intussusception (IS) [22]. The increased risk of (IS) had been reported as 1 in 10,000 to 1 in 32,000 children [23].

After the withdrawal of Rotashield, two new oral live attenuated vaccines were rapidly brought to market. Because of the prior association of IS, vigorous safety testing was performed. Both these vaccines were developed with different immune concepts in mind. A pentavalent vaccine was developed on the premise that serotype-specific neutralizing antibodies are the major determinants of protection (homotypic immunity). Whereas the other vaccine made of a single serotype was developed with the knowledge that repeated infections even if they are of different serotypes do not lead to broadening of the humoral immune response (heterotypic immunity).

RotaTeq (RV5) produced by Merck Research Co. was licensed in 2006. RV5 is a pentavalent live bovine-human reassortant vaccine. The parent strains were isolated from both human and bovine hosts. Four of the reassortant viruses contain outer capsid proteins G1–G4 from the human parent strain and the attachment protein (P7[5]) from the bovine parent strain. Whereas the fifth reassortant virus contains the attachment protein (P1A[8]) from the human parent strain and the outer capsid protein G6 from the bovine parent strain. RotaTeq is marketed as a three-dose regime with vaccinations recommended at 2, 4, and 6 months

of age. Due to the problems associated with Rotashield, a large safety and efficacy trial was carried out on more than 70,000 infants in the USA and Finland. The vaccine was found to have a 74% efficacy protection against G1–G4 rotavirus gastroenteritis and an extremely good safety profile with only three cases of IS in 100,000 infants [24]. RotaTeq Phase III trials in the developing world are still ongoing.

The second oral Rotavirus vaccine "Rotarix" was licensed in 2008 by GlaxoSmithKline. Rotarix is a monovalent live attenuated vaccine. The strain G1P[8] was initially isolated from a 15-month-old sick patient in the USA. It was attenuated by multiple passages in cell culture, plaque purified and again passaged in Vero cells. Rotarix is marketed as a two-dose regime administered as early as 6 weeks of age followed by a second dose given before 16 weeks of age with at least 4 weeks in between each dose. Like RotaTeq, Rotarix has gone through extensive efficacy and safety testing and showed a 70–85% protective efficacy against severe disease caused by serotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [25, 26]. These clinical trials were carried out in Europe, Central and South America. However, while the pre-licensure trials showed no increased risk for IS [27], ongoing post-license surveillance has revealed a slight increase of IS [28]. In 2010 the global advisory committee on vaccine safety (GACVS) reviewed this post-license data which lead the FDA to approve a labeling change to Rotarix stating the increased IS risk.

The completion of trials in South Africa and Malawi showed an overall protective efficacy of 61% which although lower than that in the developed world is considered promising in the face of high maternal neutralizing antibodies and competing gut flora in children in these developing countries [29].

Another Rotavirus vaccine (LLR) is a lamb-derived monovalent live attenuated vaccine made by Lanzhou Biologicals and is licensed only in China. It is reported to produce neutralizing antibodies in 60% of vaccines; however, LLR was not tested against a placebo so its precise efficacy is unknown [30].

4.2.3 Cholera and Cholera Vaccines

Cholera is an acute diarrheal disease caused by ingestion of *Vibrio cholerae* serotypes O1 and O139. The majority of cases are caused by *V. cholerae* O1 organisms, which are further defined as classical and El Tor biotypes. Cholera is transmitted by ingestion of contaminated food and water and is mainly a problem in areas of the world that have poor sanitation. It is estimated that 5–7 million cases occur worldwide resulting in greater than 100,000 deaths, although the exact number is thought to be much higher due to under reporting [31]. In the USA an average of ten cases a year were reported from 1995 to 2000 [32] and all were associated with persons that had traveled to an endemic region [32]. Oral rehydration therapy has dramatically improved the fatality rates.

In 2006 Cholera reemerged as a serious health threat after the number of cases reported to the WHO rose by 79% from the previous year [33]. While improvements to sanitation and the clean water provisions are the best measures in preventing cholera this requires long-term substantial investment. While in the short term access to safe and efficacious vaccines are likely to reap immediate positive effects.

Parenteral *V. cholerae* vaccines had poor immunogenicity and high reactogenicity. Because of this and the mucosal nature of the infection, oral *V. cholerae* vaccines began to be developed in the 1980s. There are three licensed cholera vaccines on the market today. Dukoral (WC/rBS) produced by Crucell and distributed by Sanofi Pasteur is an oral killed whole cell *V. cholerae* O1 containing purified recombinant B-subunit of cholera toxoid (WC/rBS). The second vaccine ORC-VAX is a variant of WC/rBS and contains no B-subunit. It is licensed and produced by VaBiotech in Vietnam. A third vaccine Orochol (also distributed under the name Mutachol) is also licensed by Crucell, but is no longer manufactured. Orochol was an oral live attenuated vaccine (CVD 103HgR) derived from the classical Inaba 569b strain. Currently there are no licensed Cholera vaccines on the US market.

Dukoral stimulates both antibacterial and antitoxic immunity. The vaccine is given in two doses 1 week apart to children over 6 years of age and adults. To children 2–6 years of age three doses are given. Clinical trials in Bangladesh [34] and Peru [35] have shown that the vaccine is safe and has a protective efficacy of 85–90% during the initial 6 months after vaccination. One year following vaccination a protective efficacy of 62% was seen. After 3 years the protective efficacy had dropped to 50% [36]. Interestingly protective efficacy varied according to age range with the lowest efficacy 26% seen with children aged 2–5 years but this increased with age to 62% with children and adults >5 years old [36].

4.2.4 Typhoid Fever and Typhoid Vaccines

Typhoid fever (TF) is a systemic disease that is transmitted via the fecal–oral route and is generally associated with poor hygiene and a lack of adequate sanitation. Clinical symptoms follow a 10- to 14-day incubation period and are described as malaise, anorexia, myalgia, and fever. Abdominal discomfort sometimes occurs as does constipation and diarrhea. In 25% of Caucasian patients exanthem or rose spots develop on the chest abdomen and back, and in about 1% of cases intestinal perforation and hemorrhage occur. TF has more or less disappeared from the developed world but in nonindustrialized countries there are 16 million cases each year resulting in 600,000 deaths worldwide. The worst affected areas are parts of South and East Asia, Africa, South America, and several Asian nations [37]. Although antibiotics are the therapy of choice, increasing antibiotic resistance makes prophylactic vaccines a valuable public health tool.

A live attenuated *S. typhi* strain TY21a is the oral vaccine of choice to prevent typhoid fever. It was developed in the 1970s by chemical mutagenesis [38]. The vaccine strain has a mutation in the *galE* gene which results in the inactivation of the enzyme

uridine diphosphate (UDP) galactose-4-epimerase preventing the interconversion of UDP-galactose and UDP-glucose. However the gene deletion also results in a decrease in growth rate and the absence of the Vi antigen. The vaccine was first produced as a liquid formulation but is now marketed as enteric-coated capsules under the name Vivotef. The vaccine is produced by Crucell and is licensed in over 30 countries including the USA.

TY21a has an excellent safety record. The first field trials were conducted from 1978 to 1980 in Alexandria, Egypt with approximately 32,000 school children from the ages of 6–7 years. The children were given three doses of a reconstituted lyophilized formulation and were given a sodium bicarbonate capsule before hand to neutralize the stomach acid. An impressive 96% protective efficacy was seen with this trial 3 years after vaccination [39]. The next four field trials were carried out in Chile which has a higher endemicity rate than Egypt. The first contained 140,000 children and compared a gelatin capsule formulation with sodium bicarbonate to enteric-coated capsules and compared whether three doses administered 2 days apart or three doses given 21 days apart was more efficacious. The best regime was found to be enteric-coated capsules given 2 days apart this resulted in a 62% protective efficacy up to 7 years after vaccination [40]. The lower efficacy found in Chile compared to Egypt was thought to be due to the method of transmission. In Egypt *S. typhi* is thought to be water borne and thus smaller inocula whereas in Chile transmission is thought to be food borne which generally results in a higher inocula [40].The next trial was carried out between 1982 and 1986 and involved 92,356 school children. This trial investigated whether one dose or two doses of the vaccine were more efficacious. Two doses of the vaccine provided a 52–71% protection for 2 years, then dropped to 22% in the third year whereas the one dose gave only low levels of protection for 2 years and showed no protection in the third year. Thus more than two doses are required to provide adequate protection [41]. The third trial carried out from 1984 to 1987 involved 190,000 children and compared 2, 3, and 4 doses given within an 8-day period [42]. The incidence of TF was found to be considerably lower with the group given the four doses. Two other trials were carried out to compare liquid formulation versus enteric capsules. One was carried out in Chile and the other in Indonesia where *S. typhi* has an extremely high transmission rate. Both trials resulted in the liquid formulation having a slightly better efficacy. However protective efficacy was lower in Indonesia (53%) [43] compared to Chile (76%) [44]. This difference is thought to be due to the higher attack rate in Indonesia [40].

4.3 Gene-Based Oral Vaccines

Classical oral vaccines to prevent disease were successful and easy to distribute. The next section discusses the use of oral vectors to deliver antigen genes to a heterotypic pathogen to induce protection. At this point in time, all of these approaches are experimental and not approved in a marketed product.

4.3.1 Adenoviral Vectors

Early work in the 1960s and 1970s with the military oral Ad4 and Ad7 adenoviral vaccines led to the development of methods to dry and place adenovirus into enteric-coated capsules and pills. The military vaccine was very successful (greater than 95% efficacious in a boot camp setting) and safe, and the vaccine format had significant advantages over injected vaccines (reviewed in [45]). The idea that the format could be used for development of adenoviral (Ad) vectors to deliver heterologous antigens for protection against other illnesses was tried in the early 1980s. The advantage is that the delivery had already been worked out—there was plenty of experience drying and placing adenoviruses in enteric-coated dosage forms. The problem with the approach was that the replicating vectors induced better immune responses against adenoviral rather than the heterologous antigen. In studies by Lubeck et al., the immune responses to hepatitis B surface antigen in their Ad4 and Ad7 vectors were rather modest in monkeys, even after administrating booster titers of 8e9 pfu of the replicating Ad vector [46]. Experiments almost 20 years later with replicating Ad type 5 vectors were also disappointing in that the ability to elicit immune responses to the heterologous antigens was difficult with the oral route alone [47]. Anti-vector immunity induced by immunization is a significant concern if the approach is to be used repeatedly. A clinical trial was started in November 2009 with a replicating Ad4 vaccine to prevent influenza (clinicaltrials.gov NCT01006798). No results have been posted as of mid-2011, but an expanded phase I trial with higher dose levels (up to 1e11 particles) has recently been added. The prior results with replicating Ad vectors suggest other approaches were needed to develop a platform that could be used over and over again.

4.3.2 Non-replicating Oral Adenoviral Vectors

Several vectors and formats have been tested by investigators over the last 20 years. One significant advantage of using non-replicating vectors is that neutralizing anti-vector immunity does not appear to be induced compared with either injected adenovirus or replicating virus [46, 48]. Results show that these approaches could induce meaningful antibody titers in mice, but the results were still modest compared to an i.m. injection. In a paper by Sharpe et al., antibody titers to measles NP were obtained after administrating 5e8 pfu adenovirus expressing measles virus NP [49]. In studies by Xiang et al., 2e7 pfu of rAd5 expressing rabies virus glycoprotein (rGP) was able to induce significant antibody titers to GP in C57BL6 and ICR mice. Antibody titers to rGP appear to be an order of magnitude less for oral immunization than for i.m. injection, but oral immunization was able to induce neutralizing antibody titers to rabies [48].

Some investigators have attempted to improve immune responses to payload antigens by the addition of adjuvants to the adenoviral vector. As an example, investigators have recently used a TLR agonist of the *Eimeria tenella* (rEA) antigen to improve the immune responses to payload antigen [50]. rEA antigen signals

through an MD88 pathway and may signal through TLR11 [51]. Ad expressing the rEA antigen induced improved cellular immune responses after i.m. injection. Surprisingly, the immune responses were still modest when given orally even though rEA antigen was selected based on inducing high levels of IL-12 in bovine intestine [50]. Another similar approach by Vaxart has recently entered clinical trials (May 2011). This approach is using an adenoviral vector with a TLR3 agonist to improve immune responses to antigen. (Clinical trials.gov no. NCT01335347.) Preclinical data suggests that the adjuvant can improve immune responses to antigen when the vector is given orally (Tucker, US Patent 7,879,602).

Ko and colleagues at the Vaccine Research Center proposed using enteric adenoviral vectors as a way to improve immune responses following oral delivery. Enteric adenoviruses naturally infect the intestinal space, and may be more resist to acidic environments of the stomach. In the study by Ko et al., an E1-deleted Ad41 vector was used to deliver the antigen (HIV envelope gp140) [52]. The vector was compared to Ad5 and not found to significantly improve the immune responses to gp140. The immune responses without i.m. boosting were extremely modest, even after substantial adenoviral doses [52]. The authors did suggest that oral priming followed by i.m. boosting may be beneficial because of the lack of induction of neutralizing antibodies to adenovirus by the oral route compared to i.m. injection [52], which presumably would afford better overall immune induction than multiple i.m. injections.

4.3.3 Other Oral Viral Vectors

Several investigators have evaluated the use of vaccinia as an oral vaccine vector. Vaccinia is a potent vector when injected, and there was the possibility that it could be given orally. An early study suggested that intranasal administration of modified vaccinia Ankara (MVA) could circumvent preexisting immunity to vaccinia [53]. A study with MVA covalently linked to cationic liposomes shows a similar observation [54]. However, the use of MVA where oral was the only route of delivery was not particularly successful [55]. In a study with prime-boost administration, oral priming with a Vesicular Stomatitis Virus (VSV) vector expressing the SIV gene plus i.m. injection of an MVA appeared to improve the quality of the immune response versus i.m. alone [56]. In the same study, VSV-SIV given orally alone induced no significant immune response. Two investigators showed in early 2000 studies that Adeno-Associated Virus could deliver Abeta protein and protect mice against experimental Alzheimer's disease [57, 58].

4.4 PLGA and Chitosan Microparticles

Another approach that has been attempted is to use microcarriers of approximately 1 μm in size to deliver DNA itself. In terms of oral delivery, the theory is that small microparticles can protect the DNA in the harsh environment of the stomach, but are

the correct size to be endocytosed by dendritic cells or by M cells, allowing the DNA to pass through the epithelial cell layer of the intestine. Certainly, particles less than 10 μm were able to pass through M cells and induce immune responses [59]. Several investigators have published on this approach, but the immune responses have been rather modest. In published studies by Jones et al., mucosal and systemic antibody responses to luciferase could be induced when mice were given PLGA microspheres by oral gavage [60]. Kaneko et al. used the PLGA microsphere's to elicit T-cell responses to gp160 [61]. Antibody responses to gp160 were extremely modest, either systemically or mucosally. In contrast to the rather modest results following oral delivery, injection of DNA coated on microspheres or contained within microspheres were able to induce substantial immune responses when injected [62].

The results of these studies suggest that immune responses could be elicited, but they were lacking in magnitude compared to injected approaches. The safety of these approaches is likely better than injected vaccines, but immune responses need to be improved for gene-based oral vaccination to work in clinical studies.

4.5 Bacterial Vectors

Over the last two decades there has been significant interest in using bacteria as vaccine vectors particularly for oral delivery. There are many reasons for the interest in oral bacterial vaccine vectors though these vectors are not without their challenges (summarized in Table 4.1): (1) bacteria are adept at surviving through the gastric environment of the stomach and intestine and propagating on and within mucosal surfaces where they can replicate to huge numbers [63]; (2) intestinal delivered bacteria can infect at mucosal surfaces and induce both mucosal and systemic immunity which can be exploited by bacterial vectors to provide protection at mucosal surfaces which are often the sites of pathogen entry; (3) many bacterial components (LPS, Flagelin, CpG DNA) can be recognized through pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and stimulate the innate immune system enhancing inductive immunity to delivered antigens. Many of these PRRs have being exploited as research and clinical adjuvants [64]; (4) they can be produced at industrial scales and lyophilized to aid room temperature storage and to facilitate delivery (Dietrich, Guido, Collioud, A and Rothen, SA, 2008, BiopharmInternational.com/http://biopharminternational.findpharma.com/biopharm/article/articleDetail.jsp?id=557306&sk=&date=&pageID=5). (5) Bacteria can be utilized either as homologous vaccines to elicit immunity to their own antigen(s) [65] or as heterologous vaccine vectors to deliver non-self antigen(s) [66]. These heterologous vaccines can be one of either two types; bacteria that deliver bacterial expressed antigen(s) (antigen vaccines) [67] or those that deliver DNA (DNA vaccines) [68, 69] coding for vaccine antigen(s) under the control of promoters (i.e., the CMV promoter) that permit expression in the host cell. (6) Different bacterial strains have different properties, which can be utilized to induce different types of immunity and to minimize pathogenicity.

Table 4.1 Summary of advantages and disadvantages of bacterial vaccine vectors

Advantages	Disadvantages
Bacterial production well characterized and routine	Pathogenicity
Ability to survive and propagate in the intestine	Environmental containment
Capable of eliciting both local and distal mucosal and systemic immune responses	Antibiotic selection genes carried on plasmids might be picked up by other bacteria in the gut
Choice of bacteria can dictate type of immune response. Cellular vs humoral, TH1 vs TH2	Problems of immunity directed to the vector rather than the vaccine antigen
Can deliver large or multiple antigens or DNA from which antigens are expressed	Bacterial glycosylation patterns might limit vaccine use for nonbacterial antigens
Bacteria act as adjuvants themselves i.e. Flagellin, LPS etc.	Efficacy of bacteria vaccines maybe affected by other gut microflora
Many bacteria such as Lactic acid bacteria regarded as GRAS and have probiotic, pro-immune qualities	Limited clinical success despite years of research

The first oral bacterial vaccines were homologous vaccines and were developed as attenuated strains to provide protection against the parent pathogenic bacteria from which they were derived. They were attenuated to reduce their virulence and disease affects. There are now two commercial live oral bacterial vaccines in clinical use; a typhoid vaccine using *Salmonella enterica typhi* serovar Ty21A and a cholera vaccine that utilizes a *V. cholerae* strain CVD 103-HgR [70] (see also sections 4.23 & 4.24). Both have been used successfully to reduce the incidences of these diseases and they demonstrate the utility of oral bacterial vaccines at least to themselves (homologous vaccines). Many heterologous vaccines exploited these same bacterial strains as vaccine vectors. *S. enterica* serovar *typhi* is probably the most published vaccine vector. There is now a wealth of publications in which oral bacteria vectors have been used as vectors for heterologous antigens ranging from vaccines for pathogenic organisms such as *Streptococcus pneumoniae* [71], *Helicobacter pylori* [67], Influenza [72], and for anticancer vaccines [69] and antimalaria treatment [73]. Some of these approaches have been tested in the clinic [74] but as yet there are no commercial heterologous oral bacterial vaccines for human or veterinary purposes. In this section we will discuss some of the challenges to the use of bacterial vaccine vectors and approaches to addressing these issues.

4.5.1 Bacterial Containment and Pathogenicity

There are a number of limitations to live bacterial vectors and one of the major issues is bacterial containment. This includes individual, environmental, and genetic containment. Lack of individual and environmental containment can lead to increased pathogenicity depending on the characteristics of the bacterial vector in question. A desirable vaccine vector will deliver the transgene to the desired intestinal region, preferably to immune inductive cells, and elicit a vaccine-specific immune response

with minimum reactogenicity to the vector. The vector ideally would not be long lasting, would be noninvasive, and would not be shed into the environment or be transmitted to contacts of the original recipient. It should not transmit plasmid DNA bearing transgenes or selective markers into the environment. Traditionally containment has been achieved through the use of attenuated vectors, which typically have deletions in genes necessary for virulence, regulatory or metabolic pathways.

4.5.2 Virulence and Auxotrophic Mutations for Containment

Virulence mutations must balance decreased virulence with maximal immunogenicity. An example of virulence attenuated bacterial vectors is *Salmonella* strains that contain deletions in the phosphate sensing genes *PhoP* and/or *PhoQ* [65]. Mutations in these genes result in *Salmonellae* that are attenuated in mice and afford partial protection against *S. enterica* serovar *typhi* challenge. Similarly mutations in the ATP-dependent protease ClpXP and Lon results in loss of virulence in mice but protects against oral challenge with *S. typhimurium* challenge [75]. *V. cholerae* attenuated vaccine strains utilize deletions in a number of toxic genes including cholera toxin A [76].

Another class of attenuated vectors are auxotrophic mutants that require an essential metabolite for survival, thus when bacteria deleted in these genes are administered as a vaccine these bacteria have a limited capacity for survival and will be cleared quickly. Sometimes these mutations can be complemented with a plasmid expressing the gene for the missing metabolite. The same metabolite expressing plasmid can also be used as a vector for the vaccine transgene, and the dependency on this expressed metabolite for survival provides a means of selection of the transgene DNA. Deletions in the aromatic amino acid (aroA) biosynthetic pathway have been utilized for attenuation in several bacterial strains (*Salmonella*, *Shigella,* and *Listeria*) [70]. Epaulard et al. used a *Pseudomonas* vaccine vector against ovalubumin and demonstrated that an aroA mutated strain was a 100-fold less toxic than that of the non-mutated parenteral strain [37]. Combination of aroA with a quorum sensing-deficient strain resulted in maximum efficiency in protection in a prophylactic melanoma mouse model. Similarly, deletion of aspartate-semialdehyde dehydrogenase (*asdA*) gene in *Salmonella* means an obligate requirement for diaminopimelic acid (DAP), which is an important component of the gram-negative bacterial cell wall constituent Peptidioglycan. DAP can be supplied in vitro for bacterial production but *in vivo* its absence will lead to loss of the bacteria [77]. In *asdA* chromosomal mutants plasmid complementation with this gene permits survival *in vivo* where this metabolite is normally absent. Eventually the plasmid will be lost during replication and the cells will die thus this “balanced lethal” system limits the capability of the bacteria to survive. Furthermore the *asdA* deleted mutation has been combined with a regulated delayed lysis system using the *murA* gene (enzyme involved in muramic acid another essential cell wall

component) to confer attenuation and biological containment [71] and has been exploited in a oral *Salmonella*-vectored vaccine to deliver influenza nucleoprotein and provide protection to mice against lethal challenge [78]. Immunity is also enhanced when antigens are secreted outside the cell as opposed to being retained in the cytoplasm [79]. Other auxotrophic mutants include Alanine racemase in Lactic acid bacteria [80] and thymidylate synthetase (*thyA*) whose absence leads to cell death in *Lactobacillus lactis* [81]. If the essential gene is replaced with a transgene of interest on the bacterial backbone, this can dispense with the requirement for plasmids and selective biomarkers such as antibiotic resistance. An added advantage is if reversion to the original wild-type genotype did occur, then the transgene would be lost. Reversion to wild-type genotype can be reduced by multiple gene mutations.

One way to limit fear of bacterial spread is to use bacteria as DNA vectors rather than for antigen delivery from expressed bacterial genes [69]. Using this approach, vector success is not based on bacteria replicating but on the bacteria remaining viable for sufficient time that they can express the antigen in vivo. Bacteria can either be programmed for dell death i.e. as described above based on obligate metabolite requirement (*asdA*, *murA* mutants) or potentially bacteria could be treated with antibiotics [82]. However use of antibiotics can lead to bacterial resistance and an alteration of the natural flora of the gut. *S. typhimurium* has been used as a DNA vaccine with efficacy in a beta-galactosidase expressing fibrosarcoma model [69]. In this study Paglia et al. demonstrated that in the fibrosarcoma model *S. typhimurium* targets antigen presenting cells (APCs) and that expression in splenocytes was observed with a eukaryotic promoter but not with a prokaryotic promoter. Bacteria as DNA delivery vectors offer advantages over direct DNA approaches as bacteria act as natural adjuvants being potent inducers of TNFalpha, IFN-gamma, and IL-12. In addition the bacteria themselves can act as DNA carriers providing protection against the hostile environment of the intestine and allow for specific uptake by APCs or other target cells that allow antigen expression and presentation. There is the potential that DNA carried on bacterial plasmids can be taken up by other bacteria resulting in horizontal transmission. This is of particular concern where the plasmid may contain antibiotic resistance markers. A number of groups have developed antibiotic-free plasmid selection systems such as the earlier discussed balanced lethal plasmid system based on the *asd* gene [83]. An additional method to limit bacterial survival and also to facilitate DNA release from *Listeria monocytogenes* bacteria is the expression of a phage lysin [84]. After the bacteria are internalized they escape from the vacuole into the cytoplasm where they express a phage-specific lysin gene from a cytoplasmic-specific promoter *Act*A. Expression of lysine leads to destruction of the bacteria and release of DNA into the cell's cytoplasm and eventual uptake into the nucleus. In a macrophage cell line this was demonstrated to lead to cell genome integration at a rate of 10^{-7} [84].

4.5.3 *Bacterial Ghosts for Containment*

Another system that has been evaluated to avoid bacterial spread is the use of non-living bacteria as a delivery system. This technology has been coined bacterial ghost technology. The bacterial ghosts are empty envelopes of gram-negative bacteria such as *Escherichia coli* which are produced by controlled expression of the lysis gene E of bacteriphage lamda PhiX174 [85]. Expression results in a transmembrane-specific tunnel structure which, due to the high internal osmotic pressure within the bacterial cell, causes expulsion of the cytoplasmic content through the tunnel and out of the cell. This results in the formation of an empty bacterial envelope that can be used as a vaccine against the cell wall contents of the same bacteria or as vaccine vectors. Bacterial ghosts can be used as homologous vaccines as described for *S. entiridis* [86] and for *V. cholerae* [87] and enterohemorrhagic *E. coli* [88] or they can be used to transfer antigen mixed with the ghosts or antigens expressed within the ghosts exported to the periplasmic spaces or anchored in the membranes. They can also be used to package DNA, drugs, and other compounds [89]. Packaging of antigens or DNA can be exploited for vaccine delivery. An elegant technology for immobilization of DNA to the inner membrane of bacterial ghosts was described by Mayrhofer et al. [90]. In this system, a plasmid DNA carries a tandem repeat of a modified lactose operator, which is recognized by a fusion protein consisting of a lactose operon repressor fused to a hydrophobic sequence MS2 that can anchor the fusion protein in the cytoplasmic membrane of *E. coli*. The Lac repressor recognizes the tandem repeat of the lactose operator thus linking the DNA to the bacterial membrane. During the E-gene-specific lysis process most of the cell contents are expelled from the cell; however, the anchored DNA is retained and is available for delivery. Despite their promise as vaccine vectors, there are only a few examples of bacterial ghost technology for heterologous antigen delivery described in publications [91, 92], and there are few examples of their use as DNA vaccines. One promising study demonstrated that bacterial ghosts carrying plasmid DNAs are efficiently taken up by APCs. They also demonstrated that BGs are more efficient at inducing immune responses than naked DNA by intradermal and i.m. routes of administration and are capable of modulating immune responses from a mixed Th1/Th2 response to a more dominant Th2 response. This data suggests that in addition to DNA targeting bacterial ghosts act as natural adjuvants.

4.6 Immunological Properties of Different Bacterial Vectors: *Salmonella*-, *Shigella*-, *Vibrio*-Based Vaccines

Different bacterial species and strains have unique characteristics that make them appealing as vaccine vectors. Pathogenic strains such as *Salmonella*, *V. cholerae,* and *Shigella* strains tend to aggressively colonize the gut and can elicit potent immune responses. The downside is that these vectors can induce disease symptoms and these side effects are undesirable in a vaccine vector. Reduction of pathogenic symptoms necessitates attenuated vectors as discussed in the previous section.

Alternatively commensal/symbiotic strains such as lactic acid bacteria are not pathogenic and readily colonize the gut. However, these may not be as potent at eliciting immune responses as the pathogenic strains. The challenge with using these various strains is to balance undesired reactogenic responses with immunogenic responses.

4.6.1 Salmonella

Salmonella are adept at intestinal infection. They can invade through the M cells of Peyer's patches (PP), infecting the intestinal epithelium and subsequently colonize deeper tissue such as the liver and spleen. Bacteria can be found within the mesenteric lymph nodes and blood within hours of infection [93]. In addition to infecting PP, *Salmonella* can infect solitary intestinal lymphoid tissue [94]. Once taken up by phagocytosis, the bacteria remain in vacuoles called "*Salmonella* containing vacuoles." Some of the bacteria are killed and then processed by the endosomal pathway and are presented by MHC class II molecules stimulating a $CD4^+$ response. They can also elicit $CD8^+$ responses [73] though they are less efficient at doing so against heterologous antigens [95]. To enhance $CD8^+$ responses some researchers have utilized the gram-negative type III secretion system (TTSS). Fusion of antigens to type III secretion signals allows bacteria to secrete bacterial proteins directly into the cytosol of infected cells. Delivery in this way can result in antigen presentation by MHC class I molecules and induction of $CD8^+$ response [95, 96]. As proteins need to be unfolded prior to secretion by this pathway, this system does not work for all proteins and modifications to the antigen maybe necessary [96]. Another system to enhance delivery of antigens into the cytosol utilizes a $SifA^-$gene whose deletion permits escape of *Salmonella* from vacuoles into the cytoplasm [97]. An oral *Salmonella*-vectored influenza vaccine combining the $SifA^-$ mutation with a delayed lysis bacterial phenotype elicited a Th1 response against NP as shown by a skewed IgG2A/IgG1 levels in mice. This was sufficient to provide protection after lethal challenge. Other methods to induce protective $CD8^+$ responses with *Salmonella*-based vectors include the expression of secreted protective proteins such as *L. monocytogenes* listeriolysin which induced efficient $CD8^+$ responses sufficient to protect mice from Listeriosis [98]. Alternatively expression of *E. coli* hemolysin by *Salmonella* permitted bacterial escape from phagosomes and enhanced delivery of recombinant DNA constructs to the cytosol of macrophages [99].

4.6.2 Shigella-, Listeria-, and Vibrio-Based Vaccines

Shigella and *Listeria* both have the ability to escape the endosome and to move and reproduce in the host cells cytosol, which means that it can directly access the MHC class I molecules not normally available to intra-vacuolar bacteria such as *Salmonella* and *Mycobacteria*. In addition the tropism of these organisms for intestinal mucosa has generated interest in them as bacterial vectors. There are few animal models (Guinea Pig and primates) which makes preclinical studies with *Shigella* vaccine

vectors difficult [69]. Given that *Shigella* only infects the GI tract of a limited host set means that much of the work with *Shigella* has involved intranasal delivery where its efficacy for antigens and DNA delivery has been demonstrated [100]. In addition *Shigella* is highly virulent even despite attenuation.

Listeria, though a common food-borne pathogen, is less virulent and is normally only associated with disease in the immunocompromised. *L. monocytogenes* has been used in a number of vaccine vectors including a vaccine against HIV gag which induces $CD8^+$ Gag-specific immunity [101] and as a vaccine capable of eliciting $CD4^+$ and $CD8^+$ T-cell responses against human papilloma virus 16 sufficient to reduce viral titers upon challenge [102].

V. cholerae has also been researched as a heterologous vaccine vector [103]. *V. cholerae* is attractive as a vector as infections are noninvasive being restricted to the intestinal tissue. The bacterium adheres to M cells and presents antigens to the associated lymphoid tissue [104]. In addition there is significant clinical experience with attenuated *V. cholerae* as a homologous vaccine against cholera but despite this experience there are no published clinical studies with *Vibrio* as a heterologous vaccine vector.

4.6.3 Lactic Acid Bacteria

In addition to the pathogenic strains discussed to date, there has been significant interest in Lactic acid vector vaccines. These bacteria include lactobacilli, lactococcus, and streptococci. Many of these organisms are nonpathogenic common bacteria of the GI tract and are found in high levels in the small intestine. A number of lactobacilli species are found in milk-derived products and in dietary supplements where they are consumed in large quantities 10^7–10^8 organisms/g. Their probiotic qualities, their safety record (regarded as GRAS) plus their ability to colonize the intestine make them attractive oral vaccine vector candidates. Many immune studies have demonstrated that LAB can successfully elicit immune responses in man. Examples of *Lactobacillus* vaccines include *Lactobacillus planturum* which has been used as an oral vaccine vector to protect mice against tick borne lyme disease [105]. *Lactobacillus casei* vaccine vector expressing entertoxigenic *E. coli* fimbrial protein protected mice against challenge [106]. Some novel approaches to enhance immunity include expression of fusion antigens with dendritic cell homing proteins and has been exploited to protect mice from an anthrax challenge using an oral *Lactobacillus acidophilus* bacterial vector [107].

4.7 Bacterial Vectors in Clinical Trials

Successful preclinical vaccine studies in animal models have been translated into a number of clinical studies using different bacterial vaccine vectors for heterologous vaccines.

The data to date indicate that these vectors are in general well tolerated though the immune response to the heterologous antigen tends to be weak and generally

lower than that of the vector. This was demonstrated in an oral *Salmonella typhi* vaccine vector for hepatitis B where most of the patients developed anti-*Salmonella* antibodies but none developed anti-hepatitis B antibodies though the vaccine was well tolerated [73]. Similarly oral delivered *Salmonella* expressing *H. pylori* urease has been used as a *H. pylori* vaccine and while most individuals seroconverted to *Salmonella* vector (10/12), no humoral response and a weak T-cell response was seen in a couple of patients (3/12) to the target antigen [108]. A follow-up study with the same vaccine vector in patients failed to provide protection against *Helicobacter* challenge [109]. While most clinical studies have utilized *Salmonella typhi* vaccines based on the original attenuated strain other potential oral bacterial attenuated vectors including *L. monocytogenes* have been utilized and deemed safe [110]. In addition despite being highly attenuated the vaccines can have reactogenic properties. A recent study using *L. monocytogenes* expressing influenza nucleoprotein resulted in mild asymptomatic elevation of serum transaminase in 4 out of 12 patients, 100% mucosal immune response to *Listeria* but no immune response to the influenza nucleoprotein [111].

To date despite the encouraging preclinical data there exists no effective bacteria vector expressing a heterologous transgene that elicits significant immunity in human subjects. As discussed one of the major challenges is to balance vector reactogenicity with transgene immunogenicity. While much has been learned and vectors have been improved further improvements in bacterial vectors and expression systems will need to be developed and advancements made in understanding of bacterial–human interactions and on host cell immunity before commercial heterologous oral bacterial vaccine vectors become a reality.

4.8 Summary

Oral vaccines are marketed products and in clinical development. The ability to simplify vaccine delivery and development, and potentially improve vaccine performance will drive more products to be made on an oral basis. There are technical challenges left to solve for some of the platform approaches using bacterial or viral vectors, but the advantages of the format will push developers to make technological improvements to overcome these obstacles.

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Section II
Design and Development of Next Generation Vaccines

Chapter 5
Development of Biophysical Assays to Better Understand Adjuvanted Vaccine Formulation Potency and Stability

James Chesko, Thomas Vedvick, and Steve Reed

5.1 Introduction

The essential requirements that a vaccine be safe and efficacious can be restated as a formulation that it is made with the appropriate potency that persists over the duration of conditions until it is administered to the patient [1–3]. When a vaccine elicits an immune response of sufficient valence and strength to induce protection against a pathogenic threat without unacceptable adverse effects and the critical biomolecular factors contributing to that response are well defined and characterized, the formulation can be reliably given with a strong assurance of both safety and efficacy. Historically such tests for potency have been functional assays such as infectivity titers and in vivo induction of antibodies [4–8], though these tests are generally time and resource intensive; more importantly, they are "black box," empirical verification that whatever was tested either worked or didn't, offering little insight into why the system behaved as it did, and how it could be improved if desired. When a rapid response is needed such as production and distribution to combat an emerging pandemic, such tests are often unfeasible or untimely, as the case of the recent H1N1 flu pandemic demonstrated [9, 10], when several batches of influenza vaccine were produced, shipped, and administered to patients before it was determined the vaccine potency had dropped below specified levels. There is considerable effort and potential reward in both time and cost savings to develop in vitro tests that show correlation to in vivo potency [11–19]. With increased experimental understanding of structural immunology, it may become possible to establish a set of specifications that establish vaccine performance based upon higher order structures, i.e. biophysical critical quality attributes as described in ICH Q6b [20, 21].

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5.2 Biomimetic Hypothesis

Figure 5.1 shows an example of representative vaccine formulations that are now being developed and approved for both viral (influenza) and bacterial pathogens. What are very distinct about these systems are the phase separated nature of the components and the organization of the surfaces, interfaces, and internal structures. Such morphologies are telltale signs of viruses and bacteria that the immune systems have evolved over millennium to sense, recognize, and mount protective responses against. From a practical point of view this means that classical vaccine formulations with adjuvants such as alum should interact strongly enough with other biomolecular components through processes such as surface physisorption and chemisorption and maintain structural stability for the shelf life of the product. The biophysical properties confer a microenvironmental context that molecular patterns often described in isolation may rely upon to inherit the properties of adjuvancy, such as the immunological requirements of co-stimulation. In the case of alum, for example, if the interaction induces significant changes in antigen structure or does not allow the release of the protein properly into the processing and presentation pathways the adjuvant effect may be lost and antigenicity reduced. The interaction between delivery vehicle and antigen must be properly tuned through preformulation or screening methods, as will be discussed later in this chapter.

5.3 Historical Perspective

Scientific research describing connections between biophysical properties and vaccine potency stretches back over 65 years, when it was reported that centrifugation could be used to enrich high molecular weight components in a Japanese encephalitis vaccine and double its potency [22]. More recently, the link between immunogenicity and oligomeric forms of monoclonal antibodies has been fueling renewed interest and investigation, since recombinant proteins such as growth factors and monoclonal antibodies comprise the majority of biological products. As incidence of unwanted immunogenicity became apparent, a concerted effort was taken to examine factors causing such adverse events. Although the role of chemical amino acid sequence, glycosylation, and pegylation was considered along with chemical decomposition like oxidation, physical degradation through aggregation pathways was singled out as an especially relevant attribute [23, 24].

The connection between protein aggregates and adverse antibody-mediated events had been shown earlier for intravenous immune globulin [25, 26] and recombinant growth hormone [27]. Other studies have gone on to identify both the complexity and heterogeneity of associated protein species, noting physicochemical differences in aggregates created by mechanical stress, chemical oxidation, and thermal degradation [28–30]. The immunogenic behavior of the resulting protein lots subjected to different stresses showed considerable variation in transgenic mice and more uniform, increased immunogenicity in outbred

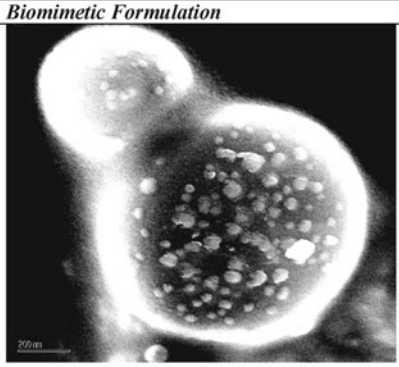
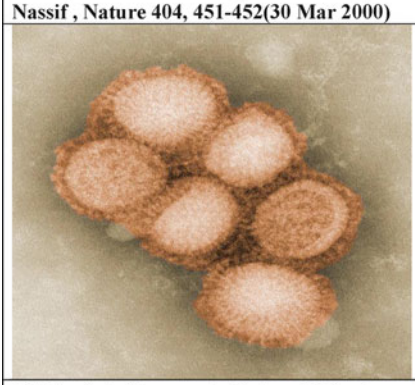
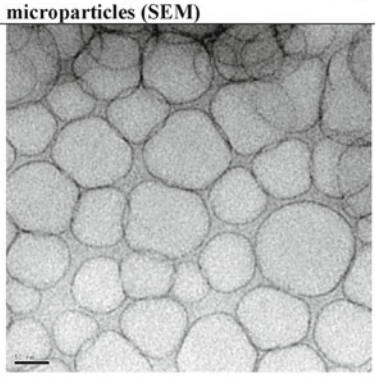
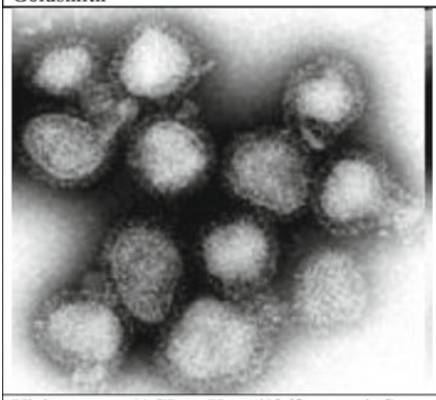
Pathogen	*Biomimetic Formulation*
	
SEM picture of *N. meningitidis* courtesy of Xavier Nassif , Nature 404, 451-452(30 Mar 2000)	**Protein Antigen Adsorbed on biopolymer microparticles (SEM)**
	
TEM of Influenza A (avian flu) virus, CDC Public Health Image Library #11214, C.S. Goldsmith	**TEM Oil in water emulsion, used in adjuvanted flu vaccines**
	
Virion (A/HongKong/1968 influenza,) http://www.ncbi.nlm.nih.gov/ICTVdb/Images/Murphy/ F. A. Murphy, UC Davis.	**TEM of Virus Like Particles (VLPs)**

Fig. 5.1 Biomimetic (biochemical and morphological mimicry) of vaccine formulations and the pathogenic infection they represent. Properties such as spatial dimension and chemical composition, temporal persistence and local concentration (depot effect), the presence of pathogen associated molecular patterns, epitopes and co-stimulatory molecules are present in both constructs and thought to be important biophysical aspects conferring potency and influencing stability

mice [31]. It has been hypothesized that B- and T-cell epitopes may possess overlap with sequences that have a propensity for aggregation [32]. Given the variation possible in protein degradation and association products, it is perhaps not surprising that some aggregated forms do not seem to show enhanced immunogenicity, as studies with recombinant factor VIII have suggested [33]. Using phase contrast microscopy to assess the extent of aggregation of DTP and Hib adsorbed on alum, large aggregated structures resulting from freeze–thaw showed a decrease in potency [34], suggesting that aggregation can sometimes result in loss in activity. Biophysical test methods capable of discerning differences in association that correlate with immunogenic variance are necessary for elucidating these connections.

5.4 Experimental Methods

Classical analytical techniques often involve disruption of the component organization, removing interactions that define the chemical microenvironments that give the vaccine formulation many of its essential properties [35]. Commonly utilized methods such as high performance size-exclusion chromatography may not be sensitive enough to detect fractions of aggregates that can have significant biological activity [36, 37], presumably due to secondary interactions and binding to the solid phase [38]; thus, there is a need for complementary biophysical methods. Analytical ultracentrifugation-sedimentation velocity (AUC-SV) [38–40] is the gold standard for measuring molecular weight distributions, though it is a time-consuming technique requiring sophisticated, expensive equipment. Asymmetric flow field flow filtration (aF4) combined with light scattering can separate and detect particulate forms of formulation components if suitable membrane materials, solvents, and running conditions can be found [41, 42]. Microflow imaging [43, 44] allows individual structures that show optical contrast to be digitized and analyzed, permitting some classification and discrimination between various scattering species such as solid protein particles, silicone oil droplets, and glass fragments [45]. Dynamic light scattering is more rapidly and easily performed, but tends to overestimate cluster sizes and does not distinguish between populations of similar sized scatterers that differ by approximately threefold size, when not overwhelmed by the contributions of the largest species present in the mixture [46–48]. Nano-tracker follows trajectories of individual particles and can measure heterogeneity more readily, though it possesses limits on both the smallest and largest particles that are either too dim or too large and bright for the detector to localize [49, 50]. Fluorescence single particle tracking (fSPT) uses a similar principle to the nano-tracker, with the difference of using fluorescence emission rather than elastic scattering to measure the particle position and extract particle radius through Brownian motion analysis using the Stokes–Einstein relation [51]. Taylor dispersion analysis [52, 53] utilizes changes in diffusivity due to shear forces in fluids that can depend on channel geometries. The extent to which

even small amounts of particulate forms can alter formulation immunogenicity is so dramatic that this property has been labeled a critical quality attribute [54].

Underlying the higher order (i.e., quaternary) structure of such proteins [55, 56] are the lower order conformations and interactions which lead to association and assembly, properties that are best characterized by classical spectroscopic methods such as circular dichroism [57, 58], fluorescence [59, 60], Fourier-transform infrared spectroscopy [61, 62], Raman spectroscopy [63, 64], derivative ultraviolet absorbance [59, 65], NMR [66, 67], and diffraction methods [68, 69]. Microscopic methods that show particulate morphology and organization such as electron microscopy are valuable for nanodispersions including virus-like particles [70, 71], oil-in-water emulsions [72, 73], alum [74], and other biomimetic complexes [75]. Correlations between immunological activity and protein conformation and association can suggest structure–activity relationships that guide specifications for stability and discrimination between antigenic forms that have differing degrees of potency.

5.5 Applications

5.5.1 Protein Antigens

An example of protein structure that shows distinct correlation with activity is hepatitis B surface antigen (HbSAg). As the self-assembling particle matures it undergoes changes in structure and conformation driven in part by redox-coupled reactions, resulting in maturation of affinity towards different antibodies that bind to epitopes [76]. The use of surface plasmon resonance methods effectively monitors protein higher order structure (conformation) in a biophysical measurement that correlates to a functional (in vitro binding) assay that is mirrored in an increase in potency [77, 78]. Structural changes that are observed in HbSAg also can be seen on protein released from alum [79], and the antibody titers generated through immunizing mice show an inverse correlation to the binding strength of the antigen to the alum [80]. The relatively weak cell-mediated response of HbSAg adsorbed to alum could be increased through using a polymeric carrier PLGA [81], and a two-dose regimen with alum possibly replaced with a single shot of PLGA-HbSAg [82].

Formulating and monitoring stability of vaccine formulations can have both a molecular (maintaining chemical covalent structures present in the primary sequence [83]) and biophysical aspect (preservation of higher order structural elements and organization [84]). Studies with recombinant proteins including monoclonals and antigens have elucidated mechanisms responsible for loss of stability and suggested ways to stabilize through excipients and modifications to process and product [85]. When intrinsic properties of protein antigens do not confer required stability [86], peptidomimetics to chemically create epitope structures of greater stability can be attempted [59].

5.5.2 *Antigen–Adjuvant Interactions*

Compatibility of antigens with adjuvants such as alum can be studied systematically based upon the observed biophysical trends of how potency is modified by adsorption strength and stability modulated by perturbation of structural elements [87, 88]. Biophysical techniques have been applied to the formulation and characterization of HIV-env gp120 [89] as an example of how vaccines may be developed in a rational manner [90, 91] through a methodology of preformulation screening of adjuvants using biophysical techniques [92, 93]. Biomimetic structures with lipids on alum have been formulated and tested, with encouraging results in dose-sparing and potency enhancement [94].

5.5.3 *Adjuvant Structure, Biophysical Form, and Biological Activity*

The higher order molecular arrangements known as phases that biological amphiphiles such as Toll-like receptor four agonists can adopt is dependent upon details of their chemical microenvironment including pH, ionic strength, the presence of certain cationic species such as $Ca^{2+,}$ concentration/activity, temperature, and the presence of other molecular species [95]. The supramolecular structure consisting of associated lipopolysaccharides in different conformation and arrangements (polymorphs) can dramatically modulate the biological response [96–98] and release of inflammatory cytokines that can lead either to a protective immune response, or in some cases adverse events such as septic shock. From the biophysical characterization of the structures, it may be possible to discover amphipathic molecules that can bind and effectively neutralize the cytokines that cause sepsis [99, 100].

5.6 Summary

Biophysical testing is emerging as a valuable tool for the development and testing of adjuvanted vaccines, both to explore immunological structure–activity relationships and supplant onerous biological assays when sufficient understanding permits strong correlation between physicochemical properties and in vitro/in vivo potency and stability testing. Biophysical measurements may characterize higher order biomolecular organization that mirrors biomimetic structures and properties that classical analytical methods typically do not see, but are a critical component of complex interactions with the immune system that lead to an effective vaccination response.

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Chapter 6
High Throughput Screening for Stabilizers of Vaccine Antigens

C. Russell Middaugh, David B. Volkin, and Sangeeta B. Joshi

6.1 Introduction

The formulation and stabilization of vaccines remains one of the most problematic steps in their development [1, 2]. Once an antigen has been identified, its conversion into an efficacious, safe, convenient, and stable dosage form that can be effectively delivered to a target population is often beset with numerous difficulties. Not the least of these is the many different types of vaccine antigens, each with its own individual chemical identity, the presence of associated multiple physical characteristics and degradation pathways as well as accompanying delivery problems. Types of vaccine antigens in rough order of their complexity include peptides, peptide conjugates, natural and recombinant proteins, DNA-based systems, carbohydrate and carbohydrate-conjugates, virus-like particles, live-attenuated and inactivated viruses and bacteria among others. Thus biological macromolecules such as proteins, nucleic acids, polysaccharides, and lipids all may play roles as key components in vaccines and require appropriate attention. Furthermore, many vaccine formulations include an adjuvant which itself can be quite complex in nature (e.g., aluminum salts and oil-in-water emulsions) as well as in terms of adjuvant interactions with antigens and other solution components.

Traditionally, the formulation of vaccines has primarily been an empirical process. Using immunogenicity of antigens in test animals (usually mice), stability and efficacy have been examined in the presence of potential excipients usually selected from the GRAS (generally regarded as safe) libraries and the FDA inactive ingredient guide. Thus due to the time-consuming and labor-intensive nature of these experiments, the formulation space evaluated is often rather limited with resultant failures as well as less than optimal final formulations. This situation has, however,

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begun to change with improvements in our ability to characterize vaccines at a molecular level, a better understanding of the degradation pathways that occur, and the advent of high throughput (HTP) technologies which permit a much broader formulation design space to be examined [1, 2]. Although these three topics are interconnected, we focus here on the use of HTP methods in conjunction with the first two developments. In this sense, the uses of HTP methods in vaccine formulation studies are somewhat different than those employed in drug and vaccine discovery, which usually focus on the identification of novel chemical entities and antigens, respectively.

In vaccine formulation development, HTP technologies are primarily being used to (a) characterize the antigen and (b) screen for stabilizers. To a lesser extent, these approaches are also being used in adjuvant compatibility studies [3, 4]. In the case of characterization, the usual approach is to stress the antigen using a variety of environmental perturbations over a wide range of their accessible experimental values. The most common of these are temperature, pH, ionic strength, antigen concentration, agitation and freeze–thaw stress with temperature and pH, usually the initial two examined. Such studies are often described as "accelerated stability studies." An ongoing and still unsettled debate concerns the extent of the immediate relevance of such accelerated studies to pharmaceutical phenomena of interest such as their ability to predict critical results of real-time, long-term storage studies. Although no definitive answers to these questions can be given, several comments are in order. First, there is no practical alternative to their use if vaccine formulations are to be developed in a reasonable amount of time. Second, it is clear that in many (but not all) instances, they are predictive, especially in their ability to rank order different formulations. And third, accelerated stability studies are an effective way to identify and understand the physicochemical "weak spots" in a macromolecular antigen, and thus they can be used to design HTP experiments that result in improved vaccine formulations, despite any theoretical objections to their use.

There has been an explosion in the availability of methods that can be used to characterize antigens and vaccines in the last several years [5–7]. In the sections below, when we discuss the use of such analytical approaches with specific vaccine antigens, we will evaluate and comment on their specific applications. We will first discuss some of the key biophysical techniques in more general terms here. Most often we are concerned with the physical properties of vaccine antigens which include their size, shape, internal structure (conformation), and aggregation state. In the case of better defined macromolecular systems such as highly purified protein and nucleic acid molecules, we can also consider their chemical changes induced by degradative processes such as oxidation and deamidation. The latter are difficult to study in more complex systems such as some virus-like particles (VLPs), viruses and bacteria, although the increased availability of mass spectrometry-based proteomic approaches may eventually permit such analyses to be more routinely used for vaccine formulation development.

One key to the use of biophysically based approaches is their recent availability in HTP formats. To the vaccine formulation scientist, HTP usually means hundreds of samples rather than thousands to hundreds of thousands of samples that would be

tested by the small molecule medicinal chemist. This ability to run HTP experiments is reflected in new technologies that have become available which are often based on robotic autosamplers, conventional microtiter plates, and multiple sample cuvette holders in the case of spectrophotometers.

The secondary structure (helix, sheets, turns, and less ordered structure) of polypeptides and nucleic acids are usually examined by far UV circular dichroism (CD), infrared spectroscopy (typically FTIR based), and Raman methods. Modern spectrophotometers are available with temperature controlled four to six sample cell holders and autosamplers. Although CD is generally restricted to solution states, both infrared (IR) and Raman methods can also examine solids. Various deconvolution procedures are available for all three methods focusing primarily on the 180–250 nm region, the Amide I band and the Amide III signals, respectively. The precision and accuracy of estimates of secondary structure content tend to be in the 2–3% range for these three techniques, and they can be used over a wide range of concentration. We find that using our laboratories multiple CD instruments and three thermal runs a day (10–90°C), it is possible to test approximately 40 samples per day. Using our multiple FTIR spectrophotometers, however, we can analyze less than ten samples a day. There are instruments available with multiple FTIR sampling technology for solid samples and capillary-based solution sample devices for measurement of tens of simultaneous measurements are in development. These secondary structure sensitive biophysical methods can be applied to complex samples such as VLPs, viruses, and DNA vaccines in which the nucleic acid is associated with delivery agents such as cationic lipids and polymers. In cases where multiple (different) proteins are present, one sees a weighted signal from the different components, which is often dominated by the majority species present. Such signals can still be used as measurements of structural stability if they are sufficiently sensitive to relevant changes as described below.

Absolute determinations of macromolecular three-dimensional structure are usually performed by X-ray crystallography or NMR, but these approaches are not generally directly relevant to vaccine formulation issues. Therefore, measurements of tertiary structure changes are most often based on changes in the environment and consequent spectroscopic alterations of intrinsic aromatic groups (e.g., Trp, Tyr, and Phe amino acid residues in proteins and peptides). Proteins containing Trp usually have significant fluorescence and changes in the intensity and wavelength of indole emission are commonly used to follow structural alterations. Phe and Tyr fluoresce much more weakly and are not as commonly employed. In contrast, both near UV absorption and CD tend to manifest multiple peaks from each of these three amino acids that are environmentally sensitive. Raman spectra also manifest distinct side chain peaks (from both aromatic and a few nonaromatic residues), but these are usually hidden by other peaks in infrared spectra. In the case of fluorescence and absorbance, microtiter plate technology is readily available making these convenient HTP techniques. In fact, instruments with both near UV and visible lasers permit fluorescence and light scattering data to be simultaneously obtained. The latter may also be detected by simply scanning through excitation wavelengths. If high-resolution absorption spectra are desired, diode array or CCD detection is

preferred to obtain sufficient resolution to analyze the small changes (e.g., peak shifts) of interest. Such studies are typically performed in a derivative mode and are quite rapid.

Fluorescent dyes can also be used to obtain tertiary (and other) structural information from a variety of macromolecular components found in vaccines. When protein structure is altered, certain apolar dyes whose fluorescence is normally quenched can bind such regions with a subsequent relaxation of this quenching and a resultant dramatic increase in fluorescence. A common example is the naphthalene-based dye, ANS. Other dyes bind well to associated/aggregated protein in which intermolecular beta structure is formed [8]. Nucleic acids bind dyes between their bases (intercalation) as well as in both their major and minor grove in their double-stranded forms. Again, strong fluorescence is introduced which can be quite sensitive to changes in structure of nucleic acids. Similarly, many colorimetric probes bind to various environments in lipid bilayers including such locations in viruses, bacteria and various delivery vehicles containing bilayers. Although the extensive variety of probes available for such a wide variety of molecular sites in vaccine antigens makes them quite useful, the potential perturbation of the system by the probe itself requires caution in interpretation of the data obtained.

A number of methods for the characterization of a macromolecule's quaternary structure have been available for some time [9], and several of them have been adapted to HTP formats. Chief among these are size exclusion chromatography and light scattering-based methods, both of which are capable of measuring sizes in the nanometer to hundreds of nanometer range with good precision and accuracy. Both are also capable of dealing with sample heterogeneity to varying extents. A superior method for analysis of tertiary structure is analytical ultracentrifugation (AUC) in both its velocity and equilibrium modes, but the low throughput of these techniques have primarily relegated them to research use at this time. A number of methods span the nanometer to micron size range and can be discussed in the context of monitoring aggregation as well. There has, in fact, been a dramatic evolution of new methods to examine protein aggregation and subvisible particle formation [10–12] because of the increasing recognition of the importance of these degradation pathways [13, 14]. In conjunction with the appearance of these new methods is their development in HTP formats. Among the most commonly used methods for monitoring particles of sizes greater than 500 nm to hundreds of microns are field flow fractionation (often combined with multi-angle light scattering), laser diffraction, electrical sensing zone methods, light obscuration, flow through microscopic technologies with digital imaging as well as various manual and automated visual inspection methods. In addition, some quite novel techniques based on various physical phenomena such as the vibrating microbalance, pressure/frequency/duration blockade events and differential sedimentation in a fluid (the disc centrifuge) are becoming available. Use of these methods in combination allows a wide range of particle sizes, from the nanometer to visible (over 100 μm) size range, to be quantitatively characterized for properties such as size, density, number, and shape. The inclusion of infrared and Raman spectrometers with microscopes also permits compositional analysis to be performed. A major limitation with many of these

methods is lack of a wide range of temperature control, although standard techniques such as light scattering do permit submicron particles to be examined over a reasonable range of thermal gradients. If not already available, however, HTP adaptations of these particle techniques should soon become commercially routine.

The strength of having such a wide range of methods to probe vaccine antigen structure and its response to environmental stress is our ability to construct a more detailed physical picture of vaccine behavior. This information can then be used to develop HTP screening assays for vaccine stabilizers and ultimately the creation of more optimal formulations. The large amount of data created, however, requires ways to evaluate it that allows one to see the forest from the trees. For example, if data are acquired at seven different pH values from 4°C to 90°C at 2°C intervals by seven different methods, this results in over 2,000 individual pieces of information. Thus, a data analysis methodology that permits one to view such large datasets in an intuitive but comprehensive manner would be helpful. There are a number of approaches that might be employed for this purpose, but we will confine ourselves here to a description of one with which we developed in our laboratory and are particularly familiar. This is known as the empirical phase diagram (EPD) approach. Several recent reviews describe this procedure in detail so we will limit ourselves to a general discussion of it here [7, 15].

The basic idea is to represent the physical state of a macromolecular system as a vector in a highly dimensional experimental space. In an EPD, the physical state of a system is defined in terms of experimental conditions such as temperature, pH, ionic strength, or concentration. Thus, for example, an individual vector for a macromolecular antigen (protein, VLP, virus, bacteria, DNA, etc.) at an individual temperature (say 30°C) and pH (e.g., 5.0) is represented as a vector in which the components of the vector are the experimental measurements (e.g., CD ellipticity, fluorescence emission peak position, and diameter from DLS) at the values of those particular variables. The data are usually normalized to facilitate comparisons but this need not be the case. For visual convenience, each vector is then reduced to the three components of greatest magnitude (again, this is not necessary, but simplifies the analysis) and assigned a color based on a red/green/blue (RGB) scheme. The sum of these colors then represents the physical state of the vaccine antigen in terms of the experimental measurements that have made the biggest contribution to the behavior of system under that condition. A plot of (for example) temperature and pH as the independent variables versus the color vectors then results in a form of stimulus/response diagram which is called an EPD. These diagrams typically present themselves as extended regions of color which in turn represent distinct physical states of the system. In simpler cases such as peptides, proteins, and naked DNA molecules, it may be possible to define these regions in terms of recognizable physical states by reference back to the original measurements. In the case of proteins, these might be native forms, minor conformational and molten-globule-like states, soluble aggregates or more highly aggregated particles.

Examples of EPDs will be shown below as we provide examples of the application of HTP methods to various vaccine types. As we will show, the major application of EPDs is to identify “apparent” phase boundaries that define conditions under which

the structure of the vaccine is altered and thus present one potential (and possible relevant) pathway of physical degradation. For example, a very common degradation pathway is aggregation with possible development of analytical screens for stabilizers based on methods such as SEC and light scattering (static, dynamic, turbidity). The availability of HTP formats for these methods is obviously a factor in their ultimate selection as screening methods. To better illustrate application of HTP technology to formulation development of vaccines, we have selected the following three categories of vaccine antigens: proteins, viruses, and DNA. Similar HTP approaches applied to the other macromolecular vaccine antigens such as peptides, bacterial cells, and carbohydrate containing vaccines are described elsewhere [15, 16].

6.2 Vaccines Based on Proteins and VLPS

6.2.1 Protein-Based Vaccines

In one of the earliest studies employing EPDs and HTP screening methods, region II of the erythrocyte-binding antigen of *Plasmodium falciparum* (EBA-175-RII) was examined [13]. A nonglycosylated version of this protein (EBA-175-RII-NG) was previously identified as a promising candidate for a malaria vaccine and was the target of this investigation. In initial studies, a second derivative near-UV absorption method was used to characterize the effect of temperature and pH on protein antigen's structure and stability. By monitoring the positions of the five negative absorption peaks between 250 and 300 nm, changes in structure induced by these environmental perturbations are easily seen. Because the aromatic side chains tend to be dispersed in varied types of locations throughout protein structure (Phe buried, Tyr interfacial, Trp varied), a somewhat global picture of conformational response can be obtained. Using diode array detection for rapid spectral acquisition and an eight position cuvette holder, it was possible to obtain the data to construct an EPD in only a few days [17] (Fig. 6.1). At least five distinct "apparent" phases are clearly visible corresponding to the native-state, several conformationally perturbed forms as well as versions in which both soluble and insoluble aggregates are observed. An EPD of EBA-175 RII-NG in which 5% sucrose was present showed the apparent phase boundaries shifted to higher temperature and lower pH, illustrating the ability of this form of structural data presentation (EDP) to visually present the stabilizing effect of the sugar on the protein antigen's conformational integrity.

To probe additional elements of structure, an EPD was also constructed using multiple biophysical techniques such as CD as well as intrinsic (Trp) and extrinsic (ANS) fluorescence spectroscopy [17]. The EPD (Fig. 6.2), while very similar to that obtained from UV absorption measurements alone, showed additional detail including the presence of molten-globule (MG)-like states. Based on the EPDs, a microtiter plate aggregation-based turbidity assay was developed to screen for stabilizers at pH 6 and 45°C. A library of approximately 70 GRAS compounds was screened and a variety of sugars, polyols, nonionic surfactants as well as guanidine

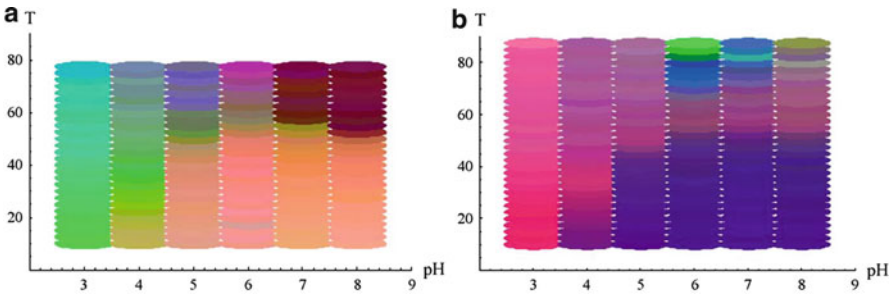

Fig. 6.1 Empirical phase diagrams created using high-resolution second derivative UV absorbance spectroscopy data for a candidate malaria vaccine protein antigen EBA-175 RII-NG (**a**) in the presence of 5% sucrose and (**b**) in the absence of sucrose. Each area of similar color represents a different physical state of the protein [17]. Reproduced with permission from Elsevier, Inc. (Vaccine)

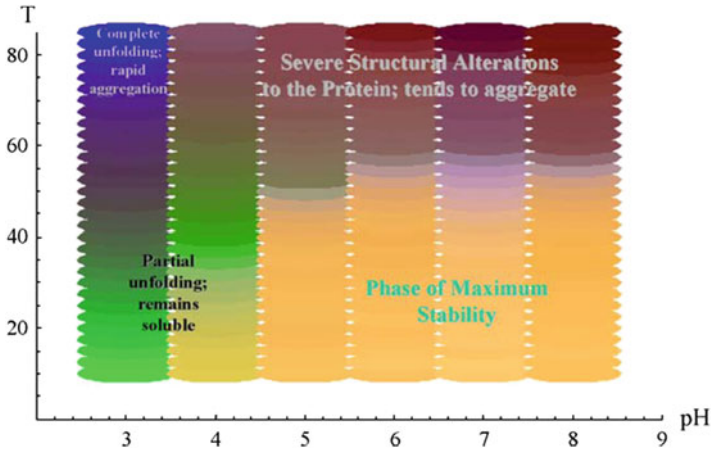

Fig. 6.2 Empirical phase diagram generated for a candidate malaria vaccine protein antigen EBA-175 RII-NG in the presence of 5% sucrose using ANS fluorescence intensity, CD molar ellipticity at 222 nm and intrinsic fluorescence intensity spectral center of mass data. The labels indicate the state of the protein within the same region of color based on observations concluded from transition temperatures obtained by these techniques. The region of greatest stability lies within pH 5.0–8.0 at lower temperatures [17]. Reproduced with permission from Elsevier, Inc. (Vaccine)

hydrochloride and arginine were identified as inhibitors [13]. In a secondary screen, intrinsic fluorescence was used to identify the effect of these agents on the conformational stability of EBA-175 RII-NG. Combinations of sugars and Brij®-35 were found to increase the experimentally observed thermal melting curves and were selected for further development. Adjuvant studies with aluminum salts (adsorption isotherms, desorption studies, stability analysis) were then conducted with the stabilizers to select final formulations. The use of HTP methods for both the characterization and screening aspects of this work is estimated to have reduced the completion time from 4 to 6 months to less than a month with a more extensive coverage of the formulation space and therefore improved optimization of the final formulation.

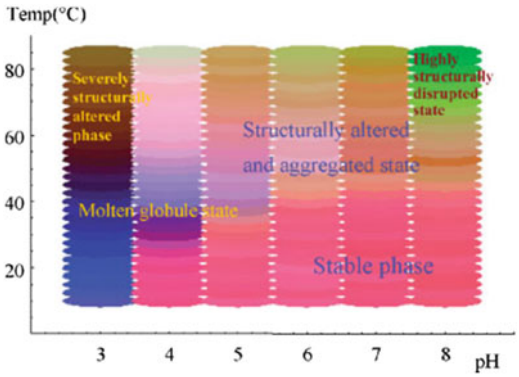

Fig. 6.3 Empirical phase diagram of recombinant protective antigen (rPA) based on intrinsic, ANS dye-binding fluorescence, and CD results. Distinct phases are observed: (1) most stable phase [red-colored region in the lower, right-hand corner]; (2) molten globule-like state [blue/purple area at pH 3, <45°C, pH 4, 25–40°C and pH 5, 30–45°C]; (3) severely structurally altered phase [dark brown area at pH 3, >45°C]; (4) structurally altered and aggregated state [light purple–light brown region at pH 4–7, >50°C and pH 8, 50–65°C]; (5) highly structurally disrupted form [green area at pH 8, >65°C] [18]. Reproduced with permission from John Wiley and Sons (J. Pharm. Sci.)

A second recombinant protein-based antigen example in which these HTP methods were used involves development of powder formulations for nasal delivery of an anthrax vaccine based on the protective antigen (PA) of *Bacillus anthracis*. It has been a major effort of many groups for some time to develop a recombinant version (rPA) of the current non-recombinant PA vaccine. In this case, EPDs of the protein in solution were developed using intrinsic Trp and ANS fluorescence, CD and light scattering [18]. The EPD revealed rPA to be a relatively unstable protein as a function of temperature and pH [18] (Fig. 6.3). Besides identifying native, stable forms of rPA at moderate temperature and neutral pH, a variety of different structurally disrupted, MG and highly associated states were also identified by reference back to the original data. Based on the location of the apparent phase boundaries, an initial screen for stabilizers was developed using a microtiter plate turbidity method at pH 5 and 37°C. A number of good stabilizers were identified including malic acid, sodium citrate, several detergents, and trehalose. These excipients were in turn tested using CD and intrinsic Trp melting experiments and were found to conformationally stabilize rPA by as much as 10°C, with the exception of the detergents which had little effect on the protein's thermal stability. After concentration optimization studies, these results were used to prepare dry powder formulations using both freeze drying and spray freeze drying. The final formulation contained trehalose, a CpG oligonucleotide as an adjuvant and chitosan as an adhesive. The average particle size obtained was about 70 μm, appropriate for nasal deposition but large enough to minimize deep lung entry. The resultant vaccine protected rabbits in an anthrax lethal aerosol challenge and was remarkably stable compared to the solution vaccine in long-term stability studies. Again, the entire project was completed in a few months compared to the anticipated year of work.

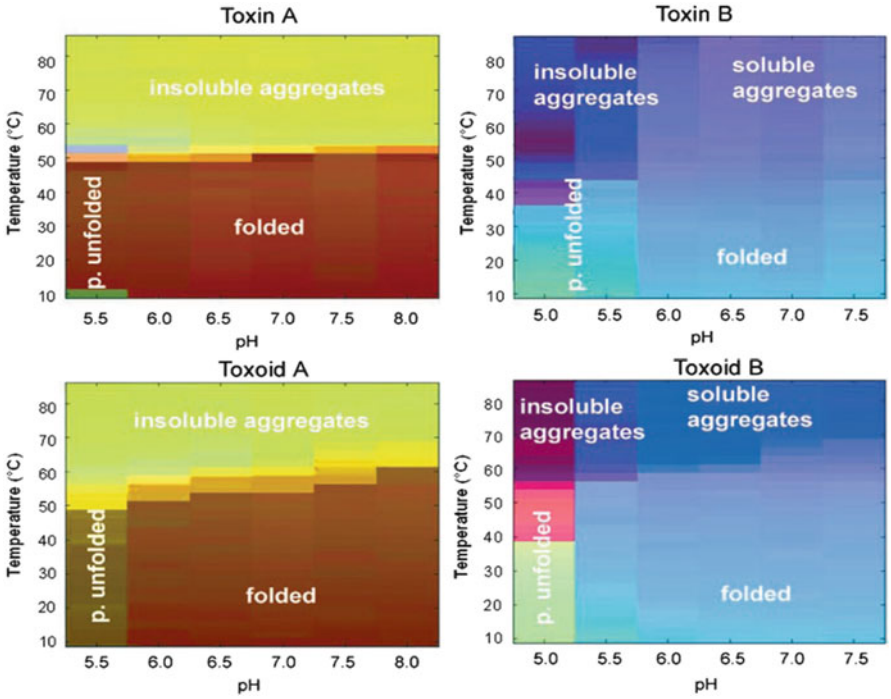

Fig. 6.4 Empirical phase diagram created using OD 350, Trp and ANS fluorescence, and CD data for *Clostridium difficile* toxins/toxoids A and B (p. unfolded stands for partially unfolded). Data were normalized simultaneously for the corresponding toxin and toxoid [19]. Reproduced with permission from John Wiley and Sons (J. Pharm. Sci.)

A somewhat more complex application of HTP methods to stabilize recombinant protein vaccine antigens is to *Clostridium difficile* toxins and toxoids. Acute inflammatory colonic mucosal events caused by this class of organisms are a major problem in hospitalized patients, thus development of an effective vaccine is a vital need. The two proteins that are primarily responsible for disease are designated toxins A and B. Both are high molecular weight (308 and 260 kDa, respectively) and are fairly well studied. For use as vaccines, the proteins have been cross-linked with formaldehyde to inactivate them and are subsequently identified as toxoids A and B. EPDs of both the toxins and toxoids based on high-resolution UV absorbance, CD, intrinsic and extrinsic (ANS) fluorescence, DLS and turbidity measurements have been obtained as a function of pH and temperature [19] (Fig. 6.4). Both toxins are relatively thermally unstable with enhanced stability at higher pH. Crosslinking of both proteins markedly improves their conformational stability, raising the melting temperatures by approximately 10°C. The resulting EPDs were used to develop HTP screening assays for both toxoids [20]. Initially, a microtiter plate aggregation assay based on OD_{350} was used employing elevated temperatures and slightly acidic pH. A GRAS library of excipients was tested and compounds which stabilized both toxoids were examined for their ability to inhibit unfolding at higher pH using CD, ANS fluorescence, and

DSC. Results were then optimized for excipient concentration. The compounds identified (sucrose, sorbitol, dextrose, glycerol, Tween 80, and Pluronic F68) were then tested for their ability to stabilize both proteins on the surface of an aluminum hydroxide adjuvant [20]. A number of compounds continued to possess stabilizing properties under these conditions and have served as a basis for commercial formulations of a promising candidate *C. difficile* vaccine.

An even more complex example concerns the development of formulations for vaccines based on the components of the Type III secretion systems of gram-negative bacteria. These molecular complexes which are found on the surface of most gram-negative organisms contain a wide variety of different proteins (more than 25) that form a syringe-like structure which is involved in the secretion and translocation of virulence factors into host target cells. Recombinant versions of both "needle" and "tip" proteins currently serve as a basis for vaccines in development against pathogens such as *Yersinia enterocolitica*, *Burkholderia pseudomallei*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Extensive biophysical characterization studies of both types of proteins from the above indicated organisms have been performed using the EPD approach. As in the previous examples, a combination of CD, fluorescence, second derivative UV absorption, and light scattering were used as a function of temperature and pH to create EPDs (Figs. 6.5 and 6.6). Of special note was the lack of stability of certain proteins in the needle class [21, 22]. Again, the EPDs served as a basis for the development of HTP screening assays with which to identify stabilizers for each protein in vaccine formulations. In the case of the needle antigens, CD was used as the screening tool, while the tip proteins were examined using an aggregation assay [23, 24]. A number of compounds were identified which stabilized both classes of proteins, and these excipients were tested with the protein antigens on the surface of an aluminum hydroxide adjuvant. Sucrose and dextrose were found to stabilize both types of proteins on the adjuvant surface based on spectroscopic measurements and served as a basis for final formulations of these vaccine candidates. Formulations of these protein antigens from several microorganisms were found to be both stable and highly immunogenic in mouse models [24].

The EPD characterization and excipient screening approaches are also applicable to membrane proteins. A good example includes the temperature and pH stability of recombinant major outer membrane protein of *Chlamydia trachomatis* (Fig. 6.7), which is in early development as a vaccine against various diseases [25].

6.2.2 VLP-Based Vaccines

A somewhat more complex situation occurs when recombinant proteins assemble into defined particles to form VLPs. The power of the VLP approach is well known with the two most successful recombinant-based vaccines, hepatitis B (HBV) and human papilloma virus (HPV), both consisting of self-assembled particles of this type. As a consequence, the VLP approach is now a common one for designing new vaccine antigens and we consider HTP applications for VLP vaccine formulation development with three examples.

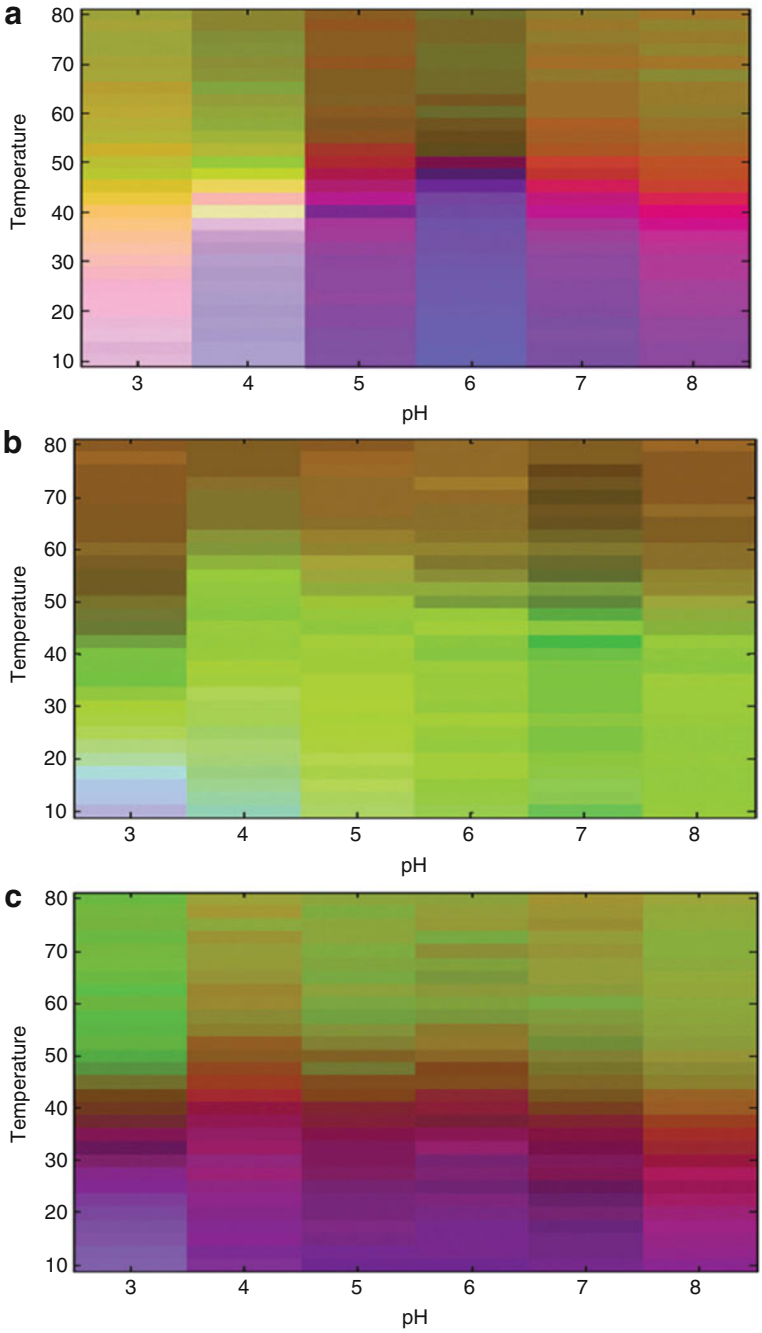

Fig. 6.5 Empirical phase diagrams (EPDs) for three recombinant type III secretion system needle proteins (**a**) MxiHD5, (**b**) BsaLD5, and (**c**) PrgID5 generated from Tyr second derivative near-UV absorbance peak positions, ANS fluorescence intensity, and CD molar ellipticity at 222 nm data as a function of temperature and pH [21]. Reproduced with permission from John Wiley and Sons (Proteins: Structure, Function and Bioinformatics)

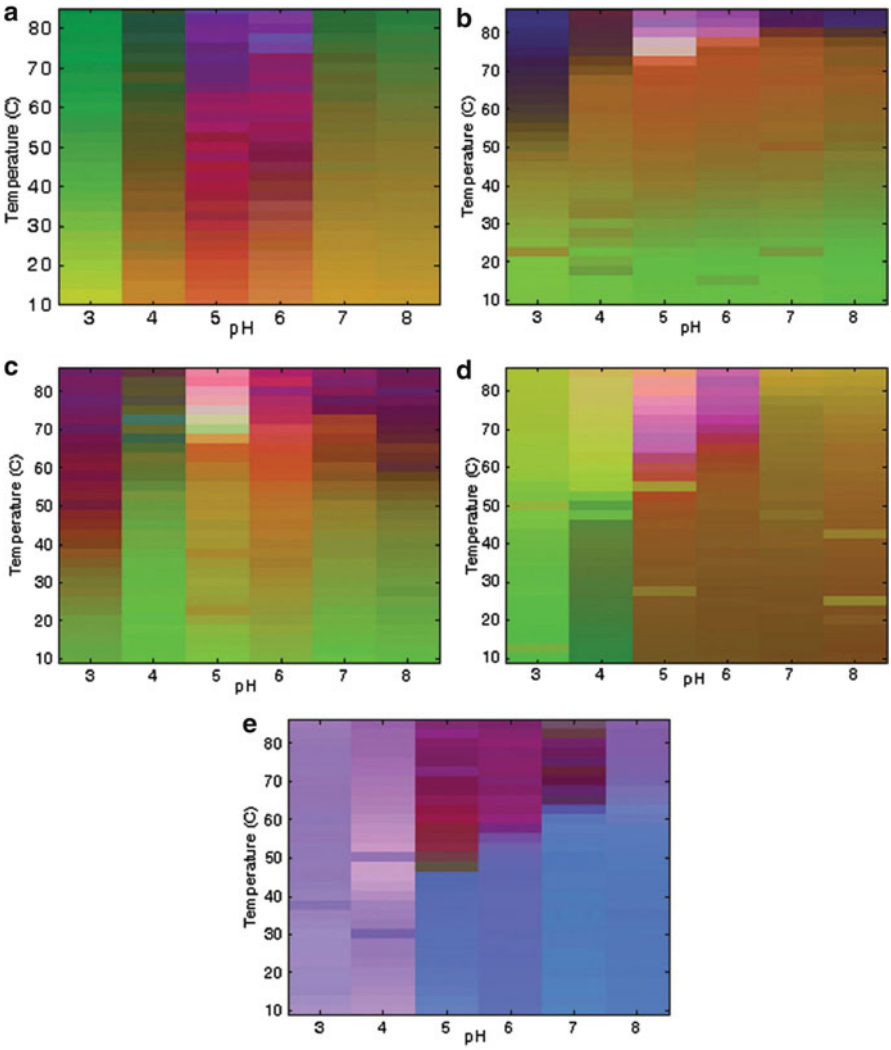

Fig. 6.6 Empirical phase diagrams of five recombinant type III secretion system tip proteins. Empirical phase diagrams for IpaD (**a**), BipD (**b**), and SipD (**c**) show similar responses to pH and temperature. LcrV (**d**) and PcrV (**e**) phase diagrams exhibit similar trends as well [22]. Reproduced with permission from John Wiley and Sons (Proteins: Structure, Function and Bioinformatics)

Norwalk virus and related noroviruses are major causes of viral gastroenteritis worldwide. Consequently, vaccines are much needed for these pathogens. The capsid of Norwalk virus consists almost entirely of a single 58 kDa protein. When expressed recombinantly, this protein self-assembles into icosahedral VLPs which are highly immunogenic. The structure of these VLPs was characterized by a combination of UV absorption, CD, intrinsic and extrinsic fluorescence, DLS and DSC [26]. In addition, transmission electron microscopy (TEM) was used to further analyze the struc-

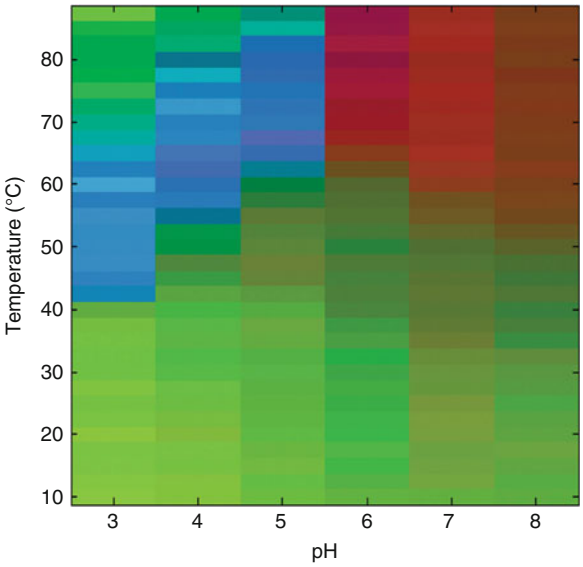

Fig. 6.7 Empirical phase diagram (EPD) of major outer membrane protein of *Chlamydia trachomatis* (nMOMP). The EPD was generated using an averaged CD signal at 216 nm, intrinsic fluorescence peak position, static light scattering intensity, and OD350 measurement. Data were normalized within each technique, and the presence of changes in the spectroscopic measurements is manifested by color change in the EPD over the indicated pH (3–8) and temperature (10–87.5°C) range [25]. Reproduced with permission from the American Chemical Society (Mol. Pharm.)

ture of the VLPs in the different apparent phases seen in an accompanying EPD (Fig. 6.8). The particles are very stable over the pH range of 3–7 up to 55°C. At higher pH (Fig. 6.8), however, capsid dissociation was seen and manifested in all of the various measurements. Aggregation at pH 5 and 60°C was used as an initial screen to identify potential stabilizers [27]. Compounds providing positive results were then screened using CD, DSC, and ANS fluorescence to examine conformational stability. Sucrose, trehalose, and chitosan glutamate were all found to significantly stabilize the VLPs based on all of these criteria. This information was used to construct a number of effective formulations, one of which is currently being used in initially successful human clinical challenge studies.

In addition to the self-assembly of recombinant viral proteins, another type of particle has become a promising vaccine target. A recent example of this type is the formulation of flu vaccines based on expression of "triple gene" products in recombinant baculovirus, with the usual flu hemagglutinin (HA) and neuraminidase (NA) used as the primary antigens with self-assembled murine leukemia virus (MLV) gag protein serving as a core to the particle. Thus, the final form of this type of VLP (also sometimes referred to as virosomes) consists of membrane-bound HA and NA surrounding an MLV gag core. Despite the complexity of this approach, an HTP EPD has been successfully applied to an H1N1 version of such a vaccine [28]. The usual complement of spectroscopic and light scattering techniques was applied to

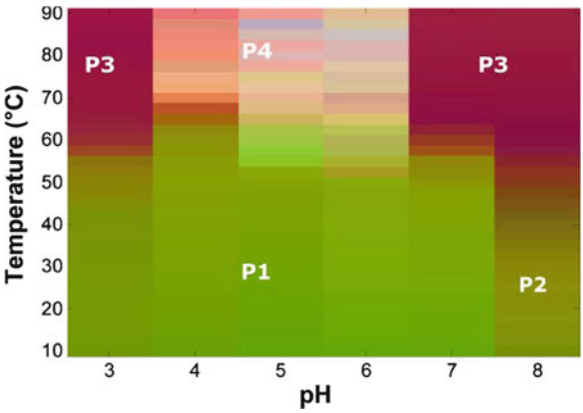

Fig. 6.8 Empirical phase diagram for Norwalk virus-like particles (NV-VLPs) based on UV, intrinsic and extrinsic fluorescence, and CD results. Four distinct phases (*P*) of the NV-VLP were observed; *P1*, native, intact form; *P2*, disassembled; *P3*, soluble VP1 oligomers; *P4*, aggregated [26]. Reproduced with permission from the American Society for Biochemistry and Molecular Biology (J. Biol. Chem.)

this enveloped VLP. In addition, the membrane-sensitive dye laurdan was used to monitor membrane fluidity as a function of temperature and pH. The major difference between this study and those previously described is the fact that the various signals measured reflect a combination of the various contributions of the various components (i.e., HA, NA, gag, lipid membrane) of the particle. Nevertheless, such composite signals manifest multiple structural transitions that reflect temperature and pH-dependent destabilization processes that may reflect physical degradation events that are manifested during long-term storage conditions. The EPD produced from the accelerated data reflects the complexity of the response of the H1N1 VLP to pH and temperature stress (Fig. 6.9). An aggregation (OD_{350}) assay was used as an initial screening technique for stabilizers followed by intrinsic and laurdan fluorescence to confirm physical stabilization. On this basis, trehalose, sorbitol, and glycine were all found to provide stabilization of both viral protein tertiary and membrane structure.

As another example of a VLP vaccine antigen, we consider the hepatitis C viral envelope glycoprotein E1. A truncated form of E1 (E1y) forms VLPs when expressed in yeast and has been used as a basis for a candidate hepatitis C vaccine. In this study [29], detergents were found to have profound effects on the structure and stability of the particles. A temperature/pH EPD study of the particles was performed (Fig. 6.10) using CD, intrinsic and ANS fluorescence and static and dynamic light scattering. A series of EPDs were then constructed in the presence and absence of the two dissociative detergents and have served as a basis for formulation development of an E1-based VLP hepatitis C vaccine.

6.2.3 Live Virus Vaccines

Perhaps surprisingly, the HTP EPD approach can also be applied to complex biological entities such as viruses. Attenuated, live viruses serve as a basis for many of our most effective vaccines including measles, mumps, rubella, varicella, and rotavirus. HTP approaches have been applied to both already available live virus

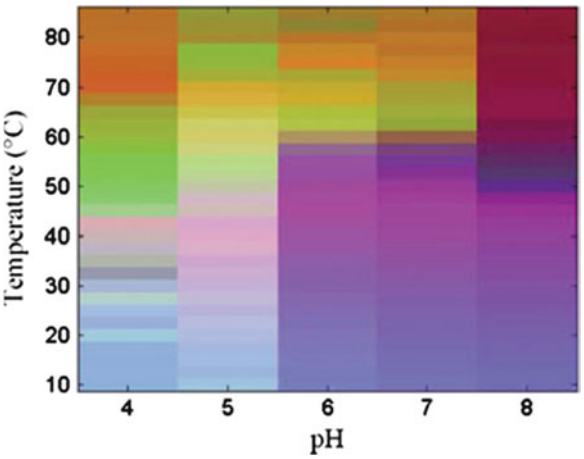

Fig. 6.9 Empirical phase diagram (EPD) derived from biophysical characterization of influenza virus-like particles containing membrane-bound HA and NA surrounding an MLV gag core. The EPD is prepared from temperature-dependent effective diameter, static light scattering, polydispersity, circular dichroism at 227 nm, intrinsic fluorescence (peak position and relative intensity at 330 nm), 8-anilino-1-naphthalene sulfonate fluorescence (peak position and relative intensity at 485 nm), and generalized polarization (GP) of laurdan fluorescence data collected across the pH range from 4 to 8 [28]. Reproduced with permission from John Wiley and Sons (J. Pharm. Sci.)

vaccines and a number in development. A major problem with applying the previously described methods to such vaccines is that they typically consist of crude preparations of virus which contain host cell impurities. Therefore, the following procedure has been used. The virus(es) is (a) first purified (typically by centrifugation and/or sucrose density gradient centrifugation), (b) studied by the various physical methods, (c) screening performed to identify stabilizers and (d) finally, the stabilizers are added back to the crude (vaccine) viral preparations and stability studies undertaken to confirm the effectiveness of the stabilizers in the actual vaccine.

A second problem concerns the fact that many live attenuated vaccines contain a mixture of infectious and noninfectious particles in the crude preparations. We have generally found, however, that the processes which initially result in the vaccine containing inactivate virus particles are different than those that are responsible for loss of viral activity during storage. Thus, this permits the EPD approach to be applied to the stabilization and formulation of the vaccine. If it is not the case, the EDP procedure may fail to identify effective stabilizers.

As an initial example, we consider the measles virus. This live attenuated viral vaccine is one of the most important available, given the continuing problem with this disease, especially in the developing world. Lack of stability is an ongoing problem with this vaccine despite its strong efficacy. In a recent study, measles virus particles were purified and then examined as a function of pH and temperature using a combination of static and dynamic light scattering, CD, intrinsic, ANS, and laurdan fluorescence [30]. The data were combined in an EPD (Fig. 6.11) which served

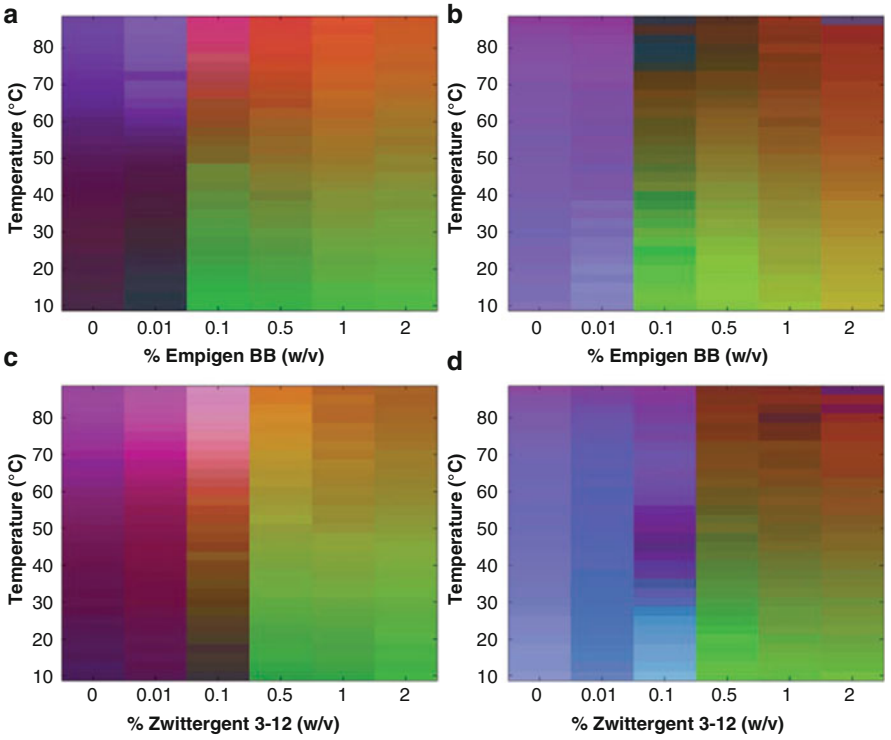

Fig. 6.10 Empirical phase diagrams (EPD) of E1-based VLP candidate hepatitis C vaccine antigen (HCV E1y) in the presence of detergents. The EPDs with Empigen BB show similar trends at pH 5 (**a**) and pH 7 (**b**) while those obtained in presence of Zwittergent 3–12 display similarity between pH 5 (**c**) and pH 7 (**d**) for the E1y protein/particle [29]. Reproduced with permission from John Wiley and Sons (J. Pharm. Sci.)

as a basis for designing HTP screening studies. Aggregation at pH 5.5 and 55°C was used for the initial screen with laurdan fluorescence employed as a secondary membrane integrity assay and CD as a protein structure probe. Porcine gelatin, lactose, mannitol, malic acid, proline, and myo-inositol were all identified as effective stabilizers of the measles virus based on these methods. The ability of the six stabilizers was then tested during storage by plaque-based infectivity assay. After 24 h at 21°C, five of the six compounds were able to protect the virus from inactivation, with lactose the exception. This information has been used to formulate more stable versions of measles vaccines including dried versions for inhalable administration.

No vaccine currently exists for human respiratory syncytial virus (RSV), although this virus is universally the leading cause of lower respiratory tract infections among children. Thus, a live attenuated version of this virus is being explored as a critically important new vaccine candidate. The virus has been purified by discontinuous sucrose density gradient centrifugation and examined by DLS and optical density measurements, second derivative absorption spectroscopy, CD as well as intrinsic and extrinsic (ANS) fluorescence over a wide range of pH and temperature [31].

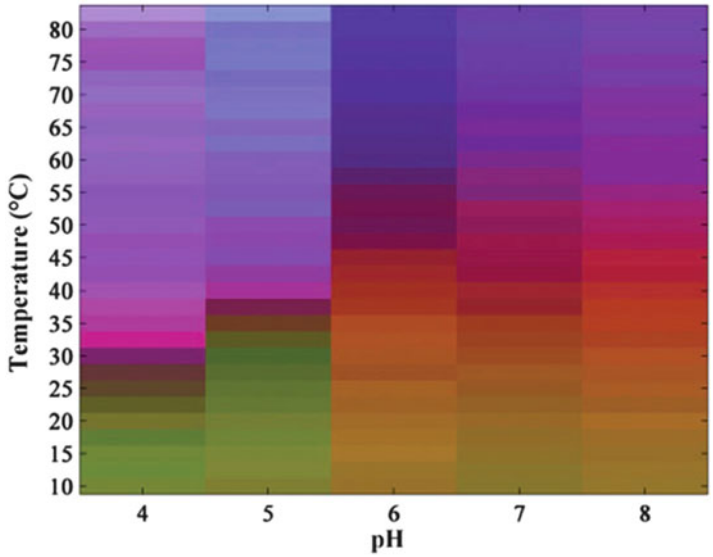

Fig. 6.11 Empirical phase diagram (EPD) of measles virus. Data used to generate the EPD were measurements of mean effective diameter, intensity of (562 nm) light scattered at 90°, CD at 222 nm, intrinsic fluorescence intensity at 322 nm, ANS peak position, ANS fluorescence intensity at 469 nm, and general polarization (GP) of laurdan fluorescence [30]. Reproduced with permission from Landes Bioscience, Inc., Human Vaccines

The resulting EPD displayed a well-defined series of apparent structural phases (Fig. 6.12). Based on this stimulus/response picture of the virus, an HTP screen of the virus was developed employing its aggregation behavior at 56°C [32]. A number of amino acids, sugars, and polyols were found to have significant inhibitory effects on light scattered by the viral particles. Strikingly, a number of polyanions (heparin, sucrose octasulfate, polysialic acid, dextran sulfate) had the most dramatic stabilizing effect, presumably due to the presence of a polyanion binding site on the virions. Again, structural stability was confirmed by a series of biophysical measurements including CD, UV absorption, and light scattering with membrane integrity examined by the generalized polarization of laurdan fluorescence. As in the measles virus example, it is not yet possible to resolve the structural origin of the effects seen, but they have served as an empirical basis with which to develop more stable formulations of live attenuated RSV vaccines.

It is also possible to use these approaches with live virus vaccines containing multiple serotypes. For example, serotypes G1, G3, and G4 of rotavirus have been characterized and compared as a function of temperature over the pH range of 5–8 using CD, fluorescence, and DLS [33]. The EPDs derived from these data for each of the three serotypes (Fig. 6.13) have been compared and show clear differences in stability. Using a tissue culture infectivity assay, it was found that partial correlations exist between temperature and pH-induced structural alterations and biological activity. Again, this demonstrates the utility of the HTP EPD approach to

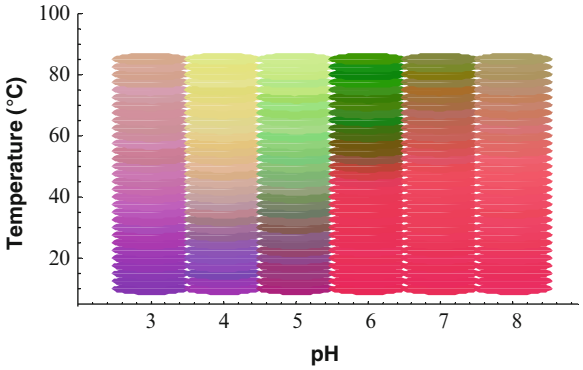

Fig. 6.12 Empirical phase diagram (EPD) of human respiratory syncytial virus (RSV) over the pH range 3–8. Data included in the generation of the EPD are all negative second derivative UV peaks, CD signal at 222 nm, optical density at 350 nm, intrinsic fluorescence peak position, intrinsic fluorescence intensity at 330 nm, and ANS fluorescence intensity at 485 nm [31]. Reproduced with permission from the American Chemical Society (Mol. Pharm.)

preformulation and formulation work with live attenuated virus vaccines. Similar studies have been performed with adenoviruses and confirm the ability of this approach with multiple types of adenoviruses [34–37]. An example of an EPD of adenovirus type 4 is shown in Fig. 6.14.

6.2.4 DNA Vaccines

Although no human DNA vaccines have yet become commercially available, the promise of this form of vaccine continues. In fact, several veterinary vaccines employing this technology are on the market. Although HTP approaches are in their infancy with regard to DNA vaccines, some preliminary results have been reported. The methods used to characterize nucleic acids are by and large similar to those used to characterize proteins, VLPs, and viruses (which of course contain nucleic acids). Thus absorption spectroscopy, CD, FTIR, DSC, DLS, and the other techniques discussed above are all applicable to nucleic acids, although interpretation of the resultant data does differ [38–41]. A major difference is the utility of fluorescence spectroscopy since DNA is not intrinsically fluorescent. Extrinsic dyes, however, which bind to the grooves in DNA as well as between the bases are very useful in structural studies. The EPD approach has been applied to plasmid DNA in the context of DNA complexed to delivery vehicles such as cationic lipids and polymers [41]. In contrast to most of the other systems discussed, the types of stress employed were pH and ionic strength. Temperature was not employed as an environmental stress since DNA plasmids tend to melt at very high values. The methods used were CD, DLS, and extrinsic dye fluorescence. Although the DNA plasmid examined produced only three distinct apparent phases in the calculated EPDs, an increase in

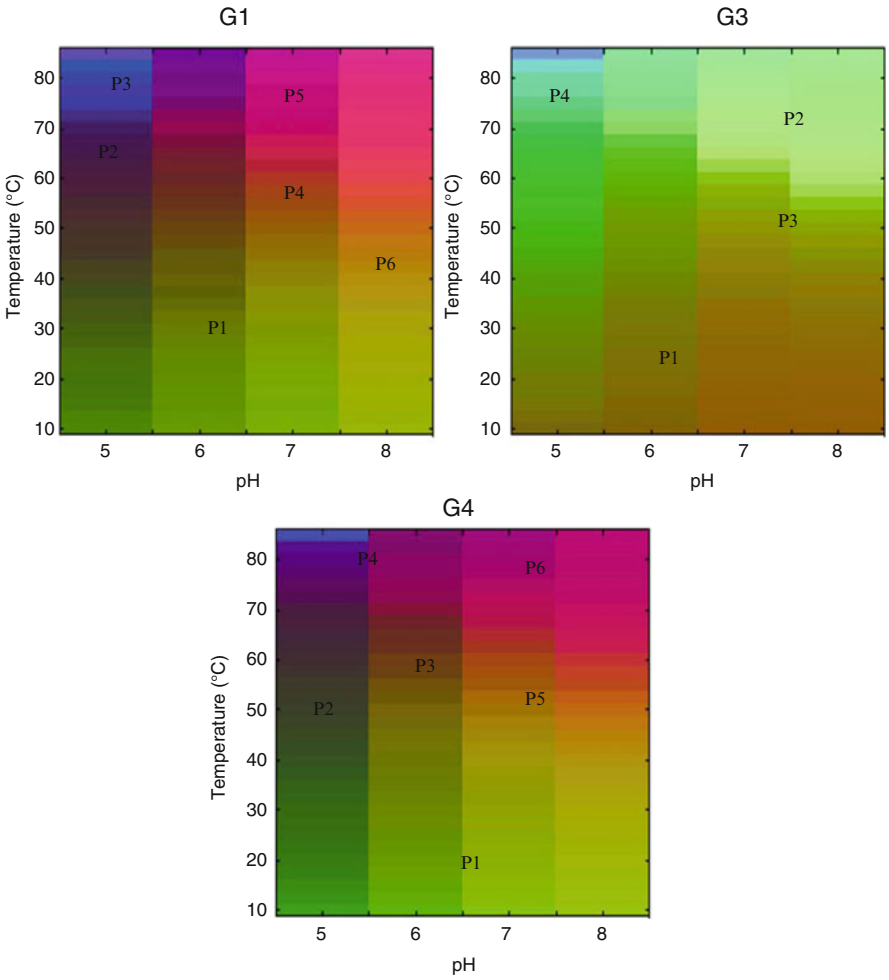

Fig. 6.13 Temperature/pH empirical phase diagrams of rotavirus strains G1, G3, and G4 based on intrinsic fluorescence, CD thermal melts, and static and dynamic light scattering data [33]. Reproduced with permission from Landes Bioscience, Inc., Human Vaccines

the number of phases was seen in the eight phase diagrams produced by different complexes (Fig. 6.15). Nevertheless, the EPDs are not as well defined as those obtained from protein-based systems. Initial analyses of stabilizers for plasmid DNA itself have been based on the conversion of the supercoiled plasmid to open circular and linear forms as determined by gel or HTP HPLC-based methods [42, 43]. A number of stabilizers have been identified including ethanol, EDTA, DTPA, sodium citrate, malic acid, and the detergent Pluronic F68. The combined data suggest oxidation as the major degradation pathway that will be encountered in future work to develop commercial formulations of DNA vaccines. Although work on

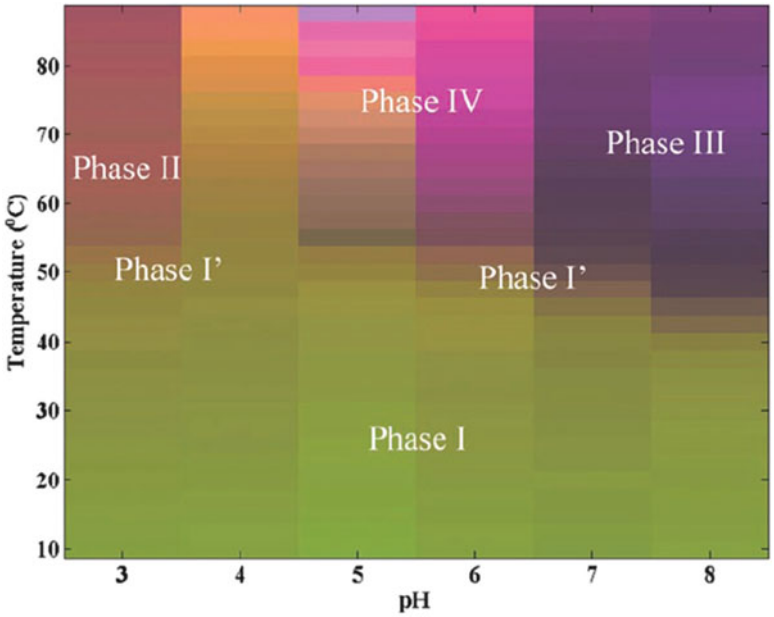

Fig. 6.14 An empirical phase diagram (EPD) of adenovirus Ad4. The EPD was constructed using circular dichroism at 218 nm; circular dichroism at 208 nm; second derivative UV peaks for overlapping Tyr/Trp and Trp alone absorption; peak position in intrinsic (Trp) fluorescence; propidium iodide fluorescence intensity at 613 nm; optical density at 350 nm; effective hydrodynamic diameter from dynamic light scattering; and static light scattering intensity at 295 nm. Phase assignments are: phase I, native form; phase II, partially altered form; phase III, highly altered form; phase IV, extensively aggregated form; phase I′, transition state [34]. Reproduced with permission from Landes Bioscience, Inc., Human Vaccines

DNA and associated vaccines should still be considered preliminary, HTP approaches should prove useful in future formulation advances.

6.3 Summary and Future Directions

There are a variety of ways in which HTP methods can be used to further the development of vaccines. In this brief review, we have described two of them. In the first, physicochemical data are generated in an HTP mode and synthesized into a comprehensive stress/response picture of the vaccine antigen in the form of an "EPD." In the second, the "apparent" phase boundaries observed in a vaccine EPD are used to develop HTP screening assays to identify potential excipients (stabilizers) for a vaccine formulation. Optimization of the latter, in terms of concentration and combinations of excipients, is also performed with a variety of HTP methods as well. In addition, there are many other approaches that are facilitated by HTP modalities during vaccine discovery and development. For example, two of the best

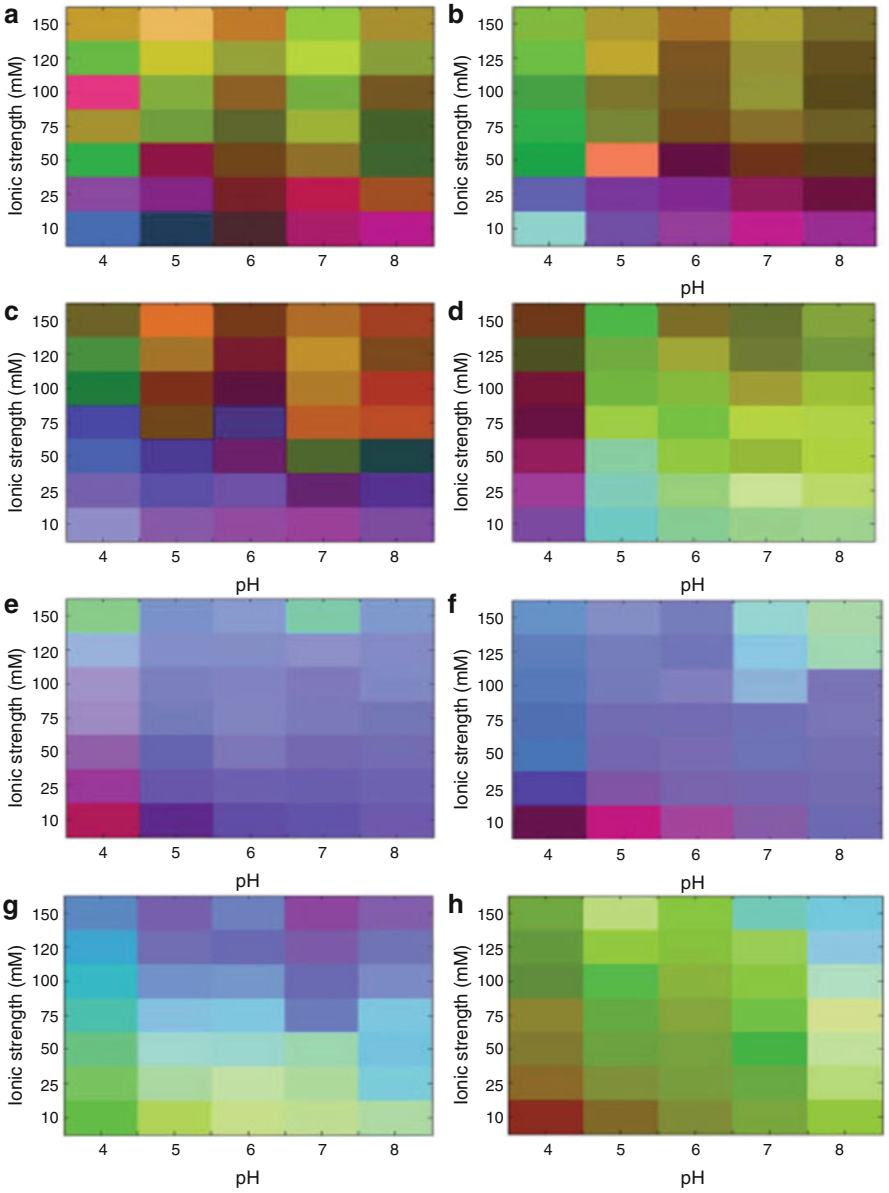

Fig. 6.15 Ionic strength-pH empirical phase diagrams of various nonviral (plasmid DNA) gene delivery complexes with cationic lipids and polymers (**a** and **e**, 1,2-dioleyl-3-trimethylammonium-propane (DOTAP); **b** and **f**, DOTAP/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (D/D); **c** and **g**, poly-L-lysine (PLL); **d** and **h**, polyethyleneimine (PEI) at positive/negative charge ratios of 0.5 (**a**–**d**) and 4 (**e**–**h**). Phase diagrams are generated from DLS (the size of the complexes), CD (changes in the secondary structure of DNA), and fluorescence (condensation of the DNA) studies [41]. Reproduced with permission from John Wiley and Sons (J. Pharm. Sci.)

known of these HTP applications at present are vaccine antigen identification and optimization of manufacturing process parameters (e.g., cell culture and purification conditions). No doubt other HTP applications will soon be forthcoming.

New approaches to vaccine development often follow advances in the sister world of therapeutic proteins. Much of what was described above was originally developed for application to therapeutic proteins. Currently, the largest class of therapeutic proteins is that of monoclonal antibodies. Thus, an ongoing inspection of this rapidly growing field is worthwhile for the vaccine formulation scientist. For example, a number of techniques including differential scanning fluorimetry [44, 45], the use of an increasing number of extrinsic dyes [8, 46] and flow cytometry [47], characterization of aggregation [45, 48–50], and PEG precipitation for solubility studies [51] have all been recently performed in HTP modes as part of formulation development of monoclonal antibodies. The use of more standard biochemical techniques such as HPLC and capillary IEF has also been adapted to HTP setups to examine the stability of these biopharmaceutical formulations [52, 53]. In addition, characterization of the glycosylation state of monoclonal antibodies [54] and influenza viruses [55] has been performed using HTP methods. Among many other HTP applications, more theoretical approaches are also underway [56].

It has also been recently become evident that the internal motions (often referred to as flexibility or dynamics) of macromolecular systems are related to their stability [57]. In this regard, a wide variety of methods such as isotope-exchange, ultrasonic velocimetry, pressure perturbation DSC, red-edge excitation fluorimetry, UV absorption derivative slope spectrometry, and fluorescence lifetime anisotropy, which are all adaptable to HTP applications, can be used to characterize dynamic motions in macromolecular systems and thus are applicable to examine vaccine antigen dynamics [57–60]. Such data have already been analyzed by the EPD methods [58].

There is little doubt that the use of these new HTP approaches to examine monoclonal antibody stability and flexibility will be adapted to the study of vaccine antigens in the near future. Thus, the availability and utility of various HTP methods will only continue to increase over the next few years, making the vaccine formulation development process an increasingly effective and efficient way to provide thermostable vaccines that maintain potency during manufacturing, storage, and administration.

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Chapter 7
Exploring Novel Analytical Tools to Improve Characterization of Vaccine Formulations

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7.1 Introduction

7.1.1 Approved Vaccines and New Trends

Safety and tolerability are probably the two most important characteristics of a modern vaccine beyond efficacy [1]. This is particularly relevant as vaccines have proved to be the most successful medical intervention after water sanitization [2].

Based on their preparation vaccines can be classified into three major categories: live vaccines, killed or attenuated vaccines, and component vaccines. Each one of these categories has very different and peculiar advantages and disadvantages.

Combining previous points with the need to use vaccines as a tool to protect weak segments of the population and with a more refined knowledge on the root causes of the reactogenicity caused by vaccines of the early days we now understand why vaccines are evolving to a specific direction which has a much lower complexity in terms of components [3]. Nowadays the 14 vaccines routinely administered to kids contain about 200 viral or bacterial components with respect to the over 3,000 present in the 7 routine vaccinations of the 1980s, meaning a component's complexity reduction of around 30 folds in 30 years. This huge reduction in vaccines components complexity has gone hand in hand with a reduced immunogenicity and the need to restore it by different means, so that the majority of the vaccines currently under development try to take advantage of novel technologies and recent knowledge aiming at this [4].

The discovery that the particles of the hepatitis B surface antigen (HBsAg) found in infected people were immunogenic and protective but noninfectious prompted the efforts to purify those particles from chronic carriers and later stimulated the

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idea to produce the antigen in a recombinant way [5, 6], which was finally licensed in 1986. Later the recombinant approach was followed for a recombinant Lyme disease vaccine [7] and for a quadrivalent human papillomavirus (HPV) vaccine [8] and many others in the pipeline paving the way to one of the most widely used vaccine preparation approaches employed nowadays.

Components-based vaccines are characterized by highly purified proteins, recombinant or not, or glyco-conjugates and are often combined with an adjuvant. The role of the latter is to boost the immunogenicity of the antigens [9, 10] that is per se much lower outside of a context in which other components of the pathogen are not present in a live attenuated or killed fashion as it happens in the preparation of different categories of vaccines.

This brings to the conclusion that vaccines are evolving to specifically drive immune system's attention only towards those components for which it is necessary to mount the appropriate immune response.

Furthermore the technology advancement that characterizes this vaccine generation is not only important for its impact on the quality of the components used in each formulation but also for the improvements achieved in the control and definition of the characteristics of each vaccine component.

Thus vaccine characterization serves to address comparability of product characteristics throughout its lifecycle as a whole and in each of its components. In conclusion we will see how vaccine characterization can now be more precise than ever because of the nature of vaccines' preparation, because of the growing knowledge in several fields ranging from immunology to biochemistry and physics and because of the sophisticated analytical tools available.

7.1.2 Characterization of Vaccines

Focusing our attention onto components vaccines and on the characterization of the final drug product without deliberated consideration for the characterization needs of the single drug substances that make up the final drug product, we can make a first distinction between the challenges associated with the characterization of an adjuvanted vs a non-adjuvanted formulation.

All vaccine formulations need to be characterized at least by the analysis of some basic parameters like pH, osmolality, appearance, endotoxin content, pyrogenicity, sterility, identity, and integrity of the antigens. All these parameters need to be constant or within a narrow range for acceptance and possibly for the longest time possible thus determining the expiration characteristics of the product. Each assay is specifically relevant to assure specific qualities of the product, pH, and osmolality for example, contribute significantly to control pain following vaccination, this typically occurs immediately or within minutes after inoculation. Pain can be caused by many components in the vaccine such as stabilizers, high or low pH, high osmolality, or preservatives. The pyrogenicity assay is fundamental to determine the potential to induce fever, endotoxin monitoring is important because of its strong

pro-inflammatory potential while sterility, antigen identity and integrity are of obvious relevance. If these assays appear easy for a non-adjuvanted formulation, the challenge of these formulations may reside in making a long-term stable antigen solution with a strong need of excellent formulation and biochemical skills.

The presence of an adjuvant instead significantly increases the skills and efforts necessary to evaluate some of these parameters. Even if the role of the adjuvant is well recognized, regulating authorities pose very strict requirements before approving any new adjuvant for human use; there is no surprise then in finding only a handful of them. Currently approved adjuvants for human use can be divided into mineral salts, oil-in-water emulsions and liposomes [11, 12]. Aluminum hydroxide and phosphate or calcium phosphate are the safest and by far those with several decades of use history, while oil-in-water emulsions, MF59 and AS03, have been introduced much more recently and the eldest of them (MF59), now counts on nearly 15 years of use in humans. To this list it was recently added the adjuvant system 04 (AS04) from GlaxoSmithKline, a combination of aluminum hydroxide and monophosphoryl lipid A (MPL), a microbial-derived TLR4 agonist.

7.1.3 Characterization of Mineral Salts Adjuvanted Vaccines

Mineral salts adjuvants are characterized by at least three major properties, a highly charged surface, positive or negative, a very large surface area, and by a pretty defined particle size distribution [11, 13]. Combination of these characteristic gives them a very high adsorptive capacity for a broad set of antigens. Adsorbed antigens are attracted to the surface of the adjuvant surface and adsorb to it with different strengths, described by a second parameter, the adsorptive coefficient.

These characteristics then introduce the need to analyze and control many more parameters with respect to a non-adjuvanted formulation, like particle size, antigen adsorption, and adsorption stability, impact of adsorption on antigen identity and integrity and possibly surface charge potential. In the case of adjuvant mixtures like AS04 there are, as an additional layer of complexity, assays required for the characterization of the MPL and its impact on the other formulation's components. While some of these characteristics may be simply measured with appropriate techniques, some other may require development of assays "on purpose."

The analysis of antigen adsorption, for example, is of paramount importance [14]. It can be divided into the analysis of antigen adsorption over a specified time and temperature range and in the analysis of the antigen post-adsorption. There are no guidelines specifying whether the antigen needs adsorption or not, although all components present need to be justified and more so the adjuvants. There are at least a few examples where it is recommended, but there are clear guidelines specifying that whatever the adsorption extent is it needs to remain constant for all the points of the analysis which is pretty intuitive if we consider that the characteristics of the product and of the components need to remain constant as we saw earlier. Due to the nature of the interactions occurring between the antigen/s and the mineral salts

adjuvant, it is pretty common to observe complete or nearly complete antigen adsorption, this leads to the conclusion that the analysis needs to be further extended. As an example we can consider a common way to look at antigen adsorption to highlight the pros and cons of basic adsorption characterization. Aluminum hydroxide containing formulations, for example, can be centrifuged to separate the adjuvant from the liquid phase and then the amount of antigen in the liquid phase can be quantified by different means with different degrees of precision determining the level of un-adsorbed antigen/s and indirectly the amount of adsorbed antigen/s. To determine the amount of adsorbed antigen, there are currently two major possibilities, dissolve the adjuvant gel or treat the adjuvant in order to release the bound antigen/s. This step can be easily performed by treating the aluminum pellet with agents that either dissolve the gel or interfere with antigen/s binding, respectively. Knowing the nature of the force/s driving antigen adsorption onto the surface of the adjuvant is critical to determine how to interfere with adsorption and attempt antigen desorption. Antigen adsorption onto the surface of aluminum hydroxide is mostly driven by ligand exchange, electrostatic forces, hydrophobic interactions, van der Waals forces and hydrogen bonding, thus, to interfere with adsorption the most commonly used techniques employ high salt or phosphate concentrations and/or pH that does not support antigen adsorption to interfere with all charge-related contributions, ethylene glycol or detergents to interfere with hydrophobic interactions or combinations of these agents. As an alternative it is possible to attempt dissolution of the aluminum gel by sodium citrate solutions [11]. In all these cases it is very relevant but often neglected to understand the impact of the formulation and desorption conditions used, on the stability of the antigen/s per se, as a nonsuccessful antigen recovery from the formulation supernatant or desorption process may be confused with a strong adsorption of the antigen while it could instead be antigen precipitation. Thus after assessing antigen stability under formulation conditions in absence of the adjuvant, it is important to understand the impact of desorption conditions on the bio-physic-chemical stability of the antigen; once conditions maintaining appropriate antigen stability are established desorption can be attempted. It derives that antigen desorption is not as straightforward as it seems as it depends on the combined nature of the antigen/adjuvant interaction and on the stability of the antigen under the desorption conditions, so that starting from a restricted panel of conditions interfering with the basic nature of each of the factors influencing adsorption each antigen may need development of its special "desorption cocktail." It is also important to consider that adsorption and formulation aging may themselves induce changes in protein conformation impacting antigen stability as reported in a few recent reports. As a matter of fact it has been reported in several papers and for different antigens that desorption becomes more difficult as the formulation ages; this phenomenon has been associated with antigenic structural changes interpreted as indication of partial unfolding. Such unfolding would result in a greater degree of contact between the antigen and the adjuvant surface and so resulting in an increased resistance to desorption [15, 16].

Particular attention needs to be dedicated also to the characterization methods used to quantify the antigen/s because of the different nature of the interference that the

excipient combination used for desorption may cause in different assays. A very common semi-quantitative method largely employed because of the low impact of various interferences is SDS-page, the method is simple and fast and allows Western blotting to determine identity of the antigen/s but has limitations in terms of an accurate quantization and detection of little qualitative modifications. Other methods for quantization rely on UV absorbance or immunoassays but taken alone are more prone to misinterpretation as the amount of information they provide is lower with respect to SDS-page, optimally they would need to be combined especially in those cases where more than one antigen is present and recoveries are not complete.

In conclusion, antigen desorption can be challenging and several parameters need to be kept under consideration for an accurate evaluation; furthermore, this needs to be considered in conjunction with the limitations posed by the desired analysis on the desorbed antigen/s. We will see how depending on the level of accuracy desired in the analysis of the desorbed antigen/s, different scenarios can be envisaged with an increasing level of complexity and challenge directly proportional to the increasing level of the desired characterization.

7.1.4 Characterization of Oil-in-Water Emulsion Adjuvanted Vaccines

Both oil-in-water emulsions approved for human use are a combination of squalene oil and Tween 80 surfactant with either a second surfactant like SPAN 85 (MF59) or α-tocopherol (AS03) in mildly acidic citrate buffer or in neutral PBS, respectively. Oil-in-water emulsions appear very different from the mineral salts discussed earlier and are characterized by a narrow oil droplet size and by the presence of larger particles that are used as an indicator of aging [17]. Several techniques are available to monitor interactions among emulsion components, other to determine component concentration, particle size, charge, and other to ases interfacial properties.

As in the case of formulations containing mineral salts also in the case of emulsions the antigen/s and the adjuvant need to retain their characteristics upon formulation, the presence of the emulsified oil droplets and of some spare detergent offers the antigen/s a very different environment with respect to other formulations. The hydrophobic droplets and the residual free detergent could be viewed from a biochemical stem point as more challenging to generate a long-term stable protein formulation, thus impact on antigen stability needs to be carefully addressed.

Also in this case the analysis of antigen post-formulation needs particular attention. Usually an ultracentrifugation step is required to separate the oil droplets from the remaining solution, if no strong interaction occurs between antigen/s and the droplets they should be found in the clear subnatant solution and SDS-page should be again of great help for a basic semi-quantitative evaluation. Also in this case there are various pitfalls that need to be considered especially if the desired final analysis' accuracy is high.

Table 7.1 Recently described techniques used for direct vaccine characterization

Direct	Suspension	Emulsion
Isothermal calorimetry (ITC)	x	x[a]
Differential scanning nano calorimetry (nDSC)	x	x
FACS	x	–
Dynamic light scattering	x	x
Electron microscopy (EM)	x	x
Zeta potential	x	x
Static light scattering	x	x
Direct alhydrogel formulation immunoassay (DAFIA)	x	–
Fourier transformed infrared (FTIR) spectroscopy	x	–
Front face fluorescence spectroscopy	x	–
Raman spectroscopy	x[a]	–
Single particle optical sensing	–	x
NMR	–	x
Surface tension	–	x

[a]Theoretically possible but not tested

7.1.5 Characterization of Vaccines: Novel Tools

We have seen the general principles that apply to component vaccine's characterization adjuvanted with mineral salts or oil-in-water emulsions, now let's focus attention on the most recently described analytical tools reported in literature. These techniques can be distinguished in two main classes based on their ability to provide information directly on the entire formulation (Table 7.1) or indirectly after appropriate formulation treatment. All of them have been applied with some success mostly for research purposes on exploratory vaccines to deepen comprehension of the basic mechanism governing antigen–adjuvant interaction and stability.

7.1.6 Calorimetry

Both ITC and nDSC can provide very useful informations on the thermodynamic parameters that characterize protein adsorption or interfacial interactions. It should also be mentioned immediately that these techniques are not very user-friendly especially when working in the presence of an adjuvant and that the instruments are quite expensive, for these reasons not so many research groups provide regular characterization data via calorimetry and even less are applied to vaccine characterization.

Nonetheless, thermal stability of proteins in the presence or absence of the adjuvant can be followed deriving important parameters like the transition temperature (T_m) and the shape of the thermogram, both parameters provide the most immediate information on increased or decreased stability of the protein and can be used in pre-formulation work to scout the best formulation conditions to improve protein

stability, although not conclusive, it is worthwhile mentioning that a few studies highlight how the excipients that are used to stabilize a protein in solution maintain a stabilization effect also on the adsorbed protein [18].

Assessing reversibility of the melting reaction under study offers the unique opportunity to understand the point of no return in stressing a protein and so could in theory be used to determine the limit stress conditions and have a ranking of the shelf-life of different formulations. Free energy, enthalpy, entropy can also be derived and provide information on the mechanism driving the interaction with the adjuvant and the importance of protein conformation effects versus surface or interface/protein interactions but require an extensive and demanding work; finally stoichiometry determination and binding constant (K_a) can be derived and roughly compared with the adsorptive capacity and coefficient respectively, giving a measure to the amount and strength of the interaction. Interestingly calorimetric techniques take into consideration the antigen and its interactions as a whole. It should also be noted that calorimetric assays have also the potential to be successfully used to asses protein stability of vaccine's components to demonstrate that antigens after desorption posses similar T_m and thermogram profile as the parental antigens before adsorption on aluminum hydroxide. Last but not least, both techniques are sensitive enough to work at 0.1 mg/mL protein concentration that is commonly found in many vaccines.

7.1.7 FACS

FACS is probably the best-suited technique to characterize a suspension as it is of incredible success in many areas of biology but is also among the most neglected characterization techniques employed in the vaccine field with only a handful of publications. Recently the use of FACS has been rescued to determine the presence of silicone oil droplets released from siliconized vessels [20], and it has also been positively evaluated in a POC experiment to characterize antigen loading in an aluminum hydroxide formulation [21]. The assay takes advantage of an antibody directed detection of the antigen on the surface of alum after a blocking step. The advantages demonstrated are first of all the possibility to have an in situ detection method with a tool like an antibody that can recognize structurally relevant protein portions, secondly the sensitivity and accuracy of antigen determination combined with information on antigen distribution and morphological analysis, opening up the possibility to analyze in a very fine way how the adsorption process and formulation parameters impact antigen distribution. It goes without saying that a specific antibody binding must be prevented at all times to avoid data misinterpretation. Finally it is important to stress again the possibility to probe structural integrity and accessibility of the antigen directly on the surface of the adjuvant.

Based on its own characteristic that combines light scattering and fluorescent detection, this technique may prove very useful also to characterize protein aggregates as well as other types of adjuvanted formulations.

7.1.8 Light Scattering

When it is necessary to measure particle size, the static or dynamic light scattering (DLS) techniques are the most immediate choice, depending on the expected size to be measured one prevails on the other with the static technique suited to measure particles above 0.5 μm and up to the mm range, while the dynamic is more suited to measure particles below 5–10 μm down to a few nm. As with all techniques there are critical parameters that deserve attention for an appropriate use, so care should be paid to apply the most appropriate mathematical model to analyze the data and extrapolate results especially in the DLS where also the choice of the other parameters needed for the final calculation should be carefully based on experience only if the parameters cannot be measured. This is particularly true in this case as the results can be completely overturned in case of an unwise choice. DLS can also be applied with the necessary adjustments to derive information on zeta potential. Zeta potential is measured using a combination of the measurement techniques: electrophoresis and laser Doppler velocimetry, sometimes called laser Doppler electrophoresis. This method measures how fast a particle moves in a liquid when an electrical field is applied—i.e. its velocity. Once we know the velocity of the particle and the electrical field applied we can, by using two other known constants of the sample—viscosity and dielectric constant, work out the zeta potential. This parameter is important to understand how surface charge changes as a function of antigen adsorption and formulation conditions possibly anticipating the behavior and stability of nanoparticles in solution, in fact the development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the surface. Thus an electrical double layer exists around each particle and influence how each particle interacts with the neighboring ones, pH is probably the most important parameter that needs to be highlighted when discussing zeta potential data that are otherwise useless.

7.1.9 Direct Alhydrogel Formulation Immunoassay

This technique was described very recently and is basically an ELISA [22]. The antigen content of an aluminum formulation is measured by a specific antibody in a multi-well plate after appropriate washing, blocking and a final step of signal amplification via a fluorescently labeled secondary antibody. The advantages of this approach derive from the high sensitivity and by the use of antibodies to detect the antigen directly in the final formulation without the need to desorb the antigen and with the further opportunity to detect structurally relevant epitopes with appropriate monoclonal antibodies, the limit of this approach is mostly represented by the absence of further characterization of the formulation during the analysis, so that it is not known what happens to the antigen during the process, does it remain adsorbed on the surface of the adjuvant or falls off and sticks to the well. In any case this technique is amenable to further development and is potentially interesting.

7.1.10 Electron Microscopy

Negative staining electron microscopy was recently applied to the characterization of Cervarix by Deschuyteneer and colleagues [19]. In this experiment the technique becomes very handy as the vaccine antigens are virus-like particles (VLPs) whose structures were shown to be similar to that reported for the native virions by a number of techniques, the great majority of the particles for both antigen types present in the final formulation of VLPs appeared sub-spherical and single-shelled. The size of the HPV-16 VLPs ranged between 35 and 50 nm with a main peak at 40 nm, while the size of the HPV-18 VLPs ranged from 40 to 55 nm with a peak at 50 nm, electron microscopy of the adsorbed VLPs formulations showed direct structural retention. Clearly this technique may not be always useful because most antigens do not behave like a VLP but shows very nicely that under peculiar circumstances it may well be.

7.1.11 Front Face Fluorescence

Fluorescence spectroscopy has been applied mostly to follow the signal of those natural tags like tryptophan, tyrosine, and phenylalanine that are natural components of most proteins.

Because of their aromatic nature these amino acids are able to fluoresce as a consequence of appropriate excitation. Interestingly, polarity changes in the environment surroundings these residues has an impact on maximum and intensity of emission of these residues. To this purpose tryptophan are probably the most useful due to the well-separated excitation and emission spectra and the yield of signal upon excitation. In this case an emission maximum between 325 nm and 330 nm is usually considered to derive from a tryptophan embedded in a hydrophobic environment, while an emission maximum around 350–355 is usually considered deriving from a tryptophan embedded in a hydrophilic environment, like a free amino-acid in solution [23, 24]. Thus, the above-mentioned characteristic of these amino acids serves as a very sensitive natural probe of changes more or less pronounced that can happen around these residues as a consequence of formulation with different adjuvants. It should also be mentioned that the fluorimeters employed for these measures are not conventional as in these cases the fluorescent radiation would scatter on each particle encountered while crossing the volume of the cuvette and would be completely lost before arriving to the detector, so the geometry of the instrument in this case is such that the exciting radiation hits the sample at an angle of 45° and the derived signal is finally detected at 90° so that the resulting fluorescent radiation does not need to travel across the entire suspension with the advantage of minimizing losses because the measured fluorescence is the one bouncing out of the cuvette. This technique is usually carried out in combination with a slow thermal scan and has the advantage of working at the low protein concentration often used in commercial vaccines. Finally, this technique highlights general changes in the environment surrounding aromatic residues with implications on tertiary and quaternary structure of protein antigens [15, 16].

7.1.12 Fourier Transformed Infrared Spectroscopy

Fourier transformed infrared (FTIR) spectroscopy provides an estimate of the changes in secondary structure composition. This method takes advantage of special deconvolution methods to separate and integrate overlapping amide I infrared adsorption bands associated with α-helix, β-plated sheet and random structures. In this method the spectrum is related to the small effects of the regular secondary structure on the vibrational frequency of amide groups in the protein linkage. Unfortunately the technique is not very sensitive and cannot be easily applied at protein concentration close to those usually employed in vaccines, thus even in those cases where some limited data have been generated, the results are highly impacted by the high protein concentration adsorbed on alum so secondary effects on observed protein structure could influence the observed results. Similar to FTIR another technique that is very useful to look at protein secondary structure is CD; unfortunately, this technique is also highly impacted by the presence of the adjuvant or formulation excipients so both the major techniques that provide information on the secondary structure of proteins are of little help in the case of adjuvanted formulations.

7.1.13 Single Particle Optical Sensing

This technique [25] is an extremely useful tool to evaluate the number of large particles (≥5 μm) in an emulsion. This parameter is extremely important as oil droplets of that size or larger could result in potentially dangerous particles once injected into a patient, for this reason much care should be taken to control and possibly avoid them. Large particles are the result of emulsion destabilization via coalescence, which is the inevitable outcome of these thermodynamically unstable dosage forms. For this reason it is extremely important to outline the globule size limits and to ensure that the dosage form does not prematurely progress to a stage where the process of coalescence advances to a critical point before the end of its shelf-life, where the safety of the infusion is compromised.

7.1.14 Surface Tension

When incorporating a small molecule in an emulsion another parameter that needs attention is surface tension [26] as changes in this parameter may result in profound effects on the size of the emulsion itself and on the distribution of the added drug. It goes without saying that also this parameter is of paramount importance to control emulsion's stability.

7.1.15 Indirect Detection

We have seen at the beginning of the chapter that indirect detection is the base of most of the assays commonly accepted for release. These assays need at least one step in which the adjuvant is separated from the antigen, as discussed, attention should be taken to minimize all those reagents and conditions that could have a profound effect on the stability and integrity of the antigen. If appropriate precautions and controls are taken, then the desorbed sample can be considered as only minimally impacted by the desorption or separation process, so that the differences observed could be tentatively associated with the formulation step occurred before desorption/separation and analysis. Although not easy to set up on samples that have been desorbed with the aim of high salt concentrations, high ionic strength, detergents and extremes of pH or combinations of the above, all chromatographic techniques should prove very useful, given careful choice of excipients that do not interfere with the downstream analysis. Reverse phase chromatography, for example, is a very good and reliable technique to quantify precisely the amount of desorbed antigen and its identity thus coupling well with the more qualitative results obtained by SDS-Page and Western blot. Drawback of the technique is the strong need to have samples prepared in a very accurate way and the big impact that minor variations can have on the final outcome together with the generally negative impact of salts and detergents on the analysis so that each time desorption conditions should be chosen carefully to avoid unwanted interference.

Size exclusion chromatography would also be of extreme value to understand what changes have occurred on the desorbed antigens in the process of adsorption and desorption in comparison with the original untreated sample, particularly it would prove useful for multimeric antigen to monitor their quaternary structure and to probe the presence of aggregated protein in general.

Similarly, all other chromatographic techniques from ionic exchange to affinity interaction would be appropriate tools to probe changes in the characteristics of the antigen post desorption, with ionic exchange being very suited to monitor charge differences possibly deriving by deamidation events. It should be mentioned here that deamidation is one of the most common post-translation modification occurring in recombinant proteins, it usually involves asparagine and glutamine residues with the first being by far more frequently deamidated with respect to the second [27, 28]. The process results in generation of an aspartate or isoaspartate residue thus adding a negative charge to the protein with consequences on the immunogenic side. The reaction can be either enzymatic or not, in the case of highly purified proteins, as is the case of vaccine components, the nonenzymatic reaction is usually responsible for the deamidation process, thus care should be taken to avoid those circumstances that favor the reaction, above all slightly basic pH, the second most relevant factor affecting the rate of deamidation is the primary sequence of the protein with small residues like glycine at $N+1$ position being by far the most frequent, followed by small polar amino acids [29], other factors favor the reaction like $pH < 2$, accessibility to the solvent and some degree of conformational flexibility in the

region of the molecule [30]. On the other side, another very common chemical degradation occurring in proteins is oxidation.

Oxidation may arise from a number of well-characterized sources, reactive oxygen species responsible of most oxidation events depend on the availability of molecular oxygen dissolved in any aqueous formulation followed by contaminant traces of metal ions, ultraviolet light and peroxides [31–33]. The most commonly oxidized residues in a protein are methionine, cysteine, tryptophan, histidine, phenylalanine, and tyrosine also in the case the oxidation reaction the modified residue results in substantial modification of its function or immunological properties with implication on protein instability as well.

Both chemical modifications described above are potentially very well recognizable by mass spectrometry approaches combined with peptide mapping. This approach is very nicely described in the paper of Vessely et al. [34], where the authors analyzed chemical modifications of peptides derived by proteolytic digestion of vaccine candidates after desorption and separation from alum salts. Desorbed proteins were digested with a specific protease and the peptides mixture was analyzed by mass spectrometry directly [i.e., matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)] or after chromatographic separation (i.e., liquid chromatography–mass spectrometry, LC–MS). Changes in the mass-to-charge ratio (m/z) of the peptides can be indicative of a chemical modification, which can be further explored by other analytical techniques such as tandem mass spectrometry (MS/MS).

7.2 Challenges, Limitations, and Opportunities

To date there are a number of studies on model formulations with nonclinically relevant antigens that in the last 10 years have been used to test hypothesis on the mechanism of antigen adsorption on mineral salts adjuvants or to study the stability of emulsion-based formulations. Although these studies exist, some fundamental mechanistic questions are still open and will attract much attention in the near future. For example, it is still not clear what the effects of adsorption are on antigen stability, are they stabilizing or destabilizing? Are these effects reversible or irreversible? How do they impact immunogenicity and protection? All these are crucial questions to answer in a regulatory environment. Clearly the major limitations rely on the need to assess the antigen while still interacting with the adjuvant. If the progresses of recent years have started shedding light on these interactions, a final answer will require development of new assays or integration of characterization data from different techniques all at once. So the limitation becomes an opportunity for the future not only because there is wide space to improve the characterization of novel adjuvants but also for new delivery systems and formulations. In nearly 80 years since the first vaccine adjuvant was approved by the FDA (United States Food and Drug Administration), no other adjuvant has been approved by the FDA for use in humans, while EMA has been more prone to introduction of novel adjuvants

Table 7.2 Categories of adjuvants used in vaccines in development, testing or use

Name	Class	Stage
Alum	Mineral salt	Licensed
MF59	O/W emulsion	Licensed (EU)
Liposomes	Lipid vesicles	Licensed (EU)
Montanide	W/O emulsion	Phase III
PLG	Polymeric microparticle	Phase I
Flagellin	Flagellin linked to antigen	Phase I
QS21	Saponin	Phase I
AS01	MPL + liposomes + QS21	Phase II
AS02	MPL + O/W emulsion + QS21	Phase II
AS03	O/W emulsion + α-tocopherol	Licensed (EU)
AS04	MPL + alum + HBV	Licensed (EU)
RC-529	Synthetic MPL + alum	Phase II
Iscom	Saponins + cholesterol + phospholipids	Phase I
IC31	Peptide + oligonucleotide	Phase I
CpG 7909	Oligonucleotide + alum, MF59 + oligonucleotide	–
ISS	Oligonucleotide alum	Phase II
MF59 + MTP-PE	Lipidated MDP + O/W emulsion	Phase I

licensing three new one in recent years. Nevertheless, substantial investment in adjuvant technology continues and new patent application filings progresses together with new clinical studies.

It is clear from Table 7.2 [12] that the characterization of work described in recent past and in this chapter covers only a minor part of the characterization necessary to characterize these new adjuvants and the associated formulations, so new developments are to be expected.

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Chapter 8
Immunobioengineering Approaches Towards Combinatorial Delivery of Immune-Modulators and Antigens

Ankur Singh, Pallab Pradhan, and Krishnendu Roy

8.1 Introduction

Vaccines have been one of the most important discoveries of modern medicine. They are the primary mode of protection against a wide range of infectious diseases and, if effective, can provide long-lasting immunity. Despite recent advances in our understanding of the immune system, prophylactic vaccines against chronic infectious diseases and immunotherapeutic vaccines against cancer remain elusive. Unlike preventive vaccines that have virtually eradicated fatal diseases like polio and smallpox, immunotherapy of chronic diseases and established or unexpected infections, for example human immunodeficiency virus (HIV), has yet to demonstrate global clinical success. Even for diseases where preventive vaccines are available, for example influenza, the protection is transient and requires multiple administration and yearly immunizations. In addition, most cancers and emerging infectious diseases, like the H1N1 influenza, and drug resistance infections like tuberculosis, need new transformative strategies to increase protective immunity many folds over currently available vaccines. Successful immunotherapy using vaccines requires effective strategies to penetrate tissue barriers, efficiently target antigens, adjuvants and immune-modulators to immune surveillance cells, provide strong stimulatory effects to activate those cells, and modulate the cellular response

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appropriately and efficiently in order to generate potent antiviral or anticancer immunity. The emerging field of immunobioengineering provides new concepts and strategies to design materials, antigens, and adjuvants to induce potent immune response; and engineer vaccine delivery systems to modulate the behavior of immune cells [1]. In this chapter we review the emerging approaches in immunobioengineering with specific focus on delivery formulations for multiple immune-modulators and antigens.

8.2 Need for Combinatorial Delivery Systems

Live-attenuated or inactivated vaccines from whole microorganism have antigens and stimulatory components (like bacterial CpG motifs, RNAs, and glycolipids) that can readily activate the host immune system. However, these are not absolutely safe to vaccinate with. Alternatively, new generation vaccines have focused on safer DNA and recombinant protein-based vaccines where the antigens are purified from microorganism and delivered. Since these purified antigens often lack danger signals that prime immune cells, successful immunotherapy using DNA or protein vaccines, especially in humans, requires effective strategies to penetrate tissue barriers, activate immune cells as well as enhance antibody and T-cell-mediated immune response. Failure of vaccines against chronic targets can be attributed to several factors that independently, or in combination, influence the extent of immune response [1]. These include the low numbers of immature DCs sampling the bloodstream, skin, and tissues at any time (approximately 1% of cells) [2, 3] as well as intrinsic inability of conventional vaccines to induce a potent T-cell response (Fig. 8.1). Immune modulation can be characterized by the interplay of secreted cytokines. For example, Interleukin (IL)-12 fosters development of T helper 1 (Th1) cells, whereas up-regulation of IL10 and IL6 promotes a Th2 lymphocyte response [4]. For viral, cancer, protozoal, and fungal infections, a strong Th1 cellular response plays an important role in destroying the disease-causing cells [5–7] while a strong Th2 response is essential for fighting against extracellular pathogens, allergies, and helminthic diseases [8]. Thus it is critical for an immunotherapeutic strategy for infectious and cancer diseases to either "drive" the antigen-specific immune response strongly towards Th1 phenotype or create an appropriate balance of T helper response for effective protective immunity. Thus, there are several components to a successful vaccine, including (a) recruitment of a large number of immune cells and efficiently target antigens, adjuvants and immune-modulators to professional antigen presenting cells (APCs) (b) provide strong stimulatory effects to activate those APCs (c) modulate the APC response to generate desirable potent antigen-specific immunity and (d) provide these functionalities in an integrated delivery platform that can be scaled up and translated into clinical practice.

Commonly used antigens include plasmid DNAs, recombinant protein antigens, and purified lystates from diseased cells that can alarm the immune system but are limited in their efficacy to meet the aforementioned characteristics. Plasmid DNA encoding for particular antigens can generate long-term humoral and cellular immunity with efficient generation of CD4+ T helper cells and CD8+ cytotoxic T cells in

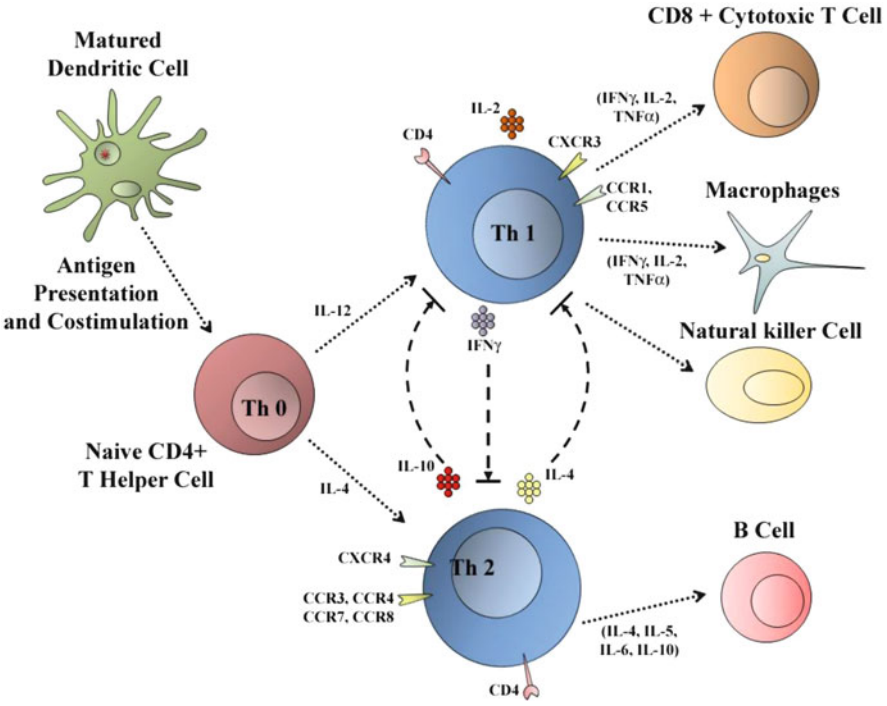

Fig. 8.1 Antigen presentation and differentiation of naïve T helper cell into Th1 or Th2 phenotype

animal models of allergies [9–11], and infectious diseases as reviewed by Gurunathan et al. [12] and Donnelly et al. [13]. Administered alone, both DNA and protein antigens are weakly immunogenic in humans, with short half-life, require high antigen doses, and so far have been insufficient in eliciting significant cellular and antibody response [14, 15]. Although unmethylated bacterial sequences (known as CpG motifs) in plasmid DNAs can act as strong immunostimulatory entities, especially for T helper 1 (Th1) mediated cellular immunity [16–19], the efficacy of the approach is limited to the animal models of infectious diseases [20–22] and allergy [10]. Similarly, synthetically derived peptide sequences and proteins offer the opportunity to design a particular epitope that can be used in vaccines to mount a specific and desirable immune response. Interestingly, protein vaccines are weakly immunogenic in absence of any stimulatory entities and often tend to degrade and get cleared from the body. Nevertheless, plasmid DNA and protein vaccines offer safer alternatives to viral vaccines and additional strategies to boost the antigen mediated immune response are required.

The need for combinatorial system arises from several primary challenges faced by protein or DNA-based human vaccines. For efficient activation of DCs and priming of T cells, it is necessary to co-deliver antigens and adjuvants (immune-modulators) to a large number of antigen-presenting cells which must efficiently process and present the antigens through MHC molecules while producing a favorable milieu of cytokines. One possible way to meet these complex and interconnected needs is through development

of synthetic vaccine delivery systems that can efficiently deliver multiple immunomodulatory molecules and antigens to the target cells in single delivery system [23].

8.3 Polymeric Delivery Systems for Modulation of Immune Response

Biodegradable carriers provide an efficient way to protect the vaccine antigen from the surrounding environment prior to delivery [24–27]. These carriers could be polymeric micro-and-nano-particles, polypolexes, lipid-based carriers, and degradable scaffolds. The simplest alternative to a bolus DNA vaccine would be a DNA–cationic polymer complex that could significantly improve the cellular uptake, prevent DNA integrity and the immunogenicity of the antigen. Cationic polymers like poly-L-lysine (PLL) [28], polyethylenimine (PEI) [29, 30] have been extensively used to study DNA vaccine immunizations. The outcome from such complexation is variable, for instance, mucosal administration of PEI-DNA vaccine has been shown to induce a mixed Th1/Th2 response including Jak-3, a marker for interferon gamma and IL10, a Th2 cytokine. Interestingly, PEI has immunostimulatory effect in the absence of formulated plasmid DNA [29, 30]. Another cationic polymer that has been used to deliver plasmid DNAs for vaccine purposes is chitosan [9, 31–33]. Roy et al. have demonstrated the effectiveness of orally delivered chitosan–DNA nanoparticles in inducing protective immunity in the peanut allergy mouse model [9].

8.3.1 Particulate Delivery Systems as Vaccines

Micro- and-nano-particles made of polyesters [34, 35], polyanhydrides [36], liposomes [37] can be used for antigen delivery to induce cellular and humoral immunity. Such systems offer opportunity to conjugate DC-specific ligands for targeted delivery, encapsulate or surface tether antigens and immunostimulatory adjuvants, increase the availability of antigen, and can also deliver both antigen and adjuvant to same cells [38]. Numerous polymers have been investigated as micro- or nano-particle systems and as injectable/implantable hydrogels for antigen delivery, the most commonly reported being synthetic polyesters, polyanhydrides, and natural polymers. The polyester, poly-lactic-*co*-glycolic acid (PLGA), is considered one of the front-runners in particle-based vaccine research since it is already approved by the FDA for use in other human applications (surgical sutures), has a well-characterized degradation profile, and is easily decorated using common conjugation chemistries. Typically, antigens are formulated in PLGA microparticles through water–oil–water (w/o/w) double emulsion process where an organic phase of PLGA is mixed with an aqueous phase of protein/DNA antigens and finally emulsified in a surfactant (like polyvinyl alcohol) to generate antigen encapsulated particles. The emulsification process can be detrimental to the antigen and thus results in denatured components and low encapsulation of antigens.

PLGA-based delivery systems can trigger a Th1 or Th2 immune response depending on the incorporated immunostimulants and antigens. Importantly, imma-

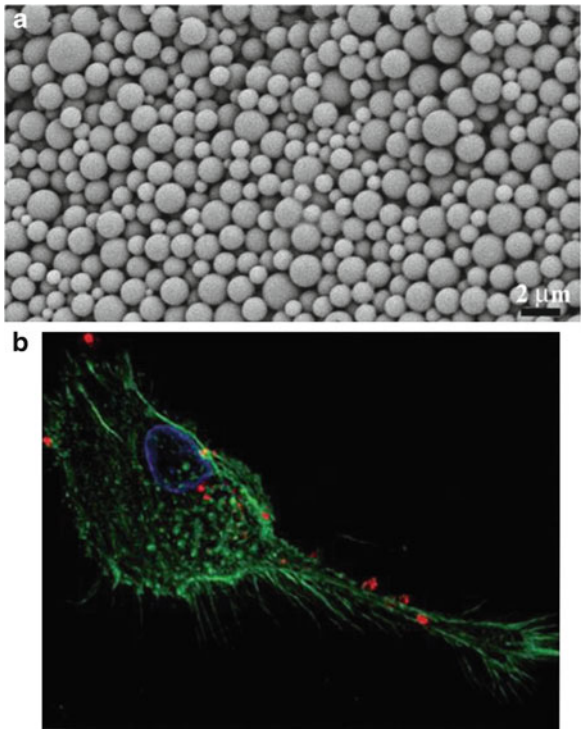

Fig. 8.2 DCs phagocytose microparticle formulations of PLGA in vitro. (**a**) Scanning electron micrograph (SEM) of PLGA microparticles (scale = 2 μm). (**b**) Peripheral blood mononuclear cell-derived DCs were incubated with rhodamine-conjugated dextran-encapsulated microparticles (*red*) for 5 h, fixed, and then stained with Hoechst dye for nucleus (*blue*) and phalloidin–Alexa Fluor 488 for actin (*green*). 3D fluorescent microscopy images indicate uptake of PLGA microsphere formulations. Intracellular rhodamine signals were seen as bright, localized spheres in PLGA-treated DCs. Adapted with permission from [39]

ture DCs can selectively internalize microparticulate system based on foreign material composition and size dimensions in nanometer to submicron to 10 μm range (Fig. 8.2) [27, 40–43]. Of these, particle size smaller than 5 μm is more favorable because of easier transport to lymphatics [44] and earlier studies have indicated generation of Th1-specific immune response [45].

Studies with immunization of HLA-A2/Kb transgenic mice with human cytochrome P450 CYP1B1 (CYP1B1), a weekly immunogenic, tumor-associated antigen encoding plasmid DNA formulated in poly(lactide-*co*-glycolide) (PLGA) microparticles elicited generation of human CYP1B1-targeted CD8+ T cells [46]. Numerous studies have been conducted using different combinations of DNA and protein antigens encapsulated in microparticles with successful demonstration in small animals or even nonhuman primates; however, a successful clinical translation is yet to be seen. Several research groups have developed nanoparticles with combinations of antigens and polymers and one advantage nanoparticles have over microparticles is higher antigen–polymer ratio; however, immune stimulation efficacy of the nanoparticles is debatable [47]. A few selected antigen delivery systems are summarized in Table 8.1.

Early studies have shown that combination of an antigen with polymeric particles elicits stronger immunity compared to bolus antigen [44]. Initial hypothesis was based on microparticles working as adjuvants that stimulate immune response; however, the adjuvancy could be attributed to denatured components of the vaccine

Table 8.1 Polymer based antigen delivery systems

Delivery system	Antigen	Comment	Reference
PLGA	Ovalbumin	Th1 type immunity with high IFNγ, low IL-4, IL-10 compared to antigen-alum	[48]
		Stronger Th1 type immunity with high IFNγ, low IL-4, IL-10 compared to antigen-microparticle and antigen-alum	[48]
		Predominant Th2 response	[49]
	Malaria	Intranasal immunization resulted in mixed Th1/Th2 with significant IFNγ, IgG2a, IgG1, IgE compared to alum and s.c, oral route	[50]
	HIV-1 (gp120)	Significantly high IgG, IgG2a production with encapsulated MPL administration	[51]
		Significantly high IgG, IgG2a production with encapsulated RC529 administration	[51]
	MenB	Significantly high IgG, IgG2a production with encapsulated MPL administration	[51]
		Significantly high IgG, IgG2a production with encapsulated MPL administration	[51]
	Tetanus toxoid (TT)	TT-CpG co-loaded nanospheres showed mixed Th1/Th2 response; high IFNγ, IgG2b, IgG3, and IgG1	[52]
	HBsAg	Stronger Th1-type immunity with 60 old higher IFNγ, low IL-4, compared to only DNA loaded microparticle and PBS	[38]
Chitosan	Peanut allergen	Oral delivery resulted in high IgG, IgG2a, and reduced IgE	[9]
Alginate	Tetanus toxoid (TT)	High IgA with TT-CpG co-delivery using alginate microparticles as compared to alum-TT or soluble TT-CpG; low IgG with microparticles than alum-TT	[53]
Chitosan-alginate	HBsAg	High IFNγ production with co-injection of HBsAg	[54]

during formulation process [55]. A critical limitation associated with encapsulation of antigens in microparticles is the damage caused by the homogenization-induced shear stress, unfavorable exposure to organic phases, and possible acidic environment of degrading microparticles. In addition, incorporated doses of antigen within microparticles are often low and thus impose a significant challenge in clinical translation to meet an effective vaccine dose.

8.3.2 Surface Presentation of Antigens Induce Stronger Innate Immunity

Recently, a major shift in particle-based immunotherapy strategy has been that presentation of nucleic-acid or protein antigens on microparticle surface (instead of entrapped inside) may significantly enhance their bioavailability and the resulting immune response [56–58].

8.3.2.1 Cationic Particulate Delivery Systems

Cationic microparticles have been used as DNA vaccines by adsorbing plasmid DNA on the surface of positively charged microparticles [59–61]. Cationic PLGA microparticle vaccine was first developed by Chiron Vaccines (now Novartis, Inc.), where a modified w/o/w double emulsion process was used by incorporating a positively charged surfactant cetyltrimethylammonium bromide (CTAB) to the aquaeous phase [61]. The resulting micropaticles had surface coating of cationic CTAB molecules and the negatively charged DNA could readily be adsorbed through ionic interaction. The potential of these cationic microparticles has been established in mice and nonhuman primate models with enhanced immune response compared to naked DNA. The ability to surface adsorb a wide range of plasmid DNA antigens resulted in successful demonstration of vaccine efficacy in tumor protection model of rat colon cancer [62], protective infectious model of tuberculosis [63], as well as humoral and cellular immunity in a hepatitis B surface antigen (HBsAg) [64]. Cationic microparticle-based vaccines can also be generated by simply blending other cationic polymers like poly(ethyleneimine) PEI or chitosan with PLGA [65, 66]. Most of the blended vaccines have shown potential in small animal models and await successful trials in larger nonhuman primate models. Recently, cationic CTAB and dimethyldidocylammonium bromide (DMAB) blended PLGA nanoparticles were reported; however, further preclinical evaluation is needed to ascertain their potential as effective vaccines [67].

Premature release of adsorbed DNA or the polycation/DNA complex from blended formulations can have unfavorable consequences, in vivo. Leaching out of free polycations like PEI imposes toxicity issues and difficulty in renal clearance [68]. To overcome these drawbacks, Kasturi et al. used conjugation chemistry instead and covalently bonded positively charged PEI to the carboxylic acid moieties on the surface of PLGA microparticles [59]. PEI has an excess of primary amines such that every third atom in the backbone of this polymer is a nitrogen atom, which could be protonated across varying pH. Using EDC/NHS bioconjugation chemistry, the carboxylic acid groups are activated and conjugated to the primary amines of PEI. The technique ensures covalent conjugation of minimal amount of PEI on the surface thus minimizing any potential toxicity issues. These covalently modified PEI-PLGA microparticles carrying tumor idiotype DNA antigen induced, even in the absence of any adjuvants, significant protective antitumor immunity in mouse model of B-cell lymphoma [60].

8.3.2.2 Anionic Particulate Delivery Systems

Anionic particulate systems have been explored as an alternative approach for the delivery of protein antigen to APCs to overcome the problem of antigen delivery by microencapsulation methods where loss of integrity of antigen structure and their functionality occurs due to exposure to harsh environments like shear forces, sonications, organic

solvent during the fabrication procedures. A novel anionic PLG microparticles was thus developed by Kazzaz et al. by inclusion of an anionic detergent sodium dodecyl sulfate (SDS) during the PLG microparticle formation [69]. The PLG-SDS microparticles were able to absorb p55 gag protein from HIV-1 by electrostatic interaction with 100% loading efficiency and induced CTL response and adjuvant effect by inducing antibody against p55 following intramuscular immunization in mice. Singh et al. [70] developed similar kind of anionic PLG microparticles replacing SDS with dioctyl sodium sulfosuccinate (DSS), which is used in many licensed pharmaceutical product. These PLG-DSS microparticles absorbed recombinant antigens from Neisseria meningitides type B (Men B) by electrostatic and hydrophobic interaction and showed potent bactericidal antibody response against recombinant Men B antigen following immunization in mice through intramuscular route. When anionic DSS microparticles were compared with their nanoparticulate form as vaccine delivery systems for two different antigens (recombinant antigen from Men B and antigen from HIV-1, env glycoprotein gp140), a comparable immune response in mice was observed for both the micro- and nano-particulate forms [71]. A novel biodegradable polymer, poly(2-sulfobutyl-vinyl alcohol)-*g*-poly(lactide-*co*-glycolide)-based anionic nanoparticles were prepared by Jung et al. by solvent deplacement procedure and was used for the mucosal delivery of tetanus toxoid in mice [47]. A surfactant free anionic PLA nanoparticles system was developed by Ataman-Önal et al. [72], and these nanoparticles were prepared by dialysis method and used for the delivery of HIV-1 p24 protein in different animal models. Following subcutaneous immunization of p24 adsorbed PLA nanoparticles, a strong CTL response and high antibody titers were observed in mice, rabbit, and macaque. Thus, the different anionic particulate systems described here are promising delivery systems for antigen delivery in vivo.

8.3.3 Combinatorial Delivery of Antigens and Soluble Adjuvants Using Micro-and-Nano-particles

Modulating the type and strength of an immune response during immunotherapy is of critical importance for generating effective protection against various infectious diseases and cancer. At the cellular level, this is controlled through antigen presentation by DCs and by the resulting T-cell response (Th1 for viral infections and cancer). Particle-mediated delivery of antigens allows the opportunity to co-deliver (in the same particle and thus to the same DCs) encapsulated adjuvants and thus further modulate or enhance the immune response. Immunostimulatory adjuvants, as reviewed by O'Hagan and Valiante, represent "diverse components co-administered with vaccine antigens that enhance antigen-specific immune response, in vivo" [73]. Over the past decade several adjuvants have been discovered; however, classification of these molecules in specific class categories is intricate [74]. In this chapter we focus on most commonly used adjuvants in microparticles-based strategies, like monophosphoryl lipid A (MPL-A), CpG oligonucleotide, and small interfering RNA (siRNA).

Adjuvant administration strategies can greatly influence the immunogenicity and safety of adjuvants. Early adjuvants included heat-inactivated or attenuated bacteria. Consequently, such bacterial-based adjuvants like Freund's complete adjuvant (FCA) result in toxicity-related side effects including granuloma formation, pyrogenicity, and allergic reactions [75]. FCA and several low toxicity derivatives have been evaluated in human clinical trials; however, none of them have been approved by FDA as commercial vaccines [75, 76]. Alum, first introduced in 1920s, remains the only FDA approved vaccine adjuvant to date in North America [73, 77]. Alum, however, induces a Th2 type response with production of IL4 and IgE neutralizing antibodies and thus cannot be potentially used with antiviral and anticancer vaccines that require Th1 response with enhanced production of IFN-γ [15, 74, 78, 79].

The limitations of alum administration and the severe toxicity associated with other adjuvants like FCA prompted discovery of new classes of safer adjuvants and design of controlled delivery systems to create a balance between Th-type immunity. Although bolus or soluble forms of adjuvants have been shown to enhance immune response [80, 81], it has been recently emphasized that formulated delivery of adjuvants with antigens results in a superior immune response as compared to bolus or soluble forms of adjuvants with antigens [82, 83]. Formulating vaccine components can protect degradation of antigens and adjuvants, prolong the release and immunogenic affect of antigen and adjuvant, localize the adjuvancy of adjuvant to specific target site thus minimizing toxicity concerns, and co-deliver both antigen and adjuvant to same cell type and, occasionally, to the same cell [38].

Early studies with encapsulation of muramyl dipeptide (MDP) within gelatin microspheres reduced the required adjuvant dose by 2000-fold, to activate macrophages for antitumor response, as compared to soluble MDP supplemented in the macrophage culture medium [84]. Microencapsulation of lipopolysaccharide (LPS) into lipid-based systems has significantly reduced the toxic effects of LPS, but not low enough to qualify for human applications. Consequently, tremendous effort is being devoted to discover and develop adjuvants and formulations to establish protective immunity. A few selected immune-modulators are discussed here.

8.3.3.1 Monophosphoryl Lipid A

MPL-A is detoxified derivative of Lipid A portion of lipopolysaccharide obtained from *Salmonella minnesota* Re595. MPL-A is obtained by removing the phosphate and fatty acid group from Lipid A thus resulting in significant reduction in toxicity to ~ 0.1% of its parent molecule LPS [85–87] and is recognized by TLR4 through PAMPs [87]. The immunomodulatory effect of MPL-A is Th1 specific with production of IFNγ and IL2, and it has been suggested that MPL-A induces T- and B-cell immunity through Toll-interleukin 1 receptor domain-containing adapter inducing interferon-β (TRIF) mediated signaling of TLR4 [87].

MPL-A has been used as an adjuvant by itself or in combination with other adjuvants as alum or trehalose dimycolate, or with delivery systems as liposomes [88, 89], micro emulsions and polymeric micro- and nano-particles [90].

Immunomodulatory effect of MPL-A has been reported with numerous antigens including the hepatitis B surface antigen (HBsAg) [91, 92], HBcAg [90], the HIV soluble protein (gp 120) [51, 93], antigen from *Neisseria meningitidis* serotype B (Men B) [51], and tuberculosis subunit vaccine [94]. A recent study highlighted the significant potential of using biodegradable delivery system for multi-modal delivery of MPL-A with hepatitis B virus core antigen, to synergistically induce a robust Th1-specific immune response [90]. MPL-A has been co-encapsulated with HBcAg in PLGA nanoparticles and administered subcutaneously in C57BL/6 J mice. The study illustrated that nanoparticle mediated delivery of antigen and MPL-A was necessary for the production of high IFNγ by T cells [90].

The effect of encapsulating MPL and RC529 in microparticles was investigated by Kazzaz et al. where gp120 protein antigen was surface adsorbed on anionic PLG microparticles and administered with soluble MPL, soluble RC529, PLG encapsulated MPL or RC529, and compared to soluble CpG co-administration. Encapsulation of MPL and RC529 resulted in significant induction of IgG and igG2a antibody titers, higher than that observed with soluble adjuvants including CpG [51].

8.3.3.2 CpG Oligonucleotides

Cytosine-guanosine (CpG) oligonucleotide (CpG-oligo) sequences are pathogen-associated molecular patterns (PAMP) that are present in bacterial DNA (not in humans). Their presence in plasmids itself or in combination with plasmid DNA or proteins can significantly enhance the immune response [95, 96]. In fact, DNA vaccines composed of plasmid DNA produced in bacterial cells have several CPG motifs (~ 1 per 20 bases) and add to immunostimulatory ability of DNA vaccines. Most immune cells that lack TLR9 and CpG oligos are preferably taken up by plasmacytoid DCs and B cells through adsorptive endocytosis in humans and are primary source of TLR9-mediated immune response [95]. Endocytosed CpG engages with intracellular TLR9 on the endolysosomes [97]. Binding to TLR9 induces the activation of DCs with enhanced production of pro-inflammatory and antiviral cytokines like IL12, IL6, TNFα and specifically induces differentiation of naïve CD4+ T cells into Th1 phenotype with enhanced production of IFNγ [96, 97].

The TLR9-mediated specificity of action of CpG has made it an extremely promising candidate for immunotherapy as monotherapy in combination with vaccines in human clinical trials [95]. The capability of CpG to induce Th1 response is not compromised even in presence of delivery systems or adjuvants known to induce Th2 response (alum or IFA) [95, 98]. When administered with respiratory syncytial virus vaccine in mice, CpG preferably diverted towards Th1 response even though the vaccine itself was Th2 biased [99]. Th1 response was marked with high levels of IFNγ and low levels of IL4 detected in mice immunized with both DNA vaccine and CpG oligos. Approaches to combinatorially deliver CpG oligos with antigens include liposomes [100], muticomponent nanorods, biodegradable polymeric microparticles [52, 58, 61, 101, 102], and other relatively advanced designs such as polydimethylsiloxane (PDMS) chips with PLGA seals [103]. Alginate is another class of biomaterial that has been used to co-deliver CpG with vaccine antigens [53].

Since most purified subunit proteins lack the PAMPs and other "danger-signal" hallmarks of the original organism, combinatorial delivery of the protein antigen along with an adjuvant has been shown to induce robust immune response compared to the antigen–particle system alone [104]. The HIV-1 recombinant gp120 protein adsorbed to anionic PLG-DSS microparticles was co-delivered with cationic particles containing surface-adsorbed CpG oligos as an adjuvant; this dual delivery resulted in the most potent immune response amongst the experimental groups [58].

Use of synthetic delivery systems facilitates delivery of multiple copies of antigens and in some cases, multiple adjuvants. Recently, Kasturi et al. demonstrated that using PLGA nanoparticles (~300 nm) containing antigens and TLR ligands (MPL-TRL4, R837-TLR7) synergistically induced antigen-specific neutralizing antibodies compared to a single adjuvant system [43]. Immunization with OVA-PLGA nanoparticles containing MPL and R837 induced persistent germinal centers in draining lymph nodes (Fig. 8.3). These nanoparticles with whole inactivated viral antigen and combination of MPLA/R837 induced robust antibody-mediated immune response in a 2009 pandemic H1N1 influenza A rhesus macaque model.

8.3.3.3 RNA Interference and siRNA as Immune-Modulators

Small interfering RNAs are 21–24 nucleotide sequences present in all mammalian cells that direct RNA-induced silencing complex (RISC) to specifically identify mRNA sequences and cleave then. Recently, RNA-based therapeutics has gained tremendous interest especially with the discovery that RNA sequences can effectively "silence" protein expressions with high specificity. Delivering synthetic, short interfering RNA (siRNA) into the cell cytoplasm interferes with the messenger RNA (mRNA) and prevents protein translation. The unique ability of siRNA to specifically silence desired genes within the cells constitutes a new opportunities to target genes that play critical roles in immune modulation [38]. Although the functioning of siRNA is sequence specific and presumably less toxic, being inherent and conserved to the multicellular organism, siRNA with certain sequences can also induce innate cytokine response in mammals [105]. The immunostimulatory activity of siRNA is sequence specific; however, very few sequences have been well identified. One such sequence is presence of poly(U) or GU-rich sequences in the siRNA [105], particularly 5′-GUGUG-3′ can be immunostimulatory and cause cytokine production irrespective of their gene silencing efficacy. Since double-stranded siRNA can induce a nonspecific immune response, a judicial choice of siRNA sequence and other processing such as chemical modification (as reviewed by [105]) should be taken into consideration to restrict the immune response to the specific gene silencing effect [106]. The immunostimulatory effect of siRNA is similar to double-stranded (ds) RNA as it acts as a virally associated signal, recognized by TLR 3 in DCs. TLR3-mediated activation stimulates DCs to produce type I interferons (IFNs) and other cytokines initiating innate immunity [107, 108].

siRNA-mediated gene knockdown is a potential immunomodulatory tool for modulating DC activation and function. Immunomodulatory effects of siRNA have been less explored for vaccines. Very few studies have shown

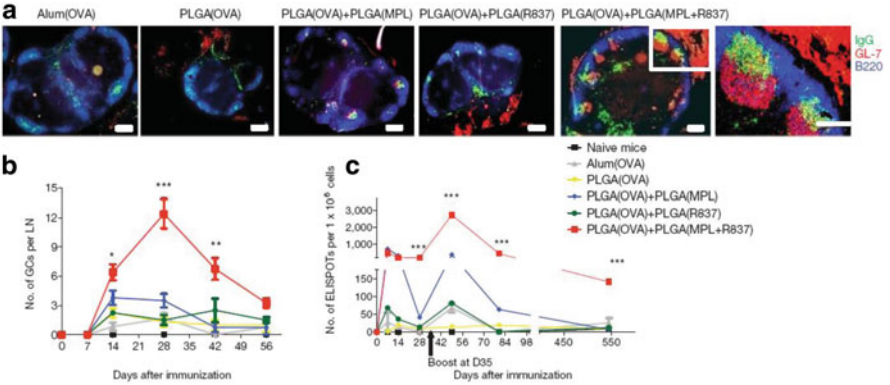

Fig. 8.3 Immunization with nanoparticles containing MPL and R837 induces persistent germinal centers and long-lived antibody-forming cells in draining lymph nodes. (**a**) C57BL/6 mice were immunized with OVA encapsulated in nanoparticles with MPL1R837 plus antigen. Four weeks after primary immunization, draining lymph nodes were excised, tissue sections prepared and stained for germinal centers (GL-7, *red*; B220, *blue*; and IgG, *green*). Images are representative of two independent experiments with draining lymph nodes obtained from 2 to 3 mice per treatment condition per experiment. Scale bars, 200 μm for first five panels from left and 100 μm for right-hand panel. (**b**) Germinal centers (GCs) were counted in lymph node (LN) sections at the time-points indicated and represented as mean ± s.e.m. from 4 to 6 draining lymph nodes from $n = 2$, 3 mice per treatment group. (**c**) ELISPOT assay. Combination of TLR4 and TLR7 ligands has no effect on the short-lived antibody-secreting cells, relative to single TLR ligands, but stimulates long-lived antibody-secreting cells that persist for 1.5 years. *Graph* represents average spots per 1 × 10E6 total lymph node cells ± s.e.m. from duplicate cultures per treatment group. Data are representative of at least 2–3 independent experiments per time point indicated. Adapted with permission from [43]

potential to use siRNA with vaccine antigen for therapeutic use and most of these studies utilize cytokines as targets. A few recent studies have successfully demonstrated this concept in vitro. Liu et al. [109] showed that lipid vector-based delivery of IL10 targeted siRNA into DCs can successfully prevent IL10 expression and "switch" the DC phenotype to generate Th1 type T-cell activation. IL10 is crucial for down regulation of the Th1 response after inflammatory infection. Similarly it has been shown that siRNA against IL12 effectively silences protein expression and enhances secretion of Th2 cytokines by stimulated T cells [110]. Th1 cells produce IL2 and interferon gamma (IFNγ) which preferentially stimulate cell-mediated immunity and prevents proliferation of Th2 cells, respectively. Production of IL10 (Th2 response) inhibits Th1 response but stimulates B cells.

Our group has recently developed a novel combinatorial delivery system to co-deliver siRNA together with plasmid DNA to specifically divert the immune response towards Th1 type. Singh et al. reported that simultaneous delivery of cytokine-silencing siRNA with DNA antigens could modulate the functionality of target APCs in vivo and thereby control the type (Th1 versus Th2) and strength of immune response (Fig. 8.4) [38].

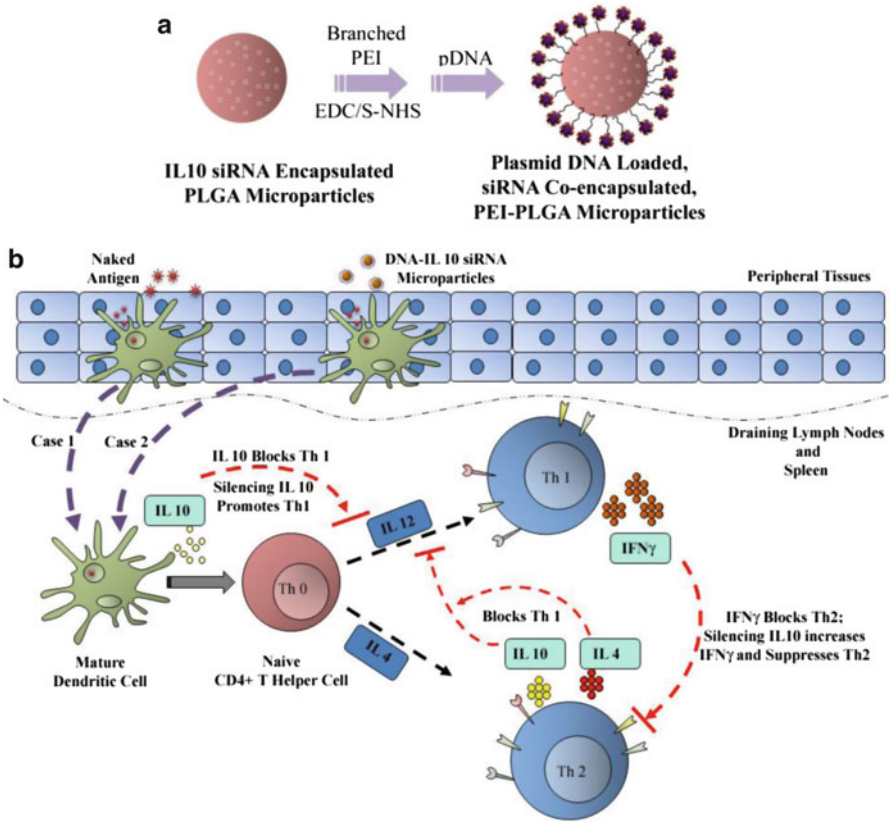

Fig. 8.4 Interleukin-10 modulation using IL10 siRNA loaded microparticles. (**a**) siRNA encapsulated, microparticles are surface modified to attach PEI and plasmid DNA is electrostatically loaded. (**b**) The professional DCs ingest antigen at the site of injection (or infection) and activated migrate to draining lymphoid tissues. The activated DCs present antigen to naïve T cells. The Th0 cells can then proliferate into two of its phenotypes: Th1 and Th2. The activated DCs produce IL 10 cytokine which acts as Th1 suppressor and tends to divert the pathway towards Th2 response which further suppresses Th1 through IL10 and IL4. Naked DNA vaccines have less control over IL10 production by DCs and thus the resulting immune response may be compromised. We hypothesize that if we co-deliver IL 10 specific siRNA to these immature DCs, it would prevent the production of IL 10 through gene suppression. In the absence of IL10 the pathway will be more favored towards Th1 response, desirable for antiviral and antitumor responses. Adapted with permission from [38]

8.3.4 Hydrogels and Scaffolds-Based Systems for Immune Modulation

Hydrogels are three-dimensional, hydrated networks of crosslinked polymers. Injectable hydrogel systems have been more recently explored as another avenue for delivery and sustained localization of vaccine formulations. These hydrogels could be used as bulk gels, microparticle-shaped gels, or even nanogels as discussed later in the

section. Injectable hydrogel system has been recently much explored for delivering multiple biomolecules (nucleic acids, proteins, and peptides, e.g. APC chemoattractants, tumor antigens as well as immune modulators) in a single injectable formulation which can create an in situ "immune priming center" and thus help recruiting a large number of APCs (especially DCs) at the site of injection and delivering antigen to the migrated DCs. Recent approaches in this interesting field include work by Darrell Irvine's group where polyethyleneglycol (PEG)-based protein-loaded microparticles were created and then functionalized with CpG oligonucleotides for TLR stimulation. DCs stimulated with CpG-functionalized Ovalbumin-loaded particles induced a tenfold increase of IL2 secretion from activated T-cells than DCs stimulated with soluble OVA and CpG oligos [111]. Degradable scaffolds have also shown positive results against tumor development through simultaneously delivery of antigens, adjuvants and the cytokine GM-CSF using a PLGA-based scaffold [112]. These scaffolds, called infection mimicking centers, can be tailored with components to represent key aspects of an infection and can control immune-cell trafficking and priming in the body. The formulation has shown significant potential in murine model of melanoma with 90% survival compared to untreated animals that die within 25 days. The infection mimicking center enhanced Th1-specific immune response leading to tumor protection. However, these scaffolds required surgical implantation into subcutaneous pockets [112] and may have limited translation into clinic.

Injectable self-gelling hydrogels, on the other hand, offer a more suitable immunization alternative over implantable scaffolds. Limited effort has been made in developing situ crosslinking hydrogels as innovative immunoengineering tool for vaccination. Roy et al. reported an in situ injectable, PEG-based hydrogels for nucleic acid delivery that underwent gelation once injected [113]. These hydrogels gelled in ~18 minutes, maintained DNA bioactivity, however, degraded over a long period of 31 days. Hori et al. developed a cell-based therapy using injectable self-gelling alginate hydrogel formulation comprising of calcium loaded alginate microspheres and soluble alginate solution [114]. When injected along with pre-activated DCs subcutaneously in mice, the solution gelled within 60 min at the site of injection and thus creating a "Vaccination Node" locally which attracted host DCs and T cells in large number for over a week. Further, to enhance the immunotherapeutic efficacy, co-delivery of immunomodulatory factor IL-2 and CpG oligonucleotides along with BMDCs using an in situ alginate hydrogel systems was evaluated [115]. In this work, CpG was loaded onto the surface of poly-L-lysine adsorbed alginate microspheres and mixed with IL-2 and BMDCs containing soluble alginate solution to form in situ alginate gel. When injected subcutaneously in mice, microporous gel was formed and infiltration of phagocytic cells into the in situ formed gel was demonstrated. The approach could also be used for cell-based therapies where antigen primed DCs could be encapsulated in an in situ crosslinking alginate hydrogel and delivered as DC vaccines [114].

Singh et al. recently developed a fast degrading material-based synthetic immune priming center as a protective vaccine against weakly immunogenic non-Hodgkin's lymphoma by in situ amplifying the number of functional, antigen-specific T helper 1 (Th1) type cells

following immunotherapy [116]. An in situ cross linking hydrogel was developed using polymers like dextran vinylsulfone and tetrapolyethyleneglycol thiol (PEG-4-SH) for combinatorial delivery of chemokines (i.e., MIP3α) and nucleic acids (i.e., IL10 siRNA and pDNA antigen) [116]. By controlling the chemistry of polymers it was shown that a fast-degrading (2 days) or a slow-degrading (7 days or greater than 1 month) immune priming center could be created thus controlling the release of immature DC attracting chemokines and antigen/adjuvant loaded microparticles to the migratory DCs in a sustained manner. When used with weakly immunogenic idiotype lymphoma antigen and IL-10-silencing siRNA, the delivery system eliminates nonspecific delivery of pro-inflammatory cytokine targeted siRNA by localizing it to phagocytotic DCs. Using a murine model of A20 B-cell lymphoma, it was demonstrated that the combination of DNA antigen and IL-10 silencing synergistically activated recruited iDCs and caused a strong shift towards Th1 response while suppressing Th2 and Th17 cytokines. The synthetic immune priming center-based immunotherapy showed 20% greater CD8+ cytotoxic T cell (CTL) response and 53% stronger CD4+ CTL activity against murine A20 B-cell lymphoma compared with control naked DNA vaccine. Further, in vivo immunization induced significant protection ($p<0.01$) against subsequent lethal lymphoma challenge.

Not only bulk hydrogels but nanometer-sized hydrogels have been explored for their potential as vaccines. Nochi et al. developed an intranasal-mucosal vaccine with nanogel consisting of a cationic cholesteryl-group-bearing pullulan (CHP), which forms physically crosslinked nanometer-sized gels by self-assembly in water and has the ability to physically trap proteins through hydrophobic interactions (Fig. 8.5) [117]. Nanogel mediated intranasal delivery of a nontoxic subunit fragment of Clostridium botulinum type-A neurotoxin BoHc/A resulted in robust botulinum-neurotoxin-A-neutralizing serum IgG and secretory IgA antibodies in absence of mucosal adjuvant. Finally, the CHP nanogel generated robust tetanus-toxoid-specific systemic and mucosal immune responses when used in combination with the toxoid [117].

8.4 Conclusive Remark and Future Challenge

Immunotherapy, as a strategy for treating a variety of cancers, has been explored for several decades. Unfortunately, despite significant success in animal models, very few strategies have shown clinical efficacy in human applications. In this chapter we have discussed various biocompatible and biodegradable polymeric delivery systems used in controlled release technology for delivery of vaccine antigens and adjuvants. Injectable biomaterials-based vaccines that are capable of simultaneous but controlled delivery of multiple vaccine compinents (antigen, adjuvants, immune-stimulators) could significantly improve the efficacy of next generation DNA, protein and peptide-based vaccines. Innovative immunobioengineering strategies not only allow us to balance or bias the requirement of Th1 and Th2 responses, but also facilitate robust recruitment of naive DCs at the site of immunization. Micro- and nano-particles have shown the potential to deliver antigen and adjuvant alone or in combination and have targeted immune cells in peripheral tissues or lymph nodes. Hydrogels

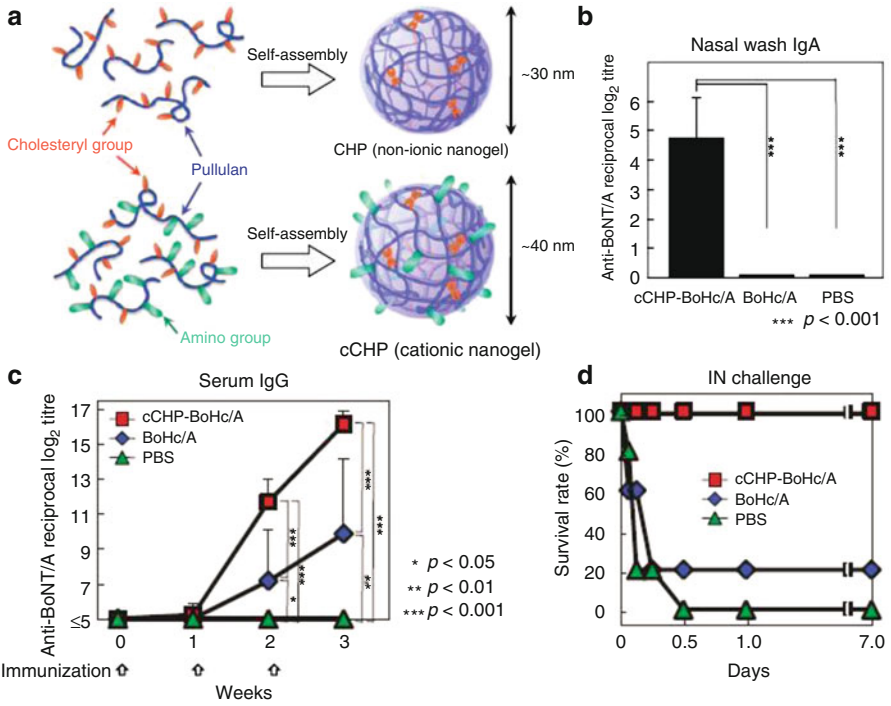

Fig. 8.5 Use of cCHP nanogel as a new antigen-delivery vehicle for intranasal vaccination. (**a**) cCHP nanogel was generated from a cationic type of cholesteryl-group-bearing pullulan. (**b**) Vigorous BoNT/A-specific IgA antibody responses were observed in nasal washes collected from mice intranasally immunized with cCHP–BoHc/A, but not from those given naked BoHc/A or control PBS. (**c**) Strong BoNT/A-specific serum IgG antibody responses were induced by intranasal immunization with cCHP–BoHc/A. (**d**) Mice intranasally vaccinated with cCHP–BoHc/A were completely protected from intranasal exposure to the progenitor toxin. Adapted with permission from [117]

and liposomes are emerging delivery systems for combinatorial delivery of multicomponent vaccines. A crucial development could be towards evaluation of the efficacy of hydrogel vaccine preserved as lyophilized formulations. This would potentially make them more favorable towards clinical applications. Of interest would be to evaluate the efficacy of particle-based vaccines or injectable hydrogel depots on a broad-spectrum of infectious, cancerous, or parasitic diseases.

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Section III
Novel Delivery Technologies for Vaccines

Chapter 9
Current Status of Electroporation Technologies for Vaccine Delivery

Claire F. Evans and Drew Hannaman

9.1 Introduction

With the first demonstration in the early 1990s that plasmid DNA could be taken up by somatic cells in vivo, resulting in expression of genes encoded by the plasmid and controlled by mammalian promoters, the stage was set for the investigation of the range of compelling applications for endogenous expression of proteins in animals and humans [1]. The finding that simple injection of purified plasmid DNA into a target tissue could induce sustained endogenous production of proteins from the recipient's own cells precipitated a flurry of research that was hoped would quickly lead to therapies for genetic, metabolic, and infectious diseases, as well as cancer. Unfortunately, while the past two decades have seen substantial progress in understanding the advantages and limitations of nucleic acid-based interventions for human disease as well as licensure of multiple veterinary products, there are currently no DNA-based products approved for human use. With well over 100 human clinical studies of DNA-based product candidates conducted to date, the overarching conclusion from these studies is that, for the vast majority of applications, conventional injection of plasmid DNA into tissues at clinically feasible dose levels is unable to produce consistent, biologically meaningful responses, especially when scaled up from rodent models into larger animal species (including humans) [2]. One key factor contributing to these results is the relatively low efficiency with which DNA crosses the cell membrane to reach its intracellular site of action [3]. To address this issue, many approaches for improving the intracellular uptake of DNA have been evaluated, with electroporation-mediated DNA delivery being one of the most promising. This chapter will discuss the utility of DNA vaccines, and the promise that electroporation delivery systems bring to the use of nucleic acid-based

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vaccine strategies, as well as the potential impact of electroporation on the field of vaccines in general.

9.2 DNA Vaccines

9.2.1 Overview

DNA vaccines are typically composed of plasmid DNA encoding one or more antigens of interest. The antigen sequences incorporated into a DNA vaccine can range from a single peptide epitope all the way to the entire coding sequence for a protein target. There is tremendous flexibility in antigen design, making it possible to encode antigens using strategies such as consensus or mosaic sequences, inclusion of multiple epitope sequences, incorporation of specific mutations, or as fusion protein sequences. Most DNA vaccines now use antigen sequences that have been adapted to optimize codon usage to the codon bias of human genes and modified to optimize production and stability of RNA by removing such elements as cryptic splice donor or acceptor sites [4]. The coding regions are positioned between a strong mammalian promoter (e.g., CMV) and a polyadenylation signal, and may include upstream enhancer and/or intron sequences. If multiple antigenic sequences are expressed by the same plasmid, the coding sequences may be separate and controlled by different promoters, or a single open reading frame may be used with expression of the individual proteins controlled by an IRES (internal ribosome entry site) sequence. Alternatively, processing signals may be interspersed between different antigen sequences that lead to proteolytic cleavage of the various protein components into separate entities. DNA vaccines may also encode immunopotentiators designed to enhance resultant immune responses, such as a cytokine, chemokine, or pathogen-derived adjuvant (e.g., tetanus toxin). These may be included on a separate plasmid or may be encoded on the same plasmid as the antigen of interest. In some cases, the immunopotentiator and antigen may be expressed as a synthetic fusion protein.

9.2.2 Advantages of DNA Vaccines

DNA-based vaccines offer a number of potential advantages compared to the conventional immunization strategies which are utilized in the majority of vaccines currently licensed for human use (i.e., delivery of a live attenuated pathogen, an inactivated pathogen, or a protein subunit). The foremost of these advantages is the ability to simultaneously induce both cellular and humoral immune responses against the encoded antigens even in the absence of a live pathogen or a potent adjuvant. The ability of DNA vaccines to target both arms of the adaptive immune

response while maintaining a favorable safety profile has been demonstrated in numerous studies in both animals and humans (reviewed in [5]).

A second advantage of DNA vaccines is that the manufacture of plasmid DNA is a straightforward, well-defined process that takes advantage of the scalability of *Escherichia coli* fermentation and the ease of isolating DNA from bacterial cultures. Importantly, the production processes for DNA vaccines do not require access to or production of the pathogen itself and are largely independent of the sequence encoded within a given plasmid. Thus, DNA vaccines constitute a true manufacturing platform in which the production processes developed for one product candidate can be readily and predictably leveraged for subsequent product candidates with the attendant reductions in cost and technical risk as multiple candidates progress in clinical development.

Third, DNA also offers tremendous flexibility in design of the antigen and/or immunopotentiator sequences encoded by DNA vaccines. Antigen sequences can be rapidly and simply synthesized, greatly facilitating vaccine strategies based on rational antigen design/modification. As previously discussed, changes in the DNA sequence of the encoded antigen exhibit minimal impact on the DNA manufacturing process, thus allowing target antigens to be readily and quickly modified in response to new variants or to developments in selection of vaccine targets.

Fourth, the plasmid DNA platform is conducive to inclusion of multiple antigens/components in the vaccine product. As discussed above, the antigens may be encoded on separate plasmids or combined into one or more plasmids encoding multi-antigens. Since all components of the DNA vaccine have the same basic chemical structure and properties, there is virtually no risk of unwanted interactions among vaccine components during the manufacturing process. Fifth, the stability profile of DNA is generally superior to that of protein or attenuated/inactivated vaccines, making it attractive with respect to shelf life and reduced need for maintenance of the cold chain during product transportation and storage. Finally, in contrast to vaccines based on live viral vectors, preexisting or vector-induced host immune responses to DNA plasmids themselves have not been described and therefore do not limit vaccine efficacy. As has been well documented in recent years with viral vector delivery platforms, host immune responses to virus-encoded proteins can raise potential safety concerns [6] and/or prevent effective re-administration of the same viral serotype, thereby diminishing or preventing the desired biological effects [7, 8]. The lack of interference by host immune responses to the vector allows the use of the DNA-based platform for a variety of indications.

Currently, there are three DNA vaccines licensed for use in veterinary indications, including vaccines for prevention of infection by West Nile virus in horses, hematopoietic necrosis virus in farmed salmon, and a therapeutic melanoma vaccine for dogs [9–11]. Despite these successes, the initial high level of enthusiasm for human clinical applications of DNA vaccines waned because of the limited success in extrapolating favorable results from small animals to humans. Even with promising nonclinical proof of concept, clinical trials testing a range of DNA vaccines delivered by conventional needle injection were disappointingly unable to demonstrate consistent, target levels of immune responses [5]. One significant

factor contributing to the success of DNA vaccines in small animals is the relatively high DNA doses and volumes of injection used in these species. It is recognized that the injection of comparatively large volumes of fluid in a small rodent muscle can generate hydrostatic forces which can improve the efficiency of intracellular DNA uptake. On a body mass basis, the volume that is typically injected into mouse muscle (50 μl) would scale up to an (infeasible) injection volume of 150 ml in a 60 kg human. Thus, the hydrostatic pressures generated in the mouse muscle under these conditions are unlikely to be recapitulated in larger species. However, as illustrated by the robust immunogenicity of the West Nile virus DNA vaccine in horses, even relatively low efficiency delivery characteristic of conventional low volume injection in a large animal species can be sufficient to confer protective immunity if the target antigen is sufficiently immunogenic.

In spite of the suboptimal immunogenicity observed in a majority of DNA vaccine clinical trials conducted with conventional needle injection, the numerous advantages of the DNA-based vaccine platform spurred investigations into means to enhance the potency in large species, including humans. A number of approaches have been evaluated as a means to resurrect the DNA vaccine field, including improvements in vaccine design; formulations, such as cationic lipids or polymers; adjuvants, such as toll-like receptor agonists; and delivery techniques such as jet injection, ballistic (gene gun) delivery, and electroporation. Among these, electroporation-mediated DNA delivery has proved to be very promising, with substantial evidence of increased potency of DNA vaccines delivered with electroporation in multiple large animal species and, more recently, in several human clinical trials. Note that there are a number of excellent, recent reviews on DNA vaccines that provide depth beyond the scope of this chapter [12, 13].

9.3 Electroporation-Mediated Delivery of DNA Vaccines

9.3.1 Clinical Electroporation Devices for DNA Vaccine Delivery

With respect to in vivo DNA delivery, electroporation refers to the propagation of electrical fields within tissues resulting in a transient increase in cell membrane permeability. This enables increased intracellular uptake and expression of DNA molecules present in the local tissue at the time of electroporation application. Most commonly, the electroporation-inducing electrical fields are applied in tissue using arrays of slender rod or needle electrodes contacting the target tissue. Alternatively, larger “plate” style electrodes have also been used for application in surface accessible tissues. The requisite electrical fields are generated using a power source that supplies a series of pulses with a defined waveform, duration, frequency, and field strength. The power source may be configured to generate electrical fields using either constant voltage or constant current. Initial studies in rodents clearly demonstrated that electroporation greatly enhanced expression of proteins encoded by the transferred plasmid DNA [14–16], and this was soon expanded to include applicability

of the procedure to larger species [17, 18]. Building on these initial observations, it was rapidly demonstrated that administration of DNA vaccines with electroporation to multiple species, including rodents, rabbits, cattle, and nonhuman primates, resulted in increases in humoral and cellular immune responses of 10–1,000-fold compared to conventional DNA injection alone [19–22]. These enhanced responses were initially and primarily described following intramuscular vaccine delivery, but in recent years, a number of studies have also demonstrated improved responses following electroporation-mediated DNA delivery in skin [23–25].

A variety of device technologies have been developed to support the in vivo application of electroporation for DNA vaccine delivery, and they have been described in detail in other publications [26, 27]. Briefly, the primary objective of these device development efforts is the provision of devices and associated administration procedures capable of achieving safe, effective, and reproducible DNA vaccine delivery within the target tissue. Since the enhanced DNA delivery characteristic of electroporation occurs only in tissues where the DNA is present contemporaneously with the electrical fields, this is best achieved through the provision of devices which can facilitate co-localization of the DNA injection and subsequent electrical field application in the target tissue. Additionally, early clinical experience indicates that the degree of discomfort perceived by the recipients of electroporation is highly correlated with the volume of tissue exposed to the electrical fields [28]. Thus, propagation of electroporation should ideally be confined to tissue in which the DNA has been distributed, making devices which have sufficient precision to reliably achieve this likely to be favored from a tolerability perspective.

As the technology is further refined for human clinical applications, additional considerations that are likely to play a role in the commercial feasibility of the approach include further emphasis on the tolerability profile of the device, the implementation of appropriate safety features, the simplicity of its use, and finally, the upfront and recurring cost of use. A summary of the electroporation devices in development for DNA vaccine delivery in the clinical setting is provided below.

9.3.1.1 Devices for DNA Vaccine Delivery in Skeletal Muscle

One of the first devices to enter clinical testing for DNA vaccine delivery was the "MedPulser" device developed by Inovio Biomedical [29]. Adapted from devices designed for intratumoral delivery of DNA and chemotherapeutics, the MedPulser utilizes a three-step, manually controlled administration procedure. The procedure is initiated with the intramuscular injection of the DNA into the target tissue using a conventional hypodermic needle and syringe. The withdrawal of the injection syringe is followed by the intramuscular insertion of four needle electrodes arranged in a rectangular array at the site and depth at which the operator judges the DNA to be distributed. The procedure concludes with the user activating the pulse generator connected to the electrodes and then withdrawing the electrode array from the tissue. Although the MedPulser device has generated encouraging results in at least one study to date, it is recognized that the success of this device is largely dependent

on the ability of the operator to ensure that the electroporation is reliably applied at the tissue site in which the DNA has been distributed. While perhaps suitable for use in small-scale clinical trials with well-trained operators, it is unclear whether a manually controlled approach can sustain the degree of reproducibility across operators and recipient populations that will be required for product commercialization.

Prompted by these concerns, the field has made significant strides in the development of devices which facilitate co-localization of the DNA administration with the application of the electrical fields. Of foremost importance has been the development of devices in which the means for DNA vaccine administration and electroporation application are integrated into a single device. By integrating the procedural steps required for electroporation-mediated DNA vaccine administration, these devices have the potential to provide more consistent administration while reducing the need for operator training. Moreover, the simpler and more rapid procedure application achievable with these integrated devices is likely to improve acceptance among both recipients and operators. Several integrated device technologies have progressed into clinical testing for DNA vaccine delivery.

The first integrated device to be utilized in clinical testing was the "Twinjector" device (Inovio Biomedical Corporation) [30] followed recently by a more refined version called the "Elgen" (Inovio Biomedical Corporation). Each of these device configurations comprise a multi-use handheld applicator device that is connected to a constant voltage electrical signal generator. In the Twinjector/Elgen device configurations, the DNA dose to be delivered is drawn up into two conventional injection syringes which are affixed with an appropriate injection needle and then inserted into the applicator. The device is placed against the skin of the recipient above the target muscle site. Operator actuation of a gearing mechanism driven either manually (Twinjector) or by motor (Elgen) causes the device to slowly insert the injection needles while simultaneously depressing the syringe plungers to distribute the agent. The resulting DNA distribution comprises a roughly circular column of DNA centered each injection needle. Once the injections have been completed, the electrical signal generator is activated with the two injection needles serving as a bipolar electrode array for propagating the electrical fields. Following completion of the electrical field application, the two injection needles/electrodes are withdrawn from the tissues.

Another integrated device currently in clinical testing for electroporation-mediated DNA vaccination is the TriGrid™ Delivery System for intramuscular administration (TDS-IM) developed by Ichor Medical Systems (San Diego, CA) [26, 31]. The TDS-IM comprises three components: an Application Cartridge, a handheld Integrated Applicator, and a Pulse Stimulator. The Application Cartridge is a sterile, single use component that contains an array of four electrodes and a syringe/injection needle with the DNA dose to be administered. The electrodes are arranged in two equilateral triangles with a common base, forming a diamond shape around the central injection needle. Prior to administration, the Application Cartridge is inserted into the reusable Integrated Applicator which is then connected to the Pulse Stimulator which controls the administration procedure sequence and generates the required signals for induction of electroporation. Prior to administration, a depth

gauge on the Application Cartridge is adjusted based on the skin thickness of the recipient to ensure intramuscular administration. The device is then placed against the skin of the recipient above the target muscle site. It is aligned so that the major axis of the diamond-shaped electrode array parallels the orientation of the underlying muscle fibers so that the propagation of the electrical fields will correspond to the ellipsoid fluid distribution characteristic of an intramuscular injection. Upon device activation, mechanisms housed in the Integrated Applicator deploy the recessed electrodes and injection needle into the target muscle. Brief signals from the Pulse Stimulator are used to verify proper deployment of the injection needle and electrodes, followed immediately by administration of the DNA at a controlled rate of injection. Once the injection is completed, constant voltage electrical signals from the Pulse Stimulator are relayed to the deployed electrodes, inducing the electroporation effect. Importantly, the application of electroporation is confined to the site of DNA administration through the aforementioned diamond-shaped configuration of the electrodes combined with the use of electrical insulation limiting electroporation application only to the depth of DNA injection. At the conclusion of the procedure, the device is withdrawn and an automatic sharps protection shield deploys in order to minimize post procedure visualization and exposure to the electrodes and injection needle. Overall, the automated, user-independent TDS-IM device has been designed to perform the administration procedure in a rapid and reproducible fashion while ensuring that the application of electroporation is confined to the site of DNA distribution.

The final device in clinical testing for intramuscular DNA vaccine delivery is the CELLECTRA device originally developed by Advisys, Inc. and now owned by Inovio Biomedical [32]. Developed originally for use in veterinary applications, including the porcine growth hormone releasing hormone (GHRH) product currently licensed for use in Australia (see Sect. 9.3.2 for discussion), the CELLECTRA utilizes a five electrode circular array connected to a constant current pulse generator through a reusable handle device. Prior to the administration procedure, the electrode array is inserted into the handle device. The handle is then manually depressed over the skin at the target site of administration, inserting the electrodes into the underlying muscle tissue. A syringe and injection needle containing the DNA to be delivered is inserted through a central injection port in the handle and into the tissue. Once full insertion is reached, a depth control stop prevents further needle insertion. The user then initiates the injection of the agent into the tissue followed by activation of the pulse generator. The procedure concludes with the withdrawal of the device following completion of the electroporation sequence.

9.3.1.2 Devices for DNA Vaccine Delivery in Skin

The encouraging findings with electroporation-mediated intramuscular delivery of DNA vaccines combined with the potential advantages of skin delivery for certain DNA vaccine applications have prompted substantial efforts to develop device technology to support the application of electroporation for DNA delivery in skin.

This includes adaptation of device platforms developed originally for intramuscular delivery (e.g., CELLECTRA, TDS) as well as development of dedicated platforms for skin delivery (Cyto Pulse DermaVax™, Inovio Minimally Invasive Device). While the basic principles and requirements for electroporation delivery in skin are the same as those for intramuscular delivery, the ability to directly visualize the site of DNA distribution following skin injection has reduced the impetus to develop integrated devices to facilitate co-localization of the DNA and electrical fields. As a result, most of the current device configurations use a manually controlled multi-step procedure comprising user-performed injection followed subsequently by electrode array insertion and activation at the site of DNA injection (the lone exception being the TDS-ID device).

The first device to enter clinical testing for electroporation-based delivery in skin was the DermaVax [33] device originally developed by Cyto Pulse Sciences, Inc. and now owned by Cellectis, Inc. The DermaVax device utilizes a single use electrode array comprising two parallel rows of 2 mm length needle electrodes that are connected to a constant voltage pulse generator through a reusable connector cord. Of note, the DermaVax pulse generator device is capable of varying the amplitude and duration of the pulses within a given sequence, potentially allowing for the identification of more complex stimulation conditions that may be beneficial for certain applications. Electrode arrays with four or six electrodes per row are available to accommodate varying tissue volumes. The DermaVax administration procedure is initiated with a user-controlled injection of DNA into the skin of the recipient. Clinical studies have been performed using both needle injection using the Mantoux technique as well as using needle-free injection with the Biojector device. Following completion of the injection, the electrode array is manually inserted into the site the user judges to be the site of DNA distribution (typically demarcated by a skin weal) such that the two rows of electrodes span the injection site and the pulse generator is activated to generate the electrical fields across the administration site.

Another device in early phase clinical testing is the CELLECTRA skin device derived from the intramuscular CELLECTRA device described in Sect. 9.3.1.1. The CELLECTRA skin device utilizes an array of three penetrating electrodes arranged in a triangular configuration which interfaces with a reusable handle connected to the CELLECTRA constant current pulse generator. Consistent with the DermaVax device, the procedure is initiated with a manual DNA injection in the skin followed by insertion and activation of the electrode array at the putative DNA distribution site.

The TriGrid Delivery System for skin administration (TDS-ID) [28] is scheduled to enter clinical testing in 2012. Designed to function analogously to the TDS-IM device, the TDS-ID device is based on an integrated, automated three-component design. At the initiation of the procedure, the user inserts a single use Application Cartridge into the handheld Integrated Applicator. The Application Cartridge houses an array of four electrodes configured to form two isosceles triangles with an adjoining base around a central needle-free injection port. This configuration is designed to accommodate the circular injection distribution characteristic of a skin injection. Placement and activation of the TDS-ID device at the target skin site result in the

automated deployment of the electrode array and verification of insertion followed by the injection of the DNA by needle-free jet injection into the skin. Immediately after injection, the electroporation-inducing electrical fields are propagated at the site of DNA administration by the constant voltage TDS Pulse Stimulator device. At the conclusion of the procedure the device is withdrawn and a sharps protection shield deployed over the electrodes. Consistent with the rationale described for the TDS-IM device, the TDS-ID device is designed to support a consistent, user-independent administration procedure in which the electrical fields are co-localized with the site of DNA distribution.

In addition to the devices either in clinical testing or on the cusp of initiation, there are several novel minimally invasive skin delivery device concepts for which initial proof of concept for epidermal DNA delivery has been established. These include two similar systems which utilize a closely spaced grid of non-penetrating "point" electrodes configured in a 4×4 arrangement; one was developed at Inovio utilizing a 1.5 mm intraelectrode spacing [34] and the other in the laboratory of Richard Heller utilizing a 2 mm intraelectrode [35]. In both cases, the administration procedure is initiated with a user-controlled injection into the skin followed by the placement and activation of the electrode arrays at the site of DNA administration. Inovio has also assessed the initial feasibility of a non-contact electrode system which utilizes repeated activation of a piezoelectric spark generator placed above (i.e., not in contact with) the site of DNA administration resulting in localized electric field propagation [36].

Finally, Inovio has published results from the initial testing of a device capable of simultaneous electroporation application at multiple depths (e.g., muscle and skin) [37]. The device utilizes the 4×4 minimally invasive skin device described above, where the four corner electrodes of the grid are extended to a 1.5 cm length. Following independent but overlying DNA injections in both the skin and the muscle, the electrode array is then inserted into the skin, with the four corner electrodes penetrating into the muscle tissue. The electrodes are then activated in a staged fashion using a pulse generator device capable of applying the required stimulation conditions for application in skin and muscle, respectively.

9.3.2 Animal Studies and Vaccination Strategies

Electroporation-mediated delivery of DNA vaccines has been evaluated for a number of indications, including prophylactic and therapeutic applications for infectious diseases, cancer, and allergy. This delivery technology has been used for DNA vaccines in numerous mammalian and avian species, including mice, rats, hamsters, guinea pigs, ferrets, rabbits, woodchucks, dogs, pigs, goats, sheep, cattle, rhesus macaques, cynomolgus macaques, chimpanzees, baboons, chickens, and ducks [19, 22, 38–47]. Electrode arrays are readily adaptable to different species, with the intraelectrode spacing and depth of penetrating electrodes typically adjusted to suit the target muscle or skin.

In addition to its use for DNA vaccine delivery, electroporation has also been used to deliver DNA encoding therapeutic proteins that have systemic and/or local tissue effects, such as interferon-beta [48] or VEGF [49]. In fact, the only DNA product that is currently licensed for delivery by electroporation is a veterinary product in Australia consisting of a DNA plasmid encoding porcine GHRH that is delivered intramuscularly to sows to improve the viability of offspring [50]. Electroporation-mediated plasmid delivery has also been used to provide long-term expression of DNA-encoded monoclonal antibodies [51–53], indicating that DNA-based monoclonal antibody products could potentially replace the need for frequent injections of recombinant monoclonal antibody therapies. Electroporation has also been widely used to deliver DNA vaccines as a means of generating antibodies against antigens, such as self-proteins, for which high affinity antibodies were difficult to generate with traditional vaccination methods [54, 55]. In addition, intratumoral delivery of plasmid DNA encoding immunotherapies such as IL-12 and AMEP (anti-angiogenic metargidin peptide) for melanoma have shown intriguing antitumor effects in animal models [56, 57] and is now being evaluated in human clinical studies. In a Phase I clinical trial, intratumoral delivery of plasmid encoding IL-12 resulted in complete regression of metastases in 10% of subjects and disease stabilization or partial response in 42% of subjects [58]. This approach is undergoing further evaluation in several clinical trials. A Phase I trial of intratumoral AMEP is in progress (ClinicalTrials.gov Identifier NCT01045915), and another Phase I evaluated AMEP antitumor activity using intramuscular delivery with electroporation has recently been initiated (NCT01664273).

One key issue related to vaccine product development is whether the immune response generated following vaccination is capable of protecting the host from the pathogen (infectious disease vaccines) or tumor (cancer vaccines). The ability of DNA vaccines delivered with electroporation to provide protective immunity has been demonstrated in several animal models. Electroporation-mediated vaccination with various influenza DNA constructs followed by challenge with homologous and/or heterologous virus strains has clearly shown that protective levels of antibodies can be generated by this vaccination approach in mice, ferrets, and/or nonhuman primates [20, 39, 59–61]. Multiple studies of electroporation-mediated delivery of SIV or SHIV DNA vaccines in macaques have also shown some degree of viral control following viral challenge [62–64]. Protection of macaques from anthrax spore challenge or Venezuelan equine encephalitis virus has also been demonstrated following electroporation-mediated delivery of DNA vaccines encoding proteins specific to each pathogen [45, 65]. Interestingly, electroporation-mediated administration of the anthrax DNA vaccine generated neutralizing antibodies and protection from challenge at levels comparable to those provided from the currently licensed recombinant anthrax vaccine, supporting the robustness of the DNA vaccine/electroporation delivery platform [65]. Other indications for which electroporation-mediated delivery of DNA vaccines have provided protective immunity include infection with *Clostridium difficile* (mice) [66], chikungunya (mice, macaques) [67], lymphocytic choriomeningitis virus (mice) [68], monkeypox (macaques) [24], bovine viral diarrhea virus (cattle) [69], *Schistosoma* (mice) [70], *Mycobacterium*

tuberculosis (mice) [71], foot-and-mouth disease virus (mice) [72], *Pseudomonas aeruginosa* (mice) [73], Japanese encephalitis virus (mice) [74], or challenge with tumor cells (mice, rat, hamster) [75–79]. In addition to protective efficacy, the ability to elicit therapeutic responses has also been described in several animal models, including chronic viral infection [80] and cancer [81, 82].

Mucosal immune responses are likely to play an important role in protection against initial infection from many pathogens. With electroporation-mediated delivery, strong mucosal responses, particularly T cell responses, have been detected in macaques following intramuscular administration of plasmids encoding SIV antigens [83], especially when a plasmid encoding the CCL27/CTACK chemokine was co-administrated with the SIV DNA vaccine [84]. In addition, mucosal IgA responses have been detected in mice following electroporation-mediated delivery of plasmid-encoded influenza antigens [20]. The inclusion of biological response modifiers, such as CCL27/CTACK, that enhance T cell migration to mucosal surfaces may be an effective strategy to elicit antigen-specific immunological responses at the primary site of pathogen entry.

9.3.3 Nonclinical Safety of Electroporation-Mediated DNA Vaccine Delivery

A number of issues are involved in assessing the safety of DNA vaccines delivered with electroporation. First, the clinical electroporation device and procedure should have safeguards that avoid accidental discharge, prevent administration of the incorrect electrical field, and protect the operator from the electrodes or the syringe used to administer DNA. For the most part, these issues have been addressed in the clinical electroporation devices currently being evaluated in the clinic [26]. A second important safety issue is that the antigen produced by the vaccine should not produce unwanted or harmful biological effects in the subject. For example, when selecting a bacterial toxin as the antigen in a vaccine, the gene encoding the toxin must be modified to reduce the potential for toxicity to the host (e.g., inactivation of *C. difficile* toxin A [66]). If a vaccine includes a gene encoding a protein with potential toxic systemic effects, the risk of potential adverse side effects may be mitigated by performing a dose escalation in initial human testing, starting with delivery of low quantities that are unlikely to produce high amounts of the protein, and increasing levels if no severe adverse events are observed. Toxicity due to the DNA vaccine and/or the administration method typically has been evaluated by repeat dose toxicity studies in a species predicted to have biological responses (including immune responses) similar to those of humans. With electroporation-mediated delivery of DNA vaccines, no significant systemic effects have been reported in animals. In the first few days following the procedure, low to moderate levels of inflammation have been observed at the injection site tissues (muscle and/or skin) due to recruitment of macrophages, T cells, B cells, and dendritic cells, probably in response to antigen expression and

tissue damage [85–87], as evidenced by the presence of muscle fibers with centrally located nuclei, suggestive of regeneration. The inflammation and associated tissue damage are typically rapidly resolved, with tissue appearing normal within several weeks following delivery of the DNA vaccine [32, 88–90], indicating that electroporation-mediated DNA vaccine delivery does not lead to long-term deleterious effects to the tissue.

The biodistribution and persistence of DNA vaccines following administration have been of particular interest to regulatory agencies, since integration of the DNA into host genomic DNA is a potential safety concern, especially in the gonads. Biodistribution studies examining the fate of the DNA vaccines delivered with electroporation have demonstrated that, similar to conventional injection, the DNA is found primarily at the injection site, with only low levels detectable in other tissues [89–91]. Although electroporation delivery results in higher initial levels of DNA vaccines in transfected tissue compared to conventional needle injection, the levels drop dramatically over time [89–92]. The decrease in plasmid DNA with time is probably the result of immune-mediated clearance of cells expressing the vaccine antigen [85, 86, 93], and the exact kinetics of clearance depends on host immune responses to the antigen. According to FDA Guidance for plasmid DNA vaccines for infectious disease indications, evaluation of whether the plasmid DNA has integrated into the host DNA is currently recommended when there are >30,000 copies of plasmid DNA detected per μg host genomic DNA [94]. Integration studies typically involve separation of high molecular weight genomic DNA from low molecular weight plasmid DNA using various gel electrophoresis methods. The level of plasmid DNA remaining associated with genomic DNA is then measured by qPCR using primers specific for the plasmid DNA. In published studies examining persistence of plasmid DNA vaccines delivered with electroporation in rats or macaques, levels of plasmid DNA that co-purified with genomic DNA were extremely low and similar to those described following conventional DNA injection [90, 91, 95]. Currently, there are no published studies demonstrating that plasmid DNA vaccines delivered with electroporation into animals have actually integrated into host chromosomal DNA. The only study that has found direct evidence of plasmid DNA integration into host DNA following delivery with electroporation was with a plasmid encoding a self-protein, erythropoietin [96]. In this study, the very sensitive RAIC–PCR method used for detection of integration events identified only a few examples of plasmid DNA/host genomic DNA junctions, and was a non-quantitative assay, making it difficult to reach conclusions about the propensity of the plasmid DNA to integrate, other than that it was a rare event. Thus, concerns about integration of plasmid DNA vaccines delivered with electroporation have largely been assuaged by the lack of data indicating that significant levels of plasmid DNA actually integrate into host genomic DNA. Overall, studies of DNA vaccines delivered to multiple animal species with electroporation have not identified any general safety issues that would preclude testing of this approach in humans.

9.3.4 Human Clinical Trials of DNA Vaccines Delivered with Electroporation

The dramatic enhancement in potency of DNA vaccines delivered with electroporation in animal models has sparked great interest in testing this approach in humans (reviewed in [97]). Currently, there are more than 25 Phase I and II clinical trials ongoing or completed worldwide using electroporation to deliver DNA vaccines for multiple indications (see Table 9.1) (www.clinicaltrials.gov and www.abedia.com/wiley). In addition to evaluating the safety, tolerability, and feasibility of electroporation-mediated DNA delivery, these trials are testing a variety of strategies for inducing potent immune responses. These include the use of vaccines encoding multiple antigens, codon and RNA transcript optimization, inclusion of DNA-encoded adjuvants, use of xenogeneic or consensus antigen sequences, targeting of the antigen to antigen presenting cells, inclusion of T helper cell epitopes, use of epitope or poly-epitope-based antigen designs, or DNA priming followed by boosting with viral-encoded antigens. In addition to the primary characterization of safety, many of the trials are focused primarily on generation of antigen-specific T cell responses (e.g., for therapeutic vaccines), although several also include assessment of antibody responses (e.g., for influenza vaccines) or initial evaluation of efficacy endpoints (e.g., for control of viremia or disease progression).

The first clinical trial using electroporation for delivery of a DNA vaccine was a Phase I/II trial initiated in 2005 in HLA-A2+ subjects with prostate cancer [98]. The vaccine encodes an HLA-A2 restricted tumor-derived epitope from prostate-specific membrane antigen (PSMA) fused to a portion of the tetanus toxin fragment C that provides CD4+ T cell epitopes to enhance responses to PSMA. The vaccine was delivered intramuscularly by conventional injection or with the Inovio Twinjector, with crossover between study groups allowed after safety and immunological data were generated from the initial series of vaccine administrations given at increasing doses. Study results published thus far have indicated that electroporation delivery significantly enhanced antibody responses to the tetanus toxin portion of the vaccine compared to those generated following conventional needle injection [98]. Since the subjects receiving the PSMA vaccine had all previously been immunized with a tetanus vaccine, it is possible that the observed antibody responses were due to activation of tetanus memory responses. Analysis of CD4+ responses to the tetanus toxin fragment and CD8+ responses to the PSMA epitope showed increased responses in vaccinated subjects [99], but there was no clear distinction between subjects that received conventional delivery versus electroporation delivery, although interpretation of the results was complicated by the crossover design of the trial. Importantly, this trial provided safety and tolerability data that indicated that the electroporation procedure was tolerable, with no reports of serious device or vaccine-related adverse events.

Additional trials evaluating electroporation-mediated intramuscular DNA vaccine delivery were initiated in 2007 for cancer, hepatitis C virus (HCV), and

Table 9.1 Human clinical trials of DNA vaccines delivered with electroporation[a]

Indication and *encoded antigens/genes*	Clinical phase	Electroporation device	Target tissue	Location	Trial identifier[b]
Prostate cancer *PSMA epitope—tetanus toxin fragment*	I/II	Inovio Twinjector	Muscle	England	GTAC No. 89[c]
Melanoma *Xenogeneic tyrosinase*	I	Ichor TDS-IM	Muscle	USA	NCT00471133
CEA and/or HER-2 positive cancer *CEA* and *HER-2*	I	Inovio MedPulser	Muscle	USA	NCT00647114
HIV (prophylactic) *Env*, *gag*, *pol*, *nef*, *tat*	I	Ichor TDS-IM	Muscle	USA	NCT00545987
HCV (therapeutic) *NS3/NS4a*	I/IIa	Inovio MedPulser	Muscle	Sweden	NCT00563173
Cervical intraepithelial neoplasia *HPV E6* and *E7* (*HPV types 16* and *18*)	I	Inovio CELLECTRA	Muscle	USA	NCT00685412
Cancer (selected solid tumors) *Telomerase*	I	Inovio MedPulser	Muscle	USA	NCT00753415
Prostate cancer *Xenogeneic PSA*	I/II	Cyto Pulse/Cellectis DermaVax	Skin	Sweden	NCT00859729
HIV (prophylactic) *Env*, *gag*, *pol* ± *IL-12*	I	Inovio CELLECTRA	Muscle	USA	NCT00991354
Hepatitis B virus (therapeutic) *Middle surfact antigen* + *IL-2/IFN-γ*	I/II	Custom made (two-needle electrode)	Muscle	China	NCT01189656
Colorectal cancer *CEA-tetanus toxin epitope* ± *recombinant GM-CSF*	I/II	Cyto Pulse/Cellectis DermaVax	Skin	Sweden	NCT01064375
Various leukemias *WT1 epitope—tetanus toxin fragment*	II	Inovio ELGEN-1000	Muscle	England	NCT01334060
Melanoma *TRP-2 epitope/monoclonal antibody*	I/II	Ichor TDS-IM	Muscle	England	NCT01138410

Influenza virus *HA, NA, m2E-NP* (*H5N1*)	I	Inovio CELLECTRA	Muscle	USA	NCT01142362[d]
Malaria *Plasmodium falciparum polyepitope*	I	Ichor TDS-IM	Muscle	USA	NCT01169077
Influenza virus *HA*, *NA*, *m2E-NP* (*H5N1*)	I	Inovio CELLECTRA	Muscle	Republic of Korea	NCT01184976
HIV (therapeutic) *Gag*, *pol*, *nef*, *tat*, *vif*±*IL-12*	I	Ichor TDS-IM	Muscle	USA	NCT01266616
HIV (therapeutic) *Env*, *gag*, *pol*	I	Inovio CELLECTRA	Muscle	USA	NCT01082692
HCV (therapeutic) *NS3/NS4a*	II	Inovio MedPulser	Muscle	Sweden	NCT01335711
HPV-16 or-18 associated cervical intraepithelial neoplasia *E6* and *E7* (*HPV-16* and *-18*)	II	Inovio CELLECTRA	Muscle	USA, Australia, Canada, Republic of Korea, South Africa	NCT01304524
Influenza virus *HA* (*H5*)	I	Inovio CELLECTRA	Skin	USA	NCT01403155[d]
Influenza virus *HA* (*H1*, *H5*)	I	Inovio CELLECTRA	Skin	USA	NCT01405885
HIV (prophylactic) *Env*, *gag*	I	Inovio CELLECTRA	Muscle	USA, Kenya, Uganda	NCT01260727
Hepatitis B virus (therapeutic) *Middle surfact antigen* + *IL-2*/*IFN-γ*	II	Custom made	Muscle	China	NCT01487876
Hepatitis B virus (therapeutic) *S and L envelope*, *core*, *polymerase* + *IL-12*	I	Ichor TDS-IM	Muscle	Republic of Korea	NCT01641536
HIV (prophylactic) *Gag*, *pol*, *nef*, *tat*, *vif*±*IL-12*	I	Ichor TDS-IM	Muscle	Kenya, Rwanda, Uganda	NCT01496989

(continued)

Table 9.1 (continued)

Indication and *encoded antigens/genes*	Clinical phase	Electroporation device	Target tissue	Location	Trial identifier[b]
Hantaan and Puumala viruses *Gn*, *Gc*	I	Ichor TDS-IM	Muscle	USA	NCT01502345
HPV-16 associated head and neck cancer *E7*, *calreticulin*	I	Ichor TDS-IM	Muscle	USA	NCT01493154
HIV (prophylactic) *Gag*, *pol*, *nef*, *tat*, *vif*±*IL-12*	I	Ichor TDS-IM	Muscle	USA	NCT01578889
Influenza virus *HA* (*H1*)	I	Inovio CELLECTRA	Skin	Canada	NCT01587131
HPV-16 or -18 associated cervical intraepithelial neoplasia *E6* and *E7* (*HPV-16* and *-18*)	I	Ichor TDS-IM	Muscle	Republic of Korea	NCT01634503[e]

[a]Arranged in order of approximate initiation date based on www.ClinicalTrials.gov as of August 17, 2012
[b]ClinicalTrials.gov Identifier
[c]UK Gene Therapy Advisory Committee number
[d]NCT01403155 is a follow-on study of participants in NCT01142362
[e]Not yet recruiting as of August 19, 2012

HIV vaccines. The cancer studies included Phase I evaluation of a DNA vaccine encoding the tumor-associated antigens carcinoembryonic antigen (CEA) and HER-2 delivered with the Inovio MedPulser to patients with cancers known to express the CEA and/or HER-2 antigens (NCT00647114), and Phase Ia/Ib evaluation of a DNA vaccine encoding a xenogeneic melanosomal antigen (murine tyrosinase) delivered to Stage IIB–IV melanoma patients by Ichor's TDS-IM (NCT00471133). Although results have not yet been published for these cancer studies, the data from the HCV and HIV studies have been encouraging. The HCV vaccine is designed for therapeutic use in patients with chronic HCV infection, and encodes the viral NS3/4A protein complex that is critical for viral replication. Designed as a Phase I/II dose escalation study in 12 treatment-naïve patients with genotype I chronic HCV infection, subjects received 0.167, 0.5 or 1.5 mg of plasmid (NCT00563173). Transient reductions in viral load were detected in a subset of subjects in the two higher dose cohorts, and persisting T cell responses to the vaccine HCV proteins were observed in four of nine patients in the two highest dose groups [100]. In addition, the MedPulser electroporation procedure was well tolerated by the subjects. Interestingly, following completion of the vaccination schedule and placement onto an HCV standard-of-care regimen of interferon-alpha plus ribavirin, the majority of patients had rapid responses to the antiviral treatment, with loss of viral RNA occurring much earlier than expected when compared to historical outcomes in these populations [100]. The intriguing finding that therapeutic vaccination followed by standard-of-care therapy may speed control of chronic viral infection is being further investigated in a recently initiated Phase II trial of the HCV vaccine (NCT01335711). In this randomized trial, patients will receive an interferon-alpha plus ribavirin standard-of-care course of therapy alone, or the HCV DNA vaccine delivered with electroporation followed by the same standard-of-care regimen.

The HIV study initiated in 2007 was the first trial of electroporation-mediated DNA delivery in healthy volunteers (NCT00545987 [101]). Initiation of this trial was significant to the field in that it indicated that the safety risks associated with electroporation-mediated delivery of DNA vaccines were considered acceptable in a healthy adult population by regulatory authorities. The HIV vaccine was composed of two plasmids encoding multiple HIV-1 subtype C antigens and was delivered intramuscularly by conventional injection or with the TDS-IM electroporation system. The randomized, placebo-controlled trial included three vaccine doses delivered with electroporation (0.2, 1.0 or 4.0 mg) plus a conventional delivery group at the highest dose. Although the study was initially designed to include only two administrations of vaccine or placebo, following favorable safety and tolerability results after the two administrations, the protocol was amended to include a third immunization in the subjects that received the highest vaccine dose with electroporation (4 mg) or placebo. HIV-specific T cells were detected in 75% (4 mg), 63% (1 mg), and 13% (0.2 mg) of volunteers receiving two vaccine administrations with electroporation, in contrast to 0% in the conventional delivery group. The response level with electroporation was increased to 88% following the third vaccine administration. Notably, the antigen-specific T cell responses were sustained

throughout the course of the study and were directed toward multiple antigens encoded by the vaccine [101, 102]. The electroporation procedure was well tolerated, and no participants discontinued the study as a result of adverse events. These results provided strong evidence that the enhancement of immune responses observed in animals with electroporation-mediated plasmid DNA delivery could also be generated in human subjects.

In 2008, a Phase I trial of a human papilloma virus (HPV) DNA vaccine was initiated in subjects diagnosed with CIN (cervical intraepithelial neoplasia) 2 or 3 that had undergone surgical or ablative treatment (NCT00685412). This vaccine targets the E6 and E7 oncogenic proteins of HPV subtypes 16 and 18, and is composed of two plasmids encoding E6/E7 fusion proteins that were generated using consensus sequences. It was delivered intramuscularly with the Inovio CELLECTRA device three times at 0.6, 2.0 and 6.0 mg. The trial is completed, and although results have not been published yet, several meeting abstracts and presentations have indicated that the vaccine was generally well tolerated, and no serious adverse events were reported [103, 104]. Cytotoxic T lymphocyte (CTL) responses were observed in 13 out of 18 subjects, and responses were detected to all four target antigens. Humoral responses to the HPV antigens were also detected, with 15 out of 18 subjects developing antibody responses to at least one antigen. Encouraged by these results, a fourth administration of the vaccine with electroporation was added to the trial design using the highest dose (6 mg) to evaluate whether responses could be boosted and whether broader responses could be generated. Although results from this trial have not been published, a randomized, placebo-controlled Phase II study of the vaccine in 148 CIN 2/3 or 3 patients that have not received surgical or ablative interventions was initiated in the USA and will include study sites in at least four other countries (NCT01304524). The primary endpoint for this trial is efficacy, as measured by the number of subjects with confirmed HPV 16 or 18 that show regression of cervical lesions to CIN 1 or less by 36 weeks after study initiation. A secondary endpoint is whether subjects responding to the vaccine also exhibit clearance of the HPV 16 or 18 infection. This Phase II study will provide valuable information about the ability of a DNA vaccine delivered with electroporation to promote immune responses capable of therapeutic activity, and if viral clearance is observed, will provide a strong impetus for further studies not only of this vaccine but also of other therapeutic DNA vaccines. Other recently initiated trials evaluating HPV DNA vaccines for cancer indications include a trial in the US evaluating calreticulin fused to HPV-16 E7 for head and neck cancer associated with HPV-16 infection (NCT01493154), and a trial in the Republic of Korea for HPV-16 or -18 associated CIN (NCT01634503). Ichor's TDS-IM is being used in both trials for vaccine delivery.

The first trial using electroporation to deliver a DNA vaccine to skin was initiated in 2008. The vaccine uses a xenogeneic approach for prostate cancer and encodes the rhesus macaque prostate-specific antigen (PSA). It is being delivered with the Cyto Pulse (now Cellectis) DermaVax electroporation system at one of four dose levels (0.05, 0.1, 0.4 or 1 mg) to patients with relapsing, non-metastatic prostate cancer (NCT00859729). Besides safety, the trial is monitoring whether the vaccine

induces PSA-specific immune responses, as well as whether antitumor effects are observed.

A number of other cancer DNA vaccine trials have been initiated. A DNA vaccine encoding a portion of the human telomerase reverse transcriptase was evaluated in multiple solid tumors including various stages of non-small cell lung carcinoma, breast cancer, melanoma, upper gastrointestinal tract carcinoma, colon carcinoma, renal cell carcinoma, bladder carcinoma, and prostate carcinoma (NCT00753415). The study has been completed, but results of the primary (safety) and secondary (immune responses to the vaccine) endpoint measures have not yet been published. Another cancer trial using the DermaVax delivery system is a Phase I/II trial evaluating a plasmid DNA-encoded fusion of human CEA and a T helper epitope from tetanus toxin in stage I or II colorectal cancer (NCT01064375). This trial includes intravenous cyclophosphamide administration 3 days prior to each vaccine administration. One treatment group receives two doses of vaccine delivered with electroporation at weeks 0 and 12. A second group that had already received the CEA/tetanus toxin DNA vaccine delivered by conventional needle injection receives the vaccine at weeks 0 and 12 with electroporation to test the ability of electroporation delivery to boost responses. The third group receives the vaccine with electroporation at weeks 0 and 12 and in addition, recombinant GM-CSF is administered intradermally/subcutaneously for 4 days starting the day before vaccination. The primary endpoints are safety and immunogenicity of the vaccine, with secondary endpoints including evaluation of the ability of electroporation to boost responses or GM-CSF to enhance immunogenicity. Another ongoing cancer DNA vaccine trial is for acute or chronic myelogenous leukemia. The vaccine consists of HLA-A2 restricted epitopes from the zinc finger transcription factor, Wilm's tumor protein (WT1), which has been associated with the development of various leukemias, fused to a portion of the tetanus toxin to provide CD4 help. In the Phase I/II trial, the DNA vaccine is delivered intramuscularly with the Inovio MedPulser (NCT01334060). The objectives of the trial are to evaluate whether the vaccine alters mRNA levels of the oncogenes BCL-ABL or WT1, immune responses to these proteins, time to disease progression and survival, as well as whether there is a correlation of changes in oncogene transcript levels with immune responses. One additional cancer vaccine trial that is currently in progress is a Phase I/II trial that was initiated in the spring of 2011 in HLA-A2+ patients with malignant melanoma (NCT01138410). The vaccine contains an HLA-A2-restricted CTL epitope from the melanosomal antigen, TRP-2, and helper T cell epitope sequences encoded within a human antibody molecule. This approach takes advantage of the ability of the Fc region of the antibody to bind to Fc-gamma receptors expressed on antigen presenting cells, thus targeting the vaccine to cells responsible for promoting vaccine responses [105]. It is being delivered by the Ichor TDS-IM device to resected Stage IIIb or Stage IV malignant melanoma in doses of up to 4 mg of plasmid DNA.

Several trials have been initiated in the last few years to evaluate DNA vaccines for infectious diseases. These include four Phase I HIV trials assessing whether addition of a plasmid encoding IL-12 enhances immune responses to the HIV proteins

encoded by the vaccines. A randomized, double-blind trial evaluating 3 mg of a multi-plasmid HIV vaccine coding for consensus sequence gag, pol, and env proteins with or without 1 mg of a plasmid coding for human IL-12 delivered intramuscularly with Inovio's CELLECTRA to healthy volunteers has been completed (NCT00991354). Interim results presented in a press release [106] showed that a higher number of subjects developed T cell responses to the vaccine when IL-12 was included (91%) versus the vaccine encoding only HIV antigens (67%), indicating that IL-12 may improve vaccine responses when included as a gene-based component of the DNA vaccine. This HIV vaccine, without the IL-12 plasmid, is now being evaluated in a trial in HIV-infected subjects on stable HAART (Highly Active Antiretroviral therapy) with the vaccine delivered intramuscularly with the CELLECTRA system (NCT01082692). Another trial evaluating the ability of IL-12 to enhance responses has been initiated in HIV-infected subjects on HAART (NCT01266616). In contrast to the previous trial, one of the trial objectives is to determine the most favorable dose of IL-12 plasmid delivered in combination with plasmids encoding HIV antigens. The HIV portion of the DNA vaccine (called HIV-MAG) consists of two plasmids encoding an HIV-1 clade B gag/pol fusion, a nef/tat/vif fusion, and the env protein. A third plasmid codes for human IL-12. The two HIV plasmids are being delivered at a total dose of 3 mg, with an escalation of the IL-12 plasmid including 0.0, 0.05, 0.25, or 1 mg. All administrations are intramuscular at weeks 0, 4, and 12 by either conventional needle injection or with the Ichor TDS-IM, and include saline placebo controls. Delivery of this vaccine with the Ichor TDS-IM is also being evaluated in two trials in healthy subjects. The first study (NCT01496989) is a randomized, double-blind placebo-controlled trial taking place in three countries in Africa. The HIV-MAG vaccine regimen includes groups that will receive a replication-defective adenovirus (Ad35) encoding HIV-1 antigens (gag, reverse transcriptase, integrase, nef and env) following or preceding administration of the DNA vaccine. The second prophylactic trial utilizing the HIV-MAG construct also includes a dose escalation of the IL-12 plasmid, and in addition, will evaluate the effect of a boost with a recombinant vesicular stomatitis virus (VSV) encoding HIV gag (NCT01578889). Another trial evaluating a DNA plus electroporation prime with viral boost for HIV prophylaxis is underway in healthy adults (NCT01260727). In this trial, the DNA vaccine encodes env and gag and is delivered intramuscularly with the CELLECTRA device or the Biojector 2000 jet injector followed by administration of a modified vaccinia Ankara (MVA) vaccine encoding env, gag, and pol. Altogether, these HIV trials of DNA vaccines will provide a solid basis for determining whether DNA vaccine-based strategies for HIV are worth pursuing, and whether enhancing technologies such as electroporation and immune modulators such as IL-12 are of benefit.

Another trial for an infectious disease indication was initiated in the fall of 2010 for malaria caused by the parasite, *Plasmodium falciparum*. This prophylactic vaccine is being evaluated in healthy subjects not previously exposed to malaria, and is a polyepitope DNA vaccine consisting of multiple CTL and helper T lymphocyte (HTL) epitopes from antigens expressed during the pre-erythrocytic stage of the parasite life cycle (NCT01169077). The vaccine is being delivered on days 0, 28,

and 56 by Ichor's TDS-IM device, at doses of 0.25, 1.0, and 4.0 mg. The primary outcome is safety, with additional objectives including immunogenicity of the poly-epitope vaccine and tolerability of the electroporation administration procedure.

A paper was published in early 2012 describing the results of a Phase I/II trial in China that evaluated electroporation-mediated delivery of a therapeutic vaccine for chronic hepatitis B virus (HBV) infection [107]. This study used a two-needle electroporation device that was designed specifically for the trial. The ED-DNA vaccine consists of two plasmids; one plasmid codes for the HBV envelope middle protein and the second plasmid codes for a human IL-2/interferon-γ (IFN-γ) fusion protein. In the first part of the trial, six subjects with chronic hepatitis B infection and serum alanine aminotransferase (ALT) values of one to two times the upper limit of normal (and therefore not indicated for antiviral treatment according to current guidelines) received the ED-DNA vaccine with electroporation at weeks 0, 4, 12, and 24. Increases over baseline of HBV-specific IFN-γ T cell responses were detected in five of six subjects, and the two subjects that demonstrated the highest HBV-specific T cell responses also exhibited reductions in viral load. The second part of the trial was double-blinded, randomized, and placebo-controlled in subjects chronically infected with HBV with ALT values of two to ten times the upper limit of normal. The subjects received the ED-DNA vaccine with electroporation as well as concurrent lamivudine antiviral treatment (20 completed the study) versus lamivudine only (9 completed the study). The lamivudine was administered to both groups for 72 weeks beginning at week 0, with vaccine delivered at weeks 12, 16, 24, and 36. A significantly higher proportion of subjects on the combined therapy exhibited reduced viral loads at week 60 compared to lamivudine monotherapy, fewer mutations associated with lamivudine resistance were found in the combined therapy group, and T cell response rates were significantly higher with the combined therapy. The vaccination regimen, which included administration at four different sites in the deltoid muscles per time point, was well tolerated. A larger, second Phase II trial using the same vaccination approach (lamivudine monotherapy versus ED-DNA vaccination with electroporation plus concurrent lamivudine therapy) is currently underway in China to further investigate the antiviral effects of ED-DNA vaccination added to standard of care nucleoside therapy (NCT01487876). A similar approach is currently being tested in the Republic of Korea using a vaccine consisting of three plasmids that encode the HBV core, polymerase, and S and L envelope proteins as well as human IL-12 (NCT01641536). The chronically infected HBV subjects are also receiving nucleoside antiviral therapy. The trial includes a plasmid DNA dose escalation, with delivery provided by Ichor's TDS-IM.

Multiple trials for influenza DNA vaccines delivered with electroporation have been initiated and/or completed. Previously, encouraging results were obtained with influenza DNA vaccines delivered with other enhancing technologies (gene gun or cationic-lipid-based Vaxfectin) in Phase I clinical trials [108, 109]. Although target hemagglutination-inhibition (HI) responses were detected in subjects in both trials, overall, the responses to these vaccines were not as robust as would be needed for a commercial product. These results indicated the continued need for enhancement of responses to DNA vaccines, possibly by electroporation. The first trials of an

influenza DNA vaccine delivered with electroporation were located in the USA (NCT01142362) and Korea (NCT01184976), with administration using the CELLECTRA intramuscular device. The vaccine consists of three plasmids encoding consensus sequences for the hemagglutinin (HA), neuraminidase (NA), and M2e-nucleoprotein (M2e-NP) antigens of the H5N1 avian influenza virus. Healthy subjects received 0.2, 0.67, or 2.0 mg of each plasmid at two time points separated by 1 month. According to a press release (http://ir.inovio.com/index.php?s=43&item=443), the results from the US trial indicated that ELISA analyses detected high levels of antibody responses in 96% of the subjects, with antibody responses generated to all vaccine antigens. However, HI titers greater than the indicator of protection titer for influenza virus of 1:40 were detected in only 3 out of 27 of subjects against the Vietnam (A/H5N1/1203/04) strain. Two of these subjects also exhibited HI titers greater than 1:40 against the Indonesia (A/H5N1/5/2005) strain, indicating cross-protection in these subjects. To evaluate effects of a boost with a DNA vaccine encoding a single influenza antigen, as well as to test electroporation-mediated skin delivery in the prophylactic setting, an additional trial was initiated in the subjects from the first influenza trial in which a single administration of 0.9 mg of a plasmid encoding H5 was delivered using the CELLECTRA skin device, with the goal of increasing HI titers (NCT01403155). It is not known why the vaccine evaluated in the initial trial generated strong binding antibody responses yet low HI titers. The use of consensus sequences in the vaccine design, antigen competition between multiple vaccine components, and/or preexisting T cell responses to vaccine components may be factors contributing to this finding; elucidation of the mechanism will lead to improved vaccine and clinical trial designs that should be applicable to other indications as well.

In July 2011, a Phase I trial was initiated using the Inovio CELLECTRA skin device for an influenza vaccine targeting H5N1 and H1N1. Three plasmids encoding two different H1 HA sequences and one H5 HA sequence, designed using consensus sequences, are being administered individually and in combination to healthy volunteers (NCT01405885). This trial will allow an analysis of whether administration of multiple antigens inhibits responses to individual components, and will provide valuable information regarding the safety and tolerability of the new skin electroporation device. Recently, a clinical trial was initiated in Canada (NCT01587131) investigating whether administration of a plasmid DNA-based influenza vaccine expressing two different H1 HA proteins and delivered intramuscularly with Inovio's CELLECTRA enhances responses to a trivalent inactivated seasonal vaccine in the elderly (>65 years of age). If enhanced responses to the seasonal influenza vaccine are observed with the DNA prime, this could be an important strategy for improving immune responses to vaccines in the elderly.

Finally, DNA vaccines encoding hantavirus antigens from Hantaan and Puumala viruses, pathogens that can cause the sometimes fatal hemorrhagic fever with renal syndrome (HFRS), are being evaluated in healthy subjects in a Phase I clinical trial (NCT01502345). The Haantan and Puumala antigens are encoded on separate plasmids. Studies in hamsters using either gene gun or electroporation delivery of the DNA vaccines showed that immune interference (i.e., reduced immune responses to

Hantaan antigens) was detected when the DNA for both viruses was delivered to the same cells [110]. In the current clinical trial, the vaccines are being delivered with Ichor's TDS-IM either separately or together, and the results should indicate whether immune interference by the Puumala antigens is also observed in human subjects.

9.4 Challenges and Future of Electroporation-Based DNA Vaccine Delivery

Although initial clinical trial results indicate that DNA vaccines delivered with electroporation generate more robust immune responses than DNA delivered by conventional needle injection, strategies for further enhancing immune responses to DNA vaccines delivered with electroporation in humans would increase the likelihood of successful development of product candidates. Based on studies showing marked enhancement of antigen-specific responses in animals, multiple approaches for improving responses to DNA vaccines are currently being evaluated in clinical trials, as described in Sect. 9.3.4. In addition, a better understanding of the role that regulatory T cells may play in vaccine responses will be important, since there is evidence that these cells can affect the magnitude of primary CD8+ T cell responses as well as the quality of memory T cell responses following DNA vaccination [111]. Other cells that can reduce immune responses are myeloid-derived suppressor cells, which can be potent suppressors of T cell functions and are present in greatly increased numbers in cancer patients (reviewed in [112]). For therapeutic vaccines, it is also becoming more apparent that combining disease-modifying drugs (e.g., chemotherapeutics or antivirals) with vaccination may be an important strategy for controlling disease [100, 107, 113].

The results of tolerability assessments of the electroporation approach conducted in clinical trials to date have been encouraging, with generally favorable responses to the procedure by the subjects even in the prophylactic setting [101]. Although anesthetics are typically used prior to electroporation application in animals to enable precise location of the injection and to avoid the startle reflex, local anesthetics are not typically used in human subjects, supporting general feasibility of the approach for vaccine applications. However, to support the broadest range of applications for electroporation-mediated DNA vaccine delivery, including prophylactic mass immunization and pediatric use, further modifications in device designs and administration procedures may be necessary. Initial clinical experience indicates that electroporation-mediated DNA vaccine delivery in skin exhibits a more favorable tolerability profile than intramuscular delivery, likely due to the smaller volume of tissue in which the electroporation-inducing electrical fields are propagated. Additionally, administration in skin is conducive to a simple application of a topical anesthetic which could further improve the tolerability profile of the procedure.

The design of a DNA vaccine can make a huge impact on the overall ability of the vaccine to promote immune responses. Vaccine features that must be carefully selected so as to maximize expression in target tissues include choice of target

antigens, DNA sequence of the antigen (e.g., codon optimization), regulatory regions such as promoter/enhancer and polyadenylation signal, secretion signal, and total number of plasmids. The ongoing accumulation of clinical data for electroporation-based delivery of a broad range of DNA vaccine designs is expected to support identification of specific vaccine strategies and components that promote strong immune responses, enabling subsequent incorporation into other candidate vaccines in development.

One issue confounding interpretation of clinical trial results that is not unique to DNA vaccines but is certainly a factor affecting trials for DNA vaccines delivered with electroporation is that often a true correlate of protection (i.e., an immune response responsible for protection [114]) is not known for a specific indication, particularly for vaccines designed primarily for induction of T cell immunity. In addition, nonclinical findings in animal models may not be predictive of human responses, further complicating the vaccine development pathway. Nevertheless, often a surrogate correlate of protection can be identified that can be used to monitor human responses; whether this surrogate is also an appropriate measure of vaccine efficacy in animal models has to be tested empirically.

The plethora of data generated in animal models and the initial results generated in human clinical trial results have produced continued enthusiasm for further investigation of DNA vaccines delivered with electroporation. Improvements in vaccine design, electroporation delivery devices, and clinical trial designs will continue to bring this field closer to licensure of a DNA vaccine/electroporation-based product for human use. The results of clinical trials currently in progress, as well as results generated by several more trials of DNA vaccines delivered with electroporation that are expected to be initiated in the next year, will be critical for maintaining strong interest in this approach to vaccine development.

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Chapter 10
Microneedles for Intradermal Vaccination: Immunopotentiation and Formulation Aspects

Alexander K. Andrianov

10.1 Introduction

Microneedle systems can open ample possibilities for the development of new generation vaccines and even revolutionize the practice of vaccination [1]. Ease of administration, improved immune protection, antigen dose sparing, and independence of cold-chain distribution are among the many potential benefits that the technology can introduce in the field [2]. Due to the same advantages, microneedle-based vaccines, and intradermal vaccination in general can also open new prospects for the development of low cost vaccines for the developing countries [3, 4].

The primary basis for the use of microneedles in vaccine administration lies in the exceptional role of skin, which has long been recognized as an attractive target for vaccination [5]. Intradermal administration of vaccines appears to be preferable to conventional intramuscular injection because the skin contains large population of dendritic cells, potent antigen-presenting cells, which are important in immune surveillance [6, 7]. Despite these significant immunological advantages, practical realization of the approach has been slow, in part due to the lack of devices that can accurately and reproducibly administer vaccines to the skin [5]. Although much progress has been achieved in the development of various devices for transdermal and intradermal delivery, a number of significant challenges remain [8–11]. Compatibility of existing methods and devices with one of the most important components of contemporary vaccines, immunoadjuvants, is one of the issues that can be particularly important for the successful commercialization of the technology [12–16].

This chapter provides a brief overview of various techniques and systems employed for intradermal immunization with a focus on microneedles, formulations, and the use of intradermal immunoadjuvants. Moreover, it offers a compre-

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hensive review of one of the potentially important approaches under development, polyphosphazene microneedles, in which synthetic biomaterial is employed as both an immunoadjuvant and a microfabrication material.

10.2 Intradermal Immunization: A Brief Overview of Techniques, Formulations, and Intradermal Immunoadjuvants

Standard intradermal immunization techniques, which typically make use of fine gauge hypodermic needles and syringes at a low angle of insertion (Mantoux technique) [17] or bifurcated needles [18], do not fulfill pharmaceutical industry's needs in clinical development of intradermal vaccines due to difficulties of using these techniques in clinical settings, inconsistency of results, dosage limitations, and significant wastage of vaccines [17]. To address these issues, a number of alternative approaches have been contemplated. A Micro Injection System, featuring a 30 gauge microneedle specifically designed for a perpendicular insertion into skin up to the depth of 1.5 mm and containing a syringe for the delivery of solution formulations, has been developed [17, 19]. Needle-free liquid jet injectors have been employed for decades with excellent bioavailability data, but occasional pain and bruising, as well as some past incidents of transmission of disease between users have limited their wide acceptance [20]. Sophisticated ballistic particle delivery systems or "gene guns" have been also engineered to allow the delivery of solid micro- and nanoparticles containing vaccine formulations [21, 22]. Moreover, needle-free patches containing solid adjuvanted vaccine formulations have demonstrated potential, as they can be easily applied to the skin (often with some pretreatment by a gentle abrasion) to carry out transcutaneous immunization [23]. Finally, a great number of intradermal or transdermal techniques utilize solid microneedles—submillimeter structures capable of penetrating the stratum corneum, the outer layer of the skin [2, 8, 10].

Designs and materials, which are used to construct microneedles, vary depending on the application techniques. The approaches range from simply using microneedles for piercing or scraping microscopic holes in the skin with subsequent deposition of the liquid vaccine to solid antigen containing formulations microfabricated in such way that the resulting shape and material properties are suitable for their application as microneedles. The latter typically requires substantial efforts on the reformulation of existing vaccines; however, such microneedles offer significant additional advantages, which include a considerable extension of shelf-life and potentially reduced reliance on temperature-controlled distribution chains [2]. Vaccine containing microneedles can be further distinguished into "coated microneedles," in which there is an inert metal or plastic support that is "coated" with vaccine formulation, and "dissolvable microneedles," in which the entire structure is prepared on the basis of the formulation; therefore, no solid material is left after such microneedles are applied to the skin [2]. The first design is attractive due to its

simplicity as it does not require extensive process optimization in an effort to balance the required mechanical strength and dissolution time, and also due to the minimal stress on vaccine antigen during the microfabrication process. The second is highly desirable when no "sharps" should be left after the application and avoidance of microneedle reuse is a priority. In both cases, delivery of vaccines to the dermal or epidermal compartments of the skin is based on a simple and dependable strategy—vaccine formulation relies on a water-soluble binding material, frequently polymer, which quickly dissolves when applied to the skin as part of a band-aid like patch. The approach has been shown to be robust and effective in vivo, with immunological results generally superseding ones generated using traditional parenteral injections [12, 24–29]. However, the potential lack of compatibility between the existing microfabrication methods and many promising vaccine adjuvants, which frequently include biphasic or particulate systems, may create a significant obstacle for the commercial development of the technology.

The importance of formulation excipients and especially immunoadjuvants in the intradermal immunization is still largely under discussion. Although there is a general consensus that intradermal immunization can be more efficient at eliciting immune responses due to the presence of a high frequency of dendritic cells in the dermal layers of the skin [6, 7], the effect may not be as pronounced or even inadequate when compared with parenteral adjuvanted vaccine formulations. This is especially important as contemporary vaccines increasingly rely on immunoadjuvants to provide the required quality of the immune response and satisfactory protective immunity [30]. Unfortunately, some of the most popular adjuvants may not be the best candidates for intradermal immunization. For example, alum, which is the most common adjuvant currently used in the vaccine market globally [30], was shown to induce serious adverse effects, such as formation of granuloma, when administered intradermally [14].

A substantial number of studies have been focusing on the evaluation of promising immunoadjuvants for intradermal immunization. These results can provide a general guidance on the suitability of these adjuvants or their relative activity using various administration routes. However, it is also important to note that given the variety of application techniques discussed above, adjuvants can be administered in the skin at different depth, in the diverse physical form, such as liquid, solid, or hydrogel, in the presence of relatively high concentration of surfactants or other excipients, or even facilitated by the use of physicochemical methods, such as electroporation, which can certainly impede the accuracy of a comparison.

Delivery of solution formulations by intradermal injections appears to be a dominating technique in the majority of such studies. Monophosphoryl lipid A (MPL®) [31], emulsion-formulated toll-like receptor agonists, including TLR4, TLR9, TLR7 agonists [32], oligodeoxynucleotides containing the CpG motif (CpG ODN) [33, 34], cholera toxin (CT) [34], granulocyte-macrophage colony-stimulating factor (GM-CSF) [35], and mast cell activator compound 48/80 [34] are a few examples of adjuvants that have been successfully investigated using such methods. Facilitation of intradermal immunization using electroporation produced mixed results—hyaluronidase, imiquimod, and MPL adjuvants were reported to have minimal

effect on the immune response [36], whereas micron-size gold particles enhanced the percentage of responding animals [37]. Alhydrogel [16, 38, 39] and unmethylated, phosphorothioate-linked, CpG-containing oligonucleotides [39] were reported as potential adjuvants for a Microinjection System. Solution formulations containing *N*-trimethyl chitosan, but not its nanospheres [40], lipopolysaccharide (LPS), Quil A, CpG, CT [41] have been effective topically after distortion of stratum corneum using solid microneedles. Finally, integration of adjuvants and needle-free jet injection devices has been also realized to deliver vaccines adjuvanted with plasmid DNA-coated nanoparticles [42], alum [42], imiquimod [43], and GM-CSF [43].

Solid-state vaccine formulations containing immunoadjuvants have been also studied. Heat-labile enterotoxin (LT, derived from *Escherichia coli*) has been successfully tested in needle-free patches with multiple vaccines including anthrax [44, 45], influenza [46], tetanus toxoid [47], and demonstrated potential in humans [15, 48]. Adjuvants have been also tested in coated microneedles. Glucosaminyl muramyl dipeptide (GMDP) [13] was incorporated in microneedles containing model antigen, Ovalbumin. Although the enhanced immune response has been detected for the GMDP containing microneedles compared to non-adjuvanted microneedles, an increase was about tenfold lower than the adjuvant effect observed for intramuscular injections indicating inferior performance of the adjuvant when delivered intradermally. Moreover, contrary to microneedle results, conventional intradermal injection did not show a significant difference between adjuvanted and non-adjuvanted formulations, which may potentially suggest the advantage of the solid-state delivery of adjuvanted vaccines.

In the following sections we will discuss adjuvanted microneedle systems, which utilize a macromolecular immunoadjuvant of a polyphosphazene class, the approach that makes use of a single compound to construct microneedles and to provide potent immunoadjuvant properties.

10.3 Polyphosphazene Immunoadjuvants

Polyphosphazene immunoadjuvants are long-chain molecules, which are designed around the biodegradable inorganic backbone and organic side groups containing anionic moieties. Despite the apparent simplicity, multiple molecular structures can be created by synthetic assembly of various pendant groups around such template with a rational design focusing on macromolecules with environmentally controlled degradation profiles, benign breakdown products, and desired interaction characteristics [49–54]. Poly[di(carboxylatophenoxy)phosphazene] (PCPP; Fig. 10.1) is the most popular representative of this class, which has been successfully advanced to the development state. A potent immunoadjuvant effect of PCPP has been well documented when administered with a variety of bacterial and viral antigens in more than a dozen of animal models [50, 55–60]. Its track record in human clinical trials includes significant improvement in seroconversion and seroprotection rates for a seasonal influenza vaccine [61] and fourfold rise in neutralizing antibodies

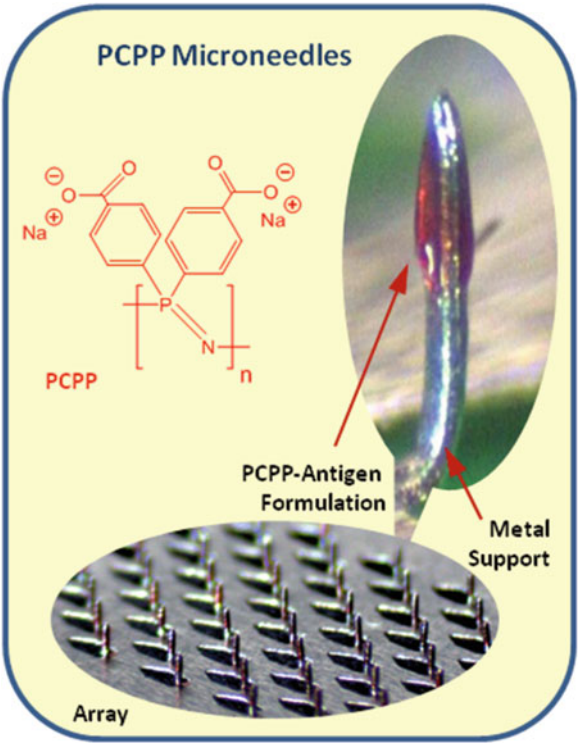

Fig. 10.1 Schematic presentation of polyphosphazene microneedles and chemical structure of PCPP

against respiratory syncytial virus (RSV) strains in greater than 75% of participants [62]. Vaccine formulations containing PCPP have been reported to be safe and immunogenic [62, 63].

Immunopotentiating effect of polyphosphazenes can be characterized by modulations in the onset, magnitude, quality, and duration of immune responses. It has been reported that polyphosphazene adjuvants induced significant increases in antibody responses as early as 2 weeks after immunization for a number of antigen including influenza and hepatitis B [56, 58, 60, 64]. Sustained levels of antibody titers for the length of the experiment (up to 41 weeks) were observed for X:31 influenza antigen and HBsAg [56–58]. Interestingly, it was also found that depending on the type of polyphosphazene, and possibly on the structure formation in solution, the IgG isotype profiles can vary. PCPP primarily enhances IgG1 antibody responses, which are typically associated with Th2-type response, whereas its "sister" polymer poly[di(carboxylatoethylphenoxy)phosphazene] (PCEP) [51] or PCPP copolymers containing oxyethelyne side groups [65] have been shown to also enhance IgG2a [58, 65], which can be associated with Th1-type immune responses providing protection against intracellular pathogens [66].

Another important feature of PCPP—antigen sparing effect—has been demonstrated in lethal challenge studies with H5N1 influenza vaccine using highly relevant preclinical model (ferrets). In these experiments PCPP formulated vaccine afforded

100% protection from mortality, whereas non-adjuvanted formulation was not protective at a dose of at least tenfold higher [55]. In regard to quality and magnitude of immune responses, PCPP adjuvanted X:31 influenza formulations injected in mice were as potent as their non-adjuvanted counterparts containing 25 times higher dose of the antigen [58]. Benchmarking of polyphosphazene adjuvanted vaccines against those containing alum suggests either greatly superior responses for polyphosphazenes in animal studies [57, 58] or at least equal performance in humans [62].

Polyphosphazene immunoadjuvants are generally easy to formulate as they have excellent solubility in neutral and basic aqueous solutions; however, conformation–activity and molecular weight–activity relationships [56, 67], as well as ionic sensitivity [53] may play an important role and formulations have to be characterized and optimized accordingly to achieve superior results. Similar to other polyelectrolyte adjuvants [68], biological activity of PCPP strongly depends on its association with the antigen; however, contrary to their conventional counterparts, polyphosphazene adjuvants form stable water-soluble, non-covalent complexes with antigenic molecules spontaneously and thus do not require chemical conjugation [67]. As it will become evident from discussion below, intermolecular complexation provides important advantages also for the use of polyphosphazenes as microneedle fabrication materials.

10.4 Polyphosphazene Microneedles

Microneedles containing vaccine formulations, such as coated or dissolvable microneedles, should satisfy at least two fundamental requirements. First, they should have sufficient mechanical strength and appropriate shape to be able to penetrate the outer layer of the skin. Second, microneedles, or their formulation compartment (in case of coated microneedles) should be able to dissolve and release vaccine within the predetermined application time, which is typically in the range of several minutes. Water-soluble polymers, which provide good cohesion and adhesion properties, adequate tensile modulus, appropriate solution viscosity for the microfabrication process, and satisfactory dissolution profiles are viable candidates as microfabrication materials. Sodium salt of carboxymethylcellulose (CMC) is just one example of a water-soluble polymer that has been thoroughly investigated as a microfabrication material [69].

A unique position of PCPP as a microfabrication material for vaccine containing microneedles results from a combination of already mentioned immunoadjuvant activity with its high molecular weight characteristics, water-solubility, and excellent microencapsulation properties [70, 71]. Initial studies of PCPP as a microfabrication agent have been focused on the preparation of coated microneedles. Figure 10.1 shows a representative array of microneedles composed of a titanium structural support and a solid PCPP-antigen formulation coated on its external surface. Arrays, each including fifty 600 μm long microneedles, were prepared using a micro-dipcoating process, in which an aqueous antigen-PCPP formulation was

deposited on the tip of a metal support from a custom-designed micro reservoir via a series of dipping-drying cycles [69, 72]. Polyphosphazene microneedle arrays were then integrated into adhesive patches, which can be easily applied to the surface of the skin so that the formulation is dissolved and the vaccine is released.

10.4.1 In Vivo Potency of Polyphosphazene Microneedles

The potency of polyphosphazene microneedle vaccine in vivo has been studied with a focus on its ability to release the payload when applied to the skin and immunogenicity of the formulation [12]. As the technology is centered on intradermal administration, the similarity of animal and human skin was considered to be most relevant criteria, which resulted in the selection of a swine animal model [73].

Microneedle application and antigen release studies were designed to investigate the following parameters: penetration of the stratum corneum as examined by optical and histological inspection of the application site and physicochemical analysis of the residual material on microneedles following the application. The results of such studies, which were enabled by the use of fluorescently labeled antigen and co-formulated dyes, showed the release of active components in excess of 90% within 15 min application period [12]. These results were also independently confirmed in vitro in a study, which used porcine cadaver skin and resulted in an approximately the same delivery efficiency [12].

Polyphosphazene microneedle formulations were benchmarked against parenteral formulations and non-adjuvanted microneedles in the immunogenicity study in pigs using recombinant hepatitis B antigen (HBsAg) [12]. Although selected antigen proved to be very weakly immunogenic when delivered intramuscularly without an adjuvant (Fig. 10.2, curve 1), its PCPP adjuvanted formulation induced immune responses that were at least tenfold higher (Fig. 10.2, curve 3). Interestingly, practically no improvement in immune responses induced by HBsAg was achieved when non-adjuvanted microneedles composed of antigen and CMC as a construction material were employed (Fig. 10.2, curve 2), or as a result of intradermal injection of antigen without PCPP (data not shown). Polyphosphazene microneedles were tested at two different doses of antigen and both further outperformed PCPP adjuvanted parenteral formulation for approximately one order of magnitude (Fig. 10.2, curves 4 and 5). This demonstrated an impressive synergy between PCPP and intradermal delivery, which can be hardly predicted on the basis of responses for adjuvanted parenteral injection and non-adjuvanted microneedles. A substantial potential for antigen dose sparing was also demonstrated. Polyphosphazene microneedles containing ten micrograms of HBsAg induced tenfold higher antibody titers than 20 micrograms of PCPP-HBsAg formulation delivered intramuscularly [12]. No significant reactogenicity issues were reported in the study [12].

These results, obtained in the large animal model, which is highly relevant in terms of preclinical development of intradermal delivery systems, demonstrate the potential of the system from the bioavailability and immunogenicity standpoints.

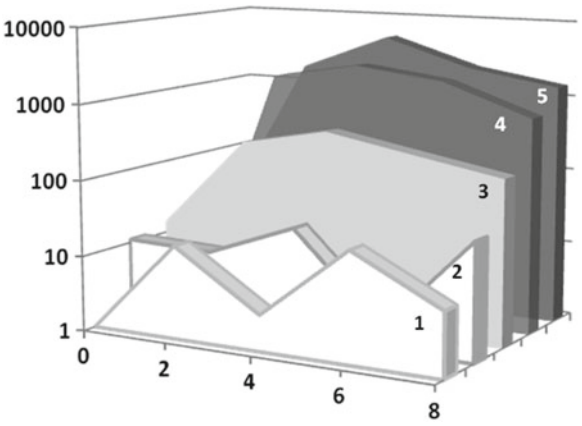

Fig. 10.2 Serum IgG-specific mean HBsAg titers after a single dose immunization of pigs with HBsAg. Ten micrograms of HBsAg was administered intramuscularly (*1*) and intradermally using CMC microneedles (*2*). PCPP adjuvanted formulation containing 10 μg of HBsAg and 66 μg of PCPP was also delivered intramuscularly (*3*). Finally, PCPP microneedles containing 20 μg (*4*) and 10 μg of HBsAg (*5*) were administered intradermally. Polyphosphazene microneedles contained 66 μg of PCPP. Seven animals were used per group

10.4.2 PCPP Formulations: Added Value for the Microfabrication Process

Microneedle fabrication using a dip-coating process provides simplicity and implies relatively minimal constraints on the formulation [72]. Antigen and PCPP doses can be also well controlled as the required amount of the formulation supplied to a microneedle array can be conveniently measured volumetrically [12]. Nevertheless, the antigen can be subjected to a significant stress as the coating and drying processes are conducted at ambient temperature, a large numbers of coating cycles are typically required, and additions of certain excipients to the formulations, such as surfactants, are desirable to provide even distribution of the formulation [69, 72]. To that extent, polyphosphazenes can offer some additional advantages that can minimize potential negative impact of the production process on the vaccine formulation.

10.4.2.1 Antigen Stabilization in PCPP Formulations for the Microfabrication Process

The ability of PCPP to improve stability of antigens and proteins in aqueous solutions [54, 55] can be of critical importance for the manufacturing of microneedle vaccines, especially when unstable antigens are concerned. The stabilizing effect of PCPP, which was observed in a broad range of polymer concentrations, provides a dramatic effect in prolonging protein half-life in accelerated stability studies [54], and is even more pronounced in the presence of surfactants, which are frequently used in vaccine and

microfabrication formulations. The effect is linked to macromolecular interactions in the system and the formation of PCPP–protein and PCPP–protein–surfactant complexes. It has been recently demonstrated that PCPP was able to facilitate incorporation of H5N1 influenza antigen or other proteins into microneedles without any detectable loss of activity [55, 74], whereas other studies emphasized the need for a stabilizing agent, such as trehalose, when CMC was employed as a microfabrication material [75].

10.4.2.2 Accelerated Microneedle Fabrication and the Use of Surfactant-Free Systems

Coating process is a key stage of microfabrication technology as biological activity and other functional properties of microneedles are essentially defined at this phase. A large number of dipping-drying cycles, excessive use of surfactants or buffer salts can compromise either immunogenicity of the formulation or mechanical strength and dissolution rates. Thus, coating formulations, which should provide adequate viscosity, surface tension, and ionic strength without negatively affecting the antigen, are the focal point in the production of potent microneedle vaccines.

PCPP has been compared with one of the most investigated microfabrication polymers, CMC [69], for its ability to produce microneedles with a minimal number of coating cycles and reduced amounts of surfactants and salts [12, 74]. It has been found that the formation of complexes between PCPP and the antigen led to a marked increase of solution viscosity resulting in a fewer coating cycles needed to achieve target antigen/adjuvant loading on microneedles compared to CMC formulations [74]. This signifies a significant reduction of the stress on the antigen imposed by the process. Moreover, it was also established that intermolecular interactions in PCPP formulations reduce the dependence of the coating procedure on surfactants, which can be important as the content of surfactant in vaccine formulations may need to be controlled [76]. It appears that the highest efficiency of PCPP as the microfabrication agent is observed in solutions with low ionic strength, which is highly desirable for coating formulations [74].

Overall, in addition to already described antigen stabilizing effect in the formulation, polyphosphazene immunoadjuvant is capable of further decreasing the stress on the vaccine, through minimizing or eliminating undesirable factors in the manufacturing process.

10.4.3 PCPP Microneedles with Ionically Cross-Linked and Multilayer Coatings

Antigen containing polyphosphazene microneedles are typically produced using a single coating formulation, which, as discussed above, is generally sufficient to induce potent immune responses and afford reliable intradermal delivery. However, polyelectrolytic nature of PCPP allows additional modulation of the formulation through ionic

complexation with small or large molecules, which can result in the formation of cross-linked networks or layers of polyelectrolyte complexes. These supramolecular assemblies can be produced using additional formulations after the main coating process is completed and can result in a generation of other desirable properties, such as sustained antigen/adjuvant release or improved moisture sensitivity.

10.4.3.1 Ionically Cross-Linked, Sustained Release Microneedles

PCPP has been shown to form protein compatible hydrogels when treated with physiologically benign ionic compounds, such as calcium chloride or spermine hydrochloride, in aqueous solutions [71, 77, 78]. This property has been proven to be useful for microencapsulation of proteins under mild conditions and the resulting networks provided potential for creating sustained release systems [70, 71, 79]. In vaccine formulations, slow erosion of such matrices can provide modulated release of both antigen and the immunoadjuvant, PCPP [80, 81]. Although PCPP microneedles are generally designed to dissolve practically instantaneously upon their contact with aqueous environment (Fig. 10.3a), it can be also beneficial to prolong the release of the antigen as it can potentially result in a more potent and persistent immune responses [30]. Such sustained release can be achieved, for example, if the dissolution rate of the coating is reduced due to ionic cross-linking (Fig. 10.3b), and the coating can be deposited in the skin in the insoluble hydrogel state and release the antigen over an extended period of time.

The approach of ionic cross-linking of polyphosphazene immunoadjuvant by calcium or spermine salts has been also successfully realized for microneedles [74]. Treatment of protein containing PCPP microneedles using various formulations of such ionic cross-linkers results in a significant decrease of release rates in vitro offering simple approach to the development of sustained release formulations [74]. Variations in a number or duration of treatments with the cross-linker or combinations and sequences of such cross-linkers provide additional tools in the modulation of antigen or immunoadjuvant release rates [74].

10.4.3.2 Microneedles with Multilayered Structures

Another feature of polyphosphazene immunoadjuvants, which can be of considerable value for the use in microneedle technology, is their ability to form interpolymer complexes with polyelectrolytes of the opposite charge. Polyelectrolyte complexes of PCPP have been investigated as part of microsphere formulations and were demonstrated to be capable of modulating surface properties and permeability characteristics [77, 79]. The concept of microneedles, in which moisture sensitive vaccine coatings are protected by the alternating layers of more hydrophobic and less sensitive to aqueous environment polyelectrolyte complexes, was recently elaborated [82]. As PCPP and CMC, which constitute the main component of the coating, are negatively charged molecules, chitosan was employed as the macromolecular counterion [82]. The microfabrication process

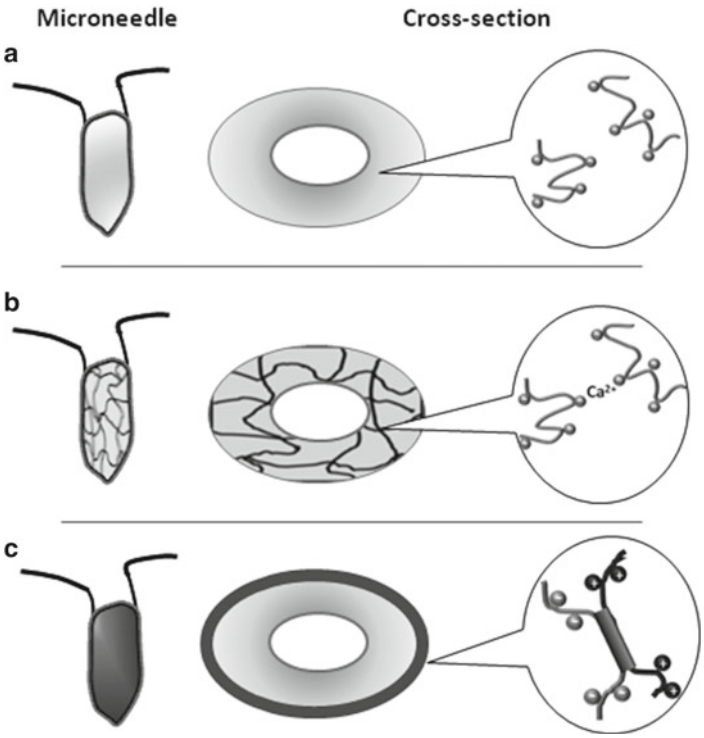

Fig. 10.3 Various designs of polyphosphazene microneedles including microneedles with standard antigen-PCPP formulations (**a**), ionically cross-linked formulations for modulated antigen release (**b**), and formulations containing semi-permeable polyelectrolyte complexes for reduced moisture sensitivity and modulated release, surface-coated version is shown (**c**)

was carried out under the conditions that enable formation of polyelectrolyte complexes between these oppositely charged macromolecules and resulted in the deposition of multilayer assemblies in the outer section of the coating (Fig. 10.3c). The results demonstrate that microneedles containing multilayer formulations display suppressed moisture sensitivity in water vapors compared to their unmodified counterparts [82]. PCPP microneedles modified with PCPP-chitosan polyelectrolyte complex layers demonstrated an uptake of water of only a 5% after exposure to 100% relative humidity environment for 6 h. For comparison, their unmodified counterparts displayed a water uptake in excess of 30% (A. Marin and A.K. Andrianov, unpublished data). Despite this impressive reduction in moisture sensitivity, which can be important for the improvement of microneedle shelf-life and also during the administration process, multilayered microneedles maintained quick protein release characteristics required for their use. The approach also showed potential for sustained protein release applications, as such layers can be assembled in the internal sections of the formulation to fabricate multi-compartment microneedle coatings with delayed release characteristics [82].

10.5 Conclusion

Intradermal immunization remains one of the most promising and, nevertheless, challenging approaches in the development of new and improved vaccines. The use of immunoadjuvants, their compatibility with microneedle systems, is one of the potential obstacles the technology faces on a path to successful commercialization. The development of polyphosphazene microneedles constitutes one of the potential solutions to this challenge. PCPP, the lead polyphosphazene immunoadjuvant, displayed excellent immunopotentiating and dose-sparing properties in immunization studies as part of microneedles. In addition, the material characteristics of this synthetic polymer appear to be ideally suited for the microfabrication and microneedle application requirements. The system also provides an important protein stabilizing effect in the production process, as well as ability to easily modulate release and stability characteristics.

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Chapter 11
MicroCor® Transdermal Delivery System: A Safe, Efficient, and Convenient Transdermal System for Vaccine Administration

Parminder Singh, Guohua Chen, and Wade Worsham

11.1 Introduction

The development of convenient, safe, efficient, and effective delivery methods for large molecular weight drugs (including biologicals and vaccines) by non-parenteral administration routes continues to be an objective for a number of academic institutions and commercial organizations. Transdermal delivery of drugs is generally limited to small and potent molecules using conventional patch technology and passive diffusion mechanisms. Large molecular weight drugs are generally administered by intramuscular or subcutaneous routes of administration using the traditional needle and syringe, or in some cases using needle-less injector devices. Fear and pain are universal concerns in needle-based drug delivery, particularly in the pediatric population, and can make the experience of vaccination rather unpleasant. "Needle-phobia" is one of the major reasons for vaccine noncompliance, which can lead to less than optimal preventive outcomes. Noncompliance also contributes significantly to the direct and indirect economic burden on the patient and the health-care system.

Another limitation of biologicals and vaccines is their inherent instability in a liquid state at room temperature. Most biologicals and vaccines must be stored under refrigerated conditions for their intended shelf-life. This requires specialized manufacturing, transport and storage requirements, which create additional burden and cost to the manufacturers, health-care providers, and patients. The potential for accidental needle-sticks and the resulting cross-contamination, as well as the need for trained professionals to administer the injections, are some of the other main obstacles surrounding needle-based vaccine delivery. Several novel noninvasive or minimally invasive technologies are currently being explored as alternatives to the needle injection for the delivery of large molecular weight drugs.

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The transdermal route of administration offers advantages like easy access, relatively large surface area, painless and bloodless delivery, and a rich immune environment for antigen uptake and resulting immune response. The use of microstructure-based devices (also known as microneedles) for transdermal delivery of small organic molecules and large molecules (peptides, proteins and vaccines) has been extensively reviewed in the literature [1–3]. These devices have been fabricated from a number of materials ranging from metals to silicon dioxide to thermoplastics and water-soluble polymers, as either solid or hollow microstructures and in numerous geometries (e.g., varying shape, height, angle, and aspect ratio) [4–8]. The hollow microstructures have not only the advantage of delivering liquid vaccines without reformulation but also the disadvantages of needing trained personnel for administration, stabilizing a liquid formulation during storage, reconstituting the vaccine before injection, or potentially leaking or being blocked during administration. Coated microstructures might be able to be administered with little or no training using a solid formulation with good stability during storage; however, they may require specific formulation work, the coating process may be complex and inefficient, drug delivery may be limited depending on coating uniformity, and there are safety issues due to sharps left on the device after use. A more elegant approach to designing microstructure-based systems involves the integration of the drug or vaccine directly into a biocompatible and biodegradable polymer matrix, then fabricating the microstructures to be capable of skin penetration with only a slight external force. Corium has developed this technology, MicroCor® Transdermal Delivery System (TDS), which uses solid-state biodegradable microstructures that penetrate the stratum corneum barrier layer of the skin with the application of slight external force and dissolve to release the drug or vaccine for local or systemic uptake, without leaving any medical sharps. The MicroCor technology can be formulated and designed to deliver a wide variety of small and large molecular weight drugs and achieve a range of drug delivery profiles, from a rapid, bolus delivery to a more sustained and continuous delivery. The MicroCor technology is simple to use, convenient, minimally invasive, efficient, safe, and effective for the transdermal delivery of small and large molecules.

11.2 MicroCor Design Considerations

The MicroCor TDS is designed to deliver a topical or systemic dose of a drug or vaccine across the stratum corneum barrier layer of the skin using an array of microstructures. The MicroCor TDS is formed by integrating two key parts: the microstructure array (MSA) containing the drug or vaccine and an applicator device. These two integrated parts are packaged together for a single use application.

The MSA consists of a drug layer and a backing layer. The drug layer comprises the dried vaccine and other inactive ingredients in a biocompatible, biodegradable, and water-soluble matrix. The backing layer supports the drug layer and consists of a biocompatible, non-water-soluble matrix. The shape and size of the microstructures allow them to penetrate the stratum corneum barrier layer of the skin and release the drug or vaccine into the skin for local or systemic uptake. The applicator device con-

sists of a plastic shell with skin contact adhesive covered by a release liner, and a spring to provide the energy needed to penetrate the microstructures into the skin. The MSA is mounted to the applicator device. The final assembled MicroCor TDS is the drug or vaccine containing MSA integrated with the applicator device.

The MicroCor TDS is activated by depressing an activator button on the applicator device. When activated, the energy from the spring penetrates the microstructures into the skin. The drug layer dissolves into the skin delivering the drug or vaccine and the device is removed and discarded.

11.2.1 Applicator Design Considerations

Considerations for the primary applicator design include usability by the target population under normal operating conditions, ensuring reproducible penetration of the microstructures. Within each of these areas there are key design factors that can be adjusted for the specific application. These factors may be impacted by the target indication/population and the geometry of the microstructures in the array. The applicator components and materials are selected to be biocompatible.

To achieve usability, the steps needed to apply and use the MicroCor TDS must be minimized. The MicroCor TDS has three user steps: (a) remove the release liner from the skin contact adhesive, (b) apply to the skin and push the button to activate, and (c) remove the TDS after few minutes and dispose. The force required to activate the applicator device is selected such that different population demographics will be capable of easily activating the device. The diameter and height of the TDS is selected to provide flexibility in placing the TDS at different body sites and to simplify transport by a user.

Sufficient energy must be available to cause the microstructures to penetrate the skin. The applicator device has a spring which provides energy to cause the microstructures to penetrate into the skin. The available energy is used to penetrate the microstructures to the desired depth and to compress the skin tissues, with the remaining energy lost through various mechanisms.

The delivered energy, by its inherent correlation with the depth of microstructure penetration into the skin, is closely related to drug delivery. The applicator device performance has been measured experimentally by the released energy, skin penetration efficiency (SPE), the residual drug remaining on the TDS after use, and in vivo pharmacokinetics. Experimental data has shown consistent delivery of drug or vaccine into the skin with as low as 0.15 J of released energy for the current MSA configuration.

11.2.2 MSA Design Considerations

The MSA was designed so that the dissolving portion which contains the drug or vaccine will penetrate at a minimum the stratum corneum barrier layer of the skin, but will not reach the underlying deep tissue containing blood vessels and

nerves. The sharpness of the tips helps to reduce the energy required to penetrate the microstructure into the skin, making a sharper tip more desirable. The inactive ingredients and microstructure shapes were chosen so the microstructures would have the required mechanical properties of stiffness, toughness, and compressive strength. The strength and stiffness are optimized to ensure penetration into the skin. The ability of a material to absorb energy and plastically deform before fracturing defines its toughness. The drug layer was designed to provide the microstructures with sufficient toughness so they remain intact during shipment, handling, and application. The desired dose, drug solubility, and amount of inactive ingredients needed to create a strong, tough, and stiff microstructure define the required microstructure volume. A film forming material is used as a backing to support the microstructures for ease of assembly into the applicator device.

11.3 MicroCor Formulation Considerations

The MSA consists of a drug or vaccine containing a solid-state biodegradable microstructure layer and a backing layer. The backing layer, which supports the microstructures, is made of a biocompatible and non-water-soluble matrix. The microstructures contain the dried drug or vaccine and the excipients in a biocompatible and water-soluble matrix. Upon penetration into the skin, the drug or vaccine containing microstructures dissolves and delivers the vaccine into the skin. The inactive ingredients used to form the microstructures comprise a polymer(s) which provides required mechanical strength and optionally a sugar which stabilizes the biologic or vaccine. The polymers used in the microstructures are biocompatible, water soluble, and able to form mechanically strong microstructures. The sugars used are well-characterized stabilizing excipients for various biologics and vaccines.

The vaccine active ingredient is first formulated with a water-soluble matrix in a liquid solution. The inactive ingredients, polymer and sugar must be compatible with the vaccine in the liquid formulations. The liquid formulations should have minimal to no impact on the integrity of the vaccine for the duration of the MSA fabrication process (hours to days) at room temperature or at refrigerated temperature.

The vaccine containing liquid solution is then cast into polydimethylsiloxane-based mold and dried to form the drug layer of solid-state microstructures. The purity of drug or vaccine is closely monitored to ensure minimal to no impact on the drug or vaccine integrity during the fabrication process. The process parameters and formulation compositions are optimized for an individual drug or vaccine, depending upon its physicochemical properties. The backing layer is then cast and dried to complete the MSA. The MSA in its dried form is integrated with the applicator device and then packaged in a poly foil pouch under an inert gas such as nitrogen to allow prolonged room temperature stability of the drug or vaccine.

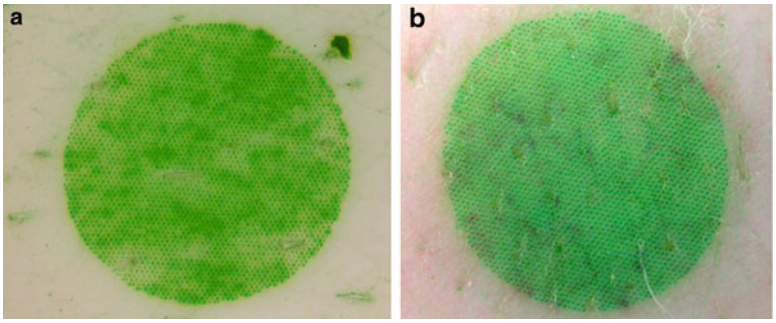

Fig. 11.1 *In vitro* (**a**) and *in vivo* (**b**) light microscopic images of microstructure penetration into pig skin

The functionality of the MSA is characterized by an *in vitro* or *in vivo* skin penetration efficiency (SPE) test and a residual drug or vaccine analysis. The SPE test is conducted to evaluate the mechanical properties of the microstructures to ensure they are strong enough to effectively penetrate the skin. The residual drug analysis is conducted to estimate the apparent dose delivered and the drug delivery efficiency.

The SPE test is performed by applying the MSA on the excised skin *in vitro* or *in vivo* using a force applicator. The application sites are dye stained and photographed to visualize the penetrations. The penetrations are quantified using a custom developed image analysis program and the percent SPE is calculated as follows:

$$\%\ \mathrm{SPE} = 100 \times (\#\ \text{penetrations} / \#\ \text{microstructures})$$

Following the SPE test, the MSA is extracted and analyzed for the residual drug or vaccine. The apparent dose delivered is then calculated as follows:

$$\text{Apparent dose delivered} = \text{initial drug content} - \text{residual drug content}$$

$$\text{Apparent delivery efficiency} = 100 \times \left[1 - \frac{\text{residual drug content}}{\text{initial drug content}} \right]$$

The MSA containing a human parathyroid hormone (1–34) [(hPTH(1–34)] formulation is illustrated as an example to characterize the *in vitro* and *in vivo* performance. The hPTH(1–34) MSA was applied to either excised Yorkshire pig skin *in vitro* or *in vivo* using a force applicator. The application sites were dye stained and photographed to visualize penetrations. Penetrations were quantified using a custom developed image analysis program. Figure 11.1a, b shows the light microscope images of *in vitro* and *in vivo* SPE tests, respectively. The dark green

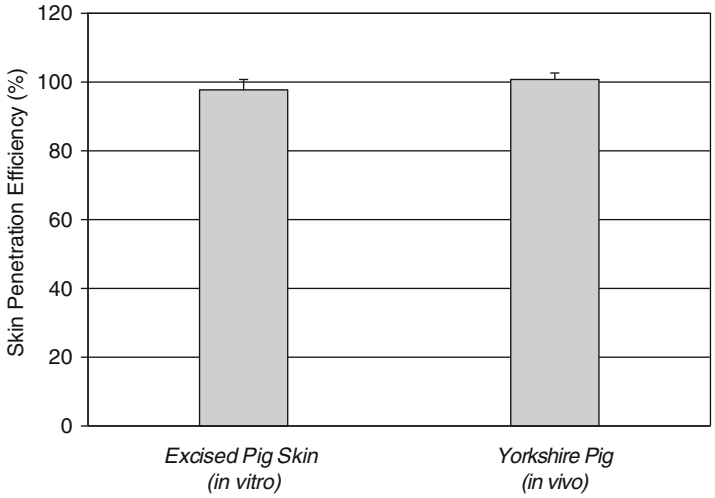

Fig. 11.2 In vitro and in vivo skin penetration efficiency in pigs

dots are indicative of successful penetrations. hPTH(1–34) MSA demonstrated >95% SPE both *in vitro* and *in vivo*, as shown in Fig. 11.2, indicating good mechanical performance of the MSA.

The microstructures containing hPTH(1–34) dissolved within 5 min of skin insertion. The nearly complete dissolution of the microstructures highlights an important safety feature of the MicroCor technology. Once the microstructures have dissolved within the skin, there is minimal risk of cross-infection due to accidental reapplication or needle reuse. This is consistent with the World Health Organization directive on developing safe drug administration practices. In addition to safety benefits, this feature of the MicroCor technology also provides economic benefits by eliminating significant direct and indirect costs to treat needle-stick injuries. There are no sharps left after use and the system can be disposed in a normal waste receptacle, further minimizing disposal costs.

To quantify the amount of hPTH(1–34) delivered, after the SPE test, the MSA was extracted and residual hPTH(1–34) quantified. The apparent delivery efficiency of hPTH(1–34) from the MSA was estimated to be >70% *in vitro* and >80% *in vivo*, respectively, as shown in Fig. 11.3. In addition to hPTH(1–34), the MicroCor technology has also shown high drug delivery efficiency for a number of other drugs, biologicals, and vaccines.

11.3.1 rPA Delivery in Rats

Recombinant protective antigen from *Bacillus anthracis* (rPA) was obtained from List Biologicals (Campbell, CA). The MSA containing rPA was fabricated using a casting and drying fabrication method. A given volume of rPA formulation

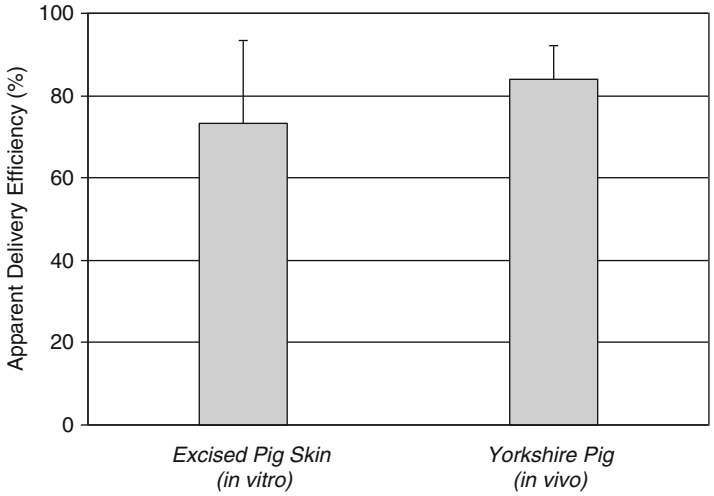

Fig. 11.3 In vitro and in vivo apparent drug delivery efficiency in pigs

Table 11.1 Composition of rPA-containing formulations (percentage of solids, w/w)

Component	Formulation I	Formulation II	Formulation III
rPA	2.5%	5%	10%
PVA	20%	20%	20%
Trehalose	31%	30%	28%
Maltitol	31%	30%	28%
HP-β-CD	15.5%	15%	14%

was spread over the surface of a silicone mold and the formulation was then pressurized followed by convection drying. Next, a given volume of the backing solution was spread on top of the dried rPA containing layer, was pressurized and then dried. The rPA containing MSA was demolded and packaged with desiccant in a heat-sealed barrier foil pouch and stored under refrigerated conditions. Three rPA formulations were fabricated into MSAs according to compositions shown in Table 11.1. To verify the microstructure quality, the MSA was inspected on a stereoscope and only arrays with greater than 95% intact microstructures were used in the *in vitro* and *in vivo* studies. A solid-state dry film without microstructures was fabricated as a control for the rPA immunogenicity study using rPA formulation III.

The integrity of rPA after being processed into MSA and solid-state films was verified using SDS-PAGE gel analysis. MSAs of each formulation, solid-state film, and their associated liquid casting solutions were analyzed and found to be consistent with a stock rPA standard solution, thus indicating that rPA is stable during MSA processing (data not shown).

To prepare the primary immunization liquid formulation for the intramuscular (IM) and intradermal (ID) administrations, rPA was mixed with phosphate-buffered

Table 11.2 rPA immunization study design

		Immunization 1	Immunization 2
Group	Animals	Day 0	Day 28
1	5	Liquid, IM 10 μg rPA, alum	None
2	5	Liquid, IM 10 μg rPA, alum	Liquid, ID 1 μg rPA, no alum
3	5	Liquid, IM 10 μg rPA, alum	Liquid, ID 10 μg rPA, no alum
4	5	Liquid, IM 10 μg rPA, alum	Liquid, IM 10 μg rPA, no alum
5	5	Liquid, IM 10 μg rPA, alum	rPA solid-state film (10% rPA)
6	5	Liquid, IM 10 μg rPA, alum	MicroCor I (2.5% or ~5 μg rPA)
7	5	Liquid, IM 10 μg rPA, alum	MicroCor II (5% or ~10 μg rPA)
8	5	Liquid, IM 10 μg rPA, alum	MicroCor III (10% or ~18 μg rPA)

saline containing aluminum hydroxide adjuvant. The three liquid formulations for the second immunization were prepared in phosphate-buffered saline with no alum.

11.3.1.1 *In Vivo* rPA Delivery and Immunization Study

Female, Sprague Dawley rats (approximately 220 g) were chosen because they were the smallest animal model compatible with the size of the MSA and the method of array application. All animal husbandry and handling procedures were performed in accordance with approved IACUC procedures. Each animal received a primary IM injection immunization on day 0 followed by a second ID, IM, or transdermal immunization using MicroCor TDS on day 28 as described in the overall study design in Table 11.2. Five animals were assigned to each treatment group.

The solid-state film or the MSA was each die cut into 16 mm diameter discs and attached to the tip of the spring-loaded impactor with a thin layer of petroleum jelly. The impactor device was activated to apply the test articles to the skin. Within 15 min of removing the device, the skin sites were photographed and scored for edema and erythema using the 0 (none) to 4 (severe) scale of the Draize scoring system [9].

Blood samples were obtained from all animals before immunizations to establish a baseline IgG antibody level. On day 0, each rat received the primary immunization of 10 μg rPA with adjuvant delivered via IM injection. Blood was drawn two weeks (day 14) after this injection and the serum, isolated by centrifugation of the whole blood, was stored at −80°C for later analysis of antibody levels. The animals received the second immunization in the form of one of the treatment groups listed in Table 11.2 on day 28. Again, blood was drawn two weeks after immunization on day 42 to assay the serum for IgG levels.

Serum anti-rPA IgG titers were measured by an enzyme-linked immunosorbent assay (ELISA) as described in [10].

Mean values reported in this study represent the average of at least five replicates. Error bars are the standard deviations (SD) of the means. When a comparison between two means was required, a Student's *t*-test with a 95% level of confidence

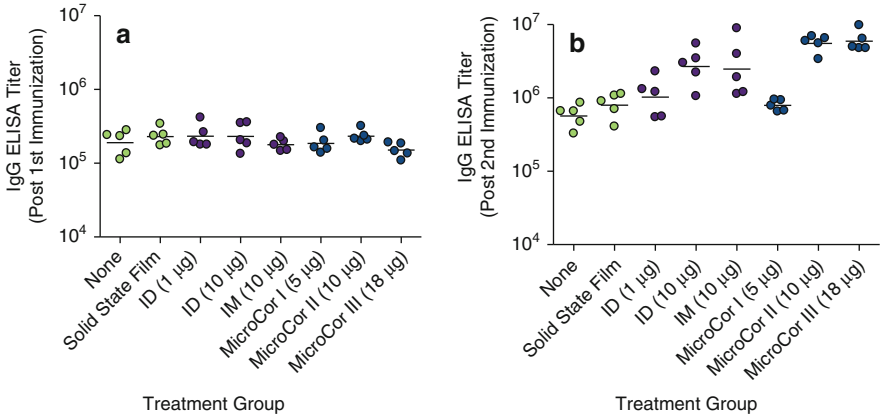

Fig. 11.4 Individual serum anti-rPA IgG antibody titers measured two weeks after the first immunization (**a**) and two weeks after the second immunization (**b**) for each treatment group. Geometric means are also plotted for each treatment group. The estimated amount of rPA delivered is reported in parentheses for immunization groups

($\alpha = 0.05$) was used. Comparisons between two or more means were performed using a one-way analysis of variance at the same level of confidence (ANOVA, $\alpha = 0.05$). A p-value < 0.05 was considered to indicate statistical significance.

11.3.1.2 IgG Antibody Titers After Immunization

Figure 11.4 shows the anti-rPA antibody titers for the individual animals in each treatment group. The estimated amounts of rPA delivered rounded to the nearest microgram are reported in parentheses for the immunization treatments. All groups received the same primary immunization and as expected and shown in Fig. 11.4a, the antibody titers at 2 weeks post the primary immunization were comparable for all eight treatment groups. After the second immunization, there were several differences seen among the various groups, with results depicted in Fig. 11.4b.

An increase in antibody titer was observed for the control (none) group after the second two-week period even with no second immunization. The increase was likely due to the presence of the alum adjuvant in the initial immunization which may have caused the primary immune response period to be extended. Nevertheless, antibody titers for the 10 µg ID and IM treatment groups, though not statistically different from each other ($p = 0.88$), were higher than the control ($p = 0.001$ and 0.009, respectively) and the solid-state film groups ($p = 0.007$ and 0.03, respectively). Likewise, the MicroCor TDS II (10 µg) and III (18 µg) groups had significantly higher antibody titers than the control (none) and solid-state film (all $p < 0.0001$). The MicroCor TDS II and III groups also had statistically higher antibody titers than the ID (10 µg) group ($p = 0.04$ and 0.03, respectively) and higher, but not statistically different IgG titers than the IM (10 µg) group ($p = 0.09$ and 0.07,

respectively). This is likely due to the higher variability seen in the IM group. The antibody titers were comparable for the MicroCor TDS II (10 μg) and III (18 μg) groups indicating a plateau in the dose–response curve above a certain threshold antigen concentration ($p = 0.73$).

The MicroCor TDS 5 μg group had antibody titers that were significantly lower than both the II and III MicroCor TDS groups ($p < 0.0001$). The 5 μg group was comparable to the 1 μg ID group and the solid-state film ($p = 0.39$ and 0.98, respectively), indicating a minimal threshold concentration required for eliciting an increase in the IgG response (typical of an S-shaped dose response curve). The 5 μg group was expected to have a higher titer based on the robust response of the 10 μg group II. However, the doses for the MicroCor TDS groups are indirect estimates based on microscopic evaluation of microstructure dissolution, and as a result there could be some error with these dose estimations.

The immune response expressed in terms of anti-rPA titers, which has been shown to be a good indicator of overall protective immunity [11], was higher after the transdermal delivery of rPA with the MicroCor TDS than after the ID and IM routes of administration. Also the immune response after the transdermal delivery with the MicroCor TDS was more consistent and reproducible as compared to the ID and IM routes of delivery. Interestingly, there was no statistical difference observed between the 10 μg IM and ID groups in this study. In some literature reports, the ID route has been shown to have a better antibody response than the IM route for certain antigens, although this may not always be the case [12–14]. The overall improved response seen with the transdermal route versus traditional injections may indicate that different aspects of the immune system are responsible for the anti-rPA antibody response, and it is possible that the balance of antigen presentation by dermal dendritic cells in the dermis and/or Langerhans cells in the epidermis determine the overall antibody response.

11.3.1.3 Local Skin Tolerability

Evaluations of the dissolution of the microstructures indicate that penetration depths of at least 100 μm, and possibly more than 150 μm, can be achieved. At this depth, the skin irritation effects observed in rats were mild and transient. After administration of the rPA-containing MicroCor TDS and the solid-state film, there was no observable edema on any of the treatment sites (all scores = 0) and erythema ranged from none (score = 0) to slight (score = 1) for both the film treatments and the array treatments. Small numbers of petechiae were visible; however, light blotting of the skin revealed no blood on the skin surface. Figure 11.5 shows representative images of the rat skin after application of the solid-state film and MicroCor TDS (2.5% rPA formulation) treatment. These results show that the MicroCor technology has excellent skin tolerability, and

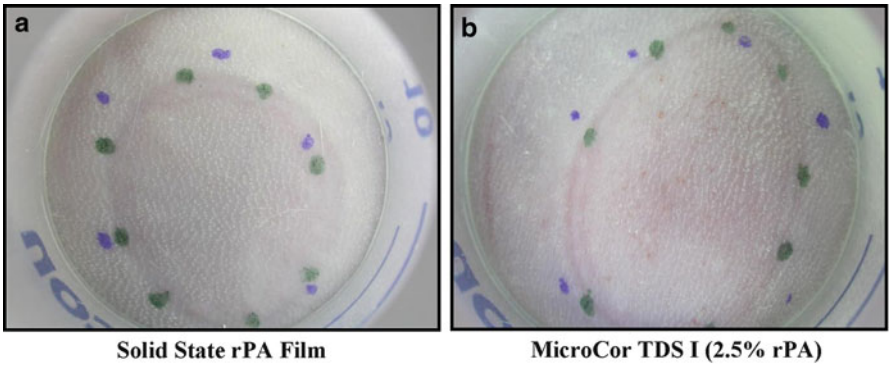

Fig. 11.5 Representative photographs of skin sites after *in vivo* application of the solid-state rPA film and an rPA-containing MicroCor TDS array in the rat model. *Note*: Treatment sites have been outlined with *blue* and *green* marker. The microstructures were well tolerated as evidenced by the lack of edema and no more than mild erythema

these results have been confirmed in further *in vivo* studies with a number of compounds and in a Phase 1 clinical study.

11.4 Conclusions

The transdermal route is a well-accepted route of administration as is evident from patient acceptability and the commercial success of patches in several therapeutic areas, such as pain management, hormone replacement, nicotine cessation, hypertension, and neurologic diseases. The transdermal route is limited, however, to small molecule delivery due to the excellent barrier properties of the skin. The surge in development of biotechnologically based macromolecule drugs and a renewed interest in vaccines has created an opportunity for the development of needle-free transdermal delivery systems for painless, safe, and effective delivery of these drugs.

The results with rPA presented here and Corium's experience with a number of other molecules tested have demonstrated the success of using the MicroCor technology for simple, convenient, efficient, safe, and effective transdermal delivery of a wide variety of drugs, biologicals, and vaccines. Leveraging the MicroCor technology will allow the continued development of therapeutic drug and vaccine candidates that are cost-effective and amenable to commercial scale-up.

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Chapter 12
Mimopath™-Based Vaccine Delivery

Kees Leenhouts

12.1 Introduction

Vaccines are considered one of the most valuable public health intervention tools. Nevertheless, the performance of many existing vaccines is far from optimal and there are still diseases for which no vaccine is available. A key issue in the development of improved and new vaccines is safety, since most vaccines are given to healthy individuals. In order to improve safety profiles, the use of well-defined (recombinant) purified antigens for the generation of subunit vaccines has become leading in vaccine development programs. In addition, there is an increasing interest to explore other modes of vaccine administration besides the use of needles. Since purified soluble antigens are usually poorly immunogenic, even more when delivered through the mucosal (nasal, oral) routes, the addition of safe adjuvants to increase the efficacy of vaccines is needed.

The success or failure of an antigen-specific immune response depends on the interface between innate and adaptive immunity. The innate immune system comprises mechanisms and cells (e.g., macrophages, dendritic cells) that defend the host in a nonspecific manner from infection by other organisms. This means that the cells of the innate system recognize and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. On the other hand, the recognition of dangerous entities by the innate immune system is a prerequisite for the stimulation of pathogen-specific adaptive immune responses. It is now known that adaptive immunity dependency on the innate immune system results from the need for antigen processing and presentation. These functions are displayed by professional antigen presenting cells (APC),

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such as dendritic cells (DC). These latter cells establish the cross talk between T and B cells, which may lead to the stimulation and activation of active immunity.

The recognition of pathogenic microorganisms is in part performed by the presence of pattern recognition receptors (PRR), such as the Toll-like receptors (TLR), on cells from the innate immune system. The initial uptake and phagocytosis of microbes by APCs is facilitated by recognition of microbe-associated molecular patterns (MAMP) by PRRs. Conserved MAMPs that are recognized by TLRs are, e.g., lipopolysaccharides (LPS) of Gram-negative bacteria and peptidoglycan (PGN) of Gram-positive bacteria. It is known that LPS is a specific agonist for TLR4 and PGN likewise for TLR2. The recognition of MAMPs leads to the activation of DC maturation, a process entailing upregulation of major histocompatibility complex (MHC; class I and class II) and co-stimulatory (e.g., CD40, CD80 and CD86) molecules, together with the production of different pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), which in turn results in optimal antigen processing and presentation to naïve T cells. Adaptive responses may result in productive activation of T helper type 1 (Th1), type 2 (Th2) or type 17 (Th17) cells. A well-orchestrated innate and adaptive immune response usually leads to pathogen eradication and long-lasting immunity against the specific agent.

The properties of MAMPs make them inherent attractive for their use as adjuvant in (subunit) vaccines in order to establish the activation of the innate immune system that is pivotal to the proper induction of adaptive immune responses. Many bacterial TLR agonists like LPS-, lipopeptide-, flagellin, and DNA derivatives have been or are being developed as new adjuvants [1]. Another approach is to take advantage of a repertoire of MAMPs present in a certain microbe and use live bacterial vectors for the delivery of antigens. Attenuated strains of pathogens like *Salmonella typhi*, *Escherichia coli*, *Vibrio*, *Mycobacterium*, and *Listeria* are under investigation for the development of prophylactic and therapeutic vaccines [2]. However, administration of live bacterial vaccines derived from pathogens, albeit from attenuated strains, poses some risks. In addition, vaccination using recombinant bacteria results in the release of live recombinant organisms into nature. To overcome these potential risks, several containment strategies have been developed including one that generates nonliving Gram-negative delivery vectors [3–5].

Innocuous Gram-positive lactic acid bacteria (LAB) may present a safer alternative for attenuated pathogenic bacteria as vaccine delivery vehicle [6]. Dietary LAB have a long history of beneficial and safe use in the food industry and are known for their widespread use in fermented foods, including probiotics and as such have obtained the "generally regarded as safe" (GRAS) status [7, 8]. The most prominent member of the LAB family that is being investigated as live vaccine vector is *Lactococcus lactis* [9]. Recent work of Yam et al. [10] demonstrated the immunomodulatory activities of *L. lactis*: in vitro production of pro-inflammatory cytokines by murine macrophages upon incubation with *L. lactis*; the in vitro maturation of murine DCs (CD11c+); and the in vivo induction of the cytokines IL-1β, IL-12, and IL-10 in DCs. IL-1β is a pro-inflammatory cytokine that has been shown to be an effective mucosal adjuvant [11]. IL-12 functions to induce Th1 responses and is known as an effective adjuvant to promote cell-mediated immunity [12]. IL-10 is a

Table 12.1 Comparison of BLPs with HA (split virus) and HBsAg (VLPs) intranasal vaccines in mice and rats

	HA (HI titer in mice)		HbsAg (mUI/mL titer in rats)	
Formulation	Pre-immunizations	Post-immunizations	Pre-immunizations	Post-immunizations
Ag	0	23	0	1
Ag + BLP	0	283	0	350

cytokine that exhibit dual functions: it activates humoral immune responses by stimulating B-cells and it is also known as a down-regulator of the immune system [13]. In the same study, it was shown that *S. typhi* and *E. coli* failed to induce in vivo expression of IL-10 in murine DCs. Taken together, the study of Yam et al. [10] demonstrated that *L. lactis* induces both Th1 and Th2 immune responses and that this bacterium may play a homeostatic role by dampening inflammatory immune responses.

The use of live recombinant LAB like *L. lactis* may limit the risks associated with the use of live bacterial vectors in vaccines. In addition, strategies have been developed that contribute to the containment of live recombinant LAB in the environment [14]. Nevertheless, regulatory hurdles and the perception of the lay public may hamper the development of vaccines based on such bacterial vectors. The use of a bacterial carrier based on nonliving and nonrecombinant *L. lactis* that has preserved immunostimulatory capacities may be useful to overcome these obstacles.

12.2 The Mimopath™ Concept

12.2.1 Binding and Mixing

The Mimopath™ concept was designed to use nonliving *L. lactis* bacterium-like particles (BLPs) as a backbone that is to be externally loaded with surface attached antigen to mimic a pathogen. This concept is supported by the general view in the vaccine field that antigens are best presented to the immune system as particles. During the Mimopath™ development it was noticed that in a number of cases binding of the vaccine antigens was not necessary in order to obtain improved and protective responses. The mixing of, e.g., the regular split virus influenza vaccine with BLPs elevated the HI titers to levels above the protective limit of 40 after intranasal delivery in mice. Similarly, BLPs mixed with hepatitis B surface antigen (HBsAg) also enhanced titers to a level that is considered to be protective (>10 mUI/mL) after intranasal administration in rats (Table 12.1). In both cases the antigens used had no (non)specific binding interaction with the BLPs, but it should be noted that these antigens have already some kind of particulate nature by themselves. Importantly, these examples show that BLPs may be a suitable immunostimulant to improve existing vaccines and/or enable efficacious administration of the vaccine through a mucosal route.

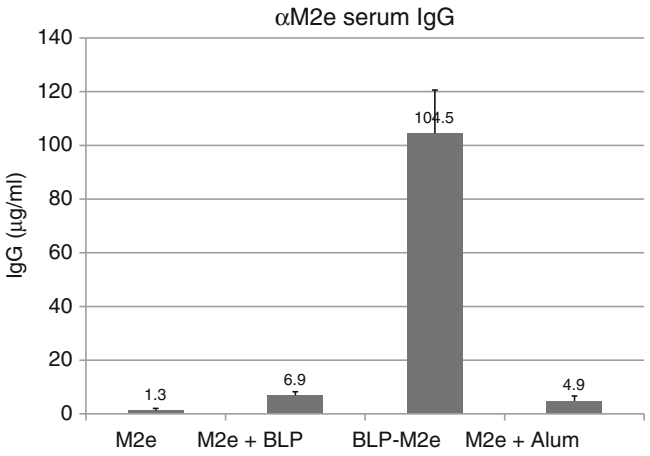

Fig. 12.1 M2e-specific serum IgGs after intramuscular immunizations of mice (three times) with a tandem repeat of 3 copies of the 24 amino acid M2e peptide, either without adjuvant (M2e), mixed with BLPs (M2e + BLP), bound to BLPs as fusion to Protan (BLP – M2e), or adsorbed to aluminum hydroxide (M2e + alum)

For novel soluble recombinant protein vaccines the approach is to bind the antigen to the surface of the BLPs through the use of a proteinaceous binding domain called Protan. The binding of such antigens provides a considerable improvement compared to just mixing. An example is given in Fig. 12.1 which summarizes the results obtained with the influenza virus conserved M2e peptide that was used as a tandem repeat of three copies in intramuscular immunizations in mice. A more than 15-fold improvement in the M2e-specific serum IgG response was observed when the peptide was bound to the BLP instead of mixed with the BLP. In general, the nature of the antigen and the route of administration may influence the fold of improvement.

In summary, there are two formulation formats for Mimopath™, one in which the antigens are simply mixed with BLPs and one in which the antigens are bound to the surface of the BLPs as a Protan fusion protein (Fig. 12.2).

12.2.2 The BLPs

The Gram-positive bacterial cell surface consists of a single membrane on the inside and a thick cell wall on the outside. The cell wall is made up of multiple layers of PGN with various other components that may protrude both inside and outside. A simple pretreatment in hot acid destroys all cellular components, including intracellular components like DNA. Cell-wall components other than the rigid PGN matrix are also degraded. The result is a nonliving particle that still has the same shape and size as the bacterium before treatment (Fig. 12.3a). The procedure is applicable to all Gram-positives,

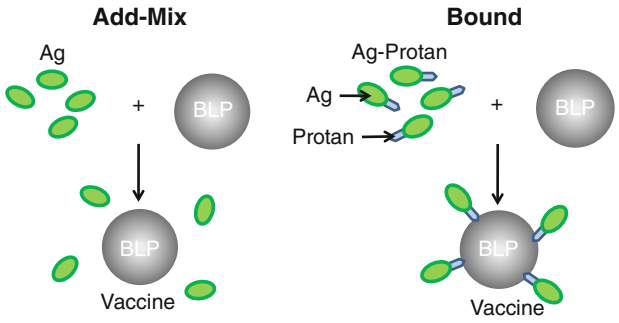

Fig. 12.2 The two formats of Mimopath™-based vaccines. The panel on the *left* summarizes the admixing format that is especially useful to improve existing vaccines. The panel on the *right* summarizes the binding approach that is in particular suitable for soluble recombinant proteins. *Ag* antigen

hence the name that was formerly used: Gram-positive Enhancer Matrix (GEM). However, due to the harsh nature of the chemical treatment some Gram-positive species may be prone to lysis and are therefore less suitable for the manufacturing of BLPs. A bacterium that does not lyse using this treatment is the lactic acid bacterium *L. lactis*. The safe background of this bacterium makes it also very suitable for use in vaccines. *L. lactis* was therefore used to develop the procedure to generate BLPs. Suitable acids that can be used to generate BLPs include acetic acid, hydrochloric acid, sulfuric acid, monochloroacetic acid, trichloroacetic acid, and trifluoric acid. The acid treatment is followed by extensive washing with buffer to remove acid and degradation products. The procedure results in nonliving spherical-shaped BLPs that have a diameter of approximately 1–2 μm and consist predominantly of a PGN outer surface (Fig. 12.3 [15, 16]). Several GLP and cGMP BLP lots have been made and stability studies indicate that *L. lactis* BLPs formulated in PBS are stable for more than 2 years during storage at various temperatures (−80°C, +5°C, +25°C).

Temperature, pH, and incubation time in acid determine the characteristics of the BLPs generated. Important characteristics of BLPs are the stimulation of the innate immune system and the binding of Ag–Protan fusions. Since the procedure to generate BLPs results in particles with mainly PGN on the outer surface and PGN is a known agonist for TLR2 [17] that is also present on innate immune cells, the availability of PGN in a suitable format for immune stimulation is measured in a TLR2 cell-based assay. HEK-Blue™-hTLR2 cells (Invivogen) are being used for this purpose as reporter-based system that monitor TLR2-induced NF-κB activation. *L. lactis* cells that have not been treated with hot acid show a modest activation of the NF-κB pathway in this assay. Treatment of *L. lactis* in 10% trichloroacetic acid (pH 1) at 70°C results in an increase in the activation through TLR2 in a time-dependent manner. A similar observation is made for the binding of Protan. Non-treated *L. lactis* cells show binding of only modest amounts of Protan, and the increase in the amounts of Protan that is bound by the BLPs depends on the treatment time in the acid (Table 12.2). Experiments of Steen et al. [18] showed that

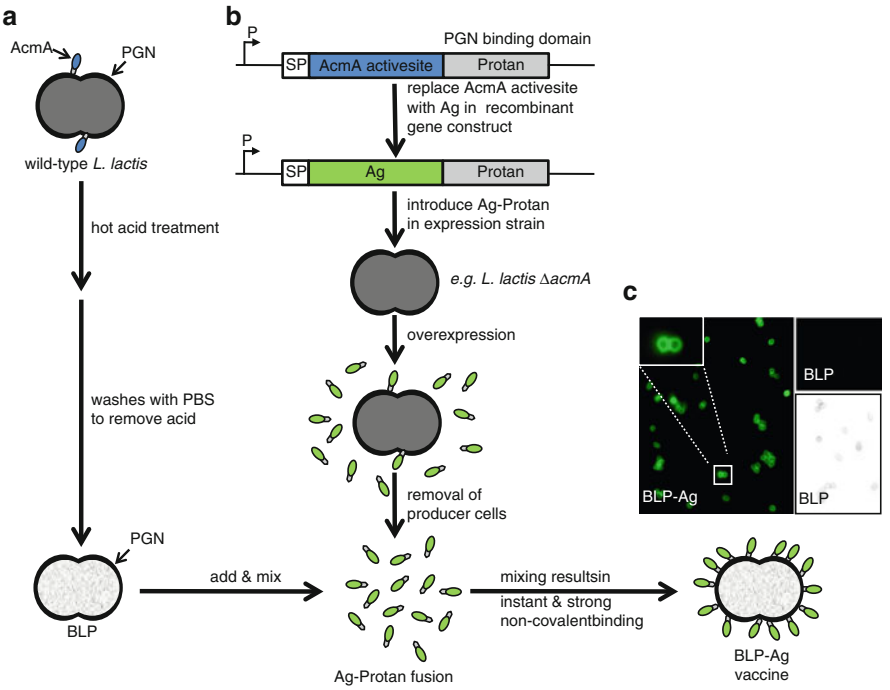

Fig. 12.3 Overview of the manufacturing of a Mimopath™-based vaccine with bound antigens. (**a**) Production of BLPs. After treatment in hot acid, degradation products and acid are removed by washing with phosphate buffered saline (PBS). The BLPs are finally formulated in PBS. (**b**) Gene constructs that enable the expression of antigen (Ag)–Protan fusions are introduced in a suitable expression host. Preferably the constructs also enable the secretion of the fusion product in the growth medium. Suitable hosts are, e.g., *L. lactis* or *E. coli* for bacterial antigens and mammalian cells for viral antigens. For *L. lactis* an *acmA* mutant strain is used because the AcmA enzyme also contains the Protan domain. After overexpression and secretion of the Protan fusion of interest, producer cells are removed by centrifugation or microfiltration. The growth medium with the Ag–Protan fusion may be used as such for binding to BLPs or an additional purification step may be introduced prior to binding. (**c**) Mixing of an Ag–Protan solution with BLPs results at instant and strong non-covalent binding, resulting in BLPs that are completely covered at the surface with the antigen

Table 12.2 Influence of incubation time in 10% trichloroacetic acid at 70°C on TLR2 activity and Protan binding of the generated *L. lactis* BLPs

Incubation time, *t* (h)	Relative TLR2 activity (%)	Relative Protan binding (%)
0	100	100
0.5	75	227
1	138	414
2	243	751
4	1,218	1,001

cell-wall components hinder the binding of Protan to untreated *L. lactis* cells. It was shown that lipoteichoic acids and surface proteins localize to parts of the cell surface to which Protan does not bind. The removal of cell-wall components with acid resulted in binding of Protan to the entire surface of the bacterial particles and consequently higher amounts can be bound. The above described results suggest that the acid treatment removes cell-wall components which improves the availability of the PGN at the particle surface for binding to Protan or TLR2.

12.2.3 Protan Fusions

The PGN-binding domain Protan is derived from the C-terminal part of the lactococcal cell-wall hydrolase AcmA. It consists of three Lysin motif (LysM) homologs of 43 amino acids that are separated by non-homologous spacer sequences of 28–33 amino acids highly enriched in serine, threonine, and asparagines [19]. The LysM motif, usually 42–65 amino acids in length, is a ubiquitous modular cassette (Pfam PF01476) found across prokaryotes and eukaryotes in more than 4,000 proteins. In prokaryotes, LysM seems to have evolved into a general PGN-binding motif, whereas in eukaryotes it is a chitin-binding motif. *N*-acetylglucosamine (GlcNAc) seems to be an important constituent of the LysM ligand and has been shown to interact with LysMs of some bacterial and eukaryotic proteins. Whether GlcNAc is the sole moiety recognized by LysM remains to be elucidated (LysM domains are reviewed in [20]).

The three-dimensional structure of the LysM motif has been determined for only two bacterial proteins, *E. coli* membrane-bound lytic murein transglycosulase D [21] and *Bacillus subtilis* YkuD [22]. The LysM domain has a βααβ secondary structure with the two α-helices packing onto the same side of an antiparallel β-sheet. An extensive mutational analysis revealed a highly robust folding pathway with no detectable transition state plasticity, indicating that LysM is an example of an ideal two-state folder [23]. Based on the available structural information van Roosmalen modeled the Protan LysM domains [24]. The model indicated the presence of a shallow groove opposite of a hydrophobic side of the domain. Docking experiments with *N*-acetylglucosamine-*N*-acetylmuramic acid-alanine as a minimal PGN module on the Protan LysM model predicted the groove as the most favored binding site of this compound. Intriguingly, two of these LysM domains could be modeled as a dimer which would allow an exact fit of the two grooves that could potentially accommodate a PGN strand (Fig. 12.4). Oligosaccharide binding sites are often grooves with a platform of aromatic residues, capable of forming stacking interactions with sugar rings. The proposed binding site of the Protan LysM domains features a groove and an aromatic platform containing one tryptophan and two tyrosine residues. Amino acid residues near the putative binding groove in the Protan LysM domain were identified that could be used to insert fluorescently labeled tryptophan residues allowing sensitive spectroscopic measurements of PGN-binding events [24]. This technique could be a valuable tool in addition to the

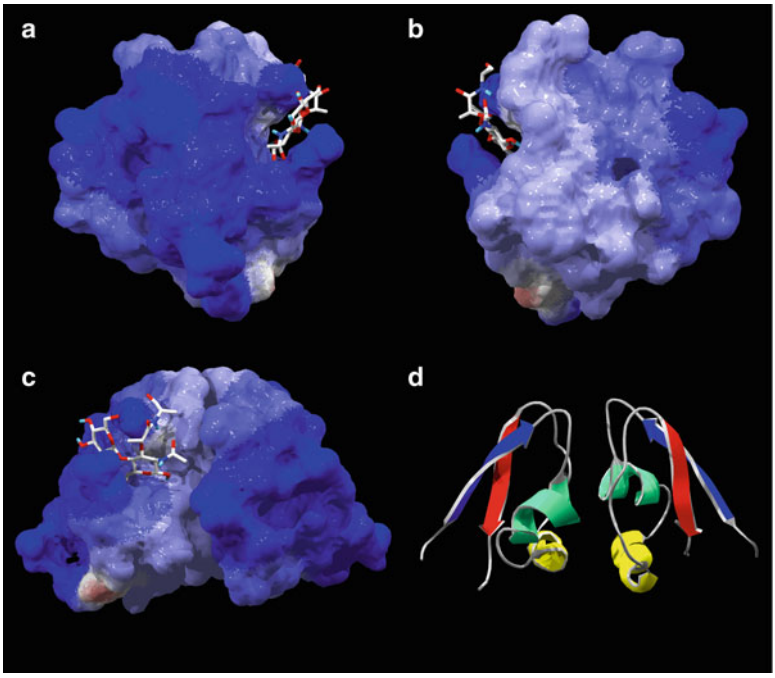

Fig. 12.4 Modeling and computational docking of an AcmA LysM motif. Electrostatic surface potential of LysM in which *red* and *blue* represent negative and positive potentials, respectively. The *stick* figure represents the structure of a possible bacterial peptidoglycan ligand *N*-acetylglucosamine-*N*-acetylmuramic acid-alanine as the top-scoring free binding energy compound docked in the groove formed between the β_1 sheet and α_1 helix of LysM. (**a**) Front-side view. (**b**) Back-side view. (**c**) Theoretical model of a dimer consisting of two LysM domains. The contact surface consists of the hydrophobic side of each domain. (**d**) Ribbon representation of the dimer: β_1 *blue*; α_1 *green*; α_2 *yellow*; β_2 *red*. AutoDock 3.05 [25] was used for docking studies, Swiss-PdbViewer 4.01 and Pov-Ray 3.7 for generating the pictures

single-molecule force spectroscopy technique to measure and localize individual LysM–PGN interactions that was used by Andre et al. [26], which revealed a bimodal distribution of binding forces for Protan, presumably reflecting the occurrence of one and two LysM–PGN interactions, respectively.

The native Protan domain consists of three LysM motifs. Buist et al. [19] and Bosma et al. [16] showed that one LysM has only a very low binding affinity. The latter investigators demonstrated that two LysM motifs are sufficient to obtain binding affinity that is similar to the native Protan domain. Using immobilized BLPs on a gold surface of a surface plasmon resonance instrument (Biacore), the binding of Protan to the BLPs was quantified with a K_D of 3.4×10^{-5} M. Although this binding is strong, it is not as strong as a typical Ab–Ag binding ($K_D = 10^{-9}$–10^{-13} M). Bound Protan was released from the BLPs in these experiments with 100 mM glycine-HCl (pH 1.5) regeneration buffer. Zeng et al. [27] used near-field scanning optical microscopy and

atomic force microscopy and also demonstrated strong and highly stable binding of Protan to BLPs at physiological pH and abrogation of this specific binding in an acidic environment (pH 4.4) equivalent to the biochemical pH in phagolysosomes of APC in which immunogens are processed for antigen presentation.

Ag–Protan fusions have been produced in prokaryotic (*L. lactis*, *E. coli*) and eukaryotic hosts (CHO and HEK cells). To date, over 40 different antigens of bacterial, viral, or parasitic nature have been successfully overexpressed as Protan fusions using these expression hosts. The Protan fusions are preferably secreted by the expression cells, allowing easy removal of the production cells. Conventional protein isolation techniques are then used to purify the Protan fusion. The purified fusion is subsequently mixed with BLPs to allow binding. The BLPs with bound Ag–Protan fusion are subsequently recovered, washed, and formulated in a suitable buffer (Fig. 12.3b). *L. lactis* as an expression host is attractive because it does not produce toxins and it has the advantage of secreting the Protan fusion into the growth medium that contains very little other secreted *L. lactis* proteins, allowing easy purification. Nevertheless, very little experience with this production host exists in the pharmaceutical industry. A conventional choice as a bacterial production host is *E. coli*. However, Protan fusions show a tendency to be incorporated in inclusion bodies in this organism. Experiments with a yellow fluorescent protein (YFP)-Protan fusion showed that buffer conditions can be found allowing proper refolding of YFP and restoring the binding ability of Protan (van Roosmalen, personal communication). A disadvantage of this approach is that refolding conditions are likely to be antigen specific and therefore the proper conditions need to be determined for each Ag–Protan fusion. Secretion into the periplasm or even into the growth medium may circumvent these challenges. Expression and secretion of Protan fusions using animal host cells is especially attractive for, e.g., viral antigens that need to be glycosylated. The Protan sequence contains a few putative glycosylation sites in the spacers that flank the LysM motifs. It is presently not known whether Protan is indeed glycosylated if expressed in animal cells, but if so, this does not seem to affect the binding affinity of Protan for BLPs in a negative way. Experiments with HEK cell produced influenza hemagglutinin (HA)-Protan showed proper binding of this fusion to BLPs. Viral surface proteins of enveloped viruses are often glycosylated multimeric proteins. The expression of such proteins in their native multimeric form as Protan fusion is a particular challenge that has been solved recently. The native lipid embedded hydrophobic transmembrane region (multimerization domain) of HA, which is essential for correct multimerization and folding (requires a lipid environment for proper trimerization), was substituted by a hydrophilic heterologous coiled-coil motif with similar conformation-inducing properties. A single Protan LysM motif appeared to be sufficient to obtain efficient and strong binding to BLPs of these trimeric HA (HA^{tri})-Protan fusions. Apparently, the three single LysM motifs brought together into one trimeric HA protein act together as in three LysM motifs present in a single linear polypeptide. Like influenza virus, the BLPs with bound HA^{tri}-Protan were able to agglutinate red blood cells (in contrast to monomeric HA bound to BLPs), demonstrating the functionality of the trimeric HA bound to the surface of the BLPs. In this way, BLPs

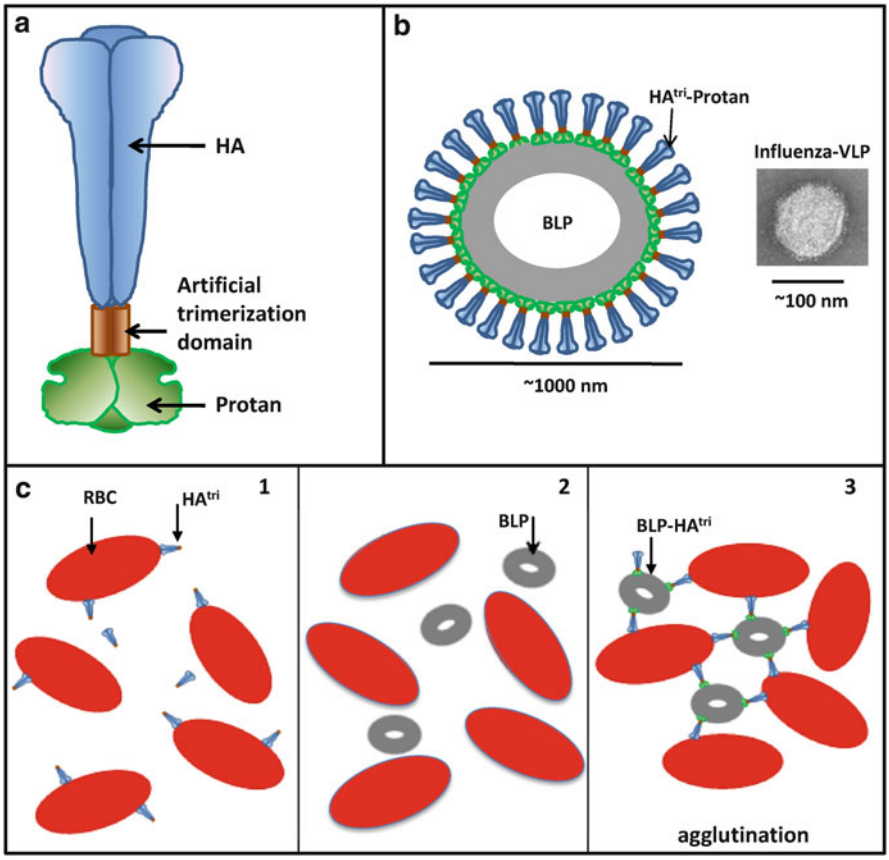

Fig. 12.5 (**a**) Schematic presentation of trimeric HA-Protan (HA^{tri}-Protan). (**b**) BLPs with bound HA^{tri}-Protan represent giant VLPs. (**c**) Agglutination test: (*1*) trimeric HA (HA^{tri}) binds to red blood cells (RBC), but this does not cause agglutination; (*2*) BLPs do not bind to RBCs and do not cause agglutination; (*3*) BLPs with bound HA^{tri}-Protan (BLP-HA^{tri}) bind to RBCs and cause agglutination

with bound HA^{tri}-Protan act like giant VLPs (Fig. 12.5). BLPs with bound HA^{tri}-Protan generated robust and also much higher HA-specific serum IgG responses after intranasal immunization of mice compared to other HA formulations (Haijema and de Haan, data to be published elsewhere).

12.3 Mode of Action

Cells of the innate immune system, such as macrophages and DCs, produce TNF-α and IL-6 upon incubation with BLPs and efficiently take up these particles [28, 29]. A TLR screening was performed by in vitro stimulation of HEK293T cells

transiently transfected to express human TLR2, 3, 4, 5, 7, 8, 9 and mouse TLR7 and 9 (human and mouse TLRs are mainly the same, only mouse TLR7 and 9 differ from human TLR7 and 9). This study consistently showed that *L. lactis* BLPs are a TLR2 agonist and do not interact with TLR3, 4, 5, 7, 8, and 9 [29]. In the same study it was shown that cell surface markers CD40, CD80, CD86, and MHC-class II of murine neonatal and adult DCs, as well as CD80, CD83, CD86, and HLA-DR of neonatal and adult human DCs are upregulated upon incubation with BLPs. Upregulation of these markers is a hallmark of DC activation/maturation and is a prerequisite for efficient antigen presentation and interaction with T-cells (adaptive immune system). Murine DC activation results in the production of IL-12, TNF-α, IL-10, IL-6, IFN-γ, and MCP-1 and human DCs start to secrete IL-12, TNF-α, IL-10, IL-6, IL-1β, and IL-8, again both in neonatal and adult DCs. In addition, BLP-primed DCs were shown to have an enhanced capacity for T cell stimulation. Moreover, mice intranasally immunized with a BLP-based vaccine (*Yersinia pestis* LcrV antigen bound to BLPs) showed enhanced levels of antigen-specific antibody secreting cells in the nasal-associated lymphoid tissue (NALT), spleen, and bone marrow. The latter two are believed to represent reservoirs of vaccine-induced plasma cells that support the production and maintenance of circulating antibodies.

In vivo, the first step in the induction of immune responses after mucosal administration is passage of the vaccine through the mucosal epithelial cell lining. M cells (microfold cells) are cells found in the follicle-associated mucosal epithelium that have the unique ability to sample antigen from the lumen and deliver it via transcytosis to APC (macrophages and DCs) and lymphocytes (T- and B-cells) located in a unique pocket-like structure on their basolateral side. It is known that particles of 1–10 μm are efficiently taken up and transcytosed unprocessed to the underlying cells of the immune system. The uptake of fluorescently labeled BLPs by M-cells was demonstrated after intranasal administration in mice (Fig. 12.6).

Considering the particulate nature, its size and bacterial origin of the BLP, uptake and activation of the immune system through M-cells after mucosal delivery is highly likely. Nevertheless, other ways of uptake and activation may contribute to the stimulation of the immune system. By using an in vitro human nasal epithelial cell activation assay, it was demonstrated that BLPs evoked not only production of IL-6 and IL-8 by these cells but also expression of CCL-20 and TSLP, which are chemotaxis and activation related factors of DCs (Yeh et al., data to be published elsewhere). This could mean that upon contacting mucosal epithelial cells interleukins and chemokines are secreted to attract DCs which sample the lumen for antigen. In the same work it was shown that BLP-primed DCs triggered the induction of total IgG and IgA production in B cells.

After uptake by M-cells or by DCs at the mucosal lining, particles are presented to the underlying cells of the immune system, including DC, B-, and T-cells. These cells may migrate to draining lymph nodes and this results in local (mucosal) and systemic responses even at distant sites. Such responses have been measured for antigens bound to or mixed with BLPs [30, 31]. The antibody isotyping profiles and the cytokines released upon immunization with BLP-based vaccines all indicate that the immune response is well balanced and that formulation

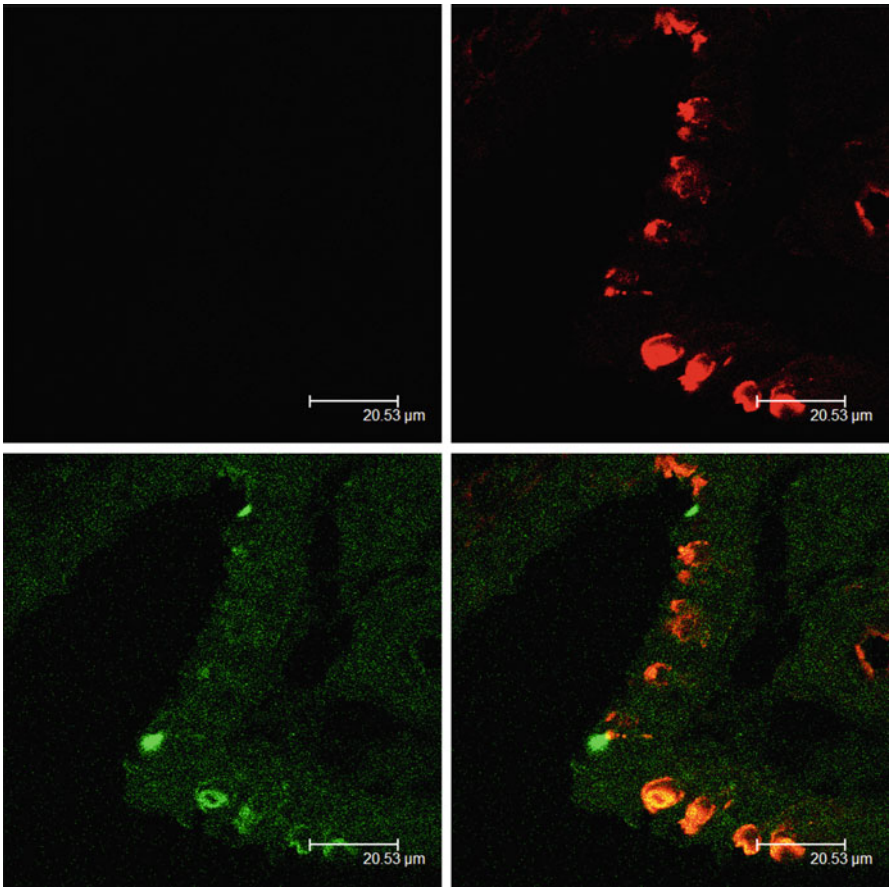

Fig. 12.6 Alexa-labeled BLPs (*green*) were administered intranasally to adult BALB/c mice. Cryosections of the nasal cavity were obtained 15 min after dosing and stained with Rho-UEA-1-lectin specific for M-cells. The figure shows images of the nasal section stained with fluorescent antibodies visualized under fluorescent microscopy. M-cells are stained *red* in the *upper right panel*. Alexa-labeled GEM particles (*green*) can be observed along the nasal epithelium (*lower left panel*). After coming in contact with *red*-labeled M cells, the *green*-labeled GEM particles are taken up resulting in *orange–yellow* staining inside the M cells (*lower right panel*)

with BLPs drives the response away from the typical Th2-skewed responses observed for antigen alone or formulated with aluminum salts [29, 31]. Recently, we demonstrated by using TLR2$^{-/-}$ knockout mice that the serum IgG and mucosal IgA responses elicited by BLP-based vaccines are almost exclusively attributed to the activation of the immune system through the TLR2 pathway (Table 12.3; Haijema et al., similar representative data is being communicated for publication elsewhere).

Table 12.3 Influence of TLR2 on HA-specific serum IgG response in mice

Route of administration	Vaccine	$TLR2^{+/+}$ mice ratio HA/HA+BLP (fold increase)	$TLR2^{-/-}$ mice ratio HA/HA+BLP (fold increase)
Intranasal	HA	1	1
	HA + BLP	3.2	1.1
Intramuscular	HA	1	1
	HA + BLP	19.3	1.8

12.4 Mimopath™-Based Vaccines

The Mimopath™-based vaccine system has been shown to enable mucosal delivery of antigens in animal models and to raise robust systemic and local antigen-specific responses. The vaccine system is also suitable for parenteral administration after which improved humoral and systemic immune responses are measured compared to aluminum salts. Both routes of administration induce a balanced immune response, and the *L. lactis* BLPs seem to drive the response in favor of a Th1-type response as compared to non-adjuvanted and aluminum salts adjuvanted vaccines. The latest indications are that also Th17-type lymphocytes are activated through the use of BLPs. These types of immunity have been demonstrated to play important roles in the clearance of viral and bacterial infections. To date, Mucosis has evaluated several mucosal and parenteral BLP-based prototype vaccines in animal models to protect against bacterial, viral, and parasitic disease in order to generate proof of concept for its vaccine technology (Table 12.4).

The most advanced BLP-based vaccine is a seasonal influenza vaccine, FluGEM™, that is currently in clinical phase of development. FluGEM™ contains the regular commercially available trivalent split influenza vaccine (TIV; per dose: 15 μg HA per strain) admixed with different doses of BLPs. The preclinical evaluation of the vaccine included the intranasal and intramuscular route of administration in various animal models. In mice, intranasally delivered monovalent FluGEM™ formulations resulted in hemagglutination inhibition (HI) titers in serum at a level equivalent to the conventional vaccine delivered through the intramuscular route, well above the protective titer of 40 (2 log 5.3). Serum IgG titers were also similar, whereas serum IgA and secreted IgA responses were much stronger with intranasal FluGEM™ (Table 12.5). The phenotypic characterization of the responses generated with intranasal FluGEM™ showed a better balanced Th1/Th2-type ratio of the serum antibody response with lower IgG1 and higher IgG2a components and a higher number of Th1-type cytokines producing spleen cells, while Th2-type producing spleen cells were reduced [31]. Taken together, the data showed that intranasal FluGEM™ elicits superior Th1-type responses compared to conventional intramuscular influenza vaccine. A Th1-type response is considered to contribute to better protection from infection and to help in virus neutralization by secretion of IFN-γ [35–37]. Moreover, the natural infection also induces Th1-type responses.

Table 12.4 Summary of preclinical proof-of-concept studies performed with BLPs

Pathogen	Antigen	Bound (b) or mixed (m)	Route of admin.	Animal	Mode of generating PoC	Observed outcome
Influenza virus	HA (split virus)	m	i.m.	Mouse, rat, rabbit, ferret	Correlate of protection	Serum HI titers >40 strong increase compared to benchmark[a] [32][b]
				Mouse	i.n. challenge	100% survival; complete abrogation of viral replication in lungs [32][b]
			i.n.	Mouse, rat, rabbit, ferret	Correlate of protection	Serum HI titers >40 comparable to i.m. benchmark[a] [31][b]
				Mouse	i.n. challenge	100% survival; strong reduction of viral replication in lungs[b][32]
	HA (subunit)	b	i.m.	Mouse	Correlate of protection	Serum HI titers >40 strong increase compared to i.m. benchmark[a,b]
			i.n.	Mouse	Correlate of protection	Serum HI titers >40 comparable to i.m. benchmark[a,b]
	M2e	b	i.n.	Mouse	i.n. challenge	100% survival; strong reduction of viral replication in lungs[c]
Hepatitis B virus	HBsAg	m	i.n.	Mouse, rat	Correlate of protection	Serum titers >10 mIU/ml comparable to i.m. benchmark[d,e]
Plasmodium berghei	CSP epitopes	b	i.m.	Mouse	i.v. challenge (bites of infected mosquitoes)	100% survival; sterile immunity[b][34]

Streptococcus pneumoniae	Iga1protease SlrA PpmA	b, m	i.n., i.m.	Mouse	Pulmonary challenge (pneumonia model)	50–75% survival; strong reduction in bacteremia [28]
					i.n. challenge (colonization model)	Strong reduction in nasalpharyngeal colonization[b]
Yersinia pestis	LcrV	b	i.n.	Mouse	i.v. challenge	100% survival [29]
			o.g.	Mouse	i.v. challenge	Up to 85% survival[f]

Protective immunity raised by various BLP-based vaccines
PoC proof of concept, *i.m.* intramuscular, *i.n.* intranasal, *i.v.* intravenous, *o.g.* orogastric
[a]Regular i.m. unadjuvanted influenza split virus vaccine
[b]Mucosis, data to be published elsewhere
[c]Saelens and Leenhouts (unpublished data)
[d]Regular i.m. HBsAg vaccine with aluminum salt adjuvant
[e]Mucosis (unpublished data)
[f]Pasetti and Leenhouts (unpublished data)

Table 12.5 Immune responses elicited with FluGEM in mice

	Serum					Mucosal		Spleen (spots/10^6 cells)		
Vaccine	HI titer (2 log)	IgG (10 log)	IgG1 (10 log)	IgG2a (10 log)	IgA (10 log)	Nose IgA (2 log) [% responders]	Nose IgA (2 log) [% responders]	IL-4	IL-2	IFN-γ
Benchmark[a]	8.3	4.4	4.8	2.8	1.1	1.9 [12.5]	0.0 [0]	73	25	6
FluGEM	8.0	4.2	4.2	3.6	2.2	4.2 [100]	4.6 [100]	12	53	22

[a]Unadjuvanted split virus vaccine

Table 12.6 Lung viral replication and survival of mice immunized with FluGEM

Vaccine	Rout of admin	Lung viral repl. (10 log)	Survival (%)
PBS	i.n., i.m.	6.3	0
Benchmark[a]	i.m.	4.5	100
FluGEM	i.n.	2.5	100
	i.m.	0.0	100

[a]Unadjuvanted split virus vaccine

Table 12.7 Duration of immunity and recall response

	Relative HA-specific serum IgG response		
Vaccine	Four weeks post final immunization	Eighteen weeks post final immunization	One week post booster immunization[a]
Benchmark[b]	100%	28%	248%
FluGEM	100%	109%	389%

[a]Animals received booster immunization at 18 weeks post final immunization
[b]Unadjuvanted split virus vaccine

The intramuscular administration of FluGEM™ resulted in a three to fivefold increase in the HI titers as compared with TIV. Interestingly, intramuscular FluGEM™ also generated local sIgA responses in the nasal pharyngeal tract. In a murine PR8 challenge model, mice prime-boost immunized with intramuscular conventional split vaccine are completely protected. This is also the case for mice immunized with intranasal and intramuscular FluGEM™. However, a marked difference was noted in the levels of viral replication in the lungs of challenged mice. Intranasal FluGEM™ lowers the viral replication levels with several logs and intramuscularly administered it even completely abrogates lung viral replication (Table 12.6; [33]). Therefore, FluGEM™ elicits robust immune responses that potentially can contribute to reduce viral shedding by infected individuals and thereby improve herd immunity.

In addition to robust systemic and local responses, FluGEM™ generated immunity seems to wane slower than conventional split virus vaccine. Table 12.7 summarizes the serum IgG responses after prime-boost immunization of intranasal FluGEM™ or intramuscular split virus vaccine (benchmark). FluGEM™ generated IgG levels remained constant over a period of 3.5 months post final immunization, whereas the IgG levels elicited by the intramuscular benchmark reduced to almost a quarter of the response over the same period of time. Although the FluGEM™ IgG levels remained high, they could still be boosted after this period of time (Haijema et al., similar representative data is being communicated for publication elsewhere).

The immunostimulatory activity of BLPs in FluGEM™ was not only evident in mice. Similar observations were made in rats, rabbits, and ferrets. Table 12.8 summarizes HI titers in rabbits and ferrets obtained with FluGEM™ (Haijema et al., unpublished data).

Table 12.8 HI titers in rabbits and ferrets obtained with FluGEM

Animal	Vaccine	Route of administration (number of doses)	HI titers (2 log values)[a]		
			H1N1	H3N2	B
Rabbit	Benchmark[b]	i.n. (3)	6.2	4.8	3.2
	FluGEM	i.n. (3)	7.9	6.9	4.7
Ferret	Benchmark[b]	i.m. (2)	3.9	2.8	2.9
	FluGEM	i.m. (2)	7	6	5.6

[a]Pre-immune sera were negative
[b]Commercially available split trivalent influenza vaccine

The FluGEM™ vaccine is intended for use in a population ranging from adults at risk to elderly adults. In view of the intended use in vaccine clinical trials, a GLP safety evaluation was conducted according to the guidelines of the Committee for Medicinal Products for Human Use (CHMP), among which the "Guideline on adjuvants in vaccines for human use" (July 2005). BLPs and FluGEM™ formulations were evaluated for local and systemic toxicity after intranasal or intramuscular administration in rats and rabbits (Table 12.9). The number of administrations reflected the number of administrations in the clinical study plus one ($n+1$). A full human dose was used in the rabbits and one-fifth of that dose in rats, which is equivalent 20- and 70 times the human dose per kilogram body weight, respectively. The repeated-dose and local tolerance toxicity study evaluated effects after a single, two (intramuscular), and three (intranasal) administrations. There was no evidence of systemic and local toxicity (special attention was paid to the olfactory bulb and the brains in the intranasally dosed animals). There were no significant symptoms reported in rats and in rabbits symptoms were limited to the injections site. These effects in rabbits were transient with full recovery within 14 days post administration. Such observations are well expected to occur with compounds that elicit an inflammatory immune response.

The preclinical efficacy, safety, and tolerability data generated with BLP-based intranasal and intramuscular vaccines have been encouraging and suggests that BLPs may be a suitable immunostimulant for use in humans. Mucosis started in 2011 a first Phase I clinical study for its lead BLP-based vaccine, intranasal FluGEM™.

Acknowledgments I would like to acknowledge my colleagues at Mucosis and the collaborators of Mucosis for sharing unpublished data and for their valuable contributions to the work and ideas described in this chapter.

Table 12.9 Preclinical safety studies performed with BLPs

Study	Animal	Formulation	Dose	Route of admin.	Admin. Regime (days)	Observed outcome
Local tolerance and repeated-dose toxicity	NZW rabbits	BLP	Up to 1.5 mg/kg	i.n.	0, 21, 42	No treatment-related effects. No toxic effect level equivalent to up to 20 times the human BLP dose
			Up to 0.15 mg/kg	i.m.	0, 21	Well tolerated. Transient symptoms limited to injection site. No evidence of systemic toxicity in terminal studies (up to 20 times the human BLP dose)
		FluGEM	BLP; up to 1.5 mg/kg HA: 15 μg/strain	i.n.	0, 21, 42	No treatment-related effect. No toxic effect level equivalent to up to 20 times the human BLP dose
			BLP: up to 0.15 mg/kg HA: 15 μg/strain	i.m.	0, 21	Well tolerated. Transient symptoms limited to injection site. No evidence of systemic toxicity in terminal studies (up to 20 times the human BLP dose)
	Wistar rats	BLP	Up to 5 mg/kg	i.n.	0, 21, 42	No treatment-related effect. No toxic effect level equivalent to up to 70 times the human BLP dose
			Up to 0.5 mg/kg	i.m.	0, 21	No treatment-related effect. No toxic effect level equivalent to up to 70 times the human BLP dose
		FluGEM	BLP: up to 5 mg/kg HA: 15 μg/strain	i.n.	0, 21, 42	No treatment-related effect. No toxic effect level equivalent to up to 70 times the human BLP dose
			BLP: up to 0.5 mg/kg HA: 15 μg/strain	i.m.	0, 21	No treatment-related effect. No toxic effect level equivalent to up to 70 times the human BLP dose
				i.m.	0, 21	No treatment-related effect. No toxic effect level equivalent to up to 70 times the human BLP dose

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Section IV
Novel Particulate Delivery Systems for Vaccines and Adjuvants

Chapter 13
NanoBio™ Nanoemulsion for Mucosal Vaccine Delivery

Tarek Hamouda, Jakub Simon, Ali Fattom, and James Baker

13.1 Introduction

Nanoemulsions (NEs) are high-energy oil-in-water emulsions smaller than 1,000 nm that disrupt the outer lipid membrane of pathogenic microbes [1–4]. Building on studies that demonstrated the potential of NE to prevent influenza infection [5], it was recognized that NE inactivated influenza virus generated greater immune responses than formalin inactivated influenza virus when administered intranasally [6]. Subsequent studies have demonstrated this material acts as a mucosal adjuvant with numerous antigens including recombinant anthrax protective antigen (PA) [7], killed-vaccinia virus [8], recombinant human immunodeficiency (HIV) gp120 [9], recombinant hepatitis B surface antigen (HBsAg) [10], and purified *Burkholderia cenocepacia* outer membrane protein (OMP) [11]. In addition, it was recognized that the surfactant in the NE is locked at the interface between the oil droplets and water and does not appear to denature proteins. This confers thermostability to the antigen [10, 12–14] and potentially allows for the elimination of the cold chain required for all currently available vaccines [15]. The mechanisms by which adjuvants enhance the immune response are starting to be elucidated and include improved antigen delivery as well as innate immune activation [16]. The NE adjuvant acts via both mechanisms as it enhances antigen uptake by dendritic cells (DCs) as well as activating Toll like receptors (TLR) 2 and 4; this enhances both humoral and cell-mediated Th1 and Th17 immune responses [17]. Importantly, NE mucosal adjuvant activity occurs without damaging the mucosal epithelium [10] and has been demonstrated to be safe and well tolerated in early phase human clinical trials (Stanberry, submitted). In this review, we will delineate the physical and chemical properties, mechanisms of activity, preclinical studies, and clinical experience available regarding mucosal NE adjuvants.

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13.2 Physical and Chemical Properties of NE Adjuvants

13.2.1 Composition

NE adjuvant is a high energy oil-in-water emulsion readily made of pharmaceutically approved ingredients that are included on the Food and Drug Administration (FDA) inactive ingredient list for approved drug products that are generally regarded as safe (GRAS). These include oil, water, surfactants, such as Tween 80, solvents, such as ethanol, as well as quaternary ammonium compounds, such as cetylpyridinium chloride (CPC). One particular formulation employs a specific combination of CPC, Tween, and ethanol that appears to have several advantages for use as a vaccine adjuvant. The cationic surfactant CPC produces positively charged oil droplets that are attracted to the negatively charged mucous membranes, facilitating mucosal uptake. Remarkably, the irritation normally associated with cationic surfactants appears to have been substantially reduced by the addition of Tween and the locking of surfactants at the oil–water interphase [18]. Finally, ethanol is important due to its role in localizing antigen in the oil phase. $W_{80}5EC$, the most extensively tested NE formulation to date, is made of 53% water, 38% soybean oil, 4% Tween 80, 4% ethanol, and 1% CPC. Emulsification results in the formation of stable droplets that are 400–500 nm in diameter. Simple mixing of the desired antigen with the adjuvant results in integration of antigen into the lipid core with approximately 90% of the antigen localized within the lipid phase (Fig. 13.1) [14].

13.2.2 Physical Characteristics

13.2.2.1 NE Zeta Potential

NE adjuvants are positively charged due to the presence of quaternary ammonium chloride. Mixing the NE with negatively charged antigens, such as HBsAg, results in an HBsAg-NE mixture that remains positively charged, with the magnitude of the positive charge decreased. This suggests that there is an electrostatic association between HBsAg and NE adjuvant. Importantly, thermodynamic analysis of the interaction between the HBsAg and the NE using isothermal titration calorimetry (ITC) reveals a spontaneous exothermic reaction that is energetically favorable [10].

13.2.2.2 Thermostability of NE Adjuvant Alone

NE adjuvant is stable for several years under many conditions including autoclaving. Like most emulsions, freezing will disrupt the emulsion by crystalliz-

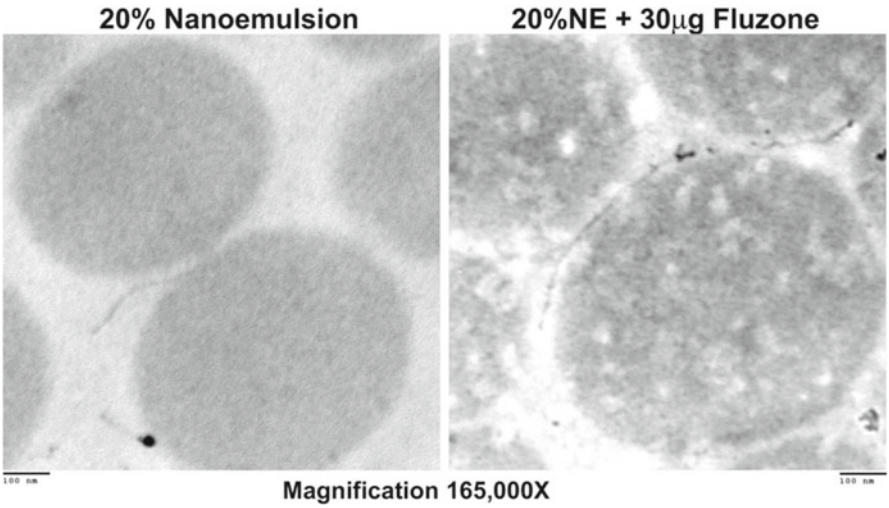

Fig. 13.1 Cross-section trasmission electron microscopy (TEM) of 20% W_{80}5EC NE adjuvant with 30 μg total HA from the 2008–2009 Fluzone® performed by fixing with 1% (w/v) osmium tetroxide solution and mixing with histogel in a 1:10 ratio to form a solid mass. The solid mixture was sliced into 1 mm slices, rinsed, and dehydrated with using the Durcupan® 159 kit (Fluka, EM #14020)

ing the water phase, which causes separation of the oil and aqueous phases. Thermostability of the W_{80}5EC adjuvant manufactured under current good manufacturing practice (cGMP) procedures has been assessed at 5 ± 3°C, 22 ± 3°C, and 40 ± 2°C prior to addition of antigen. There was no change in physicochemical properties of the key parameters of emulsion stability (including appearance, particle size, and pH) for the maximum duration assessed for 2 years.

13.3 Formulation of Antigens with NE Adjuvant

13.3.1 Formulation

Antigen is incorporated into the NE by simple mixing. As evidenced by electron microscopy (Fig. 13.2, left column), this simple mixing procedure results in incorporation of the antigen into the oil phase of the nanoemulsion droplet. Complete association is also evidenced by the fact that the particle size of the HBsAg-NE mixture creates a single peak ~330 nm, whereas the size of HBsAg alone is ~30 nm and the size of NE droplets alone is ~300 nm. (Fig. 13.2, right column).

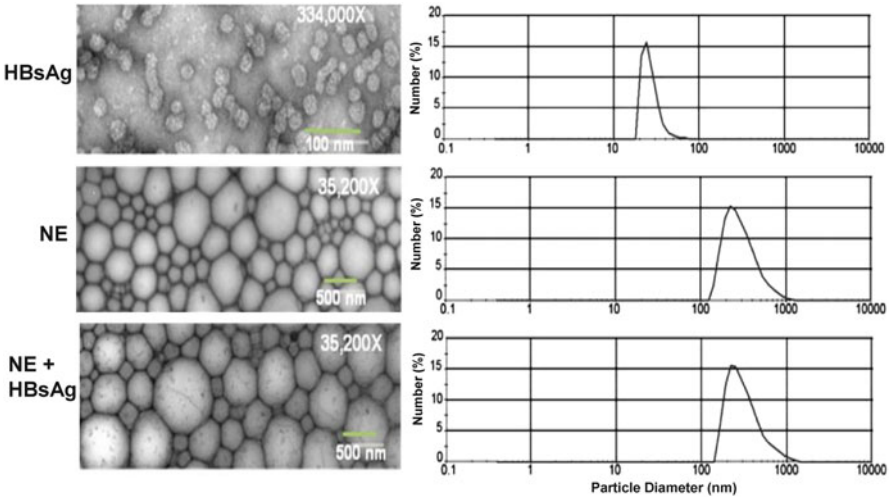

Fig. 13.2 HBsAg alone, NE alone, and HBsAg extemporaneously mixed with NE. Images were obtained using scanning electron microscopy (SEM). Particle diameter was measured using a laser diffraction particle-sizer

13.3.2 Thermostability of Antigen in NE

The combination of NE adjuvant with antigen is also thermostable. Anthrax PA was prevented from degradation by mixing with NE adjuvant [7]. HBsAg combined with NE adjuvant was assessed for up to 12 months at 40°C [10]. Vaccine antigen was stable and immunogenic at room temperature for up to 6 months (Fig. 13.3) [10]. Finally, influenza antigen with NE adjuvant was stable and immunogenic at room temperature for 3 months, the maximum temperature and duration studied [12]. Thermostability of vaccines is very advantageous as it allows for rapid vaccine distribution without refrigeration or breaks in the cold chain in routine vaccine distribution. This is particularly useful in pandemic response where large amounts of vaccine need to be distributed quickly, as well as vaccination campaigns in developing areas where the cold chain is not reliable.

In summary, NE adjuvants are composed of GRAS products that are easy to manufacture and readily compatible with a variety of antigens. Alone, NE adjuvants are thermostable up to 40°C for several years. Mixed with antigen, NE adjuvants are thermostable up to 40°C for weeks to months. Mucosal, easily manufactured, thermostable adjuvants provide significant advantages to vaccine development and implementation programs.

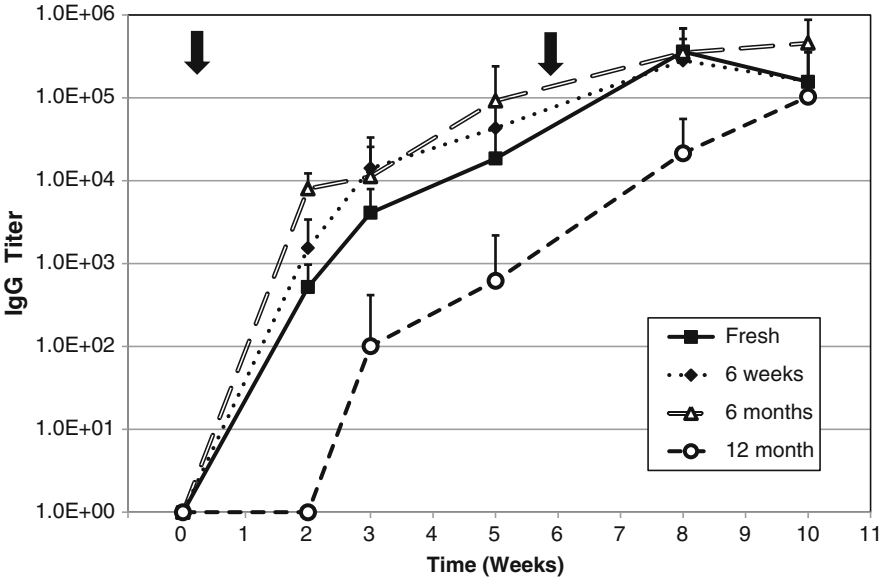

Fig. 13.3 Immune response of mice to HBsAg-NE vaccine formulations prepared fresh and stored at 25°C for 6 weeks, 6 months, and 12 months. Animals were immunized at 0 and 6 weeks. *Error bars* depict standard deviation

13.4 Mechanisms of Action

The mechanisms by which adjuvants enhance antigen-specific immune responses are multifactorial, but have been traditionally categorized as either enhancing antigen delivery or innate immune potentiation [16]. Although the term delivery may refer to the process that is utilized to deliver the vaccine to a particular tissue, the term antigen delivery typically refers to the delivery of antigen at the molecular level, i.e. delivery of antigen to antigen-presenting cells (APC) [19]. Immune potentiation may also refer broadly to enhancement of the immune response via multiple mechanisms, but has been focused in adjuvant literature on the stimulation of innate immune responses involving pathogen associated molecular patterns (PAMPs) that are conserved between pathogens, such as lipopolysaccharide (LPS) and flagellin. PAMPS stimulate the innate immune response via TLRs that are specific for subsets of PAMPs (i.e., TLR 4 binds LPS whereas TLR 5 binds flagellin) [20]. NE adjuvants utilize both mechanisms, by loading DCs and stimulating TLRs 2 and 4. However, it appears this activation occurs without a specific ligand but is the result of membrane perturbations induced by interactions with the NE.

13.5 1NE Adjuvant Enhances Antigen Internalization into DCs In Vitro

Delivery of antigen into DCs plays a pivotal role in the initiation of antigen-specific immune responses [21, 22]. NE adjuvant enhances antigen internalization as demonstrated in vitro in the murine DC line JAWSII and in the primary bone marrow-derived DC (BMDC). Mixing with NE increased internalization into DCs of such diverse antigen proteins as ovalbumin (Ova), recombinant PA (rPA), recombinant HBsAg (rHBsAg), and enhanced green fluorescent protein (EGFP) (JAWSII) (Makidon, in preparation). NE also enhances antigen uptake into nasal mucosal epithelium and lymphoid tissues in vivo. In antigen uptake and trafficking studies, the EGFP-NE (enhanced green fluorescent protein mixed with W_{80}5EC) was applied to the mouse nasal mucosa. At 24 h post-treatment, green fluorescence was detected throughout the nasal epithelium, submandibular lymphoid tissue, and thymus (Makidon, in preparation). Intense green fluorescence was detected in the majority of epithelial cells (including the M cells) after administration of the NE-EGFP mixture, as compared to the less intense fluorescent signal seen when EGFP was delivered alone. No adverse effects on the nasal mucosa, body weight, or temperature of the animals were seen. Other studies also assessed internalization and trafficking using highly fluorescent silica-based quantum dots (QDOTs) as a surrogate antigen. Owing to their neutral coating, the QDOTs do not interact non-specifically with cell membranes [23]. Thus, the level of cell fluorescence in the nares of animals treated with QDOTs in PBS was essentially equivalent to naïve controls (Fig. 13.4, middle and right columns). In contrast, nasal administration of QDOTs in NE adjuvant produced significant internalization, trafficking and QDOT distribution into the mouse lymphatic tissues during first 24–72 h (Fig. 13.4, left column), which subsequently dissipated over the 5–8 days following nasal application (data not shown). These biodistribution studies confirm that antigens incorporated in W_{80}5EC nanodroplets have enhanced uptake in nasal mucosa and efficient presentation to systemic lymphoid tissues.

13.5.1 Molecular Features of NE-Antigen Presentation

Activation of transcription factor NF-κB is essential for sustaining the molecular signaling cascade and for regulation of genes critical for the innate and adaptive immunity [24, 25]. NEs were evaluated for their ability to induce NF-κB activation in vitro using a modified human monocyte cell line, THP1-Blue; results show that the Tween80-based NEs induced significant NF-κB activation (Bielinska, personal communication). As the TLR family of innate immune receptors plays a pivotal role in the activation of both innate and adaptive immune responses via NF-κB-mediated transcription [26, 27], the role of TLRs in NE adjuvant activity was investigated using human HEK293 clones engineered to express only a single specific TLR (InvivoGen, San Diego, CA). Evaluation of W_{80}5EC and W_{80}5E NEs was performed

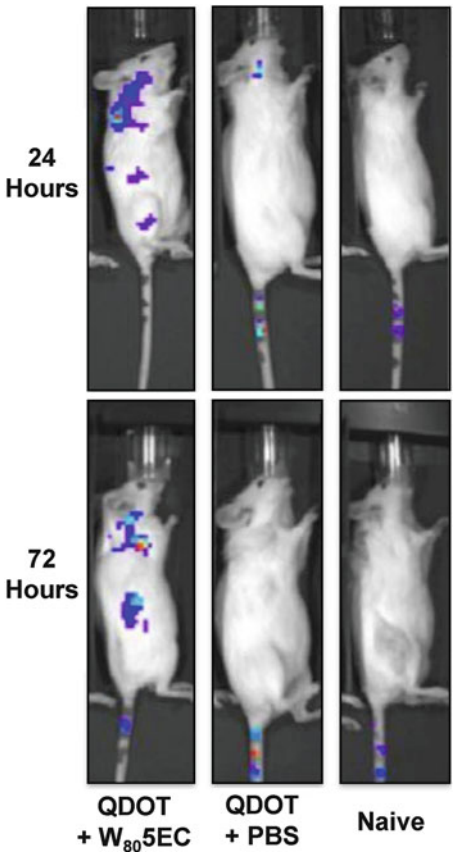

Fig. 13.4 Representative mice after IN administration of W_{80}5EC NE adjuvant with QDOTs, PBS, with QDOTs, or nothing (naïve mice). QDOT fluorescence was detected and imaged at 24 and 72 h using the Xenogen Bioluminometer

using seven different HEK293 TLR clones (TLR2, 3, 4, 5, 7, 8, and 9). Each of these cell lines were incubated for 24 h at 37°C with 0.001% concentration of either NE, and with TLR-specific ligands as positive controls. NF-κB activation was detected by measurement of SEAP secreted into the cell culture medium. Results indicate that both NEs activate transcription factor NF-κB through a mechanism dependent on interaction with either the TLR2 or the TLR4 receptor (Bielinska, personal communication).

The role of TLR4 activation by NE adjuvant was further studied in vivo using TLR4-deficient mice. The TLR4 mutant mice (TLR4–/–) and their wild-type counterpart (WT, TLR4+/+) were immunized intranasally with two doses of either HBsAg-W_{80}5EC or HBsAg-PBS formulations. Serum anti-HBsAg IgG titers were measured 4–5 weeks after primary (day 0) and booster (day 28) immunizations. There was no discernable difference in the total anti-HBsAg IgG or subclass distribution response in TLR4–/– mutants and TLR+/+ controls. However, the analysis of the cellular response indicated that, in contrast to WT, the TLR4–/– mutants did not mount an effective antigen-specific cellular response. In vitro stimulation of splenocytes isolated from WT mice produced a robust induction of IFN-γ and other

Th1-type and Th17-type cytokines, while no significant induction of Th1-type and Th17-type cytokines was detected in the TLR4−/− mutants. No antigen-specific activation was observed in naïve splenocytes of either strain of mice, and response to LPS was only significant in WT animals [17].

In the context of this data, the role of NE adjuvant in stimulating cellular immune responses should be emphasized. Th1 responses are important in killing infected cells [28], while Th17 has been recognized to play an important role in mucosal cell-mediated immunity (CMI) [29]. Inducing the appropriate immune response is critical, as poor quality immune responses to vaccines such as killed measles and killed respiratory syncitial virus (RSV) can result in enhanced disease upon pathogen exposure [30]. The mechanisms of enhanced disease include the generation of poor avidity antibodies [31, 32] as well as Th2 skew associated with eosinophilia and reactive airways [33]. It is important to note that these pathologic immune responses can actually be reversed by adding TLR4 agonists to the killed vaccine, as demonstrated with monophosphoryl lipid A (MPL) [34] as well as NE adjuvant [35].

In summary, the mechanisms of NE adjuvant activity include the loading of NE with antigen into epithelial cells and dendritic cells for presentation to T cells in lymph nodes, as well as TLR 2 and 4 activation. The resultant immune responses are qualitatively different from unadjuvanted responses and able to induce mucosal, high avidity humoral, as well as Th1 and Th17 CMI. This unique immune response is characterized in the preclinical and clinical sections and may be important in the development of vaccines against pathogens that have traditionally eluded vaccine developers.

13.6 Preclinical Studies

NE adjuvant has been used to stimulate immune response when mixed with several different antigens and administered either intranasally (IN) or intramuscularly (IM) in a number of animal models. IN studies are summarized in Table 13.1 and expanded upon for a number of antigens in this section.

13.6.1 Influenza

Initial studies demonstrated that placing NE into the nares of mice could protect mice for several hours from respiratory challenge with a lethal dose (LD)90 of influenza virus. This was initially presumed to be due to inactivation of the virus before it reached the lungs [5]. Animals who survived the respiratory challenge, however, were shown to have high titers of anti-influenza antibodies. Subsequent studies documented that mixing influenza virus with NE and placing it in the nares on a single occasion produced strong protective immunity [6]. In subsequent mouse immunogenicity studies, 5–20% W_{80}5EC adjuvant was used to inactivate influenza A/Puerto Rico/8/34/05 (H1N1). Mice immunized with the NE adjuvanted influenza virus IN showed a robust influenza-

Table 13.1 Summary of vaccines and animal models tested with IN administration of NE adjuvant

Vaccine	Species
Seasonal influenza	Ferrets, rabbits, mice, humans
Pandemic influenza	Mice, rats
Respiratory syncitial virus	Mice, cotton rats
Hepatitis B	Mice, rats, guinea pigs, dogs, and non-human primates
Anthrax	Mice, guinea pigs
Streptococcus pneumoniae	Mice, rabbits
HIV	Mice
Smallpox	Mice
Dengue	Mice
Burkholderia cepacia	Mice

specific humoral immune response as demonstrated by ELISA as well as hemagglutination inhibition (HAI) assays. Serum HAI titers were more than 10,000 following two vaccinations. Splenocytes from vaccinated mice were assayed for cytokine production following virus stimulation. The cytokine profile demonstrated a robust cellular immune response with enhanced Th1 and Th17 immunity that is balanced against both intracellular and extracellular forms of the virus. All vaccinated mice were protected against challenge with an LD80 of live influenza virus [12]. Experience with the W_{80}5EC adjuvant also has been extended to ferrets, the recommended preclinical model for influenza [36]. The adjuvant was tested with two different split-commercial vaccines (Fluvirin® and Fluzone®) and was compatible with both preparations. The nasal NE adjuvanted vaccine was superior to IN as well as IM immunization with non-adjuvanted influenza vaccines. Additionally, the adjuvanted vaccine was dose-sparing, using up to 50-fold less hemagglutinin (HA) antigen than IM non-adjuvanted vaccines. Importantly, W_{80}5EC-adjuvanted vaccine resulted in the production of antibodies against heterologous influenza strains not present in the vaccine and sterilization of the nasal secretions and turbinates in ferrets following challenge with live virus [14].

13.6.2 HBV

The immunogenicity of HBsAg-NE vaccine was evaluated in mice, rats, guinea pigs, dogs, and primates. Animals immunized IN developed robust and sustained systemic IgG, mucosal IgA, and strong antigen-specific cellular immune responses. Serum IgG reached titers $\geq 10^6$ and was comparable to intramuscular vaccination with alum-adjuvanted vaccine (HBsAg-Alu). Normalization showed that HBsAg-NE vaccination correlates with a protective immunity equivalent or greater than 1,000 IU/mL. Th1 polarized immune response was indicated by IFN-γ and TNF-α cytokine production and elevated levels of IgG2 subclass of HBsAg-specific antibodies. Vaccines stored for a year at 4°C, 6 months at 25°C, and 6 weeks at 40°C retained full immunogenicity.

This data suggests that needle-free nasal immunization with HBsAg-NE could be a safe and effective hepatitis B vaccine, or provide an alternative booster administration for the parenteral hepatitis B vaccines. The Th1 associated cellular immunity may also provide therapeutic benefit to patients with chronic hepatitis B infection who lack cellular immune responses to adequately control viral replication [10].

13.6.3 Anthrax

Mice and guinea pigs were immunized IN with *Bacillus anthracis* rPA mixed in NE adjuvant. rPA-NE immunization was effective in inducing both serum anti-PA IgG and bronchial anti-PA IgA and IgG antibodies after either one or two IN administrations. Serum anti-PA IgG2a and IgG2b antibodies and PA-specific cytokine induction after immunization indicate a Th1-polarized immune response. rPA-NE immunization also produced high titers of lethal-toxin-neutralizing serum antibodies in both mice and guinea pigs. Guinea pigs immunized nasally with rPA-NE vaccine were protected against an intradermal challenge with ~1,000 times the 50% lethal dose (~1,000× LD50) of *B. anthracis* Ames strain spores (1.38×10^3 spores), while all the control animals died within 96 h. Nasal immunization also resulted in 70% and 40% survival rates against intranasal challenge with 10× LD50 and 100× LD50 (1.2×10^6 and 1.2×10^7) Ames strain spores [7].

13.6.4 Smallpox

Incubation of vaccinia virus (VV) with 10% NE for at least 60 min caused the complete disruption and inactivation of VV. Simple mixtures of NE and VV (Western Reserve serotype) (VV/NE) applied into the nares of mice resulted in both systemic and mucosal anti-VV immunity, virus-neutralizing antibodies, and Th1-biased cellular responses. Nasal vaccination with VV/NE vaccine produced protection against lethal infection equal to vaccination by scarification, with 100% survival after challenge with 77 times the 50% lethal dose of live VV, although animals protected with VV/NE immunization had somewhat more extensive clinical symptoms after virus challenge than animals vaccinated by scarification [8].

13.6.5 HIV

Mice and guinea pigs immunized IN by the application of recombinant HIV gp120 antigen mixed in NE demonstrated robust serum anti-gp120 IgG, as well as bronchial, vaginal, and serum anti-gp120 IgA. The serum of these animals demonstrated antibodies that cross-reacted with heterologous serotypes of gp120 and had

significant neutralizing activity against two clade-B laboratory strains of HIV (HIVBaL and HIVSF162) and five primary HIV-1 isolates. The analysis of gp120-specific CTL proliferation, INF-γ induction, and prevalence of anti-gp120 IgG2 subclass antibodies indicated that nasal vaccination in NE also induced systemic, Th1-polarized cellular immune responses [9].

13.6.6 RSV

Nasal immunization with NE adjuvanted RSV in a mouse model induced durable, RSV-specific humoral responses, both systemically and in the lungs. Vaccinated mice exhibited increased protection against subsequent live viral challenge, which was associated with an enhanced Th1/Th17 response. NE-RSV vaccinated mice displayed no evidence of Th2-mediated immunopotentiation or enhanced bronchopulmonary disease, as has been previously described for formalin-inactivated RSV vaccines. There was decreased mucus production and increased viral clearance [35].

13.7 Animal Toxicity

No significant NE-related toxicity has been identified in nonclinical studies involving mice, rats, rabbits, ferrets, dogs, and primates. Good Laboratory Practices (GLP) toxicity studies of 130 New Zealand White rabbits were immunized IN with Fluzone® (2008–2009), phosphate buffered saline (PBS), NE-adjuvant, or Fluzone® (15 or 30 μg total HA) mixed with 10% or 20% NE-adjuvant. Rabbits received two doses on study days (SD) 1 and 15. Safety assessments included clinical observations, ophthalmoscopy, body weights and food consumption, body temperatures, serum chemistry, hematology, coagulation and urinalysis, organ weights and organ weight ratios, gross and microscopic pathology. Multiple sections of the nasal turbinates including the cribiform plate, olfactory bulb, brain, pituitary, and cranial nerves were examined in light of the occurrence of Bell's palsy observed with NasaFlu an intranasal vaccine that employed a bacterial toxin adjuvant, *Escherichia coli* heat-labile toxin that was withdrawn from the market in Switzerland. The immunogenicity assessment included neutralizing antibodies against all the strains present in Fluzone® 2008–2009 vaccine on SD 1 (prior to vaccination), 16, and 28. Clinical, clinical laboratory, gross, and histopathological observations were unremarkable. The maximum administered dose (30 μg total HA + 20%NE) was considered the no observed adverse effect level (NOAEL). Robust immune responses were elicited on day 16 to A/Brisbane (H3N2). Day 28 results showed robust immune responses to all the vaccine strains with 90–100% seroconversion for all groups administered NE-adjuvanted vaccine. No immune response was detected in the rabbits vaccinated IN with Fluzone® alone, NE alone, or PBS alone.

In summary, NE adjuvant administered IN is capable of adjuvanting numerous antigens including whole virus, split virus, recombinant protein, as well as purified protein in a number of animal models. The resultant immune responses include mucosal, humoral, and cell-mediated Th1 and Th17 components that are important in protection against a wide variety of pathogens. The safety and toxicology testing of NE in animals has not raised safety concerns, allowing the progression to human clinical trials.

13.8 Clinical Experience

13.8.1 Safety and Tolerability

NE components have well-known safety profiles that have allowed for extensive human use in many products. In particular, CPC has been used in humans for over 40 years in mouthwashes, toothpastes, lozenges, and various throat, breath, and nasal sprays. Dermal antimicrobial NEs products have been applied to the skin in over a 1,000 subjects in a number of Phase 1–3 clinical trials. Importantly, there was no evidence of permeation into the bloodstream from topical use, including application to open lesions. While vigilant monitoring for safety continues, there have not been any safety concerns reported to date with respect to the use of NE as antimicrobials or mucosal adjuvants.

Safety and tolerability of the NE applied as a mucosal adjuvant has been assessed in a randomized, controlled, Phase I clinical dose escalation study in which the study product was administered by dropper; a second Phase 1 study in which the study product is administered by dropper and sprayer is ongoing. In the completed Phase 1, NE (W_{80}5EC) mixed with commercial Fluzone® (2008–2009 formulation) antigen was administered IN by dropper in volumes of 200 or 500 microliters (μL) to healthy adults 18–49 years of age (Stanberry submitted). A total of 140 subjects received a single administration of study product composed of 5%, 10%, 15%, or 20% W_{80}5EC extemporaneously mixed with 4 or 10 micrograms (μg) of strain-specific HA; IN PBS, IN Fluzone®, and IM Fluzone® served as controls.

There were no serious adverse events or adverse events leading to withdrawal from the study. Reactions were generally mild to moderate and of short duration. The most frequently reported adverse events in recipients of the highest dose of study product (20% W_{80}5EC with 10 μg strain-specific HA) included oropharyngeal pain (55%), rhinorrhea (50%), as well as headache (40%) and were similar to those reported by recipients of 10 μg strain-specific HA alone (47%, 33%, and 47%, respectively). There did not appear to be a dose response in reactogenicity and dose-limiting reactogenicity was not reached.

Given the history of Bell's palsy associated with intranasal administration of *E. coli* heat labile toxin (LT) adjuvanted influenza vaccine in Europe [37], volunteers were carefully monitored by targeted neurological exams for up to 1 year. There

were no reports of cranial nerve palsy or any other neurologic or immunologic sequelae throughout the study. Although there is no theoretical reason why NE adjuvant should bind ganglioside as does LT, vigilant monitoring throughout the clinical development program will continue.

13.8.2 Mucosal Immune Response

Antigen-specific mucosal immune responses were assessed in a subset of volunteers receiving 10%, 15%, or 20% W_{80}5EC. Nasal wash (NW) specimens were obtained pre-vaccination as well as days 28 and 60 post-vaccination and assessed for IgA against Fluzone® antigen. All volunteers that received any concentration of NE adjuvant had a statistically significant increase in antigen-specific IgA from pre-vaccination to day 28 or 60 post-vaccination. Day 28 anti-Fluzone IgA geometric mean titer (GMT) in volunteers that received the highest concentration of NE (20% W_{80}5EC with 10 μg of strain-specific HA) was similar to or greater than anti-HA NW titer reported for adult volunteers with prior natural infection who were protected following challenge with wild-type influenza virus [38].

13.8.3 Humoral Immune Response

Antigen-specific serum immune responses were assessed by HAI pre-vaccination as well as days 28 and 60 post-vaccination. Similar to the mucosal immune response, all volunteers that received any concentration of NE adjuvant had a statistically significant increase in antigen-specific HAI from pre-vaccination to day 28 or 60 post-vaccination. Day 28 HAI GMT in volunteers that received the highest concentration of NE (20% W_{80}5EC with 10 μg of strain-specific HA) was 71 (95% CI 33–155) for H1N1, 83 (95% CI 42–166) for H3N2, and 86 (95% CI 42–179) for B, respectively, well above the seroprotective level of 40 (Fig. 13.5). In comparison, the only currently licensed intranasal influenza vaccine elicits HAI GMT of 12, 61, and 12 for H1N1, H3N2, and B, respectively [39]. It is well known that the current intranasally administered vaccines elicit lower systemic immune responses than parenterally administered vaccines, yet provide similar protective efficacy, possibly due to their more robust mucosal immune responses [40]. In addition to the expected mucosal immunity, NE adjuvanted vaccines appear to produce robust humoral responses after a single intranasal dose.

In summary, the clinical experience to date demonstrates that NE adjuvant is safe, well tolerated, and does not result in adverse events such as cranial nerve palsy that have been associated with other nasally administered mucosal adjuvants. IN administration results in antigen-specific mucosal IgA as well as serum HAI levels that are associated with protection. CMI data in humans is pending at the time of submission of this manuscript.

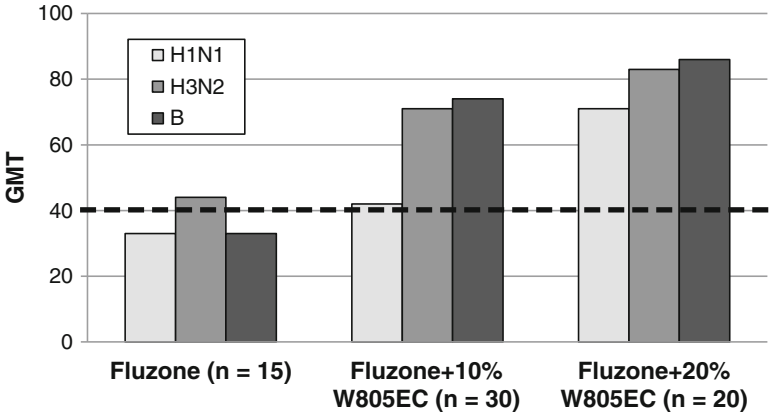

Fig. 13.5 Human serum HAI GMT 28 days after a single intranasal administration of 30 μg total HA in the 2008–2009 formulation of Fluzone® alone or with 10% and 20% W_{80}5EC; *dashed line* is seroprotective titer ≥40

13.9 Unique Immune Effects of NE Adjuvants

The vast majority of available vaccines are delivered via the parenteral route to generate primarily systemic humoral antibodies through activation of the Th2 immune response.

IN immunization with NE-formulated vaccines enhances mucosal immunity as evidenced by an increase in IgA response in the mucosa compared to parenteral immunization, a feature that may prove to act as first line of defense at the port of entry of many pathogens, as the majority of pathogens enter the body via the mucosa [41, 42]. Moreover, in contrast to parenteral immunization with alum, vaccines administered with NE generate enhanced Th1 response as evidenced by a shift in IgG2:IgG1 ratios as well as elicitation of unique cytokines such as IFN-γ and IL-17. CMI is particularly important with respect to pathogens that are intracellular or complex [43, 44], while Th17 responses in particular have recently been recognized to be a critical component of the mucosal immune response [45, 46].

Direct delivery of antigens to APCs via administration of vaccines in the nasal cavity allows for circumventing the unfavorable interaction with preexisting systemic antibodies that may interfere with the immune response to these antigens. This epitopic suppression phenomenon was observed several pediatric vaccines such as *Haemophilus influenzae* [47], pneumococcus [48], as well as Vi conjugates to prevent typhoid fever [49]. The features of mucosal immunity, Th1-biased immune response, and avoidance of preexisting immunity, may play a decisive role in therapeutic as well as prophylactic vaccines such as hepatitis B in chronic carriers and RSV in children and the elderly.

The durability and quality of a systemic humoral antibody response is also important [50]. After vaccination with NE adjuvant, antibodies increase to high levels and persist at high levels for months without a significant decline. This is in

contrast to observations with the majority of parenterally administered vaccines, where a typical antibody response is a fast rise in antibody IgG levels following immunization followed by a fast decline, especially in immunocompromised patients [51, 52]. The rapid decline in antibody may result in levels below what is needed for efficacy [52, 53]. Dagan and colleagues [54] reported a decrease in pneumococcal nasopharyngeal carriage acquisition in the first year post-immunization with conjugated pneumococcal vaccine compared to children immunized with polysaccharide vaccine. However, they observed that nasopharyngeal carriage in these children caught up with the rates in non-immunized peers which coincided with decline in specific antibodies induced by the vaccine. In another growing field of immunotherapies against drug addictions, sustained levels of antibodies are of great importance in maintaining the positive effect of anti-addiction vaccines. Antibodies to nicotine spike after each boost of the vaccine but decline fast after each immunization, requiring multiple monthly booster injections to maintain therapeutic levels of specific anti bodies [53].

In summary, the immune response afforded by formulation of vaccines with NE adjuvants for mucosal delivery is unique and involves the following components: (1) mucosal immunity, (2) Th1 immune response, (3) Th17 immune response, (4) avoidance of epitopic suppression, and (5) persistent and sustained antibody levels. This approach to vaccine development is a significant contribution to the field of vaccinology and may permit vaccines not feasible with other approaches.

13.10 Conclusion

In conclusion, NE adjuvants, such as W_{80}5EC, are oil-in-water emulsions containing nanometer-sized droplets that improve antigen delivery to dendritic cells and enhance immune activation of TLR 2 and 4. The combined effect produces mucosal, systemic humoral, Th1, and Th17 cellular immune response to enhance overall disease protection. Nanoemulsions are easy to manufacture from commonly used pharmaceutical ingredients and confer thermostability to obviate the need for refrigeration during distribution. The NE adjuvant platform is versatile with respect to the types of antigen that can be incorporated, including whole virus, spit virus, recombinant protein, or purified protein. Antigens can be incorporated into the adjuvant by simple mixing at time of manufacture or right before administration. Mucosal administration results in the generation of early innate as well as late adaptive mucosal immune responses that are particularly important for the pathogens that invade via the mucosa. In addition, nasal mucosal administration allows for user-friendly nasal administration without the pain and anxiety associated with injections. These benefits could be particularly useful for pandemic responses in developed countries or general distribution in developing countries, where the cost of manufacturing, lack of refrigeration, and scarcity of health-care personnel for vaccine administration are potential barriers to successful disease prevention.

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Chapter 14
Influenza Virosomes as Antigen Delivery System

Christian Moser and Mario Amacker

14.1 Introduction

Over 15 years of clinical experience with vaccines based on Influenza virosomes has generated a considerable track record, featuring an excellent safety and tolerability profile as well as convincing immunogenicity and efficacy data. In the past decade, a second generation of Influenza virosomes has been developed and validated as a versatile, standalone carrier and adjuvant system for heterologous subunit antigens.

Influenza virosomes represent a unique type of virus-like particle (VLP) which is applied in commercial vaccines and in several vaccine candidates in clinical development. Originally, VLPs were defined as nonreplicating, recombinant virus capsids [1, 2], but in recent years the definition has been expanded to include virus-like structures derived from enveloped viruses [3–5]. Common to all VLPs is their particulate structure that mimics a virus combined with their complete inability to replicate. These features ensure a high safety level but retain the advantages of a particulate structure which enhances the stability and the immunogenicity of the co-delivered antigens. Therefore, virosomes are VLPs by all structural and functional criteria, even though they are assembled in vitro, not by a host cell like most other VLPs.

Influenza virosomes are homogenous, spherical, and unilamellar vesicles with a mean diameter of 150 nm. They are assembled from lipids and purified Influenza virus envelope components in a tightly controlled in vitro process. Both the particle

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structure and the incorporated Influenza proteins are essential for the function of virosomes as a combined antigen carrier and adjuvant system for heterologous antigens. Virosomes do not only provide classical carrier functions, such as antigen stabilization and delivery in a repetitive array, but include also adjuvant-type functions such as the activation of immune cells. A particular feature of virosomes is the immune-enhancing effect due to preexisting immunity against Influenza. These properties of Influenza virosomes make them an attractive standalone vaccine delivery system which is suitable for various types of subunit antigens, in particular for small, poorly immunogenic molecules.

Originally, the term virosome was proposed for oncogenic subviral ribonucleoprotein particles of Rous sarcoma virus. However, the current definition of virosomes was established in 1975 as a preparation of unilamellar liposomes with integrated viral envelope proteins, using Influenza A as an example [6]. Morein et al. expanded the method of a controlled disassembly/reassembly procedure to other enveloped viruses and proposed the use of the resulting proteoliposomes/virosomes as vaccines [7]. Over the following decades, virosomes have been generated successfully from a wide variety of enveloped viruses (Rubella, Semliki Forest, Measles, Herpes simplex, Rabies, Sindbis, Vesicular Stomatitis, Epstein–Barr, Sendai, Hepatitis B, Respiratory Syncytial, Human Immunodeficiency). These artificial virus envelopes were prepared at lab scale and mostly used for functional studies, or as homologous vaccines or gene delivery vehicles. Although the methods to formulate virosomes from different viruses vary considerably, the basic concept is common to all, namely the controlled in vitro assembly of a VLP from solubilized viral envelopes. Because this assembly process is only efficient at high protein and lipid concentrations, milligram amounts of purified virus are required, even for lab scale formulations of virosomes. Accordingly, the availability of sufficient virus material often represents a major technical hurdle for the preparation of virosomes from a specific virus species.

14.2 Influenza Virosomes

To date, virosomes derived from Influenza virus remain the only virosome type, which has been clinically tested and is applied in commercially available human vaccines. While the unique biological properties of the envelope protein hemagglutinin (HA) provide a convincing scientific rationale for using Influenza, another very practical reason is the availability of large amounts of clinical grade virus produced according to good manufacturing practice (GMP). Influenza virus is produced for several hundred million doses of seasonal Influenza vaccines every year. The same material can be used for the generation of Influenza virosomes. Notably, Influenza virus grown in cell culture is at least equally suited for the production of virosomes as virus produced by the conventional method, in embryonated chicken eggs [8].

A major breakthrough was achieved by Stegman et al. in 1987 with the establishment of a formulation method, which yielded functional Influenza A virosomes, featuring the same pH-dependent, hemagglutinin-mediated membrane fusion activity as the parental Influenza virus [9]. A key element to HA-functionality was the use of octaethyleneglycol mono(*n*-dodecyl)ether (OEG; C12E8) as a detergent. However, in order to obtain homogenous particle preparations, a purification step via a sucrose gradient was necessary after the particle assembly, a procedure associated with substantial losses of the precious Influenza envelope proteins.

An efficient formulation process at industrial scale became possible when an excess of egg-derived phospholipids were added to the purified envelope fraction before virosome particle assembly [10]. Under optimized conditions, the process yielded a homogenous particle population upon detergent removal, thereby rendering further purification steps obsolete. The addition of purified lipids led to an increased lipid to protein ratio of the particles, and therefore, a reduction of the density of the viral envelope proteins on the virosome surface. Nevertheless, these so-called immunopotentiating reconstituted Influenza virosomes (IRIV) retained the biological properties of the parental virus with regard to cell interaction and pH-dependent fusion activity [10, 11].

The technical and biological features of Influenza virosomes made them attractive to explore novel applications within and beyond the vaccine field:

- The controlled particle assembly in vitro from defined components allows for rational design of the particle composition and efficient empirical optimization of the particle properties. This is a clear advantage over conventional VLP platforms, where the particles are assembled by a genetically engineered host cell and thus, the control over the particle composition is indirect and rather limited.
- The VLP structure in the form of a unilamellar lipid membrane vesicle with an aqueous lumen and a protein-decorated surface provides various options to integrate additional molecules, either onto the surface, or into the membrane or the lumen.
- The virus-like interaction of virosomes with a wide variety of cell types ensures a rapid uptake by target cells.
- The HA-mediated, pH-dependent membrane fusion activity enables escape of molecules encapsulated in the lumen of virosomes from lysosomal degradation and access to the cytoplasm of target cells.

14.2.1 IRIV: First Generation Influenza Virosomes

IRIV represent the core technology behind the first generation of commercial, virosome-based vaccines, adapted from the formulation process developed in the early 1990s (Table 14.1). Epaxal® and Inflexal® V (Crucell) were launched in 1994 and 1997, respectively. To date, over 70 million commercial doses of these virosome-

Table 14.1 Products based on first generation immunostimulating reconstituted Influenza virosomes (IRIV)

Products	Composition and assembly	IRIV functions	References
Hepatitis A vaccines intramuscular Epaxal® (Crucell), 1994 Epaxal® junior (Crucell), 2008	Inactivated hepatitis A Influenza A/ Singapore/6/86 (H1N1) Lipids: EYPC, EYPE HAV adsorbed to IRIV after assembly Liquid formulation in syringe	Carrier and adjuvant for non-Influenza antigen (HAV)	[10, 12–14]
Seasonal Influenza vaccines intramuscular Inflexal® V (Crucell), 1997 Invivac® (Solvay Pharamaceuticals), 2004	Three seasonal Influenza vaccine strains (A/ H1N1, A/H3N2, B) according to WHO recommendations Lipids: EYPC IRIV assembled from each strain separately Blend of three IRIV Liquid formulation in syringe	Carrier: VLP structure for Influenza antigens	[15–18]
Seasonal Influenza vaccine adjuvanted, intranasal Nasalflu® (Berna Biotech), 2000	Three seasonal Influenza vaccine strains (A/ H1N1, A/H3N2, B) according to WHO recommendations Lipids: EYPC HLT from *E. coli* (mucosal adjuvant) IRIV assembled from each strain separately Blend of three IRIV HLT added to blend Liquid formulation in intranasal application device	Carrier: VLP structure for Influenza antigens Mucosal delivery	[19–21]

EYPC phosphatidyl-cholin purified from egg yolk (lecithin), *EYPE* phosphatidyl-ethanolamine purified from egg yolk (cephalin), *HLT* heat labile toxin, *HAV* hepatitis A virus

based vaccines have been distributed and their safety and efficacy has been documented in various clinical studies [12, 15].

In the following years, additional IRIV-based vaccines reached the market. Nasalflu® (SSVI, Berna Biotech), a virosome-based, trivalent Influenza vaccine for intranasal administration was licensed in Switzerland in 2000. However, the product had to be withdrawn from the market in 2001 due to rare but serious side effects related to the mucosal adjuvant, which had been added to enhance the immunogenicity of the vaccine [19, 20, 22].

In 2004, the trivalent, virosome-based Influenza vaccine Invivac® (Solvay Pharmaceuticals) was registered in Europe, but the product was commercially available only for a limited period of time. Finally, in 2008 Epaxal® junior (Crucell) was launched as a pediatric follow-up product to the successful adult Hepatitis A vaccine.

Among the licensed IRIV-based vaccines only the Hepatitis A vaccines Epaxal® and Epaxal® junior represent true carrier applications of Influenza virosomes, where the IRIV functions as a delivery vehicle for a heterologous antigen of interest (AoI), Hepatitis A antigen. Thereby, the response to the Influenza antigens impacts positively on the response against the AoI. Several direct comparisons with alum-adjuvanted Hepatitis A vaccines have consistently demonstrated the superior tolerability of the virosome-based product Epaxal® and at least equal immunogenicity [12, 23, 24].

Virosome-based Influenza vaccines essentially contain the same antigen composition as non-particulate, subunit vaccines. Here, the role of virosomes is reduced to providing Influenza antigens a VLP structure, which in theory could improve the immunological properties [16, 25]. However, comparative clinical studies with conventional Influenza subunit vaccines showed that the virosome formulation did not significantly increase immunogenicity against Influenza, but rather improved tolerability [15, 26]. Whether the improved tolerability results from the particle structure or from the higher purity of the product remains unclear [27].

14.3 Second Generation Influenza Virosomes

Ever since the first functional Influenza virosomes were described, and the first generation of products were licensed, the methods for assembly and characterization of virosomes were continuously refined and novel applications explored. These efforts resulted in several types of second generation of Influenza virosomes, which were specifically designed either as B- or as T-cell vaccines, and beyond conventional vaccines, for nucleic acid and drug delivery (Table 14.2).

Common to all vaccine applications of second generation virosomes is that they are not used as Influenza vaccines but as a carrier and delivery system for defined molecules unrelated to Influenza. In contrast to the only example of a first generation virosome applied as a carrier system (Epaxal®), the payload is integrated in most cases during the particle assembly process, not adsorbed to readymade virosome particles (Fig. 14.2). Accordingly, the most prominent improvements for second generation virosomes comprise various novel methods to integrate payload molecules, subunit vaccine antigens, nucleic acids or drugs. These aspects are discussed in more detail in Sect. 14.4.

Furthermore, modifications in the lipid composition were explored in various directions. The egg yolk derived phospholipids used in the first generation virosomes were increasingly replaced by synthetic analogues. Although this change had no impact on the properties of the resulting virosomes, the use of precisely defined,

Table 14.2 Second generation virosomes and their applications

Function and effect	Virosome type	Composition and assembly	Applications	References
B-cell vaccines	GIRIV	Membrane-anchored antigens	Clinical:	[28–31]
Antigen display on surface		Influenza A/Singapore/6/86 (H1N1)	PEV3A—bivalent Malaria	[32–34]
B and CD4 cell stimulation		Lipids: EYPC, EYPE or PC, PE	PEV6—trivalent Breast cancer	
Humoral immunity		Co-assembly with antigen	MYM-V101—HIV	
		Liquid formulation in syringe	Preclinical: RSV, Leishmania, Alzheimer's	
	GTIRIV	Membrane-anchored antigens	Clinical:	[35–37]
		Influenza A/Singapore/6/86 (H1N1)	PEV3B—bivalent Malaria	
		Lipids : PC, PE, TC-Chol	PEV2—HCV (CD4 peptide)	
		Sucrose	PEV7— monovalent Candida	
		Co-assembly with antigen	Preclinical: various targets	
		Lyophilized product in vial		
T cell vaccines	TIRIV	Unmodified antigen	Clinical:	[38]
Cytoplasmatic delivery of encapsulated antigen		Influenza A/Singapore/6/86 (H1N1)	PEV2—HCV (2 CD8 peptides)	
MHC I presentation		Lipids : EYPC, EYPE, TC-Chol		
CTL induction		Sucrose		
		Assembly and lyophilization of empty TIRIV		
		Reconstitution with peptide solution prior to injection		
	Virosomes without additional lipids	Unmodified antigen	Preclinical	[39, 40]
		Influenza A/Johannesburg/33/94 (H3N2) or A/Panama/2007/99 (H3N2)	Ovalbumuin, peptides	
		No additional lipids		
		Passive encapsulation during assembly		
	CIRIV	Unmodified antigen	Preclinical	[41]
		Influenza A/X-31/1968 (H3N2) and A/Singapore/6/86 (H1N1)	HCV, Ovalbumin peptides	
		Lipids: PC, PE		
		Chimeric virosomes composed of two strains		
		Two-step encapsulation via liposomes		

Nucleic acid delivery Cytoplasmatic delivery vehicle CTL induction Gene silencing	DNA/RNA IRIV	siRNA, plasmid DNA Influenza A/Singapore/6/86 (H1N1) or A/Panama/2007/99 (H3N2) cationic lipids, optional EYPC Inulin for lyophilization Cationic lipids to interact with nucleic acids DNA packaging during or after IRIV assembly Lyophilization possible	Preclinical Various targets	[42–46]
Targeted drug delivery Cytoplasmatic drug delivery Antibody on surface as targeting device	Fab-IRIV	Doxorubicin Lipid-anchored antibody fragment Influenza A/Singapore 6/86 (H1N1) Lipids: EYPC, EYPE Co-assembly with antibody pH-driven drug loading after assembly	Preclinical Breast cancer	[47]

The order from top to bottom reflects the stage of development of the application and virosome type
EYPC phosphatidyl-cholin purified from egg yolk (lecithin), *EYPE* phosphatidyl-ethanolamine purified from egg yolk (cephalin), *PC* synthetic phosphatidyl-cholin, *PE* synthetic phosphatidyl-ethanolamine, *TC-Chol* cholesteryl *N*-(trimethylammonioethyl) carbamate chloride, *CIRIV* chimeric IRIV with two types of Influenza HA and with antigens inside non-leaky IRIVs, *GIRIV* IRIV formulated with antigens containing a membrane anchor and thus integrated in the virosomal membrane, *GTIRIV* lyophilizable GIRIV, *TIRIV* lyophilizable IRIV where the antigens are either integrated in the virosomal membrane or located inside of IRIVs

synthetic components instead of purified lipids represents a highly relevant improvement from a regulatory and GMP manufacturing point of view. The inclusion of cationic lipids originated from the intention to use virosomes as nucleic acid delivery vehicles [42–46] but later on proved also critical as a stabilizer in the development of lyophilizable virosomes, in combination with the addition of sucrose [38] or inulin [48]. The use of 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) instead of OEG as a detergent in the formulation process enabled detergent removal via dialysis as opposed to the generally used a batch chromatography method [45].

Last but not least, the biochemical and immunological properties of virosomes were investigated to better understand their mode of action [25, 35, 49–52], as further discussed in Sect. 14.5. The continuously accumulating knowledge on how virosomes function keeps feeding back into the design of novel vaccine compositions.

14.4 Applications of Second Generation Virosomes

Second generation virosomes were applied to numerous antigens at preclinical stage up to proof of concept (Malaria, Breast cancer, HCV, HIV, Candida, DiTe, Alzheimer, Melanoma, RSV, Leishmania). Most of these approaches are by design B-cell vaccines with the intention to induce antibodies. A T-cell vaccine approach was explored for a therapeutic Hepatitis C vaccine based on two CD8 peptides and one CD4 peptide [38].

Four candidate vaccines (Malaria, HCV, Breast cancer, HIV) were tested in a total of six clinical trials, all of them with synthetic peptides as antigens (Table 14.2, [35]). A seventh phase I trial is currently ongoing, with a therapeutic vaccine against recurrent vulvovaginal candidiasis, based on a recombinant protein derived from *Candida albicans* as AoI [36].

Notably, all trials have confirmed the excellent safety and tolerability profile observed with the first generation of virosome-based vaccines. Three applications of 10–50 μg peptide antigen proved sufficient to induce robust antibody responses in five trials with three different B-cell vaccines (Malaria, Breast cancer, HIV).

The bivalent Malaria vaccine PEV3 was shown to induce robust, long lasting, and functional antibody titers in a phase I clinical trial [53]. Indications of protective effects were observed first in a clinical phase II challenge trial [28], and recently in a phase Ib trial in an endemic area [37].

PEV6, a trivalent peptide vaccine against breast cancer, was shown to induce antibodies against an auto-antigen, Her2/Neu, even though the subjects in this study were breast cancer patients of advanced age and disease stage [29]. Notably, these results were achieved with peptide doses of 10 μg.

A virosome-based bivalent HIV vaccine had impressive protective effects in a monkey challenge study, in particular in a systemic prime/mucosal boost regime [30]. In a subsequently performed phase I clinical trial with a monovalent form, the HIV vaccine has proven safe immunogenic, also when applied via the intranasal route [54].

The trivalent, peptide-based HCV vaccine PEV2 designed for CTL induction failed to generate significant responses in a phase I clinical trial, despite encouraging preclinical data [38]. It remains unclear whether this shortcoming is due to the peptide design, or indicates a limitation of the virosome technology.

14.5 Mode of Action

The goal of any vaccination is to generate populations of mature, antigen-specific effector and accessory cells. An important aspect is the induction of immunological memory for a sustained, long-lasting effect. Subunit antigens are preferable from a safety point of view but often are poorly immunogenic by themselves, and therefore require adequate carrier and/or adjuvant systems to achieve an effective level of immune response. A vast number of adjuvants have been identified that are capable of enhancing the immunogenicity of weak antigens, but aside from aluminium salts, only very few are actually applied in licensed human vaccines, namely MF59 (Novartis), AS03 and AS04 (GSK), and Influenza virosomes (Crucell, Solvay). Over the past decade, substantial progress has been made in understanding the interplay between innate and adaptive immunity, which has provided explanations for the mode of action of established adjuvants [55–59] and allowed for a more rational selection of novel adjuvant candidates. Profound knowledge of the mode of action provides a rational basis for the risk assessments of adjuvants, and for the design of conclusive toxicology studies and clinical trials. Safety concerns are more difficult to address in clinical trials than immunogenicity since severe side effects are not acceptable even at very low frequencies [60–62]. Therefore, a thorough safety assessment is also the key issue in regulatory guidelines for clinical testing and market approval of novel adjuvants [62–64].

Influenza virosomes applied to deliver an antigen unrelated to Influenza virus exert their multiple effects through their particle structure as well as through their individual components. An important aspect is the effect of preexisting immunity against Influenza which significantly enhances on the immune stimulating function of virosomes. Therefore, virosomes represent an excellent example for a multifunctional carrier and adjuvant system (Fig. 14.1).

14.5.1 Carrier Function

The carrier function relates to the particle structure of virosomes and plays a key role in the early events after administration. The incorporation of the AoI in the virosome particle protects it from premature degradation by extracellular proteases. Upon intramuscular injection, virosomes can access the draining lymph node via free lymph drainage, or in association with migrating cells, in particular antigen-presenting cells (APC). The free draining hypothesis is supported by a recent report, demonstrating

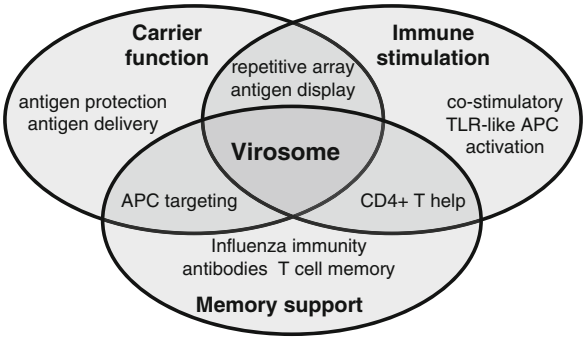

Fig. 14.1 Multifunctionality of virosomes. The overlapping functions of virosomes are depicted schematically to highlight the central role of the virosome structure. The carrier function is mainly based on the VLP structure. The immune stimulating effects relate to both the particle structure and the Influenza-derived virosome components with stimulatory effects and synergistic potential. Preexisting immunity against Influenza impacts on both the carrier and the immune stimulatory functions. Antibodies interact directly with the particle structure, while cellular immunity expands the pool of helper T cells supporting the development of effector cells. This figure is adapted from [35]

that nanoparticles up to 200 nm and non-enveloped VLPs are detectable in APC resident in the lymph node, but larger particles only in macrophages originating from the injection site [65]. Virosomes are rapidly taken up by cells, both in vitro and in vivo [51, 52, 66]. Cell entry of Influenza virosomes has been described to occur both via two independent endocytosis pathways, clathrin-dependent and clathrin- and caveolin-independent endocytosis, and to a very similar extend as active Influenza virus [66]. Within the lymph node, virosomes are thought to interact with APC as well as with B cells as intact particles, as observed with viruses and particulate antigens [67, 68]. In this situation, the repetitive display of the AoI on the virosome surface acts as a strong activation signal for AoI-specific B cells via cross linking of Ig receptors [69, 70]. Upon initial activation by contact, those B cells process the entire virosome for MHC II presentation, including the Influenza components, to obtain their signal 2 from CD4 helper T cells. As a consequence, AoI-specific B cells are amenable to T-cell help with two specificities, AoI and Influenza.

14.5.2 Immune Stimulation

Influenza virosomes facilitate the antigen uptake, and enhance processing and presentation by APC. Direct activation of dendritic cells (DC) by virosomes was demonstrated in the murine system [39], while a human plasmacytoid DC line did not increase expression of activation markers upon in vitro stimulation with virosomes [51]. In the experimentally confirmed absence of classical activators, nucleic acids and bacterial activator molecules, the activation signal induced by virosomes may

be mediated by components derived from Influenza virus, e.g. the HA, via so far unidentified signaling pathways. This hypothesis is further supported by the finding that recombinant Influenza HA was reported to activate both murine and human DC in vitro [71–73].

Human peripheral blood mononuclear cells (PBMC) exposed to Influenza virosomes produce cytokines (TNF-α, GM-CSF, IFN-γ, IL2, but not IL4). Interestingly, TNF-α and GM-CSF were rapidly secreted at high levels, suggesting that at least these cytokines do not originate from the proliferating, Influenza-specific memory cells present among the PBMC but from a more abundant, so far unidentified cell type present in PBMC preparations, which is responsive to virosomes [49].

Further investigations are needed to clarify to which degree virosomes are capable to provide a danger signal and to directly activate DC, and by which mechanisms.

14.5.3 Memory Support

Preexisting immunity against the carrier is known to impair the function of viral vectors and VLPs, and quench the immune response against the heterologous AoI [74–76]. In sharp contrast, the immune response against an AoI in the context of an Influenza virosome is enhanced by preexisting immunity against Influenza. This attribute is of particular importance because preexisting immunity against Influenza is widespread among the human population and can be detected in nearly every individual [49, 77, 78].

Preclinical studies in mice have clearly demonstrated early on that preexisting immunity against Influenza enhances the antibody response against heterologous antigens administered subsequently in the context of virosomes [79]. Notably, this enhancer effect is strain independent. The same positive effect was observed for preexisting immunity against either Influenza B, or against the homologous Influenza A/H1N1 strain used to reconstitute the virosomes [35]. However, the magnitude of CTL responses against virosome-encapsulated peptides was not significantly increased by preexisting immunity against Influenza [38, 80].

Both humoral and cellular elements of preexisting immunity against Influenza are thought to contribute to the immune-enhancing effects. Anti-Influenza antibodies are thought to target virosomes to Fc receptors of APC, thereby accelerating and enhancing uptake by APC. For this purpose, the antibodies do need neither virus neutralizing nor hemagglutination inhibiting capacity. Mere binding to any of the Influenza proteins present on virosomes is sufficient. This hypothesis is consistent with the observed strain type-independence. A speculative explanation for the lack of enhanced CTL responses is interference of antibodies with the HA function necessary for the cytoplasmatic delivery, which might be sufficient to compensate for the positive effects of improved APC targeting.

Reactivation of Influenza-specific memory cells, as demonstrated in human PBMC is considered the main cause for cell-mediated immune enhancement [49]. B cells specific for the AoI are activated upon contact with the antigen displayed on the surface of virosomes. Subsequently, these cells process and present all virosome components in the context of MHC-II, including the Influenza proteins. As a result, these cells can obtain their signal 2 not only from antigen-specific but also from Influenza-specific CD4+ T helper cells. In a situation of preexisting immunity, these Influenza-specific CD4+ T cells are more abundant and faster activated than naïve CD4+ T cells reactive to the AoI, and therefore, can significantly contribute to the differentiation and proliferation of AoI-specific effector cells.

Multiple infections by constantly drifting virus strains result in a complex immunity against Influenza in the human population. For that reason, no conclusive clinical data are available on the positive effect of preexisting immunity on the response against a heterologous AoI administered in the context of virosomes. The Hemagglutination inhibition (HI) titer against the virus strain used in virosomes [A/Singapore/6/86 (H1N1)] was measured in several clinical trials. Consistently, no significant correlation was found between the response against Hepatitis A virus and the baseline HI titer against Influenza, thereby excluding at least a negative effect [12, 81]. However, the value of these data is limited because HI titers do account neither for humoral immunity against heterologous Influenza strains nor for any cellular immunity. No correlation was detectable either between preexisting cellular immunity against Influenza and the response against the malaria-derived peptides formulated on virosomes [78]. Without truly naïve subjects available as negative controls, it appears extremely difficult to clinically verify the positive correlation between baseline anti-Influenza immunity and response to the AoI, as it has been clearly demonstrated in animal models [35, 79].

Further preclinical and clinical studies are necessary to dissect the contributions of the different elements and to determine, whether the positive effect directly correlates with the magnitude of preexisting immunity or rather requires threshold levels of preexisting humoral and cellular immunity.

14.6 Virosome Production

The assembly of virosomes is in essence a three-step process, starting from inactivated, purified Influenza virus, lipids, and the AoI (Fig. 14.2). The general method has remained largely unmodified when comparing first to second generation virosomes, except for the membrane integration of the heterologous antigens designed for B-cell induction (Fig. 14.2, A vs B).

Initially, the Influenza virus is concentrated by ultracentrifugation. The virus pellet is dissolved in detergent, and the insoluble complex of viral RNA and internal proteins is separated from the solubilized envelope components by ultracentrifugation.

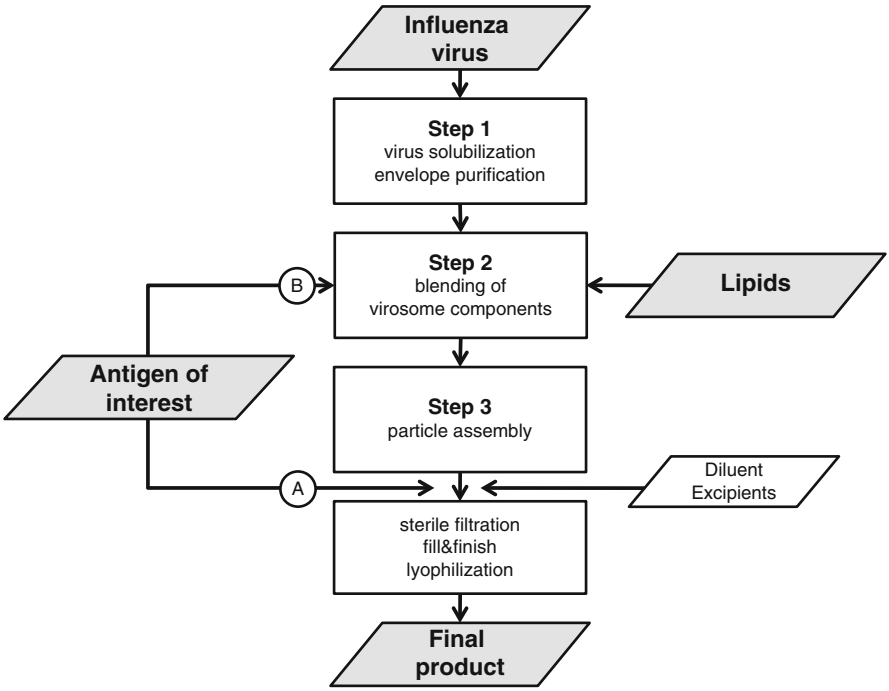

Fig. 14.2 Schematic production process of Influenza virosomes. The three-step procedure for assembly of virosomes is shown, starting with the purification of the Influenza envelope components from inactivated virus (Step 1), followed by blending in the other constituents of the particle (Step 2). Finally, the particles are assembled by controlled detergent removal to yield the intermediate bulk (Step 3), which is then processed to the final product. With regard to the integration of a heterologous antigen of interest, two options are depicted. In the first generation HAV vaccine Epaxal®, the heterologous HAV antigen is adsorbed to the previously assembled empty virosomes (**A**). In second generation B-cell vaccines, the antigen is anchored in the virosomal membrane via a lipid anchor, and thus, is added prior to particle assembly (**B**)

As a next step, the lipids to be incorporated are dissolved in the same detergent and added to the purified viral envelope fraction. For second generation virosomes and in case that membrane integration is envisaged, the AoI is also added at this step.

Finally, the detergent is removed by batch chromatography on polystyrene beads, thereby triggering the formation of the virosome particles. This step is critical as the homogeneity of the particles depends on the proper ratio of both the virosomal components and the detergent adsorbent [17].

After assembly, the formulation is diluted to the desired concentration and sterile filtered. Multivalent vaccines are blended at this point from monovalent intermediate products to yield the final vaccine formulation, as established for the trivalent seasonal Influenza vaccine Inflexal® V [17]. The most recent second generation vaccines can be stored in a freeze-dried form instead of the conventional liquid form

[37]. The presentation of the vaccine in a dry powder form led to increased stability and allowed the development of innovative administration forms like vaginal capsule for direct mucosal immunization (manuscript in preparation).

14.6.1 Basic Virosome Components

14.6.1.1 Influenza Component

The inactivated, purified Influenza virus used as a starting material for the production of virosomes is identical to the material used for subunit Influenza vaccines [17]. In that context, the method of virus inactivation plays a role with regard to the functionality of hemagglutinin. Virus inactivation with beta-propiolactone (BPL) is preferable to formaldehyde (FA), because BPL leaves proteins largely unaffected while FA treatment does modify proteins, including the HA. Virosomes generated from virus inactivated with BPL proved a higher fusion activity than comparable formulations from FA-inactivated virus [45].

The purified envelope fraction of Influenza virus used for the assembly of virosomes contains predominantly HA, about fivefold less neuraminidase (NA), traces of M2e tetramers, and lipids originating from the viral membrane [35, 82]. The ratios between the proteins remain assimilable between virosomes and parental virus, but the overall lipid to protein ratio is increased in virosomes formulated with additional lipids. For first generation virosomes, egg-derived, purified phospholipids were used, while second generation virosomes are composed of synthetic lipids, including phospholipids, cholesterol-derivates, and other lipid molecules.

14.6.1.2 Lipids and Sugars

The physicochemical and biological properties of virosome particles can be modulated not only by the amount but also by the type of lipids added [83, 84]. The addition of charged lipids has proven useful to associate and deliver negatively charged molecules such as nucleic acids [44, 46, 50].

Conventional liquid formulations of Influenza virosomes, including all first generation virosomes, feature a limited stability unless stored at +2 to 8 °C. They are particularly sensitive to freezing, which can lead to the loss of fusion activity and particle aggregation. In addition, subunit antigens, recombinant proteins or synthetic peptides, are prone to oxidation, deamination or other modifications when stored as aqueous solutions. Therefore, the option to freeze-dry virosomes represented an attractive potential for improvement of the technology. By addition of sugars and, optionally, cationic lipids, virosomes could be protected from the negative effects of freezing described above [38, 48]. Upon reconstitution in water, the lyophilized formulations were shown to retain their particle characteristics, their fusion activity, and their immunological func-

tions [38, 80]. At the same time, lyophilization also increased the stability of subunit antigens integrated in the virosomes (Pevion Biotech, unpublished data). Furthermore, lyophilization enabled a novel approach to efficiently encapsulate small antigens like peptides into virosomes, by reconstituting empty lyophilized virosomes with a peptide solution [38].

14.6.2 Integration of Heterologous Antigens

The physical association of any AoI with a virosome particle is essential to exploit the full carrier and adjuvant effect of the platform. The most suitable approach to associate a non-Influenza antigen depends on both, the specific biochemical properties of the molecule and the intended positioning in the context of the virosome particle. The positioning determines the fate of the AoI and thereby, the character of the resulting immune response.

14.6.2.1 Surface Display

AoI displayed in a repetitive array on the outer surface of the virosomes primarily induce a humoral immunity, through direct interaction with antigen-specific B cells. In addition, induction of antigen-specific CD4 T cells is achieved via APC which process and present antigen-derived peptides in the context of MHCII. Surface display is achieved by anchoring the antigen in the virosome lipid bilayer, either via a hydrophobic protein domain, or via a synthetic phospholipid covalently conjugated to the antigen. For peptides used in clinical trials, the N- or C-terminal conjugation to phosphatidyl-ethanolamine (PE) was performed at the end of peptide synthesis [29, 32, 53]. In similar fashion, recombinant proteins and carbohydrates can be conjugated to a lipid anchor during the virosome manufacturing process [30, 33, 79]. In contrast, robust integration of the envelope protein F of RSV was achieved without modifications of the antigen due to the presence of a transmembrane domain, in analogy with the Influenza envelope proteins [34].

14.6.2.2 Encapsulation in the Virosome Lumen

If cytoplasmatic delivery is envisaged, the payload needs to be encapsulated in the lumen of the virosomes. With respect to vaccine antigens this approach is chosen to target APC with the intention to induce antigen-specific cytotoxic T-lymphocytes (CTL). The pH-dependent fusion activity of HA enables virosomes to fuse with the membrane of the late endosome upon acidification. As a result, the content of the virosome lumen is released into the cytoplasm, thereby providing access to the endogenous antigen processing pathway, and presentation in the context of MHC I, which is a prerequisite for CTL induction.

The straightforward method, adding the antigen before particle assembly, will lead to entrapment of a small fraction of the antigen [40]. More efficient approaches are the encapsulation into chimeric Influenza virosomes (CIRIV) via a liposome intermediate [41] and the use of lyophilized TIRIV for encapsulation upon reconstitution [38].

Beyond the application for vaccine antigens, different encapsulation approaches have been developed for nucleic acid [42, 44–46, 50] and drug delivery [47].

14.6.3 Quality Control

Retaining the authentic conformation and functionality of the viral proteins in the context of the particle structure is a key aspect of the virosome formulation process, as exemplified by the pH-dependent fusion activity of HA [50, 66, 85]. As a consequence, a thorough quality control of virosomal vaccines goes far beyond quantitative content analysis, and includes particle characterization with regard to size, homogeneity, composition, and the assessment of the fusion activity in vitro [66, 85].

The pH-dependent fusion activity per se is not required in all applications of virosomes to fulfill their carrier function. For instance, when applied to a B-cell vaccine, access to the cytoplasm is required neither for antibody induction nor for the generation of CD4 helper T cells. However, the fusion assay in vitro can be seen as a comprehensive quality control for Influenza virosomes, since it demonstrates that HA has retained its authentic, functional conformation and is positioned accurately and in sufficient amounts on the virosome surface.

Furthermore, the association of the AoI with the virosome structure is essential to exploit the full potential of the carrier and adjuvant system, and therefore, analytical assays are needed to demonstrate that all components are present in a single particle, and that the particles are homogenous within tight specifications.

14.6.4 GMP Manufacturing Process

The basic formulation method has been successfully translated into a robust industrial scale process compliant with GMP guidelines for the first generation IRIV products Epaxal® and Inflexal® V [17]. However, neither product requires the integration of additional, non-Influenza antigens at the stage of virosome assembly, since the Hepatitis A antigen is adsorbed to Influenza virosomes after particle formation (Fig. 14.2). For the trivalent seasonal Influenza vaccines, the virosomes are generated separately from each Influenza strain, and subsequently blended at equal HA concentrations to yield the final product [17]. Since the launch of Inflexal® V in 1997, virosomes have been generated from all the virus strains recommended for seasonal Influenza vaccines (Influenza A/

H1N1, A/H3N2, B), each compliant with the specifications regarding size and HA content and with confirmed fusion activity in vitro.

The second generation virosomes (GIRIV, GTIRIV, Table 14.2) require the integration of the non-Influenza AoI during the particle assembly process, in particular those anchored in the lipid bilayer (Fig. 14.2). Although the overall formulation process remains the same as for first generation virosomes, the process is optimized for each novel composition, in order to obtain homogenous particles with minimal material losses during formulation and the subsequent sterile filtration process. The feasibility of GMP grade production for second generation virosomes has been demonstrated by the successful production of vaccines for seven clinical trials, including nine different peptide antigens (two Malaria, three HCV, three Breast cancer, one HIV) and one recombinant protein (Candida).

For multivalent vaccines, a similar approach as applied to the trivalent seasonal Influenza vaccines was chosen. First, a separate virosome is assembled for each heterologous antigen. Subsequently, these monovalent intermediate products are blended to yield the multivalent vaccine containing the desired antigen concentrations.

To date, several multivalent vaccine candidates based on second generation virosomes have been successfully manufactured, released, and tested in clinical trials (Table 14.2), among them were several lyophilized vaccines (Malaria, HCV, Candida). The successful development of temperature-insensitive, lyophilized forms of virosomes illustrates the practicability of lyophilization under GMP compliance and the flexible application of the Influenza virosome.

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Chapter 15
Matrix M Adjuvant Technology

Karin Lövgren Bengtsson

15.1 Introduction

Vaccine adjuvants are substances or procedures that when added to or applied in vaccines aim at substantial improvement of the quality and/or magnitude of immune responses to the vaccine antigens. The term "adjuvant" is derived from the Latin term *adjuvare*, which means "to help." The resulting immunological benefit of adjuvant addition depends not only on what kind of help the adjuvant can offer but also on what kind of help that is applicable to the antigen in question.

The need for vaccine adjuvant addition was substantially increased by the introduction of highly purified subunit vaccine antigens. Contrary to older antigen preparations, these antigen preparations contain only relevant antigens or epitopes and are substantially free of other microbial molecules or substances such as LPS, dsRNA, unmethylated CpG motifs, flagellin, or other pathogen-associated molecular patterns. These molecules, unnecessary contaminants from an antigen point of view, contribute substantially in a positive way to the immunogenicity of antigen preparations but they also contribute to the generation of vaccine side effects. The immune stimulation exerted by these microbial "contaminants" is beneficial for initiation of immune response and today refined preparations of such molecules constitute the basis of several modern adjuvant preparations [1], e.g., lipid A, LPS, bacterial toxins, and flagellin. Another substantial difference lies in the antigens themselves. They have changed from being larger microbial entities to single proteins or even parts of protein antigens. Due to various reasons implied, e.g., by production feasibility, recombinant antigens are often smaller in size compared to its parent native antigen, a fact that also contributes to reduction of immunogenicity [2]. Also here we see that efforts are made to apply what has been learnt, by

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expressing antigens as larger multimeric particles such as various VLP constructs. As a consequence, today's efforts in making more efficient vaccines include both antigen design and adjuvant addition.

From being added to vaccine antigen as a general immune response enhancer like $Al(OH)_3$, with the purpose to increase mainly the antibody response, modern adjuvants are becoming more integrated in the vaccine design processes, though their presence mostly being motivated by the urge of improving on cellular immune responses.

15.1.1 Adjuvant History

The effect of different adjuvant formulations with different antigens in various animal species has been thoroughly reviewed over the years. It is challenging but not always possible to extrapolate results from one antigen–adjuvant combination to another and from one experimental setup or species to another. Cox and Coulter [3] introduced a fairly broad definition of adjuvant as "any substance or procedure that results in a specific increase in immunogenicity of a vaccine component." This definition captures to a great extent also the diverse achievements in adjuvant development during the last decades. The "old" classical adjuvant formulations such as aluminum salts ($Al(OH)_3$ and $AlPO_3$) and water/oil emulsions (FCA and FIA) primarily regarded as "physical adjuvants" interact with antigens to increase their immunogenicity by e.g., creation of slow-release depots, antigen particulates or precipitates. The physical formulation of antigens resulted in increased antigen presentation, local inflammation, and recruitment of antigen-presenting cells. These effects are likely achieved also by modern antigen design, decreasing the needs or the benefits of physical adjuvants.

However, the area of adjuvant research is diverse and full of surprises and several attempts to generalize and extrapolate general properties have failed due to the complexity and variety of immune response mechanisms.

15.2 The Immune-Stimulating Complex Technology

15.2.1 Quillaja saponin Adjuvants

The potent adjuvant effects of saponins have been known for decades [4] and saponins are used as adjuvant in several animal vaccines. In the literature, a whole range of saponins from various sources, not limited to plants, have demonstrated immune stimulatory properties [5]. However, the most potent and useful saponins are still those isolated from the plant *Quillaja saponaria* Molina. The core adjuvant activities exhibited by *Q. saponin* are characterized as enhancement of a combination

of antibody and cellular immune responses [6]. Despite the very good adjuvant profile, saponin-based adjuvants took long time to enter into human clinical developments and so far no human vaccine contaning a saponin adjuvant is registered. The major objections to saponin adjuvants for human vaccines have been associated with the lytic properties of saponins, local pain at the injection site and a general molecular instability leading to degradation at physiological pH at ambient temperatures [7]. However, these properties are strongly influenced by the formulation of the saponin adjuvant and must not be regarded as typical for saponin containing adjuvant formulations in general.

15.2.2 The Immune-Stimulating Complex

The first immune-stimulating complex (ISCOM) was created in the early 1980s [8]. They were conceived by a combination of rational scientific experiences and the spur of luck. In his struggle for making efficient subunit vaccines, Morein concluded that subunit vaccines were not satisfactory immunogenic to perform as vaccines without some additional potentiation. He tried multimeric particulate formations of antigens to increase immunogenicity but was not satisfied with the results, additional immune stimulation was required [9].

His choice of adjuvant substance became *Q. saponin*, or Quil A, since these molecules like extracted purified viral membrane protein are amphipathic and form micelles in aqueous solution. A combined micelle, a multimeric particle consisting of antigens and adjuvant was the goal. The resulting product did not have the physical characteristics expected from a protein micelle, the sedimentation coefficient was lower and a very special regular appearance was visualized by negative-staining electron microscopy [8, 10]. Due to its potent immunogenicity, the product was called ISCOM. ISCOMs were in the following years demonstrated superior to most other competing adjuvant formulations at the time [11–14]. Antigens formulated into ISCOMs were not only inducing high and long-lasting antibody responses, the antibodies were also biologically more active. Along with the antibody responses, strong cellular responses including CTL were also demonstrated. The induction of Class-I restricted CTL was indicated already in 1988 [15] and was proven some years later with gp120 of HIV virus and influenza [16]. The amount of antigen required for the potent immune stimulation with ISCOMs was surprisingly low; hence, dose-sparing effects were demonstrated long before the threats for pandemic influenza infections demanded such features of potential adjuvants [17, 18].

ISCOM-technology has become a collective phrase encompassing “classical” ISCOMs, the 40 nm complex composed of *Q. saponin*, cholesterol and phospholipids with antigens physically incorporated into the structure, as well as other particulate saponin-based developments such as the ISCOM–Matrix which does not contain the antigen physically integrated into the complex. Not only the terminology this area is unclear, the physical characterization of ISCOM formulations is difficult or and preparations in literature are often incompletely described and numerous

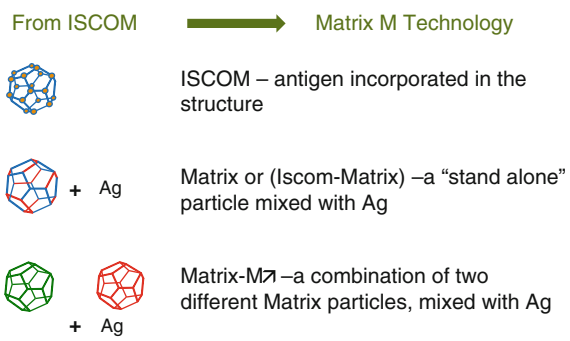

Fig. 15.1 A schematic overview of the different ISCOM technology formulations. On *top*, the classical ISCOM structure formed by *Quillaja saponins* and lipids with antigens physically incorporated into the structure. The Matrix structure (*middle*) formed by semipurified or fractionated *Q. saponins*. The Matrix (or ISCOM–Matrix) is used "stand-alone" simply mixed with antigen(s). No physical incorporation of the antigen(s). The Matrix M formulation (*bottom*), consisting of two different Matrix structures each produced from a different fraction of purified saponin

preparations denoted ISCOMs in literature are most likely not ISCOMs but to a great extent mixtures of ISCOM–Matrix and antigen(s) in solution. A schematic overview of the ISCOM technology area is shown in Fig. 15.1. Ongoing Mode-of-Action studies will most likely reveal the essential differences in terms of adjuvanticity between ISCOM and Matrix adjuvant formulation. One thing is clear though, the "classical" ISCOM formulation provides also with a particulate formulation of the incorporated antigens that is not provided by ISCOM–Matrix simply mixed with antigens [19, 20].

15.2.3 The ISCOM–Matrix

As it became clear that ISCOM particles were built up by a strong and specific interaction between the saponins and cholesterol and that amphipathic antigens were only co-incorporated during their formation [21], it also became clear that so-called empty ISCOMs [22, 23], "ISCOM–Matrix" [21] or simply Matrix, could be produced without protein antigen(s). Matrix particles without incorporated antigens were surprisingly excellent adjuvants mixed with protein antigens. It took some time to accept the fact that Matrix in simple mixture with antigen(s) could constitute a potent adjuvant, often as good as the ISCOM since the core adjuvant effects of the ISCOM were thought to rely on the co-presentation of adjuvant and antigen in a multimeric particle with efficient targeting and antigen delivery to antigen presenting cells as a main feature. From this view, the potent adjuvant activity of Matrix is puzzling. Apparently the ISCOM and Matrix formulations share other crucial immune stimulatory activities that are not primarily dependent on a physical interaction with the antigen(s).

The formulation of saponins into Matrix particles is superior to the use of saponins freely in solution. The complex formation with cholesterol neutralizes the inherent lytic properties of saponins and also provides improved chemical stability to the saponin molecules. The nano-particulate nature of Matrix-formulated saponin may also contribute to the positive adjuvant activity in a similar way as other nano-particulate matters has general immunostimulating effects [24, 25].

Matrix adjuvant formulations are immunologically potent. They induce antibody responses and cellular responses constituting the basis for protective immunity to [26].

The adjuvant activities of Matrix formulations roughly parallels those of ISCOMs, inducing high and long-lasting levels of broadly reacting antibodies supported by a balanced Th1/Th2 response including multifunctional T cells [27–29] and CTL [12, 16]. The adjuvant properties of Matrix fulfill the demands of a modern adjuvant, a formulation with dose-sparing capacity, improved quality of immune responses, and an acceptable safety profile.

In contrast to most adjuvant formulations, ISCOMs and Matrix adjuvant do not exert adjuvant activity through depot or slow-release mechanism. On the contrary, adjuvant and antigen is rapidly removed from the site of injection to be found in blood, draining and distant lymph nodes, spleen and liver [30].

Matrix formulations perform well with most types of antigen, with the exception of low molecular weight hydrophilic antigens. Such antigens probably require some "physical" help in terms of size and/or multimericity and are best adjuvanted as "classical" ISCOMs.

The formulation of vaccines with Matrix adjuvant is convenient as the vaccine antigen(s) is simply mixed with a suspension of Matrix particles in physiological buffers not requiring adsorption or emulsification.

Due to practical reasons, the Matrix formulation became the preferred ISCOM-technology derived adjuvant for human and animal vaccine developments. A major problem with the classical ISCOM technology is reproducible antigen incorporation. To control and assay for incorporation rate and final composition is laborious and costly whereas Matrix can be produced in large batches as a bulk substance for subsequent mixing with antigen.

However, with native membrane derived antigens the classical ISCOM technology works very well also at industrial scale. The Equine influenza vaccine Equip [31, 32] an ISCOM formulation was launched more than 10 years ago, a competing product Equilis Prequenza [33, 34], which has a Matrix adjuvant was launched 5 years ago. Both products are regarded as efficient vaccines.

15.2.4 Saponins for Use with ISCOM and Matrix Technology

The first type of saponin that was used with the classical ISCOM technology was semi-purified preparations of saponins from *Q. saponaria* Molina like Quil-A (from Superfos, Denmark now Brenntag Biosector, Denmark). Similar preparations from other suppliers were also used. However, there are variations between batches and

suppliers in the concentration and functionality of saponins for use as ISCOM or Matrix adjuvant. Crude saponin extracts contains more than 20 different but related saponin compounds and a variety of other substances extracted from the tree, such as phenols, tannins, and waxes [35]. *Q. saponins* are triterpene glycosides and though structurally related, they exhibit different biological effects. Most of the components have some degree immune stimulatory activity, some are very active. The saponin components also differ in terms of reactogenicity and in physical properties such as hemolytic effect.

Preparative and analytical RP-HPLC is used to obtain and characterize purified fractions of saponins. The major saponin constituents have been denoted QS-7, QS-18, QS18, and QS-21[36]. QS-7 (also called Fraction A) is a nontoxic saponin with low hemolytic activity and low to moderate adjuvant activity. QS-17 and QS-18 contain the most toxic and hemolytic saponins, they are potent adjuvants but are rarely used due to their toxicity. QS-21 (also known as Fraction C) has a much better safety profile and a potent adjuvant activity and is the most used saponin species. Applications of QS21 in free forms and in various mixtures (e.g., AS01) are pursued by GSK, whereas CSL (ISCOMATRIX) and Isconova (Matrix C) use Matrix formulations of Fraction C type of material in vaccine development.

15.3 The Matrix M Formulation

15.3.1 Improvements by Formulation: Retained Activity and Increased Safety

The development of Matrix adjuvant technology started with purification and characterization of saponin fractions.

Among the purified saponins that were most extensively evaluated for Matrix formulation were *Q. saponin* Fraction A and Fraction C. The Fraction A was characterized as Matrix structure forming but of weak adjuvant activity. Similar differences were noted also when the two fractions were used in "classical" ISCOMs [37]. Comparatively higher doses were required to obtain adjuvant activity also when compared to semi-purified saponin or Fraction C saponin. Considering the differences noted when comparing the two fractions (see Table 15.1), it was hypothesized that a different approach on formulation would increase the possibility of fine-tuning the adjuvant activity. The usual way of formulation was to blend purified saponin preparation together and then formulate the mixture into Matrix particles. Instead, it was decided to make individual Matrix preparations from the purified fractions and mix the readymade Matrix particles. It was anticipated that such a procedure would let the individual Matrix particles act independent to each other and thereby possibly exert different adjuvant properties. The most striking effects noted in the initial experiments were, however, not related to immune modulation but to acute toxicity (see Table 15.2). Further studies in laboratory animals confirmed

Table 15.1 A comparison of acute toxicity at high dose, between the traditional Matrix concept containing Fractions A and C saponins together in the Matrix structure and the Matrix M concept with different saponin fractions in separate Matrix particle

Dose	Composition (Fractions A and C, % by weight)	Formulation	Lethality
50 μg	80% + 20%	Separate	2/8
50 μg	90% + 10%		0/8
50 μg	95% + 5%		0/8
50 μg	80% + 20%	Together	8/8
50 μg	90%+ 10%		6/8
50 μg	95% + 5%		5/8

The two different formulations are given at identical dose and composition, the difference is how they are formulated

Table 15.2 Some major differences in adjuvant activity and biological properties of Fractions A and C saponins purified from a crude extract of *Quillaja saponaria* Molina

	Fraction A	Fraction C
Antibody[a]	+ IgG1, weak IgG2a	+++ IgG1 and IgG2a
T cell	+++ IL-2, IFN-γ	+++ IL-2, IFN-γ
Toxicity	None	Some
Hemolysis	– (+)	++ (+)

[a]In mice

the initial findings. Formulations based on a mixture of separately formed Matrix particles made from different saponin fractions are dramatically less reactogenic in mice than the corresponding formulation with mixtures of saponin in Matrix particles. Adjuvant activity is retained or slightly enhanced.

A combination of Matrix particles, produced from Fraction A and Fraction C material, called Matrix-M, with each saponin type in separate particles, was developed by Isconova [38]. A graphic illustration of the Matrix-M formulation is shown in Fig. 15.1. Preclinical studies have demonstrated potent adjuvant activities along with a significantly enhanced safety profile of Matrix-M compared with earlier formulations. Matrix-M has just entered into human clinical trials.

A comparison of reactogenicity of Matrix-M formulated saponin compared with Matrix-Made from a mixture of Fraction A and Faction C in mice is shown in Table 15.2. A specific comparison on the reactogenicity of Matrix-M and Matrix C is shown in Fig. 15.2.

The Matrix-M adjuvant is dosed in micrograms blended from stock solutions of Matrix A and Matrix C with vaccine antigens at vaccine production. Since Matrix-M is an additive and not part of the vehicle or vaccine excipient, the optimum dose and composition for specific vaccine antigen(s) is readily adjusted during development. Typically Matrix-M composition range within 5–15% Matrix C and 85–95% Matrix A.

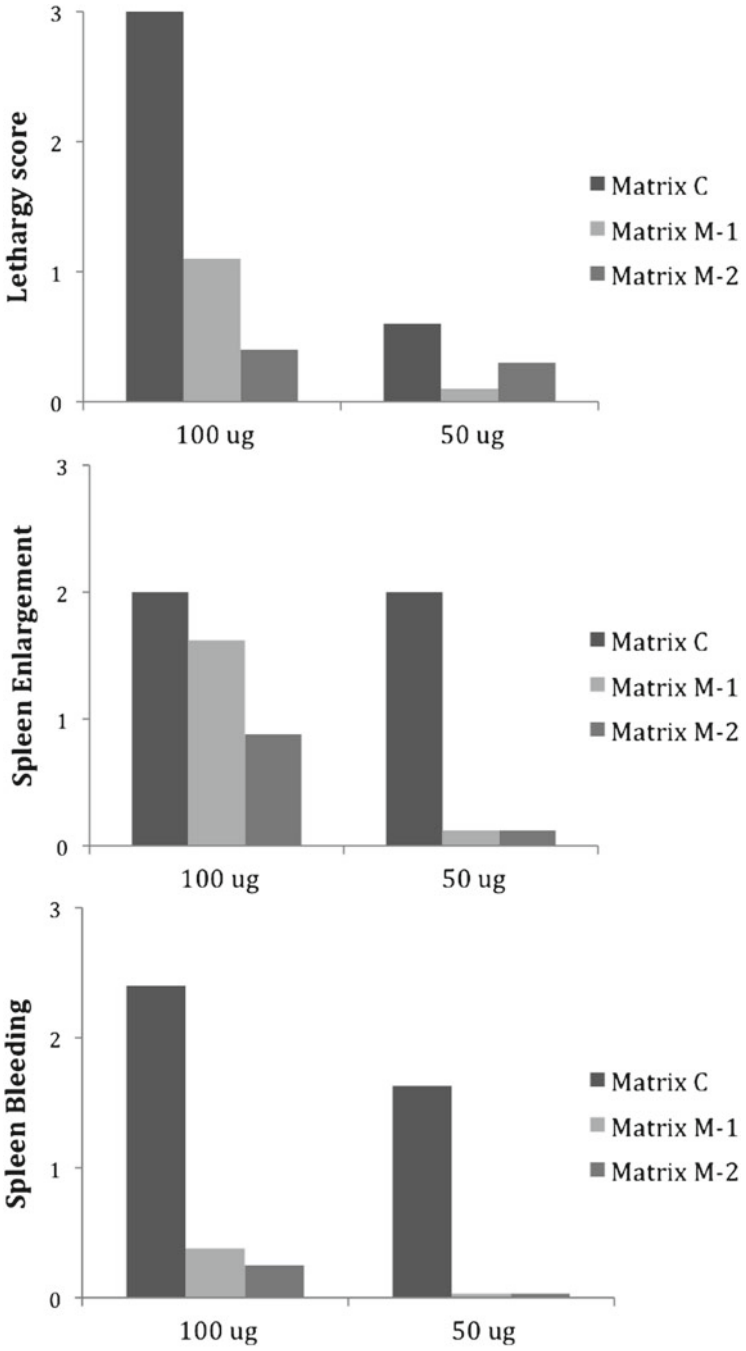

Fig. 15.2 Demonstration of the reduced reactogenicity of Matrix-M formulations compared to Matrix C. A total amount (saponin content) of 100 or 50 μg of each Matrix was administered subcutaneously to Balb/C mice. Lethargy score (cumulative days 0–4), spleen enlargement, and spleen bleeding at autopsy day four are shown in the figure. Matrix M-1 contains 15% Matrix C and Matrix M-2 contains 10% Matrix C

In the pharmaceutical industry it is well recognized that a new formulation of the same substance is a new drug as the effect and performance might be significantly influenced. Likewise, new formulations of *Q. saponin* resulted in altered and remarkably improved adjuvant properties.

The concept of the Matrix-M, i.e., a combination of Matrix A mixed with a stronger immune modulator has been verified also with non-saponin adjuvants such as cholera toxin and MPL. In analogy with Matrix C, the amount of the stronger immune modulator can be substantially reduced while obtaining conserved or even enhanced immune potentiation. Similarly, AbISCO-100 (Isconova AB, a mixture of Matrix A and Matrix C for use in mice) has been used to potentiate the adjuvant properties of Toll-like receptor agonists [39–41].

15.3.2 Clinical Developments with Matrix M

The improved safety profile of Matrix-M over other saponin adjuvant preparations noted in laboratory animals encouraged to expansions into human vaccine adjuvant applications. Matrix-M adjuvant has been applied in two human Phase-I studies. The first study with a pandemic influenza vaccine (H5N1) was performed within the PANFLUVAC consortium (www.panfluvac.org). Virosomal HA (30, 7.5 or 1.5 μg) was mixed with 50 μg Matrix-M. A control group received 30 μg of virosomal HA alone. Adults were given two intramuscular administrations at days 0 and 21. The serum antibody response was evaluated by three serological assays; single radial hemolysis (SRH), microneutralization (MN) assay, and hemagglutination inhibition assay (HI). The vaccine was well tolerated in all groups but mild local pain was more frequent in the Matrix-M adjuvanted groups.

In this study [28], a good safety record along with substantial increase of both homologous and heterologous antibody responses was demonstrated for Matrix-M. A dose-sparing potential down to 1.5 μg/dose was recorded. It seems that the potent adjuvant activity and safety, recorded of Matrix-M in murine models, are valid also in humans [27, 28].

In another presently ongoing study (Jan 2012), performed by Isconova, Matrix-M is tested for efficacy in a seasonal influenza vaccine for elderly, 65–75 years old. (http://clinicaltrials.gov/ct2/show/NCT01444482). This study is still blinded and under evaluation. Primary endpoint for this exploratory study is safety; secondary endpoints are immunological assessments of B- and T-cell response enhancement in the elderly.

15.4 Concluding Remarks

Adjuvant development is driven by the need to improve immune responses to vaccine antigens without increasing side effects beyond the acceptable. Adjuvant use in human vaccines has been limited and cautious and until the last decade, only

aluminum salt adjuvants were approved. In animal vaccines however, several adjuvants and antigen presentation systems have been used, including both ISCOMs and Matrix formulations.

It is well appreciated that Matrix-M exhibit all properties of a modern, safe, and effective adjuvant. It induces well-balanced and potent cellular and humoral immune responses. A human vaccine dose will probably not contain more than maximum a total of 100–200 μg of material, antigen and adjuvant combined. In contrast, conventional adjuvant formulations usually contain tens of milligrams of these materials combined.

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Section V
Safety Assessment of Next Generation Vaccines

Chapter 16
Strategies for the Nonclinical Safety Assessment of Vaccines

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16.1 Introduction

Over the past century, vaccines have made a large impact on public health. Prophylactic vaccines prevent disability and disease, saving millions of dollars in potential health-care spending. Since prophylactic vaccines are administered to healthy individuals, including infants and children, it is important to demonstrate the safety of vaccines preclinically prior to testing the vaccine in clinical studies. A benefit-to-risk profile is considered for each individual vaccine and depends on many factors including preclinical and clinical toxicities that are observed, frequency of administration and intended target population. For prophylactic vaccines, in particular, the concerns about potential risks often outweigh the perception of benefit [1]. Therefore, over the past decade, there has been an increased focus on nonclinical safety assessment of vaccines, including toxicity testing.

Traditional vaccines have focused on prevention of infectious diseases by eliciting humoral immune responses, and are typically composed of whole, inactivated, or attenuated microorganisms (bacteria or viruses) that have lost their disease-producing properties [2]. Next generation vaccines are being designed not only for prevention of infectious diseases but also for treating chronic diseases such as hepatitis C or cancer. Next generation vaccines aim to induce strong humoral and cell-mediated immune responses and include both prophylactic and therapeutic vaccines. Next generation vaccines are often produced synthetically or purified from pathogens, and include antigens (proteins, peptides or carbohydrates) capable of inducing humoral and cellular immune responses. These new epitopes are often weak immunogens; therefore, they need to be presented in multimeric form, conjugated, or formulated with immune potentiators such as adjuvants in order to elicit a stronger immune

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response [2]. Next generation vaccines include virus-like particles (VLPs) that are noninfectious but are immunogenic, or can act as carriers to linked peptide-antigens. Next generation vaccines also include DNA vaccines which transfect cells in order to express the antigen of interest, and are delivered either in a plasmid form or vectored using an intact bacteria or virus.

Safety concerns for traditional and next generation vaccines include the potential to induce local and systemic reactions. Local reactions are often observed with vaccines administered via the intramuscular or subcutaneous routes of administration, and there are concerns about the severity of pain, redness, swelling, in addition to formation of granulomas and abcesses at the injection site, necrosis and regional lymphadenopathy [3]. Vaccines might induce systemic reactions, including nausea, diarrhea and general malaise. Potentially severe responses might include anaphylaxis, pyrogenic fever responses, organ specific toxicity, or immune-mediated toxicities (such as cytokine release, immune activation or suppression, and autoimmune diseases). Other potential concerns include effects on reproduction and development, and carcinogenicity. For live or attenuated pathogen-based vaccines, there is a risk of reversion to virulence in addition to concerns regarding administration of the vaccine to subjects who have an impaired immune system. For next generation vaccines which include adjuvants, there are potential synergies and interactions between the mechanisms of action for vaccine antigens and adjuvants. Adjuvants typically act by enhancing the immune response, and might cause excessive amounts of pro-inflammatory and pyrogenic mediators leading to an exacerbation of both local and systemic effects [3]. Next generation vaccines that are DNA-based or vectored have specific risks of recombination and integration into the host genome. Therefore, the biodistribution, integration, and persistence of the DNA or vector are important evaluations for DNA-based and vectored vaccines.

Prior to starting clinical studies with next generation vaccines, adequate information about the pharmacological and toxicological effects of the vaccine should be available [1]. This includes in vitro and in vivo studies to examine the mechanism of action and potential efficacy of the vaccine, in addition to a thorough evaluation of the safety of the vaccine. This chapter will focus on the nonclinical safety assessment of vaccines, and will include a discussion of the toxicology studies that need to be performed for new vaccines in clinical development and quality control tests that are needed to demonstrate that the vaccine product is safe for use in humans.

16.2 Overview of Toxicology Studies for Vaccines

Nonclinical testing of traditional vaccines was focused mainly on efficacy studies in animals and “safety pass” of vaccine formulations. Over time, the extent of nonclinical safety testing has been greatly increased and a requirement for full toxicology studies of vaccine candidates have been implemented according to current guidelines (Table 16.1). Presently, nonclinical safety studies with vaccine candidates, including the next generation vaccines, are aligned with overall principles of

Table 16.1 Regulatory guidelines

Vaccine type	Regulatory agency	Guideline
All vaccines	World Health Organization (WHO)	WHO guidelines on nonclinical evaluation of vaccines [4]
	European Medicines Agency (EMA)	Note for guidance on preclinical pharmacological and toxicological testing of vaccines [5]
Prophylactic vaccines	Ministry of Health, Labour and Welfare, Japan	Guideline for nonclinical studies of vaccines for preventing infectious diseases [6]
	State Food and Drug Administration, China	Technical guidelines for preclinical research on preventive vaccines [7]
DNA vaccines	Food and Drug Administration (FDA), United States of America	Guidance for industry: considerations for plasmid DNA vaccines for infectious disease indications [8]
	WHO	Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines [9]
Viral vector and cell-based vaccines	FDA	Guidance for industry: guidance for human somatic cell therapy and gene therapy [10]
	EMA	Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [11]
		Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products [12]
		Points to consider on the manufacture and quality control of human somatic cell therapy medicinal products [13]
Recombinant protein/peptide vaccines	FDA	Points to consider in the production and testing of new drugs and biologicals produced by recombinant DNA technology [14]
	International Conference on Harmonization (ICH)	Preclinical safety evaluation of biotechnology-derived pharmaceuticals [15]. Note: this guideline is more applicable for biological drugs, not vaccines
Adjuvants	EMA	Guideline on adjuvants in vaccines for human use [16]
Combination vaccines	EMA	Note for guidance on pharmaceutical and biological aspects of combined vaccines [17]

(continued)

Table 16.1 (continued)

Vaccine type	Regulatory agency	Guideline
Vaccines for women of childbearing potential	FDA	Guidance for industry: considerations for developmental toxicity studies for preventative and therapeutic vaccines for infectious disease indications [18]
Therapeutic cancer vaccines	FDA	Draft guidance for industry: clinical considerations for therapeutic cancer vaccines [19]
Cell substrates for viral vaccines	FDA	Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications [20]
	WHO	Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks [21]
	ICH	Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin [22]

Table 16.2 Types of toxicology studies

Study type	Purpose	Comment
Single-dose toxicity	To determine the acute effects after vaccination by examining general parameters (mortality, clinical signs, body weight, food consumption)	These acute evaluations are often incorporated within repeat-dose toxicity studies, and separate single-dose toxicity studies do not need to be performed
Repeat-dose toxicity	To determine the effects of repeated administration of the vaccine in animals	This is typically the pivotal toxicology study that is performed prior to clinical trials
Local tolerance	To determine the potential irritation at the injection site	To reduce animal use, a local tolerance evaluation can be incorporated within the repeat-dose toxicity study
Safety pharmacology	To evaluate the potential for undesirable effects on the cardiovascular, respiratory, and central nervous systems	Separate safety pharmacology studies are generally not performed for vaccines [1], and endpoints are incorporated in the repeat-dose toxicity study instead
Developmental and reproductive toxicity studies	To examine potential effects on fertility, fetal development, and postnatal development of the offspring	Required for vaccines that will be indicated for women of childbearing potential [18]
Biodistribution studies	To examine tissue distribution following administration	Performed for nucleic acid and viral vector-based vaccines

toxicology evaluation, that is, the detection of their potential for local and systemic toxicity. At the same time the guidelines allow for appropriate flexibility in study designs according to the type of the vaccine candidate, the human population to be treated, and the dosing regimen to be applied in the clinical use.

The purpose of the nonclinical toxicology evaluation is to examine the toxicity of all the components present in the vaccine formulation in addition to the toxicity of the induced immune response. Toxicology studies provide information that might help to determine a safe starting dose in the clinical study and identify any potential toxicities or target organs [23]. It should be noted that there are some limitations of safety evaluation in animals, since effects in animals are not always indicative of the effect that might be seen in humans, and rare toxicities that appear in certain subpopulations are only detected in clinical studies. Nevertheless, toxicology studies provide important safety data for vaccine development.

The toxicology program for each vaccine varies depending on the type of vaccine and intended use in humans. An overview of the main toxicology study types is provided in Table 16.2. In general, all vaccines need to be evaluated in a repeat-dose toxicology study prior to the start of Phase 1 clinical studies. Developmental and reproductive toxicology studies are needed for vaccines that will be administered to women of childbearing potential and are performed in parallel with Phase 3 clinical studies [23]. Biodistribution studies are needed for DNA-based and viral-vectored vaccines.

Toxicology studies are performed in animals and need to be performed in compliance with national and international laws for the protection of laboratory animals. Toxicology study protocols are reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Toxicology studies are usually conducted in compliance with Good Laboratory Practices (GLP) [24]. The vaccine lots used in GLP studies should be from lots that are manufactured with a similar production process, formulation, and release specifications as the lots intended for clinical use. Stability data are needed, supporting the use of the vaccine for the duration of the toxicology study.

16.2.1 Repeat-Dose Toxicity Studies

Repeat-dose toxicity studies are generally needed for all vaccine types. A single species is typically used for the evaluation, which must be shown to be a relevant species based on the immunogenicity or efficacy of the vaccine in the selected species. In many cases, rodents or rabbits are used for the toxicology evaluation. Nonhuman primates are only used if no other relevant species exist. Disease models are typically not used for toxicology studies, but supplementary studies in disease models could be used to address specific toxicology concerns. For example, a transgenic mouse model of Alzheimer's disease, which over-expresses the human β-amyloid protein, could be used to demonstrate that Alzheimer's disease vaccine candidates do not cause meningoencephalitis or microhemorrhage in the brain [25].

In repeat-dose toxicity studies, the same route of administration as the clinical route is used in animals; however, a more intensive dosing regimen is applied in animals when compared with the planned regimen for humans. This "overdosing" approach based on the number of doses administered, i.e., one more dose is administered in animals when compared with the number of doses administered to humans, and greater dosing frequency, i.e., every 2–3 weeks in animals compared to typically every few months in humans, is driven by the intent to maximize potential hazard identification in nonclinical safety studies [1]. In addition, the full-human dose of the vaccine or the maximum amount that can be injected into the selected animal species also results in much higher exposure to the vaccine in animals based on their smaller body weight compared to humans. Importantly, the vaccine formulation used in toxicology studies should be representative of the proposed clinical formulation. Therefore, for adjuvanted vaccines, the vaccine antigen(s) and adjuvant are tested together based on the evidence that immune response to adjuvanted vaccine can only be evaluated within the confines of immunogenicity of the vaccine antigens [26].

Control groups that are included in the repeat-dose toxicity study include adjuvant alone, if applicable, and a saline-treated group. The group size varies depending on the animal species used, but for rodents, 10 per gender per group are usually included for each necropsy. For non-rodents the number per group is typically 3–5 per gender per group for each necropsy. Antemortem parameters evaluated include

daily clinical observations, weekly body weights, food consumption, and physical examinations. An assessment of local reactogenicity is performed after each vaccine dose is administered. Specific safety pharmacology evaluations (e.g., body temperature, electrocardiogram, and central nervous system evaluations) could be incorporated within the repeat-dose toxicity study [27]. For vaccines that are administered intramuscularly, particular attention is focused on redness and swelling at the injection site and impairment of limb use after the injection. Clinical chemistry (urinalyses, hematology, serum biochemistry, coagulation) evaluations are typically performed a few days after the first vaccination and at the scheduled necropsies. Immunogenicity assessments are performed at the end of the study (as described in Sect. 3.1). Ophthalmic examinations are included after the first vaccination and prior to the first necropsy. Necropsies are performed on two occasions: (1) 1–3 days after the last dose is administered and (2) after a treatment-free period of 2–4 weeks (to determine whether any effects detected at the first necropsy have started to recover with time). Postmortem evaluations include gross examination of all major organs, organ weights for selected organs, and histopathology evaluation of a standard list of tissues [4].

Treatment-related effects that are typically observed in repeat-dose studies with vaccines administered parenterally, include inflammation at the injection site, hyperplasia of the draining lymph nodes, increases in spleen weight and clinical chemistry changes that are indicative of an inflammatory response. These are typically not severe and are transient changes, and they are therefore not considered to be an adverse effect.

16.2.2 Considerations for Prophylactic and Therapeutic Vaccines

The general approach to the toxicology evaluation for therapeutic and prophylactic vaccines is very similar; however, there are a few small differences in the repeat-dose toxicity study designs. For example, for a therapeutic vaccine, the interval between dose administrations in animals would follow the clinical study design very closely, including the total number of doses and dosing intervals; whereas for a prophylactic vaccine, the dosing interval could be condensed in the animals (e.g., clinical dosing frequency of once-every-3-months could be condensed to once-every-3-weeks in the toxicology study) and one more dose is administered in animals when compared with the number of doses in the clinical regimen. There is a perception that there is a potential difference in the tolerance for adverse effects for therapeutic vaccines when compared with prophylactic vaccines, since therapeutic vaccines address life-threatening conditions for which there might be no other treatment options. However, the benefit-to-risk ratio needs to be carefully evaluated depending on the target population. It should be noted that certain target populations for therapeutic vaccines might be immunosuppressed due to other concomitant medications. Therefore, caution is needed when evaluating the benefit-to-risk ratio for both therapeutic and prophylactic vaccines.

16.2.3 Considerations for Inclusion of Adjuvants in Vaccine Formulations

Novel adjuvants are being incorporated in next generation vaccine formulations in order to reduce the amount of vaccine antigen and increase both the magnitude and duration of the immune response, thereby reducing the frequency of booster immunizations needed. Adjuvants can be used to modify a desired immune response and activate both the innate and adaptive arms of the immune system [2]. Novel adjuvants that are being tested in clinical trials currently include mineral salts (e.g., aluminum hydroxide), oil emulsions (e.g., MF59), particulate adjuvants (e.g., virosomes and ISCOMS), microbial derivatives (e.g., monophosphorylated lipid A), and endogenous immunomodulators (e.g., human GM-CSF). Some adjuvants that were developed in the past (e.g., Freund's adjuvant) were not found to be acceptable for large scale vaccination campaigns due to safety concerns, which included severe local reactions, acute toxicity, and delayed hypersensitivity. EMA's Committee for Medicinal Products issued a guideline on adjuvants in vaccines for human use, which covers the nonclinical and clinical aspects for consideration [16].

The safety profile of an adjuvant alone would be typically impacted (positively or negatively) by its interactions with vaccine antigen(s) and needs to be evaluated in the context of the full vaccine formulation. A theoretical concern about increased toxicities due to synergy between adjuvant-induced responses and vaccine-induced responses has been raised by regulatory authorities. It is proposed that toxicological characterization of chemical-based adjuvants in a manner similar to all new chemical entities (NCEs) is desired in order to understand their unique toxicity profiles [6, 16]. If the adjuvant is not species-specific, it is tested in two species (one rodent and one non-rodent). If the adjuvant is species-specific, then testing in one species might be justified.

New adjuvants are typically assessed for local tolerance and systemic toxicity in a repeat-dose toxicity study. The repeat-dose toxicity study design could reflect the proposed clinical use of the vaccine, and the number of administrations in animals should be higher than the number planned for humans [16]. In general, dose ranging toxicology studies do not need to be performed on the adjuvant alone. The doses tested would reflect the targeted clinical use, which is typically much lower than the maximum tolerated dose. The purpose of the toxicology studies with adjuvant alone is to establish a margin of safety rather than a maximum tolerated dose. Full necropsy and histopathology are included in the repeat-dose toxicity study. Similar to other NCEs, an assessment of genotoxicity potential of novel chemical adjuvants is also recommended using the standard battery of tests (e.g., potential for gene mutation, chromosome aberrations, and primary DNA damage) [28]. Carcinogenicity studies are not required for adjuvants, since they are only used a few times at low doses. An evaluation of the adjuvant's effect on reproductive toxicity is needed for inclusion of the adjuvant in vaccines that will be administered to women of childbearing potential or during pregnancy.

Although the value of studies with an adjuvant alone for risk assessment of a vaccine as a whole is still being debated, in light of these guidelines, it is anticipated that the rigorous and comprehensive toxicology programs for novel adjuvants alone will be required in the future. Perhaps, a practical approach to compliance with these expectations by developers of vaccines containing novel adjuvants could include a generation of a "Master File" for a given adjuvant. With this approach, nonclinical safety studies with an adjuvant alone would be included in the Master File and potentially repetitive safety studies of that adjuvant alone could be omitted.

16.2.4 Considerations for New Approaches to Administration of Vaccines

Different delivery systems are being used to incorporate immunopotentiators and focus the immune response through a desired path. Several types of delivery devices for vaccines are also being evaluated in order to more efficiently target the vaccine to a specific area in the body and reduce the pain associated with needle-based injections.

Delivery systems include emulsions (e.g., MF59) and microparticles (e.g., liposomes and biodegradable polyesters), which might have immunostimulatory capabilities, by themselves [2]. This type of delivery system encapsulates and protects the antigen from degradation, and acts as a vehicle that mimics the structure of natural lipid bilayer membranes, allowing them to enter into the reticulo-endothelial system by endocytosis. Delivery systems could also stabilize the antigen and result in formulations that are thermostable. Delivery systems that are present in vaccine formulations need to be included in the formulation that is used toxicology study in animals. A group of animals that are dosed with the delivery system (e.g., lipsome or emulsion), by itself, could be included in the toxicology studies to compare the effects of the delivery system by itself or in combination with vaccine antigens.

Delivery devices are used to target the vaccine antigens to the proper location in the body and include less painful ways to deliver vaccine antigens parenterally, such as microneedle patches and autoinjectors [29]. Oral and intranasal vaccines are also being developed, in order to have a less invasive method of administering vaccines. Toxicology studies of vaccines that are intended for delivery in the clinic using a specific device should include the use of the clinical delivery device in the animal study [23]. This is particularly important for new types of injection devices, since local irritation is a concern. If the device has already been cleared for use in humans, then a cross-reference to the Investigational Device Exemption or Master File could be listed in the Investigational New Drug application for the vaccine. The manufacturer of the device usually performs biocompatibility testing to evaluate the interaction between the device and tissues. Biocompatibility studies utilize analytical chemistry, in vitro tests, and animal models [30, 31]. Specific types of tests that might be performed on the device, by itself, include cytotoxicity in tissue culture, sensitization assays, irritation tests, acute and systemic toxicity tests, intracutaneous tests, implantation tests, and hemocompatibility tests.

For DNA-based vaccines, electroporation has been used to deliver the DNA into cells. Toxicity studies examining the specific method of electroporation are needed. Electropermeabilization may leave the target tissue damaged depending upon the electrical parameters associated with the electroporation [32]. For the technique to be clinically acceptable for use in gene/DNA delivery, there should be no permanent damage to the skin [33]. Electroporation devices that are intended for administration might need to be adjusted for use in animals; for example, the needle length of the injection array could be different in animal and human studies.

16.2.5 Considerations for Safety Assessment of Excipients, Residuals, and Contaminants

Excipients, such as buffer components and preservatives, are added to vaccine formulations to improve the stability of vaccine components. When selecting excipients for inclusion in vaccine formulations, it is preferable to use excipients where toxicology data are available and that have been previously used in other marketed vaccines and products, for which clinical safety has been already demonstrated. Such excipients should not be regarded as being "novel," and a scientific review of the available toxicology data would provide sufficient toxicology evaluation for the excipient. For novel excipients, toxicology studies are required. A study of the excipient within the repeat-dose toxicity studies for the vaccine is more relevant with respect to the interaction of the excipient with other vaccine formulation components.

Residuals and contaminants are substances that are used in the manufacturing process and may be present in the final formulation in residual amounts (e.g., formaldehyde, toxins, viral growth media). No specific regulatory guidelines are available for the safety assessment of vaccine residuals or contaminants. A determination or estimation of the "worst case" mass of the residuals or contaminant per vaccine dose could be made or measured directly if assays exist. ICH guidelines on impurities (ICH Q3A/B) do not cover biological or biotechnological products; however, the general principles of the guidelines could be applied [34, 35]. For example, ICH Q3B states that for drugs that are administered <1 g per day, the maximum reporting threshold for impurities or degradates should be 0.1% [35]. ICH Q3C on residual solvents discusses an approach for establishing permitted daily exposure (PDE) limits [36]. Both these concepts might be considered applicable to residuals and contaminants in vaccines.

The threshold of toxicological concern (TTC) concept that was developed for risk assessment of human exposure to even the most harmful of chemicals could be applied for excipients and residuals in vaccines. The goal of the establishment and application of acceptable TTC values was to avoid unnecessary toxicity testing and safety evaluations when human intake was below a threshold amount that would be safe even for harmful chemicals. In developing the TTC concept, an Expert Group under the International Life Sciences Institute (ILSI) considered a wide range of toxicological concerns including metabolism and accumulation, structural alerts,

endocrine disrupting chemicals, genetic toxicity, carcinogenicity, neurotoxicity, teratogenicity, developmental toxicity, allergenicity, and immunotoxicity [37]. This published work has been adopted by the European Medicines Agency for establishing a guideline on the limits of genotoxic impurities in pharmaceutical development [38]. Although the impurity guideline addresses TTC levels for potent genotoxic carcinogens and was not intended to be applied for residuals and contaminants, it could be an approach that is taken to address the levels of residuals and contaminants in vaccines.

16.3 Adequate Design of Toxicity Studies with Next Generation Vaccines

16.3.1 Approaches to Measurement of Immune Response in Toxicology Species

Animal species that are used in nonclinical safety studies with vaccine candidates should be able to mount an immune response, for example, antibody levels to the vaccine antigens. At the same time, toxicology studies are expected to be conducted in a laboratory animal species, for which historical control data exist to help distinguish true toxicity caused by the tested vaccine from potential background (not test article-treatment related) lesions occasionally found during a thorough histopathological examination of most organs and tissues involved in such studies. Rats are most commonly used in toxicology studies with a broad range of chemical entities and they are typically the species of choice for toxicity studies with vaccine candidates. However, rats are rarely used in nonclinical pharmacology studies that are focused on protective or therapeutic immune responses to a vaccine candidate. Instead, mice, rabbits and/or nonhuman primates (NHPs) are typical species in vaccine pharmacology studies based on available models of diseases and attempts to predict immune responses from animals to humans. In order to "bridge" the toxicology and pharmacology animal species, a measurement of immune responses in species selected for nonclinical safety evaluation (e.g., rats) to a vaccine candidate is included in a separate study or within a repeat-dose toxicity study [39]. This approach provides indirect evidence of the exposure and activity of the vaccine and is aligned with the general principle of all toxicology studies, in which the demonstration of the animal exposure to a test article following the administration of this test article in the course of a study is required. However, based on recent discussions and some regulatory guidelines [6], there are additional expectations (if not requirements) for toxicology studies of vaccines, that is, the animal species should be sensitive to the pathogenic organism or toxin targeted by the vaccine-induced immune response. Addressing this expectation in toxicology studies may be problematic when the "disease-sensitive species" are different from the "routine species" because the former are not well characterized to provide reliable data to

distinguish between "background" lesions relative to what may be considered a vaccine-related effect. Typically, a solution to this problem would involve the use of more than one species to evaluate safety of such vaccine candidate; for example, using a routine species in a well-controlled toxicity study (i.e., compliant with Good Laboratory Practice regulation) and a nonroutine species in an exploratory safety study, which is likely less comprehensive but with endpoints focused on pathogen-specific concerns [23]. In both types of studies, the immune response to the vaccine should be demonstrated.

The evaluation of the immune response to the vaccine relies on immunoassays that are developed in order to measure the most relevant endpoint, i.e. antibody response or cellular immune response. For the measure of specific antibodies, standard ELISA formats or multiplex assays for multiple antigens vaccine candidates are often applied [40]. When the candidate vaccine targets the cellular arm of the immune response, assays measuring cytokine-secreting antigen-specific T lymphocytes (such as γ-interferon ELISpot) can be utilized [41]. These assays are typically developed and performed to support nonclinical pharmacology studies (e.g., using mice, rabbits, or NHPs), and then are adopted for the use in toxicology selected species (e.g., rats).

16.3.2 Incorporation of Additional (Nonroutine) Endpoints in Toxicology Studies

Immune stimulation is an intended pharmacological effect of vaccines, and thus effects on various immune system parameters are expected and desirable. Such effects may include changes in hematology (various white blood cell types) and serum biochemistry (e.g., protein and globulin) parameters, local irritation and inflammation at the injection site, lymphoid enlargement and hyperplasia, and spleen weight increases [42]. These effects are generally modest and reversible, and, as consequences of the intended pharmacological activity of the vaccine, are usually not considered adverse.

Traditional vaccines containing aluminum salts as adjuvants have predominantly functioned via local rather than systemic mechanisms, and a systemic inflammatory response to these vaccines has generally not been a concern. However, as indicated above, the theoretical concern of synergistic immune stimulation seems to be heightened for novel, particularly "molecular" adjuvants such as Toll-like receptor (TLR) agonists [43] and cytokines [44, 45]. The safety concerns for the use of immunostimulatory adjuvants include potential excessive pro-inflammatory and pyrogenic responses (IL-6, TNFα, IL-8, IL-1β, PGE2); stronger or unexpected organ specific toxicity (local inflammation, cell death, immuno-dysregulation); severe local reactogenicity (increased vascular permeability, cellular infiltration, fluid accumulation); and break-down of self tolerance (dysregulation of T cells and other host cells). Therefore, the potential for a systemic inflammatory response for vaccines, particularly those containing

immuno-active adjuvants, is expected to be assessed within the nonclinical toxicology studies. While there are several examples of animal safety studies conducted with a novel adjuvant alone or included as a control group for the toxicology study with a vaccine containing that adjuvant, including oligonucleotides (e.g., CpG DNA sequences [46]), oil emulsions (e.g., MF59 [47]), and saponin-based (e.g., QS-21 [48]) adjuvants, they generally confirmed the expected dose-dependent effects based on mechanisms of action, but did not reveal any findings of toxicological concerns. In contrast, there are examples of unwanted immune responses in patients treated with experimental therapeutic vaccines in clinical trials. One example of adverse T cell-mediated toxicity induced by a therapeutic vaccine involved an amyloid-β vaccine AN1792, consisting of Aβ1-42 amyloid antigen and the QS-21 adjuvant, used in clinical studies for treatment of Alzheimer's disease [49]. Symptoms of meningoencephalitis that were observed in some trial patients were not predicted by nonclinical safety studies and the cause of the unwanted immune responses was difficult to establish. Based on some retrospective nonclinical studies using transgenic mouse models of the disease, it was suggested the inclusion of the QS-21 adjuvant in the AN1792 vaccine might have contributed to the adverse Th1 response, involving significant IFN-γ, IL-4, and TNF-α expression [50].

This example illustrates a great challenge we currently face in the development and safety evaluation of vaccine candidates, regarding the ability to prospectively identify potential overt immune stimulation in the presence of the desired immune responses to vaccines. A great effort is put in place by vaccine developers into the identification of biomarkers of adverse immune stimulation in nonclinical studies. A recent review of extensive studies on saponin-based adjuvant ISCOMATRIX™ [51] reflects this line of work and progress made in the characterization of both physicochemical properties and biological activity as well as markers of immune responses induced by this novel adjuvant. The described work on exploratory assessment of serum markers of auto-immunity, inflammation, and allergy is based on clinical studies with HPV16E6E7 and HCV Core vaccines containing ISCOMATRIX™. In these studies, measurements of anti-cardiolipin antibodies, anti-B2 glycoprotein 1 and IgE levels were evaluated. The results are not necessarily conclusive at this stage but help to direct future work on biomarkers in both animal and clinical studies.

Another line of current research on potential biomarkers of immune stimulation and inflammation includes genetic profiling in response to adjuvanted vaccines [52]. Metagene- and pathway-based analytical approaches were adopted to provide quantitative readouts with biological relevance which can be used to study mode of action and rank vaccine and adjuvant candidates under development [52]. Gene profiling was performed on peripheral blood from monkeys treated with several vaccines with known clinical adverse effects. A gene module data analysis approach was used to demonstrate that one of the gene modules could be used as a classifier to predict vaccine/adjuvant reactogenicity. The classifier gene set was then applied in subsequent monkey studies to predict reactogenicity associated with experimental vaccines.

While work focused on establishing reliable biomarkers to apply them to nonclinical and clinical safety evaluation is ongoing, some steps to address concerns about undesired immune stimulation in toxicology studies can be put in place by including additional parameters that are not routinely measured in these studies [53]. For example, potential prolonged systemic inflammatory responses can be assessed by measuring acute phase proteins, e.g. C-reactive protein (CRP), IL-6 levels, complement components and/or coagulation factors as part of enhanced clinical chemistry analysis of serum and/or plasma samples collected from vaccinated animals at appropriate time after the administration of the vaccine. When using an immune potentiating adjuvant, the development of anti-DNA or anti-RNA antibodies could be monitored. Potential pathogenic autoimmune responses against a particular tissue could be evaluated by targeted immunohistochemistry evaluation of this tissue.

16.4 Adequate Design of Developmental and Reproductive Toxicity Studies with Next Generation Vaccines

To date there is no documented evidence of reproductive toxic effects in humans caused by any approved vaccine. However, the regulatory authorities do not presume a product is safe until it has been directly tested using appropriate preclinical test methods and well-designed, adequately powered clinical trials [1]. Therefore, to address potential developmental hazards of vaccine candidates, developmental toxicity studies in animal models are currently required for vaccines indicated for maternal immunization and/or immunization of women of childbearing age, according to the FDA's guideline titled "Considerations for Reproductive Toxicity Studies for Preventive Vaccines for Infectious Disease Indications" [18].

16.4.1 Design According to Guideline Recommendations

Developmental and reproductive toxicity (DART) studies provide information on potential effects of the vaccine on fertility, fetal development, and postnatal development of the offspring [18]. Since the primary concern for preventive and therapeutic vaccines is safety during development and growth of the embryo and fetus, the evaluation is focused on effects on the pregnant/lactating female and embryo-fetal development following exposure of the female to the vaccine from implantation through the end of pregnancy, with follow-up of the offspring through weaning. A postnatal follow-up of the pups from birth to weaning is also included to assess normal growth, nursing activity, body weights, and viability which are established as reliable indicators of normal development. Design of vaccine DART studies has been reviewed by Wolf et al. [39] In brief, female animals are immu-

nized a few weeks before mating in order to ensure peak immune responses during the critical phases of pregnancy (e.g., organogenesis). Vaccine booster doses are then administered during gestation (embryo-fetal period) and lactation (postnatal period) to evaluate potential direct embryotoxic effects of the components of the vaccine formulation and to maintain an immune response throughout the remainder of gestation. If an adjuvant is included in the vaccine, an adjuvant-alone control group could also be included, similar to the approaches to general repeat-dose toxicity studies discussed above.

16.4.2 Considerations for Vaccines Containing Immune Potentiators

As for general toxicology studies, for next generation vaccines, and particularly vaccine containing immunopotentiating adjuvants, questions and concerns have been voiced regarding the design of DART studies as delineated in the FDA guidance. These questions were discussed at a workshop on nonclinical evaluation of vaccines [54]. It was reported that participants generally agreed that the primary objectives and design of current DART studies performed according to the existing guidelines are appropriate and no specific changes were recommended. Furthermore, it was confirmed that no specific immunotoxicological endpoints are necessary since the evaluation of antibody response to the vaccine antigen(s) in DART studies is adequate to assess an effect of the vaccine on the immune system in the treated mother and indirectly on the developing immune system of the offspring. Additional immune parameters should only be evaluated on a case-by-case basis where there is an increased concern for potential immunotoxicity. Also, if DART studies would reveal vaccine-induced adverse effects on either the pregnant/lactating animal, the embryo/fetal development or development of the offspring, further nonclinical studies to evaluate the cause of the effect should be conducted. Follow-up studies would include broader immunological evaluations, e.g. histochemical analysis for antibody depositions, evaluation of lymphoid organ weights, histology and hematology of the F1 generation.

16.5 Quality Tests for Biological Products and Cell Substrates

Quality control of biological materials involves analytical and biological testing to identify quality attributes such as identity, purity, potency, and mass, and assess safety including sterility, pyrogenicity, and adventitious agents. In vivo quality testing is conduced not only in support of marketed products but clinical materials as well as preclinical materials. The main goal of this type of testing is to identify issues that may have arisen during manufacturing (i.e., introduction of adventitious agents/contaminants, changes in potency and/or properties of cell substrates or other

biological starting materials over time). The testing may also be used as a screening tool for biological materials that may have inherent characteristics that may affect their safety and/or tolerability (e.g., pyrogenicity testing on a vaccine candidate with bacterial components).

Potential sources of contamination of biotechnology products include the original source of the cell lines or from adventitious introduction during the manufacturing process. Some examples of these sources of contamination (as listed in ICH Q5A [22] and USP <1050> [55]) include viruses introduced into the Master Cell Banks (MCB) via: (1) derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; and (4) contamination during cell handling. In the case of the introduction of adventitious viruses, sources include: (1) the use of contaminated biological reagents such as animal serum components; (2) the use of a virus for the induction of expression of specific genes encoding a desired protein; (3) the use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) the use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

16.5.1 Regulatory Guidelines for Quality Testing

General guidelines available for this type of testing include the European Pharmacopoeia (EP [56]), the United States Pharmacopeia (USP [57]), the United States Code of Federal Regulations (US CFR [58]) and the Product License. Other countries may also have specific Pharmaopoeia (e.g., China, Japan, Brittan) so manufacturers should also consult these guidelines prior to marketing vaccines in these regions. Typically, the details of the testing contained in the Product License supersede the most current guidance documents unless specific regulatory approvals are sought to update the license in question. For more specific guidance for particular types of vaccines, cell substrates, or stages of production, additional guidelines are available (Table 16.1). Guidance documents are also available for Regulatory Submissions [59]. Testing supporting release of product generally follows current Good Manufacturing Processes (cGMP) guidelines [60, 61] though some tests are conducted per GLPs (e.g., tumorigenicity testing).

Testing requirements vary by region so manufacturers who market biologics worldwide typically design the assays such that the criteria for all markets may be satisfied in a single assay. For example, the specifications for the General Safety test listed in the CFR [62] differ from those in the EP [63]. Therefore, one could design the general safety test to satisfy all markets by using the greatest number of animals specified (5 mice and 2 guinea pigs) and a weight range inclusive of the ranges in all regions (17.0–21.9 g for mice and 250–350 g for guinea pigs). The duration is the same for both regions but if one were longer, presumably the longer duration would be selected. If the specifications selected are outside the range for one of the markets, it may be necessary to gain regulatory approval based on the rationale of reduction in animal use.

16.5.2 In Vivo Quality Control Tests

The specific in vivo tests include various species, as each species is more or less sensitive to particular adventitious agents and the most sensitive species should always be used. Adult and suckling mice are utilized to detect adventitious viruses. Adult mice detect lymphocytic choriomeningitis virus (LCMV), coxsackieviruses, flaviviruses, and rabies virus. Suckling mice detect coxsackievirus types A and B and other picornaviruses such as polioviruses and echoviruses, alphaviruses, bunyaviruses (including phleboviruses and nairoviruses), arenaviruses, flaviviruses, rabies, and herpesviruses (such as herpes simplex virus). Guinea pigs are sensitive to *Mycobacterium tuberculosis* and adventitious viruses including paramyxoviruses (including Sendai virus), reoviruses, and filoviruses, and rabbits are used to screen for simian Herpes B virus. Eggs are also utilized via various injection routes for the detection of herpesviruses, poxviruses, rhabdoviruses, rickettsiae, mycoplasmas, bacteria, orthomyxoviruses (influenza virus), and paramyxoviruses (mumps, measles, parainfluenza viruses), alphaviruses, and vesiculoviruses. In the antibody production test, hamsters, rats and mice are utilized to detect specific viruses. The hamster antibody production (HAP) test is utilized to detect lymphocytic choriomeningitis virus (LCMV), pneumonia virus of mice (PVM), reovirus type 3 (Reo3), Sendai virus, and simian virus 5 (SV5). The rat antibody production (RAP) test is specific for Hantaan virus, Kilham rat virus (KRV), LCMV, mouse adenovirus, mouse encephalomyelitis virus (Theilers, GDVII), PVM, rat coronavirus (RCV), Reo3, sialodacryoadenitis virus (SDAV), Sendai virus, and Toolan virus (HI). The mouse antibody production (MAP) test detects Ectromelia virus, mouse rotavirus (EDIM), Hantaan virus, LCMV, lactic dehydrogenase virus (LDM), minute virus of mice (MVM), mouse adenovirus (MAV), murine cytomegalovirus (MCMV), mouse encephalomyelitis virus (Theilers, GDVII), mouse hepatitis virus (MHV), PVM, polyoma virus, Reo3, Sendai virus, thymic virus, and K virus [20].

The endpoints vary by test and include physical signs, survival, body weight, body temperature, antibody levels, and/or gross necropsy and/or histopathological evaluation (Table 16.3).

16.5.3 Alternatives for In Vivo Release Tests

16.5.3.1 In Vitro Alternatives

Alternatives for some of the in vivo tests have been developed. For example, in vitro alternatives to the Rabbit Pyrogen Test include the Limulus amebocyte lysate (LAL) and the Monocyte Activation Test (MAT). The LAL assay is a well-established in vitro test widely used for the detection of pyrogenic endotoxins in biologic products. While this assay has utility for its intended purpose, it is unable to detect non-endotoxin pyrogens and false positive results may be obtained for vaccines that contain bacterial components. Therefore, the rabbit pyrogen test is still used for all products for

Table 16.3 In vivo quality control tests

Purpose	Test	Products tested	Endpoint	Method	Acceptance criteria
Test for extraneous contaminants	General safety/ abnormal toxicity[a]	Final product	Physical signs/ survival/body weight	At least 2–5 mice and 2 guinea pigs are injected IP in a volume of 0.5–1 or 5 mL for mice and guinea pigs, respectively. Animals are observed daily for 7 days [62, 63]	EP: none of the animals die or show signs of ill health. If >1 animal dies, the preparation fails. If no more than one animal fails to meet these criteria, the test may be repeated once US CFR: (1) they must survive the test period, (2) they do not exhibit any response which is not specific for or expected from the product and which may indicate a difference in its quality and (3) they weigh no less at the end of the test period than at the time of injection. The test may be repeated twice, in the species that failed only, if any of the acceptance criteria are not met with the caveat that the second repeat test must include twice the number of animals in the first repeat test
Purity	Pyrogen test	Final product	Body temperature	Rabbit body temperatures are monitored for 3 h at intervals not more than 30 min following IV injection of the product into three rabbits. Temperature rise is determined by the difference between the maximum temperature rise versus the initial/control temperature [64–66]	USP: if any of the three initial rabbits have a body temperature equal to or exceeding 0.5°C, the test is repeated in five additional rabbits. The test passes provided the sum body temperature increase of all eight rabbits does not exceed 3.3°C US CFR: per the USP with the exception that if the material requires testing in five additional rabbits, the test passes if not more than three of the eight rabbits show individual rises in temperature of 0.6°C or more, and if the sum of the eight individual maximum temperature rises does not exceed 3.7°C EP: the test passes if the cumulative temperature in the three rabbits does not exceed 2.65°C. If the initial test does not pass, further groups of 3 rabbits are tested up to a total of 12 rabbits. The test fails if the cumulative temperature rise in all 12 rabbits is greater than 6.60°C

Adventitious agents	Adult and suckling mice	Cell banks and vaccine seeds/ bulks	Survival, evidence of transmissible agent or other viral infection	No less than 10–20 adult mice are injected IP and also IC in some cases or IM only and animals are observed for 21–28 days. No less than 10–20 suckling mice are injected IP and also IC in some cases or SC only and animals are observed for 14–28 days. If any animals die or show signs of disease after the first 24 h, autopsy and in some cases prepare and inject a suspension of appropriate tissues from the first group of animals into a second group of animals. In some countries a suspension of appropriate tissues from all survivors of the suckling test is injected into a second group of animals [20, 21, 55, 67]	At least 80% of inoculated mice must survive and show no evidence of infection attributable to the test material
	Guinea pigs			Five guinea pigs are injected IP and in some cases also IC and observed for 42 days to 6 weeks. Any animals that die or show signs of disease after the first 24 h are autopsied and all surviving animals are autopsied at the end of the test. Tissues are evaluated for signs of infection attributable to the test article [20, 21, 67]	
	Rabbits			Five rabbits are inoculated with product ID and also SC in some cases and observed for 4 weeks. Animals that die or show signs of sickness are investigated for cause of death via necropsy [20, 21]	
	Embryonated chicken eggs		Survival	Two routes of administration are utilized for the egg test. 10 eggs each are inoculated via yolk sac and the allantoic route. Eggs are incubated for not less than 5 or 9 days and evaluated for hemagglutinins. In some countries it is also required that passage of allantoic fluids and yolk sacs into a second group of eggs be conducted [20, 21, 55, 67, 68]	The test is valid if greater than 80% of test and control eggs are free of hemagglutinins and do not show gross evidence of viral infection.
	Antibody production tests: performed when potential exists for exposure to rodent viruses.	Cell banks	Antibodies to species-specific agents	The test article is inoculated into specific-pathogen-free (SPF) animals that are subsequently tested for antibodies to specific agents. Hamsters, rats, and mice are utilized as each detects multiple, specific viruses. [20, 21, 55, 68]	Absence of detectable antibodies to specific viruses

(continued)

Table 16.3 (continued)

Purpose	Test	Products tested	Endpoint	Method	Acceptance criteria
Cell properties	Tumorigenicity[b]	Cell banks	Tumor formation	Ten nude (nu/nu) mice or other suitable animals are inoculated SC or IM with cells at or beyond the end-of-production passage level. A group of animals inoculated with positive (e.g., HeLa) control cells is included. Animals are observed for a minimum of 16 weeks, up 7 months in some cases. An interim necropsy is included in some cases. Endpoints include tumor formation and gross and histological evaluations [20, 21, 68]	A test fails if progressive tumor formation with histopathic or genotypic confirmation of inoculated cells is observed at the site of injection in at least 2 out of 10 animals. At least 9 out of 10 animals injected with positive control cells should show progressively growing tumors in order for test to be valid
	Oncogenicity. To assure that agents that could immortalize cells and endow them with the capacity to form tumors are not present in cell substrate evaluated	Lysates of cell banks	Tumor formation	Typically test cellular DNA and cell lysates in newborn nude mice, newborn hamsters, and newborn rats. FDA/CBER and other regulatory agencies can be consulted for methods [20]	

Neurotropisms	Neurovirulence[c]	Viral vaccine seeds/ bulks	CNS effects	Not fewer than ten suitable *Macaca* or *Cercopithecus* monkeys are inoculated with 0.5 mL of material to be tested in thalamic region of each hemisphere. Control monkeys included as cage-mates or in immediate vicinity. Inoculated monkeys are observed for 17–21 days. At the end of observation period, autopsy and histopathological examinations of appropriate areas of brain are evaluated for evidence of CNS involvement [69]	Material complies with test if no unexpected clinical or histopathological evidence of involvement of CNS attributable to inoculated virus
Potency[d]	Immunogenicity	Final product	Vaccine titer	Product-specific [70]	Product-specific

IP intraperitoneally, *IC* intracerebrally, *SC* subcutaneously, *IV* intravenously, *IM* intramuscularly, *ID* intradermally, *US CFR* United States Code of Federal Regulations, *EP* European Pharmacopoeia, *USP* United States Pharmacopeia

[a]It should be noted that some products elicit expected physical signs based on components in the test sample itself so the dose and/or duration may be altered to allow for reversal of the expected signs before assessing for extraneous contaminants. In addition, some products may be exempt from the test based on the mode of administration or nature of the product

[b]In general diploid cell lines are considered to have low potential for tumorigenicity and well-established cell lines may not require repeated testing

[c]Neurovirulence testing is required, for example, if inadequate data on neurovirulence of a virus for which a novel vaccine is being developed, if neurotropism or neurovirulence is apparent, or if a novel vaccine has been attenuated by passage in neuronal tissue

[d]Tests may be conducted in vivo and/or in vitro

which the LAL is inappropriate. In an effort to reduce animal use, the MAT, a novel in vitro assay has been developed and validated for the detection of both endotoxin and non-endotoxin pyrogens [71]. The assay involves the stimulation of a lymphocyte population in whole blood, peripheral blood mononuclear cells (PBMCs), or a reproducible cell line (MonoMac 6) by the analyte of interest and the measurement of cytokine release (IL-6, IL-1β, TNF-α). This assay is also listed as an alternative to the rabbit pyrogen test in the EP [72].

Some of the tests for adventitious agents also have in vitro alternatives. In vitro tests such as culture and PCR for the identification of *Mycobacterium* are, in some cases, acceptable as an alternative to the test in guinea pigs and the test in rabbits for the presence of herpes B virus in primary simian cultures may be replaced by a test in rabbit kidney cell cultures [20, 21].

It is also possible to replace in vivo potency tests with in vitro methods. The design of potency studies is flexible and generally product-specific and if an in vivo model is available, one may start with an animal (typically mouse) test with plans to move to an in vitro model proven to correlate with in vivo data in an effort to reduce animal use. It should be noted, however, that in vitro tests may not always correlate with clinical experience due to their ability to detect chemical changes that may not lead to functional effects on potency.

16.5.3.2 In Vivo Alternatives

In vivo assays designed to detect neurovirulent potential in live virus vaccines have traditionally required the use of nonhuman primates (NHPs) in the Monkey Neurovirulence Test (MNVT). While this type of test remains appropriate for some neurotropic virus strains (e.g., polio and yellow fever), at a workshop in 2005 jointly organized by the International Association for Biologicals (IABs), the EP and the WHO, it was recommended that MNV testing no longer be required for established strains with proven safety records such as measles, mumps, rubella, and varicella [73]. In fact, the EP monographs for measles [74], mumps [75], rubella [76], and varicella [77] now require that only new strains of these viruses be evaluated for neurovirulence and that the test, in an appropriate animal model, be conducted during preclinical development only. In addition, FDA has removed the requirement for neurovirulence testing from the CFR [73]. In some cases, neurovirulence testing is required, for example, if inadequate data on neurovirulence of a virus for which a novel vaccine is being developed, if neurotropism or neurovirulence is apparent, or if a novel vaccine has been attenuated by passage in neuronal tissue. However, novel models for neurovirulence testing that do not involve the use of NHPs are being pursued. For example, Rubin et al. have developed a neonatal rat model which has shown better predictive value than the MNVT in distinguishing between neurovirulent and attenuated strains of mumps virus [78]. Other test systems such as marmosets have also been evaluated and show some promise. Nonanimal-based testing methods have also been considered but full replacement of animal testing may not be feasible as the complexity of neurovirulent viruses may not be adequately reflected [79].

16.5.4 In Vitro Quality Tests

In addition to the in vivo tests described in Table 16.3, there are several in vitro tests that also detect adventitious viruses. The Cell Culture Safety Test in human diploid or monkey kidney cells detects a variety of adventitious viruses that include cytopathic viruses, hemadsorption viruses, and hemagglutinating viruses. The use of Human Diploid Cells identifies a variety of human viruses (such as herpesviruses, adenoviruses, coronaviruses, reoviruses, alphaviruses, rubella, flaviviruses, rabies, enteroviruses, certain strains of hepatitis A virus, poliovirus, coxsackie B virus, echovirus, rhinoviruses, orthomyxoviruses, paramyxoviruses) and simian viruses (such as simian cytomegalovirus). The use of Monkey Kidney Cells could identify human viruses [such as enteroviruses, coxsackie B viruses, echoviruses, orthomyxoviruses, paramyxoviruses, HSV, poxviruses, polyomaviruses, rotavirus, alphaviruses, rubella, flaviviruses, rabies viruses, vesiculoviruses, filoviruses, influenza viruses, bunyaviruses (including phleboviruses and nairoviruses), arenaviruses, and reoviruses, polioviruses, rhinoviruses, adenoviruses (some strains)] and simian viruses (such as herpes B virus) [20].

Other tests for detection of adventitious viruses include: (1) Transmission Electron Microscopy (TEM) which can detect viral particles in a cell substrate, including those from endogenous retroviruses; (2) Reverse transcriptase (RT) assays which can detect any retrovirus, as all retroviruses encode and contain RT; (3) Infectivity Tests for retroviruses which can be performed on a case-by-case basis; and (4) PCR or Southern Blot which can be performed to detect specific viruses. In vitro tests for nonviral adventitious agents include tests for mycoplasma, mycobacteria, and bacterial and fungal sterility.

Other safety tests for product release include tests for the presence of residual cells and DNA. Residual cellular or nuclear material in the final product poses a potential risk because of oncogenic and/or infectivity potential. DNA can be removed, digested, or inactivated to lessen these risks.

16.6 Conclusion

The development of a broad range of novel or next generation vaccines containing more synthetic and/or recombinant components rather than microorganism-derived components has a clear advantage from the manufacturing and process control perspective. With the continuous progress in technology, especially molecular and genetic methodologies applied both in discovery of vaccines and formulation sciences, the development of well-defined specific antigens for use in vaccines enables production of next generation novel vaccines to prevent and/or treat diseases which have been refractory to vaccination in the past. Scientific and technological advances have led to improved vaccine products aiming at elimination of potentially virulent or carcinogenic components and reduction of impurities.

However, vaccines containing "purer" antigens are often not very immunogenic and therefore require addition of more effective adjuvants and other excipients. Paradoxically, while the next generation vaccines are more defined and better characterized than the traditional vaccines, they are viewed as "less natural." Also, advances in molecular engineering leading to the use of novel adjuvants and other components as well as novel delivery systems are not always paralleled by a full understanding of biological mechanisms of action of these components. A limited understanding of mechanism of action of newly available vaccine adjuvants leads to increased concerns about their safety [3]. Nevertheless, many recent scientific and regulatory discussions dedicated to this topic seem to indicate that currently recommended and applied approaches to nonclinical development, including toxicology studies, of vaccine candidates are appropriate and adequate [54]. The principles for the nonclinical safety assessment reviewed in this chapter should provide a foundation for the evaluation of next generation vaccines.

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Chapter 17
Safety Challenges Facing Next Generation Vaccines and the Role for Biomarkers

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17.1 Introduction

This chapter will provide the reader with a framework of how vaccine safety is currently assessed and will review the strengths and limitations of tools utilized preclinically to determine the safety of new vaccines (with a focus on adjuvanted vaccines). The specific safety challenge that has faced vaccines recently and will undoubtedly face the next generation vaccines is that of the infrequent and delayed adverse event of autoimmune disease which shares ingredients of the immune response that are being modulated by next generation vaccines (e.g., T-cells, B-cells, tolerance, TLR, inflammasome). Therefore, the following sections will provide an introduction to the challenges facing preclinical and clinical studies utilizing novel adjuvants. Finally, this chapter will conclude with a "translational" section related to biomarkers and their potential to predict outcome in subjects receiving vaccination. Examples of vaccine-dependent signatures with the potential to predict subject responses, such as immunogenicity, efficacy and safety, as well as subject-specific signatures (e.g., genetic makeup) that may modulate these responses will be provided to emphasize the dual-edge of how increasing the immune response may be favorable for efficacy but potentially detrimental for safety. This is one of the key challenges for the next generation of vaccines and the identification of reliable biomarkers that have the potential to overcome this challenge. The chapter will thus conclude with the large quantity of information that is likely to result from biomarker research and what will need to be addressed to enable biomarkers to fulfill their promise.

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17.2 Current Vaccine Safety Assessments

Because vaccines are usually administered to subjects who are otherwise healthy, they are placed under higher levels of scrutiny regarding related toxicity/adverse events than other pharmaceuticals. Furthermore, vaccines are composed of numerous components (Table 17.1) which, theoretically, have the potential to increase the chances for eliciting an associated toxicity compared to small molecule therapies that consist of single compounds.

Furthermore, there is a diverse range of adjuvants (e.g., inorganic salts such as alum, oligonucleotides, emulsions such as MF59, and agonists of various Toll-like receptors and other components of the innate immune system) that can be formulated with certain vaccines to enhance the immune response. Each of the ingredients comprising a vaccine, particularly novel adjuvants, requires the same careful evaluation that a new chemical entity undergoes. There are clearly defined guidelines from the US Food and Drug Administration, European Medicines Agency, and the World Health Organization [1] that form the basis of current preclinical toxicology studies for vaccines to assess the risk for human toxicity, including the following: single-dose studies, repeat-dose studies, local tolerance, safety pharmacology, development and reproductive studies, specialized toxicity, and toxicology for new chemically synthesized adjuvants. The signals assessed by these studies focus on changes in physiology, histopathology, injection site irritation, specific organ systems, fertility/fetal development, theoretical concerns, and hypersensitivity/genotoxicity.

These preclinical toxicology studies are designed to assess the intrinsic toxicity of vaccine formulations (including vaccine antigens, adjuvants and excipients), as well as toxicity resulting from the induced immune response. However, such routine studies have their limitations as dramatically illustrated by the unanticipated serious adverse events produced by anti-CD28 therapy in humans that was not observed in animal testing [2]. Furthermore, preclinical safety evaluations may not be adequate for identifying certain rare but potentially serious vaccine-associated adverse events, such as intussusception, hypersensitivity, febrile seizures, or anaphylaxis, and these evaluations are limited in predicting certain types of acute toxicities in the absence of

Table 17.1 Vaccines may be based on various platforms each with its own potential for toxicity

Vaccine components
Attenuated/live organisms (including bacteria, viruses, or parasites)
Living irradiated cells
Virus-like particles
Recombinant viruses
Plasmid DNA
Synthetic peptides
Polysaccharides
Purified/recombinant proteins

a relevant species for toxicity assessment. For example, with adjuvanted vaccines in particular, species-specific differences in cells, receptors, signaling pathways, and the tissue distribution of common components of the immune system targeted by adjuvants may lead to erroneous conclusions regarding human safety. One can imagine the difficulty in accurately predicting toxicities that are rare and that might occur acutely (such as viscerotropic disease and neurotropic disease after yellow fever vaccination) or arise months or years after vaccination (as seen with autoimmune diseases).

17.3 Focusing on Adverse Events

Potent stimulation of the immune response with adjuvanted vaccines has led to concerns regarding possible induction of autoreactivity. However, such concerns need to be methodically and thoroughly investigated due to the negative consequences to the welfare of the public when a safe and effective vaccine is incorrectly linked to an adverse event. For example, the false association between the measles/mumps/rubella (MMR) vaccine and the development of autism [3] led to a decrease in vaccination in the United Kingdom with subsequent increases in cases of measles. So why are adjuvants used in vaccines? The main reason is to enhance the effect of antigens or antigenic preparations for vaccines. For example, the use of highly purified subunit and recombinant antigen vaccines has resulted in less immunogenic second generation vaccines due to the absence of ill-defined immunostimulatory contaminants contained in earlier vaccines. Such was the case for whole-cell diphtheria, pertussis, and tetanus (DTwP) vaccine [4], where traces of immunostimulatory components [5] that may have been ligands for TLR and other innate immune receptors [6, 7]. Thus, the addition of adjuvants may compensate for the reduced immunogenicity of purer antigen preparations. Other important reasons to include adjuvants are to overcome hyporesponsiveness in certain populations (e.g., infants, elderly, immunocompromised), reduce the dose of vaccine required, reduce the number of injections, and enhance the quality or breadth of the immune response [8]. However, as more insights have been gained about the pathways by which the innate immune system recognizes pathogens, there have been increasing concerns that potent adjuvants may trigger unwanted inflammation and autoimmunity.

17.3.1 Historical Concern for Adjuvants Triggering Autoimmunity

There have been only a few documented cases of autoimmune disease induced by vaccines (Table 17.2). The rabies vaccine used in the 1920s contained phenolized sheep brain and induced encephalomyelitis in 0.1% of vaccinees. This vaccine preparation is no longer used in humans. The influenza vaccine used in the 1970s

Table 17.2 Documented cases of vaccines inducing autoimmunity

Vaccine preparation	Autoimmune disease
Nerve cell rabies vaccine (1920)	Encephalitis [9]
Swine flu vaccine (1970)	Guillain–Barre syndrome [10]
Measles vaccine or measles, mumps, rubella (MMR) vaccine	Immune-mediated thrombocytopenic purpura [11]
Inactivated virosomal-subunit vaccine (*E. coli* toxin adjuvant)	Bell's palsy [12]

contained the high-yielding influenza recombinant X-53 and had an estimated risk of one case per 100,000 of Guillain–Barre syndrome. Subsequent influenza vaccines based on different strains have a much lower risk of 1 in 1 million [13]. Both the measles vaccine and the MMR vaccine are associated with a risk of immune-mediated thrombocytopenia (1 in 30,000). However, the risk induced by the vaccines is substantially lower than after contraction of the natural diseases of measles or rubella (1 in 3,000 to 1 in 6,000, respectively) [14].

17.3.2 Utility of Preclinical Studies for Adjuvant-Triggered Human Autoimmune Disease

Strategies that are currently approved for assessing adjuvant toxicity preclinically have been insightful for more general and acute adverse reactions including skin eruptions at the injection site, musculoskeletal symptoms, and those of systemic inflammation (e.g., fever). However, preclinical models for predicting autoimmune diseases remain elusive for the reasons related to the differences in genetics and physiology between animals and humans, and the uncertainties and differences regarding the etiologies of autoimmune diseases in both species. There are numerous animal models of autoimmune disease, but their ability to predict human disease is limited in several respects. First, these models typically mimic the human disease that converge at the levels of end-stage pathology but differ in their pathogenetic mechanisms of action. For example, the MRL-FAS^{lpr} mouse strains [15] show similarities in the high titers of autoantibodies and renal disease but lack other clinical manifestations typically identified in the human disease. Second, the use of animal models that develop autoimmune disease at a high frequency (e.g., 90% incidence) may not be able to predict the incidence of rare autoimmune disease events in humans. Third, animal models induced to develop autoimmune disease may have an induction phase with very little relevance to the disease development in humans (e.g., experimental autoimmune encephalomyelitis [16] in rodents for predicting human multiple sclerosis development). Fourth, spontaneous animal models of autoimmunity may have similar pathologies to humans, but the genetic basis and mechanism of pathogenesis may be quite different. The MRL-*Fas^{lpr}* mouse model, which spontaneously develops lupus nephritis, has a mutation in the Fas gene responsible for defective lymphocytes apoptosis [15]. Polymorphisms in the

Fas gene, however, have not been reported to be linked to lupus susceptibility in genome-wide association studies in humans [17, 18]. Finally, it is also important to consider species-specific differences related to an adjuvant's mode of action. For example, studies in humans have demonstrated that TLR9 is expressed in plasmacytoid dendritic cells and B cells [19–21]; in mice, however, this TLR is additionally expressed in macrophages, myeloid dendritic cells, and activated T-cells [22–25]. Another important component of the innate immune system, TLR8, appears to utilize different agonists in humans versus other species [26]. These species differences in agonist usage, expression, and cellular distribution make inferences from preclinical toxicology studies assessing novel adjuvants difficult and highlight the need for more preclinical models that can better reflect human physiology (e.g., humanized, non-rodent species, or transgenic models, or in vitro cell- and organ-based systems based on human tissues).

17.3.3 Hypersensitivity: A Rare but Relevant Safety Signal

Induction of hypersensitivity reactions by today's vaccine preparations is rare but can still happen. In fact, although the exact figures are unknown, it is estimated that at least one per 500,000 individuals develop immediate or late hypersensitivity reactions to vaccines [27, 28]. Of these, most reactions occur to vaccine components used to formulate, produce, or potentiate their action including but not limited to ovalbumin, aluminium, yeast proteins, gelatin, antibiotics or some preservatives and stabilizers such as 2-phenoxyethanol and thiomersal [29–44]. While the cumulative experience in the field of vaccine research and development has allowed us to predict and prevent the occurrence of severe immediate hypersensitivity reactions, most allergic reactions are confined to the local site of injection and are also limited in time. Immediate and local reactions are mostly represented by erythema, swelling, and local pain accompanied infrequently by symptoms derived from the activation of the systemic immunological system (e.g., fever and irritability) [45]. Most common delayed-type hypersensitivity reactions may manifest as eczema which extend beyond the area of inoculation or the typical Arthus-like reaction classically seen in hyperimmune individuals receiving the tetanus vaccination [46, 47]. Although these are all clinically self-limiting, the prediction of adverse events related to occurrence of hypersensitivity reactions have been and continue to be one of the first considerations in the design of clinical trials.

Both local and systemic reactions to adjuvants can be somehow attributed to the activation of immune inflammatory mechanisms triggered by tissue damage and mediated by a range of cytokines such as IL-1, TNFα and IL-6. These are responsible for commonly seem flu-like symptoms, acute vascular responses, worsening of autoimmune or allergic diseases or other manifestations of immunotoxicity [45]. Delayed-type hypersensitivity constitute the basis of the histopathological mechanisms that participate in these reactions and are clinically involved in diseases such as MMF (macrophagic myofasciitis), which has been associated with the local

administration of aluminum salts, although evidence of its etiological link remains controversial [48–50].

Perhaps one of the key safety issues to consider in terms of the potential development of hypersensitivity and toxicity reactions is the predictability of safety signals in adjuvanted vaccines administered to pregnant women. The potential advantages of providing ante-natal protection to diseases for which no natural immunity develops within the first 6–8 months of life is of paramount importance. Immunotoxicity studies in this context are limited and preliminary. For example, it has been shown in animal experimental models that injection of high doses of CpG adjuvant can increase the incidence of fetal resorption and craniofacial and limb defects, while a contrary effect can be obtained when lower doses are administered [51–54]. This effect was attributed to the development of Th1-mediated immune responses that correlated with the development of cellular necrosis and a mixed inflammation reaction and calcification in the spongiotrophoblast layer of the placenta [51]. Induction of secretion of cytokines by adjuvants is a well-known and expected mechanism responsible for the recruitment of both innate and adaptive immune cell mediators to the site of injection and for the systemic effects of all adjuvanted vaccine formulations. In this sense, it has been proposed an active role of the placenta in the development and regulation of gene expression related to immunoregulatory component (i.e., cytokines) during organogenesis and hence the potential involvement of the fetal tissue in modulatory effects to vaccines [52, 54]. These and the issues discussed above has prompted the need for biomarker discovery to predict such adverse reactions and design safer, more effective vaccines.

17.3.4 Clinical Challenges

Given the above-mentioned limitations in translating preclinical findings to responses in humans, it will be necessary to develop alternative preclinical tools to better assess risk in humans. To tackle the issue of autoimmune disease, the clearest answer would be derived from clinical studies that include patients with active autoimmune disease or at high risk of developing disease. However such scientifically and medically driven strategies face obvious ethical issues when considering treatments that could trigger or exacerbate underlying disease. A more feasible approach could investigate whether markers of biological processes that are known to occur in patients with autoimmune disease are seen in normal subjects after vaccination. Such a signature in response to vaccination (that would be safe in normal individuals with normal regulatory processes of the immune system) could highlight a potential risk if the vaccine was given to a “susceptible” subject with a defective regulatory mechanisms. For example, the gene signature regulated by interferon type 1 is consistently associated with systemic lupus erythematosus and other autoimmune diseases [55]. However, similar signatures are also elevated after natural viral infections and immunization by live vaccines regarded to be safe for humans (e.g., yellow fever) [56, 57]. Therefore, other factors such as location and duration

of the induced signature are likely to be important with local and transient signatures being of less concern than systemic and continuously elevated signatures.

17.4 Translational Studies: The Promise of Biomarkers

Vaccine biomarker efforts have typically involved measurement of basic immune responses, but it is becoming evident that there is a wide variability in the human immune and other host responses, which will make the interpretation of these vaccine-induced immune responses challenging. These variables include age-related differences in the quality of the immune response, subtleties related to approaches for prophylactic vs. therapeutic vaccines, and how differences in the genetics and general health status of the host can alter the resultant immune response. To begin to address these variables, efforts are underway to benchmark next generation vaccines (e.g., with novel adjuvants) against natural infection or licensed vaccines that have demonstrated acceptable immunogenicity and efficacy and are generally considered to be safe. Such studies will collect detailed information on a wide variety of early and late human host responses and will form the basis for creating a comprehensive database of information from which biomarkers and signatures may be generated. The goal of such biomarkers would be to enable the development of vaccines that safely mimic the host response to natural infection or demonstrate in early clinical studies an immunogenicity or safety profile similar to a licensed vaccines. An extension of the accumulated biomarker data to late stage clinical studies testing efficacy provide the opportunity to develop correlates of protection and safety.

17.4.1 Vaccine-Dependent Signatures

17.4.1.1 Predicting Efficacy

While gene expression signatures found their niche in cancer prognosis [58], their successful application to vaccine development will require a better understanding of the human immune response (innate and adaptive immunity) in general, in the setting of immunization, and in response to various types of infection. The feasibility of such an approach was demonstrated in an elegant study [57] where gene signatures were explored in peripheral blood mononuclear cells obtained from subjects immunized with the yellow fever vaccine (YFV-17D). This licensed vaccine serves as a useful benchmark as it is considered to be one of the most effective vaccines (e.g., broad immune response with single injection) that has been administered to more than half a billion people. The authors of this study applied a systems biology approach and extracted distinct gene signatures correlating with the magnitude of the CD8 T-cell response induced by immunization with YFV-17D or those that are associated with increased antibody titers. These signatures were validated in an

independent study and were almost 90% accurate in predicting response rate within a few days after vaccination versus those subjects likely to develop protective levels of antibody. Although the dream of many is that a single vaccine modality should protect against a particular infectious disease in all people, the reality is that certain individuals will be predisposed to respond differently to a vaccine, particularly as has been documented by the relatively poor responsiveness to the hepatitis B vaccine in subjects with specific HLA haplotypes [59] or polymorphisms in cytokine-related genes [60]. Recently, it was demonstrated that individuals possess distinct populations of commensal microorganisms (or microbiota) [61]. It is probable that such differences could influence the course of an infection or response to an immunization, particularly those involving mucosal administration. These subject-specific differences may be exploited by future technologies to enable the efficacy of the next generation of vaccines to be tailored to populations as a function of their genetic makeup and microbiota. Both of the above-mentioned examples highlight the potential for gene expression signatures in predicting responses and eventually safety provided that such signatures undergo extensive validation.

17.4.2 Subject-Specific Signatures

17.4.2.1 Predicting Safety

As alluded to earlier, continuously evolving technologies may eventually provide the tools to exploit genetic and environmental differences among subjects to improve vaccine efficacy but the same approach can be utilized to improve vaccine safety. For example, serum samples obtained from subjects who have experienced adverse events during a vaccine clinical trial (if timed appropriately) may enable the identification of safety biomarkers. A study illustrative of this personalized medicine approach was the prediction of fever after administration of the smallpox vaccine according to genetic predisposition [62]. This detailed study performed genotyping and sequencing of DNA obtained from the whole blood of more than 300 subjects immunized with the Dryvax smallpox vaccine. Data from this study demonstrated that specific haplotypes in the interleukin-1 (IL-1) gene complex and in interleukin-18 (IL-18) could predict those subjects developing fever after immunization. Similarly, another haplotype in the interleukin-4 gene was associated with protection from the development of high fever. These data illustrate that even simple signatures could potentially be used to identify safety risks in individuals receiving other types of vaccines.

Another study also targeting smallpox vaccination [63] utilized a systems biology approach to identify biomarkers for adverse events. High-dimension genetic studies to examine 1,442 single nucleotide polymorphisms were coupled with more functionally relevant proteomic data to probe associations with adverse events to components of the immune system that were stimulated excessively or prolonged after vaccination. Sixty-one subjects had five clinical visits for adverse event assess-

ments (fever, generalized rash, or lymphadenopathy) in the month following immunization and collection of serum samples for cytokine measurements prior to and in the 5–7 day period post-vaccination evaluation period. The investigators used the Random Forest method to develop a decision-flow algorithm based on three proteomic variables (intracellular adhesion molecule 1 [CD54], interleukin-10, and colony-stimulating factor 3) and a genetic polymorphism in the interleukin-4 cytokine gene. Utilizing these variables, this algorithm was capable of correctly classifying 89% of individuals with respect to their risk for particular adverse events. This systems biology approach visualized complex interactions among multiple factors including genetics (SNPs in and around genes having various immunological functions) and proteomics (cytokines responsible for mediating inflammation), but was limited in scope to a few specific genes. One could anticipate similar genotyping and sequencing efforts on a broader scale on DNA obtained from responders and non-responders to highly efficacious vaccines like yellow fever and hepatitis B to see whether a genotype was associated with the quality of the immune response (good vs. poor). While a comprehensive approach may seem unrealistic today, enabling technologies in the rapidly advancing field of deep genome sequencing will make it feasible in the future.

17.5 Challenges Facing Biomarkers

The challenges facing biomarkers are related to limitations in current technologies, our understanding of the human immune response, and the limited information that can be translated from vaccines that were licensed in the past and have withstood the test of time with regards to efficacy and safety. These challenges fall into two basic categories: interpretation of information, as previously discussed, and collection of samples from which the information is measured. Ideally, one will need to understand host responses both locally at the injection site and draining lymph nodes and systemically. This will be true for early responses (within minutes) and late responses (days to months). To this end, we will need (1) noninvasive means of frequent sampling (e.g., implantable devices for continuous feedback), (2) miniaturization of assays to maximize the amount of information gathered from small tissue samples, and (3) high throughput means to assess safety and efficacy of vaccine candidates. Together, advances in these areas will allow us to keep pace with our ability to generate better antigens, adjuvants, delivery systems, and combinations thereof.

A recent perspective [64] illustrated that biomarker signatures (e.g., gene array) have failed to translate into clinical relevant tools due to the failure of performing adequate validations in independent clinical trials and the overestimation of a signature's performance. A key challenge facing biomarkers is that knowledge of how to read the messages in the genome remains elusive. This genetic illiteracy coupled with the absence of tools to reliably interpret how genetic data translates into biological pathways greatly complicates the prediction of cause and effect. The development of the next generation of vaccines would be greatly facilitated if comparable biomarker

data from different vaccine developers were available in a standardized format that preserved proprietary interests. Such an effort would require the establishment of repositories requiring public funding and agreement on standardization. Furthermore, as mentioned previously, even the creation of a well-stocked repository of "omics" data about novel adjuvants and next generation vaccines may be of limited value if there is no frame of reference for interpreting the data and generating hypotheses about factors that predict safety. Therefore, "benchmark" studies will need to be undertaken to compare human and animal model responses with next generation vaccines to those elicited with approved vaccines for which the safety profiles and the adverse events are well known. The regulatory considerations for first-in-human clinical trials with vaccines are elegantly presented in a recent publication [65] and should serve as the frame of reference for next generation vaccines. As more data is obtained on the human response to licensed vaccines and adjuvants with established safety records, the need for guidelines on how to apply these data to evaluate novel adjuvants and next generation vaccines is paramount. Along these lines, the U.S. Food and Drug Administration has created a Genomics Evaluation Team for Safety to develop approaches for the application of new technologies to the safety of biologics. Using the existing structure of the Voluntary Exploratory Data Submission (VXDS) process [66], one could envision the submission of biomarker data from vaccines with known benefit–risk profiles that would facilitate future biomarker development, clinical translation, and regulatory qualification.

17.6 Conclusion

Vaccines are a critical component of preventive medicine, and delays in the implementation of next generation vaccines due to the risks of rare adverse events (real or perceived) will need to be balanced with the larger disease-associated morbidity and mortality caused by infection in the general population not receiving the vaccine. The highest hurdle for future vaccines will be the same challenge that has faced older vaccines—safety! For next generation vaccines (especially those containing adjuvants), there will be a need for carefully designed preclinical studies that decipher the mode of action according to standards set by regulatory agencies working in conjunction with industry. Furthermore, as our knowledge of the mode of action becomes more sophisticated, paradoxically, so will the public perception of safety as links will be drawn to potentially rare or irrelevant diseases based on commonalities in the immune system, the vaccine mode of action, and pathways of human disease. Therefore, there will also be a need for large-scale epidemiological studies in human populations to confirm the safety of the next generation of vaccines (especially for the risk of rare or delayed adverse events, such as autoimmune disease). The reader should appreciate that in addition to the challenges of vaccine-related adverse events, there will always be the delays to safe and effective next generation vaccines caused by coincidental associations (not vaccine-related) that will occur with longer follow-up periods of vaccinated subjects due to factors related to the

environment, diet, age-related changes in the immune system, and conditions associated with triggering autoimmune disease in susceptible subjects (e.g., pregnancy or exposure to natural infections).

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