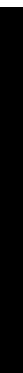


DEVELOPMENT OF VACCINES

From Discovery to
Clinical Testing

EDITED BY
MANMOHAN SINGH
INDRESH K. SRIVASTAVA

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Manmohan Singh
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PREFACE

Rapid advances have been made in the last two decades toward understanding the disease biology and developing preventive and therapeutic vaccines against infectious and parasitic diseases, cancer, allergies, fertility and immune disorders by both academia and the pharmaceutical industry. The journey has been punctuated by successes and challenges. But there is hope that with development of new technologies and better understanding of the disease biology, most if not all debilitating diseases will be *prevented*, or at the very least, cure serious infectious diseases, reduce pain and suffering of mankind, and improve the quality of life.

For development of a successful vaccine, we need to: 1. identify a target, 2. design an immunogen, 3. develop strategies for expression and purification of the immunogen in an appropriate structure and conformation, and 4. select an adjuvant and delivery system for inducing long-lasting T and B cell immune responses. Each of these aspects is critical for the successful development of a vaccine, and has its own challenges. This book provides conceptual ideas, case studies, and examples to understand these challenges and how to address them.

Immunogen design could be a rate-limiting step in the development of vaccines. Significant technological advances have been made in identification and design of novel immunogens through the genome sequencing of pathogens and identification of a plethora of antigens through reverse vaccinology. Similarly, advances in crystallization techniques have allowed for availability of structural information for viral and bacterial immunogen used to rational design of vaccines.

Once an immunogen has been identified/designed, then the next big question is: How do we use these immunogen(s) for inducing appropriate immune responses? Several platforms could be used to induce a potent immune response, such as DNA delivery, recombinant protein, VLP, and vector. Many variables exist within each platform, for example in the recombinant subunit vaccine platform, the challenges are to select which system to use for the expression of proteins or VLPs, and how to purify them efficiently and in correct conformation. Each of these platforms has its own advantages and challenges, and not every platform may be relevant or appropriate for every target; therefore platform selection for vaccine development needs to be clearly thought through. The success or failure of a vaccine depends on the quality of the immune responses induced and, to a certain extent, the quality of the immune response is platform dependent. Combination of different platforms, also known as prime-boost regimen, allows overcoming some of these challenges and broadening immune responses. This concept is more effective for difficult targets, such as HIV.

Another aspect critical for vaccine development is characterization of vaccines; better characterization increases the possibility of inducing better responses. In addition to immunogen, an integral component of a successful and effective vaccine is adjuvant. It is important to mention that to-date, aluminum mineral salts remain the most common adjuvant approved for use in vaccine products in humans. Apart from its safety profile, use had expanded due to the lack of availability of a suitable alternative. However, over the last few years, awareness about how some of these vaccine adjuvants work has led to a dramatic increase of focus in this area. Whether it is through activation of the innate immune responses or delivery to the targeted site, these novel adjuvant formulations can now be more well-characterized and optimized for their function. Formulations can now be designed to induce both cellular and humoral responses. Local responses in nasal and oral routes can now be generated using selective mucosal adjuvants. Evaluation of synergistic effects and repeated use are also being explored. However these new technologies will have to demonstrate a safety profile that is acceptable for mass immunization and prophylactic use. After a vaccine is designed, expressed, purified, characterized, and formulated, vialing the vaccine is not a trivial task and does involve significant efforts in selecting appropriate vials, quality control, and final fill-finish of the vaccine for clinical evaluation. The final step in the Phase I clinical evaluation of a prototype vaccine is regulatory compliance and filing the IND.

This book covers all the critical aspects for developing a successful vaccine, and highlights some of emerging vaccine technologies which will be a part of licensed products in the future. The book provides in-depth evaluation of all factors that govern the induction of an optimal immune response. Chapters on immunogen design, such as reverse vaccinology, and structure-based rational design of vaccines provide conceptual insights and application of concepts and tools for immunogen design. Chapters on vaccine technology platforms are organized to provide in-depth review of each platform, and its application for production and purification of immunogens. Chapters on vaccine characterization summarize beautifully the available technologies for characterizing vaccines and their applications.

Adjuvant history, antigen presentation, mechanism of action, and safety profile build a sound base for addressing some specific vaccine formulation issues. Detailed descriptions of all leading vaccine technologies with their limitations should greatly help researchers and students enhance their understanding of these technologies, and how to apply them for developing successful vaccines. The book also has chapters on clinical and nonclinical safety evaluation of vaccine formulations, which serve as a guideline for moving vaccines from research to clinic. Overall, the book highlights the most recent and up-to-date advances in the field of vaccines development.

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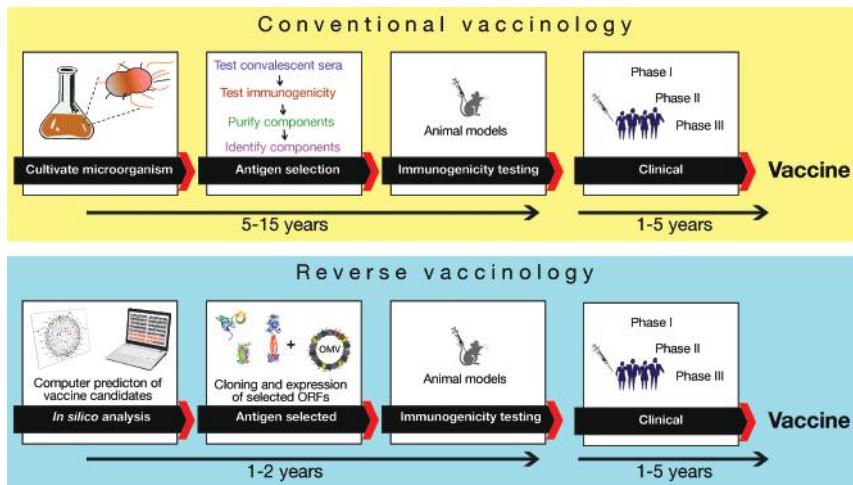


Figure 1.1. Schematic representation and time lines of classical vaccinology in comparison to the reverse vaccinology approach.

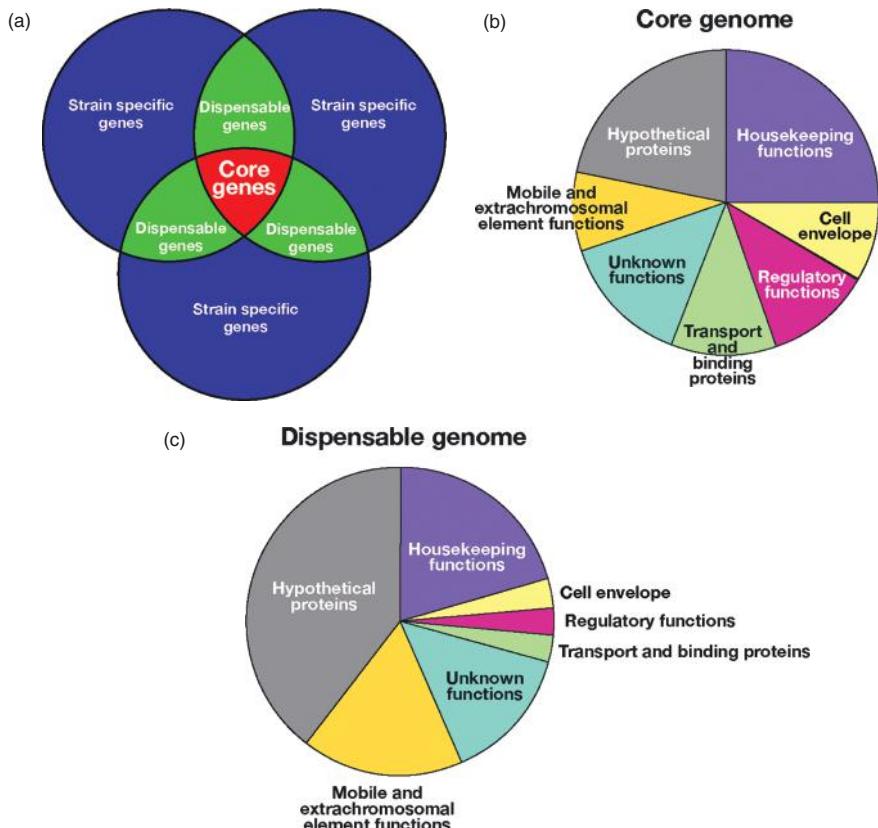


Figure 1.2. See page 10 for full caption.

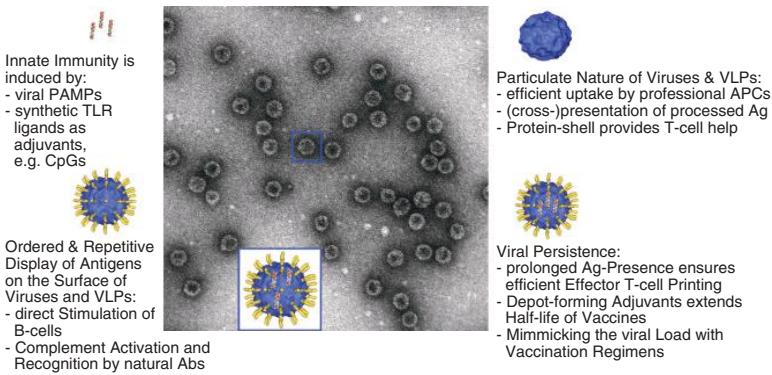


Figure 2.1. See page 22 for full caption.

EpiMatrix Cluster Detail Report												
Accession: FLU-HA Cluster: 305												
Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
305	CPYVKQNT	313	-0.29									0
306	PYVKQNTL	314	-0.26					1.45				0
307	YVKQNTLK	315	-0.32									0
308	YVQNTLKG	316	-0.56	3.06	2.28	3.18	2.81	2.43	2.81	3.11	2.55	8
309	VRQNTLKG	317	-0.05		1.51			1.62	2.01	1.7	1.41	2
310	KNTLKLATG	318	-0.16								1.34	0
311	QNTLKLATG	319	-0.08									0
Summarized Results												
Maximum Single Z score			3.06	2.28	3.18	2.81	2.43	2.81	3.11	2.55	2.55	--
Sum of Significant Z scores			3.06	2.28	3.18	2.81	2.43	4.82	4.81	2.55	25.94	
Count of Significant Z Scores			1	1	1	1	1	2	2	1	10	
Total Assessments Performed: 56				Hydrophobicity: -0.53				EpiMatrix Score: 20.17				EpiMatrix Score (w/o flanks): 21.41

Figure 3.1. See page 77 for full caption.

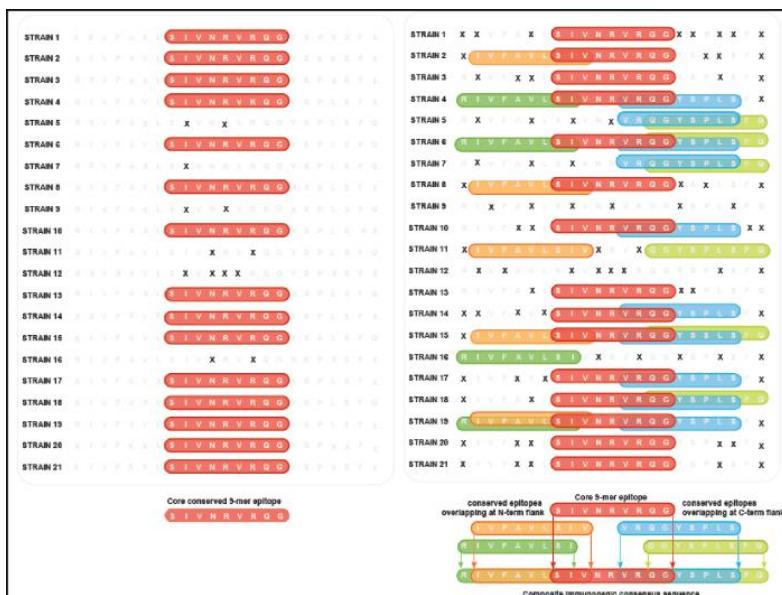


Figure 3.2. See page 78 for full caption.

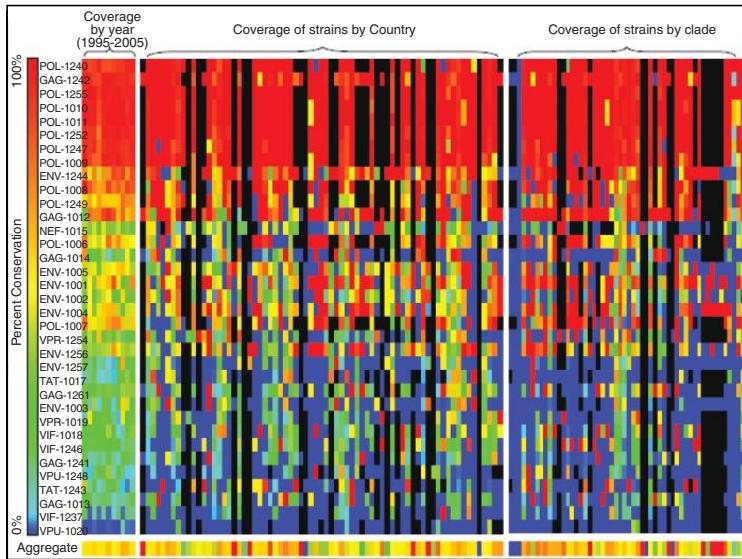


Figure 3.5. See page 83 for full caption.

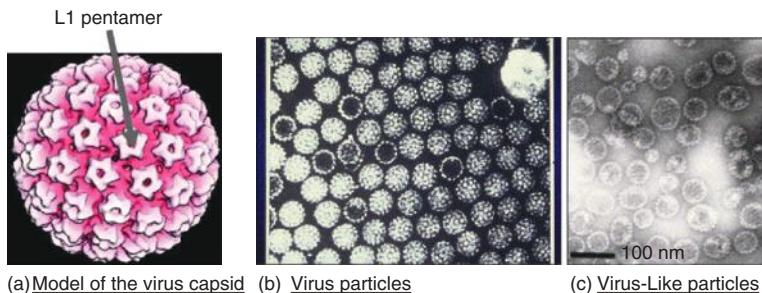


Figure 4.1. See page 101 for full caption.

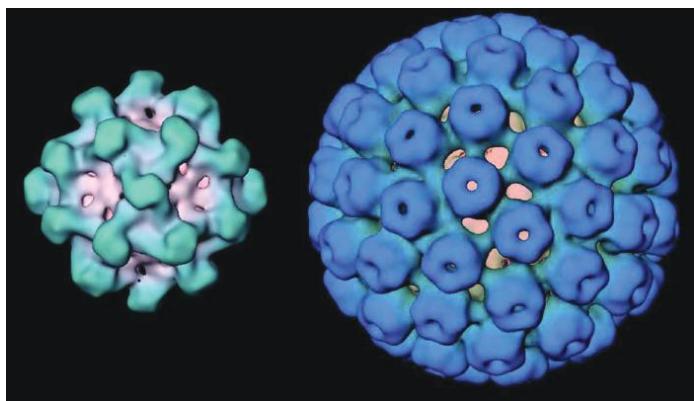


Figure 5.1. See page 129 for full caption.

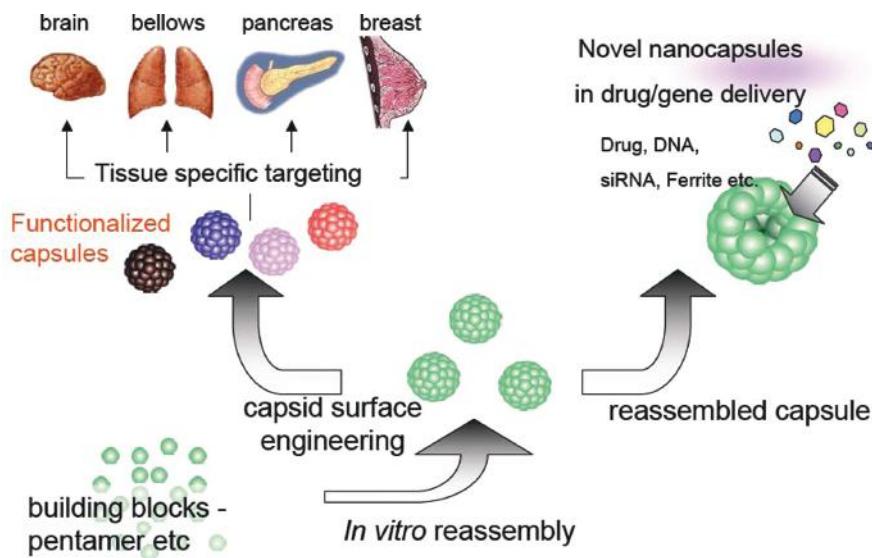


Figure 5.3. See page 131 for full caption.

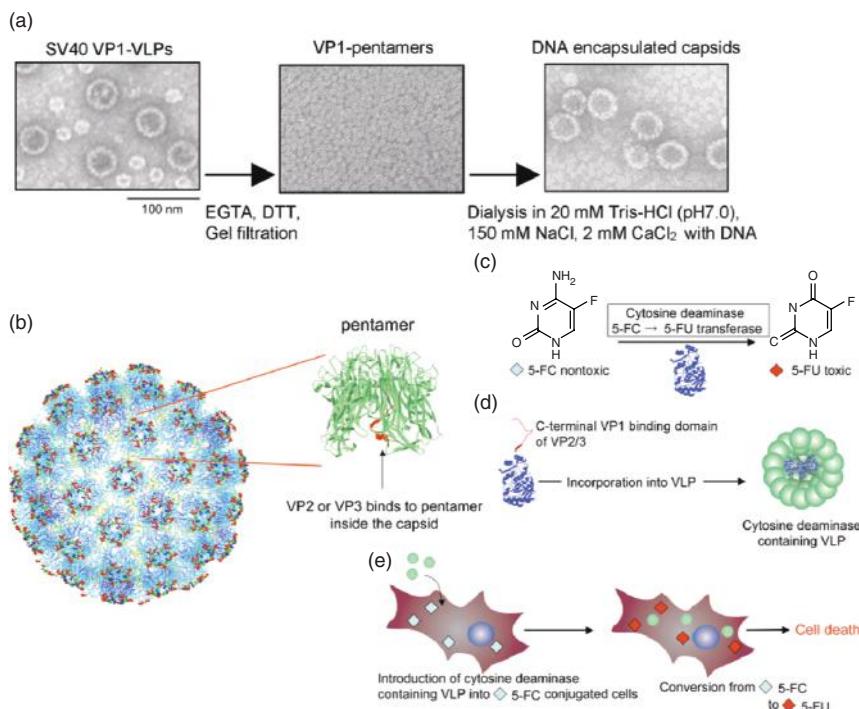


Figure 5.4. See page 134 for full caption.

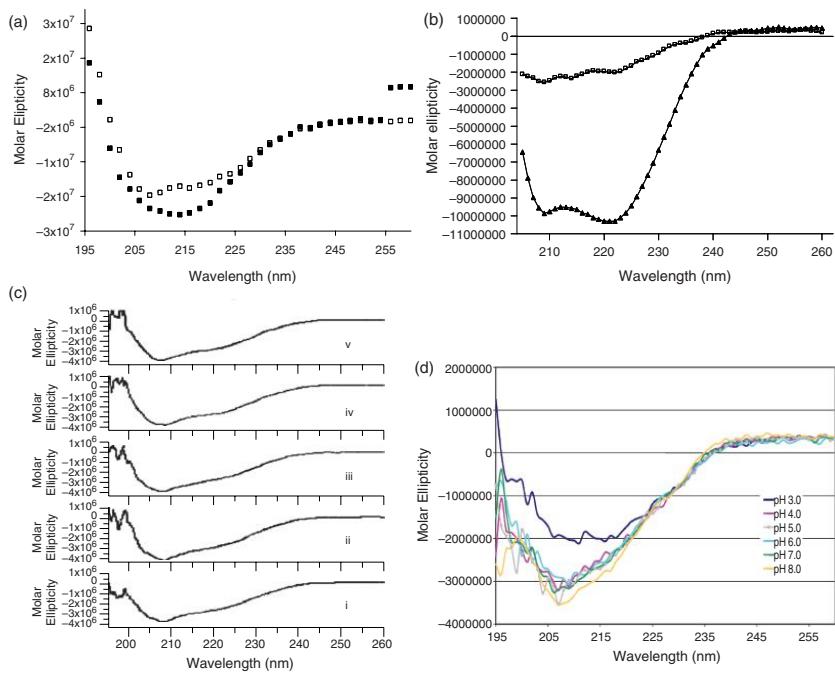


Figure 10.1. See page 266 for full caption.

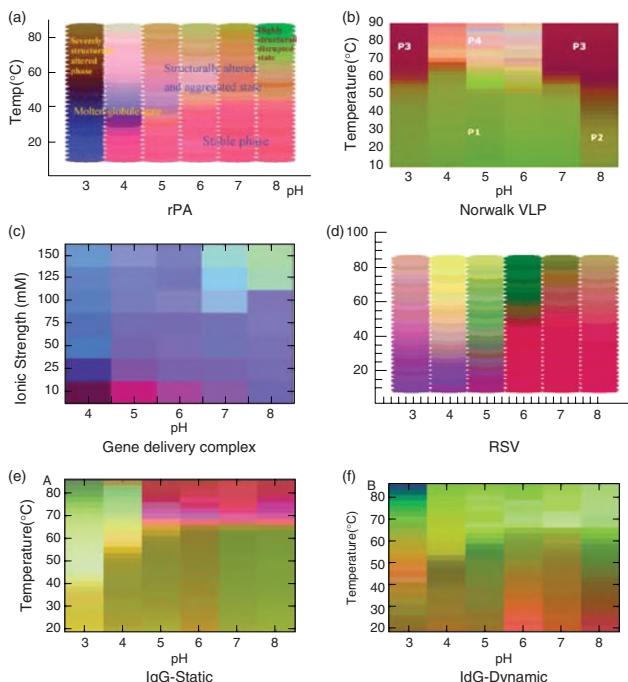


Figure 10.11. See page 285 for full caption.

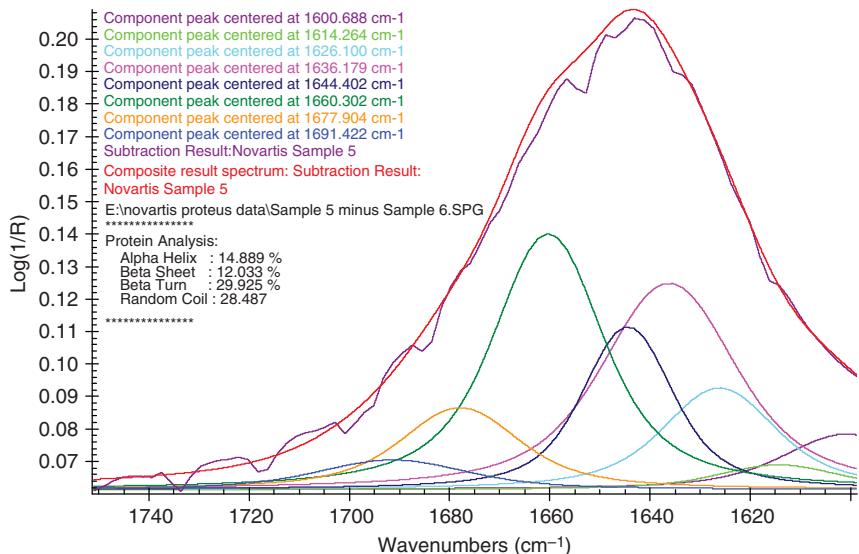


Figure 11.5. See page 301 for full caption.

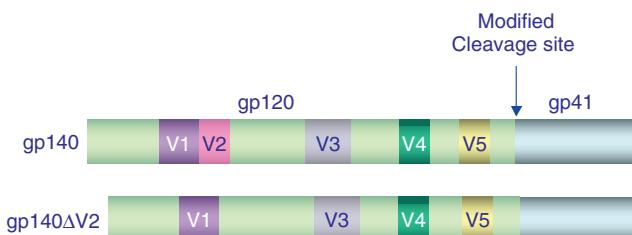


Figure 12.1. See page 310 for full caption.

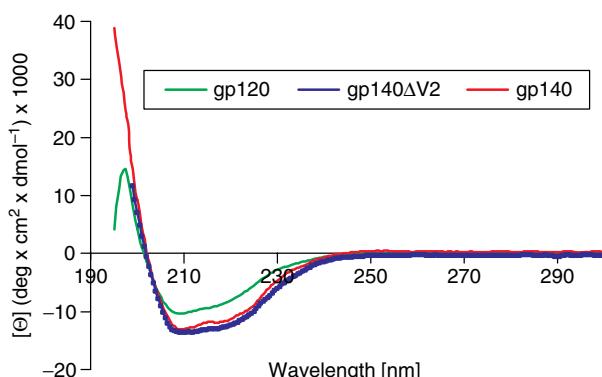


Figure 12.2. See page 311 for full caption.

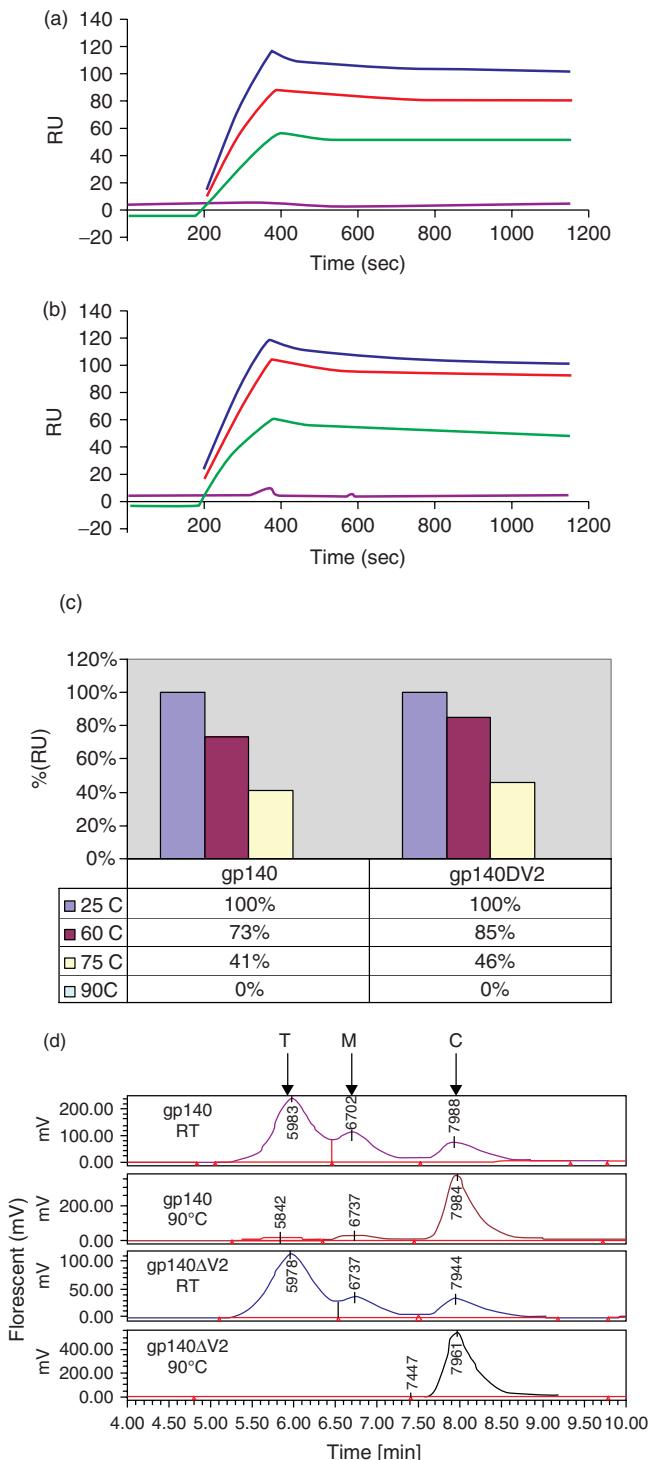


Figure 12.6. See page 315 for full caption.

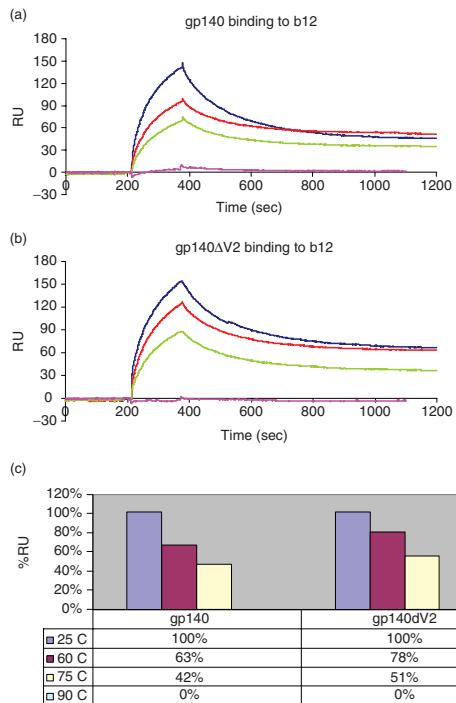


Figure 12.8. See page 317 for full caption.

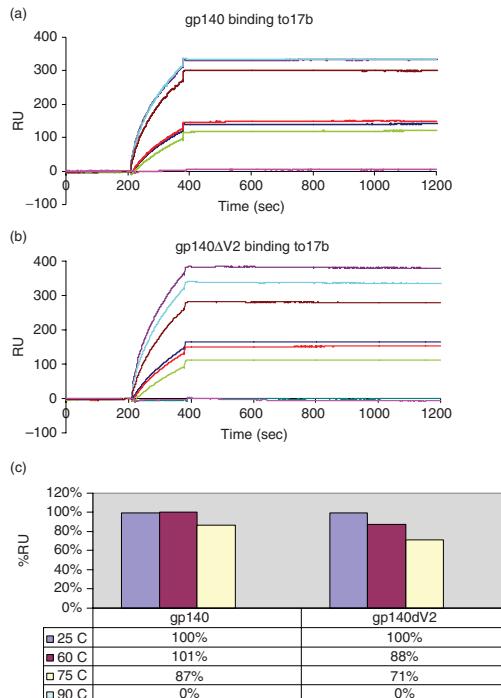


Figure 12.9. See page 318 for full caption.

PART 1

IMMUNOGEN DESIGN

MICROBIAL VACCINE DESIGN: THE REVERSE VACCINOLOGY APPROACH

Roberto Rosini, Michèle A. Barocchi, and Rino Rappuoli

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1.1 INTRODUCTION

Infectious diseases are the greatest cause of morbidity and mortality worldwide; pathogenic bacteria are responsible for approximately 50% of this burden. From a public health standpoint, prevention of diseases has a greater impact and is more cost effective than treating the infection. Vaccines are the most cost-effective methods to control infectious diseases and at the same time one of the most complex products of the pharmaceutical industry. There are several infectious diseases for which traditional approaches for vaccine discovery have failed. With the advent of whole-genome sequencing and advances in bioinformatics, the vaccinology

field has radically changed, providing the opportunity for developing novel and improved vaccines. Overall, the combination of different approaches (“-omics” approaches)—genomics, transcriptomics, metabolomics, structural genomics, proteomics, and immunomics—are being exploited to design new vaccines.

1.2 HISTORICAL VIEW OF “CLASSICAL” VACCINOLOGY

The history of vaccination is traditionally dated to the publication, in 1798, of Edward Jenner’s landmark experiments with cowpox in which he inoculated a neighbor’s boy with purulent material from a milkmaid’s hand lesion in the United Kingdom. The boy, 8 years old, was subsequently shown to be protected against a smallpox challenge. For more than 80 years, little more was done with respect to immunization, until Louis Pasteur discovered the attenuating effect of exposing pathogens to air or to chemicals. This discovery was achieved as the result of leaving cultures on the laboratory bench during a summer holiday. Thus, Pasteur developed the first vaccine made in the laboratory and also founded the terminology of vaccination [1, 2]. Since the time of Pasteur until recently, there have been two paths of vaccine development: attenuation or inactivation and the production of recombinant subunits. With regard to attenuation, heat, oxygenation, chemical agents, or aging were the first methods used, notably by Pasteur for rabies and anthrax vaccines. Passage in an animal host, such as the embryonated hen’s egg, was the next method, as practiced by Theiler for the yellow fever vaccine. After the development of in vitro cell culture in the 1940s, attenuation was accomplished by a variety of means, including selection of random mutants, adaptation to growth at low temperatures, chemical mutation to induce inability to grow at high temperature (temperature sensitivity), or induction of auxotrophy in bacteria. The second set of strategies are represented by the inactivation of the microorganism or by purifying small subunits derived from the pathogen of interest.

Late in the nineteenth century, Theobald Smith in the United States and Pasteur’s colleagues independently showed that whole organisms could be killed without losing immunogenicity. This new strategy soon became the basis of vaccines for typhoid and cholera and later for pertussis, influenza, and hepatitis A. Other approaches consisted in isolation of virulence factors from the microorganisms, such as toxins or capsular polysaccharides. In the 1920s, the exotoxins of *Corynebacterium diphtheriae* and *Clostridium tetani* were inactivated by formalin, to provide antigens for immunization against diphtheria and tetanus [1]. Extracted type-b polysaccharide capsule of *Haemophilus influenzae* was shown attractive as a vaccine antigen since the invasive disease was almost exclusively restricted to type-b organisms, and antipolysaccharide antibodies had an important role in natural immunity. However, early observations with Hib demonstrated the limitations of plain polysaccharide as a vaccine antigen. When given, during the first 2 years of life, purified polysaccharide induced relatively low levels of serum antibodies, typically insufficient to protect against invasive disease. Following further studies with a variety of bacterial polysaccharides, and in the light of the limitations of plain polysaccharide as vaccine antigens, the Hib polysaccharide

was shown to be more immunogenic when covalently linked to a protein carrier, giving additionally boosted responses characteristic of T-dependent memory [3, 4]. Overall, with the classical vaccinology approaches many infectious diseases can be prevented. Table 1.1 reports a list of vaccines licensed for immunization in the United States Food and Drug Administration (FDA) [5].

However, the above-mentioned approaches have several limitations, including the fact that, in some cases, pathogenic microorganisms are difficult to culture in vitro and, therefore, production of live attenuated, inactivated, or subunit vaccines becomes impractical and time consuming. As a result, there are several infectious diseases for which these traditional approaches have failed and for which vaccines have not yet been developed.

TABLE 1.1. Vaccines Licensed for Immunization and in the United States approved by FDA

<i>Subunit Vaccines</i>
Anthrax
Tetanus
Diphtheria
Pertussis
<i>Haemophilus influenzae</i>
Hepatitis B
Human papillomavirus
Meningococcus (groups A, C, Y, and W-135)
Pneumococcal
Tetanus
Typhoid ^a
<i>Live Vaccines</i>
Tuberculosis
Measles
Mumps
Rotavirus
Varicella
Yellow fever
Herpes zoster
Smallpox
<i>Inactivated Vaccines</i>
Hepatitis A
Influenza ^a
Japanese encephalitis
Plague
Polio
Rabies

^aAdditional live vaccine.

1.3 REVERSE VACCINOLOGY

1.3.1 Classical Reverse Vaccinology: The MenB Story

Conventional approaches to develop vaccines, as described above, are based on the cultivation of the microorganisms *in vitro*, and only abundant components can be isolated by using biochemical and microbiological methods. Although successful in many cases, these approaches have failed to provide vaccines against pathogens that did not have obvious immunodominant protective antigens [6]. With the advent of whole-genome sequencing and advances in bioinformatics, the vaccinology field has radically changed, providing the opportunity for developing novel and improved vaccines. The availability of the complete genome sequence of a free-living organism (*H. influenzae*) in 1995 [7] marked the beginning of a “genomic era,” which allowed scientists to use new approaches for vaccine design and for the treatment of bacterial infections. With this powerful sequencing technology, a new approach to identify vaccine candidates was proposed on the basis of the genomic information. This approach was called *reverse vaccinology* (Fig. 1.1). The novelty of reverse vaccinology was not based on growing microorganisms but on running algorithms to mine DNA (deoxyribonucleic acid) sequence information contained in the blueprint of the bacterium [8]. The first step of this *in silico* analysis is the appropriate combination of algorithms and the critical evaluation of the coding capacity. The predicted open reading frames (ORFs) are used for homology searches against a database with BLASTX, BLASTN, and TBLASTX programs to identify DNA segments with potential coding regions. Since secreted or extracellular proteins are more accessible to antibodies than are intracellular proteins, they represent ideal vaccine candidates and therefore the surface localization criterion is applied. The *in silico* approach results in the identification of

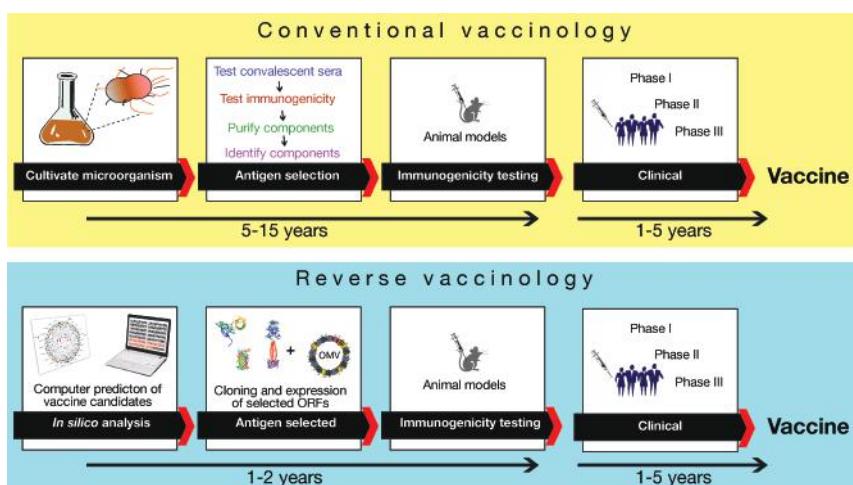


Figure 1.1. Schematic representation and time lines of classical vaccination in comparison to the reverse vaccinology approach. (See insert for color representation of this figure.)

a large number of genes. It is, therefore, necessary to use simple procedures that allow a large number of target genes to be cloned and expressed. Once purified, the recombinant proteins are used to immunize mice. The postimmunization sera are analyzed to verify the computer-predicted surface localization of each polypeptide and their ability to elicit an immune response. The direct means to study the protective efficacy of candidate antigens is to test the immune sera in an animal model in which protection is dependent on the same effector mechanisms as in humans [9].

The reverse vaccinology approach has been applied for the first time to the bacterial pathogen *Neisseria meningitidis* serogroup B. Although the use of vaccines based on the polysaccharide antigen has been successful for most of the species causing bacterial meningitis (*H. influenzae* type B, *Streptococcus pneumoniae*, and *N. meningitidis* serogroups A, C, Y, and W135), the same approach cannot be easily applied to meningococcus B. This is because the MenB polysaccharide is a polymer of $\alpha(2-8)$ -linked N-acetyl-neurameric acid (or polysialic acid), which is also present in glycoproteins of mammalian neural tissues. The poor immune response and the high risk of autoimmunity have hindered much of the research on the MenB polysaccharide [10]. An alternative approach to the MenB vaccine was based on the surface-exposed proteins contained in outer membrane vesicles (OMVs). These vaccines have been shown both to elicit serum bactericidal antibody responses and to protect against developing meningococcal disease in clinical trials [11–13]. Although these vaccines provided evidence of efficacy against the homologous strain, they show sequence and antigenic variability in their major components [14].

Thus, the genomic approach for target antigen identification was directed to develop a vaccine against serogroup B *N. meningitidis* [15]. *Neisseria meningitidis* is a Gram-negative diplococcus and an obligate human pathogen that colonizes asymptotically the upper nasopharynx tract of about 5–15% of the human population. Five serogroups (A, B, C, W135, and Y) account for virtually all of the cases of meningococcal disease [16]. *N. meningitidis* is the major cause of meningitis and sepsis, two devastating diseases that can kill children and young adults. Invasive meningococcal disease causes a significant public health burden worldwide, with approximately 500,000 cases and >50,000 deaths reported annually [6, 16]. MenB represents the first example of the application of reverse vaccinology and the demonstration of the power of genomic approaches for novel antigen identification. In 2000, an invasive isolate of *N. meningitidis* (MC58) was sequenced and analyzed to identify suitable vaccine candidates with the in silico approach described above. After discarding cytoplasmic proteins and known *Neisseria* antigens, 570 genes predicted to code for surface-exposed or membrane-associated proteins were identified. Successful cloning and expression was achieved for 350 proteins, which were then purified and tested for localization, immunogenicity, and protective efficacy. Of the 91 proteins found to be surface exposed, 28 were able to induce complement-mediated bactericidal antibody response, providing a strong indication of the proteins capability of inducing protective immunity [17]. Additionally, in order to test the suitability of these antigens for conferring protection against heterologous strains, the proteins were evaluated for gene presence, phase variation, and sequence conservation in a panel of genetically diverse MenB strains representative of the global diversity of the natural

N. meningitidis population [9]. This analysis yielded a handful of antigens, which were both conserved in sequence and able to elicit a cross-bactericidal antibody response against all of the strains in the panel, demonstrating that they could confer general protection against the meningococcus. To strengthen the protective activity of the single-protein antigens and to increase strain coverage, the final vaccine formulation comprises a “cocktail” of the selected antigens. This vaccine is currently in phase III clinical trials [17, 18].

1.3.2 Reverse Vaccinology Applied to the Pan-Genome Concept

In the last decade, microbial genomic sequencing has experienced an exponential growth. Sequencing of 1129 bacterial genomes have been completed and 2893 are currently in progress [19]. All of the genomic sequences are available in public databases, and they cover hundreds of species, as well as multiple pathogenic and commensal strains of the same species. Recently, an analysis of the *Streptococcus agalactiae* genome has led to the pan-genome definition for this pathogen [20]. It was suggested that the genomic sequence of a single strain is not genetically representative of an entire species, due to the surprising intraspecies diversity. Subsequently, in order to develop a universal vaccine with a broad range of coverage against the major circulating strains, a combination of antigens representative of different strains of the same pathogen should be included. Therefore, the classical reverse vaccinology approach has now been extended to a wider number of genomes belonging to the same species, as performed for *S. agalactiae*.

S. agalactiae (commonly referred to as group B *Streptococcus* or GBS) is an encapsulated Gram-positive coccus. GBS strains are classified into 9 serotypes according to immunogenic characteristics of the capsular polysaccharides (Ia, Ib, II, III, IV, V, VI, VII, and VIII), and approximately 10% of serotypes are nontypeable [21]. GBS was originally isolated in 1938 from animals [22], and is the main cause of bovine mastitis, an economically important problem in dairy cattle throughout the world [23]. However, invasive group B streptococcal disease emerged in the 1970s as a leading cause of neonatal morbidity and mortality in the United States [24] and represents the most common etiological agent of invasive bacterial infections (pneumonia, septicemia, and meningitis) in human neonates [25, 26]. The need for a vaccine against GBS is supported by the observation that the risk of neonatal infection is inversely proportional to the maternal antibody response specific for the GBS capsular polysaccharide. In fact, the transplacental transfer of maternal IgG antibodies protects infants from invasive group B streptococcal infection [27]. As a first approach to vaccine development, capsular polysaccharide (CPS)-tetanus toxoid conjugates against all 9 GBS serotypes were shown to induce CPS-specific IgG that is functionally active in opsonization against GBS of the homologous serotype. Clinical phase 1 and phase 2 trials of conjugate vaccines prepared with CPS from GBS types Ia, Ib, II, III, and V revealed that these preparations are safe and immunogenic in healthy adults. Although these vaccines are likely to provide coverage against the majority of GBS serotypes that cause disease in the United States, they do not offer protection against pathogenic serotypes that are more prevalent in other parts of the world (e.g., serotypes

VI and VIII, which predominate among GBS isolates from Japanese women). Hence, a universal protein-based vaccine against GBS is desirable [28]. In 2002, the whole genome sequences of 2 strains of GBS were assembled and released in the public domain. Serotype V strain 2603V/R is representative of an emerging serotype accounting for approximately 18% of early-onset cases, 14% for late-onset cases, and 31% for nonpregnant pediatric and adult cases [29, 30]. The serotype III strain (NEM316 strain) is the most common clinical serotype encountered in neonatal meningitis cases and was isolated from a fatal case of septicemia [31]. Using the sequenced strain 2603V/R as a reference, comparative genomic hybridization (CGH) was performed to circumvent the need for sequencing closely related genomes. Genomic comparisons reveal regions of loss and/or retention with respect to the reference strain. It was found that approximately 18% of the genes encoded in the sequenced strain are absent in at least one of the other 19 *S. agalactiae* strains. However, CGH experiments are limited in that they only identify the portion of the sequenced genome that is shared with the other test strains and are not able to detect genes that are absent in the reference genome. Following this work, the sequences of 6 additional strains of *S. agalactiae* were determined [31, 32]. Genome analysis of 8 clinical isolates of GBS, which are representative of the serogroup diversity and responsible for >90% of human infections in the United States, revealed the global gene repertoire of the GBS bacterial species to consist of 1806 genes present in every strain (core genome), plus 907 dispensable genes that are present in one or more but not all strains (dispensable genome) (Fig. 1.2a) [33]. In general, the core genome includes all genes responsible for the survival of the bacterium and its major phenotypic traits. By contrast, dispensable genes contribute to the species diversity and may encode supplementary biochemical pathways and functions that are not always essential for bacterial growth but that confer selective advantages, such as adaptation to different niches, antibiotic resistance, or colonization of a new host. Such genes are generally clustered on large genomic islands that are flanked by short repeated DNA sequences and are characterized by an abnormal G + C content. Investigation and functional annotation of dispensable genes reveals that hypothetical and phage- and transposon-related genes account for the vast majority of these findings (Figs. 1.2b and 1.2c). Moreover, computational predictions suggest the more genomes are sequenced, the more new genes will be found that belong to the pan-genome [20]. In fact, the pan-genome is predicted to grow about 33 new genes every time a new strain is sequenced. This profile is different from that observed for other microorganisms, such as *Bacillus anthracis*. Eight genome sequences were determined for this microorganism, but after the fourth it was verified that the number of new genes added to the pan-genome rapidly converged to zero [20, 34].

In order to identify protective antigens for GBS, computer algorithms were used to select the genes encoding putative surface-associated and secreted proteins. Among the predicted surface-exposed proteins, 396 were core genes and 193 were variable genes. Of these 589 proteins, 312 were successfully cloned and expressed in *Escherichia coli*. Then each antigen was tested in a mouse maternal immunization assay to evaluate the capacity to confer protection. No individual core proteins or a combination thereof provided high levels of immunity against a panel of GBS isolates. The best candidates, namely GBS67, GBS80, GBS104, and GBS322, identified through the reverse

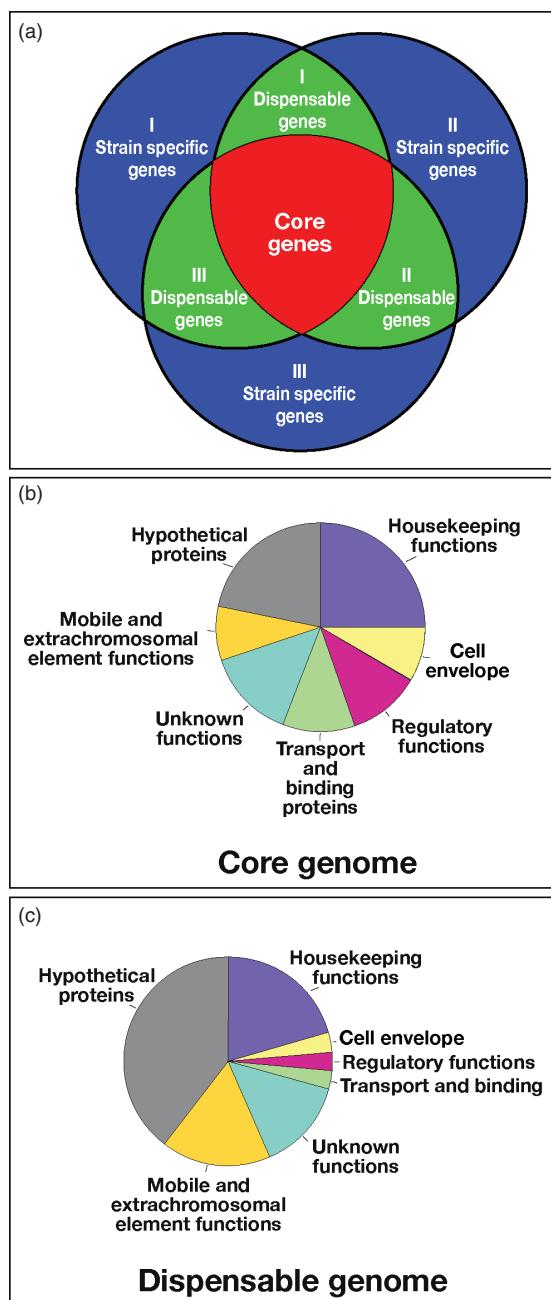


Figure 1.2. Pan-genome structure. (a) Schematic representation of the pan-genome comparative analysis performed on a set of three different strain genomes. Venn diagram overlapping circles represents core, dispensable genome, and strain-specific genes. (b–c) Gene classification of a new sequenced genome according to the pan-genome structure. (See *insert* for color representation of this figure.)

vaccinology combined with the multigenome comparison approach, belonged to the dispensable genome with the exception of GBS322, which belongs to the core genome. The most significant protection results were achieved when the four best candidates were combined together. The four-protein cocktail conferred 59–100% protection against a panel of 12 GBS isolates, including the major serotypes, as well as 2 strains from the less common serotype VIII (81 and 94% protection) [28]. Multigenome sequencing from different streptococcal species (GBS, group A *Streptococcus*, and *Pneumococcus*) has revealed to be an important approach, not only for antigen selection but also because it has led to the discovery of important virulence factors such as pili [35–37].

1.3.3 Subtractive Reverse Vaccinology: The ExPEC Experience

Many bacterial species exist in the human gut flora, both as commensal and pathogenic strains. By comparing the genomic sequences of the two types, one can identify pathogenic specific traits. In this regard, a new concept of subtractive reverse vaccinology was applied for the first time to the pathogenic *E. coli* species. *E. coli* is a commensal member of the gastrointestinal flora of most mammals. Additionally, several facultative and obligate pathogenic variants exist that cause various types of intestinal or extraintestinal infections in humans and animals. Extraintestinal pathogenic *E. coli* (ExPEC) is a facultative pathogen known to cause infections such as urinary tract infection (UTI), newborn meningitis, and sepsis [38]. Thus, the development of an efficacious ExPEC vaccine would have a significant public health and economic impact, considering the increasing antibiotic resistance among ExPEC strains and the associated mortality and morbidity. Conventional vaccinology approaches (whole cell, single antigens, or polysaccharide based) have not been successful in providing a highly immunogenic, safe, and cross-protective vaccine against ExPEC strains. Recently, Moriel et al. [39] described an innovative approach based on reverse vaccinology to develop a vaccine against the extraintestinal *E. coli* infections. As a first step, the neonatal meningitis-associated *E. coli* K1 strain IHE3034 genome was sequenced. This sequence was then compared to that of the nonpathogenic *E. coli* MG1655, DH10B, and W3110 strains since a vaccine directed against commensal or nonpathogenic strains could have potential implications for the equilibrium of the normal human intestinal flora. In particular, the comparison between IHE3034 and MG1655 revealed the presence of 19 genomic islands absent in the MG1655 genome. Many of these genomic islands contained the typical features of the pathogenicity islands (PAI), such as the presence of an integrase and transfer ribonucleic acid (tRNA) genes and a different G+C% content compared to the core genome. Subsequently, an *in silico* analysis was performed comparing IHE3034 with two other ExPEC strains, namely 536 and CFT073.

By *in silico* analysis, specific ExPEC-shared antigens predicted to be surface associated or secreted were selected, whereas the presence and the level of similarity of these antigens in the nonpathogenic strains mentioned before were used as exclusion criteria (hence the term subtractive). Among the predicted surface-exposed proteins, nine vaccine candidates were identified to confer protection. Interestingly, the gene

encoding for the most promising antigen ECOK1_3385 was found both in pathogenic and nonpathogenic strains, and located downstream to a type two secretion system (T2SS); in the nonpathogenic strains this T2SS is truncated, and consequently the antigen is expressed intracellularly but not secreted. By contrast, in pathogenic strains, the protein encoded by ECOK1_3385 is secreted into the supernatant, suggesting that it may play a role in virulence. Additionally, studies on the variability of ECOK1_3385 in a panel of 96 ExPEC strains showed a significant level of sequence conservation ranging from 86 to 100% identity. Furthermore, gene distribution analysis in 573 isolates representing diverse *E. coli* phylogeny revealed a higher proportion of ECOK1_3385 in extraintestinal and intestinal strains (67%) compared to commensal strains (30%).

Therefore, the subtractive reverse vaccinology approach used in this work has lead to the identification of nine potential antigens present in ExPEC and intestinal *E. coli*, suggesting that they may be useful for a broadly cross-reactive *E. coli* vaccine.

1.4 VACCINE DESIGN: FROM CONVENTIONAL VACCINOLOGY TO THE POSTGENOMIC ERA THROUGH REVERSE VACCINOLOGY

Despite antimicrobial therapies, infectious diseases remain the leading cause of death worldwide [40]. Vaccination is a powerful public health tool, with the global eradication of diseases such as smallpox and poliomyelitis (in the Western Hemisphere). Most of the current vaccines have been developed by adaptation of living organisms to growth conditions that attenuate their virulence, by preparation of suspensions of killed microbes or through the concentration and purification of proteins or polysaccharides from pathogens [41]. With the development of DNA sequencing technology, in the late 1990s the way for vaccine candidate identification has radically changed. The traditional reverse vaccinology approach has been integrated and flanked by the multigenomes comparison and after that by postgenomic technologies, which comprise the combination of transcriptomics, proteomics, and immunomics. Postgenomic strategies are referred to as *functional genomics*, complementing the *in silico* antigen discovery, not only by directly examining the genetic content but also the transcription and expression profiles of pathogens.

In particular, the study of global changes in bacterial gene expression is essential for understanding pathogenesis and survival in the host. For vaccine antigen discovery, it is important to know which genes are regulated *in vivo*, during infection, because they could represent protective vaccine candidates. The availability of complete genome sequences stimulated the application of complementary DNA (cDNA) microarrays in identifying the genes involved in microbial virulence, pathogenesis, and therefore useful for vaccine design. cDNA microarrays are used to study the gene expression profile of tens of thousands of genes in a single experiment. The first example where microarray-based transcriptional profiling was successfully used to identify potential vaccine candidates was in the case of MenB [42]. For the identification of new vaccine targets for MenB, transcriptional profiling studies were performed under experimental conditions mimicking certain aspects of host-pathogen interactions, such as adherence to host epithelial cells and exposure to human serum. In this

study, RNA was prepared from adherent and nonadherent bacteria, and the two RNA preparations were comparatively analyzed by DNA microarrays bearing the complete complement of polymerase chain reaction (PCR)-amplified MenB genes [42, 43]. Twelve proteins, whose transcription was found to be particularly activated during adhesion, were expressed in *E. coli*, purified, and used to produce antisera in mice. Five sera showed bactericidal activity against different strains.

While the transcriptomics approach gives a global expression profile of the gene up- and down-regulated during a particular growth condition, the proteomics approach refers to a high-throughput analysis of the complete complement of proteins, allowing the selection of a small group of antigens expressed at high levels and surface exposed [44]. In this approach, following separation by two-dimensional (2D) gel electrophoresis, the proteins are represented as fine spots on a gel that are isolated for further analysis. Mass spectrometric techniques like matrix-assisted laser desorption ionization, time of flight (MALDI–TOF) and tandem mass spectrometry (MS/MS) are used for peptide mass and sequence analysis of a given protein spot. Advancements in proteomic approaches have enabled researchers to explore this novel strategy in order to identify vaccine targets and proteins of therapeutic interest [43]. A powerful and attractive application of proteomics to identify vaccine candidates was recently reported by Grandi and colleagues, who analyzed the surface proteome of *Streptococcus pyogenes* (mainly referred to as group A *Streptococcus*, GAS) [45]. Whole cells were treated with proteases to selectively digest cell surface proteins. The resultant peptides were subsequently identified by mass spectrometry. Application of this technique to the sequenced *S. pyogenes* strain M1_SF370 resulted in the identification of 68 predicted surface-associated proteins. The approach proved to be rapid and highly selective in that the large majority (>90%) of the identified proteins fell into the categories of cell wall proteins, lipoproteins, membrane proteins, and secreted proteins. Furthermore, the method allowed for a semiquantitative evaluation of protein expression. Indeed, the number of peptides identified from a given protein correlated with the extent of its recognition by specific antibodies, as judged by fluorescence-activated cell sorting analysis. Interestingly, the list of surface-associated proteins included most of the published GAS protective antigens, as well as new protective components such as the cell envelope proteinase Spy0416, a new attracting protein for its important role in pathogenesis [45]. Recently, a similar approach has been used to identify new protective antigens in *S. agalactiae*. The authors showed that on the surface of the hypervirulent GBS COH1 strain are present 43 major proteins belonging to the families of cell wall proteins, lipoproteins, and membrane proteins. As in the case of GAS, the proteins identified comprise all protective antigens so far described in the literature as well as a new antigen, SAN_1485, which appears to be highly protective in the active maternal immunization mouse model. These data confirm the effectiveness of protease digestion coupled to mass spectrometry for the identification of surface-exposed antigens in Gram-positive bacteria and demonstrate the power of this technology for the rapid discovery of new vaccines [44]. Therefore, a combination of proteomic-based approaches and serological analysis can identify potential vaccine candidates and provide effective validation of these candidates. A number of methods have been developed to enable the high-throughput display of

the proteome of a pathogen to the host immune system. Immunomics [also known as serological proteome analysis (SERPA)] combines proteomic-based approaches with serological analysis, and it has been widely applied for antigen discovery and vaccine development. Applied to *Staphylococcus aureus*, this approach led to the identification of 15 highly immunogenic proteins, including known and novel vaccine candidates [46].

A number of infectious diseases are still waiting for an effective vaccine and new approaches are required to identify new promising vaccine candidates. Structure determination by high-throughput methods such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, or molecular electron microscopy together with the availability of a huge number of genomic sequence data has led to the growth of the field of structural genomics. The primary aim of structural genomics is to determine the three-dimensional profile of all important biological macromolecules, with a primary focus on proteins. The secondary goal is to decrease the average cost of structure determination through high-throughput methods for protein production and structure determination [47]. A complete view of protein folds may help in assigning function for hypothetical proteins, with the prediction of the protein structure by computational approaches such as homology modeling, founded on similarities with known protein structures for constructing atomic-resolution models from amino acid sequences. Structural genomics was used in the past two decades in the rational design of important chemotherapeutics, such as the human immuno deficiency virus (HIV) [48] or influenza drugs [49], on the basis of the analysis of the complex structures from the target protein. To be effective, a vaccine must induce a strong protective immune response from B and T cells. Since antibodies, by specific recognition of antigen epitopes, are an effective line of defense in preventing infectious diseases, understanding the antibody–epitope interaction establishes a basis for the rational design of vaccines, leading to the development of epitope-driven vaccines, containing only selected epitopes that have been already described, for example, in the case of cancer [50]. Overall, a complete understanding of protein structure and function and the study of functional complexes between a given macromolecule and its effectors in the host will facilitate the rational design of vaccines.

1.5 CONCLUSIONS

We have come a long way since Edward Jenner's landmark experiment with cowpox, an event that over the past two centuries has developed into the field of vaccinology. The current “-omics” revolution has provided researchers with advanced technologies that are decreasing the time it takes to identify and design target antigens. These high-throughput technologies have proved to be powerful in the discovery of vaccine candidates for group B meningococcus and now more recently many other bacterial pathogens. However, the decision of which of these large number of candidates to take forward in clinical trials still remains a challenge [43]. The future success of vaccines will not only depend on the advancement of scientific platforms but also the interdisciplinary involvement of researchers in different fields such as structural

biology, physical chemistry, epidemiology, and molecular immunology. The success of vaccines will be possible only through innovative ideas that will lead to fundamental breakthroughs.

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2

DESIGN AND DEVELOPMENT OF RECOMBINANT VACCINES WITH VIRAL PROPERTIES

Gerd Lipowsky and Martin F. Bachmann

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2.1 INTRODUCTION

Vaccination started its triumphant advance at the end of the eighteenth century with the famous breakthrough experiments and ideas of Edward Jenner who showed that inoculation with cowpox can protect against the otherwise often deadly smallpox infection. At that time, smallpox was a global threat, with more than 400,000 deaths per year only in Europe. Today smallpox is eradicated worldwide. Consequently, Jenner can be considered as the discoverer of protective immunization, and the initial empiric approach turned out to be one of the most successful medical interventions in history. By using a nonvirulent (bovine) analog of the highly pathogenic human

smallpox virus, an immune response against almost identical antigens present on the surface of both poxviruses was induced, hence conferring cross protection [1]. Later, Pasteur's seminal research then opened the possibility to selectively render pathogenic microbial strains into nonvirulent homologs that could be of potential use for safer vaccination campaigns [2].

Since then active vaccination has been proven to be protective against a large number of pathogens causing acute diseases, such as viruses (smallpox, polio, measles, rubella, mumps, etc.) or extracellular bacteria (*Haemophilus influenzae*, *Bordetella pertussis*, *Corynebacterium diphtheriae*, etc.).

Common to all simple vaccine formulations, which rely either on live-attenuated or killed microorganisms or detoxified (chemically inactivated) exotoxins, is the induction of a strong humoral immune response. In addition, vaccination with live-attenuated viruses mounts a strong T cell-mediated immunity, while their killed counterparts usually do not. Classical technologies for generating live-attenuated viruses, which are suitable as vaccines, include (i) repeated cell culture passages until mutants with desired features (e.g., reduced replication competence) could be selected (e.g., measles or polio Sabin), (ii) selection of cold-adapted viruses (e.g., influenza: FluMist), or (iii) reintroducing protective antigens from circulating wild-type strains into attenuated viruses by co-culturing both strains (leading to so-called recombinant live-attenuated vaccines such as the West Nile virus vaccine ChimeriVax).

Since living and attenuated pathogens essentially mount a similar humoral and cellular immune response in the host, these types of vaccines have been developed with great success. The provoked activation of the immune system by these vaccines is (i) sufficient to protect against infection with the live pathogen(s), (ii) in most cases long-lasting, and (iii) a cost-effective, broadly applicable live-preserving measure as often one immunization is sufficient to confer long-lasting protection. Although very efficient, the use of live-attenuated vaccines will be inevitably accompanied by safety concerns. There is the theoretical possibility that (i) the live-attenuated microbe reverts back to the virulent phenotype and (ii) that they may spread to nontarget subjects, such as immunocompromised individuals. Under these considerations, vaccines based on (chemically) inactivated or killed organisms are of a certain advantage. Still, the immune system is confronted with a complete set of antigenic determinants of the pathogen, but the virulence, that is, the capability of replication, is completely abrogated and hence the vaccine should have a better safety profile. Examples for this class of killed or inactivated microorganisms in vaccine formulations include polio (Salk), influenza, or hepatitis A virus. However, the mentioned favored safety profile comes at the expense of immunogenicity. Since killed or inactivated pathogens have lost their replication capacity, repeated booster immunizations in combination with adjuvants (like alum) are required to confer full and long-lasting protection. In addition, the virus needs to be produced in large amounts, which is not always possible. The identification of key virulence factors or toxins, which account for the pathogenesis, allows for the stepwise substitution of whole microorganisms by pathogen extracts or subunits (proteins or polysaccharides) in modern vaccines. The best established examples are antibacterial vaccines, which either consist of inactivated toxins (so-called toxoids) or pathogen-derived carbohydrates coupled to carrier

proteins [3–6]. Immunization with these vaccines induces neutralizing antibodies, which prevent severe disease (e.g., *Clostridium tetani*, *C. diphtheriae*, *B. pertussis*, *Meningococci*, etc.) without risking an unwanted spread of the infection. As discussed above for vaccines based on killed or otherwise inactivated microorganisms, the increased safety of the vaccines is linked to lower immunogenicity and, hence, booster immunizations as well as efficient and safe adjuvants are required.

In contrast, pathogens that are capable of evading recognition by the immune system are still a major challenge for vaccine development in the twenty-first century. Viruses such as the human immunodeficiency virus (HIV) or hepatitis C virus (HCV) cause chronic viral infections and appear to permanently escape recognition by the immune system. Similarly, some eukaryotic parasites have complex life cycles and continuously alter their surface (e.g., *Trypanosomes*). An alternative strategy employed by pathogens is to live and/or hide in poorly accessible cells, such as erythrocytes (e.g., *Plasmodium spec.*) or neurons (e.g., *Herpes* viruses). Some pathogens finally live in a sequestered area, for example, within granulomas (*Mycobacterium spec.*). These strategies have proven very successful ways to avoid the denunciation and clearance by the immune system [7]. Combating these pathogens with the aid of the immune system will require a novel class of vaccines, which should be able to induce both broadly neutralizing antibodies as well as cellular responses (e.g., cytotoxic T cell response). Integrating insights from virology, molecular cell biology, immunology, genetics, epidemiology, and biotechnology, and clinical research will be required to develop vaccines against these types of pathogens. One famous example where this multidisciplinary approach has already culminated in the approval of a prophylactic vaccine is the development of a recombinant vaccine with viral properties against cervical cancer, which relies on the neutralization of the causing pathogen, called the human papilloma virus (HPV) [8].

A prerequisite of designing these future types of vaccines is to analyze the molecular patterns and immunological properties of viruses, understanding the complex pathogen–host interaction(s) in molecular detail, deciphering the fundamental disease-causing mechanisms, and applying these findings to induce tailored immune responses with a safe and effective profile. Ultimately, this should not only be helpful for the development of vaccine-based therapies against complex infections but could also be employed for the treatment of other noninfectious chronic maladies, such as allergies, cancer, or other chronic disorders [9, 10].

Modern cellular immunology and transgenic animals are now providing for the first time the tools for rapid identification and testing of key molecules that are believed to be the central driving forces of these conditions. A growing number of cytokines, chemokines, growth factors, peptide hormones, and disease-linked surface antigens have been identified as potential therapeutic targets. Still, it is a challenging task to utilize this wealth of information in the development of vaccine-based pharmaceuticals. This chapter will focus on the molecular mechanisms and patterns underlying the unique immunogenicity of viruses, on the identification and usage of immunomodulating adjuvants, and discuss novel strategies that integrate these findings for the development of a new class of vaccines. Recombinant vaccines with viral properties hold a strong future promise in giving cost-effective, efficacious,

and globally applicable therapeutic/prophylactic treatment by targeting nonself and self-molecules of infectious and noninfectious disorders.

2.2 VIRAL PROPERTIES AND IMMUNE RESPONSES

The restraint in well-being by the potential health threat in the cause of microbial infections has led to the co-evolution of powerful immunological responses that guarantee that a healthy organism can deal in an appropriate manner with the invaders.

A prerequisite for an adequate response is that the responding organism is able to sense the microorganism. For that purpose, different specialized cells express certain receptor molecules recognizing the pathogen as foreign. After these cells have encountered a virus, bacterium, fungus, or helminth, they integrate the pathogen-derived signals and mount an appropriate immune response. Pathogens and host organisms have co-evolved, leading in mammals to a strong and optimal activation of the two branches of the immune system: initially the innate and subsequently the adaptive (acquired) immune response. An additional hallmark of the immune system is its ability to remember (immunological memory). With respect to our knowledge of microbial infections the immune responses against viruses are best characterized. Four characteristic features of viruses determine the host's immune response (see Fig. 2.1):

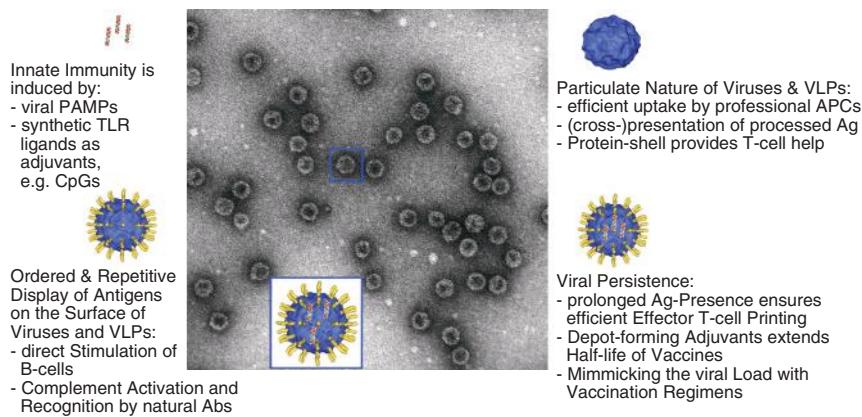


Figure 2.1. Immunogenic viral properties. Electron microscope (EM) picture of recombinantly expressed virus-like particles (VLPs) derived from capsids of the bacteriophage Q β (250,000X-fold magnification). Alongside the EM picture immunomodulating properties of VLPs are schematically illustrated. The four characteristic features of viruses, which determine the host's immune response and their translation into rationally designed vaccines are listed. Capsids forming the VLPs are displayed in blue, the antigen arrays on the surfaces are depicted in yellow. Synthetic TLR ligands, which can be incorporated into particulate vaccine delivery systems are shown as short red/blue colored helices. Note that VLPs are representative for particulate macromolecular assemblies that are developed as vaccines. Ag: antigen; APCs: Antigen-presenting cells; PAMPs: pathogen-associated molecular patterns; TLR: Toll-like receptor. (See *insert for color representation of this figure.*)

(i) they display so-called pathogen-associated molecular patterns (PAMPs), which are efficiently recognized by the host's pattern recognition receptors (PRRs), not only to trigger an innate response but also to bridge innate responses to the acquired immunity; (ii) they are of a particulate nature that facilitates their uptake by phagocytic cells of the host [including macrophages and dendritic cells (DCs)], thereby ensuring that viruses are targeted *in vivo* to an antigen-presenting cell (APC like DC), which will present viral antigens on major histocompatibility complex (MHC) molecules to trigger an optimal adaptive immune response; (iii) they carry highly repetitive surface structures that are recognized by the immune system as "danger signals" and hence can be coated by complement factors and/or natural antibodies or directly induce production of antibodies by B cells through the efficient crosslinking of specific immunoglobulins on their surface; and (iv) they replicate *in vivo* leading to a prolonged presence of viral antigens in the host. This is sensed by the immune system and greatly facilitates the generation of an effector and memory T cell response [11, 12].

In sum, an invading pathogen first induces an innate response that is followed by a humoral and cellular adaptive immune response with the accompanying attribute of immunological memory.

2.2.1 Viruses and Innate Immunity

The innate immune system has evolved to serve as a first line of defense against microbial infections [11, 13]. It can be seen as an ancient immune system that is present not only in vertebrates but also in invertebrates such as *Drosophila* or *Caenorhabditis elegans* or even in plants. In fact, our rapidly growing knowledge about the interactions of pattern recognition receptors with their cognate PAMPs and the induced signaling cascades has its origin in the discovery of *Toll* genes in the fruitfly *Drosophila*. The basic machinery underlying innate immunity as well as the order of signaling events are evolutionary conserved among invertebrates, plants, and mammals [14–18]. A hallmark of the innate immune response is its immediate activation within minutes after invasion of the host. It serves as potent defense during the initial hours and days of an infection. As for the adaptive immune system, one can distinguish between a cellular and humoral response mediated by cells such as neutrophils, monocytes, and natural killer (NK) cells or soluble factors such as complement, lysozyme, and defensins, respectively. Although the innate immune system was believed to be largely nonspecific, this dogma has been questioned by the discovery of the PRRs and their cognate pathogen-associated patterns. PAMPs reflect specific characteristics of the microbe and help to distinguish invading pathogens from cells of their hosts. These patterns include surface-exposed components of viral envelopes and bacterial or fungal cell walls, flagellar proteins, as well as viral and bacterial nucleic acids. PAMPs are essential for the survival of the microorganism, and hence their variation in structure and sequence is believed to be restricted. Consequently, a rather limited number of PRRs appears to be sufficient to sense the conserved molecular patterns [19–23].

Activation of the innate immune system by the pathogen induces a rapid burst of nonspecific toxic substances such as NO, radicals, and/or defensins (toxic peptides), as well as the secretion of inflammatory cytokines and/or chemokines. These mediators

either directly battle against the invader or recruit and activate further immune cells and are triggering the immune system toward the subsequent adaptive immune response [24].

Central to the antiviral innate immune response is the synthesis and secretion of type I interferons (IFNs). Human type I IFNs include 13 IFN- α isotypes and one type of IFN- β , IFN- ϵ , IFN- κ , and IFN- ω [25]. They are helical cytokines with high sequence homology among the a-subtypes (up to 80% sequence identity) and moderate homology to the other subtypes [26]. All share a common heterodimeric cell surface receptor called type I IFN receptor consisting of interferon (alpha, beta and omega) receptor 1 and 2 (IFNAR1 and IFNAR2) [27]. The IFNAR2 subunit alone can bind IFNs with high affinity, whereas the IFNAR1 subunit binds type I IFNs only with low affinity [28, 29]. Activation of the receptor through type I IFN binding goes along with dimerization of the receptor initiating a signaling cascade via Janus activated kinase family members, namely TYK2 or JAK1 and IFN-stimulated gene factor 3 (a trimeric complex consisting of activated STAT1/STAT2 and IFN regulatory factor 3). This complex translocates to the cell nucleus and activates transcription of target genes by binding to specific sites [IFN-stimulated response elements (ISREs)] in the promoters of IFN-stimulated genes (ISGs) [30]. Meanwhile hundreds of IFN-inducible genes have been indentified. The expressed effector molecules directly influence cellular processes such as protein synthesis, cell growth and survival, and induce an antiviral state. In addition, type I IFNs also contribute to the maturation of DCs, enhance antibody responses in B cells, mediate induction of CD8+ T cells and recruit lymphocytes and monocytes to sites of inflammation [31]. Prominent members of the ISGs are protein kinase R (PKR) and 2'-5' oligoadenylate synthetases (OAS) [32]. Both recognize double-stranded ribonucleic acid (dsRNA), which represents a molecular intermediate during viral replication of many viruses.

After binding to cytoplasmic dsRNA, PKR becomes activated and phosphorylates eukaryotic translation initiation factor eIF2-a [33, 34]. As a consequence, cellular protein translation might be repressed, which is fatal for the replicating virus that depends on the host cell translational machinery. However, it may also be lethal for the infected cell [35, 36].

In addition to PKR, OAS proteins belong to the best characterized IFN-I-induced effector molecules with antiviral activity. After their activation by binding to cytoplasmic dsRNA, they stimulate endoribonuclease RNase L through the synthesis of short oligoadenylylates. Activated RNase L eventually promotes the cleavage of both viral and cellular RNAs [37, 38].

Since both antiviral strategies have profound inhibitory effects on basal cellular processes, they rather act as suicide missions, eliminating virus-infected cells. Although PKR and OAS/RNase L become activated by viral dsRNAs, they are downstream of IFN I and are dispensable for IFN synthesis and secretion [39]. Therefore, other sensors must exist that are responsible for the initial up-regulation of type I IFNs in the course of a microbial infection.

Viral PAMPs such as dsRNA are also recognized by other cytoplasmic sensors, which belong to the family of DExD/H box RNA helicases. These intracellular sensors

are encoded by retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and LGP2 [40–42]. RIG-I (also called DDX58) and MDA5 (also called Helicard) both bear two N-terminal caspase recruitment domains (CARDs), which are also characteristic for another class of PAMPs, the so-called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) involved in the intracellular sensing of bacteria and/or danger-associated host components. In contrast, LGP2 does only feature the RNA-helicase domain. In fact, the LGP2 helicase rather prevents activation of antiviral responses, most likely through sequestration of dsRNA [43]. This might be a mechanism to limit activation of RIG-I and MDA5. When RIG-I or MDA5 are stimulated in the course of a viral infection, activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) and interferon regulatory factor 3/7 (IRF3/7) is triggered, both cooperating in the induction of type I IFN expression. Interestingly, the C-terminal regulatory domain (RD) of RIG-I is able to bind also single-stranded (ss) viral RNA in a 5'-triphosphate-dependent manner [44, 45]. The 5'-triphosphate group is added by virally encoded polymerases and is a distinctive feature of viral ssRNAs. A positively charged groove located in the RD seems to form the structural prerequisite for 5'-triphosphate binding, and this particular motif is distinct in the helicases MDA5 and LGP2. This may explain the elusive binding of 5'-triphosphate ssRNA by RIG-I [46, 47].

Beside viral RNA, viral deoxyribonucleic acid (DNA) can also be sensed by a cellular protein called DNA-dependent activator of interferon-regulatory factors [DAI; previously also known as DLM-1 or Z-DNA binding protein (ZBP)-1]. This protein is responsible for the TLR9-independent induction of type I interferon genes upon infection with DNA viruses [48]. Beside these ubiquitously expressed cytosolic sensors of viral nucleic acids, a second category of PRRs exists that primarily recognizes extracellular PAMPs. Toll-like receptors (TLRs) are type I integral membrane glycoproteins expressed either on the cell surface (TLRs 1, 2, 4, 5, and 6) or the endosomal compartment (TLRs 3, 7, 8, and 9) and hence—in contrast to the RIG-like helicases, DAI and NOD-like receptors—they are integrating signals from the extracellular environment [39, 49–51].

In mammals, at least 13 members of the TLR family exist and for most of the TLRs specific PAMPs, recognized by them, have been identified. These PAMPs derive from either bacteria, fungi, protozoan parasites, viruses, but also the host itself [51, 52]. TLRs are expressed mostly on immune cells [like dendritic cells (DCs), B cells, macrophages, neutrophils, mast cells, and natural killer cells] but also on some nonimmune cells (like fibroblasts and epithelial cells). All TLRs contain varying numbers of leucine-rich repeat (LRR) motifs building up the extracellular domain [53] and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the *Toll/IL-1R* homology (TIR) domain [54]. Consistently, TLRs share most of the signal transduction machinery with the IL-1R. After ligand binding, TLRs dimerize, undergo conformational changes, and eventually recruit TIR domain-containing adaptor proteins, including myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM) [55, 56]. In general, distinct TLR ligands can mediate different responses through the selective recruitment of these adaptor

molecules. While signaling via the MyD88 pathway stimulates production of proinflammatory cytokines, TRIF is essential for MyD88-independent signaling, which can lead to the induction of both proinflammatory cytokines and type I IFNs [57–62]. Interestingly, TLRs that are expressed on the cell surface are involved in sensing mainly bacterial products, including triacyl lipopeptides (PAMPs of TLR1/TLR2), lipopolysaccharides (PAMP of TLR4), flagellin (PAMP of TLR5), lipoteichoic acid (PAMP of TLR6), and others, whereas the TLRs located in the late endosomal compartment seem to be specialized in the recognition of viruses. The viral PAMP “dsRNA,” which is the genome of dsRNA viruses or is generated as a replication intermediate for ssRNA viruses and during symmetrical transcription in DNA viruses, is recognized by TLR3 [63]. TLR3 is the only TLR that signals solely independently of MyD88 and recognizes also a synthetic analog of dsRNA, namely polyinosine-deoxycytidylic acid (poly I:C) [64–66]. TLR3 is expressed in conventional DCs, but not in plasmacytoid dendritic cell (pDCs), and on the surface of a variety of epithelial cells, which may function as efficient barriers to infections. TLR7 and TLR8 are sensing guanosine and/or uridine-rich viral ssRNA and are located exclusively in the endosomal compartment of various cell types, including DCs, macrophages, and B cells [67, 68]. The restricted subcellular localization of TLR7 and TLR8 confine their accessibility to viral ssRNA since many enveloped viruses enter the cytosol through the endosomal compartment where acidification and enzymatic degradation is leading to ssRNA release. TLR7 can also recognize ribonucleic acid homologs such as imidazoquinoline components [Resiquimod (R-848), Imiquimod] and guanine nucleotide analogs (e.g., Loxoribine) [69–71]. Of note, self RNA released from dead or apoptotic cells is unlikely to reach the endocytic compartment since they are not sheltered in capsids and hence will be easily degraded by extracellular RNases.

A fourth receptor involved in the recognition of viral (and bacterial) nucleic acids in the endosomal compartment of pDCs, mast, and B cells is TLR9 [72–74]. TLR9 senses unmethylated 2'-deoxyribo (cytidine–phosphate–guanosine; CpG) DNA motifs that are present in genomes of microbial pathogens but are not common within mammalian genomes [75]. Synthetic CpG oligonucleotides mimicking viral or bacterial genomes are potent inducers of TLR9 signaling [76, 77].

In general, it is believed that signaling via TLRs and other PRRs does cooperate in innate immune responses. Synergy may not only exist between ligands of different TLRs but also between ligands of TLRs and other PRRs. Since different cells of the immune system express distinct combinations of PRRs, only the integrated sum of synergistic signals induced by the triggered subset of PRRs allows and enables the innate immune system to react to the plethora of different pathogens in an efficient and appropriate manner. Furthermore, the subsequent adaptive immune response will be initialized. For example, different subsets of DCs, which are essential for T cell priming, express distinct types and numbers of PRRs [78]. The specific combination of PRRs and pathogen-induced activation pattern on DCs in conjunction with the strength of the signal received from the T cell receptor-major histocompatibility complex (TCR–MHC) interaction causes a specific cytokine profile that finally determines the fate of T cell differentiation. Plasmacytoid DCs strongly express the endosomal PRRs, TLR7 and TLR9, detecting viral genomes such as ssRNA and dsDNA, respectively,

and can therefore mount a strong antiviral response by the synthesis and secretion of type I interferons such as IFN- α [79, 80]. IFN- α directly mediates antiviral responses, promotes cross presentation, as well as the induction of cytotoxic T cells (CTLs) and Th1-type responses, which are characterized by the production of IFN- γ . On the other hand, human myeloid dendritic cells express a variety of surface TLRs (TLR1, 2, 5, and 6) as well as endosomal TLRs (TLR3 and 8) and can therefore recognize bacterial, fungal, and viral pathogens [81]. By encountering these microbes, myeloid DCs mature, are able to express co-stimulatory molecules, and secrete different types of cytokines such as interleukin-12 (IL-12), tumor necrosis factor (TNF), and Interleukin-6 (IL-6). The effective stimulation of certain TLRs (3, 4, 5, 8, and 11) promote Th1 responses via the secretion of IL-12 by DCs. In contrast, activation of TLR2 or TLR1/2 and TLR2/6 heterodimers rather stimulate Th2 responses by secretion of IL-10 [82].

2.2.2 Viruses Are Particulate

In order to mount an efficient, innate, and adaptive immune response, pathogens such as viruses have to be endocytosed by phagocytic APCs such as macrophages and DCs. Within these cells, they will be processed and can be (cross) presented via MHC molecules to prime naïve T cell responses. They activate distinct signaling cascades, ultimately causing the expression and release or presentation of pro-inflammatory cytokines and co-stimulatory molecules, respectively. Immature DCs are very powerful at taking up and processing antigens. Consequently, they are located strategically throughout the peripheral tissues where invasion of pathogens is imminent, such as in the skin or mucosal tissues. In general, it is believed that the uptake of antigens by APCs is influenced by a variety of physicochemical properties displayed by the antigen, including size, shape, solubility, hydrophobicity/hydrophilicity, and surface charge [83]. The size of an antigen can be seen as a key factor for its efficient uptake by APCs. Molecular aggregates and particulate antigens have superior cell surface binding properties compared to soluble antigens and are therefore more efficiently taken up. In vitro data show that macrophages are efficient in the uptake of larger particles with the size of, for example, bacteria, while immature DCs preferentially endocytose particulate antigens such as viruses with a size between 20 and 100 nm [84]. The presence of repetitive patterns on viral surfaces can facilitate their uptake by phagocytic cells by different mechanisms: (i) binding and uptake is mediated via the mannose receptor, (ii) opsonization with natural antibodies and activation of the classical complement cascade with subsequent Fc- and complement-dependent phagocytosis, and (iii) direct triggering of the alternative pathway of complement activation [85, 86]. Once taken up by APCs, pathogens enter the endosomal compartment where they will be processed proteolytically, and the antigens gain access to endosomal localized PRRs and are loaded onto MHC class II molecules. In addition, phagocytosed pathogen-derived antigens can also be cross presented on MHC class I molecules and may induce efficient CTL responses [87, 88]. This process seems to be important for generating cellular immune responses against viruses that are not directly infecting DCs. The molecular mechanism of cross presentation is still not completely understood, but the two most prominent hypotheses presume either a

retrograde transport of antigens from the endosome to the cytosol or a direct loading of MHC class I molecules within the endosome [89–92]. The ability of particulate antigens such as viruses to be cross presented is much more pronounced when compared to soluble antigens [93]. Furthermore, cross presentation of particulate antigens is almost comparable in its magnitude and efficiency to the presentation on MHC class II molecules [94]. Interestingly, cross presentation of soluble antigens can still occur and seems to be dependent on very high antigen concentration and on mannose receptor-mediated endocytosis [95]. Activation of CD4+ and CD8+ T cells normally occurs in the T cell-rich zones of secondary lymphoid organs to where peripheral DCs migrate after encountering antigens. Particulate antigens such as viruses or bacteria can either be transported from the site of infection to lymphoid organs after being endocytosed by professional APCs (DCs and macrophages) or can directly drain to the lymph nodes [96–98]. Since the uptake and presentation of processed viral antigens by DCs is not sufficient for the induction of efficient B cell responses, the latter pathway seems to be especially important for the induction of antibodies recognizing conformation-dependent epitopes. Optimal induction of antibody responses also requires the presence and hence recognition of repetitive native conformational epitopes by B cells as present only on the surface of intact particles.

2.2.3 Viruses Display Antigens in a Highly Ordered and Repetitive Manner

After entry, viruses are able to drain to the peripheral lymph nodes without the aid of cellular vehicles [99]. Here, they interact with B cells to mount a humoral adaptive immune response. Since the genomes of viruses are rather small, they are able to encode only a limited number of gene products. In accordance, the genetic information that they can use to encode structural proteins that comprise their envelopes and cores is finite. As an inevitable consequence the outer shell of a virus will consist of multiple copies of the same structural protein(s), which can assemble into quasi-crystalline structures of the well-characterized icosahedral or rod-like shapes. These highly ordered and repetitive surface structures are recognized by the immune system as a molecular pattern associated with pathogens and are crucial in triggering its activation. The co-evolution of pathogens such as viruses and the vertebrate immune system has finally enabled the immune system to rapidly detect and respond to the ordered molecular patterns on the viral surface and discriminate foreign harmful antigens from self. These immunological responses do not only include binding of complement factors and natural antibodies but also recognition of the repetitive structures by specific immunoglobulins on the surface of B cells [100]. The subsequently induced crosslinking of the B cell receptor results in a rapid T cell-independent production and secretion of antigen-specific IgMs as well as B cell proliferation and can be seen as a crucial step in B cell activation [101]. Interestingly, those viral proteins that are displayed in an ordered and repetitive fashion are significantly more immunogenic than their soluble versions and, more importantly, this type of presentation can even break B cell tolerance. The rapid generation of high IgM titers in the course of a viral infection is often essential and sufficient for the control of an otherwise lethal infection [102–104]. Strong isotype-switched, antigen-specific IgG responses and

immunological memory result from the combined induction of germinal center formation and somatic hypermutation. These Th-cell-dependent B cell responses are possible due to the additional presence of Th cell epitopes within the viral antigens [105].

Interestingly, studies with orderly displayed synthetic or proteinaceous epitopes led to the conclusion that a minimum of 20–25 epitopes spaced at 5–10 nm is sufficient to induce strong and optimal B cell activation [106–108]. Indeed, most viral surfaces as well as virus-like particles (VLPs) present immunogenic epitopes in a similar density, corresponding well with the idea that the immune system has co-evolved to detect and classify these antigens as foreign and potentially harmful. In general, repetitiveness of epitopes on viruses and virus-like particles can be seen as optimal, strong, and antigen-specific trigger for both immediate and rapid as well as late and sustained humoral responses.

2.2.4 Host-Cell-Dependent Replication of Viruses

Viruses abuse the host for their reproduction. For the purpose of replication, they have to be present within the host for a certain period of time. Consequently, the immune system will be exposed to the viral invader and hence to virus-derived antigens for an extended time span when compared to recombinant antigens such as peptides or nonreplicating vaccine formulations. It has been shown that a relatively short, nonrecurring exposure to a bacterial pathogen (e.g., *Listeria monocytogenes*) initiated clonal expansion of antigen-specific T cells, activated effector cell differentiation, and could even generate T cell memory [109]. However, different observations were made in other experimental systems [110] where a much longer antigenic stimulation was necessary to mount an effective T cell response [111, 112]. In general, the generation of a long-lasting effector and memory T cell response in the course of a viral infection can be subdivided into three consecutive phases [113]. Here, signals from the TCR, from inflammatory cytokines, and from co-stimulation contribute to T cell activation and differentiation. During a first “expansion phase” in lymphoid tissues, naïve T cells get stimulated by a direct physical interaction with virally derived antigens presented on MHC molecules of professional APCs. Simultaneous co-stimulatory signals facilitate clonal expansion and differentiation into cytotoxic T or T helper (Th) cells. These effector T cells can migrate to virtually all tissues and help to control a viral infection within a couple of days by means of killing infected cells, activating DCs, providing help to immunoglobulin producing B cells and to CD8+ T cells, and secreting interferons and antiviral cytokines that further modulate antiviral responses.

If the clearance of the viral pathogen was successful, antigens no longer persist and the vast majority of the effector T cells (up to 95%) will be eliminated by programmed cell death during the second “constriction phase.” Finally, and in a third step, only cells that survive the constriction phase can be selected to differentiate into memory T cells providing effective long-lasting immunological T cell memory (“memory phase”) [114]. Although it has been stated that the differentiation process from, naïve to effector to memory T cells can be ultimately triggered by only a short (~24 h) antigen stimulation [109], it is widely accepted that CD4 T cell help, IL-2, inflammation signals, and the duration of antigen presence largely influence the transition from effector to memory T cells and the establishment of long-term immunity [115–118].

In terms of a kinetic view of this transition [119], it is believed that fully competent memory T cells, or so-called central memory T cells (TCM, phenotypically characterized by CD62L^{Hi}, CCR7^{Hi}, IL2+), slowly develop from a population of effector memory T cell (TEM, expressing CD62LL_{o,m} CCR7^{Lo}, IL2^{Lo}).

Interestingly, memory T cells have the potential to immediately react upon a reinfection with a strong clonal expansion and differentiate into secondary effector T cells expressing effector molecules such as perforins, granzymes, IFN- γ , and TNF- α [119]. Therefore, memory T cells can be characterized by the following features: (i) they maintain a high proliferative potential; (ii) they either maintain memory T cell fate or—after reinfection—rapidly differentiate into cells with effector functions; and (iii) they persist in a cytokine (IL-15 and IL-7)-dependent but antigen-independent homeostatic turnover. Thus, memory CD8 T cells maintain long-term stem-cell-like capabilities in the absence of antigen and show homeostatic self-renewal. Key factors influencing the degree of the initiated clonal T cell proliferation and hence also the later long-term immunity are the amount of the antigen that is present during the priming and the duration of the antigen exposure [120, 121]. For example, induction of elevated numbers of long-lived CD8+memory T cells in mice required the persistence of the viral antigen for at least 7 days. A shorter exposure produced only very low levels of CD8+T cells [122]. The magnitude of the first clonal burst has a direct impact on T cell memory since a massive clonal expansion will usually be followed by high frequencies of memory T cells.

Clearly, protective immunological memory depends on the persistence and virulence of the invading pathogen. Pathogens such as *Mycobacterium tuberculosis*, which are able to survive within the host for years and persistently present antigens in so-called granulomas, induce a strong immunological memory. T cells are constantly activated and also B cells are repeatedly stimulated, eventually leading to high levels of specific and persistent antibodies [123].

In sharp contrast, chronic viral infections such as Lymphocytic Choriomeningitis Virus (LCMV) (in mice), Human Immunodeficiency Virus (HIV), and HCV (in humans) but also cancer, may lead to a hierarchical loss in T cell effector functions like proliferative capacity, cytotoxicity, cytokine production, and eventually T cell depletion [124–126], a phenomenon called “(functional) T cell exhaustion.” It has been first described in mice after chronic LCMV infection [127, 128]. This was subsequently substantiated in studies that could demonstrate that persistent viral infections can result in a progressive loss in CD8 T cell effector functions, including deficiencies in IL-2 production, in cytolytic activity, in antigen-dependent proliferation, as well as in TNF- α and finally IFN- γ production [129]. In general, it is believed that a high level of antigen and low levels of CD4 help modulate the grade of exhaustion [129–132]. The expression of two pairs of inhibitory receptor/ligand complexes seem to correlate with T cell exhaustion during chronic viral infections. The Programmed Death-1 (PD-1)/Programmed Death Ligand (PD-L) pathway has been shown to be a negative regulator of T cell function and PD-1 expression is highly up-regulated on exhausted CD8 T cells. Interestingly, *in vivo* and *in vitro* blockade of the PD-1/PD-L interaction could restore T cell function, eventually leading to a reduction of the viral load during a chronic LCMV infection [133–136]. The second pathway, which seems

to affect a rather early time point in the establishment of a chronic viral infection and hence regulate T cell exhaustion, is the IL-10/IL-10R interaction. Blocking this ligand–receptor interaction in mice may prevent a chronic LCMV infection [137, 138], whereas blocking PD-1/PD-L interaction was beneficial also in an established viral infection. In addition, it has been suggested that during chronic viral infections a high level of regulatory T cells (Tregs) suppress CD8 T cell effector functions [139–141].

Virus-specific CD8 T cells present in chronically infected mice can be differentiated phenotypically from memory T cells (TCM) that are found after acute infections. In the course of a chronic viral infection, persisting T cells only express low levels of CD62L, CCR7, IL-7, and IL-15 receptors, resulting in low proliferative capacity and a rather nonhomeostatic fate. Furthermore, they require IL-2 for survival [142].

2.3 TRANSLATING IMMUNOGENIC VIRAL PROPERTIES INTO RATIONALLY DESIGNED VACCINES

Central to the development and rational design of novel vaccines and vaccine formulation are the following considerations: (i) the relevant protection-conferring antigen has to be identified; (ii) a vaccine has to be formulated that induces strong and specific immunological response and—if feasible and desired—immunological memory; and (iii) the vaccine candidate should exhibit optimal long-term protection against a given pathogen with a favored safety profile. Deciphering whole genomes, proteomes, and transcriptomes of pathogens as well as components of the engaged immune system is now offering for the first time in humans a rapid identification of potential new drug and antigen candidates. By such a “reverse vaccinology” approach, extensive collections of recombinant antigens could be generated and are becoming available for vaccine research. Although by now the best and most effective vaccines have been empirically derived, the profound knowledge of the molecular details of pathogen–host interactions such as the involved entry mechanisms, and of the induced innate and adaptive cellular and humoral immune responses might be useful for the rational design of recombinant vaccines. Furthermore, the development of novel adjuvants and delivery systems will be of key importance. The term adjuvant has its origin in the Latin word “adjuvare”, which means to help or to reach a goal. An adjuvant can be seen as an additive that helps and increases the pharmacological and/or pharmacokinetic features of a certain drug or supports the capability of an antigen to stimulate the immune system. Ideally, adjuvants should only induce few if any direct effects when administered by themselves.

2.3.1 Synthetic Inducers of Innate Immune Responses as Novel Class of Adjuvants

In analogy to the broadly used and quite efficient vaccines, also the licensed adjuvants for human use have been discovered empirically and are essentially limited to (i) alum (aluminum salts), (ii) MF59 (an oil-in-water emulsion), and (iii) virosomes. Although they may enhance the vaccine’s action in inducing a potent antibody response, how the molecular mechanisms of these adjuvants work in detail are still largely unknown.

It is believed that mineral salts such as aluminum hydroxide, aluminum phosphate, or calcium phosphate form antigen depots at the site of vaccination, thereby prolonging the presence of the antigen, which may have a beneficial effect on the humoral response. Furthermore, alum seems to favor a Th2 response [143, 144]. Recently, alum has been reported to induce the release of uric acid, a potent inducer of the inflammasome response [145]. For adjuvants based on emulsions, such as MF59, it is still controversially discussed if they also act as depot forming adjuvants or rather stimulate the immune system via inflammation signals [146–148]. While alum is commonly used in subunit vaccines such as Twinrix (combined hepatitis A and B vaccine), Infanrix (combined diphtheria–tetanus–acellular pertussis vaccine), or Pneumovax, MF59 is so far only approved for use in the influenza vaccine Fludac. Taken together, the empirically developed adjuvants, which are available for commercial use today, all aim to induce a strong humoral response. But the worldwide spreading of severe infectious diseases caused by HIV, HCV, *Mycobacterium tuberculosis*, *Plasmodium spec.*, and diseases such as cancer may ultimately require the development of vaccines and/or vaccine-adjuvant combinations additionally inducing strong cell-mediated immunity and Th1 responses. The acquired and fast-growing understanding of innate immunity including the involved molecules such as PAMPs and PRRs has recently boosted the preclinical and clinical testing of a number of adjuvant candidates which could be instrumental in inducing a targeted immune response. As discussed above, Toll-like receptors (TLRs) represent an important family of PRRs expressed on a variety of immune and nonimmune cells, and as such they are involved in the detection and sensing of conserved molecular patterns exposed by invading pathogens such as bacteria and viruses. The induced signaling cascade via TLR activation on innate immune cells such as DCs may facilitate generation of an effective and protective adaptive immune response with contribution of both B- and T cell immunity. The associated great expectations in using certain PAMPs as tailored modulators of innate and adaptive immune responses enhancing or, in the first place, enabling vaccine-induced protective immunity has fueled high-throughput screenings under industrial settings. They aim to identify natural or synthetic compounds useful in vaccine formulations as immunomodulating PRR ligands with improved efficacy and safety profiles. To date, several viral PAMPs and synthetic derivates thereof are in development as adjuvants for clinical use. They include immunostimulatory oligodeoxynucleotides containing CpG motifs (CpG-ODN) as TLR9 agonists (e.g., VaxImmune), synthetic dsRNA derivates such as poly(I:C), or its analogs as TLR3 agonist (e.g., Ampligen), and ssRNA-like molecules as TLR7/8 ligands (e.g., Imiquimod, Resiquimod, or Loxoribine) [70, 149, 150]. Also bacterial-derived TLR agonists like nontoxic Lipopolysaccharide (LPS) analogs such as monophosphoryl lipid A (MPL™) are in late clinical development. Cytidine-phosphate-guanosine rich oligodeoxynucleotides (CpG ODNs) are relatively advanced in their development and approval as adjuvants for human use is expected in the near future. In general, three classes of CpG ODNs can be distinguished: (i) the so-called “A-class” ODNs inducing significant IFN- α secretion after stimulation of TLR9 in pDCs and other cell types; (ii) “B-class” ODNs mainly activating B cells; and (iii) “C-class” ODNs displaying combined activities on B cells and pDCs. It has been shown that stimulation of B cells and DCs by

CpG ODNs is associated with up-regulation of co-stimulatory molecules, secretion of Th1-promoting cytokines, suppression of apoptosis, and up-regulation of chemokine receptors resulting in the induction of antigen-specific and class-switched IgG antibodies and Th1-driven immune responses as well as the generation of cytotoxic T cells [151, 152]. Beside this very potent adjuvant activity, there are certain safety issues and unfavored pharmacokinetics linked to the systemic use of naked CpG ODNs. As stated above, TLRs are expressed by a wide variety of immune and nonimmune cells. Consequently, systemically administered CpG ODN have the potential to activate TLR9 signaling in many cell types. This could lead to systemic toxicity and immunopathology. Although such unwanted side effects have been observed in pre-clinical mouse models, a simple explanation might be that rather nonphysiological high concentrations were used and/or that the animals were exposed chronically to the immunostimulatory compounds. In detail, liver necrosis, splenomegaly, changes in lymphoidfollicular architecture, suppression of germinal center formation, and of follicular dendritic cells as well as impaired humoral immune responses have been attributed to an extensive CpG ODN treatment in mice [153, 154].

CpG ODNs are DNA analogs and as such the individual nucleosides are covalently linked via a phosphodiester backbone. Nucleic acids are usually rapidly degraded in vivo by nucleases present in, for example, the serum, and in addition, due to their highly charged anionic nature, they will be easily captured by serum proteins. These poor pharmacokinetic and pharmacodynamic properties required the administration of large amounts of CpG ODNs to observe a potent immunostimulatory effect. To significantly reduce the needed concentration of certain adjuvants to fulfill a desired action in vivo, several strategies are aiming to improve the stability and half-life of administered nucleic acids within an organism. This should thereby help to solve safety issues. One prominent approach comprises the modification of the phosphodiester bridges in the backbone of CpG ODNs by substituting one sulfur for one of the nonbridging oxygen atoms to obtain a phosphorothioate backbone. This results in a nucleic acid analog with a marked improved stability in vivo. Phosphorothioate oligodeoxynucleotides are stable in vivo for up to 2 days, whereas nonmodified ODNs with a conventional phosphodiester backbone decline within a few minutes [155, 156]. However, this also changes the properties of DNA-based CpGs, which are usually of A-type to B-type CpG ODNs. An alternative strategy to improve stability and to reduce systemic toxicity might be the targeted delivery of adjuvants such as CpG ODNs directly to APCs (see below).

CpG ODNs are one class of the more advanced novel vaccine adjuvants that have been tested clinically. For example, in phase I/II clinical trials the efficacy of CpG ODNs as adjuvants for hepatitis B vaccination have been explored. One important outcome of these pilot studies was that all vaccine formulations were safe and well tolerated. The combination of two different CpG ODNs with the hepatitis B vaccine Enerix showed improved immunogenicity in terms of the kinetics as well as of the overall antibody titers [149, 157, 158]. Even in immunocompromised and hyporesponsive HIV patients, addition of CpG ODNs to the same hepatitis B vaccine was beneficial and resulted in faster, more sustained, and protective humoral immunity and antigen-specific T cell proliferation [159]. Another hepatitis B vaccine,

HEPLISAV, could also induce a rapid and strong antibody response, confer sustained seroprotection in healthy volunteers as well as in vaccine-hyporesponsive individuals, after combining with CpG ODNs [160, 161]. Beside the use as adjuvants for hepatitis B vaccines CpG ODNs have been also clinically evaluated in combination with a trivalent influenza vaccine (Fluarix), with an anthrax vaccine (BioThrax), with subunit malaria vaccines, as well as with an HIV vaccine (Remune). Although some of the results of these pilot studies [162, 163] seem to be promising (e.g., better humoral responses and more rapid seroconversion), much more work has to be done [164].

One feature of the *in vivo* action of CpG ODNs is the induction of a strong Th1-driven immune response. Relying on the hygiene hypothesis [165], this is believed to be clinically relevant for shifting (or suppressing) an allergy-prone Th2 response toward a beneficial Th1 response. In terms of efficacy and elimination of side effects, it was discussed that it may be of additional value to covalently link the CpG ODNs to the used allergen. In such conjugates, IgE binding to the allergen may be blocked and mast cell degranulation, which is triggered during the immediate allergic reaction or even in the course of vaccination, can be decreased [164]. A therapeutic effect could be observed with a vaccine (TOLAMBA) comprising CpG ODNs covalently linked to the main ragweed allergen Amb a 1 [166–168]. However, the clinical improvements were rather moderate.

Finally, novel immunotherapies including vaccination strategies aiming at inducing CD4 and/or CD8 T cells are currently evaluating the beneficial effect of added CpG ODN adjuvants. The development of therapeutic cancer vaccines was recently boosted by the identification of tumor-specific antigens and the hope that CpG ODNs may induce cytokines such as IFN- α *in vivo* and elicit strong tumor-specific effector T cell responses [164, 169, 170]. For instance, most melanomas express the melanoma-associated antigen recognized by T cells (MART1) and many of these T cells seem to be specific for a certain region of this tumor-associated antigen, the Melan A peptide. However, initial clinical trials showed no increase in Melan-A-specific T cells after vaccination with a synthetic Melan A peptide mixed with the adjuvants QS-21 (saponin) or the TLR4 agonist MPL. In contrast, when the synthetic peptide was given in incomplete Freud's adjuvant (IFA) around 50% of the vaccinated patients responded with an elevated level of MART1-specific CD8 T cells [171]. Importantly, combining this particular vaccine formulation with CpG ODNs as additional adjuvant resulted in a 100% responder rate with a mean frequency of MART1 specific CD8 T cells of more than 1% in the blood. Although this population of peripheral blood mononuclear cells (PBMCs) had differentiated to effector T cells, few tumor-infiltrating effector T cells were observed [172, 173]. Interestingly, a recent study by Buckanovich and colleagues showed that the frequency of tumor-infiltrating T cells could be modulated in mice by the expression level and activity of the endothelin B receptor (ETBR) present on the surface of endothelial cells of tumors [174]. Future preclinical work is needed to support a model in favor of ETBR blockade in cancer immunotherapies. Beside the mentioned Melan A peptide vaccines, there are currently several other vaccine formulations comprising a tumor-associated antigen combined with a certain CpG ODN in clinical testing [169]. TLR9 agonists are by far not the only PAMPs that entered preclinical and clinical development. But by now, synthetic

TLR3 ligands such as poly(I:C) and the TLR7/8 agonists imiquimod, resiquimod, and loxoribine have only been tested thoroughly as vaccine adjuvants under preclinical settings. GlaxoSmithKline is currently testing the mentioned TLR4 ligand MPL in phase III clinical trials as adjuvant for HPV and HBV vaccines.

Although poly(I):poly(C12U) (Ampligen) entered clinical testing as monotherapy for infectious and autoimmune diseases, its potential as vaccine adjuvant has only been analyzed in some preclinical studies. For example, in combination with an anthrax protective antigen, Ampligen could induce antibodies with anthrax neutralizing activity [175]. Also as a candidate adjuvant for influenza vaccination, the TLR3 agonist poly(I):poly(C12U) induced high antihemagglutinin IgA and IgG titers in mice when co-administered intranasally with an inactivated hemagglutinin vaccine. Interestingly, the induced antibodies seem to confer cross protection against a variety of influenza strains [176, 177]. To date, a phase II clinical trial is investigating the potential of Ampligen to increase the immunogenicity of a seasonal influenza vaccine and hence may permit dose titrations.

Likewise, the TLR7/8 agonists are investigated and even reached the pharmaceutical market as immunomodulating therapies in indications such as infectious diseases (e.g., HSV and HPV, respectively) and cancer [178, 179]. For example, when administered topically, imiquimode (Aldara) induces a strong antitumoral effect via cutaneous dendritic cells [180].

A recent report analyzing different PAMPs as vaccine adjuvants in combination with an LPS-deficient meningococcal (*Neisseria meningitidis* serogroup B) outer membrane vaccine showed that imiquimod and loxoribine enhanced overall immune response against the outer membrane complex. This response was Th1 biased and induced higher bactericidal antibody titers when compared to vaccine without adjuvants [181]. Also resiquimod (R848) was shown to induce a Th1-driven immune response with the induction of IFN- γ , when used as vaccine adjuvant in mice [182]. Again, conjugation of the adjuvant and the antigen before immunization seems to be superior over co-administration of simply mixed components [183]. One reason could be that conjugation facilitated the targeted delivery of the immune stimulating TLR agonists to the same APCs presenting the antigen. Based on such considerations and taking the relatively poor pharmacokinetic properties of several PAMPs into account, delivery systems have been developed that can overcome these limitations and (i) protect conjugated or packaged TLR agonists against degradation in vivo and (ii) deliver them to their favored side of action, that is, to APCs such as DCs. Such a strategy may allow reducing both the concentration of the adjuvant and of the used antigen, without losing efficacy. Instead the adjuvant–antigen conjugate is stabilized, adsorption to serum proteins can be strongly inhibited, and they can be targeted to a professional APC or even the appropriate intracellular compartment [154, 184, 185]. To date, many particulate delivery systems have been studied in detail, for example, liposomes [186], microparticles [187], virus-like particles (VLPs) [151, 154], virosomes [188], and Immune Stimulating Complexes (ISCOMs) [185, 189–191].

Cationic liposomes have been used in conjunction with MPL (TLR4 ligand) to induce protective immunity against *Mycobacterium tuberculosis* with an Ag85B-ESAT-6 subunit vaccine in mice and even resulted in a significant reduction of

bacterial load and of lung pathology in cynomolgus monkeys [192–194]. Furthermore, immunostimulatory nucleic acids presented in noncoding plasmid DNA (TLR9 agonist) packaged together with soluble leishmanial antigens into cationic liposomes induced strong Th1-driven immunity and protected to a certain degree against a challenge with *Leishmania donovani* [195].

CpG ODNs (TLR9 ligands) incorporated into cationic liposomes were shown to induce a Th1-driven immune response against the HCV antigen NS3 [196]. In a very comprehensive study, Zaks and colleagues showed that liposome–antigen–nucleic acid complexes (LANACs) could induce CD4 and CD8 T cell responses against the corresponding antigens (peptide and protein, respectively). The induced antigen-specific CD8 T cells showed effector and memory functions and could even control the growth of B16 melanomas in a therapeutic tumor model. Also under prophylactic settings, vaccination with LANACs containing the *M. tuberculosis* protein ESAT-6 resulted in protective immunity against the pathogen [191].

Another class of particulate delivery systems are microparticles consisting of poly-lactic acid (PLA) or poly-(lactide-*co*-glycolide) (PLG). By using such microparticles, it was shown in mice that co-administration of antigen and CpG ODN, both adsorbed to PLG (e.g., PLGg120/PLG-CpG ODN), induced a higher anti-HIV-1 gp120 antibody titer than without CpG ODNs [187]. When PLG nanoparticles were used that contained encapsulated tetanus toxoid (TT) as antigen and CpG ODNs as immune potentiators, immunized mice showed a strong T cell response with relatively high production of IFN- γ , TNF- α , and IL-12 [197].

There are virosomes composed of lipid membranes (liposomes) and viral envelope proteins. Essentially, they can be derived from a variety of enveloped viruses including influenza, rubella, measles, herpes simplex virus (HSV), Epstein-Barr virus (EBV), HIV, and many others. Especially influenza virosomes have been used for vaccination purposes due to both their particulate structure as well as the certain immunogenic properties of hemagglutinin. Virosomal influenza microparticles have been approved for human use as influenza vaccine but also as hepatitis A vaccine. As such, it induces strong antigen-specific and protective humoral and cellular (CD4+ T cell) responses. Virosomes are usually not used to co-deliver any further immune potentiators.

In contrast to semisynthetic particles such as virosomes, which are reconstituted *in vitro* from lipids and virus-derived envelope proteins, the production of so-called virus-like particles (VLPs) relies normally on the synthesis of viral proteins within host cells and the self-assembly of these coat proteins into VLPs *in vivo*. Both *in vivo* and *in vitro* loading of VLPs with viral PAMPs have been described. We and others have shown that, for example, bacterial RNA (TLR7 agonist) can be engulfed within the VLPs during the assembly process in *Escherichia coli* or that a certain viral PAMP can be packaged after purification into VLPs. Furthermore, the particulate nature of VLPs ensures that a covalently linked or attached antigen is displayed on their surfaces in a highly ordered and repetitive manner, hence allowing strong induction of humoral and cellular immune responses.

In general, RNA-containing VLPs induce a strong Th1-driven immune response with the typical ratio of IgG2a:IgG1 after vaccination [151]. This characteristic isotype pattern may not be only beneficial for clearance of viral and bacterial infections

but may also bear some advantage for suppressing Th2-driven immune responses, which are believed to be involved in the development and establishment of allergies. Currently, PAMPs containing VLPs undergo clinical testing as therapeutic options to treat allergies [198].

We could demonstrate that mice immunized with VLPs covalently linked to the p33 epitope derived from the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) and packaged *in vitro* with CpG ODNs responded with high numbers of antigen-specific CD8+ T cells. Furthermore, the induced cytotoxic T cells (CTLs) conferred effective and protective immunity *in vivo* as could be demonstrated by challenging with recombinant vaccinia virus expressing the p33-containing LCMV glycoprotein (Vacc-G2). Also the growth of established solid tumors expressing LCMV-GP could be eradicated in mice immunized with CpG-loaded p33-VLPs [154]. It is noteworthy to state that packaging of CpG ODNs into VLPs not only enhances their stability but also circumvents potential harmful side effects attributed to the systemic delivery of and chronic treatment with viral PAMPs in mice. In summary, VLPs are offering an optimal vaccine platform for (i) delivering viral PAMPs to their site of action, thereby further enhancing their strong inherent immunogenic properties, (ii) displaying foreign or self-antigens in an ordered and repetitive fashion to mount strong humoral and cellular immune responses, and (iii) breaking self-tolerance. Last but not least, VLPs have been proven to be safe and well tolerated from mice to humans.

2.3.2 Using Particulate Macromolecular Assemblies as Vaccines

The uptake of antigens and co-delivered immune modulators by professional APCs is central and decisive for mounting an effective humoral and cellular immune response. As discussed above, the particulate structure of many pathogens such as viruses facilitates the uptake by APCs. Therefore, many modern vaccine delivery systems are imitating this pathogen-like particulate structure—even if empirical. Although these vaccine delivery systems are quite diverse and can include particles consisting of mineral salts, oil-in-water emulsions, liposomes [186, 199], biopolymers, virosomes, or virus-like particles, the formed particles are usually within the range of dimensions similar to that of pathogens. Functionally, they all have in common that they will deliver attached, adsorbed, or encapsulated antigens and immune modulating agents in high concentrations and a target-oriented fashion to APCs to induce innate immune reactions and to efficiently present the antigen. Antigen presentation by DCs is mediated via cross presentation on MHC class I molecules or via the MHC class II pathway.

Liposomes with a mean diameter of approximately 100 nm can efficiently deliver antigens and immune potentiators to DCs with cationic liposomes being superior over anionic and neutral liposomes [200]. But also the composition of the nonpolar region of the lipid bilayer has an effect on the capacity of liposomes to stimulate DCs [201]. Liposomes have been used to co-deliver antigens from viral and bacterial origin together with immune modulators such as viral PAMPs, to protect antigen and immune modulators from clearance and degradation *in vivo*, to induce effective cross presentation on DCs, and eventually to mount a strong and protective immune response efficiently fighting the corresponding microbial infection or to generate an antitumor

response [191, 202–204]. Currently, a liposome-based tumor vaccine (L-BLP 25) is undergoing phase III clinical evaluation [205].

An attractive alternative to use liposomes might be the application of so-called immunostimulatory complexes (ISCOMs). These are also biodegradable particles, but they are smaller in size (approx. 40 nm in diameter) and seem to have superior pharmacokinetic properties when compared to liposomes. A combination of cholesterol, phospholipids, and saponin can encapsulate antigens in a spherical, cage-like structure forming the ISCOM. ISCOM-based vaccines are able to induce strong humoral and cellular responses including cytotoxic T cell activities. ISCOMs have been used as vaccine formulations in a variety of animal species and disease models. These studies highlighted their potential to induce high titers of antibodies (a predominant IgG2a response in mice) and long-lasting CTL responses with high frequencies of memory cells that conferred efficient protection in a wide variety of vaccinated animal species, ranging from mice to nonhuman primates [206]. At the molecular level, it is believed that the lipophilic nature of the ISCOM allows efficient fusion with the target cell membrane of APCs and the subsequent intracellular processing of the delivered antigen, resulting predominantly in cross presentation on MHC class I molecules [207, 208]. Interestingly, repeated administration of ISCOM-based vaccines via the oral or nasal route has been shown to be immunogenic, even in the absence of additional adjuvants such as cholera toxin (CT) or heat-labile enterotoxin from *E. coli*. Normally, the co-administration of CT or heat-labile enterotoxin is needed to mount a strong immune response after vaccine delivery through a mucosal surface [209–211]. Since ISCOM-based vaccines have proven efficacy and safety in several animal species and under different disease conditions, they have been consequently developed for human use. In clinical trials, ISCOM-based vaccines have been used to explore their safety and efficacy profiles in combination with several antigens including influenza viral proteins, human papilloma virus 16 E6/E7 fusion protein, HCV core protein, and the tumour antigen NY-ESO-1. Antibody responder rate reached almost 100% throughout the vaccinated subjects with a faster kinetics when compared to, for example, conventional influenza vaccines. Also the cellular responses seemed to be superior when compared to conventional vaccines [212–214]. In 2007, a phase II study with an influenza vaccine based on ISCOMs was commenced.

Virosomes are a further class of spherical microparticles containing lipids, albeit with a diameter of approximately 150 nm, their dimensions exceed that of ISCOMS and resemble more that of liposomes. For production purposes, the envelopes of viruses must be purified to a high grade and can then be mixed *in vitro* with virion-derived and supplementary lipids such as phosphatidylcholine or phosphatidylethanolamine. The exact dimensions and physicochemical features, which largely influence the immunological properties, are dependent on the exact composition of the virosome. Well-known virosomes that have been also approved in the meantime as vaccine or adjuvant for human use are the so-called immunopotentiating (or immunostimulating) reconstituted influenza virosomes (IRIVs), first described in 1992 [215]. They contain hemagglutinin and neuramidase as viral envelope proteins, but importantly they are presented at much lower density on the surface of the particle as compared to intact viruses or virions. IRIVs can be used as such as efficient

influenza vaccines (Inflexal® and Invivac®) or after adsorption of additional viral antigens as hepatitis A vaccine (Epaxal®). In vivo, IRIV-based vaccines bind to and are taken up efficiently by APCs via receptor-mediated endocytosis. Once reaching the endosomal compartment, a peculiar property of IRIVs, namely their HA-mediated fusion activity upon acidification, determines the further fate of antigen presentation. Antigens presented on the surface of the virosomes are retained in the lumen of the endosome/lysosome where they are subjected to proteolytic cleavage. Resulting peptides will be presented on MHC class II molecules to CD4+ T cells. This T cell help is substantial for an effective antigen-specific B cell and/or CTL response. In contrast, encapsulated antigens will end up in the cytosol and hence will be processed via the MHC class I pathway. This will result in the induction of a CTL response. Recently, virosomes have been investigated with respect to their potential use as a malaria vaccine. Two clinical trials came to the conclusion that these IRIV-based vaccines are safe, well tolerated, and induce long-lasting humoral responses in vaccinated subjects [188].

Biocompatible microparticles or nanoparticles consisting of PLA, PLG, or polysaccharides such as chitosan have been used as well as vaccine and/or adjuvant delivery system. The distinction between microparticles and nanoparticles can be simply made by defined differences in the dimensions of the particles given either in micrometers or nanometers. In general, it is assumed that particles with a mean size smaller than 5 μm are more immunogenic than particles larger than this size. Multiple reasons can account for this observation, as, for instance, (i) preferred uptake of particulate structures resembling in their dimensions that of pathogens (i.e., 20 nm up to 2 μm) by professional APCs, (ii) passive drainage of particulate structures with a size between 20 and 500 nm from the site of infection/inoculation to the lymph nodes, and (iii) cell-mediated transport for particles larger than 500 nm [98]. Although microparticles have been used as vaccine delivery systems, their general application range seems to be limited due to a rather sophisticated production process and linked unfavored properties that may destabilize antigens to be delivered [216, 217]. By using micro- and nanoparticles with a defined charge (e.g., cationic or anionic) potential antigens as well as nucleic acids can just be adsorbed by electrostatic and hydrophobic interactions to the particulate surface of the delivery system [218]. Especially cationic PLG particles have been used to adsorb and deliver DNA for vaccination purposes. Although a few studies reported successful induction of antigen-specific and protective immunogenicity in the course of a corresponding infectious disease, cancer, or allergy with these vaccine formulations in animals [219–224], limitations with respect to formulation processes as well as to potentially disadvantageous safety profiles remain.

A universal vaccine delivery platform that avoids a number of such problematic issues can be found in VLPs. A number of structural viral proteins forming the virions coat have the capability to spontaneously assemble into particulate, ordered and repetitive structures during recombinant DNA expression in a variety of expression systems, ranging from *E. coli* to mammalian host cells. Factors influencing the choice of an appropriate expression system often include up-scaling capabilities, production costs, secondary protein modifications such as glycosylation and host cell contaminations.

These considerations have made *E. coli* and yeast popular expression systems for the large-scale manufacturing of VLP-based vaccines.

By forming these particles, they inherently display a high number of the aforementioned characteristics that are crucial for a strong and potent induction of protective immunity, either under prophylactic or therapeutic conditions. One important variance, however, might be that VLPs do not contain the genetic material of the parental virus, and hence they are noninfectious and cannot cause disease. Solely by their composition one could envisage (i) nonchimeric VLP-based vaccines aiming in the induction of a protective immune response against the corresponding naturally occurring pathogen from which the VLP is derived from or (ii) chimeric VLP-based vaccines displaying antigens that are not derived from the parental virus. In the latter case these VLP-based vaccines can generally target an infectious or noninfectious disease or chronic affliction by means of inducing a tailored immune response. No matter which strategy will be preferred for a certain medical condition, VLP-based vaccines have in common that they strongly resemble viruses and, as particulate arrays with dimensions between 20 and 150 nm, are preferentially taken up by professional APCs. For instance, *in vivo* studies demonstrated that DCs preferentially take up conjugated nanoparticles with a dimension of 40 nm in diameter [84], matching approximately the size of most viral particles. Even more, the particulate structure of VLPs seems to support their direct targeting to DCs [225]. VLPs can hence be considered a good choice for delivering vaccine antigens and immune modulators with an excellent safety profile. The immune system is treating these vaccines like invading viruses, and VLP-derived antigens are consequently presented by DCs either via the MHC class II pathway or cross presented on MHC class I molecules. Cross presentation can induce potent CD8 T cell responses and was shown to work efficiently in combination with a wide variety of VLPs derived from human viruses including the papilloma virus [226], HIV [227], hepatitis B and C viruses [228, 229], parvovirus [230, 231] or derived from rather ancient and unrelated viruses such as bacteriophages [94, 232]. Interestingly, the efficiency of cross presentation is also facilitated by the particular structure of the VLP [229, 233]. Another feature that seems to be influenced by the particulate nature of VLPs is the induction of DC maturation. For example, only particulate HPV VLPs but not the unassembled protein subunit L1 can activate DCs, resulting in up-regulation of co-stimulatory molecules and cytokines [234, 235].

Importantly, VLPs consisting of recombinantly expressed small-envelope protein of hepatitis B virus (HbsAg) are licensed as hepatitis B vaccines such as Recombivax-HB® or Energix® [236] and are one of the most potent recombinant vaccines available. The recent approval of the prophylactic human cervical cancer vaccines Gardasil and Cervarix, both of which are essentially “not more” than virus-like particles composed of the major capsid proteins from 4 (Gardasil®) or 2 (Cervarix®) different human papilloma viruses, boosted the interest and awareness of vaccines based on the production of recombinant engineered VLPs. The solid and broad clinical experience with the HBV and HPV vaccines have proven their good safety profile and their robust efficacy in inducing a strong and long-lasting immune response [237–239]. Remarkably, many VLP-based vaccines do not need mixing with exogenous adjuvants to mount these strong immune responses [240, 241].

A number of additional VLPs aiming at induction of a potent immune response against the corresponding viral infection are currently in preclinical and clinical testing, including hepatitis C and E viruses, rotavirus, severe acute respiratory syndrome (SARS) coronavirus, or influenza virus [242–256].

2.3.3 Employing Repetitive Arrays for Displaying Antigens

One major advantage of VLP-derived vaccines is the optimal potential for efficient induction of a strong and long-lasting humoral immune response. On a molecular level, this can simply be explained by the geometrical arrangement of the displayed antigen in repetitive arrays on the surface of virus-like particles. When B cells expressing membrane-associated immunoglobulins that constitute the B cell receptor (BCR) encounter these ordered arrays of highly repetitive antigens, the BCRs will be efficiently crosslinked. Localized oligomerization of the BCRs within the plasma membrane of B cells constitutes BCR signaling microdomains finally resulting in B cell proliferation and up-regulation of MHC class II and co-stimulatory molecules, which are required for productive interaction with Th cells. This signaling cascade induced by antigen-mediated crosslinking of the BCRs, which is supported by T cell help, triggers class switch of Ig subtypes, antibody secretion, affinity maturation, and B cell memory. Clearly, highly repetitive and optimally spaced antigens presented by VLPs are able to induce these strong B cell responses. By virtue of genetic insertion, chemical crosslinking or even noncovalent intermolecular interactions, almost every possible and suitable antigen can be fused or conjugated to and displayed on the surface of the resulting chimeric VLPs. The variety of conceivable antigens ranges from foreign pathogen-derived molecules (e.g., M2 protein from influenza) over foreign small molecular entities (e.g., haptens such as nicotine) to self-molecules (e.g., interleukins such as IL-17 or IL-1). Likely, similar production and purification processes that had been developed for the parental VLPs can also be employed for manufacturing chimeric VLPs. Fusing an antigen by means of genetic insertion to the coat protein forming the VLP should be largely facilitated by a definite structural analysis of the parental VLP as well as the resulting chimeric VLP. Despite this awareness, most of these chimeric VLPs are still the product of rather empiric approaches where success is believed to be determined by VLP assembly and vaccination-dependent induction of antigen-specific immunogenicity in a laboratory animal. Prominent examples include aminoterminal, internal as well as carboxyterminal fusions of a great variety of protein/peptide sequences to HBV VLPs [257].

Factors such as the exact position of the insertion (e.g., NH₂- or COOH-terminal with respect to the coat protein) or restrictions in relation to the fusible size of the antigen have to be considered when designing a chimeric fusion VLP since they may have an impact on the correct assembly of the VLP and native folding of the fused antigen. These limitations can be overcome if the fusion partners are produced separately and only conjugated after purification of the antigen in its native conformation. Conjugation may be established by chemical crosslinking during which a new covalent bond between antigen and VLP is formed. Bifunctional crosslinker with reactive maleimide and/or NHS ester groups are routinely used to bridge sulphydryl groups of the cystein-carrying antigen with accessible primary amines provided by lysine residues on the

coat protein of the VLP. This technique offers the advantage to conjugate a very broad and diverse range of antigens to the VLP carrier. Not only peptides such as Der p1, angiotensin II, A β , and/or full-length proteins including IL-17, TNF- α , or IL-1 but also nonproteinaceous antigens and small haptens (e.g., nicotine) were successfully coupled to various parental VLPs [258–266]. Immunization of mice or men with these VLP-based vaccines all induced neutralizing antibodies against the conjugated antigen, which were capable of inhibiting the corresponding receptor–ligand interaction. Both the neutralizing potential and the strength of the induced antibody response could be linked to clinical efficacy of VLP-based vaccines. In a preliminary phaseII clinical trial, it was shown that immunization of addicted smokers with VLPs carrying crosslinked nicotine could induce high titres of nicotine-specific antibodies. It is believed that a high anti-nicotine-IgG-level correlate with prolonged smoking abstinence [267].

Interestingly, vaccination studies with VLP-antigen conjugates targeting self-molecules were demonstrating that even self-proteins or peptides, which are involved in the pathogenesis of chronic diseases, can be successfully neutralized *in vivo*. This medical intervention was successfully used in animal models of osteoporosis, rheumatoid arthritis, hypertension, experimental autoimmune encephalitis (EAE), autoimmune myocarditis, and allergic asthma by targeting the key molecules RANKL/TRANCE, TNF- α , IL-1, IL-15, angiotensin II, IL-17, IL-4, and IL-13, respectively [258, 262, 264, 268–274]. By displaying these self-molecules as conjugates on the surface of VLPs, they will be presented after vaccination to the immune system in a viral context and hence being considered as foreign. Since autoreactive B cells are present in surprisingly high frequencies, these can become activated when repetitive and densely ordered arrays of self-antigens efficiently crosslink the BCRs of this autoreactive B cells. Even T cell help can be provided to the B cells because the self-antigens are presented exclusively in the context of the VLP carrier that will, as potential invading and foreign pathogen, help to overcome the otherwise restrictive T cell tolerance. Specific Th cells will be induced, and activated B cells can produce class-switched and high-affine IgG autoantibodies. It is important to note that the induction of high levels of autoantibodies seems to be reversible and titers will decline with an estimated half-life between 3 and 4 months [275]. The encouraging preclinical data obtained with vaccination protocols using VLP self-antigen conjugates in a wide variety of different disease models in animals led consequently to the further development of lead candidates and their clinical evaluation. In one recent clinical trial, it was demonstrated that this concept may also holds true in men. A vaccine composed of a bacteriophage-derived VLP displaying angiotensin II induced a sustained antibody response in volunteers and resulted in a significant reduction in systolic and diastolic blood pressure in hypertensive subjects. Interestingly, this reduction was particularly pronounced in the early morning hours, the most critical time for severe cardiovascular events [265]. However, a conformational study proofed that these effects were not statistically significant.

In a few cases it has been observed that monoclonal antibodies targeting self-molecules may have rather agonistic properties instead of displaying the anticipated antagonistic effects [276–279]. However, vaccination with recombinant subunit vaccines normally induces a polyclonal antibody response against a wide variety of

different epitopes from the used antigen. Within this pool of polyclonal antibodies a certain population will have neutralizing activities leading to inactivation of the targeted self-molecule. Hence, it is very unlikely that active vaccination would result in the induction of agonistic antibodies. Conjugating or fusing self-molecules in their native conformation to carriers may result in bioactive particles, which could theoretically interfere with the recipients' metabolic pathways and activate signaling cascades through binding to the corresponding receptors. Although there is no report documenting such phenomena in the case of active vaccination, they have to be considered as an important safety issue when targeting self-molecules. Sometimes, bioactivity of the natively folded self-antigen can be reduced by the conjugation reaction itself. Another possibility is the design and use of so-called muteins instead of the wild-type protein. Muteins, which contain a single or a few mutations affecting the primary structure of the original counterpart, may still display the native conformation albeit with reduced bioactivity. Conjugating such muteins to carriers may induce antibodies after vaccinations, which are capable of neutralizing the wild-type protein. The identification of such muteins is still mainly driven by empiric approaches, although knowledge of the detailed three-dimensional structure of the parental protein should be of advantage for the rational design of muteins.

Beside the successful development of VLPs as carrier for highly ordered and repetitive antigen arrays, a number of additional vaccine platforms, both from natural or synthetic sources, are currently tested for that purpose. For example, antigens can be adsorbed via electrostatic and/or hydrophobic interactions onto the surface of charged nano- or microparticles, respectively [187, 218]. Also the ISCOM-based technology offers a further class of particulate and versatile delivery system. Antigens can either be directly incorporated into the 40-nm cage-like structure, coupled indirectly via hydrophobic carrier proteins to the ISCOMATRIX or incorporated after chemical modification such as palmitification into the particle. Clinical studies have proven that ISCOM-based vaccines are able to induce strong humoral immune responses in humans and have been reported to be safe and well tolerated [206]. Vaccine formulations based on virosomes or liposomes are successful prophylactic vaccines with a well-defined safety profile and are able to provide protection against a number of infectious diseases (see above). Currently, also a therapeutic liposomal vaccine (Stimuvax) targeting the mucin MUC1 tumor antigen, which is overexpressed on a variety of tumors, is tested in a large phase III clinical study.

VLPs as well as the other described recombinant and synthetic vaccine formulations aiming at displaying certain viral properties all have in common that they do not replicate *in vivo*. Hence the duration of the antigens' presentation to the immune system is limited. This clearly results in an advanced safety profile, but the other side of the coin may be a reduced strength of the provoked immune response.

2.3.4 Mimicking Viral Persistence

One outcome of an endured infection with a virus capable of replicating within the host might be life-long immunity. Immunization with attenuated but still live vaccines sometimes closely resembles the course of a natural infection since they are inherently able to replicate *in vivo*. Consequently, their viral antigens will be presented to

the immune system for a prolonged period of time, resulting in a strong and long-lasting humoral and cellular immune response combined with immunological memory. Often, the very potent induction of the immune system after inoculation with infectious vaccines comes at the expense of safety. In some cases, attenuated viral vaccines have the potential to revert back to the virulent phenotype. For example, there are numerous reports that the use of live-attenuated poliovirus vaccines rarely bear the risk to accumulate mutations within the vaccinated individuals generating highly pathogenic revertants that can cause neurovirulence and even epidemic poliomyelitis by getting transmitted by the vaccinees to nonimmunized individuals [280–285]. In general, there remains the possibility that the viral strains used for vaccination or revertants thereof may be transmitted from the otherwise healthy vaccinee to nonimmunized individuals or immunocompromised patients. However, a recent epidemiological study came to the conclusion that the overall benefit also to nonimmunized but immunocompromised individuals outweighs the residual risk of a secondary transmission [286]. Still, immunization of immunocompromised individuals with living vaccines requires much more attention and an elaborated risk–benefit analysis.

A current strategy to overcome these unfavorable properties in modern vaccine design is the development of recombinant subunit vaccines that do not contain genetic material of the corresponding pathogen and hence do not replicate within the host. Since a limited and rather short period of antigen exposure is ultimately linked to this feature, measures have to be taken to either prolong the presence of the antigen within the vaccinee or to ensure an efficient delivery of the vaccine to lymphoid organs. One simple method to achieve this prerequisite for a strong immune response might be to adjust the immunization regimens to frequencies that more likely guarantee persistence of antigen for some time. Repeated inoculations with a bi-weekly, weekly, or even daily administration of the vaccine have been shown to efficiently supply the antigen and more closely resemble the antigen load during a viral infection [122, 287–289].

An additional measure to prolong the persistence of the antigen after vaccination is the usage of adjuvants. As mentioned before, at least one mode of action of available adjuvants is the formation of an antigen depot at the site of inoculation. It is believed that from these depots antigen can be released over an extended period of time, thereby supporting the induction of a strong humoral and cellular immune response.

Although recombinant subunit vaccines have been proven to be generally very safe and well tolerated, their remains the possibility that the induced antibodies may rather enhance an acquired natural infection or exacerbate the disease in case of postexposure vaccination or inadequate immunization. Such phenomena have been reported for vaccinations in combination with, for example, flavivirus infections such as dengue virus [290, 291] or West Nile virus infections [292, 293].

On a molecular level, it has been shown that the entry of a number of enveloped viruses into macrophages/monocytes and granulocytes can be facilitated by the interaction of antiviral antibodies (often when they are present in only subneutralizing concentrations [293]) with Fc and/or complement receptors on the surface of the host cells [294–296]. This phenomenon was first described *in vitro* and is also termed “antibody-dependent enhancement (ADE) of infection”. Targeting viral infections with recombinant subunit vaccines must, therefore, also include a very thorough

screening and selection of appropriate antigens to prevent such antibody-dependent enhancement of infection.

2.4 CONCLUSION

Designing recombinant vaccines with viral properties is without doubt an auspicious challenge. By deciphering the molecular codes of infectious as well as noninfectious diseases, very potent tools can now be developed to design not only a future class of conventional vaccines but rather immune therapies with tailored functions. Recombinant vaccines are capable of inducing beneficial and protective humoral and cellular immune responses. In addition, they exhibit an improved safety profile when compared to vaccines based on attenuated or inactivated pathogens. The potential of specifically targeting foreign antigens as well as self-molecules with particulate vaccines such as VLPs hold strong promise in combating infectious diseases caused by viruses such as HIV, HCV, or dengue virus, chronic disorders such as hypertension or Alzheimer's disease and dysfunctions of the immune system itself, resulting in autoimmune diseases. Prophylactic as well as therapeutic vaccines with viral properties can tune the immune system in a way that for a number of medical conditions the adequate response is induced. This includes not only the blockade of key molecules by neutralizing antibodies but also the induction of relevant cellular responses and the secretion of, for example, cytokines. These novel vaccines can guide the immune system to react in an appropriate manner to the multitude of invaders or certain dysfunctions of the organism. We expect that therapeutic or prophylactic immunization with recombinant vaccines displaying favorable viral properties will open new treatment options to fight infectious as well as chronic diseases such as cancer, neurodegenerative or autoimmune disorders, and allergies.

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3

TOOLS FOR VACCINE DESIGN: PREDICTION AND VALIDATION OF HIGHLY IMMUNOGENIC AND CONSERVED CLASS II EPITOPEs AND DEVELOPMENT OF EPITOPE-DRIVEN VACCINES

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3.1 INTRODUCTION

Two types of T cells act as the adaptive immune system's second line of defense after the antibody. Class-I-restricted cytotoxic T cells (CD8+ CTL) directly engage and attack infected host cells. Class-II-restricted T helper (CD4+ Th) cells mediate the growth and differentiation of both T-effector cells and antibody-producing B lymphocytes. Both Class I and Class II-restricted T cells carry out their roles in response to T-cell epitopes, small linear peptides derived from protein antigens. These peptides are displayed on the surface of antigen-presenting cells (APC) by multiple alleles of

the major histocompatibility complex (MHC). The major histocompatibility complex is highly variable, occurring as many functionally different alleles. Humans express multiple alleles of class I and class II MHC. This diversity allows us to present a wide array of peptide epitopes to CD4+ and CD8+T cells. While B cells and antibodies generally recognize epitopes on surface proteins, T cells recognize epitopes derived from a broader range of proteins.

Once taken up by the antigen presenting cell (APC), antigenic proteins are broken down by proteolytic enzymes in the class I (CTL) and class II (Th) degradation pathways. The peptides generated in the course of the process have an opportunity to bind to human leukocyte antigen (HLA) class I and class II MHC molecules in the endoplasmic reticulum (class I) or MIIC (class II) vesicles. Any one of millions of peptide fragments of a given pathogen could be a T-cell epitope, but only about 2% of peptides generated are able to bind to pockets located in the binding groove of any given MHC molecule. Bound peptides are then transported to the surface of the APC. One of the critical determinants of T-cell epitope immunogenicity is the strength of epitope binding to MHC molecules [1]. Peptides binding with higher affinity are more likely to be selected by MHC molecules and displayed on the cell surface where they can be recognized by T lymphocytes. This T-cell epitope signal engages CTL (class I restricted) immune response to clear the epitope-presenting cell or other cells presenting the same class I epitope, or, alternatively, the T-cell epitope signals a Th cell, which expands and provides cytokine-mediated “help” to the development of humoral (antibody-mediated) and cellular (CTL) immune responses.

Following exposure to a pathogen, epitope-specific memory T-cell clones are established [2]. These clones respond rapidly and efficiently upon any subsequent reinfection, expressing cytokines, killing infected host cells, and marshalling other cellular defenses against the pathogen. It is this potential to mount a fast-acting “memory” response that vaccine designers hope to establish through the administration of pathogen derived antigens (i.e. vaccine formulations). The link between broad T-cell epitope response and protection from disease has been confirmed for human immunodeficiency virus (HIV), hepatitis B (HBV), hepatitis C (HCV) and malaria [3, 4]. Furthermore, many of the protective T cells in this broad repertoire may also target subdominant epitopes [5–7].

The T-cell response defines the induction of adaptive immune system memory (the target of vaccination) and both primary and secondary humoral response. The importance of these key determinants of protective immune response is reflected by the flurry of immunoinformatics activity over recent years. A number of T-cell epitope mapping tools have been developed to accelerate the identification of these critical components of the immune response. Using a variety of methods, including frequency analysis, support vector machines, hidden Markov models and neural networks, researchers have developed highly accurate tools for modeling the MHC-peptide interface and predicting T-cell epitopes. Available tools have been comprehensively reviewed [8–11]. What immunoinformatics tools have in common is an ability to quickly screen large volumes of genomic sequences for putative epitopes; this preliminary screen reduces the search space dramatically, typically by at least 20-fold.

3.2 APPLYING IMMUNOINFORMATICS TOOLS TO THE PROBLEM OF VACCINE DESIGN

The availability of epitope-mining tools has fueled the design and development of vaccines by a process that was first called “vaccinomics” by Brusic and Petrovsky in 2002 [12], “reverse vaccinology” by Rappuoli and Covacci in 2003 [13], and, more recently, “immunome-derived or genome-derived vaccine design” by Pederson [14], De Groot and Martin [15], and Doytchinova, Taylor, and Flower [16]. Immunoinformatics tools for developing these types of vaccines have only evolved in the last 10–15 years, and therefore, vaccines that are developed using these tools are only recently emerging from the preclinical pipeline.

Epitope-based immunome-derived vaccines (IDV) are generally considered to be safe when compared to other vectored or attenuated live vaccines. A number of IDVs have been tested in clinical trials [17–20]. In the cancer vaccine field, where epitope-based vaccines are well established, many such vaccines are currently in phase I/II clinical trials [21]. Epitope-based IDV may also provide essential T-cell help for antibody-directed vaccines; this concept has been exploited to improve existing polysaccharide vaccines such as HiB [22] and pneumococcal vaccines [23]. Thus, immunoinformatics tools can be used to improve vaccines already in the clinic; this may be one application of the tools that will progress more rapidly from concept to implementation.

Such vaccines may have a significant advantage over conventional vaccines, as the careful selection of the components may diminish undesired side effects that have been observed with whole pathogen and protein subunit vaccines.

3.3 EPITOPE-DRIVEN APPROACH TO VACCINE DEVELOPMENT

We have applied our tools to a range of pathogens such as HIV, variola, *Mycobacterium tuberculosis*, *Helicobacter pylori* and *Francisella tularensis*. In the next pages, we provide an introduction to the tools we use to develop vaccines and a step-by-step description of our approach, with a particular focus on immunogenic consensus sequences.

3.3.1 Identify Source of Raw Protein Sequence Information

The key ingredient for immunoinformatics-driven vaccines is an initial set of protein sequences that are considered to be likely targets for host immune response. A wealth of sequences, including sets of protein sequences derived from entire genomes, are now available from a range of databases. For example, the genome database at the National Center for Biotechnology Information website provides the complete genome sequences (and partial sequences) for hundreds of human pathogens, of which the majority could be used for vaccine development. Most immunoinformatics tools derive candidate epitopes directly from the protein sequence. The only requirement is that the deoxyribonucleic acid (DNA) sequence be translated to amino acids, which can be accomplished using online tools.

3.3.2 Select Protein Antigens of Interest

Public databases currently contain the complete genome sequences for more than 300 pathogens [24]. Once the raw protein sequences have been identified, the next step in the immunome-to-vaccine process is to identify, from within the target genome, a set of potentially antigenic genes/proteins. The availability of this vast array of genomes and online databases of virulence factors makes comparative analysis possible, facilitating the selection and targeting of antigens of interest. These sequences can then be screened using a variety of in silico, in vitro, and in vivo mechanisms or a combination of methods.

For example, the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) offers a wide variety of proteomics tools related to protein identification and characterization, DNA-to-protein translation, back-translation, and codon optimization, homology searching and sequence alignment, structure analysis and topology prediction, and pattern and profile searching. Following a protocol originally developed by Gomez et al. [25], researchers at EpiVax were able to use a selection of the ExPASy tools (SignalP, TMpred, and Prosite Scan, available at www.expasy.org) to triage the genome of *M. tuberculosis*, reducing a search universe containing over 4000 proteins to a more manageable set of 73 candidate antigens.

In the case of larger bacterial and viral pathogens, the traditional vaccine targets include surface proteins and secreted proteins (which can be readily found using the in silico screening programs mentioned above), toxins (which can be identified through homology matching), and virulence factors (which can be identified through the use of comparative genomics). However, many other types of proteins may also be worthy of consideration; in particular, proteins expressed in high abundance (such as viral capsid proteins), proteins overexpressed during growth or replication, or proteins overexpressed in response to stress conditions.

Using “reverse vaccinology,” a term coined by Rino Rappuoli, the immune memory of subjects who have successfully encountered and defeated a pathogen can be interrogated to identify the primary targets of a natural immune response. Rappuoli and colleagues identified novel vaccine targets using in silico techniques to screen the target genome for “surface protein-like” sequences. Candidate proteins were then expressed in *Escherichia coli* and screened against human sera isolated from pathogen-exposed subjects. Reactive proteins were deemed relevant to immune response [26].

A related approach for discovering candidate vaccine antigens involves analyzing the target pathogen’s proteome in silico, using T-cell epitope-mapping tools. Putative T-cell epitopes can then be screened against peripheral blood mononuclear leukocytes (PBML) isolated from human subjects who have been infected with the target pathogen (or who have the target cancer). A T-cell reaction to a particular epitope, typically measured by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot assay (ELISpot) assay, implies that the protein from which the peptide was derived was expressed, processed, and presented to the immune system in the course of a “natural” immune response. Using this method, measuring immune response to an epitope reveals a protein antigen. Our group describes this approach as “fishing for antigens using epitopes as bait,” an approach used to discover new antigens for *F. tularensis* (a bioterror agent) and tuberculosis vaccines [27, 28].

Additional in vitro techniques are used to identify expressed or overexpressed genes/proteins; these methods include: two-dimensional (2D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [29–31], mass spectrometry [32], and/or tandem mass spectrometry [33].

In the context of vaccines against infectious diseases, it may be prudent to exclude proteins that are highly conserved across species; such proteins (including housekeeping genes) may cross-react with unrelated virulent organisms or with self-proteins to which there may be preexisting tolerance. However, conservation across species should not be confused with conservation within species variants. Sequence conservation within species variants is a highly desirable trait for vaccine components and one that the IDV approach is well-suited to harness. Ribonucleic acid (RNA) viruses in particular are highly variable pathogens. In these cases, selecting epitopes that are conserved across variants or subtypes may allow for the development of a more broadly applicable vaccine. Alternatively, single proteins that are relatively well conserved, when compared to the balance of the target pathogen, can be selected as vaccine candidates. Recently, for example, one team has developed a hexon-epitope vaccine that may be effective against a range of adenoviruses, across serotypes [34]. Once critical antigens have been identified, the next steps in epitope-based IDV development are to select epitopes and confirm their immunogenicity.

3.3.3 Identifying B-Cell Antigens

It is important to clarify that no immunoinformatics tools currently identify B-cell antigens (recognized by antibodies). While humoral, or antibody-based, response represents the first line of defense against most viral and bacterial pathogens, the protein target of this arm of defense is usually too complex to model in silico. Antibodies recognize B-cell epitopes composed of either linear peptide sequences or conformational determinants, which are present only in the three-dimensional form of the antigen. Several B-cell epitope prediction tools, such as 3DEX, CEP, and Pepito, have been proposed and are in the process of being refined [35–38]. Unfortunately, the computational resources and modeling complexity required to predict B-cell epitopes are enormous. This complexity is due, in part, to the inherent flexibility in the complementarity determining regions (CDR) of the antibody and, in part, due to glycosylation and other posttranslational modifications that can result in modification of B-cell epitopes.

Although accurate B-cell epitope mapping tools remain elusive, the selection of potent B-cell antigens can be accelerated using T-cell epitope mapping tools. When considering B-cell antigens as potential subunit vaccines, it may also be important to consider their T-cell epitope content since the quality and kinetics of the antibody response is dependent upon the presence of T help. B-cell antigens that contain significant T help may outperform B-cell antigens lacking cognate help. In some cases, an identified T-cell epitope may contain a B-cell epitope. Although different epitopes activate T and B cells, it has been widely reported that B-cell epitopes have been shown to co-localize near, or overlap, class II (Th, CD4+) epitopes [39–41].

The ability to accurately predict T-cell epitopes from raw genomic data is fundamental to the development of an immunome-derived vaccine. T-cell epitope algorithms

now achieve a high decree of accuracy (in the range of 90–95% positive predictive value). One means of screening for “higher value” candidates is to perform HLA binding assays. A second approach is to screen for immunogenicity using blood from exposed donors. Both approaches are described in the next two sections.

3.3.4 In Vitro Assays: Peptide Binding Assays

Once identified, selected epitopes are then synthesized with lengths ranging from 9 to 25 amino acids. In vitro HLA binding assays can be used to measure the affinity of these peptides for either MHC class I or class II. These assays measure the ability of the test peptide to compete with a fluorescently labeled peptide known to bind MHC; such competition assays can be adapted for high throughput [26]. We routinely use HLA binding assays to confirm epitope predictions in vitro. A concordance between a peptide’s HLA binding and immunogenicity is often observed [27]. Therefore, HLA binding assays can be used to screen candidate epitopes when blood from exposed donors is difficult to obtain.

3.3.5 In Vitro Assays: Measuring T-Cell Responses

Both MHC class-I- and MHC class-II-restricted epitopes (targeting CD4+ and CD8 + T cells, respectively) are believed to be important for the development of effective vaccines. CD4 + T helper cells enhance and amplify immune responses by both B cells and CTLs and have been shown to be important in the development of CD8 + T-cell memory to a range of pathogens [42]. CTLs generally play a role in the containment of viral and bacterial infection [43], and the prevalence of CTLs usually correlates with the rate of pathogen clearance.

Like peptides, whole antigens can also be used to measure T-cell responses in vitro. The recognition of these antigens requires the presence of an APC that is capable of processing and presenting peptides derived from the antigen. Thus, whole PBMC (which include B cells and other potential APCs) are used in these assays.

If blood from exposed individuals is available, the putative epitopes can be tested for their reactivity with T cells, serum, or both. A positive immune response (as measured by ELISA, ELISpot, or intracellular cytokine staining) should be interpreted as a sign that the parent protein interfaces with host immune response in the course of natural infection or disease. Following confirmation, the peptides that stimulate a response can be considered vaccine candidates themselves or can be used to select the entire protein for use in a subunit vaccine.

ELISA and interleukin-2 (IL-2) and interleukin-4 (IL-4) ELISpot are related methods for detecting T-cell responses by the measurement of cytokines secreted by the T cells (IL-2 and IL-4 are examples). The expansion (proliferation) of T cells in response to stimulation by peptide:MHC can be measured by (1) the dilution of a fluorescent dye in subsequent generations of cells carboxyfluorescein succinimidyl ester (CFSE) and (2) the incorporation of a radioactive label in the proliferating cell’s DNA (tritiated thymidine incorporation assay). Cell surface and intracellular cytokine staining (ICS) are the most precise methodologies available for measuring and defining T-cell response using fluorescence-activated cell sorting (FACS) technology. T cells

that respond to a particular epitope can be directly labeled using tetramers (comprised of MHC class II: peptide complexes). Tetramer-labeled cells can then be sorted and counted, via FACS analysis. The phenotype of labeled T cells can be determined using cell surface markers and intracellular cytokine staining [44].

Validated epitopes from the same proteins or other pathogens within the same genome are sometimes useful as positive controls. A list of known epitopes can be found at the IEDB (the Immune Epitope Database and Analysis Resource); this site includes a database of T- and B-cell epitopes as well as tools for epitope discovery: www.immuneepitope.org).

3.3.6 Select Delivery Vehicle and Adjuvant

Factors extrinsic to processing, such as the cytokine milieu induced in response to a particular component of a vaccine [45] or pathogen [46], also play a role in the conditioning of the immune response. Therefore, T cell-epitopes may be necessary to drive immune response but are not sufficient. Co-stimulatory molecules that provide a second signal, the right cytokine milieu and other factors directing the nature (Th1 vs. Th2) of the immune response are also crucial [47, 48]. Adjuvants provide this added “boost” in the context of vaccines. There are many adjuvants that have been used in humans, and each adjuvant has advantages and disadvantages, as reviewed by Fraser et al. [49].

The same range of delivery vehicles that exist for conventional vaccines can be used for the development of immunome-derived vaccines and epitope-based IDVs. For example, IDVs and epitope-based IDVs can be formulated and delivered as pseudoproteins or as peptides in a carrier vehicle such as a liposome or virus-like particle (VLP); alternatively, the sequence of the IDV antigens or epitope string can be inserted into a viral or bacterial vector such as adenovirus or salmonella. Instead, a DNA vaccine construct encoding the antigen(s) or epitopes can be developed. Tools that may be helpful include BioConductor (an open-source software project for the analysis and comprehension of genomic data: www.bioconductor.org) and DNAWorks (a computer program that helps design oligonucleotides for polymerase chain reaction (PCR) based gene assembly: helixweb.nih.gov/dnaworks).

3.3.7 Animal Model for Vaccine Efficacy

The next step in the development of epitope-driven IDV is to determine whether immunization provides competent immune response. The IDV or epitope-based IDV (in its prototype delivery vehicle and with an adjuvant) is administered and immune responses to the components are evaluated following immunization. Even though a range of animal models are used for the evaluation of vaccines, results from immunogenicity studies in these models should be interpreted with caution. Although their functions may be similar, the MHC of mice, rodents, and nonhuman primates differ from human MHC (known as HLA in the context of human immune response) at the amino-acid level and these differences affect which epitopes can be presented. This is part of the reason why different strains of mice (Balb/C, C57Bl/6) have different

immune responses to pathogens as well as to vaccines for those pathogens [50]. The best approach for evaluating epitope vaccines developed for humans is to use murine models that are transgenic for HLA.

Fortunately, a number of transgenic mouse strains that express the most common HLA A, HLA B, and HLA DR molecules have been developed. T-cell responses in these mice correlate directly with T-cell responses observed in infected/vaccinated humans [51, 52]. HLA transgenic mice are now routinely used to assay and optimize (human) epitope-driven vaccines in preclinical studies [53–55]. Despite the limited number of HLA class II alleles for which transgenic mice have been developed, comparisons of immunogenicity can be done to a high degree of accuracy in the mouse model for selected HLA class II alleles (HLA DRB1 *0101, *0301, *0401, *1501). Unfortunately, it appears these mice may have difficulty breeding due to poorly understood consequences of their transgenic heritage, thereby limiting the use of this important model system.

3.3.8 Animal Challenge Studies

The final step in the development of any immune-derived vaccine is experimental validation of the immunogenicity and protective efficacy. A series of experimental immune-derived vaccines have shown efficacy in animal models and several IDVs are being tested in clinical studies. In the context of infectious disease, genome-derived vaccines that have progressed furthest along the vaccine development pipeline are generally based on whole proteins rather than epitopes [26, 56]. However, epitope-based IDVs are currently being developed for a range of infectious diseases by the authors' lab and by others [e.g. 57–59].

While it is common knowledge that subunit-based vaccines can protect against infection, similar success with epitope-based approaches is not as widely known. In addition to the studies previously cited, immunization of BALB/c mice with three doses of a peptide construct containing an H-2 (d)-restricted cytotoxic T lymphocyte (CTL) epitope from a murine malaria parasite induced both T-cell proliferation and a peptide-specific CTL response mediating nitric-oxide-dependent elimination of malaria-infected hepatocytes *in vitro*, as well as partial protection of BALB/c mice against sporozoite challenge [60]. In a separate study, immunization of BALB/c and CBA mice with CTL epitopes derived from the measles virus resulted in the induction of epitope-specific CTL responses and conferred some protection against encephalitis following intracerebral challenge with a lethal dose of virus [61]. We have published a number of studies showing protection against challenge, cited previously. Additional papers from our group demonstrating protection against challenge using epitope-based vaccines are in press as this chapter goes to print [62, 63, 64].

3.3.9 Clinical Development

A key factor bearing on the “go/no-go” decision to take a vaccine into the clinic is whether there is a clear path to clinical development. Immunome-derived vaccines and epitope-based IDVs for infectious diseases have taken longer to make the transition

from animal model to the clinic, mainly due to the novelty of the concept and the lack of a standardized test for go/no-go. That decision is hampered by the lack of suitable animal models (HLA transgenic mice can be used for live challenge but may not harbor the human version of the disease, and nonhuman primates don't have human HLA). Despite this obstacle, several epitope-driven vaccines against viral and microbial pathogens have reached the stage of phase I or II efficacy trials in humans. For example, Bionor Immuno's HIV p24 gag peptide vaccine (Vacc-4X) was demonstrated to be safe and well-tolerated in phase I trials [65] and dose-dependent and immunogenic in phase II trials in Norway [66]. Similarly, an epitope-based vaccine for malaria was demonstrated to be safe and well tolerated [67].

Cancer therapy is an exception to the go/no-go rule requiring proof of concept in animal studies [68, 69]. In the cancer vaccine field, many more peptide vaccines have successfully passed preclinical tests and are currently in phase I/II clinical trials [70]. Whereas the response rates to classical (nonpersonalized) peptide vaccines have been disappointing, responses to personalized vaccines (in a phase I trial, conducted in Japan) have been as high as 11.1% in the advanced cancers and equal to or more than 20% in malignant glioma and cervical cancers, respectively [71].

3.3.10 Role of MHC (HLA)

T cells can only recognize peptides in the context of major histocompatibility complex (MHC) molecules (HLA in humans). Thus, an important component in the epitope-driven vaccine process is the selection of pathogen-derived epitopes that have the features necessary for binding to MHC. These features, termed "MHC binding motifs," were first identified by Rötzschke and Falk [72, 73]. The MHC-binding motifs are nonrandom; any given MHC allele has a limited set of ligands that it can present. However, immunoinformatics tools can model the differences in the binding preferences of MHC alleles and can select peptides that can bind to many alleles.

When testing vaccines in laboratory strains of mice, MHC background is considered to be relatively fixed, and thus results can be compared to other mice with the same background. This is not true of humans participating in vaccine studies because humans are genetically diverse and possess multiple HLA. Because different HLA backgrounds restrict the selection of the epitope ensemble, the repertoire of possible MHC-restricted epitopes recognized by an individual's T cells has been shown to differ, even between HLA-matched individuals [74–76].

HLA variability has been considered a limitation for epitope-driven vaccine development; however, it need not be. Both DeLisi and Sette [77, 78] have addressed this issue by suggesting that epitope-based vaccines contain enough epitopes restricted by "supertype" HLA representing the broadest possible coverage of the human population. Furthermore, recent studies by Brander and Walker [79] indicate that there may be even greater flexibility in the binding of epitopes to MHC than previously recognized; this is also consistent with data presented by Frahm et al. [80]. The inclusion of "promiscuous epitopes" (epitopes that are recognized in the context of more than one MHC) [77, 81, 82] in epitope-driven vaccines may therefore overcome the challenge of genetic restriction of immune response.

3.4 VACCINE DESIGN TOOLS

3.4.1 EpiMatrix: Discovery of T-Cell Epitopes

The EpiMatrix epitope-mapping algorithm, initially developed by A. De Groot and her co-workers at Brown University and subsequently licensed to EpiVax, has been validated by over a decade of vaccine research [83–86]. Matrix motifs for 24 HLA class I alleles are available for use with EpiMatrix. In 2000, Martin and De Groot generated EpiMatrix matrices for 74 class II alleles by using an adapted pocket-profile approach first described by Sturniolo et al. [87]. This method is based on the observation that the secondary structure of MHC molecules tends to be relatively conserved, even across species. Although there are many different HLA alleles, the number of different pocket types is much smaller, and the set of binding pockets for a given HLA can often be described as a reassortment of several other HLA molecules' pockets. This observation led to the concept that different MHC have been derived by “mixing and matching” pocket profiles, whereby the binding properties of an allele can be described by the composition of its set of pockets. For this reason, while 74 class II allele matrices are available with EpiMatrix, promiscuous peptides are selected using a panel of only 8 (DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501). These alleles are very different from each other and represent the predominant types of “pockets” for the most common MHC. Considering average reported frequencies, these 8 alleles should “cover” 95% of most human populations worldwide [88].

In a typical EpiMatrix analysis, a protein antigen is parsed into all of its constituent overlapping 9-mer peptides. Each 9-mer is then scored for predicted binding affinity to one or more class I or class II HLA alleles by scoring the fit of each amino acid in the sequence to the projected pocket in the corresponding HLA molecule. In order to compare potential epitopes across multiple HLA alleles, EpiMatrix raw scores are converted to a normalized “Z” scale. EpiMatrix assessments (frame-by-allele evaluation) that score above 1.64 are defined as “hits”; that is to say potentially immunogenic and worthy of consideration. In general, we expect about 5% of all assessments to score above 1.64. These peptides have a significant chance of binding to HLA molecules with moderate to high affinity and, therefore, have a significant chance of being presented on the surface of APCs, such as dendritic cells or macrophages, where they may be interrogated by passing T cells.

3.4.2 Conservatrix: Finding Conserved T-Cell Epitopes

The genetic variability of some pathogens constitutes a significant challenge to efforts to design a vaccine driven by cellular immune response [89, 90]. Vaccines designed to protect against a given strain or clade of a quickly mutating pathogen may be ineffective when faced with a heterologous challenge. One approach to solving this problem is to design a vaccine that includes epitopes from conserved regions that may be functionally or structurally important to the alleles. EpiVax has used the

Conservatrix tool to do just this in the context of an HIV-1 vaccine program [91, 92]. The Conservatrix algorithm parses input sequences into component strings (typically either 9-mer or 10-mer segments) and then searches the input data set for matching segments. No alignment of input sequences is necessary. The program then produces a frequency table showing each unique segment in the data set and the number of times that sequence has occurred. Results of each analysis are stored in a database and may be browsed or exported to another program for analysis. Conserved sequences can be identified and then scored using EpiMatrix.

Conservatrix may also be used to compare strings derived from different strains of the same organism or to search for elements common to disparate organisms. Peptides that occur in common “housekeeping” genes may show up in many different types of organisms. In all likelihood, these common peptides have been seen repeatedly by the human immune system and are probably tolerated whereas peptides that are unique to a given pathogenic target are more likely to be immunogenic.

3.4.3 ClustiMer: Finding Promiscuous T-Cell Epitopes

We and others have observed that potential T-cell epitopes are not randomly distributed throughout protein sequences but instead tend to “cluster” in specific regions. In our experience, class II T-cell epitope “clusters” range from 9 to roughly 25 amino acids in length, and considering their affinity to multiple alleles across multiple frames, can contain anywhere from 4 to 40 binding motifs. In order to find potential T-cell epitope clusters, the ClustiMer algorithm screens the results of the EpiMatrix analysis and looks for regions with unusually high densities of putative T-cell epitopes. The significant EpiMatrix scores contained within these regions are then aggregated and normalized to create an EpiMatrix cluster immunogenicity score. Peptides with cluster scores of +10 and particularly those with scores above +15 tend to be immunogenic.

In our experience, the putative T cell epitope clusters identified by EpiMatrix perform well in ex vivo immunogenicity assays. These clustered sequences tend to be promiscuous MHC binders and frequently turn out to be reactive in significant numbers of test subjects both in terms of immune response to autologous proteins (e.g., Fab or FVIII epitopes) and in terms of response to epitopes derived from pathogenic proteins (malaria, HIV, TB). In recent experiments, 100% of subjects exposed to either tularemia or vaccinia responded to T-cell epitope pools containing approximately 20 epitope clusters. Clustering of class II binding potential is indicative of a more immunogenic therapeutic protein (biologic), and thus, for immunogenicity screening, deimmunization, and vaccine development programs, putative T-cell epitope clusters are identified.

Promiscuous T-cell epitopes also exist, to a certain degree, for class I alleles. Some laboratories have demonstrated cross presentation of peptides within HLA “superfamilies,” such as the A3 superfamily: A3, A11, A31, A33, and A68 [77]. We have also confirmed cross-MHC binding and presentation to T cells in our HIV vaccine studies [85].

3.4.4 Protein Immunogenicity Scale: Ranking the Immunogenic Potential of Whole Proteins

The EpiMatrix system has been utilized to measure the potential immunogenicity of whole proteins. In this context, EpiMatrix assesses the aggregate epitope density of a given protein with respect to the aggregate epitope density of a set of randomly generated pseudoprotein sequences of similar size [93]. By correcting for the size and expected epitope density, the potential immunogenicity candidate vaccine antigens can be directly compared. In general, proteins having higher epitope densities tend to be more immunogenic while low-density proteins tend to be immunologically inert.

T-cell epitope clusters can be very powerful. The presence of one or more dominant T-cell epitope clusters can enable significant immune response to even otherwise low-scoring proteins. For example, the well-known immunogen tetanus toxin contains no more T-cell epitopes than one would expect to find by chance, but it also contains several high-scoring T-cell epitope clusters.

3.4.5 EpiBars—Unique Pattern Predictive of High-Immunogenicity Peptides

Epitopes that contain HLA binding motifs for more than four HLA alleles exhibit a characteristic pattern on an EpiMatrix report, which we refer to as an “EpiBar.” Retrospectively, it has been determined that an affinity for more than one HLA allele is predictive of high immunogenicity. A number of well-known promiscuous epitopes exhibit EpiBars, for example, sequences that contain EpiBars include influenza hemagglutinin 30–31 (cluster score of 22), tetanus toxin 825–850 (cluster score of 46), and GAD65 557–567 (cluster score of 23). An example of an immunogenic peptide that contains an EpiBar is show in Figure 3.1. Note the horizontal bar of high Z scores at frame start 308.

3.4.6 “iTEm” Analysis—Individualized Immunogenicity Measure

As can be expected, given the variability of human HLA in any given subject group, subject-to-subject variations in T-cell response can be observed. We have observed that immune responses are closely associated with subject HLA type and with the number of matching T-cell epitopes contained in the immunogen. We developed a measure of this variability called the individualized T-cell epitope measure, or “iTEm,” which can be calculated for each subject by summing the Z scores for each positive peptide for each HLA allele in the subject’s haplotype. This calculated score allows for the differentiation between subjects of potential immunogenicity based on the number of putative epitopes contained in a protein that might be presented to their T cells, based on their HLA haplotype.

In a study of therapeutic proteins, subject iTEm scores correlated nicely with antibody responses [94]. In this study, iTEm scores were also closely correlated with both CD4+ and CD8+ T-cell responses. A significant correlation was observed between the iTEm scores for haplotypes DRB1*0701, *1501 and *1301, and antitherapeutic

EpiMatrix Cluster Detail Report												
Accession: FLU-HA Cluster: 305												
Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
305	CPRYVKQNT	313	-0.29									0
306	PRYVKQNTL	314	-0.26				1.45					0
307	RYVKQNTLK	315	-0.32									0
308	YRKQNTLKL	316	-0.56	3.06	2.28	3.18	2.81	2.43	2.81	3.11	2.55	8
309	VKQNTLKLRA	317	-0.05		1.51			1.62	2.01	1.7	1.41	2
310	KQNTLKLAT	318	-0.16								1.34	0
311	QNTLKLATG	319	-0.08									0
Summarized Results				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total
Maximum Single Z score				3.06	2.28	3.18	2.81	2.43	2.81	3.11	2.55	--
Sum of Significant Z scores				3.06	2.28	3.18	2.81	2.43	4.82	4.81	2.55	25.94
Count of Significant Z Scores				1	1	1	1	1	2	2	1	10
Total Assessments Performed: 56				Hydrophobicity: -0.53	EpiMatrix Score: 20.17				EpiMatrix Score (w/o flanks): 21.41			

Figure 3.1. EpiBar from influenza HA. Z score indicates the potential of a 9-mer frame to bind to a given HLA allele; the strength of the score is indicated by the blue shading. All scores in the top 5% ($Z \text{ score} \geq 1.64$) are considered “Hits.” Scores in the top 10% (shown but not highlighted) are considered elevated. Other scores are masked for simplicity. Frames containing four or more alleles scoring above 1.64 are referred to as EpiBars and are highlighted in yellow. These frames have an increased likelihood of binding to HLA. (See *insert for color representation of this figure.*)

antibody titers where high titers corresponded to high iTEM scores. For example, one subject who had both the *0701 and *1501 alleles had the highest antibody concentration following exposure to the immunogen “FPX”. This subject also had the highest iITEM score and the highest number of interferon-gamma and IL-4 spot-forming cells (SFCs) per million PBMCs in ELISpot assays. These two alleles might have had an additive effect in terms of processing and presentation of peptide sequences from FPX. In contrast, the DRB1*0301 allele was associated with very low iITEM scores, negligible SFCs, and a lack of an antibody response. iITEM may also explain differences between subjects in observed antibody responses to recombinant protein vaccines. We recently refined the iITEM algorithm to better balance the strength and frequency of HLA-matched motifs in a sequence, and found that the returned score more closely matched our experimental observations [95].

3.4.7 Immunogenic Consensus Sequence Epitopes

While understanding the individual’s response to an antigen can be helpful, vaccines are best developed for the population as a whole. Host MHC variability is not the only barrier to developing a globally relevant vaccine; pathogen variability also complicates the task. To address the variability of HIV, the authors developed EpiAssembler [95]. EpiAssembler identifies sets of overlapping, conserved and promiscuously immunogenic epitopes and assembles them into extended immunogenic consensus sequences (ICSSs) (see Fig. 3.2).

In order to develop the ICSSs, we first evaluated each pathogen-derived HIV 9-mer in our database for conservation using Conservatrix and for immunogenic potential using EpiMatrix. The EpiAssembler algorithm starts by selecting a single well-conserved, putative promiscuous epitope. EpiAssembler then searches for the

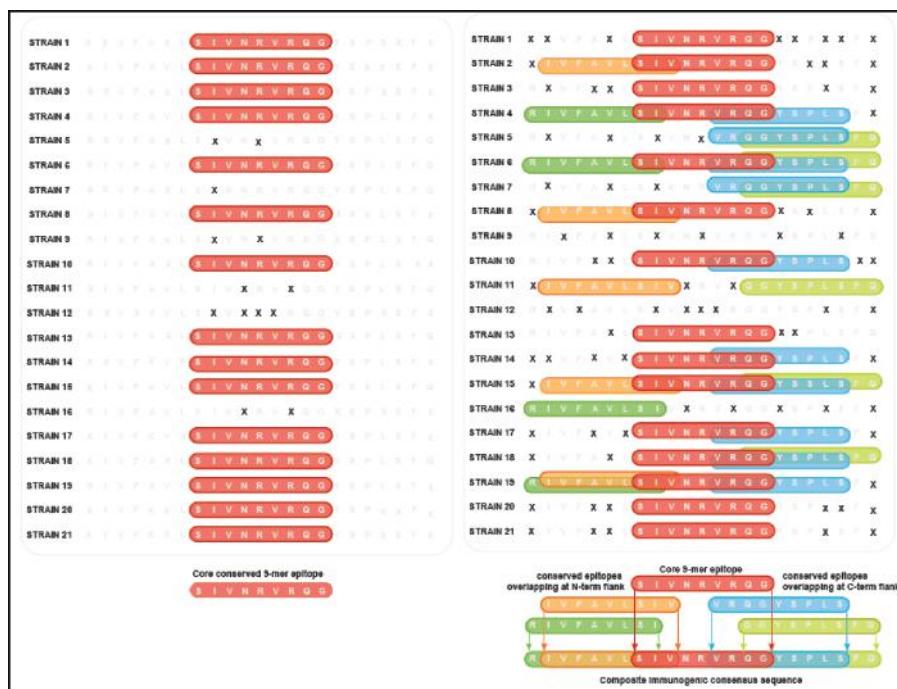


Figure 3.2. Immunogenic consensus sequence. Left panel: Each variant strain is first analyzed and a highly conserved, putatively promiscuous 9-mer is chosen as the core peptide (red). Mismatches with the selected epitope sequences are represented with the letter X. Right panel: Additional epitopes are then identified that overlap with the natural N-term and C-term flanking regions of the core 9-mer epitope. If more than one suitable overlap is identified, the overlapping peptide with the higher overall EpiMatrix rank is selected. This process is repeated using the extended peptide as the new core sequence. The cycle can be repeated for the length of an entire protein or can be truncated when the peptide reaches a length that can be easily produced synthetically. (See *insert* for color representation of this figure.)

highest scoring segments that naturally overlap either the N- or C-terminal of the core epitope. Each time an overlapping segment is identified, it is added to the core sequence. The extended sequence then becomes the new core sequence and the process is repeated.

As compared with immunogenic consensus sequences, traditional sequence-based consensus sequences, on average, contain half as many potential epitopes and cover a third fewer isolates. The ICS approach has been useful for identifying highly immunogenic epitopes for HIV vaccine design [96]. The full composite ICS peptides are exactly conserved in only a few individual strains of HIV, their sequences represent a significant percentage of circulating strains because every overlapping epitope is conserved in a large number (range 893–2254) of individual HIV-1 strains.

Of the 20 ICS peptides derived from HIV that were tested, 19 have provoked interferon-gamma release from PBMC from HIV-infected humans as measured in ELISpot assays. We found much higher immune responses to the peptides, than we had previously observed. The intensity of T-cell response ranging 200–500 SFCs per peptide was substantial, even for immunocompromised HIV-positive subjects [96]. By focusing on conserved, promiscuous T helper epitopes, the ICS approach has the potential to efficiently overcome the genetic variability of both virus and host. A method for incorporating ICS sequences in a whole protein is presented in Figure 3.3. We have proposed to use this method to make “megatope” proteins for HCV and other variable pathogens.

We have used the ICS approach to develop a number of vaccines, including our VennVax vaccine, an epitope-driven vaccine that used immunoinformatics methods to select immunogenic and conserved sequences from 7 poxvirus genomes. In studies of the epitopes using blood from Dryvax (Vaccinia)-immunized subjects, 41 out of 50 class II peptides induced T-cell responses; all 50 peptides were included

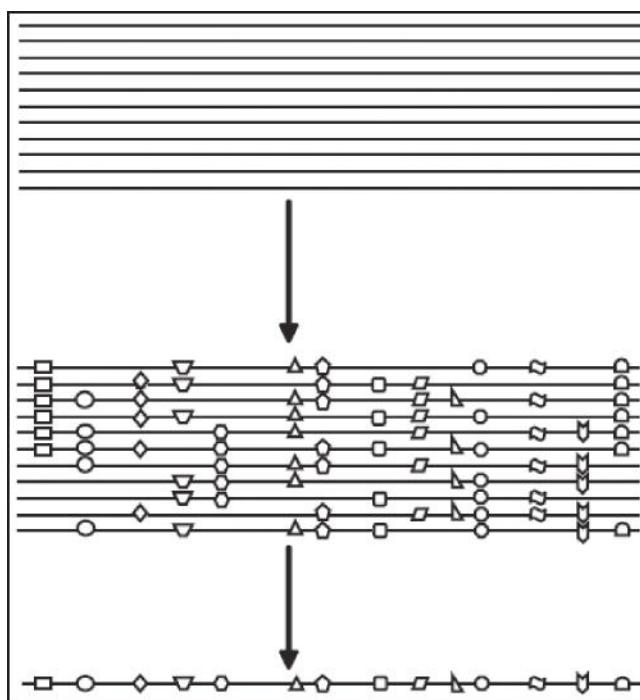


Figure 3.3. Immunogenic consensus protein. In order to obtain a consensus protein optimized for maximum immunogenic potential, putative T-cell epitopes (represented by the open symbols) that are conserved within various strains of a given pathogen are identified by Conservatrix and EpiMatrix. These conserved, immunogenic regions are then superimposed over a consensus sequence, allowing the final protein sequence to include all putative T-cell epitopes in their natural context.

in a VennVax vaccine, which subsequently was shown to protect mice against live, aerosolized vaccinia challenge [97],

The approach is also being applied to a vaccine for *Burkholderia mallei* and *pseudomallei*, comprising 31 genomes in total. EpiAssembler was used to assemble 20,427 conserved and immunogenic peptides derived from *B. mallei* into clusters of overlapping peptides. Ultimately, 1117 ICS clusters were assembled. Similarly, using the 27,026 conserved and potentially immunogenic peptides derived from the *B. pseudomallei* analysis, 1518 ICS clusters were assembled. And finally, using EpiAssembler, 40,813 peptides derived from *B. cepaciae* were assembled into 245 ICS clusters. The 2880 resulting candidate ICS clusters were then evaluated for conservation.

To our great surprise, 2 ICS clusters were found that were 100% conserved across all three species of *Burkholderia*. Two more were found to be 100% conserved in *B. mallei* and *B. pseudomallei*, and >70% conserved in *B. cepaciae*. Fifteen additional ICS clusters shared >70% conservation in all 3 species. An additional 70 ICS clusters were shown to be 100% conserved between *B. mallei* and *B. pseudomallei* but <70% conserved in *B. cepaciae*. Only one ICS cluster was found to be 100% conserved in *B. pseudomallei* and *B. cepaciae* and >70% conserved in *B. mallei*, while 12 ICS clusters were shown to be >70% conserved in *B. pseudomallei* and *B. cepaciae* but <70% conserved in *B. mallei*. Twenty-two ICS clusters were shown to be >70% conserved in *B. mallei* and *B. cepaciae* but <70% conserved in *B. pseudomallei*. The top 50 high-scoring clusters derived from this set of cross-species conserved epitopes were selected for further analysis; currently these peptides are being evaluated for immunogenicity in HLA transgenic mice.

Thus, the ICS approach is proving to be immensely valuable when designing vaccines for pathogens for which multiple strains may have been sequenced, but ultimately the proof of the principle will be demonstration of protective efficacy in humans.

3.4.8 BlastiMer: Eliminating Cross Reactivity

One of the advantages of epitope-based vaccines is that it is possible to omit deleteriously cross-reactive epitopes from their formulations. Perhaps the most famous example of an adverse effect due to cross reactivity with self was observed following vaccination for Lyme disease with the OspA protein. In this case, in some individuals, a T-cell epitope contained within the OspA protein acted as a molecular mimic for the human protein LFA-1 and promoted an arthritis-like autoimmune response in some individuals. The vaccine has been reengineered with the cross-reactive epitope removed [98]. One way to limit the possibility of cross reactivity and autoimmunity in epitope-based vaccines is to BLAST all the putative epitopes against the human sequence database at GenBank (<http://www.ncbi.nlm.nih.gov/>). Epitope sequences that are homologous to components of the human genome can be set aside while the remaining “foreign” epitopes can be safely included in vaccine formulations.

As a standard practice at EpiVax, any peptide that shares greater than 80% identity with peptides contained in the human proteome is eliminated from consideration. The BlastiMer program automates the process of submitting sequences to the BLAST engine at NCBI (www.ncbi.nlm.nih.gov/blast). By default, BlastiMer BLASTs

sequences against all nonredundant GenBank CDS translations, PDB, SwissProt, PIR, and PRF. Alternatively, the BlastiMer program can be directed against the database of patented sequences on file at GenBank. Optional parameters allow operators to limit the search to selected genomes such as the human genome. Users of the BlastiMer program may control all of the submission options available to interactive users of the BLAST engine at NCBI. Results returned by the BLAST engine are recorded in a database and can be browsed, exported, or summarized and rendered in a report format. One of the more valuable features of the BlastiMer program is its ability to produce a summarized report, allowing users to quickly separate common sequence variations from obscure ones.

3.4.9 VaccineCAD: Tool for Optimizing Epitope-Based Vaccines

One approach to developing a vaccine from epitopes is to align the individual epitopes in a protein or DNA vaccine construct as a “string of beads” without any intervening sequences or spacers between the payload epitopes [99, 100]. However, the lack of “natural flanking sequences” surrounding the payload epitopes in a string-of-beads conformation has raised concern that their proteolytic processing may be compromised and that junctional epitopes (epitopes other than the specific epitopes of interest) may be generated as a result of processing [101]. To address this concern, EpiVax has developed VaccineCAD, an algorithm that incorporates the evaluation of junctional epitopes and the insertion of spacers and breakers into the design of any string-of-beads construct.

VaccineCAD iteratively analyzes epitope assemblies and minimizes the potential for junctional immunogenicity in any string-of-beads construct. The algorithm starts by arranging the payload epitopes in a randomly selected order. Next, the peptide sequences contained in the junctional regions between the target epitopes are evaluated for potential immunogenicity with respect to the same HLA alleles used to select the payload epitopes. The “worst” junction (i.e., the junction containing the highest potential for immunogenicity) is identified. The algorithm then evaluates a large number of potential alternative sequences and selects the best available substitute.

The process is repeated until no additional reductions in predicted immunogenicity can be achieved or until all junctional immunogenic potential has been eliminated. In some cases the potential for junctional immunogenicity cannot be sufficiently reduced. In these cases a cleavage promoting spacer sequence, typically “AAY” [102–105] or a binding inhibiting “breaker” sequence such as “GPGPG” [106] will be placed in the junction between the two offending epitopes (Fig. 3.4).

3.4.10 Aggregatrix: Selecting Epitopes to Cover Multiple Criteria

In addition, EpiVax recently developed Aggregatrix, an algorithm that iteratively searches for the minimum combination of peptides that achieves maximal cross-strain, cross-subtype representation. The Aggregatrix algorithm addresses the classical time-consuming “set cover” problem faced by vaccine designers who have successfully identified a large number of MHC-restricted T-cell epitopes derived from a variable pathogen. Their goal is to find a minimal set of epitopes, which “covers” the maximum

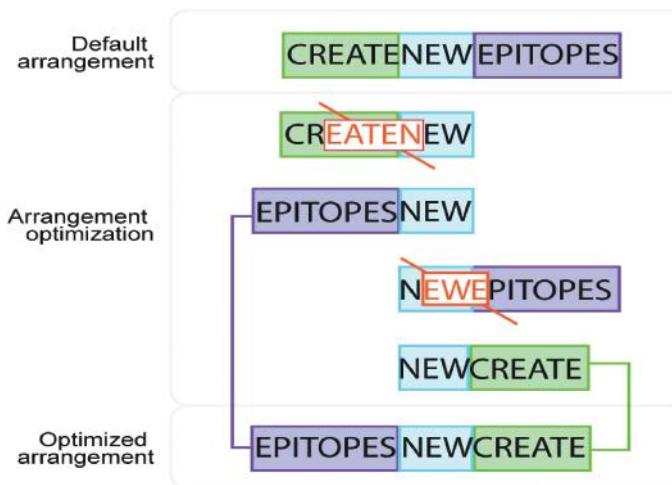


Figure 3.4. Vaccine computer-aided design (CAD). Vaccine CAD is illustrated with three example epitopes represented by the words “create,” “new,” and “epitopes.” The default arrangement of the words results in unintended sequences, represented by the words “eaten” and “ewe,” at the junctions between the intended epitopes. Reiterative modifications in the arrangement of the epitopes results in the development of a sequence that contains no “neo-epitopes” (new epitopes that were not intended) at the junctions of the juxtaposed epitope sequences.

number of HLA types and observed strains of the target pathogen. To computational experts this type of problem is known as nondeterministic polynomial time (NP-complete) and there is no known way to solve it without considering every possible combination. This brute-force approach may be possible for smaller data sets. For larger data sets, NP-complete problems will bog down even the fastest supercomputers. Fortunately, there are first-order approximations that can be applied to this type of problem. The Aggregatrix algorithm can find the minimal set of epitopes needed to “cover” a population of HLA alleles and all/most of the observed strains of the target pathogen relatively quickly and with minimal computational resources.

Consider the problem of designing a vaccine against the highly variable HIV virus. The vaccine team at EpiVax recently used the Aggregatrix algorithm to optimize an HLA-B7-restricted vaccine construct to cover multiple clades of the virus as found in multiple countries across a span of 10 years of observations [107]. The algorithm was able to identify several peptides that were conserved across clades, countries, and time. The set of highly conserved HLA-B7-restricted peptides tested and confirmed in ELISpot assay covers between 51 and 65% of strains in a given year, between 33 and 94% of strains in a given country, and between 0 and 100% of strains in a given clade. The HLA-B7 peptides cover 85, 78, 78, and 80% of clades A, B, C, and D, respectively (Fig. 3.5). This is a remarkable breadth of coverage for a limited set of epitopes given the well-known ability of HIV to mutate away from HLA [108, 109].

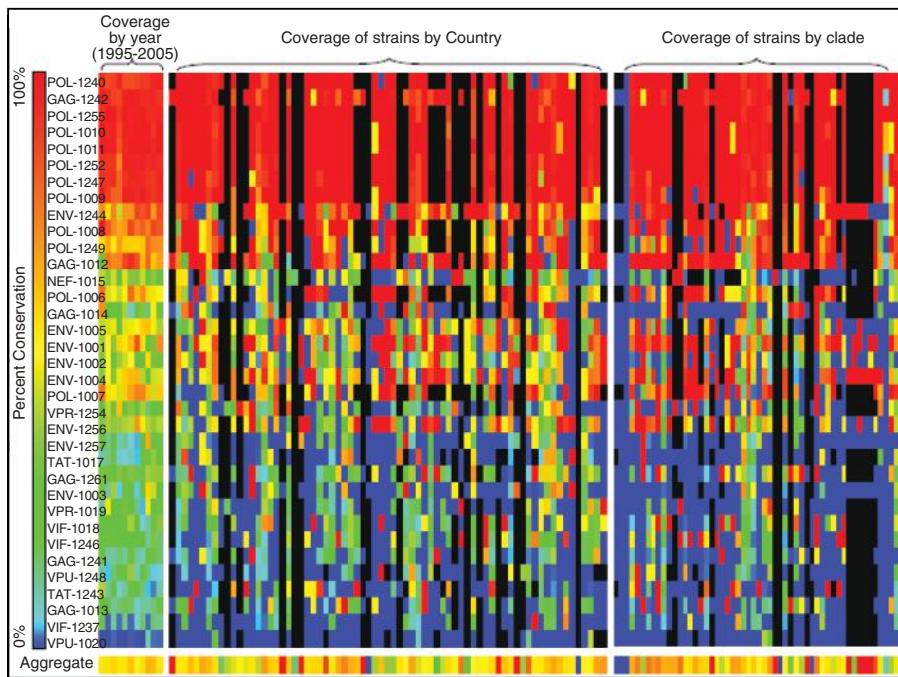


Figure 3.5. Aggregatrix. The Aggregatrix algorithm determines conservation of individual peptides across years (left set of columns), countries (middle set of columns), and clades (right set of columns). Warmer colors, such as reds and oranges, indicate a higher degree of conservation and cooler colors, such as greens and blues, indicate a lower degree of conservation. To create a vaccine that is likely to be effective across time, space, and clade, those peptides with greater conservation should be selected for inclusion in a potential vaccine. (See *insert* for color representation of this figure.)

3.5 IMMUNOGENIC CONSENSUS SEQUENCE (ICS) VACCINES

We envision two possible approaches to take advantage of T-cell help to improve the efficacy, reactivity, and duration of immune responses. The first would be to develop a peptide cocktail comprised of immunogenic consensus sequence (ICS) T helper epitopes. The second approach would be to develop composite antigens whose sequences are, in whole or in part, optimized according to the ICS approach described above (megatope proteins).

How should one deliver a Th-epitope-rich vaccine? Peptide cocktails have an excellent safety profile and can be designed to maximize viral strain coverage. Disadvantages of peptide-based vaccines include expense of manufacture and validation. To address this, we developed the ICS approach, which maximizes the antigenic coverage of each individual peptide. Peptide-based vaccines also have the flexibility of antigenic breadth. In the context of seasonal influenza, one limitation of conventional

vaccination, and of natural infection, is that the immune system often focuses strongly on the most mutable immunogen of the virus—hemagglutinin (HA). Combining conserved T-cell epitopes from HA, and from other, more highly conserved influenza proteins, may circumvent this problem, boosting antibody response despite potential viral variation, and broadening the T-cell repertoire might make it possible to impair viral escape and decrease viral loads sufficiently to disrupt transmission.

3.6 ICS PROTEIN-BASED VACCINES

By extending the approach described above, it is possible to develop complete synthetic antigens whose sequences are optimized for T helper potential. With an eye to structural considerations, even recombinant protein-only vaccines could be optimized in this way, enabling primary cognate T help to be maximized and B-cell memory to be elicited. We believe that the ideal vaccine would include whole proteins in addition to some epitopes; some or all of these antigens could be optimized using the ICS approach. Physically linking the proteins to the ICS epitopes (e.g., assembled as a VLP) would further maximize primary cognate T help, since B cells that capture the recombinant proteins would be able to process and present T helper epitopes derived from more variable proteins. Such a vaccine would likely be able to stimulate potent, relevant, and long-lasting immune responses while being safe for use in immunocompromised individuals.

As compared with immunogenic consensus sequences, randomly selected counterparts, on average, contain half as many binding motifs and cover one-third fewer isolates. To develop vaccines of equivalent antigenic “payload” using conventional methods would be prohibitively expensive as it would require including multiple different variants of each antigen. While our concept of developing ICS proteins have not yet been tested in the clinic, we believe that these and similar approaches that harness conserved T help have tremendous potential and deserve consideration.

3.7 POTENTIAL PITFALLS: ADVANTAGES AND DISADVANTAGES OF IDV

We have used the immunoinformatics tools described here to predict a large number of immunogenic epitopes from HIV, *F. tularensis*, *M. tuberculosis*, *H. pylori*, and vaccinia. Indeed, the EpiMatrix epitope-based vaccine design platform has already yielded a prototype *F. tularensis* Type A (subsp. *tularensis*) epitope-based vaccine that confers 60% protection against heterologous lethal respiratory challenge with the live vaccine strain (LVS), an attenuated subsp. *holoarctica* derivative [110]. To our knowledge no subunit vaccine for tularemia has achieved a comparable level of protection in this well-developed lethal respiratory challenge model.

To carry such a vaccine forward to the clinic will require new and better methods to be developed and validated for delivering epitopes as vaccines. In the meantime,

the IDV approach is certainly worth pursuing in tandem with other more traditional methods to developing vaccines for emerging infectious disease and biowarfare agents.

At a minimum, epitope-based vaccines must contain enough epitopes restricted by “supertype” HLA to induce a broad response in the human population. The challenge to elicit a broad immune response is illustrated by new human studies that are revealing the breadth of T-cell-mediated immune response to a range of vaccines and pathogens [111, 112]. In the case of our smallpox vaccine, this diversity of immune response may indeed be the basis for the efficacy of the vaccine. Furthermore, inclusion of diverse (nonidentical) epitopes improves paracrine help (cooperation), rather than competition, as described by Cruesot et al. [113]. This challenge can be addressed by the inclusion of peptides restricted by six class I alleles and promiscuous class II epitopes—an approach that will overcome the challenge of genetic restriction of human immune response.

There is also a growing need to predict and evaluate individual responses to vaccines, or in certain cases to develop personalized vaccine payloads. For example, development of epitope-mapping algorithms for DQ and DP class II HLA alleles will make it possible to characterize complete immunomes. This information will generate comprehensive individual T-cell epitope measures (iTEM) based on an individual’s HLA genetic makeup, allow researchers to identify *a priori* clinically important epitopes and screen clinical cohorts for subjects that are more likely to develop targeted immune responses.

Furthermore, epitope-mapping tools that are currently available are not yet useful for discovering new B-cell epitopes, whether from proteins or from nonprotein components such as carbohydrate or lipid antigens. The tools that are currently available cannot be used to accurately predict conformational (B cell) epitopes that interact with antibodies, although such tools are being refined [114]. Thus, the immunogens identified using *in silico* approaches must be evaluated *in vitro* and also in appropriate challenge models, prior to progressing to vaccine trials. It is expected that advances will be made in this field in the future.

Besides antigen identification, the success of immunome-derived vaccines relies heavily on delivery technologies. These technologies continue to mature independently and provide important lessons to epitope-based vaccine designers. The major areas of research to watch include biological macromolecule (including cytokines), lipopeptide and polysaccharide adjuvants and particulates (liposomes, exosomes, virosomes, nanoparticles), and cell-based delivery systems.

Protective immune response also involves engagement of Toll-like receptors of the innate immune system, which regulate cytokine milieu in part. In the future, it is anticipated that Toll-like receptor signaling “pathogen-associated molecular patterns” (PAMPs) [115] might also be modeled and selected using immunoinformatics tools.

And finally, the concept of epitope-driven vaccines is relatively novel. Complete genome sequences have been available for only a little more than a decade now and the tools to process the data for vaccine design are even newer. Vaccine developers relying on computational vaccine design have encountered their fair share of skepticism from

more traditional vaccine developers. However, to paraphrase Thomas Kuhn, ‘in any community of scientists, there are some individuals who are bolder than most, who seek alternatives to existing paradigms because they perceive a path forward. The new paradigm may appear to be faulty, or incomplete, because much remains to be determined. Many members of the scientific community will resist change, however, some scientists who possess an exceptional ability to recognize the potential of the new paradigm will eventually shift in favor of the challenging paradigm. In time, if the challenging paradigm is supported by additional evidence, it will replace the old paradigm, and a paradigm shift will have occurred’ [116].

By implementing immunoinformatics tools and by sharing skills and ideas so as to drive the development of novel vaccines, a paradigm shift may emerge. Thus the question is not whether to begin making immunome derived vaccines, it is *when* to begin. The need for new approaches to vaccines for human health is great, and we would argue that the time to begin is now.

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PART 2

VACCINE PLATFORMS

VIRUS-LIKE PARTICLE VACCINES: ADVANTAGES AND CHALLENGES

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4.1 INTRODUCTION

Vaccinations are the most effective way to prevent disease induced by infectious agents. The use of vaccines has successfully eradicated smallpox and reduced the incidence of measles, mumps, rubella, and poliomyelitis. However, infectious diseases such as malaria, tuberculosis, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and dengue hemorrhagic fever have been

more challenging for vaccine manufacturers. Pathogens that cause these diseases result in a large number of deaths, particularly in the developing countries. New and emerging diseases, as well as the development of biowarfare agents, are presenting new challenges for the vaccine developers.

Many of the most successful viral vaccines are based on live-attenuated or inactivated viruses. Viruses have the unique characteristic of self-assembling into particles within host cells. Upon release, they are recognized by the mammalian immune system, resulting in phagocytosis of free virions or destruction of cells producing these particles. Advances in molecular biology over the past 30 years have allowed researchers to design new viral vaccines using recombinant genetics, such as virus-like particles (VLPs). VLPs, also known as “virosomes” or “pseudovirions,” can be defined as self-assembling, nonreplicating, nonpathogenic, genomeless particles that are similar in size and conformation to infectious virions. These empty viral particles can enter target cells by receptor-mediated and non-receptor-mediated mechanisms. Compared with soluble proteins/antigens, which often require multiple immunizations and the use of adjuvants to elicit protective immune responses, VLPs are capable of inducing strong cellular and humoral responses as direct immunogens. VLPs range in size from 25 to 120 nm, depending on the viral background and resemble in structure and morphology the virus from which they are derived [1, 2]. Dendritic cells (DC) easily recognize particles of this size and uptake VLPs by endocytosis, process the particles, and present VLP antigens to innate and adaptive immune cells. These multiple viral antigens/epitopes stimulate a diverse set of immune responses with less reactogenicity associated with a live-attenuated or whole-inactivated, split vaccine [3, 4]. VLP proteins can be processed and presented on Major Histocompatibility Complex (MHC) class I molecules, therefore promoting presentation to T cells by professional antigen-presenting cells. In addition, cell-free VLPs bound with antibodies can be taken up by phagocytic cells via Fc receptors, thus facilitating MHC class II presentation [5]. Antigens expressed in their native three-dimensional conformational form can elicit more effective antibody responses compared to proteins in their nonnative forms [6]. Many neutralizing antibodies directed against viruses are elicited against conformational epitopes only present in the native form of envelopes, and some epitopes are only exposed after binding to receptors during entry [7–9].

Even though live-attenuated vaccines elicit robust immune responses, leading to lifelong immunity following a single immunization [10], these vaccines harbor safety risks since the pathogen could revert to a nonattenuated, wild-type phenotype with the potential to cause disease. Inactivated and subunit vaccines have been developed for and effectively used to fight against influenza, rabies, and hepatitis A viruses. While this vaccine strategy is safer than live-attenuated vaccines, these vaccines are less immunogenic, often require multiple immunizations, and/or the addition of adjuvants. Virus-like particles have the ability to elicit robust innate and adaptive immune responses without the safety risks associated with live vaccines. VLPs can be expressed *in vitro* from several different expression systems including bacterial, yeast, plant, insect, or mammalian cells (Table 4.1). These proteins can be directly administered as vaccines, often without the use of an adjuvant. VLPs can also be expressed *in vivo* from deoxyribonucleic acid (DNA) plasmids or viral vectors or in

TABLE 4.1. Examples of Current VLP Vaccine Strategies

Family	Virus	Genome	Envelope	Particle Composition	Expression Systems	Vaccine Status
Caliciviridae	Noro	ssRNA	No	Capsid	Baculovirus Yeast	Phase I, Preclinical
Hepeviridae	Hepatitis E	ssRNA	No	Capsid	Baculovirus	Preclinical
Flaviviridae	Hepatitis C	ssRNA	Yes	Core E1, E2	Baculovirus	Preclinical
Retroviridae	HIV, SIV	ssRNA	Yes	gag, env	Baculovirus Mammalian cells Yeast	Preclinical Phase I
Bunyaviridae	Rift Valley fever	seg. ssRNA	Yes	Capsid, G_N, G_C	Mammalian cells	Preclinical
Orthomyxo- viridae	Influenza	seg. ssRNA	Yes	HA, (NA), M1/gag	Baculovirus	Phase I
Papillomaviridae	Papilloma	dsDNA	No	L1	Tobacco Baculovirus Yeast	Preclinical Licensed Licensed
Hepadnaviridae	Hepatitis B	dsDNA	Yes	HBsAg	Yeast Potato Native SVP (plasma)	Licensed Preclinical Licensed (developing world)
					Mammalian cells	Licensed

the form of chimeric VLP with foreign antigen and/or adjuvant delivery systems [11]. In this chapter, the advantages of using virus-like particles as vaccines for a variety of viruses will be discussed.

The mammalian immune system is designed to optimally respond to microbial invaders such as viruses. Infected cells efficiently recognize viruses often because they contain pathogen-associated molecular patterns (PAMPs), which activate the innate immune system via Toll-like receptors (TLRs) and other intracellular pattern recognition receptors (PRRs) [12]. Many PRRs recognize nucleic acids, such as double-stranded ribonucleic acid (dsRNA; TLR3, Retinoic acid inducible-gene I (RIG-I), and Melanoma Differentiation-Associated Gene 5 (MDA5) agonist), single-stranded RNA (ssRNA; TLR7/8 agonist) or DNA rich in nonmethylated Cytosine-Guanine (CG) (CpG; TLR9 agonist), which leads to potent activation of the innate immune system. Even though nucleic material is not present in genomeless VLPs [13], the particles may attach or package immune stimulatory molecules onto or into VLP that can promote the activation of the innate arm of the immune response [14]. The particulate appearance of VLPs makes them attractive to professional antigen-presenting cells (APCs)

such as macrophages and dendritic cells (DCs) [15]. It is likely the highly repetitive and ordered fashion of the VLP that induces a robust humoral immune response [3].

4.2 HUMAN PAPILLOMAVIRUS

Virus-like particle vaccines against human papillomaviruses (HPV) are the first VLP vaccines approved for human use by the U.S. Food and Drug Administration (FDA) [16]. These vaccines are aimed at protecting women against cervical cancer and genital warts. Papillomaviruses are small, nonenveloped viruses ~55 nm in diameter [17]. The virus contains an 8 kilobases (kb), double-stranded, circular DNA genome that contains 10 open reading frames [17], which are divided into 8 early (E) and 2 late (L) gene products. The early genes encode the viral regulatory proteins and viral proteins needed for initiating viral DNA replication. A number of these early genes are also oncogenic leading to the cause of papillomavirus-associated cancers [18]. The late genes encode the two structural proteins, L1 and L2. L1 is a 55 (kDa) protein that forms the icosahedral capsid composed of 72 pentameric capsomers composing 80% of the total protein in the viral particle [17]. L2 is a 70 (kDa) protein that is not necessary for the formation of viral particles, but it is involved in the selective encapsidation of viral DNA [17]. Papillomaviruses infect a wide range of species, including humans, nonhuman primates, cattle, rabbits, horses, dogs, sheep, and birds [17]. However, there is no cross-species infection. There are a number of papillomavirus genotypes per species with more than 100 recognized HPV genotypes [17]. Forty genotypes are known to infect mucosal tissues [19]. These are further divided into 18 high-risk and 12 low-risk genotypes based on their relative association to cancer cases [19]. Papillomavirus infection occurs at the basal level of the squamous epithelium with viral replication limited to terminally differentiated cells as they move forward in the epithelium layer [17]. Infection can lead to the formation of warts or the induction of cancer at the site of infection [18]. HPV infection may result in cancer at a number of different sites, such as the mouth, oropharynx, anus, vulva/vagina, penis, and cervix (reviewed in [20]). Of these cancers, only cervical cancer is 100% attributable to HPV infection [20]. HPV-16 (50–60%) and HPV-18 (10–12%) account for ~70% of all cervical cancers, and, therefore, HPV-16 and HPV-18 are the major targets of vaccine manufacturers [19].

The goal of the commercially available HPV vaccine is to prevent viral infection. HPV infections evade the immune system and, if not cleared, result in a persistent infection that produces few immunological antigens [21]. The induction of neutralizing antibodies against L1 is dependent on the presentation of L1 in its native form [22–25] since the majority of neutralizing antibodies recognize conformational epitopes [24]. These epitopes are located at genotype-specific, hypervariable loops of L1 [26, 27]. L1 will self-assemble into VLPs that resemble the size and structure of native virions (Fig. 4.1) [28]. L1 VLPs are highly immunogenic and induce the production of HPV-neutralizing antibodies [28]. However, these neutralizing antibodies are type specific, with little antibody cross reactivity to closely related genotypes [29]. Therefore, a mixture (polyvalent) of L1 VLPs is needed to protect against more than one genotype.

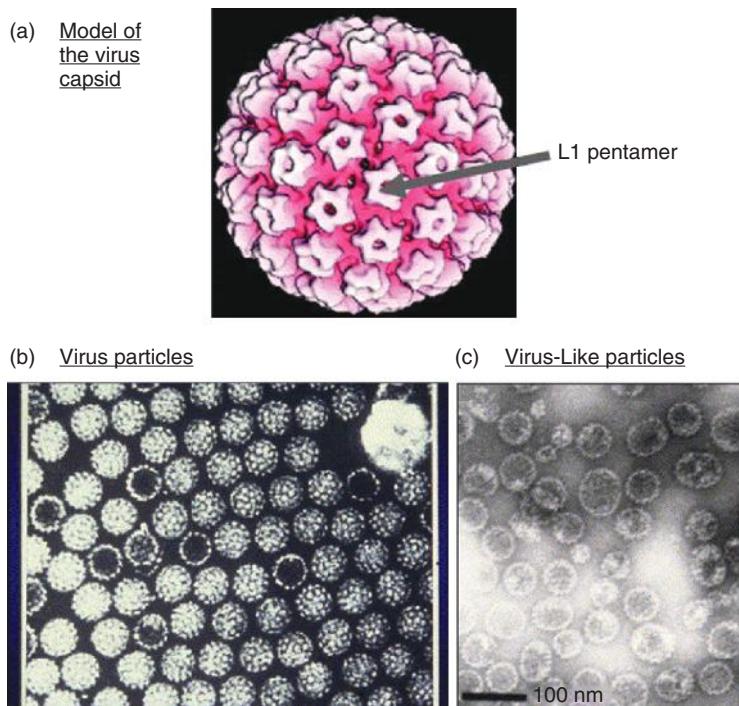


Figure 4.1. (a) A model of the papillomavirus capsid. The rosette-like surface structures (arrowed) are pentamers, each consisting of five molecules of L1; one molecule of L2 fits into the central dimple of each pentamer. (b) Papillomavirus particles; both full (contain DNA) and empty particles can be seen. (c) HPV-16 L1 virus-like particles made by expressing HPV-16 L1 in baculovirus. The L1 protein thus expressed spontaneously assembles into empty capsids or VLPs that are morphologically similar to the empty virus particles seen in panel. (See *insert* for color representation of this figure.) (Figure reprinted with permission from [22].)

Two HPV vaccines have been extensively studied for human use: Gardasil (Merck & Co. Inc., Whitehouse Station, NJ) and Cervarix [GlaxoSmithKline (GSK) Biologicals, Rixensart, Belgium]. Both vaccines are based on purified VLP platforms. Cervarix is a bivalent HPV-16/18 VLP vaccine that is administered at a dose of 20/20 µg and formulated with AS04 adjuvant (comprised of 500 µg of aluminum hydroxide and 50 µg of 3-deacylated monophosphoryl lipid A in 0.5 mL). The vaccine is delivered via an intramuscular injection given at 0, 1, and 6 months. These VLPs are produced separately using a two-component baculovirus expression system [30] with both an insect producer cell line, *Trichoplusia ni*, and a baculovirus strain engineered to carry the L1 gene. Initial trials were completed using a VLP purified via density centrifugation, but these techniques are limited in scale and are labor intensive [30]. In order to facilitate a large-scale production process, GSK developed a process utilizing multiple chromatographic and filtration techniques [30]. The vaccine

is highly immunogenic in phase III trials with 99.5% of recipients seroconverting to the vaccine [31]. The vaccine also demonstrated ~80% protection against either HPV-16 or HPV-18 6-month persistence and 76% against 12-month persistence [31]. Cross protection was also observed against the closely related HPV-45, HPV-31, and HPV-52 genotypes, albeit to a lower degree with protection ranging from ~32 to 60% [31], indicating that the optimal protection is only achieved against homologous HPV genotypes. These results demonstrate that the Cervarix VLP-based vaccine can induce strong protection against HPV-16 and HPV-18 infections.

Gardasil is a quadrivalent L1 VLP vaccine designed to protect against HPV-16/18, which cause 70% of cervical cancer, as well as HPV-6/11, the major cause of external genital warts (75–90%) [32]. The HPV-16/18/6/11 L1 VLPs are administered in three intramuscular injections, 3 months apart (0, 3, and 6 months) [22] at a dose of 40/40/20/20 µg formulated with 225 µg aluminum hydroxyphosphate sulfate in 0.5 mL [32]. These VLPs are produced separately using a yeast expression system from a recombinant *Saccharomyces pombe* vector [22]. The vaccine is highly immunogenic with 99.5% of vaccinated individuals seroconverting after receiving the full vaccine course [33]. The vaccine is also highly effective with 100% protection against vaginal, vulvar, perineal, and perianal intraepithelial lesion or warts caused by the vaccine-associated HPV types [33]. However, the vaccine was only effective if the individuals were HPV negative at the time of vaccination. There was no effect on disease outcome if a person was already positive for HPV-16/18/6/11 [33]. Cross protection was also observed with a reduction in the external anogenital or vaginal lesions (34%) and in cervical lesions (20%) associated with nonvaccine HPV types [33]. The use of the Gardasil vaccine demonstrates that an HPV VLP-based vaccine is highly immunogenic and induces protection against HPV-associated disease.

The human papillomavirus-like particles are at the forefront of VLP vaccine development. The effectiveness of Gardasil and Cervarix demonstrates that VLP vaccines can be produced on a large scale and distributed effectively. In addition, these vaccines show that it is possible to develop large-scale production methods for VLP vaccines for worldwide human vaccine manufacturing. More importantly, VLPs can be highly immunogenic and can induce protection against disease, indicating that VLP vaccines may greatly improve the effectiveness of the immune responses by better mimicking the true virus. These vaccines are the first VLP-based vaccine to be approved for human use and are a guide to the development of future VLP vaccines for other diseases.

4.3 HIV/AIDS

Human immunodeficiency virus (HIV) is a lentivirus of the family Retroviridae. The virus is composed of two single-stranded RNA genomes that, following infection, is converted into a double-stranded DNA genome that integrates into the host chromosomes, thereby setting up a lifelong infection. In 1983, HIV was discovered as the causative agent of an acquired immunodeficiency syndrome known as AIDS [34]. HIV contains the three major genes found in all lentiviruses: (1) group-associated antigen (*gag*) that encodes for the structural proteins, (2) the polymerase (*pol*) that

encodes for the enzymatic gene products, and (3) envelope (*env*) that encodes for the viral glycoproteins required for receptor binding and subsequent cell entry. At the end of 2007, the World Health Organization (WHO) estimated the number of HIV cases worldwide was 33 million with 2.7 million new cases [35].

While the use of antiretroviral drugs has lengthened the lives of HIV-infected individuals, there is currently no cure or preventative vaccine. Most of the HIV-infected patients live in sub-Saharan Africa, with limited access to life-saving antiretroviral drugs. Even in more developed parts of the world, where antiretroviral therapy is available, HIV still represents a major health concern. The drugs are expensive, require strict compliance to avoid the emergence of drug-resistant strains, have strong side effects, and need to be taken for the rest of the individual's life. The correlate of protection against HIV infection is not known, but vaccine strategies that elicit both cellular and humoral immune responses are most likely necessary to elicit protective immunity against infection. A variety of vaccine strategies have been attempted using different HIV gene products (reviewed in [36–38]), but despite major efforts, there is currently no HIV vaccine available. Using VLPs as an HIV vaccine allows for multiple viral epitopes to be presented to the immune system in their native form resulting in stimulation of the innate and adaptive immune responses [39]. Traditional vaccine approaches rely mainly on the elicitation of neutralizing antibodies and some are successful in protecting against infectious agents. However, in the case of HIV, vaccine strategies eliciting both humoral and cell-mediated immune responses appear important to limit viral replication and to protect from infection. HIV is spread mainly via sexual contact, entering the host via a mucosal surface, which requires a vaccine to elicit mucosal immune responses in order to prevent infection [40, 41].

VLP-based HIV/AIDS vaccines present multiple antigens to the immune system in order to enhance both humoral and cell-mediated immune responses. One advantage of this approach is the presentation of the Env on the surface of the particle in its native, oligomeric form, which allows for receptor binding and uptake of the VLP for antigen processing [36]. Expression of Gag alone leads to the spontaneous formation of lentiviral VLPs. However, most VLP vaccines express some form of Gag and the viral Env on the particle surface. Integration and recombination is prevented by removing the viral integrase and specific nucleotide sequence elements in the long terminal repeats [36]. These VLPs can be expressed in a variety of methods (Table 4.2). DNA plasmids or viral expression vectors delivered to a host express the VLP antigen *in vivo*. In contrast, these VLPs can be produced and purified *in vitro* (Fig. 4.2), using an insect or mammalian cell-based expressions system [42–44].

HIV VLPs composed of Gag precursors or Gag–Pol polyproteins efficiently induce both cellular and humoral responses [45–48]. In addition, Gag or Gag–Pol VLPs produced from yeast or insect cells stimulate the innate immune responses that results in monocyte and dendritic cell activation and cytokine release [53–55]. While these vaccines are effective at stimulating cellular immunity, these vaccine strategies do not appear to prevent infection in nonhuman primate models.

One major obstacle in the development of an AIDS vaccine is the genetic diversity between the different HIV-1 strains, which requires any potential vaccine to elicit broadly reactive, protective, immune responses. HIV-1 isolates are divided into three

TABLE 4.2. HIV VLP Summary^a

Particle-Forming Protein	Production System	Reference
Gag VLPs	Infection of HIGHFive insects cells with recombinant baculoviruses (baculovirus system)	Gheysen et al., 1989 [48] and Royer et al., (1992) [49]
Gag	Infection of CV-1 or SW480 cells with recombinant vaccinia viruses	Shioda and Shibuta, 1990 [50]
Gag/Pol	Spheroblasts of <i>saccharomyces cerevisiae</i>	Shakuragi et al., 2002 [51]
Carrier	Inserted/Fused Epitope	
Type I VLPs		
HIV-2 Pr41 Gag (C-terminally deleted by 93aa)	V3, HIV-2 V3+V3+CD4BR	Baculovirus system Luo et al., 1992 [75]
Insertion of epitopes at nucleotide positions 345 and 1275	V3, CD4BR, Nef epitope	Infection of SW480 cells with vaccinia virus, baculovirus system Wagner et al., 1992, [61]; 1996 [52]
Gag d211-241, Gag d436-471		
Gag d211-241/436-427, gag	Consensus V3	Baculovirus system Brand et al., 1995, [62] 1995 [63]
Gag d594-670	V3	Baculovirus system Griffiths et al., 1993, [64]
C-terminally truncated Gag		

TABLE 4.2. (Continued)

Particle-Forming Protein	Attached Polypeptide	Attachment Domain	Expression System
Type II VLPs			
GagPR	gp160	Native gp41	Infection of African green monkey kidney cells with recombinant vaccinia virus Haffar et al., 1990 [65]
Gag C-terminal truncated Gag (p50)	Chimeric gp160 gp160	Native gp41 TM Native gp41 TM	Stably transfected monkey cells Co-transfection of CV-1 cells with recombinant vaccinia viruses, expressing Gag and Env Haynes et al., 1991 [66] Vzorov et al., 1991 [67]
Gag	gp160	Native gp41 TM	Transient and stable expression in COS-7 and CV-1 cells Krausslich et al., 1993 [68]
Gag	Unprocessed gp160 gp160	Native gp41 TM Native gp41 TM	Stable transfected Vero cells Baculovirus system Rovinski et al., 1995 [69] Tobin et al., 1996 [70]
Gag	Truncated EHV-1 gp14 (gB)	None	Baculovirus system Osterrieder et al., 1995 [71]
Gag	Pseudopries virus gD protein	Native gD TM	Baculovirus system Garnier et al., 1995 [72]
Gag	gp120 and C-terminally truncated variants	Heterologous EBV gp220/350TM	Baculovirus system Deml et al., 1997 [73], 1997 [74]
Gag/Pol/Nef	gp120	Heterologous EBV gp220/350TM	Baculovirus system Buonaguro et al., 2006, [54]

^aUnless otherwise noted, all polypeptides were derived from HIV-1 isolates. Abbreviations: HIV: human immunodeficiency virus; Gag: group-specific antigen; VLP: virus-like particle; Pol: polymerase; aa: amino acids; V3: third variable loop of HIV-1 gp120; gp: glycoprotein; CD4BR: CD4-binding region of HIV-1 gp120; d: delta; Nef: negative-regulatory factor; TM: transmembrane domain; PR: protease; gp120: external HIV-1 glycoprotein; gp160: uncleaved HIV-1 envelope protein precursor; gp41: HIV-1 transmembrane protein; EHV-1: equine herpesvirus type-1; EBV: Epstein-Barr virus
Source: Reprinted from Deml et al. [46] with permission.

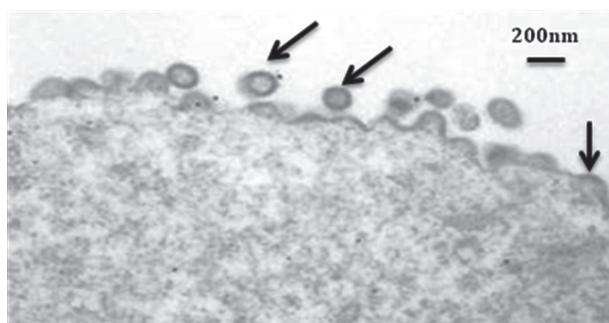


Figure 4.2. Transmission electron microscopy of HIV-like-particles budding from 293T cells following transfection with DNA vaccine plasmids expressing HIV gene products Gag and Env. The arrows indicate immunogold staining for Env on nascent VLPs and on the emerging particles on the cell surface. (Photo kindly provided by James Smith, Centers for Disease Control and Prevention.)

groups: M (major), N (non-M, non-O), and O (outlier). Group M is the most common and subdivides into several clades (A–D, F–H, J and K). Clade B isolates are the most prevalent in the United States and Europe, whereas clade C isolates are primarily found in India, China, and sub-Saharan Africa [56]. In order to address this genetic diversity, polyvalent vaccines that consist of a mixture of the different envelope antigens derived from several strains are administered simultaneously [57]. An alternative vaccine method involves the generation of centralized sequences that are based upon computational analyses of the amino acid sequences of the various HIV proteins. These consensus synthetic sequences focus on the most common amino acid in each position with an HIV protein [57]. HIV VLPs expressing envelopes using each of these methods elicit broadly reactive cell-mediated immune response [58]. These VLPs were produced in mammalian cells and were administered intranasally to BALB/c mice, adjuvanted with CpG oligodeoxynucleotide (ODNs). Both the polyvalent and consensus clade B VLPs elicited broader cell-mediated immune responses than monovalent primary clade B Env VLP vaccines. The number of specific T-cell epitopes elicited by the consensus B and polyvalent B Env VLPs was significantly higher than the monovalent clade B Env VLP.

The administration of lentiviral VLPs to mucosal surfaces is effective at eliciting mucosal immune responses. Intranasal administration of simian immunodeficiency virus (SIV) VLPs into BALB/c mice elicit neutralizing antibodies against heterologous strains of SIV (SIV1A11 and SIVmac239) [59], as well as the elicitation of cellular immune responses against the SIV Env [59]. Baculovirus-derived simian-human immunodeficiency virus (SHIV) VLPs administered intranasally to mice adjuvanted with inactivated influenza vaccine enhanced both serum and mucosal anti-Env antibodies. In addition, there was an increase in neutralizing activity against the HIV virus and increased numbers of interferon (IFN- γ) and interleukin (IL-4) producing T cells compared to SHIV VLP alone [60]. This vaccine also enhanced cytotoxic T-cell activity to the virus compared to the administration of the SHIV VLP alone [60].

Virus-like particles can also act as carrier delivery systems for exogenous proteins. Carrier HIV VLPs are produced by replacing nonessential Gag regions with the desired antigen or fusing the antigen to a truncated or full-length Gag precursor molecule. Most carrier HIV VLPs have relied on HIV proteins, such as Nef, V3 loop of envelope domains, or the CD4 binding domain of the envelope [61–64]. These carrier systems effectively elicited cellular responses to these cargo antigens, but only low neutralizing antibody titers have been detected.

4.4 NOROVIRUS

Norovirus (formerly known as Norwalk virus) is a member of Caliciviridae and is a leading cause of nonbacterial gastroenteritis [76]. There are 5 genogroups (I–V) and 32 genotypes of norovirus that are based on the sequence diversity in the VP1 capsid protein. Human noroviruses are clustered within the genogroups I, II, and III [77]. During the winter months, norovirus infections are most prevalent. The virus spreads primarily via the fecal–oral route, leading to a rapid dissemination through densely populated areas, such as nursing homes, hospitals, schools, cruise ships, and military vessels [77, 78]. Clinical symptoms of a norovirus infection include nausea, vomiting, and a nonbloody diarrhea. These symptoms persist for 2–6 days [77, 79]. Even though the symptoms induced by norovirus may subside relatively quickly, these patients can still shed virus for as long 14 days [80].

There are currently no well-suited small-animal models for human norovirus available for this difficult to grow virus. Recently, murine norovirus (MNV) strains, capable of replicating *in vitro* and *in vivo*, have been isolated [81, 82]. In addition, the ability of the self-assembling norovirus capsid protein into VLPs has spurred vaccine research. This VLP has provided a tool to study serum antibody responses to viral infection and allowed for the generation of antisera to identify a cellular receptor(s) for norovirus [83, 84]. The human histo-blood group antigens (HBGA) may act as receptors for norovirus, and they may play a role in the susceptibility of individuals to norovirus infection [85, 86]. Norovirus VLPs can be produced in a variety of systems, including yeast expression systems, the baculovirus SF9 insect system, or in transfected plant methods [83, 87, 88]. These norovirus VLP vaccines are highly immunogenic in both mice and humans following oral or parenteral administration [83, 89–91]. However, several factors may limit the development of a norovirus VLP vaccine. First, the correlates of protection are unknown. Second, there are several types of norovirus within the three genogroups that infect humans. Lastly, there may be a lack of long-term and cross-protective immunity elicited by any norovirus vaccine [77]. Mice were protected from MNV challenge following passive transfer of sera from mice immunized with the MNV VLP. In contrast, adoptive transfer of purified CD4+ or CD8+ T cells from these VLP vaccinated mice did not provide protection [92]. Therefore, antibodies induced by this norovirus VLP vaccine appear to be the main correlate of protection. In addition, a multivalent human norovirus VLP vaccine was expressed and purified from a Venezuelan Equine Encephalitis virus replicon system [92, 93]. Mice immunized with these purified VLPs via footpad injection with

alphavirus adjuvant particles elicited both homotypic and heterotypic immunity to human and murine norovirus strains [92]. The vaccines induced antibody responses that cross reacted to norovirus VLP that were not included in the vaccine formulation. Furthermore, immunization of mice with multivalent human norovirus VLP vaccines did result in reduced viral loads after MNV challenge [92]. These studies and others show that norovirus VLP vaccines can successfully induce protective immune responses following vaccination.

4.5 INFLUENZA

Influenza virus, a member of the Orthomyxoviridae family, are enveloped, negative-stranded, segmented RNA viruses, consisting of 8 individual genes that encode at least 11 proteins and are divided into three types (A, B, and C) [94]. Types A and B influenza viruses infect humans. Influenza A viruses are further subdivided by antigenic characterization of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Currently, there are 16 identified HA and 9 NA subtypes [95]. Waterfowl, such as ducks and geese, serve as a natural reservoir for all known subtypes of influenza A virus [96]. Annually, human outbreaks of influenza types A subtypes, currently H1N1 and H3N2 and influenza B, are responsible for substantial morbidity and mortality in humans [97]. High-risk groups, such as elderly, infants, and immunocompromised individuals, are most susceptible to infection and severe disease.

There are two U.S. FDA-approved influenza vaccines for human use. The traditional inactivated, whole, split, or purified influenza A and B virus vaccines are prepared by culturing live virus in embryonated chicken eggs. There are limitations to relying on an egg-based manufacturing system including egg allergies in a small percentage of the population as well as potential issues with egg supply for surge capacity or potential depletion of egg supply due to avian influenza outbreaks. The other FDA-approved vaccine is the live-attenuated influenza vaccine [98, 99]. Both vaccine strategies rely on the use of embroyinated hen eggs to produce the virus. These vaccines are labor intensive to produce and are subtype specific. Therefore, they are not effective against emerging pandemic strains. The potential H5N1 avian influenza pandemic and recently emerged swine-origin H1N1 strains emphasize the importance of developing a vaccine that is easy to scale up and can induce broader protective immunity. Virus-like particles composed of major viral structural proteins that resemble the size and shape of the virus, but lacking the genetic material, may be promising vaccine candidates for pandemic influenza subtypes. These alternative influenza vaccine manufacturing platforms, based upon scalable and recombinant approaches, could therefore be of great public benefit.

Influenza VLPs can be produced from mammalian, insect, and plant cells. Influenza virus-like particles can be produced following transfection of recombinant DNA plasmids that express the genes encoding the 11 influenza virus proteins [100]. However, influenza virus-like particles can be formed following co-expression of just three viral proteins, matrix (M1), HA, and NA. The M1 protein is essential for influenza VLP formation since it forms the core of the virus for budding from the

cell membrane. The expression of M1 alone is sufficient to drive the formation of spikeless viroids that can be released into the culture supernatant. In the meantime, the co-expression of HA can enhance M1 binding to cell membrane and thereby promote the release of VLPs [101]. Production of chimeric VLPs using the HIV Gag protein in replacement of influenza virus M1 protein appears even more efficient for VLP release than M1 (personal observation). The three-plasmid co-transfection strategy is used to produce influenza VLPs from mammalian cells since it is simple to perform and collect particles.

Another method to produce influenza VLPs are based on insect cell/baculovirus expression vector system (BEVS). The first demonstration of producing influenza VLPs in the BEVS used a single recombinant baculovirus that simultaneously expressed M1, M2, HA, and NA from A/Udorn/72 (H3N2) [102]. Electron microscopic (EM) examination revealed that these influenza VLPs had similar morphology to wild-type influenza virus. Consistent with the data from transfected mammalian cell studies, the expression of M1 protein alone in insect cell leads to the release of spikeless vesicular particles. Subsequent studies found that influenza VLPs can be generated in insect cells by self-assembly of only three structural proteins: M1, HA, and NA. These “three-protein” VLPs exhibited hemagglutination and neuraminidase activity similar to wild-type influenza viruses [103]. Recently, it was found that the expression of only HA and M1 can form VLPs in insect cell/baculovirus expression systems [104]. But the majority of influenza VLPs contains viral proteins: HA, NA, and M1. Influenza VLPs have been produced with BEVS for different influenza strains/subtypes, such as A/Puerto Rico/8/34 (H1N1) [104], A/HongKong/1073/99 (H9N2), A/Fujian/411/2002 (H3N2)[4], A/Vietnam/1203/2004 (H5N1), A/Indonesia/05/2005 (H5N1) [3, 105, 106], and the highly pathogenic 1918 (H1N1) [107].

VLPs contain the major structural viral proteins in the same conformation as the wild-type virus, so they are able to induce potent immune responses. These VLPs are highly immunogenic. However, the more interesting thing is these influenza VLP vaccines can elicit broader immune responses than inactivated viruses or purified recombinant HA proteins [3]. Mice and ferrets vaccinated with A/Fujian/411/2002 (H3N2) VLPs elicited anti-HA titers that were similar to titers elicited by inactivated virus [4]. However, VLPs induced antibodies against a broader panel of influenza isolates. These antibodies rose more quickly and reached higher titers against homologous virus than inactivated virions [4]. A/PR/8/34 (H1N1) VLPs can induce both homologous and heterologous protective immunity [107]. Similarly, vaccination of the H5N1 [Indonesia/05/05 (clade 2)] VLPs protected mice against an H5N1 clade 1 viral challenge (A/Vietnam/1203/2004). Similar results were observed between clade 1 H5N1 VLPs and clade 2 virus challenge [3, 105]. Most recently, it was reported that the 1918 H1N1 VLPs can confer H5N1 virus challenge in mice [107]. There are at least two interpretations for this phenomenon. First, the adjuvant activity induced by baculovirus may induce strong innate immune responses that non-specifically protect against H5N1 infections. Indeed, when administrated intranasally, wild-type baculoviruses can induce an innate immune response that confers protection from lethal influenza virus challenge in mice [108]. The second possible reason is VLPs cannot only elicit efficient humoral, but also cellular immune response [3,

109]. The elicitation of cross-protective immune responses by baculovirus-derived influenza VLPs is important for influenza vaccines. Since the inactivated virus vaccines elicit primarily subtype- and sometimes strain-specific immunity, the currently licensed seasonal influenza vaccine is updated annually.

Although H5N1 influenza is widespread in domestic poultry and may be the cause of a new pandemic, the recent emergence of swine-origin H1N1 in the human population has reminded the scientific community that an influenza pandemic can come from a wide variety of subtypes [110]. In addition to H5N1 and H1N1 isolates, avian influenza viruses of the H9N2 and H7N7 subtypes are also widespread in poultry and have caused human disease [111, 112]. H9N2 VLPs were generated using the baculovirus expression system and contained the HA, NA, and M1 proteins. These vaccines elicited robust immune responses and effectively protected mice and ferrets from weight loss and reduced lung viral titers [113, 114]. H7N1 VLPs are also immunogenic and protect mice against a lethal challenge [115]. These H7N1 VLPs, which contained the HA and NA from influenza and a Gag core from murine leukemia virus, were expressed and purified from mammalian tissue culture.

Interestingly, VLPs can be generated *in vivo* following DNA vaccination with a plasmid encoding the VLP proteins. These *in vivo* generated VLPs were just as immunogenic and effective as purified VLPs [115]. Additionally, the H7N1 VLP was used as a platform for generating custom HA antigens. To enhance budding of VLPs from mammalian cells, the HA was modified to reduce the receptor binding affinity, which should prevent retention at the surface of producer cells. Although introduction of this mutation enhanced VLP release into cell culture supernatants, the mutant VLP was less immunogenic than the wild-type VLP [115]. A second VLP, containing the highly conserved HA2 portion of hemagglutinin elicited robust immunity to HA2 that resulted in cross-subtype protection. This VLP also elicited neutralizing antibodies to H5N1 influenza, whereas the wild-type H7N1 VLP did not [115].

Both transfected mammalian cells and infected insect cells derived from influenza VLPs have been extensively studied in the past several years. But there are some limitations using both methods. VLPs produced via transfection of multiple plasmids are, at times, difficult to collect in large quantities. The transfection reagents are usually cytotoxic and can lead to cell lysis within 2–3 days posttransfection, which reduces the VLP yield. The baculovirus expression vector system is easy to manipulate and scale up and produces large quantities of VLPs. However, the major problem with this system is contamination of the VLP preparation with wild-type baculovirus particles; baculovirus is a rod-shaped particle roughly 70 nm × 320 nm and influenza virus is 80–100 nm in size [103, 116]. Various methods have been employed to separate VLPs and baculovirus, such as sucrose gradient, ion exchange chromatography, and size exclusion chromatography [103]. The yield can be depleted and the integrity of the VLPs can be reduced following numerous purification steps. In addition, even combining some of these purification methods, residual baculoviruses were consistently detected in VLP preparations [103]. Therefore, if inactivation of these baculovirus-derived VLPs is necessary for human use, there may be limitations of using this system for influenza VLP production. Taken together, current studies indicate that influenza VLPs are a promising vaccine candidate for influenza infection.

4.6 FLAVIVIRUSES

The viruses within the family Flaviviridae and genus *Flavivirus* are of significant medical importance. Yellow fever, Japanese encephalitis, dengue, tick-borne encephalitis, and West Nile viruses continue to infect humans every year, and many of these viruses are found worldwide [1, 2]. Most, but not all, flaviviruses are transmitted by hematophagous arthropods (primarily mosquitoes and ticks), adding another layer of complexity to outbreak control measures and often highlighting the ever-growing need for safe and effective vaccines and/or antiviral treatments. There are vaccines available to combat the diseases caused by some of the aforementioned viruses, such as yellow fever, Japanese encephalitis (JEV), and tick-borne encephalitis viruses, but despite thorough and ongoing research, there are still no licensed vaccines available for human use for others, including dengue (DENV) and West Nile (WNV) viruses.

The flavivirus RNA genome is composed of a set of structural genes [Capsid, C, preMembrane, *prM*, (which becomes mature protein following furin cleavage) and envelope, E] and nonstructural genes (*NS1*, *NS2A*, *NS2B*, *NS3*, *NS4A*, *NS4B*, and *NS5*), which are flanked by 5' and 3' untranslated regions (UTRs). The structural proteins encapsidate the RNA genome and contain most of the major immunogenic epitopes of the virus. The nonstructural proteins are required for genome replication. The RNA genome structure of flavivirus lends itself well to the production and manipulation of immunogenic particles; subviral (SVP) or slowly sedimenting hemagglutinin particles that contain no RNA genome and similar to the VLPs described for other viruses, and VLPs that contain a replication-deficient genome (Fig. 4.3).

SVPs are natural by-products of viral infection that are secreted from the cell along with infectious virions. These genomeless SVPs are generated by the co-expression of two of the structural genes, *prM* and E. During the process of virion maturation within the cell's endoplasmic reticulum and secretory pathway, *prM* and E proteins self-assemble. The resulting icosahedral structure is stable and contains a host cell-derived lipid bilayer, similar to infectious virus particles [3]. As a result, the major structural and immunologically relevant epitopes are presented on the surface of an SVP just like they are on the surface of infectious virus. This feature has made flavivirus SVPs attractive targets for vaccine development. Furthermore, since these particles contain no viral genomes and are, in essence, packets of viral protein, they are safe to use in patients with compromised immune systems.

SVP vaccine candidates have been generated for the myriad of diseases caused by flavivirus infections. SVPs can be delivered to the host by direct delivery of purified SVP protein [4–6] or by DNA vaccines [5,7–14] or viral vectors [8,15–17] encoding *prM* and E genes to produce SVPs *in vivo*. Regardless the delivery vehicle, these vaccines elicit virus-specific antibodies following vaccination, which protected most, if not all, mice from lethal flavivirus infection [5–9, 13, 14, 16, 17]. When purified JEV or DENV SVPs and DNA-encoding JEV or DENV *prM* and E genes were administered simultaneously antibody titers in vaccinated mice increased synergistically compared to vaccination with SVP or DNA alone [5]. These results suggest that altering the kinetics of delivery may increase the potency of these SVP vaccines.

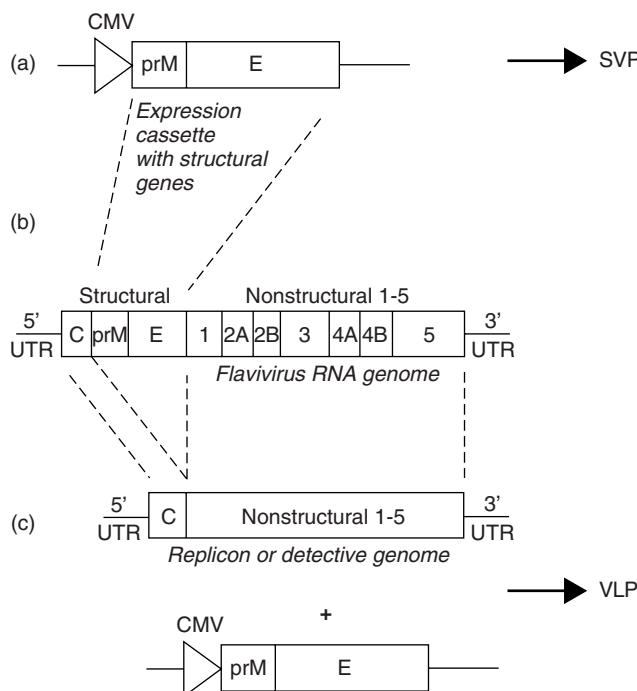


Figure 4.3. Schematic diagrams of the flavivirus genome and manipulations to create prM/E expressing constructs or replicon genomes. Panel A shows the production of SVPs from an expression vector. Classically, strong promoters like the CMV promoter have been used in DNA plasmids expressing *prM* and *E*, but other methods of expressing these genes have been employed as well. Panel B shows the flavivirus RNA genome. Panel C shows one way a replicon can be engineered by deleting the *prM* and *E* genes from the genome. By trans-complementing this replicon genome with the expression construct that produces *prM* and *E* will produce replicon-encapsidated VLPs.

DNA vaccines elicit long-lasting antibody titers and strong virus-specific memory B-cell responses in JEV SVP-vaccinated swine [12] and WNV SVP-vaccinated mice [18]. In addition to antibody production, SVP vaccination induces memory T cells [19]. Interestingly, in the absence of high titer neutralizing antibody, there was complete protection from lethal WNV infection in DNA-vaccinated mice, which was most likely from the protective effects of WNV-specific CD8+ T cells [18]. The protective effect of the virus-specific CD8+ T cells was not seen following additional vaccine boosts [18].

Flaviviral VLPs are akin to a type of defective-interfering (DI) particle enveloped in an SVP. These VLPs are expressed from a flavivirus genome that has been deliberately altered, usually by removing some or most of the structural genes, to form a replicon genome that is still capable of RNA replication but lacks the capacity

to produce infectious virions due to its lack of structural genes. However, it can be packaged into a VLP by the *in trans* complementation of the missing viral structural proteins. Flavivirus replicons also are attractive viral vectors by themselves, since they can be engineered to replicate within the cell without causing cytopathic effect, harbor foreign genes in lieu of the naturally occurring flavivirus structural genes without compromising genome replication, and can be trans-packaged into VLPs. However, since this genome is capable of replicating in cells, these vaccines are essentially live-attenuated, single-cycle, dead-end infections, unlike the subunit or recombinant protein-like SVP vaccine.

There are fewer studies using VLP than SVP vaccinations. VLPs elicit an early strong type I interferon response and drain to the local lymph node ~24 h following immunization [20]. When used as a vaccine vector to deliver other foreign genes or a murine polyepitope, these VLPs induce CD8+ T-cell responses and antibodies to the foreign protein [21, 22]. However, the antibody titer elicited to the VLP vector E protein itself remains low [22], suggesting that VLPs encoding foreign genes may be useful vaccine vectors and that the immunity to the flavivirus vector may be better achieved using another vaccine approach.

Recently, a technology called RepliVAX has blended the SVP and VLP vaccination approaches by engineering replicons that lacking C, but containing prM and E, are packaged into VLPs, and produced from SVPs from cells expressing the replicon genome (RepliVAX-infected cells) [23–25]. Since the basic construction of the RepliVAX-based vaccine is based upon manipulations of the flavivirus genome, this technology can be readily applied to create vaccines against a variety of flaviviruses. To date, the WNV and JEV genomes have been engineered to create RepliVAX WN and RepliVAX JE [23, 25]. Furthermore, a chimeric RepliVAX D2.2 to combat dengue was created by swapping the *prM* and *E* genes from the RepliVAX WN with the *prM* and *E* genes of DENV-2 [24]. RepliVAX WN and RepliVAX JE completely protect mice and hamsters against homologous lethal virus challenge with a few as 4×10^6 infectious units (particles), elicited anti-E and anti-NS1 antibody titers, and are safe and well tolerated [23, 25]. RepliVAX D2.2 elicits moderate neutralizing antibody titers and significantly delays death following lethal DENV-2 infection in the immunodeficient AG129 mice (one of the few mouse models for dengue) [24]. A vaccine based upon the technology similar to RepliVAX WN demonstrates that RepliVAX WN-like particles also elicit antibodies in both mice and horses [26].

4.7 RIFT VALLEY FEVER VIRUS

Bunyaviridae harbors more than 300 arthropod-borne viruses making it one of the largest families of animal viruses sharing antigenic and morphologic properties [117]. In humans, bunyaviruses cause severe diseases, including Rift Valley fever, Crimean-Congo hemorrhagic fever, hantavirus pulmonary syndrome (HPS), and California encephalitis. Most viruses in the family are transmitted by arthropod vectors, such as mosquitoes, sand flies, and ticks with the exception of hantaviruses, which are transmitted in aerosolized rodent excreta. Members of the family Bunyaviridae usually

cause acute febrile flu-like illness in humans with clinical features including, but not limited to, lethargy, nausea, and headache lasting for a week, but severe complications can develop in a proportion of infected individuals, including fatal encephalitis and hemorrhagic syndrome. Among various members of the family, Rift Valley fever virus (RVFV) is an important pathogen that has been extensively studied for basic research and vaccine development.

Rift Valley fever virus is a mosquito-borne pathogen of livestock and humans that historically has been responsible for widespread and devastating outbreaks of severe disease throughout Africa and, more recently, the Arabian Peninsula and Yemen. The virus was first isolated from the serum of a moribund sheep near Naivasha in the Rift Valley of Kenya in 1931 [118]. RVFV has been implicated in numerous economically devastating epizootics characterized by abortion storms and mortality ratios that can reach 100% mortality among neonatal animals and 10–20% among adult cattle and sheep [119, 120]. In humans, RVFV infection is usually benign resulting in fever, headache, and myalgia followed by a complete recovery. However, in 1–2% of affected individuals, RVFV infections can progress to more severe disease with fatal complications, such as fulminant hepatitis, retinitis, encephalitis, and hemorrhagic syndromes with a case fatality rate of 10–20% [121].

The virus genome contains tripartite RNA segments designated large (L), medium (M), and small (S) contained in a spherical (80–120 nm in diameter) lipid bilayer. The L segment encodes for RNA-dependent RNA polymerase, while the M segment typically encodes for structural glycoproteins (Gn and Gc). In some bunyaviruses, this region encodes for a nonstructural protein known as NSm. The S segment encodes two polypeptides, N and NSs in overlapping reading frames.

Immunization of susceptible livestock remains the most effective method to prevent RVFV epizootics and human infections. Currently, there are no licensed vaccines for human use. However, a formalin-inactivated virus vaccine is available but has been limited to workers in high-risk occupations [122, 123]. The live-attenuated Smithburn strain of RVFV and the formalin-inactivated virus vaccines produced are approved for veterinary use [124, 125]. Both vaccines have some limitations, thus preventing their widespread use. The Smithburn vaccine is teratogenic in cattle and sheep [126, 127]. The formalin-inactivated vaccine is safe but less immunogenic and hence requires multiple boosters [128]. Potential live-attenuated vaccine candidates under evaluation are Clone 13 and MP12, where both have shown promising results in animal trials [129, 130]. The immunogenicity and pathogenicity of these candidates have been evaluated in various animal species [129, 131]. The most extensively tested, MP12, suffers from a serious side effect inducing fetal malformations during the first trimester of pregnancy, thus limiting its use in pregnant animals [132].

Alphavirus replicons are single-hit expression vectors capable of eliciting potent systemic and mucosal immune responses against a wide range of pathogens, including hemorrhagic fever viruses [27]. Recently, our lab and others have demonstrated that expression of RVFV surface glycoproteins from alphavirus vectors based upon either Venezuelan equine encephalitis (VEE) virus or Sindbis viruses were capable of eliciting protective anti-RVFV immune response [28–30]. Another approach for vaccine development is to use VLPs that are formed when the structural (envelope and/or

nucleocapsid) proteins self-assemble to replication-deficient particles. In comparison to component vaccines based on recombinant or subunit proteins, VLPs are in general more immunogenic and more natural in presenting conformational epitopes and, hence, more likely to induce neutralizing antibodies [9]. VLP systems consisting of minireplicon ribonucleoprotein particles (RNPs) packaged by viral glycoproteins was first established for two bunyaviruses, Bunyamwera virus and Uukuniemi virus [133, 134]. Subsequently, baculovirus expression system and minireplicon RNA-based systems have been utilized to produce RVFV VLPs, which resemble authentic virus [135, 136]. VLP production was observed when Sf9 insect cells were infected with a recombinant baculovirus expressing RVFV structural proteins N, Gn, and Gc. In addition, VLPs were also produced when only N and Gc were expressed in the absence of Gn [135]. In an effort to generate RVFV VLPs from mammalian expression systems, viral structural genes were expressed along with a *Renilla luciferase* reporter minigenome system. These nonreplicating VLPs are composed of nucleocapsids consisting of N and the reporter RNA minigenome that display the envelope (Gn and Gc) proteins on the surface. These VLPs elicited neutralizing antibodies against RVFV and protected mice against a lethal dose of virulent RVFV [137].

A recent study utilized the generation of chimeric RVFV VLPs and its successful use as a vaccine candidate; 293-gag and 293 cells were utilized to generate chim RVFV VLPs [31]. The chimVLPs elicited neutralizing antibody responses and stimulated cytokine production by splenocytes from vaccinated mice leading to protection in mice and rats against lethal virus challenge. In summary, VLP vaccination seems to be a safe and effective approach to induce protective immune response against RVFV, but further studies to evaluate baculovirus and cellular protein contamination in the vaccine preparation and extensive clinical trials are needed before these VLP-based vaccines are available for commercial use as licensed vaccines.

4.8 CONCLUSIONS

VLPs represent an attractive platform for vaccine design for a variety of pathogens. They are easy to produce, noninfectious, closely mimic the native virus, can be administered via parenteral or mucosal routes, and successfully induce cellular and humoral immune responses. These vaccines have the structural advantages of live-attenuated viruses but with the safety of inactivated and subunit vaccines. The future will show whether more VLP-based vaccines will be developed and licensed, aiding in the endeavor to eradicate infectious diseases.

Abbreviations

AIDS:	Acquired immunodeficiency syndrome
APC:	Antigen-presenting cell
CTL:	Cytotoxic T cell
DC:	Dendritic cell
FDC:	Follicular dendritic cell

HLT:	Heat-labile enterotoxin
HA:	Hemagglutinin
HBGA:	Histo-blood group antigens
HIV:	Human immunodeficiency virus
HPV:	Human papillomavirus
ISCOM:	Immune stimulatory complexes
MALT:	Mucosa-associated lymphoid tissue
MNV:	Murine norovirus
NA:	Neuraminidase
NLR:	NOD-like receptor
PRR:	Pattern recognition receptor
PP:	Peyer's patch
RVFV:	Rift Valley fever virus
SIV:	Simian immunodeficiency virus
TLR:	Toll-like receptor
TIV:	Trivalent inactivated influenza vaccine
VEE:	Venezuelan equine encephalitis
VLP:	Virus-like particle

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DESIGN PLATFORMS OF NANOCAPSULES FOR HUMAN THERAPEUTICS OR VACCINES

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5.1 APPLICATION OF VIRUS-LIKE PARTICLES FOR VACCINATION

The mammalian immune system is highly attuned to recognizing and eliminating viral particles following infection. The use of particle-based immunogens, often delivered as live-attenuated viruses, has been an effective vaccination strategy for a variety

of viruses [1]. A number of virus-like particles (VLPs) for vaccination have been approved for clinical use. Clinical trials involving hepatitis E virus (HEV), influenza, hepatitis C virus (HCV), poliovirus, human immunodeficiency virus (HIV), Ebola virus, Norwalk virus, rotavirus, and severe acute respiratory syndrome (SARS) coronavirus are underway (Table 5.1). Some of those currently in preclinical testing are the hepatitis B virus (HBV) and the human papillomavirus (HPV). The structural capsid proteins of many viruses have the ability to self-assemble into VLPs. Such VLPs are 20–150 nm in diameter, and their exact size and morphology depend greatly on the particular viral proteins. These VLPs resemble intact virions but are noninfectious because they assemble without incorporating the viral genome (Fig. 5.1). Several expression systems for the production of VLPs have been reported. These include various mammalian cell lines (either transiently or stably transfected or transduced with viral expression vectors), baculovirus expression systems, and various yeast and *Escherichia coli* expression systems.

The HPV and HBV vaccines are the first VLP-derived vaccines approved by the Food and Drug Administration (FDA). Expression of the small envelope protein of HBV in yeast or mammalian cells leads to the formation of 22-nm-wide VLPs that are essentially identical to that of a natural product of HBV infection provided as the first-generation HBV vaccines. Similarly, expression of the L1 protein of HPV leads to the assembly of VLPs that are somewhat similar to the empty virus particles formed during HPV replication. These VLPs can induce strong immune responses after administration due to high-density display of repetitive epitopes on the surface of the capsid. This is further enhanced by the particulate nature of VLPs, especially in the size range of around 40 nm, which appears to be optimal for uptake by dendritic cells (DCs) [2].

5.2 INNATE AND ADAPTIVE CELLULAR IMMUNE RESPONSES AGAINST VIRUS-LIKE PARTICLES

HPV–VLPs comprised of the viral capsid protein L1 are immunogenic in mice and humans when injected intradermally or applied to mucosal surfaces in the absence of adjuvant [3–6]. Recently, the molecular mechanism of the immune response against HPV–VLP has been revealed (Fig. 5.2). Innate immune response of DC is triggered by the Toll-like receptors (TLRs)–MyD88 signaling pathway and promotes adaptive immune responses [7]. HPV–VLPs can rapidly induce specific immune responses via TLR4-mediated signaling through MyD88 adaptor molecule [8]. MyD88 then activates Nuclear factor-kappa B (NF- κ B) and activating protein 1 transcription factors for proinflammatory responses [9]. Innate immune response also induces expression of DC maturation markers. DC maturation is essential for stimulation of both innate and adaptive immune responses [2, 10]. HPV–VLP is taken up by DCs for antigen processing and presentation by the major histocompatibility complex (MHC) class I and II to activate CD8+ and naïve CD4+ T cells, respectively. Mature DCs induce the polarization of naïve CD4+ T-helper 1 (Th1) and 2 (Th2) cells through antigen presentation of MHC class II against T-cell receptor of Th1/2 cells and co-stimulatory signals between CD80/86 and CD28 [11–13]. Th1 cells produce cytokines such as Interferon-gamma (IFN- γ) and Tumor necrosis factor-alpha (TNF- α) that direct B cells

TABLE 5.1. Capsids Used for Vaccines and Vaccine Platforms

Capsid Platforms	Capsid Composition	References
Hepatitis B virus	Small envelope protein (HBsAg): Licensed	(McAleer, W.J. et al. 1984 [58], and Andre, F.E. et al. 1987 [59])
	Small envelope protein (HBsAg): Preclinical	(Kong, Q. et al. 2001 [60])
	PreS1+2 and HBsAg: Licensed	(Yap, I. et al. 1992 [61], Shouval, D. et al. 1994 [62], Madalinski et al. 2001 [63], and Yap, I. and Chan, S.H. 1996 [64])
	HBsAg: Licensed	(Krugman, S. et al. 1971 [65])
	GFP; malaria epitopes; HBV preS1; immunodominant epitopes of numerous viral pathogens, including bacterial and protozoan epitopes on hepatitis B virus core	(Pumpens, P. and Grens, E. 2001 [66], Stahl, S.J. et al. 1989 [67], Kratz, P.A. et al. 1999 [68], Birkett, A.J. et al. 2002 [69], Nardin, E.H. et al. 2004 [70], Chen, X. et al. 2004 [71], and Jegerlehner, A. et al. 2002 [17])
	Various model epitopes on woodchuck hepatitis B virus core	(Billaud, J.N. et al. 2005 [72])
	HCV HVR1; plant signal peptides; Dengue virus envelope protein; HIV gp41 2F5 epitope on hepatitis B virus S antigen	(Netter, H.J. et al. 2001 [73], Sojikul, P. et al. 2003 [74], Bisht, H. et al. 2001 [75], Bisht, H. et al. 2002 [76], Schlienger, K. et al. 1992 [77], and Eckhart, L. et al. 1996 [78])
	Core, E1, E2: Preclinical	(Baumert, T.F. et al. 1998 [79], Jeong, S.H. et al. 2004 [80], Lechmann, M. et al. 2001 [81], and Murata, K. et al. 2003 [82])
	Truncated major capsid protein (ORF2)	(Li, T.C. et al. 1997 [83], Li, T.C. et al. 2005 [84], Purcell, R.H. et al. 2003 [85] and Emerson, S.U. and Purcell, R.H. 2001 [86])
	HEV B cell epitope on hepatitis E virus	(Niikura, M. et al. 2002 [87])
Human papilloma virus	L1, major capsid protein: Licensed	(Zhou, J. et al. 1991 [88], Kirnbauer, R. et al. 1992 [4], Koutsy, L.A. et al. 2002 [89], and Villa, L.L. et al. 2005 [90])
	SHIV (HIV tat, rev; SIV gag); HPV E6/E7 on human papillomavirus	(Frazer, I.H. et al. 2004 [91] and Dale, C.J. et al. 2002 [92])
	CTL epitopes of HPV and HIV on bovine papillomavirus	(Liu, W.J. et al. 2000 [93])
Human immunodeficiency virus	Pr55gag, envelope: Preclinical	(Sakuragi, S. et al. 2002 [94], Gheysen, D. et al. 1989 [95], Shiota, T. and Shibuta, H. 1990 [96], Deml, L. et al. 2005 [97], and Doan, L.X. et al. 2005 [98])

(continued overleaf)

TABLE 5.1. (Continued)

Capsid Platforms	Capsid Composition	References
	Various HIV env epitopes on HIV; simian HIV chimera	(Deml, L. et al. 2005 [97] and Doan, L.X. et al. 2005 [98])
Influenza	HA, NA, matrix: Preclinical	(Pushko, P. et al. 2005 [99], Galarza, J.M. et al. 2005 [100], and Latham, T. and Galarza, J. 2001 [101])
Rotavirus	VP2, VP6, VP7: Preclinical	(Vieira, H.L. et al. 2005 [102], Bertolotti-Ciarlet, A. et al. 2003 [103], and Crawford, S.E. et al. 1994 [104])
Norwalk virus	Capsid: Phase 1	(Ball, J.M. et al. 1999 [105], Mason, H.S. et al 1996 [106], and Tacket, C.O. et al. 2003 [107])
Poliovirus	Capsid (VP0, 1, 3)	(Brautigam, S. et al. 1993 [108])
Ebola virus; Marburg virus	Glycoprotein (GP) and matrix (VP40): Preclinical	(Swenson, D.L. et al. 2005 [109], Warfield, K.L. et al. 2003 [110], and Warfield, K.L. et al. 2005 [111])
SARS (severe acute respiratory syndrome) coronavirus	S, E, and M: Preclinical	(Mortola, E. and Roy, P. 2004 [112])
Polyomavirus	VP1 and VP2 fused with 1–683 amino acid region of the extracellular and transmembrane domain of HER-2/neu	(Tegerstedt, K. et al. 2005 [19]; 2007 [46])
Yeast Ty	HIV V3 loop; HIV p24; malaria epitopes	(Griffiths, J.C. et al. 1991 [113], Weber, J. et al. 1995 [114], and Gilbert, S.C. et al. 1997 [115])
Phage Qbeta	Nicotine	(Maurer, P. et al. 2005 [18])

to secrete antigen-specific IgG2a, whereas Th2 cells express cytokines such as IL-4, IL-5, IL-9, and IL-13 to promote IgG1 and IgE class switch. Secretion of various cytokines by mature DCs further stimulates differentiation into B and T cells, resulting in antibody release and cytotoxic T-cell responses, respectively.

5.3 TAILORING VIRUS-LIKE PARTICLES BY ALTERING THE CAPSID SURFACE FOR VACCINE DEVELOPMENT

Through genetic fusion, VLPs can also be used to deliver immunogenic epitopes of other pathogens [14, 15]. Introduction of the immunogenic amino acid sequence to the surface region of the capsid allows the display of the immunogenic epitope with high density on the capsid surface. This technological innovation has greatly broadened the

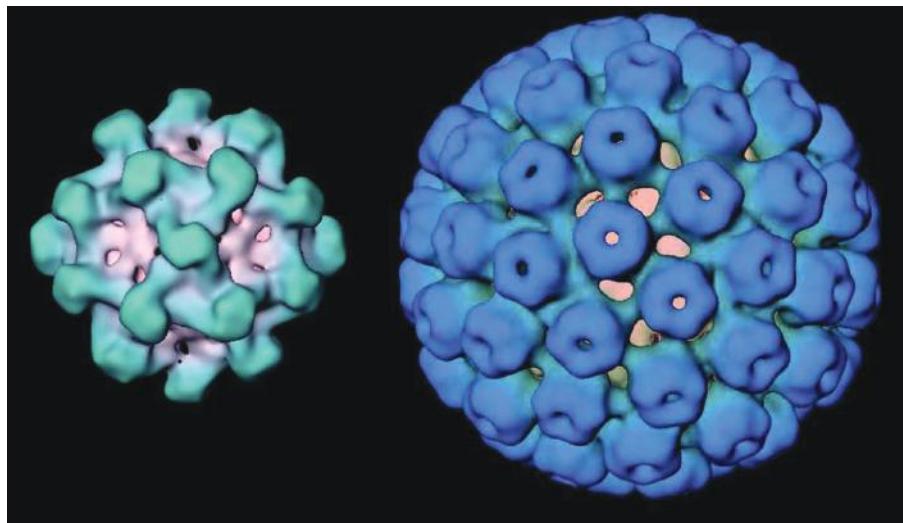


Figure 5.1. Surface morphology of VLPs. Left, T = 1 VLP of HEV. Light, T = 7d VLP of human BK polyomavirus. (See insert for color representation of this figure.)

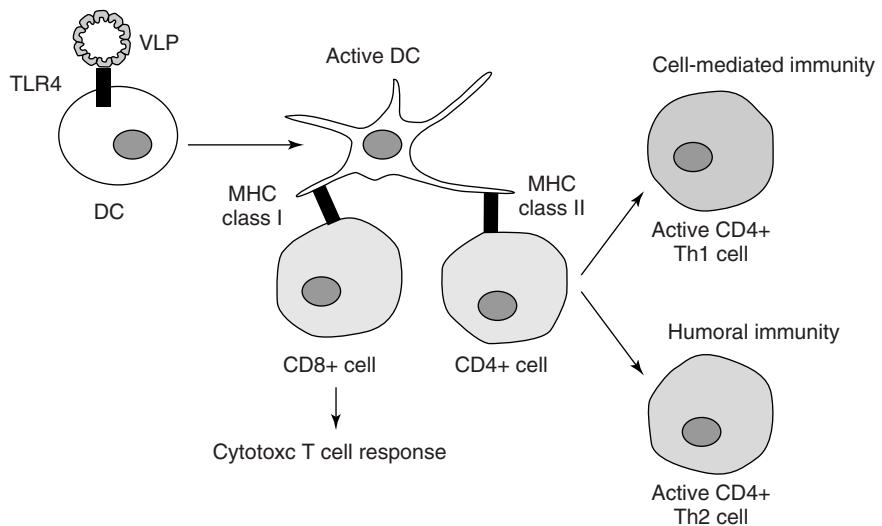


Figure 5.2. Immunoreaction against VLP. Natural and adaptive responses are induced by the association of VLP to TLR4 followed by the cell signalings of dendritic cells.

scope of their use, from immunizing against microbial pathogens to immunotherapy for chronic diseases [16]. For example, several VLPs have been developed as platforms to expose immunogens for vaccine development based on HBV, human and bovine papillomaviruses, yeast Ty, HIV/simian HIV gag, and HEV cores (Table 5.1). These capsid platforms are capable of inducing an immunogenic response against surface-exposed epitope. However, a major limitation to these VLP platforms is the small size of foreign epitopes that can be accommodated within the specific surface region of the capsid, which can preclude the presentation of large antigens such as HIV envelope or influenza hemagglutinin proteins. In order to overcome such spatial limitations, it is possible to immobilize peptide epitopes through the reactive site on the capsid platform by using chemical crosslinking. This has been done by coupling cysteine-containing peptides to the lysine residue situated in the immunodominant exposed region on HBV core particles [17]. By utilizing chemical crosslinking, it is also possible to conjugate nonprotein antigens such as nicotine on the surface of the capsid platform [18], which can induce an immunoresponse to nicotine.

Another approach to utilize the inner void space of VLPs is to encapsulate immunogens for the use of vaccination, expecting that the encapsulated immunogens would be processed and presented by MHC class I and II after the uptake into Dendritic cells (DCs). Murine polyomavirus (MPyV) VP1 is sufficient to form a spherical outer capsid structure. MPyV VP2/3 localize inside the assembled capsid. A fusion protein between MPyV VP2 and the 1-683 amino acid region of the extracellular and transmembrane domain of Human epidermal growth factor receptor 2/neu (HER-2/neu) (Her2) were encapsulated into MPyV VLP when it co-expressed with MPyV VP1 [19]. Vaccination against these Her2 containing VLPs inhibited Her2-expressing tumor growth in *in vivo* mouse models. The results show the feasibility of using MPyV-VLPs carrying Her2 fusion proteins as safe and efficient vaccines against Her2-expressing tumors.

5.4 USE OF FLUORESCENT-LABELED VIRUS-LIKE PARTICLES TO ISOLATE ROTAVIRUS-SPECIFIC B-CELL CLONES FOR HUMAN MONOCLONAL ANTIBODY PRODUCTION

Green fluorescent protein (GFP)-VLP of rotavirus (RV) has been used as antigen probe to select RV-specific B-cell clones from peripheral CD19+ cells obtained from RV-infected patients [20]. In this chapter, GFP fusions of the rotavirus VP2 protein were co-expressed with VP6 and VP7. VP2 forms the innermost core layer of the capsid and binds the ribonucleic acid (RNA) genome [21] VP6 and VP7 are major inner capsid protein and glycoproteins of the outer surface of the capsid, respectively [22]. Using a fluorescence-activated cell sorter, B-cell clones that bind to both GFP-VLP and anti-Cluster of Differentiation 19-Phycoerythrin (anti-CD19-PE) conjugate were detected and sorted into 96-well plates (one cell per well) with feeder cells. Wells with VP6- or VP7-specific immunoglobulins were identified with Enzyme-linked immunosorbent assay (ELISA), and the genes encoding the antibody Variable-region heavy-chain (V_H), and Variable-region light-chain (V_L) regions from those B-cell clones were subcloned into a Fragment antigen-binding (F_{ab}) expression

vector. This technology allows for rapid enrichment of B-cell clones that produce RV-specific antibodies.

5.5 VLP APPLICATION AS A DELIVERY CARRIER

There have been several attempts to use VLPs as a drug delivery system (DDS) (Fig. 5.3). Development of therapeutic molecules includes small chemical compounds, polymers such as DNA and RNA, and proteins. The carrier for DDS has to be functionalized in the nanoscale space in which the carrier has to hold drugs tightly but at the same time enable delivery and release of the drug at the designated target cell types. In addition, the carrier should be biodegradable following drug targeting and delivery. This is important since nonbiodegradable carriers may have toxic side effects. A variety of materials has been made from chemical-based materials to construct nanocarriers including ceramics [23], polymers [24], dendrimers [25, 26], micelles [26], nanospheres and nanocapsules [27], fullerenes and nanotubes [25], liposomes [28], and metals [29, 30]. In these carriers, therapeutic drugs are incorporated into the carriers by entrapment, adsorption, or encapsulation with both hydrophobic and hydrophilic surfaces. For active targeting to specific cell types, antibodies or ligands directed against specific tumor epitopes or receptors can be conjugated to the surface of the nanocarrier. To facilitate the release of drugs, the liposome carriers may be comprised of pH-sensitive components that degrade in a low pH environment, such as in areas of tumor hypoxia [31]. Alternatively, one may use thermo-labile liposomes,

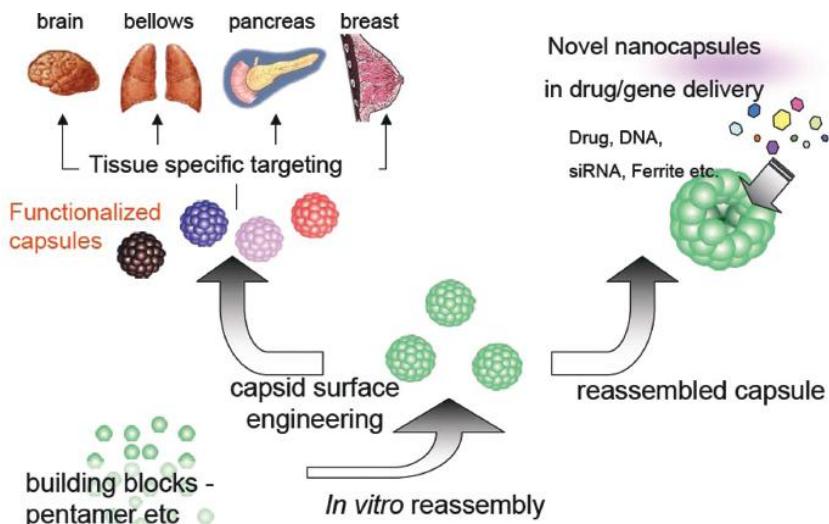


Figure 5.3. Overview of application of VLPs for therapeutic purpose of human diseases. VLPs would be applicable to deliver the encapsulated agents to the target cells for cell therapy. (See insert for color representation of this figure.)

which can release encapsulated agents in target tumor tissue by local hyperthermia [32]. Liposomal doxorubicin (Doxil) and paclitaxel-loaded human serum albumin nanoaggregate (Abraxane) are the two nanotherapeutics that have been approved by the FDA for cancer treatment.

Viruses have been used as carriers for human gene therapy because of their intrinsic cell-specific targeting properties and high transduction activity. Currently, such viruses include adenovirus, adeno-associated virus, herpes simplex virus, measles virus, Sendai virus, retro- and lenti-viruses, poxvirus, alphavirus, rhabdovirus, simian virus 40 (SV40), parvovirus, and Epstein–Barr virus (Table 5.2). However, there is great concern regarding the use of live viruses for this application. For example, recombinant viral genome may recover native viral activity and integrate into the host genome to disrupt and alter its expression, resulting in cancer formation and development of other diseases. To overcome this problem, methodologies have been developed to use the viral capsid alone, rather than live viruses, as protein-based nanocarriers. VLPs resemble intact virions, and hence they retain active specific targeting activity. The intrinsic capacity of VLPs to encapsulate nucleic acids, small molecules, and proteins make them ideal for gene and drug delivery (Table 5.2). Osmotic shock [33] and in vitro self-assembly of capsid subunits into VLPs [34–37] have been developed to encapsidate foreign deoxyribonucleic acid (DNA) into polyomavirus VLPs (Fig. 5.4). In HBV VLPs, foreign DNA and fluorescent dye were incorporated by the electroporation system for gene and drug delivery [38]. They showed that encapsidated DNA and fluorescence dye were selectively delivered into hepatocytes, which suggests that recombinant HBV VLPs still possess active targeting and high transduction activity similar to wild-type HBV virion. As for drug delivery by papillomavirus and polyomavirus VLPs, carboxyfluorescein diacetate and propidium iodide were incorporated into these VLPs [39–41]. They were able to show that hormones, vitamins, and peptides can be delivered into cells, thus suggesting that protein can potentially be delivered inside mammalian cells via these viral capsid nanocarriers. Unlike DNA transduction with viral or nonviral nanocarriers, protein delivery by VLPs avoids unexpected integration events into the genome but still enable transient and dose-controlled delivery of proteins *in vivo*. For example, it has been shown that pseudotyped lentivirions containing linamarase can deliver the enzyme to target cancer cells. As a result, the cancer cells become sensitized to linamarin, a cyanogenic glucoside substrate [42]. Similarly, in a polyomavirus system, heterologous proteins were genetically fused to VP1 proteins or fragments of minor coat proteins and successfully sequestered into VLPs [43–46]. Using this system, yeast cytosine deaminase (*yCD*), a prodrug-modifying enzyme that converts 5-fluorocytosine to 5-fluorouracil, was encapsulated into SV40 VLP. Tumor cells challenged by the *yCD*-encapsulating VLP became sensitive to 5-fluorocytosine-induced cell death [47] (Fig. 5.4b).

There have been several attempts to change or to broaden the cell-targeting activity of the viral nanocarriers by incorporating or chemically ligating cell surface-targeting peptide sequences to the viral capsid protein (Fig. 5.5). (Table 5.3). Integrin receptors are heterodimers composed of α and β subunits that play essential roles in cell–cell and cell–extracellular matrix interactions. Many investigators have reported the use of short Arginine–Glycine–Aspartic Acid (RGD) peptide (arginine–glycine–aspartate,

TABLE 5.2. Capsids Used as Carriers of Therapeutic Agents

Capsid Platforms	Encapsidated Agents	References
Adenovirus	DNA	(Douglas, J.T. 2007 [116], and Majhen, D. and Ambriovic-Ristov, A. 2006 [117])
Adeno-associated virus	DNA	(Daya, S. and Berns, K.I. 2008 [118], and Grieger, J.C. and Samulski, R.J. 2005 [119])
Herpes simplex virus	Foreign gene	(Smith, K.D. et al. 2007 [120], and Srinivasan, R. et al. 2008 [121])
Hepatitis B virus	Foreign gene and small molecule	(Yamada, T. et al. 2003 [38])
Measles virus	DNA	(Blechacz, B. and Russell, S.J. 2008 [122])
Sendai virus	DNA	(Yonemitsu, Y. et al. 2008 [123])
Retro and lenti viruses	DNA	(Lim, K.I. and Schaffer, D.V. 2008 [124], Loewen, N. and Poeschla, E.M. 2005 [125], and Sauter, S.L. and Gasmi, M. 2001 [126])
Poxvirus	Protein	(Link, N. et al. 2006 [42])
	DNA	(Arlen, P.M. et al. 2007 [127], and Moroziewicz, D. and Kaufman, H.L. 2005 [128])
Alphavirus	DNA	(Lundstrom, K. 2005 [129], and Yamanaka, R. 2004 [130])
Rhabdovirus	DNA	(Finke, S. and Conzelmann, K.K. 2005 [131])
Human papillomavirus	Small molecule	(Drobni, P. et al. 2003 [40], and Bergsdorf, C. et al. 2003 [132])
Polyomavirus	DNA	(Barr, S.M. et al. 1979 [33], Braun, H. et al. 1999 [34], Stokrova, J. et al. 1999 [133], Henke, S. et al. 2000 [35])
	Protein	(Schmidt, U. et al. 2001 [43], Abbing, et al. 2004 [44], and Boura, E. et al. 2005 [45])
Human JC polyomavirus	Small molecule	(Goldmann, C. et al. 2000 [41])
Simian virus 40	DNA	(Strayer, D.S. et al. 2002 [134], and Vera, M. and Fortes, P. 2004 [135], Kimchi-Sarfaty, C. and Gottesman, M.M. 2004 [136], and Tsukamoto, H. et al. 2007 [37])
	RNA	(Kimchi-Sarfaty, C. et al. 2005 [136])
Parvovirus	Protein	(Inoue, T. et al. 2008 [47])
	DNA	(Maxwell, I.H. et al. 2002 [137], and Srivastava, A. 2001 [138])
Epstein–Barr virus	DNA	(Komaki, S. and Vos, J.M. 2000 [139], and Sclimenti, C.R. and Calos, M.P. 1998 [140])

an integrin-binding motif) to alter the cell tropism of the virus capsids [48–52]. For example, the incorporation of the RGD motif into the maloney murine leukemia virus capsid results in the expansion of its tropism from mouse NIH 3T3 cells to human melanoma cells [51]. As an alternative approach to changing cell tropism of viral

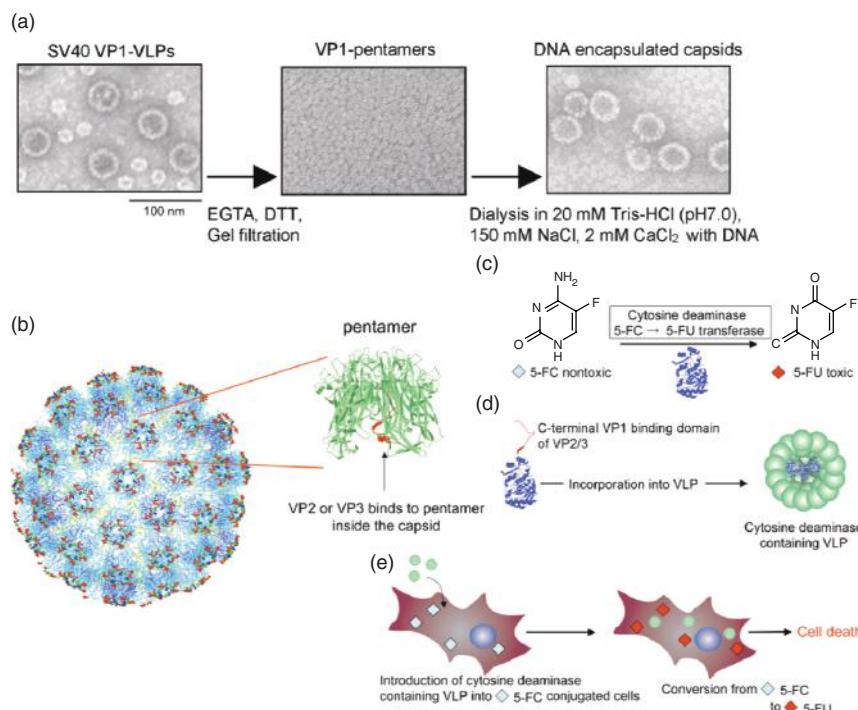


Figure 5.4. Examples of encapsulation inside the VLPs. (a) DNA-selective encapsulation into the VLP. SV40 VP1-VLPs are dissociated with calcium chelating agent (EGTA) and reducing agent (DTT), and then gel-filtrated to prepare pentamers, the building block of the VLP. In the presence of DNA, pentamer reassembled into the VLP to selectively encapsulate the DNA in physiological condition [20 mM Tris-HCl (pH7.0), 150 mM NaCl, and 2 mM CaCl₂]. (b) to (e) Protein selective encapsulation into the VLP. (b) C-terminus of VP2/3 can interact with pentamer from inside the capsid. (d) Using this fragment, cytosine deaminases were selectively encapsulated into the VLPs by fusing the C-terminal VP2/3 fragment at the N-terminus. (c) Cytosine deaminase converts the prodrug into the active form. (e) Using cytosine deaminase encapsulated VLP, prodrug administration succeeded to kill the cells only when the cells were incubated with cytosine deaminase encapsulated VLPs. (See *insert* for color representation of this figure.)

capsids, folate was conjugated to the antiadenovirus fiber monoclonal antibody [53]. Binding of this antibody on the adenovirus capsid inhibited adenovirus infection through the adenovirus receptor, but allowed folate receptor mediated entry. In order to immobilize the anti-CD4 monoclonal antibody on the capsid, the IgG-binding domain of protein A was inserted on the Sindbis virus capsid [54]. Binding of anti-CD4 antibody through protein A binding domain allowed viral entry into CD4-positive HeLa cells. It would also be possible to target specific cell types such as liver cells by introducing the preS sequence of HBV on the capsid using chemical crosslinking. Similarly, the many cancer targeting ligands that have been identified by combinatorial

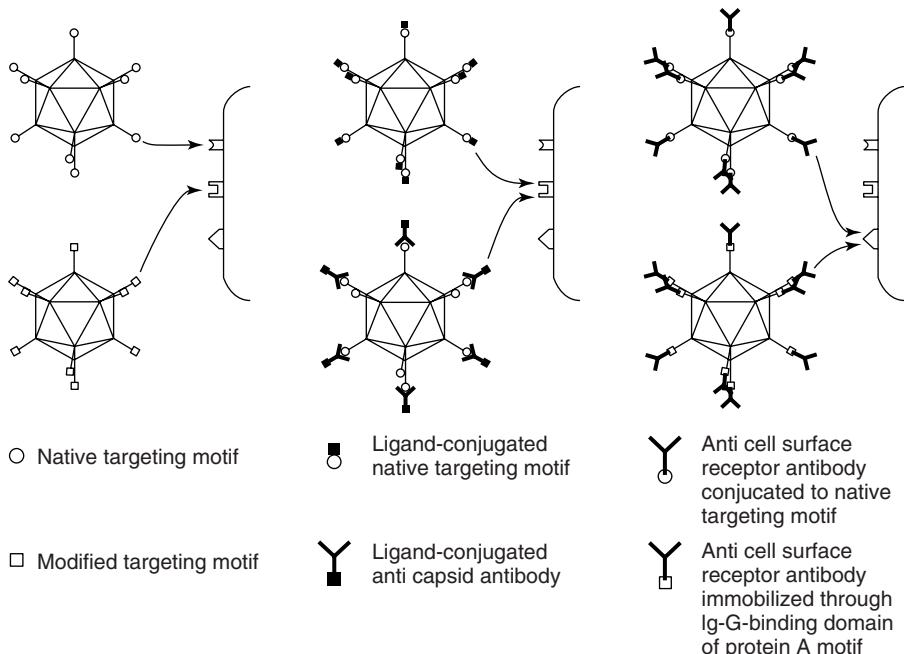


Figure 5.5. Tropism alteration using peptide sequence, ligand, and antibody. Tropism can be changed with peptide insertion at the surface residue of the capsid, ligand immobilization on the capsid by chemical crosslinking or through anticapsid antibody, and anti-cell-surface receptor antibody immobilization on the capsid by chemical crosslinking or through IgG-binding domain of protein A motif inserted at the surface residue of the capsid.

chemistry [6, 55–57] can be chemically synthesized, covalently linked to the viral capsid through site-specific ligation reactions, and the resulting targeting VLPs can be used as an efficient drug delivery system for cancer therapy.

5.6 CONCLUSION

Viral capsid was originally applied to the vaccine development using its high immunogenicity. In recent years, viral capsids have been recognized as potential nanocarriers for efficient delivery of biologically active materials to specific cell types. For this latter application, the VLPs can be genetically and chemically modified so that their targeting property can be optimized and their immunogenicity minimized.

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TABLE 5.3. Foreign Epitope Insertion to Change Capsid Tropism

Capsid Platforms	Foreign Epitopes	References
Adenovirus	(GS)5-ACDCRGDCFCG and (GS)5-KKKKKKK	(Wickham, T. et al. 1997 [50], and Vigne et al. 1999 [49])
Adeno-associated virus	RGD motif	(Shi, X. et al. 2006 [52])
Polyomavirus	protein Z dihydrofolate reductase eight glutamic acid residues and one cysteine WW domain Yes-kinase associated protein urokinase activator domain	(Gleiter, S. and Lilie, H. 2001 [141]) (Gleiter, S. et al. 1999 [142]) (Stubenrauch, K. et al. 2000 [143], and May, T. et al. 2002 [144]) (Schmidt, U. et al. 2001 [145])
Simian Virus 40	RGD motif	(Shin, Y.C. and Folk, W.R. 2003 [146])
Sindbis virus	IgG-binding domain of protein A	(Takahashi, R.U. et al. 2008 [147]) (Ohno, K. et al. 1997 [54])
Retro and Lenti viruses	RGD motif, gastrin-releasing protein	(Gollan, T. and Green, M.R. 2002 [48]; 2002 [148])

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6

DESIGNING IMMUNOGENS FOR VACCINE DEVELOPMENT IN REFERENCE TO HIV

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Indresh K. Srivastava

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6.1 SUMMARY

The human immunodeficiency virus (HIV) continues to be a major health problem worldwide; however, the situation is particularly serious in Asian and sub-Saharan countries. Therefore, development of an effective HIV vaccine could help to reduce the severity of the disease and prevent infection. Over the last two decades significant efforts have been made toward inducing potent humoral and cellular immune responses by vaccination; however, it appears that either antibodies or cytotoxic T lymphocytes (CTLs) may not be sufficient alone for the induction of sterilizing immunity or long-term control of viral replication. Therefore, it is generally believed that

both humoral and cellular responses will be needed for an effective HIV vaccine. It has been shown in passive transfer experiments using broadly neutralizing monoclonal antibodies (mAb) such as b12, 2F5, 4E10, and 2G12 that these mAbs either alone or in combination are effective in conferring protection against challenge infection to Rhesus macaques. However, for the development of an effective vaccine, it has been a challenge to induce protective antibodies of similar potency by vaccination. Therefore, efforts are being made by different groups to design an Env immunogen that may be more effective compared to the existing immunogens in inducing potent neutralizing and protective antibody responses by (i) optimization of existing Env structure to enhance the exposure of functional epitopes to focus the responses to these epitopes, (ii) obtain structural information on HIV Env and use this information for structure-based novel immunogen design, and (iii) identify novel functional epitopes and design strategies to incorporate them in potential vaccines. Once potent HIV Env structures have been identified, their effectiveness may be enhanced through the use of adjuvants, delivery systems, and prime and boost strategies to improve the quality and magnitude of neutralizing responses.

6.2 INTRODUCTION

An effective vaccine is really needed for debilitating diseases such as HIV. Acquired immunodeficiency syndrome (AIDS) has been the primary cause of death of more than 36 million people worldwide since 1981. This disease is far from being contained: In 2007 only, close to 2.5 million people were newly infected. HIV was identified in 1981 as the causal agent for AIDS [184]. In certain parts of the world, such as sub-Saharan Africa, the prevalence of HIV in the population is estimated to be as high as 34% [184]. In North America and western and central Europe, approximately 2.1 million people were living with HIV in 2007, including the approximately 78,000 who acquired HIV in the past year [184]. Moreover, the situation is continuously deteriorating as a result of the rapid emergence of drug resistance against most of the effective antivirals. Therefore, there is an urgent need for an effective anti-HIV vaccine that may be used either alone as a prophylactic vaccine or in conjunction with antiviral drugs as a therapeutic treatment.

Learning from our experiences it seems that we have been quite successful in developing the vaccines against the diseases where the correlate of protection is well established and well defined. A list of vaccines that have been successfully developed is presented in Table 6.1. The common theme is “defined correlate of protection.” Furthermore, the success rate is very high for vaccine development against diseases where correlation of protection is antibody mediated. In addition, in almost all diseases, there are cases that have successfully cleared infection, while in HIV there is no single case that has cleared a natural infection. One of the major challenges in developing a vaccine against HIV is a lack of correlation of protection. Early data suggested that the induction of the neutralizing antibodies is the key to controlling the viral replication and that which confers protection. Later based on the preclinical studies and early clinical studies, it was concluded that neutralizing antibody responses

TABLE 6.1. Correlation between the Correlate of Protection and Development of Vaccine

Pathogen/ Disease	Defined Correlate of Protection			Vaccines Available
	Antibodies	Antibodies, T cell or Mucosal	T cells	
Dietheria	Yes			Yes
Tetanus	Yes			Yes
Polio	Yes			Yes
HIB	Yes			Yes
HAV	Yes			Yes
HBV	Yes			Yes
Rabies	Yes			Yes
MMR	Yes			Yes
Influenza	Yes			Yes
Papillomavirus	Yes			Yes
Pneumococcus	Yes			Yes
Menigococcus	Yes			Yes
Typhoid fever		?		No
HIV		?		No
Malaria			?	No
TB			?	No
Cancer			?	No

induced by monomeric HIV Env are not potent enough to protect against the HIV infection [110, 112]. Therefore, the focus and the efforts shifted from induction of neutralizing antibodies to the induction of cellular responses. Previous studies using the HIV group-specific antigen (gag) have demonstrated the correlation between strong cytotoxic T-cell activity and reduced viral load. However, more recent data obtained in a macaque challenge model suggest that cellular responses focused on a single epitope may not be sufficient as well to effectively control viral replication [12–14, 190]. It remains to be seen if increasing the breadth of CTL responses will have a positive impact on the outcome of challenge infection. Hence, the working hypothesis is that humoral, cellular, and T-helper-cell responses, both at peripheral and mucosal sites, are needed for broad and durable protection against HIV. Conceptually, antibodies would serve as a first line of defense by completely preventing infection (Fig. 6.2a and 6.2b) or reducing the virus inoculum, whereas cellular responses would facilitate the clearance of HIV-infected T cells that have escaped antibody-mediated neutralization (Fig. 1.6b) and reduce the severity of the disease. However Mascola and colleagues have shown that the induction of cellular responses using DNA vaccines in conjunction with passive transfer of neutralizing antibodies did not offer additional protection compared to protection afforded by passive transfer of neutralizing antibodies alone [109]. Therefore, further studies will be needed to demonstrate the advantage of inducing potent cellular and humoral responses by an effective HIV vaccine for inducing the sterilizing immunity or effectively control the level of viral replication.

Learning from the development of successful vaccines, it is clear that we have been successful in developing vaccines against diseases where the target

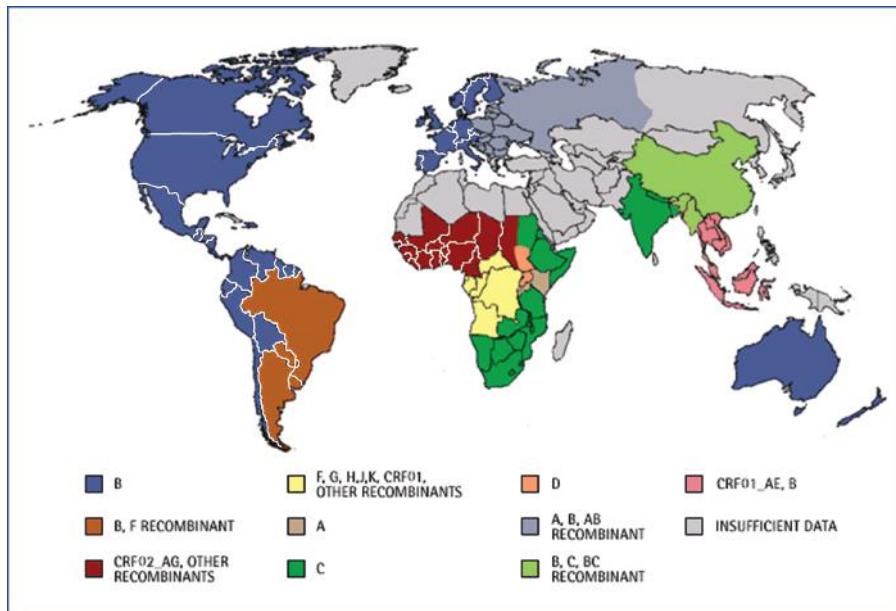


Figure 6.1. Global distribution of HIV-1.

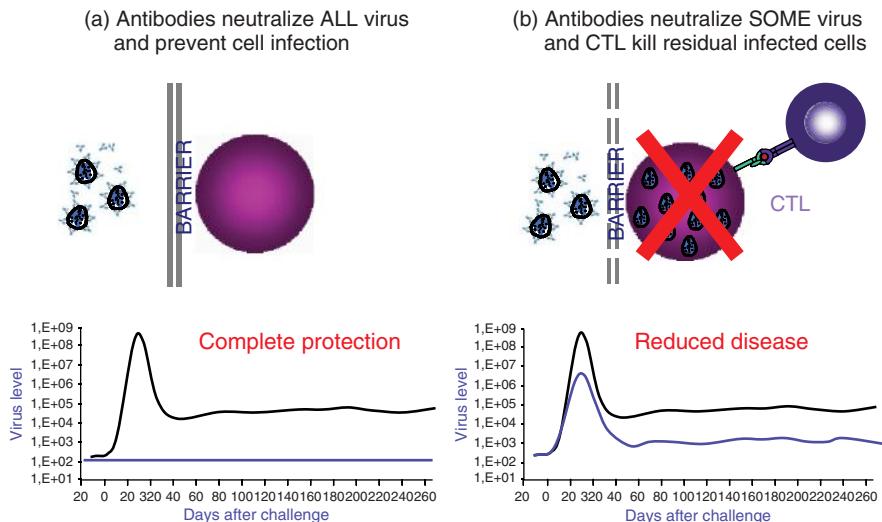


Figure 6.2. Conceptually, antibodies act as a barrier to, (a) completely prevent infection, or (b) Reduce the size of inoculum.

TABLE 6.2. Correlation between the Antigenic Diversity and Development of Successful Vaccine

Pathogen/ Disease	Antigenic Diversity			Vaccines Available
	Negligible	Moderate (10 years)	Highest High (1 year)	
Diphtheria	Yes			Yes
Tetanus	Yes			Yes
Polio	Yes			Yes
MMR	Yes			Yes
HBV	Yes			Yes
HAV	Yes			Yes
Rabies	Yes			Yes
MMR	Yes			Yes
Pneumococcus		Yes		Yes
Menigococcus		Yes		Yes
Influenza			Yes	Yes (Seasonal)
Malaria		Yes		No
HIV			Yes	No

antigen/immunogen does not have a significant degree of antigenic diversity over time (Table 6.2). Furthermore, the greater the antigenic diversity, the higher the hurdle for developing vaccine. Unfortunately HIV, Men B, and influenza, just to name a few diseases, fall in this category and developing an effective vaccine against these diseases has been a challenge. For the induction of neutralizing antibody responses, Env glycoprotein on the surface of the virion is the prime target, unfortunately, there are enormous number of variants that evolve in the infected people all over the world (Fig. 6.2). This reduces the possibility of finding broadly protective or safe HIV-1 vaccine using classical vaccine approach of chemical inactivation or live attenuation similar to other common vaccines such as polio and measles. Hence finding cross-reactive neutralizing response is one of the major targets that scientists are aiming at and did not have satisfactory achievement yet. In addition, conformation of the antigen is also a critical factor to be considered for developing the vaccine. In case of HIV, the initial efforts were focused on inducing neutralizing antibody responses of appropriate specificity against diverse primary HIV-1 strains utilizing monomeric HIV Env (i.e., gp120) glycoprotein. Strangely enough, gp120 did bind to neutralizing antibodies, however, it failed to induce antibodies of similar efficacy by vaccination. Therefore, a major challenge is to take advantage of structural information and design novel strategies to identify and expose critical epitopes that may be the target for inducing broadly cross-reactive neutralizing antibodies with protective efficacy. The focus of this review is (a) to summarize the progress made in obtaining structural data on critical broadly neutralizing antibodies and their conserved binding sites, (b) challenges in inducing these antibodies by vaccination, and lastly (c) review strategies that have been proposed to design potent immunogens that may induce broadly neutralizing antibodies.

6.3 HIV-1 NEUTRALIZING ANTIBODIES

6.3.1 Possible Steps for Immune Intervention

There are several possible steps during the virus life cycle (pre- and postattachment events) where immune intervention is possible and that may prevent the infection or control viral replication, as illustrated in Figure 6.3. Since binding of HIV to its receptor (CD4) and co-receptor (e.g., CCR5) is critical for viral entry in new CD4+ T cell, therefore, a logical and attractive strategy could be to prevent the interaction of HIV virion with its cellular receptor or co-receptor [183]. Other possible yet attractive strategies are to target other important postattachment events such as (a) the formation of a coiled-coil structure leading to virus/host membrane fusion [17, 36, 69, 76], (b) primary uncoating of the virus in the cytoplasm, (c) transcription or virus assembly, and (d) budding of the new virion. For HIV-1, inhibition of receptor binding has been seen for different types of antibodies including (i) antibodies that target carbohydrate of the virus glycoprotein [81, 156]; (ii) antibodies that target different adhesion molecules; (iii) antibodies specific for various regions of gp120 such as the CD4 binding domain, the “CD4-induced (CD4i) epitope” (located in the bridging sheet of gp120 that is created or exposed when gp120 interacts with CD4) (182), and

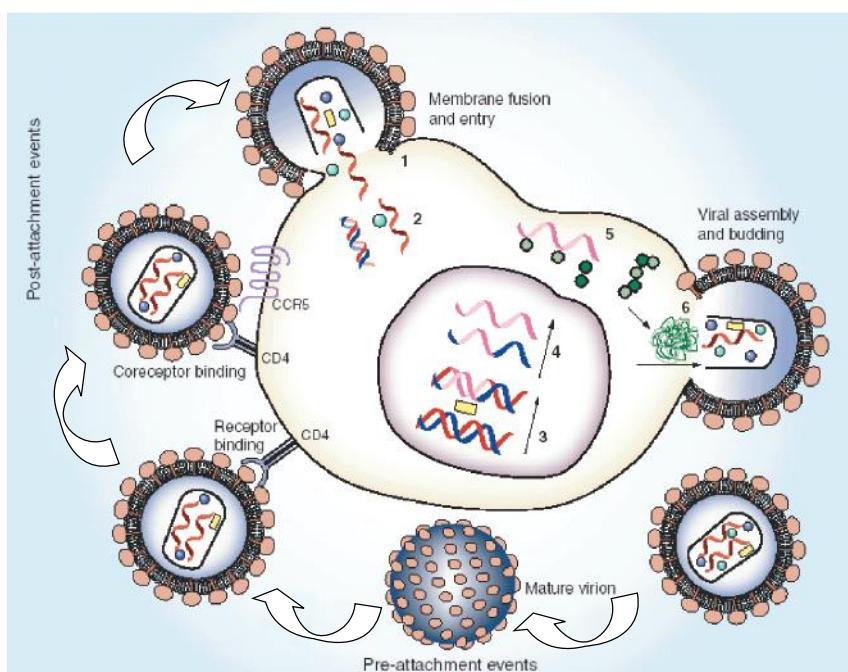


Figure 6.3. There are multiple steps vulnerable for intervention during the virus life cycle. These include 1. entry, 2. reverse transcription, 3. Integration, 4. Transcription, 5 assembly and 6. Budding.

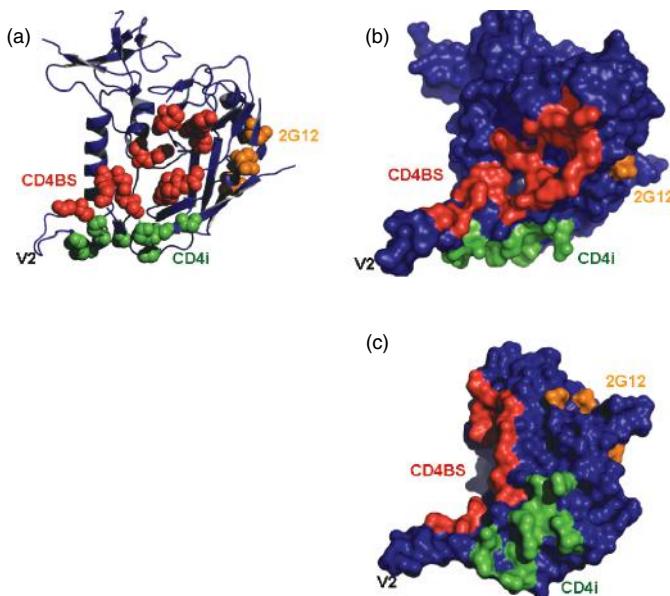


Figure 6.4. Structural representation of some of the conserved epitopes on gp120 (92). These include the CD4 binding site epitopes (CD4BS), CD4 induced epitopes (CD4i) and conserved carbohydrate epitopes (2G12). A. These epitopes are shown on Cartoon structure of gp120, and B. surface representation of gp120 in the same orientation as in A. C. The surface representation in B is rotated 90° around vertical axis, to indicate that the carbohydrate epitope (2G12) are primarily located on the outer domain, while the CDBS and CD4i epitopes are located next to each other on the inner domain of gp120.

the variable loops, V2 and V3; (iv) antibodies that target the co-receptor-binding site [101]; and (v) another class of mAbs such as 2F5, 4E10, or Z13 that may interfere with gp41 coiled-coil formation or fusion of the virus and host cell membranes. Some of these epitopes in context to gp120 structures are presented in Figure 6.4. Additional mechanism by which antibodies can mediate protection is aggregation of virus particles, thereby reducing the number of infectious virus particles and rendering the virus more susceptible to phagocytosis and subsequent destruction [52].

6.3.2 Mechanisms of Antibody Neutralization

Different mechanisms by which binding antibodies may exert a protective function is antibody-dependent cellular cytotoxicity (ADCC) where antibodies act as a bridge between Fc receptor (FcR)-bearing cells and HIV-1-infected cells or other cells that have passively absorbed HIV-1 Env onto their surface. The killing of virus-infected cells by ADCC involves cytotoxic cells [such as natural killer (NK) cells] and may contribute toward the elimination of the virus [2, 3, 59, 70]. Alsmadi and Tilley [6] studied the ability of human and chimpanzee mAbs directed against epitopes of gp41, CD4 binding site, V3 loop, and C5 domain of gp120 for their ability to induce

ADCC. They demonstrated that mAbs directed against conserved epitopes generally exhibited ADCC activity against a broader range of HIV-1 strains than those directed against variable epitopes. Furthermore, it appears that many, if not most, neutralizing antibodies are of high affinity and of IgG1 subtype, and they are capable of exerting ADCC-type activity. In a multicentered AIDS cohort study, early ADCC responses in patients were associated with higher numbers of CD4+ T cells and the absence of lymphadenopathy during the first 2 years of follow-up [155]. In two additional studies, ADCC responses correlated inversely with plasma viral load and with CD4+ T-cell counts [3, 22]. The presence of neutralizing and ADCC activity in children born to HIV-infected mothers correlated better with the clinical outcome [24]. Finally, it has been shown that ADCC antibodies were present in cervicovaginal fluids in HIV-1-infected women [15], supporting the hypothesis that this form of immunity can contribute in protection against HIV-1 at the site of virus entry. The identification of “ADCC epitopes” may help in designing strategies on means to present and enhance the potency of these epitopes to prevent or control the viral infection.

6.3.3 Conserved Antibody Binding Sites on HIV-1 Env

HIV Env is the only viral protein that presents neutralizing epitopes relevant for vaccine development. For inducing neutralizing epitopes relevant for vaccine development Env is the target. In addition, it has several known CTL and helper T-cell epitopes that may provide targets for an effective anti-HIV vaccine. The *env* gene is expressed during the late phase of viral transcription as the gp160 precursor protein. During the maturation of the virus, the gp160 is proteolytically processed by cellular serine proteases to yield (i) membrane spanning domain termed gp41 and (ii) an extracellular domain termed gp120 (Figure. 6.5a). Env proteins become heavily glycosylated during their

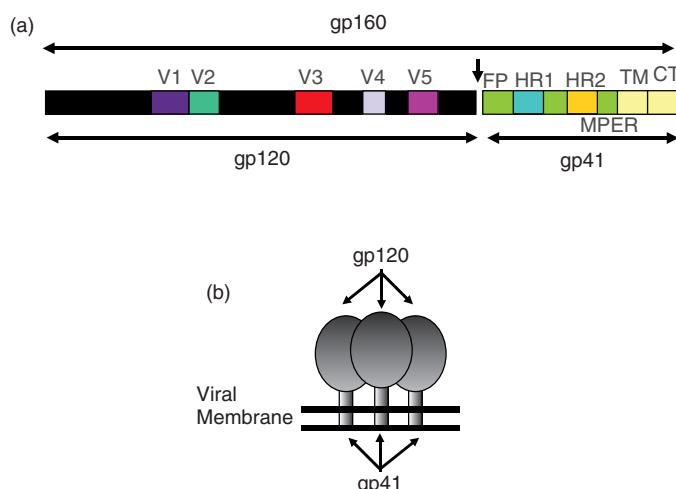


Figure 6.5. Cartoon representation of linear and 3-dimensional structure of HIV-1 envelope glycoprotein.

passage through the Golgi apparatus. gp120 and gp41 are noncovalently associated on the viral surface to form trimeric spikes (Fig. 6.5b) that can bind to the CD4 on T cells, which is the primary receptor of HIV-1. Comparison of the sequences of the *env* gene from different isolates and clades reveals that it has five hypervariable regions and five conserved regions, and a large number of cysteine residues (Fig. 6.5a). There are differences between the size of the variable loops and the number of glycosylation sites in V1 and V2 loops among virus isolates of different clades and between early and late primary viruses. This sequence variation in the hypervariable loops is due to nucleotide changes and subsequent accumulation of point mutations resulting in amino acid substitutions. These changes in Env tertiary structure are selected by the host to provide a means of escaping the immune pressure. The receptor- and co-receptor-dependent entry process of HIV into target cells is described by Moore and Doms [120].

6.3.4 CD4 Binding Sites

Interaction of HIV Env with the CD4 is an obligatory step for most circulating virus entry into the cell; therefore, as expected, the CD4 binding domain of gp120 is a highly conserved, complex, and conformation-dependent region. Hence, the CD4 binding site of Env (CD4BS) may be an excellent target for immune intervention. Consistent with the hypothesis many mAbs against the CD4BS have been developed that can neutralize T-cell line-adapted isolates [25, 139], suggesting that these epitopes are relatively well exposed on the virion surface [140]. The most potent and well-characterized monoclonal antibody against the CD4BS, b12 [8], neutralizes a broad range of primary isolates and confirms the critical role of the CD4BS in HIV-1 infection [20, 123]. Interestingly, other CD4BS monoclonal antibodies such as 559/64D, 15e, F105, b3, and b6 do not neutralize primary isolates [47, 161]. Recent crystal structure comparison between neutralizing (b12) and nonneutralizing (F105, b13) CD4BS antibodies indicated that binding of nonneutralizing antibodies is poorly compatible with viral spike [38]. The reasons for this discrepancy are not yet understood, but b12 differs from all the other CD4BS antibodies in its sensitivity to V1–V2 loop deletion [146]. It is not known if b12 contacts the V1–V2 loop or if the sensitivity is due to an indirect effect of conformational rearrangements following V1–V2 deletions. High-resolution crystal structure of b12 has been solved, and a key feature of the antibody-combining site is the protruding, finger-like long structure that can penetrate the recessed CD4 binding site of gp120. Therefore, it is quite effective in neutralizing the virus. A docking model of b12 and gp120 reveals severe structural constraints that explain the extraordinary challenge in eliciting effective neutralizing antibodies similar to b12. The structure, together with mutagenesis studies, provides a rationale for the extensive cross reactivity of b12 and a valuable framework for the design of HIV-1 vaccines capable of eliciting b12-like activity [131, 208]. Another highly potent CD4BS mAb, VRC01, is recently identified and characterized. This monoclonal antibody is shown to neutralize >90% of all the primary isolates tested [207].

6.3.5 Co-receptor Binding Sites

After primary attachment of virus to the T-cell surface, gp120 interacts with chemokine receptors CCR5 or CXCR4, which are the most common cellular co-receptors for

HIV-1. The interaction of Env to CD4 induces the conformational changes in gp120 and gp41. As a result, the co-receptor binding site and fusogenic region of gp41 is displayed, leading to the fusion of viral and cellular membranes and the release of the viral core particles in the cytoplasm of the cell. Therefore, CD4-inducible epitopes of Env may represent another target for immune intervention. Antibodies that show reactivity toward HIV Env when it is complexed with soluble CD4 (sCD4) were found in HIV-1-infected individuals [177], suggesting that these epitopes are immunogenic. Several such human mAbs have been identified, including 17b, 48D, CG10, 23E, and X5 [67, 124, 197]. The region recognized by these anti-CD4i monoclonal antibodies has now been mapped to β strands in the V1–V2 stem and the C4 region of gp120. These regions are involved in binding of Env to chemokine receptors [5, 49, 128, 197]. Surprisingly, 17b and other CD4i mAbs with the exception of E51 do not neutralize most primary isolates in their complete IgG form [197]. In contrast, Fab and single-chain fragments of these anti-CD4i mAbs readily neutralize primary isolates [51, 124] suggesting that there may be space constraints between the CD4i epitope on gp120 and the target cell membrane [95]. Therefore, the entire IgG molecule may not be able to bind and neutralize primary isolates due to steric hindrance [79, 173]. However, further studies are needed to confirm the accessibility of these conserved conformational epitopes and how to target them for vaccine application.

6.3.6 Conserved Binding Sites on Variable Loops

Contrary to earlier beliefs that epitopes in the variable domains are isolate specific and thus may not be appropriate targets for vaccine application, recent structural studies suggest that the V3 loop does contain conserved functional epitopes, which may be targeted by vaccines. The V3 loop is immunogenic and anti-V3 antibodies are induced early during infection and after immunization [78, 89, 112, 142]. However, a large proportion of these antibodies are directed against linear epitopes in the V3 loop, which serve as decoys for directing immune responses away from conserved V3 regions. These antibodies neutralize homologous isolates but have little or no neutralizing activity against diverse primary isolates [72, 161]. However, broadly neutralizing anti-V3 antibodies directed against the conserved conformational epitopes have been described [75]. The most broadly reactive of these neutralizing anti-V3 mAbs (such as 447-52D, 19b, and 2182) can neutralize a large proportion of clade B primary isolates [20, 45, 92, 141] and also have been shown to neutralize viruses from clades A, F, C, and G [73], suggesting that the epitopes recognized by these mAbs are conserved across clades.

Structural studies have shown that the V3 loop has some constant features, such as a relatively fixed size [30–35 amino acids (aa)], a conserved type II turn at its crown, a disulfide bond at its base, and a net positive charge [28, 203]. These features are required in the V3 loop in order for it to interact with the chemokine receptor [46, 174]. It has been shown by Cao et al. that deletion of the V3 loop renders the virus noninfectious, suggesting that the V3 loop is essential [31]. Increase in the length of the V3 loop by as little as a single amino acid residue leads to disruption

of the viral spike and shedding of gp120, indicating the importance of the V3 loop in maintaining stability. Recently, structural studies have demonstrated that V3 loops in viruses utilizing the CCR5 coreceptor are homologous to the unique hairpin structures of chemokine that bind CCR5, such as CC chemokines [CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES)] and CXC chemokine CXCL12 (SDF-1) in viruses utilizing the CXCR4 coreceptor [159]. In addition, Yonezawa et al. have replaced the V3 loop of the X4 virus with a 43-amino-acid region of SDF-1, which includes the β -hairpin, and demonstrated that infectivity was maintained [205]. Together, these data suggest a critical role for the V3 region in virus infectivity and provide a potential rationale for designing immunogens that induce antibodies to the conserved V3 motif. However, these concepts need to be evaluated in preclinical studies for their ability to induce cross-reactive neutralizing antibody responses.

6.3.7 Conserved Binding Sites on gp41

Passive immunization studies using combinations of anti-gp41 mAbs have resulted in protection against simian–human immunodeficiency virus (SHIV) challenge in monkeys [7, 108, 113, 135]. In addition, immunotherapy using a combination of 2F5 and/or 4E10 and 2G12 have resulted in reduced viremia in established HIV-1 infection or delay of HIV-1 rebound in individuals undergoing interrupted antiretroviral treatment [171, 178, 179].

All three gp41 directed mAbs binds to membrane-proximal region (MPER) (Fig. 6.5a) yet differ in their ability to neutralize HIV-1 primary isolates, with 2F5 being the most potent, 4E10 being the most broad, and Z13 being the least potent or broad [210]. Out of these three antibodies 4E10 and 2F5 are the most studied antibodies. The core epitope of 2F5 (ELDKWA) corresponds to residues 662–667 in gp41 of the HXB2 strain of HIV-1 [180]. Several studies, however, suggested that this antibody has a more extended epitope [9, 21, 134, 210]. The second well-defined epitope is for the 4E10 antibody. 4E10 is capable of neutralizing an extremely broad range of HIV-1 primary isolates, from virtually all subtypes, although it appears to have a modest potency [210]. The core of the 4E10 epitope, NWFDIT, maps just C terminally to the 2F5 epitope on the gp41 MPER [172, 210]. The MPER is a highly conserved region among various HIV-1 isolates and is critical for HIV-1-mediated membrane fusion and infection [125, 147]. Both 2F5 and 4E10 mAbs have long, hydrophobic Ig CDR3 regions, which could be due to additional surface of interaction with the viral membrane or with other gp41 or gp120 domains in the context of the intact virus. This information could be valuable for rational vaccine design of new antigens in the context of the membrane [33, 129].

In order to find new and better neutralizing antibodies, a selection of anti-gp41 antibodies was isolated using gp41 fragments on phage-display libraries. The selected antibodies, however, exhibited relatively modest neutralizing activity [104, 116]. Recently, similar work was done using phage-display antibody library that was panned against the gp140 (R2). A gp41-specific antibody (m46) was selected, which binds to a conformational epitope (in contrast to 2F5 and 4E10) and exhibits broad and relatively potent neutralizing activity, although the exact epitope for this mAb is yet to be identified.

6.3.8 Conserved Carbohydrate Binding Moieties on gp120

HIV Env is heavily glycosylated with approximately 50% of its mass due to carbohydrates [96]. Both N-linked and O-linked glycans are present on the HIV envelope glycoprotein. O-linked glycans are present on several unidentified serine or threonine residues, but very little is known about their actual role in the fusion process [34]. In contrast the role of the N-linked glycans is well characterized. It has been demonstrated that glycosylation is critical for the Env binding to CD4 since nonglycosylated Env protein does not bind to CD4 [1]. Thus, carbohydrate moieties on Env appear to provide a functional conformation to Env critical for its interaction with CD4. In addition, based on the crystallization studies of gp120, it appears that the exposed face of gp120 is heavily glycosylated (Fig. 6.6). Even in context of trimer, molecular modeling studies suggest that heavily glycosylated part of the molecule is exposed to the immune system [37, 196]. These data suggest that extensive glycosylation may shield critical neutralizing epitopes. Therefore, it is not surprising that antibodies recognizing carbohydrate-dependent epitopes on Env are not readily induced during the course of natural infection. However, the beststudied anticarbohydrate antibody with broadly neutralizing activity is mAb 2G12, which targets a cluster of carbohydrate moieties in gp120 [149, 156]. This mAb has broad neutralizing activity both against T-cell-line-adapted and primary HIV-1 isolates [156, 181]. However, its reactivity may be limited, as it does not neutralize subtype C isolates [27]. The unconventional configuration of this mAb [30] and the poor immunogenicity of the epitope recognized by 2G12 [181] raises questions about the mode of neutralization (since the epitope recognized by this mAb has been localized on the immunologically silent face of gp120) and how to design an immunogen with optimal exposure of this epitope. Based on oligomeric modeling studies it seems that the immunologically silent face of Env is likely to face toward the target cell membrane; therefore, it is conceptually possible to understand that 2G12 may neutralize the virus by preventing the interaction of virus with target cells.

As with most pathogens, HIV-1 induces a polyclonal antibody response to a wide array of epitopes on different viral proteins. Studies of polyclonal sera have helped

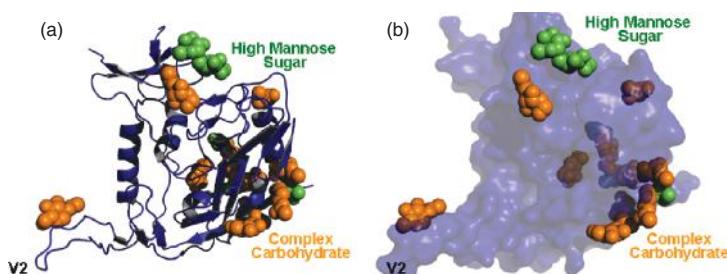


Figure 6.6. Cartoon (A) and surface (B) representation of HIV-1 gp120 with high mannose sugars and complex carbohydrates decorating the surface shown in small spherical balls. Most of these glycosylations are present on the outer domain of gp120.

to identify the specificities of antibodies that are associated with protection. It has been shown that sera collected from some HIV-infected individuals neutralize primary isolates (using CCR5 as a co-receptor; R5) [32, 169]. In addition, several investigators have generated potent neutralizing monoclonal antibodies from the bone marrow of HIV+ patients against critical functional and conserved epitopes (Table 6.3) such as the ones in the CD4 binding site (CD4BS) [8], CD4-inducible epitopes (CD4i) [177], carbohydrate-dependent epitope [181], and the epitopes present in variable loops [71, 74, 84, 89, 142] and gp41 regions of Env [18, 77, 200]. The relative position of some of these epitopes on the structure of gp120 is shown in Figure 6.4. Furthermore, neutralizing antibodies from the HIV+ patient's sera can be affinity purified on a gp120 column [169, 170], suggesting that neutralizing epitopes are present and exposed on gp120. Induction of antibodies to each of these epitopes

TABLE 6.3. Conserved Epitopes on ENV Targets for Developing HIV Vaccine

Env Region	Targeted Epitope	nAbs (neutralizing antibodies)	Non-nAbs (non-neutralizing antibodies)	Characteristics
gp120	CD4 binding site (CD4BS)	F105 b12	15e 21h 559/64D 650-D 448D 39.3 b3 b6 830D	These antibodies compete with CD4 for binding to Env. Not all of the CD4BS antibodies neutralize primary isolates.
	CD4-inducible conformational epitope	E51 X5 CG10	17b 48D 23E 49E 21C	Binding of Env to CD4 enhances the exposure of these epitopes. Most of these antibodies neutralize primary isolates as Fab and not as the whole IgG.
	Carbohydrate-dependent epitope	2G12		Poorly immunogenic and binding is dependent upon proper N-linked glycosylation.
gp41	Epitopes in close proximity to viral membrane	2F5 4E10 Z13		These antibodies interfere in membrane fusion, therefore preventing viral entry. To date, these are the most potent neutralizing antibodies identified.
	Cluster I of gp41	Clone 3 246-D		Highly immunogenic epitope, but Clone 3 is the only one of many mAbs specific for this epitope that has neutralizing activity.

may ultimately be useful in protecting individuals against HIV-1 infection; however, presentation of each of these epitopes in context to a vaccine has been a challenge.

During the past few years, considerable attention has been focused on neutralizing antibodies. Therefore, two major points need to be addressed: (i) characterizing the fine specificity of protective antibodies and (ii) means to elicit protective antibodies by immunization.

6.4 CHALLENGES IN INDUCING BROADLY NEUTRALIZING ANTIBODIES

In general the challenges in developing the vaccines involves the use of native molecules that are subject to (a) denaturation, conjugation, and modifications introduced by adjuvants; (b) viral surface proteins designed by nature to evade the immune response; (c) presence of parts of the molecule that serve as a decoy to direct immune responses to irrelevant parts of the molecule; and (d) parts of the molecule that may induce reactogenicity and instability in the vaccine. Furthermore, functional sites on the viral spike are protected from recognition by antibodies through several mechanisms that include (a) conformational masking, which requires a large amount of rearrangement of Env for high-affinity binding with a neutralizing antibody, (b) steric blockage by organizing into trimeric complex and limiting the line of access for antibodies to functional sites, (c) kinetic barrier, which restricts stable exposure of conserved functional sites, and (d) recessed binding pockets that are difficult to reach by antibodies.

During the course of natural infection, HIV triggers antibodies, cytotoxic T-cell (CTL) and CD4+ T-helper immune responses. In general, the primary peak of viremia declines before the appearance of neutralizing antibodies against HIV Env. HIV-infected individuals may generate potent neutralizing antibody responses to autologous isolates, but these responses are slow to develop and take a long time to mature [32, 85, 112, 118, 119, 122, 138, 195]. Interestingly, some long-term nonprogressors who remained disease free for more than 10 years after HIV infection developed strong, broadly cross-reactive neutralizing antibody responses, which may have contributed toward their ability to control infection [138, 145, 191, 192]. The induction of antibody responses by monomeric Env protein subunit vaccines is initially modest and requires multiple boosts to induce strong responses. In addition, these antibodies primarily recognized linear epitopes in the variable domains and neutralized T-cell-line-adapted (TCLA) virus isolates at significant dilutions [121, 185] but failed to neutralize primary HIV-1 isolates [112]. Furthermore, these nonconformational anti-gp120-specific antibodies are predominantly subtype specific and, to some extent, isolate specific. However, contrary to the earlier neutralization data, a recently published study indicated that using a modified neutralization assay with an extended incubation phase the first generation of monomeric gp120 vaccines can induce antibodies capable of neutralizing primary isolates [53]. Thus, for the fair comparisons of potential vaccine candidates, there is an urgent need to develop standardized neutralization assays [117].

Another factor that has the potential to limit the protective effect of neutralizing antibodies is the emergence of neutralization escape mutants [90]. Richman and colleagues have shown that most patients with primary HIV infection (0–39

months) developed significant neutralizing antibody responses against the autologous virus; however, neutralizing responses against the laboratory-adapted isolates as well as heterologous primary isolates are slow to develop and often low in titer [145]. The appearance of neutralization-resistant mutants indicates that neutralizing antibody responses have a negative effect on the viral replication during HIV infection. Thus, it appears that the virus is under intense selection pressure to mutate neutralizing epitopes without compromising functional aspects of the Env, such as binding to the receptor and co-receptor to evade immune pressure. This adds additional constraints in development of an effective anti-HIV vaccine. Despite this selective pressure virus replication proceeds unhindered in infected individuals. Thus, HIV Env can tolerate multiple mutations and most of these mutations are positioned in variable regions that contain several glycosylation sites. In general, the number of glycosylation sites in Env glycoprotein is relatively conserved across subtypes and isolates, suggesting a pivotal role for carbohydrates in Env function and structural integrity. This extensive glycosylation provides the virus with some flexibility to modify neutralizing epitopes to evade the immune pressure simply by altering the glycosylation profile (adding, deleting, or changing the position), without perturbing the secondary structure [192].

6.5 CURRENT STRATEGIES IN DESIGNING IMMUNOGENS TO INDUCE BROADLY NEUTRALIZING ANTIBODIES

Some of these challenges raised in the previous section can be addressed by using structural information such as (a) selectively present the conserved determinants of complex and variable antigens, (b) understand conserved architecture vs. variable façade to allow more rapid adjustment to antigenic variation, (c) identify parts of the protein that induces protective vs. disease-enhancing immunity, and (d) selectively use components of protein that are determinants of protection. These challenges are further intensified in case of HIV due to higher level of antigenic diversity and lack of clear understanding of the correlate of protection. From the available data, it is evident that Env monomer (gp120) contains neutralizing epitopes and that antibodies directed against these epitopes can protect against infection. However, the gp120 monomer has been relatively ineffective at eliciting cross clade neutralizing antibody responses. In addition, gp120 monomer formulated with alum was evaluated in a phase III efficacy trial as a protein-based vaccine. However, there was no difference in infectivity rate in individuals who received the vaccine compared to the placebo group [62, 66, 68]. The ability of this vaccine to raise functional antibody responses such as neutralizing Abs and ADCC has yet to be fully evaluated, but the information gained may help to guide further vaccine development. However, a recently concluded vector prime and protein boost (monomer) efficacy trial in Thailand demonstrated a 31% efficacy postinfection. These results rejuvenated the interest in the HIV vaccine and renewed efforts are being focused on developing HIV vaccine. It will also be critical to understand the barriers in inducing antibodies that recognize similar epitopes to those recognized by 2F5, 4E10, Z13, F105, 2G12, and b12.

Several strategies are being evaluated by various investigators to develop and evaluate novel Env immunogens to induce broadly neutralizing antibody responses by

vaccination. These strategies targeting the conserved neutralization epitopes involve several parallel but complementary approaches such as reducing complex, multidomain proteins to efficiently express neutralization domains, trim or delete variable loops to focus the responses on the conserved epitopes, eliminate sugars that are not essential for folding but may be hiding the conserved functional epitopes, alter exposed hydrophobic surfaces or protease sites, stabilize the antigen in desired conformations by engineering disulfides at appropriate positions, reinforce hydrophobic cores, and restrain with oligomerizing fusion partners. Some of these concepts have been used to design better immunogens for HIV, and in this review we will be discussing these strategies applied on (i) gp120, (ii) gp41, and (iii) soluble form of the trimer.

6.5.1 Trimer: Soluble Trimer Mimicking Native Viral Spike

6.5.1.1 Structure of Viral Spike Relevant to Antigen Design. While the efforts were directed to solve the crystal structure of the Env trimer, and addressing the technical challenges associated with it, two groups have applied a low-resolution techniques, that is, Cryo-EM, to circumvent the problems associated with crystallization and provide the structural information on the Env trimer [206, 209]. Zulletti et al. have two best-fit Cryo-EM three-dimensional structures, and both these structures suggest that (i) all the glycans are pointing outward from the surface of the trimer and solvent is exposed, which is consistent to the glycan shield model proposed by different investigators to protect the virus against the neutralizing antibodies [111, 192]; (ii) the variable regions V4 and V5 are positioned on the top surface of the complex, an orientation that would facilitate their recognition by antibodies [44]; (iii) the V1–V2 loop extends outward, implying good solvent exposure; in addition it is possible that the interaction of V1–V2 loop and the co-receptor binding site in the monomer may limit the access of co-receptor binding site to antibodies; (iv) the CD4 binding surface is exposed on the outer edge of each gp120 protomer and is orientated such that access to membrane-associated CD4 on the target cell is possible; and (v) the two models differ significantly with regard to the orientation and exposure of the V3 loop and the orientation of co-receptor binding sites. In the first model, the V3 base points toward the trimer interface run roughly parallel to the threefold symmetry axis, being partially exposed in the cavities between the lobes. In this trimer model, the V3 loop would be substantially masked by packing into the trimer axis, potentially reinforced by inter-V3 bonding. In the second model, the V3 loop is oriented outward in the solvent-exposed phase. In addition, V3 seems to be highly flexible in the CD4-unbound trimer. After Env binds to CD4, it induces a conformational change in Env that increases V3 exposure. This is supported experimentally by the increased accessibility of the V3 loop in the Env trimer to antibody binding and enzymatic proteolysis subsequent to CD4 engagement [151, 182]. It is also consistent with the highly exposed nature of the V3 loop in the structure of the V3-containing gp120–CD4–Fab complex [87]. The increased exposure of V3 in the CD4-bound conformation could be explained by a rearrangement of the trimer in the first model, or by a conformational change involving the V3 loop in the second model.

The conserved co-receptor binding site, comprising the bridging sheet [37] and associated regions, is thought to be largely inaccessible in the trimer. Experimental data

support this concept since most CD4-induced (CD4i) surface-specific mAbs cannot access their epitopes on the CD4-unliganded Env trimer, as evidenced by weak or absent neutralization. It is possible that the V3 loop may be involved in protecting the co-receptor binding sites from antibody recognition and binding.

A trimer model has been proposed previously in which the inner domain of gp120 points toward the gp120–gp41 interface and the outer domain extends outward [37]. Such a model is inconsistent with the orientation of the major axis of the ellipsoidal gp120 density within the density corresponding to gp120 in our structure. In this previous model, the V3 loop is exposed to solvent and the co-receptor binding site is buried at the trimer interface. The two fittings proposed by Zanetti et al. clearly suggest that either the V3 loop is also pointing toward the trimer interface or the co-receptor binding site is on the outside of the trimer, and protected from solvent by V3 [206]. It is likely that the Env complex exhibits some conformational flexibility.

In the context of trimeric Env, the gp41 stem appears as a compact structure with no obvious separation between the three monomers [206]. The membrane proximal region of gp41 is characterized by a highly conserved hydrophobic region, which is thought to mediate trimer self-assembly [147]. Furthermore, the density corresponding to the gp41 stem region is shorter and slightly wider than the postactivation coiled-coil conformation [29], in agreement with the hypothesis that a dramatic conformational change is required for gp41 to extend toward, and insert into, the target cell membrane. The shape of the complex suggests a new model for the Env trimer organization. The volumes are consistent with the assignment of the globular domains to gp120 and the stem to gp41. The gp120 protomers appear to fold over gp41 rather than depart radially from it, contacting each other at the top of the spike. The interaction between gp120 monomers at this contact is likely to be weak, to allow the disassembly of Env ectodomain and extrusion of gp41 for insertion into the host membrane for the membrane fusion to take place.

The two models proposed shed light on several important antigenic and mechanistic features of the Env trimer. First, they provide models consistent with the concept that glycans and immunodominant variable loops are positioned on exposed gp120 surfaces to damp the neutralizing antibody response. Second, they confirm that only a limited gp41 surface is exposed for antibody binding, as has been proposed previously [30]. Third, they provide two possible descriptions of the position of the V3 loop and the co-receptor binding.

6.5.1.2 Trimmers as Immunogen. The primary focus to developing novel immunogens has been on designing Env in trimeric conformation. Since the Env on the surface of the virion is present as a trimer, therefore, it makes sense to focus on trimeric Env and evaluate them for their ability to induce the breadth and potency of functional antibody responses. The observation that most broadly reactive neutralizing antibodies isolated so far (i.e., IgG1b12, 2G12, and 2F5) have a stronger affinity for trimeric envelope than for monomeric gp120 or gp41 provided [65, 146, 150] further impetus to evaluate trimeric Envs as immunogens. Early immunogenicity studies demonstrated that antibodies induced by oligomeric Env cross reacted with HIV Env of other subtypes and neutralized both T-cell-adapted and some neutralization sensitive primary HIV-1 isolates [186]. To use the trimeric Env as an immunogen to

elicit functional antibody responses, first challenge is to stabilize the noncovalently associated subunits of Env in the trimeric conformation without compromising the structural integrity of the molecule. Several approaches have been tried with reasonable success [19, 23, 54–56, 60, 82, 148, 149, 158, 164, 165, 202]. A schematic of the three main approaches that were tried for stabilizing Env in a trimeric conformation are presented in Figure 6.7. These approaches are (a) mutate the protease cleavage site between the gp120 and gp41; (b) mutate the protease cleavage site and also use trimerization motifs from proteins such as GCN4 (General Control Nondepressible) transcriptional activator protein from yeast or T4 (fibrin protein from T4 phage); and (c) leave the protease cleavage site intact and link gp120 and gp41 by disulfide bridge. Yang et al. used GCN4-stabilized oligomers to demonstrate that antibodies induced by oligomeric gp140 were more effective at neutralizing heterologous primary isolates, compared to antibodies elicited by the corresponding monomeric gp120 protein [204]. Earl et al. made similar observations in Rhesus macaques using a trimeric Env protein from the simian immunodeficiency virus (SIV) [56]. We reported the purification and characterization of stable trimeric Env protein from US4 (subtype B R5 isolates) [165]. We also purified and characterized trimeric protein from SF162 (subtype B) and demonstrated that it elicited strong antibody responses in rabbits, which neutralized two out of six heterologous subtype B primary isolates [11]. Other groups have purified and characterized cleaved trimers (SOSIP), Fig. 6.7E by crosslinking gp120 and

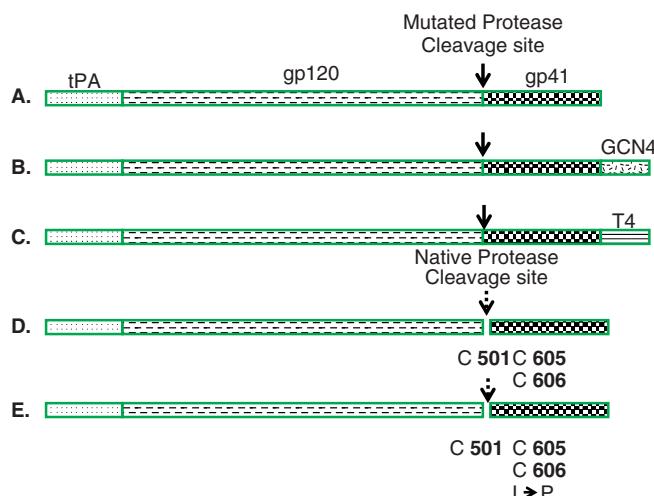


Figure 6.7. A schematic representation of three main approaches attempted to stabilize the trimeric conformation of HIV-1 envelope glycoprotein. These include 1. Mutating the natural cleavage site between gp120 and gp41, (A-C), or 2. Combing the cleavage site mutants with C-terminal trimerization motifs from proteins such as GCN4 (General Control Nondepressible) transcriptional activator protein from yeast, (B), or T4 (fibrin protein from T4 phage), (C), or 3. Insertion of artificial disulfide bond between gp120 and gp41 while maintaining the natural cleavages site, (D and E).

gp41 by disulfides. They have reported that SOSIP trimers compared to gp120 elicited approximately threefold lower titers of anti-gp120 antibodies. In the prime-boost regimen, these trimers induced antibodies that neutralized sensitive HIV-1 primary strains. In essence, both cleaved and uncleaved trimers demonstrated incremental improvement over monomeric Env in their ability to induce cross-clade neutralizing antibody responses. To further enhance the immunogenicity of trimeric Env, we have introduced a partial deletion of the V2 loop and expressed, purified, and characterized this novel Env immunogen [164]. Using biophysical, biochemical, and immunological techniques, we demonstrated that the purified Env was a trimer, had nanomolar affinity to CD4, and had critical neutralizing epitopes exposed and preserved on the trimer [164]. Deletion of the V2 loop qualitatively altered the immunogenicity of the V3 and V1 loops and rendered the C5 region more immunogenic [166]. The V2 loop deletion in the context of a trimer elicited strong antibodies in rabbits that neutralized five out of six heterologous subtype B primary isolates and conferred partial protection upon pathogenic challenge with SHIVSF162P4 to Rhesus macaques immunized with this V2 loop deleted trimer in a DNA (deoxyribonucleic acid) prime and protein boost strategy [40, 41]. These animals were followed for more than 3 years without any sign of disease. In addition, we demonstrated a reasonable correlation between the presence of neutralizing antibodies at the time of challenge in the vaccinated animals and the levels of plasma viremia during acute infection [26]. This protein is currently being evaluated in a human clinical trial (HVTN049). In a separate study, Quinnan and colleagues have evaluated the efficacy of oligomeric Env for inducing broadly neutralizing antibodies [143]. They have primed a group of Rhesus macaques with alphavirus replicon particles expressing the Env from R2 strain, and later these animals were boosted with the soluble oligomeric gp140. Concurrently, animals were also immunized with SIV *gag/pol* genes or no SIV genes to determine the additional protective benefit of inducing cell-mediated immune responses. The antibodies induced by alpha virus prime and oligomeric protein boost neutralized diverse primary isolates in vitro. The immunized animals were protected against the challenge infection with SHIVDH12 (Clone7). Furthermore the protection was associated with significantly high antibody titers and cellular immune response. We performed a challenge study in Rhesus macaques using alpha viruses expressing HIV Env as prime followed by adjuvanted trimeric Env protein administered intramuscularly as a boost. In the same study, we evaluated different routes of immunization for alpha priming such as intranasal (IN), intrarectal (IR), and intramuscular (IM). All the six animals that were IM primed and IM boosted were protected against the challenge infection. Four out of six animals had sterilizing immunity. Four out of six animals that were primed intranasally and boosted intramuscularly were partially protected against the challenge infection. In the IR-primed and IM-boosted group, only one-sixth of the animals animals had a significant delay in the onset of infection; however, the course of the disease remained unmodified [10]. In proof of concept studies with subtype C Env in rabbits and Rhesus macaques, we have demonstrated that DNA vaccines encoding for TV1 Env strain can effectively prime for humoral responses against both subtype B and subtype C primary R5-tropic HIV-1 isolates in rabbits and Rhesus macaques. Priming rabbits with DNA plasmids encoding TV1 gp140 modified in the second

hypervariable loop (gp140TV1 Δ V2), followed by boosting with oligomeric TV1 Δ V2 proteins, elicited more potent HIV neutralizing antibody responses than DNA vaccines encoding TV1 gp160 or the intact TV1 gp140. Boosting with oligomeric TV1 Δ V2 envelope proteins in MF59 adjuvant also elicited higher titers of antibodies with homologous neutralizing activity against TV1 and heterologous neutralizing activity against the subtype B SF162 primary isolate. In addition, combining subtype B and C V2-deleted immunogens resulted in an increased cross-clade neutralizing activity. Our results demonstrate that the modified subtype C gp140TV1 Δ V2 immunogen described herein elicits broad, subtype B and subtype C neutralizing antibodies [99]. In order to compare between the two immunogens derived from two different strains immunochemical studies were performed to demonstrate that the subtype C Δ V2 trimer binds to CD4 at an affinity similar to subtype B Δ V2 trimers, and its glycosylation profile is comparable to subtype B Δ V2 trimer. However, there are differences between the mAbs binding profiles for subtype B and C Δ V2 trimers, and that could cause differences in induction of the quality of immune responses [163].

6.5.2 Gp120: Design of Immunogens Targeting Conserved Binding Sites

6.5.2.1 Triggered Env: Conserved Sites Exposed upon Receptor Binding.

Another approach is to target conformational epitopes in Env that are induced upon interaction with CD4 for inducing cross-clade neutralizing antibodies. The Env protein upon binding to CD4 undergoes significant conformational changes [126, 132], which exposes co-receptor binding site, which is critical for membrane fusion and viral entry in the target cells [132]. Furthermore, mAbs such as 17b, 48d, 23e, 49e, 21c, E51, CG10, and X5 better recognize Env in CD4-bound conformation (triggered Env) [67, 124, 177, 197, 198]. Attempts were made by various groups to evaluate triggered Env (gp120–CD4 complexes) as potential vaccine candidates [50, 67]. It was demonstrated that gp120–CD4 complexes can induce broadly neutralizing antibody responses, but they need to be stabilized using crosslinking reagents [50]. In a proof of concept study, we have demonstrated that triggered Env (gp120–CD4 complexes) induced strong immune responses against both gp120 and CD4 [166], and these antibodies neutralized both a primary isolate (SF162) as well as a T-cell-adapted isolate (SF2) [162]. To elucidate the contribution of anti-Env antibodies to neutralization, we affinity purified the anti-Env antibodies from anti-CD4 antibodies and showed that these antibodies neutralized two subtype B and one subtype C primary HIV-1 isolates that were tested [162]. Since gp120 SF2 alone was not able to induce primary isolate neutralizing antibodies [11], these preliminary data suggested that certain neutralizing epitopes might be better exposed on triggered Env compared to untriggered gp120.

These studies illustrate the potential utility of this approach; however, the use of full-length CD4 raises the potential for induction of autoimmune responses. One strategy to overcome the use of CD4 for making Env complex is to identify the minimal Env binding domain of CD4 and use that for making the complexes. The reported crystallographic structure of gp120, in complex with CD4 and the Fab portion of mAb 17b [94], has demonstrated that a large surface (742 Å) of the domain D1 of

CD4 binds to a large depression (800 Å) on gp120. This CD4 interface is composed of 22 residues, contributing to gp120 binding with mixed hydrophobic, electrostatic, and H-bonding interactions. The large size and complexity of this interface makes the reproduction of such a functional domain into a small nonimmunogenic molecule a challenge, and highlights the difficulty in developing high-affinity CD4 mimics. However, despite the large number of residues present in gp120–CD4 interaction, studies on hormone–receptor systems have shown that few residues might dominate the binding energy at a protein–protein interface [42]. Thus, the design of a minimal CD4 mimics may be possible. The transfer of functional sites to small proteins acting as structural scaffolds has been proposed as a useful strategy to reproduce the structure and function of the target protein in small molecular systems [187, 189]. This approach has led to the discovery of scorpion toxin Scyllatoxin fragment as an effective mimic of CD4. A miniprotein CD4M3, was chemically synthesized, folded efficiently, and presented a circular dichroism spectrum similar to that of native Scyllatoxin. In competitive enzyme-linked immunosorbent assay (ELISA), CD4M3 bound specifically to gp120 at an IC₅₀ (50% inhibitory concentration) of 40 μM, which are four orders of magnitude higher than that of sCD4. This strategy has been recently applied to the engineering of a miniprotein that mimics the core of the CD4 protein surface, which interacts with the gp120 envelope glycoprotein of HIV-1 and, hence, inhibits virus attachment to cells and infection [188].

The biological performance of this miniprotein was further improved using “rational” structure design. In total, five substitutions were introduced (Gln20Ala, Thr25Ala, Leu18Lys, Ser9Arg, and Pro28) and the resulting miniprotein (CD4M9) bound to gp120 at 400 nM induced conformational changes in Env and inhibited infection of different virus isolates to CD4+ target cells. So far, the most improved CD4 miniprotein is CD4M33, which has an affinity for gp120 similar to CD4 [107] and induces conformational change in gp120 similar to that induced by sCD4 [107]. In an earlier study Fout and colleagues have shown that covalently crosslinked complexes of CD4 and HIV Env IIIB induced antibodies that neutralized a wide range of primary isolates [63]. Ig with neutralizing activity was recovered by affinity chromatography using Env/CD4M9 single-chain polypeptide. More recently, using CD4M9, an earlier version of the CD4 miniprotein developed by Vita and colleagues, Fout et al. prepared single-chain constructs of SCBal/M9 and performed immunogenicity studies in rabbits [64]. They showed that antibodies induced by Env–CD4M9 crosslinked complexes neutralized a broad range of primary isolates. These results demonstrate that (i) a significant portion of gp120 binding surface of CD4 can be reproduced in a miniprotein system, (ii) an engineered CD4 miniprotein contains sufficient CD4 structural elements to induce gp120 conformational changes, and (iii) these surrogate molecules may be useful in making stable complexes with envelope protein to expose envelope epitopes for the induction of neutralizing antibodies.

In another approach, Guo et al. described a small synthetic molecule (termed BMS 378806) that inhibited the interaction of gp120 with cellular CD4 and prevented viral entry [80]. This compound was bound to gp120 at a stoichiometry of approximately 1:1 with an affinity similar to that of CD4. Therefore, a molecule such as this should be evaluated for its ability to induce conformational changes in Env.

6.5.3 Rational Design to Enhance Presentation of Conserved Sites

6.5.3.1 Structure of gp120 Relevant for Rational Antigen Design. A crystal structure of HIV-1 gp120 core in complex with two N-terminal domains of CD4 and a Fab fragment of mAb 17b that binds CD4-induced co-receptor binding site was solved by Kwong et al. [93], and it is known as a liganded structure. The structure was determined using the gp120 that lacked the V1/V2 and V3 loops as well as 52 and 19 residues from the N- and C-termini. The structure provided some basic but very important information about the organization of gp120. The protein is comprised of two domains, the inner and the outer, which are bridged by a minidomain called “bridging sheet,” a four-stranded, antiparallel (β sheet). The inner domain is buried internally in the gp120–gp41 trimeric complex; therefore, as expected, functional antibodies against this region are not induced during the natural course of infection. The outer domain is extensively glycosylated and does not elicit antibody response, therefore, termed as immunologically silent. The receptor binding site, that is, CD4 binding pocket is located in a region that borders the outer and inner domains, and the bridging sheet. More recently, crystal structures of HIV-1 gp120 with intact V3 loop have been solved [87]. It appears that the V3 loop forms a rod-like structure that protrudes out from the gp120 core. This is not surprising considering its highly immunogenic properties.

The crystal structure of SIV gp120 in the unliganded state reveals a number of additional important facts. First, its overall structure is very similar to that of HIV-1 gp120, especially the outer domain of the protein [37]. Second, neither the CD4 nor the co-receptor binding sites (BS) are properly formed in the unliganded state. In the unliganded form, the two double-stranded antiparallel sheets are well separated and many residues that make up the CD4 BS are either masked or in different configuration compared to after binding CD4. Third, there is large displacement and rotation of the inner domain and bridging sheet components upon CD4 binding: (i) the three-strand antiparallel sheet of the inner domain rotates by 30°, (ii) the four-turn α -helix (type of secondary structure of protein that looks like a cork-screw) 1 shifts away from the outer domain, and (iii) the tip of the V1–V2 stem moves by over 400 Å. These events result in the formation of the bridging sheet. At present, the structure of the V1–V2 loop is not known. However, it is thought to partially mask the bridging sheet. Deeper insights into the native (i.e., untruncated) structures of gp120 could facilitate envelope-based vaccine design. Now the efforts are being made by different groups, including ours, to rationally design novel immunogens that may induce broadly neutralizing antibody responses. So far, the focus has been to optimize engineered Env structure for inducing potent antibody responses against conserved functional epitopes by structure-based targeted deglycosylation, stabilization by targeted insertion of disulfide bonds, hyperglycosylation, or by introducing mutations/deletions in the bridging sheet. In more recent study Zhou and co-workers designed and constructed stabilized gp120 molecules constrained to stay in the CD4-bound conformation in the absence of CD4 using stabilizing mutations. They measured the effect of the stabilization on the binding kinetics of CD4 and the b12 antibody and then determined the high-resolution structure of one of these gp120 stabilized variant in complex with b12 antibody. They found that b12 antibody binds to the same exposed surface initially recognized by CD4. The

main structural difference between b12 and CD4 interactions with gp120 involves the conformationally mobile β sheet 20/21. For b12, these interactions are peripheral to the binding surface as opposed to CD4 interactions with β -sheet 20/21, which form an integral part of the binding surface. Zhou and co-workers also compared between these two binding mechanism using kinetic studies and discovered that b12 is able to attach the binding surface with high affinity without undergoing any additional conformational changes in gp120 that are needed for the CD4 binding. This more flexible mechanism allows b12 to bind and neutralize primary isolates that otherwise would be protected by conformational masking [208].

6.5.3.2 Structure of gp120 Relevant for Rational Antigen Design. The extensive glycosylation of Env is likely to be involved in immune evasion. Based on crystal structure, it seems that outer domain of gp120 is heavily glycosylated as shown by Wyatt and colleagues [196] (Figure 6.5). Despite being most exposed to antibodies, this region of Env is known as “silent face” because it seldom induces neutralizing antibody responses. Thus, the sugar moieties may be shielding critical neutralization epitopes. Malenbaum et al. have demonstrated that the removal of glycosylation at position 301 resulted in an increased neutralizing sensitivity of HIV-1 to CD4 BS antibodies [105]. Furthermore, mutant virus lacking 301 glycan also demonstrated sensitivity to CD4i antibodies. It is apparent from the crystal structure that sugar moieties lie proximal to but not within the receptor binding site. In another study, elimination of N-linked glycosylation in the V1 and V2 loops of pathogenic SIV mac239 rendered the virus more sensitive to host antibody responses [144]. Koch and colleagues have performed structure-based deglycosylation of four sites flanking the receptor-binding region (i.e., 197, 276, 301, and 386). Removal of a single glycosylation site at the base of the V3 loop (i.e., aa 301) has rendered the mutant virus more sensitive to antibody-mediated neutralization by anti-CD4 binding site antibodies. Furthermore, deletion of all four glycosylation sites has made the resultant virus sensitive to neutralization by CD4i antibodies. In an other study, McCaffrey et al. [114] have demonstrated that removal of sugar moieties at positions 293, 299, 329 and also 438 and 454 made the SF162 virus more sensitive to neutralization by CD4BS mAbs and also to V3-loop-specific neutralizing mAbs. In addition, deglycosylation of V3 loop (293, 299, and 329) also made the mutant virus more sensitive to mAbs specific to CD4i epitope. Furthermore, the same group has also demonstrated that deglycosylations at positions 293 (V3 loop), 438 (C4 region), and 454 (V5 loop) also rendered the resultant virus more susceptible to the anti-gp41 mAb 2F5. In a more recent study Li and co-workers studied the effect of deglycosylation at position 197 where they have changed an N to Q. This site is located in the carboxy-terminal stem of the V2 loop. This mutant virtually showed the most pronounced increase in its sensitivity to broadly neutralizing antibodies, including those targeting the CD4 binding site and the V3 loop. In addition, the mutant virus was also sensitive to CD4-induced antibody 17b in the absence of CD4. Furthermore, sera from pig-tailed macaques immunized with mutant N7 Env had significantly higher and broader neutralizing activities than sera from WT to panel of subtype B primary isolates [97].

All of these studies suggest that carbohydrates at these positions protect epitopes located in CD4 binding site, V3 loop, co-receptor binding site, and also in gp41 [114].

Further studies will be needed to evaluate the efficacy of this approach in exposing critical neutralizing epitopes in Env immunogens.

6.5.3.3 Immune Focusing by Hyperglycosylation of Nonneutralizing Epitopes.

Burton and colleagues have taken the opposite approach of incorporating additional carbohydrate residues. They have demonstrated that four alanine substitutions on the perimeter of the Phe43 cavity of gp120 reduced the binding of weakly neutralizing CD4 binding site antibodies to gp120 [133], while increasing the binding of a potent broadly neutralizing antibody. In further studies, they focused on the reduction of binding of a wide range of nonneutralizing antibodies by incorporating seven more glycosylation sites in addition to the four alanine mutations [133]. It was interesting to note that these hyperglycosylated Env proteins were not recognized by nonneutralizing antibodies directed toward CD4 BS (such as b3, b6, CD4-IgG2, 15e, F91, F105), however, binding of the neutralizing antibody b12 remained, albeit at lower affinity, largely unaffected. Furthermore, hyperglycosylation affected the exposure of conformational epitopes recognized by mAbs 17b, 48d, and X5. It remains to be determined whether these modified molecules can alter the immunogenicity of Env and, in particular, if the increased b12 reactivity can be correlated with enhanced neutralizing activity.

6.5.3.4 Enhancing Exposure of Receptor Binding Site by Targeted Deletion of Molecule.

It has been difficult to induce antibody responses directed against the CD4 binding site by vaccination with gp120. From gp120 structure analysis, it is apparent that Env is folded into inner and outer domains (Fig. 6.8). The inner domain (with respect to the N and C terminus) comprises two helices, a small five-stranded β sheet sandwiched at its proximal end and a projection at the distal end from which the V1–V2 loop emanates. The outer domain is a stalked double barrel that lies alongside the inner domain such that the outer barrel and inner bundle axes are approximately parallel. The gp120 inner and outer domains are attached by a bridging sheet that further limits the accessibility of the CD4 binding pocket. This bridging sheet is composed of four antiparallel β strands (namely β -2, β -3, β -20, and β -21) (Fig. 6.8). The CD4 binding site is buried deep between the inner and outer domains of this molecule and, hence, possibly not accessible to antibodies. Structural data suggests that the V2 loop may fold over the bridging sheet and, due to its three-dimensional position, the bridging sheet is believed to mask the elements involved in CD4 binding and co-receptor binding. It has been shown that the deletion of V1 or V1–V2 loops does not abrogate the functional activities of the envelope glycoprotein because the recombinant viruses are fully replication competent in human PBMC [168]. Furthermore, these deletions in the context of Env protein also did not alter its ability to bind sCD4 [164, 167]. This suggests that these modifications alone preserve the integrity of important conserved epitopes on Env. Furthermore, deletion of V1 and V2 loops has made the virus more susceptible to antibody-mediated neutralization, however, no improvement was observed in terms of directing the immune response to the CD4 binding site [154, 164]. Therefore, we introduced additional deletions in the bridging sheet in an attempt to sufficiently expose the CD4 binding site without destroying the proper folding and conformation of Env. As mentioned above, there

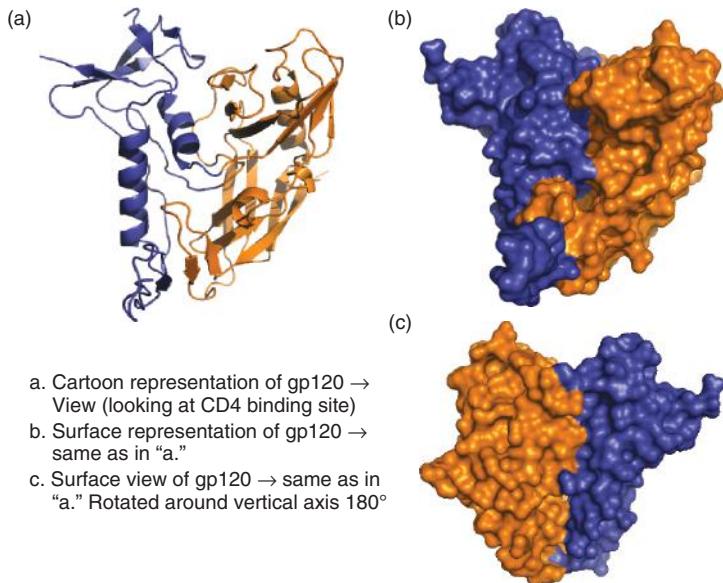


Figure 6.8. (a) Cartoon representation of gp120 → view (looking at CD4 binding site). (b) Surface representation of gp120 → same as in (a). (c) Surface view of gp120 → same as in (a). Rotated around vertical axis 180°.

are four β strands: β -2 strand corresponds to approximately amino acid residues Cys-119 to Thr-123, β -3 corresponds to approximately Ser199 to Ile-201, β -20 extends from amino acid residues 422-Gln to 426-Met, and β -21 extends from amino acid residues 431-Gly to 435-Tyr relative to HXB-2. The V1 and V2 loops emanate from the first pair of β strands (Cys-126 to Cys-196) and a small loop extrudes from the second set of β strands. This small loop extends from amino acid Trp-427 to Val-430. The H-bonds between β 2 and β 21 are the only connections between domains of the lower half of the protein (joining helix α -1 to the CD4 binding site). Based on these structural features, we have designed a series of deletions within the small loop of the bridging sheet to further expose the CD4 binding site. In addition, we are evaluating deletions in the large loop (i.e., V1 and V2 loops) either alone or in conjunction with deletions in the small loop to enhance the exposure of conserved neutralization epitopes that are shielded from the immune system (unpublished observations).

Gp41: Rational Design of gp41 Immunogens

6.5.3.4.1 STRUCTURE OF GP41 RELEVANT TO RATIONAL ANTIGEN DESIGN. The glycoprotein gp41 contains several functional domains including (1) fusion peptide (FP); (2) two heptad leucine/isoleucine repeats called HR1 (N-helix) and HR2 (C-helix) that fold into the core structure of gp41; (3) C-terminal membrane proximal external region (MPER); (4) transmembrane domain (TM); and (5) cytoplasmic tail (CT) (Fig. 4a). Extensive high-resolution structural studies are performed to fully understand the nature of the fusion mechanism. Different components of gp41 have

been described at the atomic level such as (1) the core of both HIV and SIV gp41 glycoprotein (HR1 and HR2) [29, 35, 176, 194]. (2) A variety of gp41 subpeptides [21, 88, 98, 157]; and (3) gp41 peptides in complex with antibodies (2F5 and 4E10) [33, 129]. The three-dimensional structure of the HIV-1 gp41 core, composed of HR1 and HR2 [N36 (gp41546-581) and C34 (gp41628-661) form HXB2 strain] was solved by X-ray crystallography [35, 175, 194]. The core structure of the SIV gp41 was also solved by multidimensional nuclear magnetic resonance (NMR) [29]. All structures show that the gp41 core is a six-helix bundle exhibiting structural similarity to fusion proteins of other enveloped viruses from different families such as Ebola, SARS, Flu, and paramyxovirus SV5 (Fig. 6.8) [35, 58, 102, 103, 106, 130, 193, 201]. The gp41 core is a six-helix bundle in which the N and C helices are arranged into three hairpins. The N peptides form three central helices arranged in a trimeric coiled coil, and the C peptides form three outer helices that pack in an antiparallel manner into highly conserved, hydrophobic grooves on the surface of this coiled coil (Fig. 6.9).

This six-helix bundle is assumed to be in postfusion conformation of gp41 [36]. However, the crystal structures of the gp41 core lacks both the N and C terminal regions (MPER) of the gp41 ectodomain, as well as the long loop connecting the N and C helices. The NMR structure of SIV gp41 contains the connecting loop and the core, but also lacks the N and C terminal regions. Therefore, better understanding of gp41 structure in all of its conformational states will be a valuable contribution to the field, and also will facilitate better immunogen design.

To better understand the binding mechanism of 2F5 to gp41 de Rosny and co-workers had studied the receptor-induced conformation of gp41 and showed that 2F5 binds to HIV Env in both the native and the fusion-intermediate conformations [48].

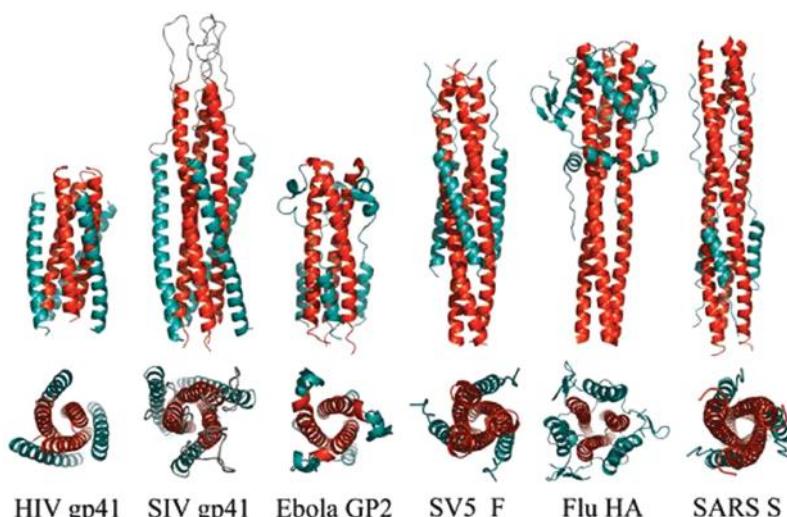


Figure 6.9. The structure of six-helix-bundle from different virus including HIV, SIV, Ebola, SV5 (Simian Virus 5), Flu (Influenza) and SARS (Severe Acute Respiratory Syndrome Virus).

These findings indicate that the 2F5 epitope is exposed in the prefusogenic form of gp41, before the formation of the six-helix bundle and is occluded or disrupted in the postfusion form of gp41 (when the six-helix bundle is formed). Further support for this finding is provided by Gorny and Zolla-Pazner. They have shown that 2F5 binding to the C peptide is inhibited by the addition of the N peptide, which forms a complex with the C [76]. Therefore, the structure of gp41 in the prefusion form could be the most relevant structure for vaccine development.

Moreover, there is evidence that exposure of the 2F5 epitope is inhibited by CD4 binding to gp120 [152], thus reducing, but not eliminating, the neutralization properties of 2F5 [127]. This is an additional support to the fact that 2F5 better binds to the prefusion or the fusion-intermediate conformations of gp41.

6.5.3.4.2 RATIONAL DESIGN OF GP41 IMMUNOGENS. Many efforts focused on generating immunogens that can induce 2F5-like mAbs. Immunization using linear peptide immunogens composed of the 2F5 epitope in different contexts failed to elicit neutralizing antibodies against diverse primary HIV-1 isolates [115, 160, 199]. Additionally, different recombinant protein immunogens displaying constrained 2F5 epitopes in the context of various protein scaffolds, for example, (1) HIV gp120 loops [100], (2) MalE protein [43], (3) conjugated with a carrier peptide (KGGG)(7)-K (K/G) [39], (4) recombinant surface antigen of hepatitis B virus [57], (5) human leukocyte antigen (HLA)-DR [86], (6) antigenic site B of influenza virus hemagglutinin [16], and (7) hepatitis B surface antigen (HBsAg) S1 protein that forms nanoparticles [137]. Most of these strategies induced high serum antibody titers against 2F5 epitope peptides, but these antibodies failed to elicit mAbs against primary HIV-1 isolates.

To further evaluate the immunogenic properties of gp41, Penn-Nicholson and co-workers constructed five soluble glutathione S-transferase fusion proteins encompassing different length of the C-terminal gp41 ectodomain from the M group consensus envelope sequence [30, 64, 100, 142, or 172 (full-length) amino acids]. The results suggested that antibody responses against different regions of gp41 varied tremendously among individual patients. Moreover, patients with stronger antibody responses against MPER exhibited broader and more potent neutralizing activity [136].

An alternative strategy is to expose the gp41 epitopes in the prefusion state of gp41, given that this state is more relevant to mAb 2F5 binding [48, 61, 153]. Kim and co-workers have constructed three different gp41 variants in a prefusion state and presented these target epitopes in the MPER on the surface of HIV virus-like particles (VLP). Following immunization of guinea pigs with these VLPs presenting gp41, the immune sera contained high titer anti-VLP antibodies, but the specific anti-gp41 antibody responses were low with no neutralizing antibodies [91].

Despite all the efforts to date, viral epitopes for the known broadly neutralizing mAbs 2F5 and 4E10 appear to be poorly immunogenic in infected individuals, and it has been a challenge to induce antibodies of similar specificities by vaccination. One possible explanation derives from recent studies that found that the 2F5 and 4E10 are polyspecific autoantibodies reactive with the phospholipid cardiolipin (self-antigen), which probably helps the HIV to avoid the immune system by autoantigen mimicry mechanism to the conserved membrane-proximal epitopes [4, 83].

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7

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS FOR VACCINE APPLICATIONS

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7.1 PROTEIN EXPRESSION

The technology used in vaccine development in the past 20 years has witnessed several major advancements; part of the breakthrough innovation concerns structure and purity of antigens and how they are obtained. The classic approaches to vaccine development require production of the antigen by the isolation of the pathogen of interest, its inactivation or attenuation, followed by injection; usually, the injected material is processed through a purification step targeted to the enrichment of one specific antigen. Even though these approaches have a history of success and the vaccines obtained are largely considered to be safe, they can have one or more of the following limitations: (i) after inactivation or attenuation some of the pathogen could still be active, especially in a background of immunosuppressed individuals, presenting a small risk of infection or other adverse reactions due to vaccination; (ii) the antigen obtained after inactivation might not be optimally presented; (iii) the production of the antigen to be injected is cumbersome, requiring large facilities and growing large amounts of a pathogen and the need for high-level biological containment facilities; (iv) the purification of the target molecule may require complex protocols and an optimal antigen can be difficult and expensive to produce; (v) the purity of the antigen is not optimal, with the possibility of eliciting undesired side effects in vaccines; and finally (vi) only a small subset of antigens among many molecules expressed on the surface of pathogens are potential protective antigens and, therefore, the target for vaccine development. In addition to the above-mentioned limitations, at times it is not at all possible to obtain an optimal antigen by the application of classic approaches. To overcome these limitations three novel concepts—recombinant subunit vaccine, reverse vaccinology [1–4], and structural vaccinology [4, 5]—have been explored and are progressively gaining more momentum, all exploiting the major advancement registered during the last 20 years in molecular biology and related techniques for gene cloning and protein expression (Fig. 7.1). The outcome of any of these three approaches is the cloning of a gene of interest and its expression in a heterologous system, followed by purification and formulation of the target antigen. With *recombinant subunit antigen production* (Fig. 7.1b), the expression of the protein in an optimized system allows the production of larger amounts of target protein, with higher purity and typically at a lower cost and in a relatively safe biological environment compared to purification of the same proteins from the pathogen itself. *Reverse vaccinology* (Fig. 7.1c) consists in the bioinformatics analysis of the genome of a pathogen to explore, quickly and effectively, all the proteins expressed by a microorganism, their potential cellular localization, and their biochemical and biophysical properties evaluate their potential immunogenicity; positive hits obtained by the initial *in silico* screen can then be cloned and expressed and further characterized for their potential as vaccine candidates. *Structural vaccinology* (Fig. 7.1d) goes one step further: Availability of large amounts of protein allows exploring the three-dimensional structure of the antigen through crystallography, electron microscopy (EM), and nuclear magnetic resonance (NMR). With detailed structural information at hand, it is possible to approach the rational design of novel, optimized antigens that potentially will elicit a more potent immunogenic response target to the protective epitopes and with fewer side effects.

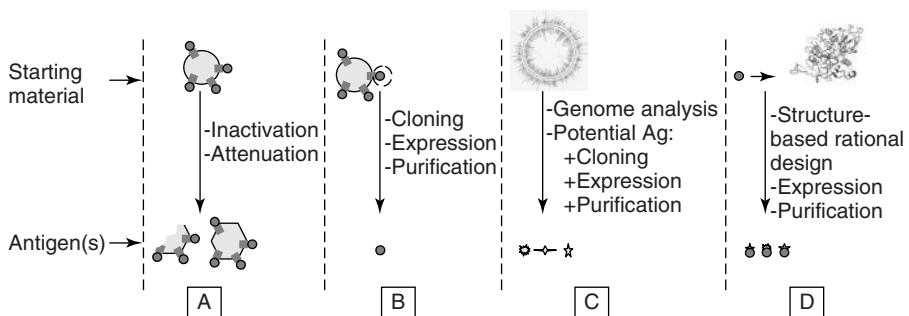


Figure 7.1. Scheme representing the classical approach for antigens production (a) and more advanced approaches (b-d) (a) The whole pathogen is isolated and inactivated, or it is produced in an attenuated version. (b) *Recombinant subunit antigens*: The molecule responsible for immunogenic response is identified, its gene is cloned, and the protein is overexpressed and purified. (c) *Reverse Vaccinology*: Starting from a pathogen's sequenced genome, many potential antigen candidates are identified, cloned, expressed, and purified. (d) *Structural Vaccinology*: This approach is aimed at improving known antigens [obtained through (b) or (c)] following rational design based on the three-dimensional structure of the protein.

Given the range of novel approaches very briefly described above, all requiring the production of relatively large (at the research scale) amounts of protein, it is easy to understand how the overexpression in heterologous systems and the purification of proteins have become increasingly important in the development of novel vaccines and for improving the efficacy of existing ones. In this chapter we will provide guidelines for selecting an expression system for the production of proteins to be used as antigens, and we will describe the techniques used for purification of the vaccine candidates.

The production of protein antigens for vaccine development faces, in addition to all the problems related with protein purification, a set of problems related to the source of the antigen and the downstream process in which the protein will be used: The proteins need to be of high purity, stable for long-term storage, formulated in an appropriate buffer that will also be compatible with adjuvants used in the immunization protocol, and should be suitable for injecting into animals. Protein preparations need to be endotoxin free, which requires an additional level of precaution during the purification process. Recently, the advent of structural vaccinology has further enhanced the requirement for extremely pure protein preparation suitable for crystallization and X-ray diffraction, for cryo-EM, or for NMR. An additional level of complication derives from the origin of the protein that is developed as an antigen: viral proteins exposed on the surface are generally heavily glycosylated, and it is important to optimize expression and purification to obtain correctly glycosylated and homogeneous protein preparation. At the same time, large amounts of proteins are required to carry out detailed characterization of the antigen and to obtain sufficient amount of material for preclinical and clinical studies.

As a consequence, a compromise between high-yield expression systems and expression systems providing the proper posttranslational modifications is required. In the following sections we will present the most common tools utilized for the expression and the purification of proteins to be used as vaccine target. We will highlight the specific requirements related to the expression of proteins for vaccines development in a context of large-scale manufacturing.

7.2 PROKARYOTIC PROTEIN EXPRESSION SYSTEMS AND EXPRESSION VECTORS

The scope of this section is to provide an overview of the many different expression vectors available; however, this is not a complete list of all the plasmids available on the market. In most plasmids used for overexpressing proteins in bacterial systems, the expression of the gene of interest is under the control of a very strong lac promoter, and synthesis of the protein of interest is induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG). In the most simple version the plasmid contains a gene encoding an antibiotic resistance, used for selection, and the target gene. More sophisticated evolutions of such plasmids contain sequences for N- or C-terminal tags (or both), N- or C-terminal fusion with proteins used to increase solubility of the target protein and simplify the purification through an affinity step; the tag can be removed by chemical catalysis or by proteolysis catalyzed by very specific proteases that recognize specific cleavage sites positioned between the protein of interest and the tag. After cleavage, an additional purification step is typically required to remove the tag itself and the protease. More specialized plasmids also allow the coexpression of genes; this is a potent and essential tool when the antigen of interest is part of a complex, and either it is not soluble without specific partner(s) or the antigen itself is formed only when the complex is fully formed.

Expression of a gene under the control of the lac promoter often allows the production of large amounts of proteins. Sometimes, the expressed protein has a toxic phenotype toward the host cell; in such a case, finely tunable plasmids can be used to obtain a stringent control of the level of protein being expressed and thus limit the toxicity and improve protein yield.

7.3 *ESCHERICHIA COLI* (*E. COLI*)

The expression of recombinant protein is widely performed in bacterial hosts, with *E. coli* being the most prominent. There are many advantages of using *E. coli*, among them are the well-known genetic information, fast growth of the cells, and high rates of protein production. Additionally, *E. coli* are easy to handle in standard laboratories, and the culture media are relatively inexpensive [6]. However, there are some inherent drawbacks with heterologous expression of target proteins in prokaryotic host cells such as *E. coli*, especially for target proteins of eukaryotic origin Table 7.1. Prokaryotes cannot perform the posttranslational modification steps (i.e., N- and O-linked glycosylation, fatty acid acylation, phosphorylation, and disulfide bond formation), which

TABLE 7.1. Summary of Prokaryotic and Eukaryotic Expression Systems

	Advantage	Disadvantage
<i>E. coli</i>	<ul style="list-style-type: none"> • High yield • Low cost • Ease of use 	<ul style="list-style-type: none"> • No posttranslational modifications • Misfolding possible
Yeast	<ul style="list-style-type: none"> • Ease of use similar to <i>E. coli</i> • Some posttranslational modification 	<ul style="list-style-type: none"> • Posttranslational modification different from mammalian
Insect	<ul style="list-style-type: none"> • Posttranslational modifications 	<ul style="list-style-type: none"> • Posttranslational modification different from mammalian
Mammalian	<ul style="list-style-type: none"> • Correct folding • Correct posttranslational modifications 	<ul style="list-style-type: none"> • More difficult to maintain • Higher cost • Lower yield
Cell free	<ul style="list-style-type: none"> • Very high yield • Shortest time • Ease of use • High throughput allows screening of many conditions • Self-induction 	<ul style="list-style-type: none"> • No posttranslational modifications • Not amenable for large scale

are often required for proper folding and functioning of the protein of interest [7]. The lack of correct posttranslational modifications and the proper eukaryotic chaperones can lead to the production of proteins in misfolded and aggregated state (inclusion bodies). Moreover, *E. coli* expressed proteins tend to retain the amino-terminal methionine, which could affect stability and produce immunogenicity [8, 9]. Also, many mammalian proteins are expressed at very low levels in *E. coli*, which can be explained by the usage of codons rarely used in eukaryotic genes and by the formation of secondary structures in the messenger ribonucleic acid (mRNA), especially in the 5' region.

One strategy for improving the yield of target proteins in *E. coli* is the use of fusion tags such as glutathione-*S*-transferase (GST), maltose binding protein (MBP), or N-utilizing substance A (NusA). Tags, preferably attached to the N-terminus of the protein of interest, can improve not only the yield but also the solubility of the protein of interest. Protein yield can also be improved by creating more favorable mRNA structures at the 5' end. Tags can also stabilize nonaggregated forms of the translated protein, thereby increasing solubility. The codon usage problem can be circumvented by transforming the host *E. coli* cells with a plasmid encoding limiting transfer RNA (tRNA) species, thereby increasing specific tRNA concentration. Usage of *E. coli* strains expressing sets of chaperones different from what is physiologically produced can increase the amount of properly folded expressed protein.

Other strategies have been used to increase the amount of soluble and active protein expressed in *E. coli*. For example, lowering the temperature during the induction phase from 37°C to around 20°C or adjusting the amount of the inducing agent, (such as IPTG), which is used to turn on gene translation in the host cell, can lead to a higher percentage of soluble protein, probably due to slower translation rate and decreased level of formation of aggregation-prone folding intermediates of the target

protein. To this purpose, *E. coli* strains able to efficiently grow at temperatures around 10°C were developed, overcoming the very long duplication time typically observed for bacteria not optimized to grow at lower temperatures. When the protein expressed within the cytosol interferes with cell metabolism, protein yield can be increased by expressing the target protein with a leader sequence, targeting it for secretion in the periplasmic space of the cell. This is an effective solution to remove the toxic protein from the cytosol with the additional advantage of expressing the target molecule in a compartment relatively poor in bacterial proteins, thus facilitating purification.

7.4 AUTOINDUCTION

Induction of protein expression by the addition of IPTG to an *E. coli* culture in logarithmic growth is one of the most commonly used approaches for obtaining soluble protein in bacteria expression systems. Recently, a protocol for autoinduction of protein expression in shaking culture at high cell density was developed by Studier [10, 11] (Fig. 7.2). Many plasmids currently used for the overexpression of exogenous genes use T7 RNA polymerase to transcribe genes under the control of the T7lac promoter. Standard protocols require the addition of IPTG to induce expression of T7 RNA polymerase, which in turn starts the transcription of the target gene. An alternative approach bypasses the requirement for IPTG addition for T7 polymerase induction, exploits the metabolic regulation of the T7lac promoter for the production of the target mRNA. This can be done by growing the bacterial culture at to high density therefore reduce the glucose level in the media. Glucose is known to inhibit transcription from T7lac, while an appropriate balance of lactose, amino acid concentration, and pH have the opposite effect. These observations allowed the definition of

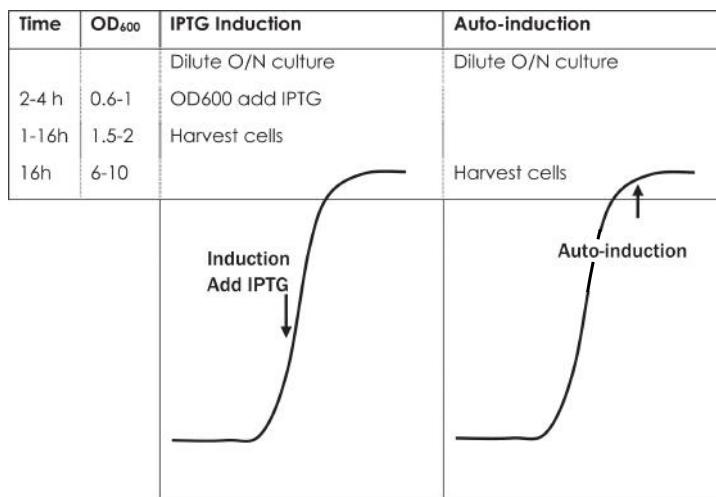


Figure 7.2. Schematic highlighting the potential advantage of auto-induction compared to IPTG induction.

growth media as either suppressor or inducer of protein expression high density [11]. This approach has several advantages compared to induction with IPTG: (1) Screening of constructs and conditions producing soluble proteins can be performed in a semi-automated set up; (2) under autoinducible condition, shaking bacterial culture will allow the cells to grow at much higher cell densities, which result in an overall higher protein yield; (3) many different conditions can be screened simultaneously; (4) growth conditions producing stable expression of large amounts of soluble protein can be further modified for the metabolic labeling of proteins for structural studies [10]; and (5) autoinduction has also an attractive economic advantage for expression of proteins at production scale because larger amounts of proteins can be produced at a lower cost.

7.5 EUKARYOTIC EXPRESSION SYSTEMS YEASTS

Yeasts are more robust compared to mammalian cells, provide high titers (often in the gram/liter ranges), and are easily adapted to fermentation processes in simple and cost-effective media. The system has been used extensively for the production of research-grade material for the preclinical studies for targets as malaria, Hepatitis B (Hep B), human papilloma virus (HPV), hanta virus, *Mycobacterium tuberculosis*, rabbit hemorrhagic disease virus (RHDV), and anthrax [12–20]. Generally, the protein expression and production were performed using *Pichia pastoris* [14, 16, 18, 21], *Saccharomyces cerevisiae* [17], and occasionally *Hansenula polymorpha* [13, 22]. The yeast system has been applied for the production of clinical-grade material for the malaria vaccine [23]. The purified protein was found to be stable for over a 1 year period, immunopotency did not diminish, and storage did not lead to alterations in the behavior of the protein upon formulation with adjuvant selected for phase 1 clinical study. Similar levels of stability, safety, and potency were reported for the Hep B vaccine produced in *H. polymorpha* and evaluated in phase 1 clinical trial [22]. Furthermore, HPV vaccine produced in yeast has been licensed for use in humans.

It has been a concern that the yeast system may not be suitable for the production of heavily glycosylated recombinant proteins or the proteins that need correct disulfide bond formation to attain their functional state. Toward this end, Miles and colleagues have shown that the malaria protein produced in the yeast seems to form correct disulfide bonds structures [21]. Barr and colleagues have expressed human immunodeficiency virus (HIV) Env proteins from gp120 ecto domain as well as from gp41 part of the proteins. These proteins were purified to homogeneity and evaluated in rabbits for their ability to induce antibodies. Antibodies raised in rabbits to env-2, a recombinant polypeptide representing the majority of the protein moiety of gp120, reacted with fully glycosylated native gp120 of HIV-SF2 virions. In addition, these env-2 antisera showed reactivity with viral gp120 of divergent HIV isolates. However, env-2 did not bind to CD4 (Srivastava and Barnett, personal communications). A 121 amino acid polypeptide (env-5), representing the region of gp41 stretching between the two hydrophobic domains of the protein, elicited antibodies in rabbits that reacted with glycosylated, native gp41. Thus,

selected domains of the HIV *env* gene expressed in genetically engineered yeast, are recognized by sera from HIV-infected humans, elicit antibodies that react with native HIV glycoproteins and provided a source of HIV envelope proteins as reagents.

Many of the therapeutic non-glycosylated therapeutic proteins are currently produced in *S. cerevisiae* and the methylotrophics *H. polymorpha* and *P. pastoris*, including insulin (and analog), growth hormone, hirudin (a leech-derived anticoagulant), and albumin. However, yeasts provide N- and O-linked high-mannose-type glycans that could be immunogenic in humans [24, 25], they are not well suited for the production of glycosylated proteins. Additionally, yeasts cannot perform tyrosine *O*-sulfation, a posttranslational modification that occurs in higher eukaryotes and in mammals [26] and has an impact on the functional characteristics of some of the proteins. For example, *O*-sulfation has been shown to increase hirudin affinity for thrombin by 10-fold compared to unsulfated analogs [27]. From the vaccine development point of view, it has been demonstrated that a sulfated tyrosine residue in the complementarity determining region (CDR) of a monoclonal antibody against HIV gp120 was crucial in antigen recognition [28, 29]. Therefore, in the present form the yeast system has limited application for the production of proteins, which need glycosylation and other posttranslational modifications for being effective either for therapeutic or vaccine application. However, more recently successful engineering of the glycosylation pathways in *P. pastoris* has allowed expression of recombinant proteins with human-type glycans [30], and high expression level of a glycoengineered monoclonal antibody was achieved [31]. *S. cerevisiae* was also genetically modified to provide O-fucosylation capabilities [32], a key modification essential for the bioactivity of many EGF domain-containing proteins such as Notch and urokinase-type plasminogen activator [33].

In summary, yeast combine the ease, simplicity, and cost-effectiveness of bacterial expression systems with the eukaryotic abilities of some posttranslational processing and secretion [34]. Therefore, yeast expression results in a product that is often similar to the native protein. Another advantage of using yeasts as the expression systems is the use of budding yeast *S. cerevisiae* in the production of bread and alcoholic beverages, resulting in a high level of confidence in the safety of the organism.

Despite these successes there remain some challenges with yeast as a host expression system of choice for viral proteins: During secretion, N-linked glycosylation occurs at the correct sites but often differs from the native pattern in that the carbohydrate chains are frequently of excessive length and are of the high-mannose type [35]. The resulting protein may, therefore, be immunogenic, which can limit the use of glycosylated proteins as human therapeutics. Alternative yeast expression systems (i.e., *P. pastoris*) allow to improve on the glycosylation limitations associated with *S. cerevisiae* overexpression and also allow to obtain more accurate folding for the expression of large proteins and overall to achieve higher yields [30, 36–38].

7.6 INSECT CELLS

The insect cell–baculovirus expression system is a powerful platform to rapidly produce high levels of recombinant proteins, viral-like particles, and pseudovirions

[39–41]. The main insect cell lines used are *Spodoptera frugiperda* (SF) 9 or 21 and *Trichoplusia ni* BTI 5B1-4 (High FiveTM). Efficient and robust production processes, able to deliver higher product concentrations, are, however, still needed to cope with increased requirements for large-scale manufacture. Carinhas and colleagues have used the combined experimental and modeling effort to quantify and environmentally manipulate the metabolism of *S. frugiperda* cells, targeting high cell density production of baculovirus vectors with potential application in human gene therapy. Culture medium was supplemented with pyruvate or α -ketoglutarate at the time of infection resulted in six- to seven-fold higher specific baculovirus yields at high cell density when compared to control cultures. Although this approach has been tried for the production of vectors, it has the potential to be effective for the production of recombinant proteins.

Baculovirus expression system has been used quite extensively for the production of vaccines either subunit-based vaccine or viral-like particles for targets such as HIV, SARS CoV, rabies, West Nile virus, HPV, and influenza [42–49]. It has been a concern that baculovirus system may not be useful for the expression and production of the recombinant proteins that need correct glycosylation because baculovirus does not add complex or hybrid carbohydrates. However, HIV Env protein is expressed in the baculovirus system, and the purified protein is evaluated for its ability to induce functional antibodies [50, 51]; the expressed protein is correctly folded, forms stable trimers, and induces strong neutralizing antibodies [50]. So, even though the system has obvious limitations, yet it is useful for the production of heavily glycosylated recombinant proteins. Furthermore, Cox and colleagues have produced the influenza vaccine in baculovirus and performed a phase I clinical trial [52]. It was very encouraging to observe that the vaccine was safe, well tolerated, and effective in inducing high titers of binding antibodies against Hemagglutinin (HA). Furthermore, these antibodies inhibited agglutination of red blood cells.

Due to the high-mannose and paucimannose type of glycosylation that is obtained in insect cells, no therapeutic protein is currently being produced in this system as this would compromise in vivo bioactivity and potentially induce allergenic reactions. Efforts are being made by different groups to engineer these cells with glycosyltransferases that will allow the production of proteins with mammalian-type sugars [53]. Insect cells also offer an interesting platform to produce vaccine antigens or virus-like particles, while engineered baculoviruses containing mammalian promoters (bacmam) show great potential as genetic vectors for mammalian cell transduction [40, 54]. One licensed HPV vaccine is composed of a truncated form of the major capsid L1 proteins of HPV types 16 and 18 expressed in High FiveTM cells (Cervarix1) and many more (e.g., Provenge, Flublok, Chimigen) are under development or in late-phase clinical trials.

Therefore in summary, the baculovirus expression system is a well-established workhorse for recombinant protein expression because it is relatively easy to handle. Infection by baculovirus is limited to insects because of its highly specific host range. Therefore, baculovirus is not harmful to humans, livestock, or poultry. The cultivation of the insect host cells requires only basic cell culture equipment compared to that used for mammalian cells, and the system is easily scalable from the milliliter to

100-L scale. The system can be operated under biosafety level 1, and in contrast to *E. coli* the insect host cells perform most posttranslational modifications required for the production of eukaryotic proteins. However, the type of glycosylation in insect cells is different than in mammalian cells. Another important feature is its ability to express multiple genes of interest at the same time from a single bi- or polycistronic vector.

This system, however, does have some limitations. For example, the host cells infected by the virus will eventually die. The heterologous gene can therefore not be expressed continuously. Each round of protein synthesis requires a new infection of fresh insect host cells. Therefore, this system is inferior to prokaryotic and yeast systems in terms of continuous production. Additionally, insect and mammalian cells have different glycosylation patterns, such as in the lengths of oligosaccharides and in mannose content [41, 55].

7.7 MAMMALIAN CELLS

Mammalian cell expression systems have several advantages over the systems discussed above because they can more accurately process mammalian and viral proteins and add proper glycosylation, essential for obtaining functional antigens; furthermore, proteins expressed in mammalian cells can be easily secreted in the culture medium, simplifying their purification. Therefore, proteins generated in mammalian cells are usually correctly folded, fully processed, and functionally active [6]. Finally, mammalian cells are often the best system for the production of viral particles to be used as immunogens. Mammalian cells can be used for either transient or stable expression, COS or Human embryonic kidney (HEK) 293 cells commonly used for transient expression and Chinese hamster ovary (CHO) cells used for stable expression [56, 57]. When mammalian cells are transfected with a recombinant plasmid containing a foreign gene, the gene will be replicated and the gene-encoded protein will be produced correspondingly. However, the transfected cell will eventually die due to the unlimited replication of the foreign DNA (deoxyribonucleic acid). In this respect, transient expression in mammalian cells is similar to the insect cell expression system where transformed cells can only produce the protein of interest transiently. Another limitation of transient expression is its scalability because of the requirement for large amounts of plasmid DNA and the need for relatively high quantities of transfection reagent. For constitutive, long-term, or large-scale protein production a stable CHO cell line expressing the gene of interest is much more desirable [56]. In this system the gene of interest is integrated into the chromosome of the host cell, and these cells can be screened and further adapted to different types of medium, even serum-free medium and suspension culture models for continuous production of protein of interest. In this section, we will review different cell substrates for the vaccine production.

7.8 BABY HAMSTER KIDNEY (BHK) CELLS

BHK cells are used quite commonly for the production of viral particles that have been used as vaccines [58–61]; however, the applications of these cells for the expression of

recombinant proteins for either therapeutic or preventive application have been limited. The recombinant proteins expressed and produced in these cells for the therapeutic applications are coagulation Factor VIIa (NovoSeven1) and FVIII (Kogenate1). Even though the list is small, however, the important information about the ability of these cells to produce highly complex proteins is gained. It seems that large proteins with considerable degree of posttranslational modifications can be expressed in these cells. Factor VIII is quite a large protein (>300 kDa), it possesses 25 potential N-linked glycosylation, many of these sites are O-glycosylation sites, 6 tyrosine sulfation sites and 7 disulfide bonds, yet it is manufactured in BHK cells to its functional state [62]. Sulfation of FVIII on tyrosine residues was shown to be required both for optimal biological activity, binding to von Willebrand factor, and efficient activation by thrombin [63]. These properties make these cells quite appealing for the expression of large complex viral glycoproteins such as trimeric HIV Env, which has 25–31 potential glycosylation sites, and 14 cysteine residues just to name a few. Efforts have been made to increase the secretion of FVIII by overexpression of the molecular chaperone Hsp70 in a BHK21-FVIII cell line [64]. Notably, apoptosis in BHK21 and BHK21-FVIII was also significantly reduced by Hsp70 expression, suggesting that Hsp70 not only helps in maturation of FVIII but also blocks intrinsic processes leading to apoptosis. Similar to that observed in CHO cells, co-expression of Aven and E1B19K also reduced apoptosis and led to enhanced productivity in batch and perfusion cultures of a BHK-FVIII cell line [65].

7.9 HUMAN CELLS

Human Embryonic Kidney (HEK) 293 cells, established by Graham and colleagues in 1977 [66, 67], are most attractive for the production of vaccines and therapeutic proteins because these cells are easy to maintain and have a higher transfection efficiency upon introduction of plasmid DNA. Furthermore, these cells can produce proteins with posttranslational modifications such as glycosylation similar to their natural counterparts. The main reason why glycosylation of the proteins produced in HEK293 cells is similar to their counterparts produced naturally is that some of the sugar transferring enzymes such as α -2-6 sialyltransferase, α -1-3/4 fucosyltransferase, bisecting *N*-acetylglucosamine transferase are present in HEK 293 cells while absent in BHK and CHO cells [68]. Overexpression of the *O*-glycosylated interferon- α -2b in HEK293 cells was found to provide a glycosylation profile highly similar to interferon (IFN) naturally secreted by human leukocyte [69]. CHO-expressed glycodelin-A (GdA), a glycoform of glycodelin found in amniotic fluid, contains only typical CHO-type glycans and is devoid of the *N,N*-diacetyllactosamine (lactoNAc)-based chains that are found naturally in human GdA. By contrast, HEK293 cells produce recombinant GdA with the same type of carbohydrate structures as natural GdA. This is significant because biological activities of glycodelin are highly modulated by its glycosylation profile [70]. The system has been applied routinely for producing protein at small scale for simple analysis [71–73]. However, more recently the HEK293 transient system is being applied for the production of recombinant proteins at the larger scales for structural studies and also for immunogenicity studies [74, 75]. It is quite surprising that

while HEK293 is probably the most widely used cell line to express research-grade recombinant proteins for various applications, however, only one licensed protein for therapeutic application (Xigris1, activated protein C), and none of the vaccines are manufactured in this host. Xigris1 is manufactured in HEK293 cells since two post-translational modifications essential for maintaining its biological activity, propeptide cleavage and γ -carboxylation of its glutamic acid residues, were not adequate in CHO cells [76]. Other approved protein therapeutics currently produced in human cells include Dynepo (epoetin delta), Elaprase1 (iduronate-2-sulfatase), and Replagal1 (α -galactosidase A), all expressed in the human fibro-sarcoma cell line HT-1080, and Alferon N1 and Sumiferon (both a mixture of 10–14 α -interferon subspecies), naturally expressed, by human leukocytes and the human lymphoblastoid Namalwa cell line, respectively. Finally, epoetin delta produced in HT1080 cells was found to be more homogeneous with a majority of tetra-antennary glycans and higher sialic acid content compared to CHO-produced erythropoietin (EPO). Epoetin delta was also devoid of *N*-acetyllactosamine repeats and of *N*-glycolylneuraminic acid (Neu5Gc), two glycan structures that are present in CHO-derived EPO [77].

It seems that CHO cells have been the cells of choice for the production of vaccines and therapeutic recombinant proteins. It is well known that CHO, BHK, and murine cell lines generate recombinant proteins with glycan structures possessing the potentially immunogenic Neu5Gc residues [78]. This immunogenicity can reduce the efficacy of a biologic because of rapid clearance by the immune system, or prevent drug re-administration as this would potentially cause an undesirable immune response. About 1–2% of circulating antibodies in humans are directed against α -1,3-galactose residues that are commonly produced by nonprimate cells [79]. On the contrary, this sugar modification for the recombinant proteins for the vaccine applications may be advantageous for enhancing the immunogenicity of the target molecule. This will be quite advantageous if the increased responses are targeted toward the epitope(s) of interest and not only to the sugar moiety. Therefore, perhaps for the production of the recombinant proteins for the therapeutic applications, human cells could be a better option as it will not only reduce the immunogenicity but will also reduce potential immunogenic reactions against nonhuman contaminants. These antibodies are clinically significant as they represent the first obstacle to xenotransplantation in humans [80] and may also recognize r-proteins produced in CHO or other nonhuman expression systems. In some areas of the United States, up to 22% of patients receiving the monoclonal antibody cetuximab produced in the mouse SP2/0 cell line have shown hypersensitivity reactions during their first infusion due to preexisting IgE antibodies against the nonhuman oligosaccharide galactose- α -1,3-galactose [81].

Efforts are ongoing in development and evaluation of new human-cell-based expression platforms for the production of recombinant proteins or production of vectors to be used in prime boost combination. For example, the human PerC.6 cell line, a human embryonic retina cell line transformed by adenovirus E1 genes [82], has been shown to generate high titers (120 mg/L) of a monoclonal antibody within 13 days following transduction with an adenovirus [83]. Diaz and colleagues have determined the quality of EPO produced in PerC.6 cells. They demonstrated that stable expression of EPO in PerC.6 cells was devoid of Neu5Gc sugar [84]. In contrast,

EPO derived from CHO cells (Eprex) had up to 3% of total sialic acids were found as Neu5Gc. The use of PerC.6 for the production of recombinant proteins for therapeutic and vaccine application is gaining popularity, and a total of 14 Per.C6-based products are in phase I/II clinical trials. Another human cell line known as HKB11, a hybrid cell resulting from the fusion of HEK293 to a human B cell line, has been used to express some recombinant proteins [85]. It has been shown that the expression of FVIII was 8 and 30 times higher in HKB11 as compared to experiments performed in HEK293 or BHK-21, respectively [86]. Human amniocytes transformed with adenoviral E1 and pIX genes also offer another platform for the production of recombinant proteins. These suspension-growing cells were shown to provide stable expression of α -1-antitrypsin in chemically defined medium at 30 pg/cell/day without the need of antibiotic selection [87].

7.10 CELL-FREE EXPRESSION SYSTEMS

The expression of proteins with a toxic effect on the bacterial cells often results in a very poor yield of the target molecule. This is often the case when overexpressing large eukaryotic proteins or enzymes with adverse hydrolytic activities (i.e., lipases, phospholipases, and kinases). The toxic effect can derive from inhibition of the transcriptional and translational mechanisms burdened by the overexpression of large proteins or as a result of enzymatic activities interfering with the bacterial metabolism. One approach to the production of toxic proteins is to mutagenize the catalytic site and produce a defective variant of the enzyme; for large proteins, the expression of subunits or domains can sometimes increase the yield of the protein that can be assembled in a functional unit starting from individual domains. When large full-length proteins or active functional enzymes need to be produced, cell-free systems are a powerful alternative. Several systems are available for cell-free transcription and transduction of genes [88–90]. The most common systems are based on *E. coli* lysates, wheat germ lysate, or pancreas reticulocyte, with the last one being the best for expression of properly folded eukaryotic proteins. One additional advantage of cell-free expression systems is the ability of carrying expression profiling and solubility of proteins starting from a large number of constructs in a short time. The time required to complete a transcription and translation reaction is as little as 2 hours. The main disadvantage of the cell-free approach is the high cost of the starting material.

7.11 PROTEIN REFOLDING

At times, proteins expressed in *E. coli* are not expressed in a soluble form due to misfolding caused by lack of appropriate chaperon proteins, rapid translation, or intrinsic low solubility. In all these situations the target protein ends up in inclusion bodies, large aggregates of somewhat misfolded. Inclusion bodies can be exploited for the purification of the protein after isolation of the inclusion bodies themselves, followed by partial denaturation of the proteins contained in it, and controlled refolding. If the protein of

interest refoldes in a functional state, further purification can be achieved using standard protocols. This approach is most effective when the protein of interest has a clear phenotype (i.e., enzymatic activity, functional epitope recognized by a monoclonal antibody) that can be used to assess the correct refolding conditions for the protein of interest. Furthermore, despite well suited and sometime powerful at the research scale, this approach has limitation at the production scale, therefore is not preferred.

7.12 PROTEIN PURIFICATION

Purification of the protein of interest in its functional and native state is important for the vaccine application. In this section, we will review the techniques that are widely used for production of vaccines.

7.12.1 Column Chromatography

Some of the commonly used techniques are summarized in Table 7.2. Column chromatography techniques can be divided in two broad groups exploiting chemical or physical properties of the protein of interest. Size exclusion chromatography separates proteins based on their size (physical property), while most other techniques separate proteins based on chemical properties or on their ability to specifically bind to selected ligands (affinity chromatography) (Fig. 7.3).

7.12.2 Size Exclusion Chromatography

Size exclusion chromatography (also known as gel filtration chromatography) is a technique for separating proteins and other biological molecules on the basis of molecular size [91]. The process employs a gel media suspended in a buffer solution that is commonly packed into a chromatographic column. These columns can vary in size

TABLE 7.2. Summary of commonly used Protein Purification Techniques at the Research or Production Scale

	Research	Production
Starting volume	Up to few L	Up to 100s L
Purification step	Technique	
Capture	Affinity	Affinity
	Immunoaffinity	Immunoaffinity
	IMAC	IEX
	IEX	Small molecule
Polishing	IEX	IEX
	IEC	IEC
Buffer exchange	SEC	TFF
	Dialysis	
	TFF	

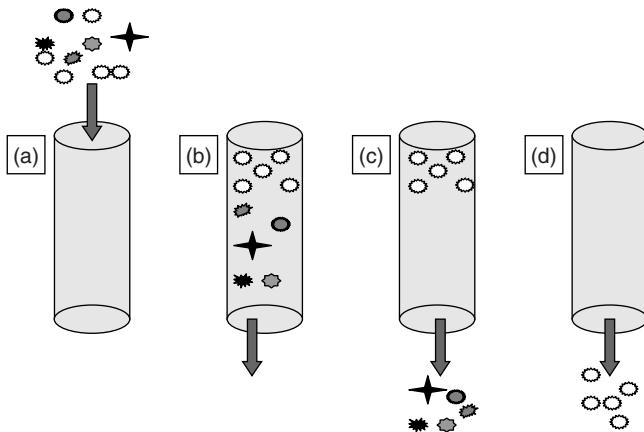


Figure 7.3. Diagram describing steps in chromatography techniques exploiting different chemical properties of proteins for purification. (a) A complex mixture of proteins is loaded on the column; (b) the target protein binds to the resin; (c) the contaminating proteins are washed out of the column; (c) elution of the protein of interest. In hydrophobic interaction chromatography (HIC) the protein is binding to the resin at a high salt concentration and eluted at low salt concentration; the initial low salt concentration in ion exchange chromatography (IEX) allows the target protein to bind to the resin through electrostatic interactions and elution is achieved with an increasing concentration of competing ions. Similarly, in affinity chromatography the protein being purified binds to the resin through a very specific interaction with a ligand, and it is eluted with a buffer exchange by decreasing the strength of the interaction.

from very small, for example, spin columns of <1 mL bed volume for analytical separations, to very large for manufacturing-scale applications. The gel media consists of spherical porous particles of carefully controlled bead and pore size through which molecules diffuse to different extents based on differences in their molecular sizes [92]. Small molecules diffuse freely into the pores and their movement through the column is retarded, whereas large molecules are unable to enter the pores and are eluted earlier. Therefore, proteins are separated in order of their molecular weight, with the largest molecules eluting from the column first.

There are two general applications for size exclusion chromatography: It is generally used for either fractionation or group separation; in fractionation applications, molecules are separated according to differences in size, as would be required in a purification or characterization protocol. A group separation separates all high-molecular-weight molecules from all low-molecular-weight molecules, effectively yielding two groups, as would be required when exchanging buffer components or salts. Commonly this method is used as a polishing step in purification schematic.

Several other methods are available to separate or characterize biomolecules based on differences in size, including sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [93, 94] and ultracentrifugation [95, 96]. The main

advantage for size exclusion chromatography over these techniques is the ability to separate analytical to manufacturing amounts of material under native, nondenaturing conditions with samples recovered in a form suitable for direct downstream processing [97]. The equipment required for size exclusion chromatography (SEC) is also relatively inexpensive and readily available. The main disadvantages of size exclusion chromatography are a relative lack of resolution, an inability to analyze more than one sample at a given time, and samples are recovered in a more dilute form than the starting material (≥ 20 -fold dilution). Regardless of these challenges, SEC has been used for many purposes, including buffer exchange or desalting [98], removal of non-protein contaminants (DNA, viruses) [99], protein aggregate removal [100], the study of biological interactions [101–103], and protein folding [104, 105].

7.12.3 Ion Exchange Chromatography (IEX)

Proteins contain charged, polar, and hydrophobic domains on their surface controlled by the amino acid sequence in the protein. Aspartic acid and glutamic acid have a negative charge at physiological pH, whereas lysine, arginine, and histidine have a positive charge at physiological pH. The combined contribution obtained averaging the individual charges of all the surface amino acids defines the isoelectric point of the protein, or the pH at which positive and negative charges are equal and the protein is electrically neutral. Ion exchange chromatography relies on the interaction of charged molecules in the mobile phase with oppositely charged groups coupled to the stationary phase [106]. The charged molecules in a buffer solution come from the buffer components (e.g., salts). The charged groups on a protein are provided by the different amino acids in the protein and can be controlled by buffering the solution above or below the isoelectric point of the target protein. Binding and elution of proteins is based on competition between charged groups on the protein and charged counter ions in the buffer for binding to oppositely charged groups on the stationary phase [107]. The higher the concentration of charged salt molecules in the solution, the greater is the competition for binding to the ligands on the matrix, so under these conditions the tendency for the protein to dissociate from the ion exchange matrix will be greater.

The protein sample is applied to the ion exchange column in a solution of low salt concentration. The counter ions with which the column has been charged are not permanently bound but are held by electrostatic interaction, and there is a continual binding and unbinding of counter ions. Under low-salt conditions, charged groups on the protein have a greater probability of binding to charged counter ions on the stationary phase. During elution, the salt concentration is increased, so that when a protein's charged domain dissociates from an ionic group on the stationary phase, there is an increased probability that ions in the mobile phase will bind to the charged group on the protein and on the ionic group on the stationary phase, keeping the protein free in solution. Thus, the proteins dissociate from the ion exchange matrix and are eluted as the salt concentration increases. The more strongly bound the protein, the greater is the salt concentration required to elute it. Ion exchange chromatography therefore separates proteins first on the basis of their charge (positive or negative) and,

second, on the basis of relative charge strength (i.e., strongly positive from weakly positive).

7.12.4 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) takes advantage of the hydrophobicity of proteins and separates them on the basis of hydrophobic interactions between the immobilized hydrophobic ligands and nonpolar regions on the surface of proteins. Protein binding to the hydrophobic ligands is enhanced by higher concentrations of neutral salts, which makes HIC an ideal next step when samples have been subjected to purification methods that generate a high-salt eluate. The bound protein is eluted by washing the column with salt-free buffer or by decreasing the polarity of the eluent [108]. One advantage of HIC is that structural damage to the biomolecules is minimal, and biological activity is maintained after purification due to a weaker interaction than affinity, ion exchange, or reversed-phase chromatography [109, 110].

Complex mixtures of proteins can be separated using HIC with different types of elution conditions. HIC has been used for purifying a variety of biomolecules such as serum proteins [111], membrane-bound proteins [112], nuclear proteins [113], receptors [114–117], cells [118], and of enzymes [119] in research and industrial laboratories [120]. HIC is even sensitive enough to be influenced by nonpolar groups normally buried within the tertiary structure of proteins but exposed if the polypeptide chain is incorrectly folded or damaged (e.g., by proteases). This sensitivity can be useful for separating the pure native protein from other forms.

7.12.5 Affinity Chromatography

Affinity chromatography separates proteins on the basis of highly specific biologic interactions such as those between receptor and ligand or between antigen and antibody [121]. This method can be used to separate a target molecule whenever a suitable ligand is available for the protein of interest. In affinity chromatography a binding substrate or ligand is covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecule and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be purified in an active form. In this section we will review different types of affinity purification on strategies, such as immunoaffinity, lectin affinity, metal affinity and small molecule affinity.

7.12.5.1 Immunoaffinity Chromatography.

Immunoaffinity chromatography uses immobilized antibodies for affinity chromatography [122]. The specificity and high affinity of the antibody–antigen interaction can lead to the highly selective absorption of proteins. By applying a protein mixture to a suitable antibody immobilized on a resin, washing off unbound or weakly bound material, and eluting the antigenic protein with appropriate elution agents, both purification and concentration can be achieved simultaneously. As a result, immunoaffinity chromatography has proven extremely useful for both biochemical laboratory scale and larger scale protein purification [123, 124].

Both polyclonal [125, 126] and monoclonal [127, 128] antibodies are used in immunoaffinity chromatography. Polyclonal antibodies are, obtained by immunizing a rabbit or goat, and purified the immunoglobulin fraction from the resulting serum. These are mixtures of antibodies with a variety of specificities and capable of binding to different regions of the protein that used as an immunogen. They are relatively easy to produce but suffer from several disadvantages for use in immunoaffinity chromatography. For example, they are heterogeneous with respect to epitope specificity and binding properties. Also, a pure antigen is required for immunization to avoid raising unwanted antibodies to minor impurities in the protein preparation. Moreover, the antibody preparation is not completely reproducible from one immunized animal to another so that it is difficult to obtain large quantities of consistent material. Monoclonal antibodies on the other hand are more difficult and expensive to produce but have several advantages for use in immunoaffinity chromatography: They can be produced with smaller quantities of immunogen that need not to be pure; once a hybridoma line is established, it can be used to produce potentially unlimited supply of antibody with reproducible properties; most importantly, the antibody binds to a single binding site or epitope and has homogeneous binding and elution properties.

Immunoaffinity chromatography suffers from one major disadvantage: sometimes due to the strength of the antigen–antibody interaction, it is usually difficult to elute the antigen from an immunoaffinity column [129]. Harsh conditions are often required for elution, such as extreme pH (pH 3 or 10) or denaturing agents (8 M urea or 6 M guanidinium hydrochloride) that denature the protein structure and results in loss of functional activity. These conditions often damage labile proteins resulting in very low yields of active, purified protein. One potential method for subverting the need for harsh elution conditions is the use of monoclonal antibodies, which bind the antigen only weakly.

7.12.5.2 Lectin Affinity Chromatography. Lectin affinity chromatography (LAC) is frequently used as a first capture step in the purification of glycoproteins. Lectins are sugar-binding proteins mostly isolated by plants with the property of binding to their sugar moiety with high specificity, and providing the possibility of fractionating proteins according to their glycosylation pattern. The most commonly used lectins are concavalin A; Gluvanthus nivalis (GNA) resin; lectil; lectin, and pea lectin.

7.12.5.3 Immobilized Metal Affinity Chromatography. Immobilize metal affinity chromatography (IMAC) resins are polymeric materials with chelating groups on their surface able to bind a divalent metal ion, typically Ni^{2+} or Co^{2+} . The metal ions, immobilized on the resin, are able to tightly and specifically bind to imidazole rings contained in hexa-histidine tags or similar histidine-rich tags. Binding of histidines to metal ions is strongly pH dependent, and it is abolished at $\text{pH}<6$. Elution of an His6 tagged protein can be achieved either by washing the column with a buffer containing high concentration of imidazole, thus effectively competing with histidine's imidazole rings for binding to the metal ions, or elution can be obtained

by lowering the pH to less than 6, where the metal ions are removed from the resin itself and the column is regenerated before a new purification can be performed.

Recombinant proteins engineered to have six consecutive histidine residues (6xHis) on either the amino or carboxyl terminus can be purified using a resin containing nickel ions (Ni^{2+}) that have been immobilized by covalently attached nitrilotriacetic acid (NTA) [130–132]. The 6xHis/Ni-NTA system is a fast and versatile tool for the affinity purification of recombinant proteins and antigenic peptides [133]. The tight association between the tag and the resin allows contaminants to be easily washed away under stringent conditions, yet the bound proteins can be gently eluted by competition with imidazole or a slight reduction in pH. The six histidine residues that comprise the 6xHis tag are uncharged at physiological pH and are very poorly immunogenic in most species. Consequently, the 6xHis tag rarely affects the structure or function of the tagged protein and need not be removed after purification [134]. When the 6xHis tagged protein fails to be satisfactorily purified after elution from a Ni-NTA column, alternative chemistries can be evaluated, such as Co-NTA or Co-TALON.

7.12.5.4 Small-Molecule Affinity. Affinity purification is not limited to proteins for which a known binding partner or antibody is available and can be performed also without the addition of a tag. Small-molecule affinity purification is based on the concept that any protein can bind to a small molecule, which can derive from three sources: (1) optimization of a known ligand, (2) *in silico* design and virtual screening of biniding partners, and (3) screening of large small-molecule libraries. Often, a combination of these three approaches leads to the development of a molecule that will bind with great affinity and specificity to the target protein. This technique has several advantages compared to other affinity techniques: (1) no additional sequence is introduced, as in the case of histidine tags or longer biological tags; (2) the production of biological ligands is often more expansive than the chemical synthesis of a small molecule; (3) higher resin capacity; (4) the resin can be sanitized, which is often not possible when biomolecules (antibodies, lectins, etc.) are used for affinity capture of the protein of interest; (5) increased safety; and (6) decreased production costs for the antigen of interest. To counterbalance the benefits listed so far is the large development cost required for each molecule and the high specificity of the interaction, which prevents from using the same modified resin for multiple proteins; as a consequence, this technique is only suitable for industrial applications after one antigen has been developed as a vaccine and large quantities of the protein need to be purified in a production process.

7.12.6 Tags Removal

The large tags used for improving solubility, stability, or to provide a tool for affinity purification, as well as the smaller 6xHis tag, oftentimes need to be removed from the target protein after purification. The most common approach to remove the tag postpurification is to introduce a highly specific protease site (Table 7.3) between the tag itself and the protein of interest. Several such sequences, and the correspondent

TABLE 7.3. Commonly Used Cleavage Sites for Removal of Tags from Fusion Proteins^a

Protease	Sequence	Comments
Enterokinase	DDDD↓K	
Factor Xa	ID/EGR↓	
PreScission	LDVLFQ↓GP	High specificity
TEV	DNLYFQ↓G	High specificity
Thrombin	LVPR↓GS	
Intein	Chemical self-cleavage (DTT)	Highest specificity

^aThe proteases listed in this table have very different consensus sequences and variable levels of specificity. The optimal cleavage site is often provided by intein, a self-splicing element that cleaves the fusion protein through an intramolecular reaction catalyzed by DTT, conferring high specificity to the cleavage reaction.

proteases, are available among which the most specific and widely utilized is Tobacco Etch Virus (TEV) protease. After proteolytic cleavage, one additional step is required to separate the tag and the protease cleavage site from the target protein. Size exclusion chromatography and ionic exchange chromatography are two techniques often used toward this goal. Alternatively, the proteases used for tag removal are immobilized on agarose beads, and they can be isolated with a centrifugation step or the agarose slurry can be packed in a small column. When the 6xHis tag needs to be removed, using a 6xHis-tagged protease allows separation of the protein of interest from the free tag and from the protease simply by eluting the hydrolyzed protein solution a second time on chelating column. One final method for in-column cleavage of the tag and concomitant separation of tag and target proteins exploits the properties of inteins (Fig. 7.4); intein are short peptide sequences that can undergo a self-cleavage reaction catalyzed by DTT. After binding of the tagged protein onto an affinity column, the column is saturated with a solution containing DTT; after cleavage, the tag will be retained on the column and the protein of interest will be eluted. This approach is not suitable for proteins containing metal ions as cofactors since DTT is a metal ion chelator and will strip any positive ion from the protein.

7.12.7 High-Pressure Liquid Chromatography

High-pressure liquid chromatography (or high-performance liquid chromatography; HPLC) is a form of column chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved [135]. This method is used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. One drawback of HPLC is the limited sample capacity and thus the inability of using this technique in large-scale protein purification protocols. On the other hand, the analytical

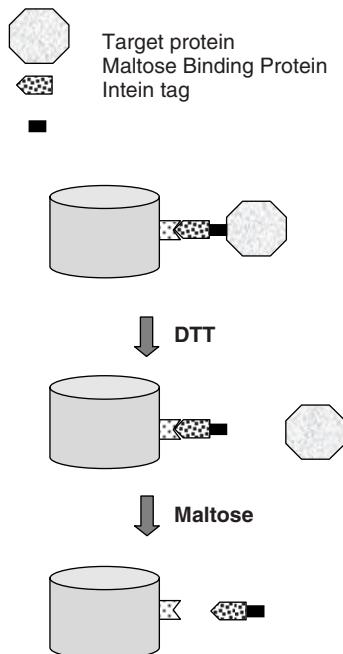


Figure 7.4. Affinity purification using the fusion tag maltose binding protein connected to the target protein through an intein molecule. Use of the intein tag allows recovery of the tagless target protein after elution with DTT; DTT catalyzes an intramolecular reaction leading to self-cleavage of intein. No protease or postcleavage separation of contaminant proteins is required.

power is far superior to any low or intermediate pressure system, both in terms of a very high number of theoretical plates that can be reached (peak resolution) and speed of analysis. One additional advantage is that analysis can be carried out on very small amounts of proteins, typically as little as 1–2 µg, thus limiting the consumption of purified protein. A recent step forward in the direction of better resolution, smaller sample size, and shorter analysis times was the introduction of ultra-high-performance liquid chromatography (UHPLC).

7.12.8 Reverse-Phase Liquid Chromatography

Like hydrophobic interaction chromatography (HIC), reverse-phase liquid chromatography (RPLC) separates molecules according to differences in their hydrophobicity. Both methods are based on interactions between hydrophobic patches on the protein surface and the hydrophobic ligands covalently attached to the gel matrix. However, in practice, the techniques are very different. The surface of an RPLC medium is usually more hydrophobic than that of a HIC medium [136]. This leads to stronger interactions that require nonpolar, organic solvents such as acetonitrile or methanol for

elution. There are many advantages to using RPLC for the analysis of polypeptides and proteins, including extremely high resolution, excellent recovery, and, therefore, high reproducibility. However the requirement for an organic solvent in the elution buffer often causes the irreversible denaturation of protein samples [137, 138].

7.12.9 Convective Interaction Media

Convective Interaction Media (CIM, BIA Separations) monoliths are composed of a single homogeneous block and not available as slurry, as typical for most other chromatographic media [139]; therefore, CIM columns are only available in predefined sizes and cannot be packed in house. The high interconnectivity and large pores of this material allow achieving higher capacity and faster flowing rates when compared with classical chromatographic materials, which translates in the ability of processing larger volumes of protein solution in shorter times. The large pore size also allows the purification of very large particles, including viruses and large DNA molecules. All the traditional chemistries are available in the monolith format: ion exchange, reverse phase, affinity, and hydrophobic interaction, activated and special applications such as immobilized enzymes, or nonstandard affinity purification molecules.

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8

DNA VACCINES FOR INFECTIOUS DISEASE

Samuel Stepenson, Manmohan Singh, and Indresh K. Srivastava

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8.1 INTRODUCTION

Compared to other conventional vaccine technologies, such as adjuvanted protein, recombinant vector, and attenuated pathogens, the concept of DNA (deoxyribonucleic acid) vaccination is relatively simple. A specific gene of interest from a particular pathogen is cloned into a plasmid containing a powerful promoter and, upon injection

into vaccinees, the antigen is produced *in situ*. The expressed protein is then processed and presented to the immune system by the major histocompatibility complex (MHC) class-I-restricted pathway for cytotoxic T lymphocytes (CTLs) induction, the MHC class-II-restricted pathway for the induction of helper T (Th) cell responses and to B cells for the induction of antibodies [1]. It has been known for quite some time that direct inoculation of linear or plasmid DNA could result in gene expression *in vivo* [2, 3]. The power of this technology for inducing potent immune responses and its potential for vaccine application was demonstrated by Tang et al. [4], Ulmer et al. [5], Fynan et al. [6], Wang et al. [7], and many others in different animal models. Using the gene gun to deliver plasmid DNA into the skin of mice, Tang et al. [4] first demonstrated the induction of antibody responses. Ulmer et al. [5] then demonstrated induction of CTL responses and proof of principle for protective efficacy of a DNA vaccine encoding influenza nucleoprotein (NP). Specifically, these animals were protected from both morbidity and mortality upon lethal challenge with a heterosubtypic strain of influenza A virus. While substantial antibody responses were also generated against NP, protection was demonstrated to be cell mediated [5]. Later that same year, DNA vaccine studies were extended to other diseases such as the human immunodeficiency virus (HIV) [7], rabies virus, and hepatitis B [8]. These early studies established several important features of DNA vaccines: (a) induction of both antibody and cellular immune responses, (b) immune responses can be induced by different routes of immunization (intramuscular and epidermal), and (c) it is possible to express foreign genes *in vivo* with proper structure and conformation, as judged by the induction of neutralizing antibodies. Since then, DNA immunization has been used successfully to induce immune responses in animal species from mice to humans with DNA encoding antigens from various sources such as HIV [9–15], herpes simplex virus 1 (HSV-1) [16–19], HSV-2 [20–22], hepatitis C [23, 24], tuberculosis [25–28], mycoplasma [29], toxoplasma [30–34], malaria [31–34], rotavirus [35–37], Ebola virus [38–40], influenza [41], and many others. In addition to infectious diseases, the DNA vaccine strategy has shown to be potentially a very powerful tool in animal models of allergy, asthma, and cancer.

This review will focus on the ability of DNA vaccines to induce humoral and cellular responses against a wide variety of infectious agents and the current approaches being developed to enhance the efficacy of DNA vaccines.

8.2 HUMORAL IMMUNE RESPONSES

Antibodies against viral proteins induced by DNA vaccines were first demonstrated by intramuscular injection of DNA encoding influenza NP and hemagglutinin (HA) [5]. The ability of DNA vaccines to induce appreciable antiviral antibodies was subsequently confirmed by several other investigators with other genes from influenza [42–47], HIV [48–53], rabies virus [7, 23, 54–56], and hepatitis B virus [57]. Since then, the DNA vaccine approach has been used extensively to induce antibody responses against various viral, bacterial, parasitic, fungal, tumor, and eukaryotic proteins.

In general, the antibodies induced by DNA vaccination are predominantly of the IgG subtype with lower levels of serum IgA and IgM [47, 58]. The quantity and quality of the antibody responses induced by DNA vaccination appear to be influenced by the nature and type of immunogen, animal species, and also upon the method and site of DNA delivery. First, as with protein-based vaccines, the inherent antigenicity of the protein expressed by DNA vaccines in part determines the potency of the immune responses induced. However, increased antibody responses can be achieved by expressing antigens as secreted proteins, or as fusion proteins with other antigens or helper T-cell epitopes [59–61]. Second, antibodies induced by DNA vaccines in small animals such as mice are durable and long-lasting as shown with influenza [54, 55], hepatitis B [56], hepatitis C [23], plasmodia [62–64], and leishmania [65–67]. However, antibodies induced in nonhuman primates by DNA vaccination are of lower titer and rather short-lived [68, 69]. This is partly due to a species-related phenomenon, since even potent protein subunit vaccines induce short-lived antibodies in primates [68]. Finally, DNA vaccines can successfully be delivered via different routes, such as intramuscular (im), intravenous, intranasal, oral, intraepidermal, intrarectal, intravaginal [52, 55, 70–75], intrasplenic [65], and intrahepatic [66]. However, the most common routes are intramuscular needle injections and gene-gun-mediated epidermal delivery. The nature of how DNA vaccines are delivered with these two modes of vaccination is fundamentally different. After im injection, myocytes are the primary cells transfected, and uptake of DNA is passive and very inefficient [67], whereas the gene gun propels DNA-coated gold beads directly into cells of the dermis and epidermis, including antigen-presenting cells (APCs). As a consequence, less DNA is required for induction of immune responses by the gene gun route, and any difference in immune priming can be overcome through injecting more DNA [6, 76, 77]. Interestingly, the type of helper T-cell response induced by the gene gun is sometimes qualitatively different. For example, in almost all cases, im injection of DNA induces dominant Th1-type (T-helper type 1) responses, as shown by the induction of IgG2a antibody responses in mice, increased levels of interferon gamma (IFN- γ) production, and little Interleukin 4 (IL-4) production [77–79]. In contrast, gene-gun-mediated epidermal delivery of DNA has been shown in some instances to induce more of a balanced Th1/Th2-type response, as shown by the induction of IgG1 antibodies, increased levels of IL-4 production, and lower levels of IFN- γ production [77, 78, 80]. The reason for these differences are not yet known but may involve the site of antigen production (i.e., myocytes versus skin cells) or differential stimulation of the innate immune system (e.g., via immunostimulatory Cytosine-phosphate-Guanine (CpG) sequences).

For many infectious diseases caused by pathogens that enter the host through mucosal surfaces, such as the human immunodeficiency virus (HIV), induction of mucosal immunity may be desirable for effective prophylaxis. So far, intramuscular injection, gene-gun-mediated delivery, or mucosal delivery of naked plasmid DNA have been rather limited in their ability to induce secretory mucosal IgA responses. However, mucosal delivery of formulated plasmid DNA, such as with cationic lipids [81], monophosphoryl lipid A [82], QS-21 [83], encapsulation in poly(lactide-co-glycolide) (PLG) microparticles [84–87], macroaggregated polyethyleneimine-albumin conjugates [88], and biodegradable alginate microspheres

[89], is effective at inducing secretory IgA responses at mucosal sites. This increased effectiveness was likely due to protection of DNA from digestion and more efficient delivery of DNA into cells of the mucosal immune system. Using a different approach, Wang et al. reported the induction of strong mucosal immunity against simian immunodeficiency virus (SIV) in primates using DNA expressing intact noninfectious virions [90]. The levels of secretory IgA observed in the rectal secretions of the immunized primates were even higher than the levels achieved through the natural infection. Thus, development of DNA vaccine technologies capable of enhancing local IgA responses will be important for the prevention of infectious diseases.

8.3 CYTOTOXIC T-CELL RESPONSES

One of the potential advantages of DNA vaccines, compared to other types of vaccines, is their ability to induce cytotoxic T-cell (CTL). DNA vaccines induce potent long-lasting cellular responses mediated by major histocompatibility complex (MHC) class-1-restricted CD8+ CTL. Effector CTL recognizing appropriate MHC-restricted peptides have been demonstrated in mice immunized with DNA encoding NP from influenza A virus [5, 44, 61, 91–93], hepatitis B surface [82] and core antigen [94], and HIV Env [95–97] and gag antigens [98–100], E6 from HPV [101, 102], among others. DNA vaccine-induced CTLs also were capable of killing virus-infected targets, thereby demonstrating recognition of endogenously processed epitopes. It has been shown that CTL induced in mice by DNA vaccination can persist for more than 2 years after immunization [70, 103, 104], demonstrating the longevity of CTL responses. DNA vaccines may also prime CTL responses in nonhuman primates where long-lived and broad responses have been observed [76, 105–109]. In several independent experiments, one or two intramuscular injections of plasmid DNA encoding HIV *env* or *gag* genes induced MHC class-1-restricted CTL in primates [110]. For example, anti-Env CTL responses were detected for at least 11 months after the last immunization [110] and Gag DNA-induced CTL responses against multiple discrete epitopes [99], demonstrating the longevity and breadth of CTL responses. Also, gene-gun-mediated immunization of Rhesus macaques with plasmid DNA encoding SIV Env (envelop) protein and *gag*, induced *env* specific CTLs [99]. Intramuscular and intravenous administration of the same plasmids combined with gene-gun-mediated immunization induced *env* and *gag* specific CTL responses. The efficiency of DNA vaccines for the induction of potent CTL responses is also influenced by the route, the site of immunization, and the immunization regimen. In general, multiple immunizations are necessary to induce strong responses, however, the number and frequency appears to be important. Fuller and Haynes [80] have shown that gene-gun-mediated delivery of HIV-1 env DNA vaccine induced strong CTL and weak antibody responses after one, two, or three immunizations. However, a fourth immunization caused a significant drop in CTL levels with a significant increase in antibody titers. A corresponding decrease in IFN- γ and increase in IL-4 was also observed along with this change in the nature and profile of the immune response. However, other investigators demonstrated that repeated immunization boosted the CTL levels against influenza

NP [111] and HIV Env [97, 110] and in both these instances the nature of response remained of Th1 type [78]. Thus, the optimum dose and regimen may be different for different DNA vaccines. In addition, differences with the quality and quantity of T-cell responses observed with different routes of DNA administration may be due to fundamental differences in how antigens are processed and presented to the immune system. After im injection, CTLs are primed mainly via cross presentation [112], where expression of antigens directly by APCs is important for CTL priming by the gene gun [113].

DNA vaccines have been tested in human clinical trials for initial safety and immunogenicity. Clinical trials using DNA vaccines either alone or in combination with a live vector boost have been conducted for herpes, influenza, hepatitis B, HIV, malaria, and several cancers. For example, phase I clinical trials were initiated to evaluate the safety and immunogenicity of HIV-1 env/rev (regulator of virion) DNA constructs in infected and uninfected human subjects [114–118]. The uninfected human subjects that received the highest dose of DNA vaccine induced antigen-specific lymphoproliferative responses and antigen-specific production of interferon- γ and β -chemokines [116]. In the infected individuals, HIV-1 env/rev DNA vaccine construct modestly boosted the env-specific antibodies; however, no consistent effect was observed on cellular responses to HIV. In a separate study, the HIV regulatory genes *rev*, *nef* (negative factor), and *tat* were evaluated in a phase I clinical trial. Immunization of infected individuals with these genes resulted in enhanced cellular responses without any consistent changes in lymphocyte subsets or viral load [114, 115, 117, 118]. The DNA vaccines were well tolerated in a broad dose range from 20 μ g up to 2500 μ g, as no significant local or systemic reactions were observed in human subjects [98, 115].

In a phase I clinical trial for a malaria DNA vaccine, it has been shown that three intramuscular immunizations of a *Plasmodium falciparum* circumsporozoite (PfCSP) construct induced antigen-specific, genetically restricted CD8+ T-cell-dependent CTL. The cellular responses were directed against multiple epitopes and were restricted by six human leukocyte antigen (HLA) class I alleles [32]. In the same study, despite induction of significant CTL responses, DNA vaccination failed to induce detectable antigen-specific antibodies in any of the volunteers [98]. Phase I trials have also been conducted with the gene gun, where DNA encoding hepatitis B surface antigen induced both humoral and cellular immune responses (CD4+ and CD8+ T cells) [99]. A phase I trial of an experimental HIV vaccine was recently initiated in Kenya, which involved a DNA vaccine encoding an HIV subtype A *gag* gene as a fusion with several discrete CTL epitopes from other HIV genes [100]. The DNA vaccine was administered by the gene gun and was the first component of a prime-boost vaccination strategy, which will be followed by a recombinant vaccinia virus vector [103]. In addition, phase I clinical trials have been initiated to compare HIV DNA vaccines delivered as naked DNA and/or by a recombinant adenovirus [104]. Phase I/II trials are also underway using DNA immunogens as potential immunotherapies for cancer, including carcinoembryonic antigen (CEA) for colon cancer, human follicular lymphoma, and a T-cell receptor V β for cutaneous T-cell lymphoma.

8.4 PROTECTION BY DNA VACCINES IN PRECLINICAL DISEASE MODELS

The influenza virus model was used to demonstrate that immune responses induced by DNA vaccination could protect mice against a lethal viral challenge [5]. The protection observed was due to NP-specific cellular responses, since passive transfer of immune serum from the immunized mice did not protect naïve mice against the challenge infection, although the serum contained high levels of specific antibodies to NP. In addition, adoptive transfer of splenocytes from the immunized mice protected naïve mice against lethal challenge infection [105]. Furthermore, both CD4+ and CD8+ T cells played a role in protection [91]. In addition to influenza, the protective efficacy of DNA vaccines has been demonstrated against many other diseases such as herpes simplex virus [17, 19, 106], rabies virus [107–109], cottontail rabbit papilloma virus [119], hepatitis B virus [99, 120], Plasmodia [62, 121–123], SIV/SIV (simian immunodeficiency virus/simian immunodeficiency virus with Envelop from human immunodeficiency virus) [53, 124–127], rotavirus [86], Lymphocytic Choriomeningitis Virus (LCMV) [128], and others.

Strong CTL responses were induced by DNA vaccines encoding HIV-1 *env* and SIV *gag* in primates and these animals were successfully protected from challenge with chimeric SHIV virus [108, 109, 129, 130]. However, strong antibody responses are not induced by DNA vaccines alone. Thus, considerable attention has been focused on prime/boost strategies involving recombinant proteins or vectors to induce protective immune responses in primates. Along these lines, Amara et al. have demonstrated that priming of Rhesus macaques with plasmid DNA encoding multiple genes of SIV (*gag*, *pol*, *vif*, *vpr*) and HIV (*env*, *tat*, and *rev*) and boosting with recombinant modified vaccinia Ankara expressing SIV Gag and Pol and HIV Env induced potent CTL, but weak antibody responses [131]. These animals were protected from AIDS against mucosal challenge with highly pathogenic SHIV. In a separate study, protection from SHIV challenge has been induced in primates primed with an HIV-1 *env* DNA and boosted with recombinant oligomeric gp140 protein [132]. Barouch et al. have demonstrated the enhanced efficacy of DNA vaccine in inducing CTL and humoral responses by co-administration of HIV-1 Env and SIVmac239 Gag encoding plasmids with a plasmid encoding IL-2/Ig in Rhesus macaques [169]. Upon challenge with pathogenic SHIV-89.6, these animals were able to control their viremia and were also protected against clinical acquired immunodeficiency syndrome (AIDS). Finally, recombinant adenovirus vectors have successfully been used to boost CTL responses and protection in primate models of SIV [133] and Ebola virus [40].

The efficacy of DNA vaccines for inducing protective responses in diseases where protection is mediated by antibodies has been shown against influenza [6, 42, 47, 68], rabies [108], HSV [16, 134], human papilloma virus [119], Ebola virus [40], and others. The effectiveness of DNA vaccines at inducing protective levels of antibodies is perhaps surprising, given the very low level of antigen produced by cells of the vaccinated host. This is particularly true for proteins that are poorly expressed (e.g., major capsid protein of papilloma virus) [119]. Possible explanations could include the duration of protein expression and the potential for

preservation of conformation-specific epitopes by production of antigen in situ. In a DNA prime-protein boost study, Barnett et al. demonstrated induction of strong neutralizing antibody responses in rabbits and Rhesus macaques by intramuscular and intradermal injection of plasmid DNA encoding an oligomeric form of HIV-1 env (o-gp140) followed by boosting with env protein (o-gp140 Δ V2 SF162) [48]. These antibodies neutralized both homologous and heterologous primary isolates, and protected Rhesus macaques from challenge with pathogenic SHIV SF162P4 [132]. In this study, the primary control of the viremia was likely exerted by antibodies as the animals were depleted of CD8+ T cells prior to challenge.

The requirements for the expression, folding, and intracellular transport of bacterial and parasite proteins compared to viral proteins are different. Nevertheless, the DNA vaccine approach is quite effective at inducing protective responses in preclinical models of bacterial and parasitic diseases. In some of these models, protection is thought to be mediated by T cells, such as tuberculosis [25, 135], *Mycobacterium pulmonis* [29], malaria [62, 122, 123, 136], and leishmania [137]. In these cases, processing and presentation of DNA-encoded antigens to T cells are unlikely to be affected by the source of the antigen (i.e., virus versus bacteria). In contrast, expression of bacterial antigens in cells of the eukaryotic host, where posttranslational modification and folding of the protein may be different than in the organism, may have an effect on the quality of antibody responses. Nevertheless, it is possible to induce protective immunity based on antibody responses with DNA encoding bacterial antigens, such as tetanus toxin C fragment [138] and *Salmonella* Type I Omp C porin [139].

In general, DNA vaccines are effective at inducing CTL but less so at inducing T-helper responses and humoral responses, as compared to other means of immunization (e.g., adjuvanted recombinant protein, inactivated virus). To overcome these limitations, several approaches have been used to increase the potency of DNA vaccines, such as (i) optimization of vectors and genes of interest, in order to achieve higher levels of expression of antigen, (ii) co-administration of plasmid DNA encoding immunologically active proteins such as chemokines, cytokines, co-stimulatory molecules, in order to enhance immune responses against encoded antigens, and (iii) targeted DNA delivery into cells by either formulated DNA, physical delivery technology, or vector systems in order to overcome some of the barriers to effective transfection of cells in situ.

8.5 VECTOR AND GENE OPTIMIZATION

Expression studies using reporter genes indicate that only picogram to nanogram quantities of protein are expressed in vivo after administration of plasmid DNA [3, 140]. While only small amounts of antigen may be necessary for presentation to MHC class 1 molecules to induce potent CTL responses, this may be a limiting factor in inducing potent humoral responses [141]. To compound this problem, certain antigens from pathogens are not efficiently expressed by mammalian cells. For example, codon usage, messenger ribonucleic acid (mRNA) instability, and dependence on co-expression of other genes (e.g., *rev* dependence in HIV) can lead to suboptimal levels of expression of prokaryotic genes in eukaryotic cells. Several approaches using

DNA vaccines to increase the potency of immune response have been successful. First, the choice of promoter, enhancer, intron, polyadenylation, and transcriptional termination vector sequences can affect gene expression. The promoter/enhancer element of cytomegalovirus (CMV) is most commonly used in DNA vaccines and offers a high level of gene expression in a variety of cells. However, one potential disadvantage of the CMV promoter is that its activity may be down-regulated by high levels of IFN- γ and TNF- α (Tumor Necrosis Factor-alpha) [142], which may be produced locally in response to DNA vaccines. Second, Haas et al. demonstrated that optimizing the nucleotide sequence of HIV genes to reflect the preferential codon usage in the mammalian cells can result in higher levels of gene expression [129, 143]. By minimizing rare codon usage and RNA secondary structures through codon design, protein expression and immunostimulation are optimized [130]. Zur Megede et al. have observed an increased HIV *gag* expression by 1000-fold *in vitro*, with a corresponding substantial increase in immunogenicity in mice and primates through the use of codon optimization and removing inhibitory sequences [100]. Third, fusion of poorly expressed sequences with highly expressed heterologous proteins can increase production of the target gene. For example, Wu and Barry showed that HIV *env* gene fused to the "C" termini of green fluorescent protein (GFP) or human protein al-antitrypsin resulted in better anti-*env* immune responses [144]. Nuclear targeting sequence (NTS) can aid uptake of DNA plasmids from the cytoplasm after physical injection to increase the efficiency of protein expression [145, 146].

Localization of the encoded protein can affect the quantity and quality of the DNA vaccine-induced immune responses. In general, secreted forms of the antigens compared to those targeted to the cytosol or plasma membrane are more effective at inducing antibody and helper T-cell responses, while cytosolic targeting may be preferred for CTL induction [147]. However, this is not always the case, as membrane-bound antigens can induce high levels of antibodies [134] and secreted antigens can be effective for CTL [148]. The effectiveness of DNA vaccine for inducing CTL responses also may be enhanced by inducing rapid degradation of antigen by proteasomes [149], which can sometimes be achieved by expression as a fusion protein with ubiquitin [150, 151]. In addition to intracellular targeting of antigen, extracellular targeting also can be effective. For example, expression of the gene of interest as a fusion protein with ligands such as CTLA4 [60, 152], L-selectin [152, 153], IgG-Fc [154], and chemokines [155], which target cell surface receptors of APCs, have been shown to enhance both cellular and humoral immunity of DNA vaccines. In a related approach, expression as a fusion protein with a heat-shock protein increased potency [156], possibly by facilitating cross priming via delivery of antigen to and activation of APCs. In addition, fusion of genes encoding microbial proteins to tumor antigen sequences have been shown to activate T helper cells and to increase immunogenicity against tumor cells [157].

These previous examples have focused on the production of most or all of the antigens. This allows for determinant selection of T-cell epitopes by the vaccinated host. In order to focus the immune response on specific T-cell epitopes, DNA vaccines encoding minimal epitopes [44, 158, 159], strings of epitopes [44, 93, 100, 160], and epitopes in the context of other proteins [44, 161] have all shown to be effective

at priming MHC class-I- and class-II-restricted responses. Epitope sequences can be optimized for MHC binding to increase the potency of a vaccine by converting a poorly processed epitope into the dominantly presented epitope by the APC [162, 163]. Therefore, for efficient induction of cellular and humoral immunity by a given DNA vaccine, the form of antigen should be evaluated on a case-by-case basis.

8.6 ADJUVANTS FOR DNA VACCINES

Even though DNA vaccines induce both cellular and humoral immune responses, high doses (5–10 mg naked DNA vaccine) are necessary for a noticeable physiological effect [164]. Therefore to enhance or modulate the immune responses induced by DNA vaccines, co-administration of biologically active molecules such as cytokines, chemokines, and co-stimulatory molecules have been used extensively [141]. The role of these biological or genetic adjuvants for DNA vaccines have been reviewed in detail [165–167]. Following are a few examples to demonstrate the potential of this approach. In general, co-administration of IL-2, a potent stimulator of cellular immunity that induces the proliferation and differentiation of T cells, B cells, and natural killer (NK) cells, enhances both cellular and humoral immune responses against DNA vaccine antigens [24, 168]. For example, Barouch et al. have shown that co-administration of IL-2 as a fusion protein with the Fc portion of immunoglobulin G significantly enhanced the DNA vaccine-induced protective immune responses against HIV and SIV antigens in mice and Rhesus macaques [12, 169]. Intramuscular and intranasal co-administration of DNA encoding another cytokine, IL-12, has enhanced cellular and T-helper responses and down-regulated humoral responses induced by DNA vaccines against various antigens [170–174]. In addition, Iwasaki et al. have shown that co-administration of IL-12 can overcome unresponsiveness to certain antigens [175]. DNA encoding other Th-1 inducing cytokines, such as IL-15 and IL-18, were also evaluated for their capability to modulate immune responses induced by DNA vaccines [170]. Interestingly, in contrast to IL-2 and IL-12, co-administration of IL-15 and IL-18 with DNA vaccines significantly enhanced antibody and proliferative responses but had little effect upon cellular responses [170, 171, 176]. In general, co-administration of DNA encoding Th-2-type cytokines, such as IL-4, IL-5, and IL-10, as might be expected significantly enhanced the magnitude of antibody responses induced by DNA vaccines [55, 176]. In addition, IL-4 DNA inhibited cellular responses [55], which may limit the application of this cytokine as an adjuvant for antiviral vaccines. Co-administration of DNA encoding Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), a proinflammatory cytokine, has been shown to enhance antigen-specific cellular, humoral, and T-helper responses [177, 178], as has been observed with antigens from HIV [81, 179, 180], influenza [175] HCV [24], encephalomyocarditis virus [181], rabies [79], and plasmodia [182, 183]. Furthermore, co-injection of GM-CSF DNA, whose protein promotes proliferation, maturation, and migration of dendritic cells as well as maturation of B and T lymphocytes, has also improved the protective efficacy in models of rabies [79], malaria [182, 183], and melanoma [184]. The effect of GM-CSF DNA appears to be dependent upon the mode and route of DNA delivery, as no enhancement was seen with gene-gun-mediated epidermal

delivery [185]. Other proinflammatory cytokines, such as TNF- α and TNF- β , were also effective at enhancing the antibody and proliferative responses in rodents [176]; however, only TNF- α DNA was effective for CTL [176].

Another strategy to enhance the potency of DNA vaccine is co-administration of DNA encoding co-stimulatory molecules such as B7.1 (CD80), B7.2 (CD86), and CD40. For example, co-injection of CD86 DNA resulted in a substantial increase in CTL responses against influenza NP [175] and HIV-1 antigens [186, 187]. Whether these effects are a consequence of rendering non-APCs capable of priming CTL or of further activation of professional APCs already expressing these co-stimulatory molecules remains to be determined.

In summary, DNA encoding many cytokines or co-stimulatory molecules have increased the potency of DNA vaccines. Further development of these genetic adjuvants will require careful evaluation of the safety of potential long-term intracellular expression of these biologically active molecules. So far, GM-CSF DNA has been evaluated in nonhuman primates and human clinical studies with a malaria DNA vaccine.

8.7 IMMUNOSTIMULATORY ACTIVITY OF DNA VACCINES

The inherent immunostimulatory activity of bacterial DNA has received widespread attention, particularly in the context of DNA-based adjuvants for proteins. It has been shown that oligonucleotides containing particular sequences surrounding CpG dinucleotides can stimulate B cells to proliferate and secrete immunoglobulins, activate APCs, and stimulate production of cytokines [188–190]. These CpG sequences signal the innate immune system through Toll-like receptor 9 [191–194], and their role in the potency of DNA vaccines is illustrated as follows. Interestingly, cotransfection of genes encoding Toll-like receptors (TLRs) show improved immunogenicity of DNA vaccines [195, 196]. First, plasmid DNA vaccines containing extra CpG motifs can induce stronger antibody and CTL responses [190, 197, 198]. However, this does not always result in more potent DNA vaccines, suggesting that context may be important. Second, methylation abrogates DNA vaccine potency [199]. This likely reflects a means by which the innate immune system recognizes pathogen-derived DNA as distinct from host DNA. Finally, the potency of plasmid DNA can also be enhanced by co-administration of noncoding plasmid DNA [200], presumably by providing additional CpG motifs. Efforts to co-deliver plasmid DNA with CpG oligonucleotides, however, resulted in reduced DNA vaccine expression and immunogenicity [195], suggesting competition between the plasmid and the oligonucleotide. Thus, appropriate DNA delivery or formulation will be required to take full advantage of CpG oligonucleotides for enhancing the potency of DNA vaccines.

8.8 DNA VACCINE DELIVERY SYSTEMS

Conventional methods of DNA vaccine delivery include direct intramuscular injection of plasmid DNA into muscle and gene gun delivery into skin, neither of which

provides optimal immune responses. Intramuscular injection is suboptimal because only a small fraction of muscle cells are transfected due to inefficient uptake of DNA by cells [3]. APCs can also be transfected but the efficiency is very low [201, 202]. Using fluorescently tagged plasmid DNA, Dupuis et al. demonstrated that after intramuscular injection (i) distribution of DNA within the injected tissue was rather limited, (ii) uptake of DNA by myocytes and APCs was low, and (iii) the majority of labeled DNA was phagocytosed and degraded by macrophages [67]. Therefore, approaches to facilitate distribution of DNA throughout the tissue, increase transfection of muscle cells or APCs, prevent DNA degradation, or enhance release of DNA out of endosomes may all be of help in the enhancing potency of DNA vaccines. With regard to DNA delivery, three promising general approaches have been shown to enhance the potency of DNA vaccines: (i) physical methods of delivery of the plasmid DNA into cells, (ii) particle-mediated delivery of plasmid DNA to APCs, and (iii) viral or bacterial vectors.

8.9 PHYSICAL METHODS

Physical or mechanical methods have been used effectively to facilitate DNA delivery *in vivo* [203, 204]. The main technologies include the gene gun (previously discussed) [41, 205–207], needle-free devices, electroporation [206, 208, 209], and hydrostatic pressure [207, 210]. Needle-free devices (such as the Biojector) are commonly used for administration of various drugs and vaccines and offer the advantages of less invasiveness (i.e., no needle) and better distribution of inoculum. For DNA vaccines, the Biojector has shown some effectiveness in enhancing immune responses in animal models [211, 212] and is being evaluated in a clinical trial involving a malaria DNA vaccine. Recently, it has been shown that electroporation treatment of tissue *in vivo* after DNA vaccination results in higher gene expression [213] and markedly enhanced cellular and humoral responses in small- and large-animal models [214–216]. Electroporation has long been used to transfect cells *in vitro* due to a transient disruption of the plasma membrane, thereby facilitating uptake of DNA. The precise mechanism by which electroporation facilitates DNA delivery *in vivo* and the factors influencing the enhancement of the immune response are not clear but may involve an increased DNA distribution within the tissue (i.e., iontophoresis) and delivery directly through membranes (i.e., electroporation) [217, 218]. It is also possible that an inflammatory response at the site of treatment may contribute to the enhanced immunogenicity of DNA vaccines [219]. Because of the physical nature of the enhanced delivery of DNA by electroporation and the effectiveness in large animals, this technology holds much promise for use in humans. However, issues related to tolerability, safety, and feasibility will need to be examined and may limit its use, in the first instances to therapeutic applications. Finally, increased DNA delivery can be achieved through brute force using hydrostatic pressure [220]. This is accomplished by intravenous (iv) injection of large volumes of DNA solution, followed by restriction of blood flow to the injected limb, and resulting in substantially higher levels of gene expression in endothelial and

muscle cells. As such, this approach would seem to be best suited to gene therapy but could conceivably be considered for therapeutic vaccination. Another intradermal technique uses the “tattoo device,” which employs nine metal needles oscillating at great speed to puncture the skin and inject DNA into skin-associated cells to elicit a strong T-cell response [221].

8.10 PARTICLE-MEDIATED DELIVERY OF DNA VACCINES

Ideally, one would prefer to have a simple formulation of DNA that could be administered by conventional means, without the need for a device. In theory, DNA formulations could provide the following: (i) increased DNA stability (i.e., protection from nuclease digestion in tissues), (ii) a depot for slow release of DNA over a period of days or weeks, (iii) facilitated uptake of DNA by cells, (iv) targeting of DNA to specialized cells (e.g., APCs), and (v) inclusion of adjuvants to stimulate immune responses. To this end, several polymers that condense or interact with DNA have been tried, with varying degrees of success. Some examples include poly(ethylenimine) [222], polyvinyl pyrrolidone [223], dendrimers [224, 225], chitosan [226], and PLG [83, 227, 228]. The best studied of these in the context of DNA vaccines is PLG, and the rationale is twofold. First, methods for encapsulation of compounds into PLG microparticles are well established, and these formulations have been used for slow-release delivery of various entities. Encapsulation of plasmid DNA in PLG microparticles has been shown to be effective for oral delivery of DNA vaccines, presumably by protection from degradation in the gut [84, 85, 229]. Second, PLG can be made into particles of uniform size and lends itself to formulation with DNA into or onto particles of $\sim 1 \mu\text{m}$ in diameter, which should be readily internalized by APCs. Indeed, it has been shown that DNA vaccines adsorbed onto the surface of PLG microparticles substantially increased humoral and cellular immune responses [227, 230], and the mechanism appears to involve targeting DNA to APCs [211]. Potential advantages of surface adsorption are that it avoids the harsh conditions of encapsulation of DNA and allows for the rapid release of DNA from the surface once inside the cell, thereby acting as an efficient delivery system [231]. Dendritic cell transfection shows increased *in vivo* immunogenicity with use of viral-sized nanoparticles [232]. Cationic polymers, such as poly-L-lysine (PLL) [233] and polyethylenimine (PEI) [234, 235], when complexed with DNA form positively charged polyplexes that neutralize the negatively charged cell membrane to aid transfection and entrance into the cytoplasm [235, 236]. Micelles, spherical capsules with a hydrophilic shell enclosing a fatty acid core, have gained ground as gene carriers in several clinical trials [237, 238]. Their thermostable and immunostable nature allow for prolonged circulation in the bloodstream. Magnetic nanoparticles such as maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and magnetite (Fe_3O_4) can be transfected using a form of magnetic guiding referred to as “magnetofection.” This system improves efficiency and delivery rate through accumulating magnetic particles on target cells through applied magnetic field gradients [239–242].

8.11 USE OF LIVE VIRAL AND BACTERIAL VECTORS FOR VACCINE DELIVERY

Use of live attenuated organisms is very effective at inducing potent immune responses. However, because of safety issues, this technology may be problematic for enhancing the immune responses against chronic deadly diseases such as HIV [243]. For example, it has been shown that an attenuated SIV can cause an AIDS-like disease in monkeys [244]. A potential alternative is to deliver genes encoding antigens using heterologous live viral or bacterial vectors. Some of the viral vectors that have been tested for DNA or RNA vaccine applications include vaccinia [100, 131, 245, 246], canarypox [247–250], yellow fever–Japanese encephalitis [251], rabies [252], canine herpes virus [253], adenovirus [133, 254, 255], variella-zoster [256], poliovirus [257, 258], and alphaviruses [259–261]. However, because the antigens are encoded by the viral genomes, these vectors are not plasmid DNA delivery systems *per se*, whereas various bacterial vectors have been used for such purposes. The ability of bacteria to readily enter cells of the respiratory and gastrointestinal mucosa provides an efficient delivery of DNA to immunologically relevant cells. For example, *Shigella* spp. are Gram-negative bacteria that enter cells via phagocytosis. To facilitate delivery of plasmid DNA vaccines out of the bacteria and into the cytosol, Sizemore et al. engineered a strain of *Shigella* with an unstable cell wall, which was shown to be effective at inducing humoral and cellular immune responses [262, 263]. *Listeria monocytogenes* is another Gram-positive bacteria that enters the cytosol of infected cells and has been successfully used to deliver plasmid DNA [264–266]. Yet another bacterial vector that has been shown to be effective for DNA delivery is *Salmonella* [267–270]. In this case, though, the bacteria remains in the phagosomal compartment of the host cell and it is not clear how the plasmid DNA is delivered to the cytosol. The use of certain bacterial vectors offers potential advantages, such as a substantial safety profile in humans (e.g., *Bacillus Calmette Guerin* (BCG) and *Salmonella typhimurium* type 21a (*S.typhi* TY21a)), the possibility of inducing systemic and local responses, and they may provide synergy with DNA vaccines in a prime/boost regimen. However, as with viral vectors, preexisting immunity may limit the effectiveness of DNA delivery.

8.12 SUMMARY

In conclusion, a substantial amount of work has been done on DNA vaccines since 1990, only a fraction of which has been cited here. During this time, work in animal models has shed light on the limitations of DNA vaccines, how they work, and how one may be able to increase their potency. First-generation DNA vaccines (i.e., naked DNA) have so far demonstrated limited effectiveness in larger animals, including humans. However, substantial advancements have been made over the past several years on improved DNA vectors and delivery, and on the use of adjuvants (biologic and genetic). The potency of these second-generation DNA vaccines in humans remains to be fully determined, but they represent significant advancements toward the development of protective DNA vaccines for humans.

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DEVELOPING STABLE CELL LINES FOR THE PRODUCTION OF VACCINE ANTIGENS

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9.1 INTRODUCTION

The economical industrial scale-up of recombinant protein production contributes significantly to today's establishments and successes of the modern biopharmaceutical industry. Mammalian cell expression systems have become the major workhorse for manufacturing the majority of marketed protein drugs [1, 2]. Accumulation of experience with cultivated mammalian cells over the past 20 years is significant in several aspects. First, it has demonstrated the suitability of mammalian cells for proper folding, glycosylation, and secretion of therapeutic proteins. Second, mammalian expression systems have been shown to express grams per liter levels of therapeutic proteins,

thus effectively addressing the cost-of-goods issue for this class of biotech products. Third, the capacity network formed in the past decades for Good Manufacturing Practice (GMP)-level mammalian cell cultivation provides easy access to biotherapeutic companies who wish to utilize it. Lastly, regulatory paths for biotherapeutic products generated from the mammalian systems are clearly paved by the Food and Drug Administration (FDA) approval of dozens of protein drugs produced in mammalian cells.

In recent years, mammalian expression systems have found wide application not only in biotherapeutic manufacturing but also in developing effective and economic vaccine antigens. Protein subunits have long been used as vaccine antigens [3–5]. Recombinant subunit vaccines have the advantage of safety and can induce desired magnitude and quality of immune responses when appropriate formulation and delivery are identified. Most recombinant subunit vaccine antigens are generally produced in *Escherichia coli* and yeast, due to their distinct advantages for protein production including growth rate, cost, safety, and ease of culturing; however, recombinant proteins produced in these organisms may lack proper folding, assembly, and certain posttranslational modifications. Therefore, *E. coli* and yeast may not be the most suitable hosts for the development of all biotherapeutics and vaccines where structure may be important for developing immunologically relevant antigens. More recently, mammalian cells are being used to create stable cell lines expressing vaccine antigens. The use of stable mammalian cell lines is especially important for the development of viral vaccine antigens that need to mimic the antigen structure encountered in an infected host. Successful invasion by virus particles is mediated by virion surface antigens, which bind to host receptors [e.g., severe acute respiratory syndrome] (SARS), human immunodeficiency virus (HIV), and respiratory syncytial virus (RSV)]. These surface antigens represent attractive targets for vaccines as antibodies induced to these antigens could be broadly neutralizing and ultimately prevent infectivity and confer protection. Thus, mammalian expression systems that maintain native protein conformation and antigenicity have been used to prepare several such recombinant subunit vaccine antigens, including SARS spike protein [6–8], HIV type 1 gp120/gp41 [9–11], Respiratory Syncytial Virus (RSV) FG glycoproteins [12], and fusion protein [13].

Mammalian expression systems comprise various expression cell lines, notable among which are Chinese hamster ovary (CHO) cells, baby hamster kidney cells, human embryonic kidney 293 cells, and mouse myeloma NSO cells [14]. To establish stable cell lines for long-term and high-volume protein production, the CHO cell line appears to be the most predominant host. In fact, about 60–70% of biotherapeutics are produced in mammalian cells with CHO cells as the host [15].

Another important application of mammalian expression systems in the development of viral vaccine candidates involves the utilization of complementing cell lines for production of replication-defective viral vaccines. Viruses are being exploited as live vaccine vectors to deliver genetic sequences for pathogen proteins, allowing the development of novel prophylactic and therapeutic viral vaccine candidates. One critical issue for development of any live attenuated viral vaccine is obtaining an appropriate balance between safety and immunogenicity. Since many replication-competent viral vectors are not considered safe to enter clinical trials in humans, a number of

deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses have been genetically engineered to be replication deficient. This is achieved by use of viruses that lack key genes and are thus unable to replicate after entering the host cell. In order to propagate such viral vectors, a helper or complementing cell line that expresses the missing gene is needed [16].

In this chapter we will describe methods and protocols that are applicable to the stable expression of secreted recombinant proteins. Protocols illustrate the manufacturing of proteins for vaccine antigen production in CHO cells and the development of complementing cell lines that support the production of replication-defective viral vaccines. These protocols can be adapted for different purposes with appropriate modifications. We assume the readers are familiar with general concepts and techniques of cell culture. We provide references to the seminal original research articles on key CHO technologies for readers to grasp the ideas behind these protocols. Additionally, key issues important for the production of a complementing cell line for propagation of replication-defective vectors as vaccine candidates will be discussed.

9.2 BACKGROUND, METHODS, AND APPROACHES—CHO CELL RECOMBINANT PROTEINS

9.2.1 Cell Lineage

The cell line CHO-K1 is the ancestor of the commonly used CHO cells. It was originally derived through extended culturing of primary ovary cells from a Chinese hamster without application of exogenous immortalization agents [17]. The CHO-K1 has a karyotype of 20 ($n = 22$, [18]). This indicates some spontaneous transformation, apparently contributing to the immortality. When the CHO-K1 cells were submitted to the American Type and Culture Collection in 1970, they had been cultured more than 400 passages from the original establishment. One of the significant developments making CHO cells very useful in recombinant protein technologies was the establishment of lineages [19] that lacked dihydrofolate reductase (DHFR). The authors were able to select DHFR-deficient lines (DUKX) after mutagenesis steps by using [^3H]-deoxyuridine to kill cells with intact DHFR.

Because of the deficient *DHFR* gene in CHO DUKX cells, one can apply methotrexate (MTX) to select for clones transfected with vectors containing a modular *DHFR* complementary DNA (cDNA) [20, 21]. Furthermore, one can culture clones arising from the initial selection with increasing concentrations of MTX, which results in new clones with many more copies of the transforming gene. This latter technique is the foundation of widely practiced amplification of recombinant protein expression in CHO cells [22–25].

With its epithelial origin, the CHO-K1 lineage of cells, including CHO-DUKX, has anchorage-dependent and serum-dependent growth characteristics. Individual cell lines established for expressing recombinant proteins can be adapted to grow in defined serum-free media in suspension [23, 26], which could be a long and unpredictable process. In order to establish CHO cell lines more quickly for research and large-scale industrial recombinant protein production, sublines such as CHO-SSF3 and

TABLE 9.1. Commonly Used CHO Cells

Name	Vendor	Catalog No.
CHO-K1	ATCC	CCL-61
CHO/dhfr-	ATCC	CRL-9096
CHO-S	Invitrogen	11619-012
DG-44 (dhfr-)	Invitrogen	12613-014

PA-DUKX were developed that have enhanced the tendency to grow in suspension culture with serum-free media [27, 28]. Other versions of suspension-grown CHO cells in serum-free media are provided by Invitrogen. The commonly available CHO lines and their sources are listed in Table 9.1.

9.2.2 Selection Markers

The deficiency of the *DHFR* gene in CHO DUKX made it a convenient selection marker with MTX for a single vector introduction, with the added benefit of possible gene expression amplification [21]. There are instances, however, when multiple selection markers are required for successful recombinant protein expression, such as proteins consisting of multiple subunits or proteins requiring additional posttranslational processing enzymes. Nowadays, there are many selectable markers to choose from, and Table 9.2 lists a few representative ones as well as the vendor information for the appropriate expression vector.

9.2.3 Cell Line Maintenance

As robust and adaptable cell lines, different lineages of CHO cells are capable of growing in attached monolayer culture or in suspension culture. This allows for ease of transfection, selection, and expression amplification of CHO cells in attached culture followed by transition to suspension culture for large-scale expression of heterologous proteins. All the basic manipulations of CHO cells should be carried out in a biosafety cabinet (BSC) or tissue culture hood. CHO cells are grown at 37°C in a CO₂ incubator with humidified air. The concentration of CO₂ is determined based on the amount of bicarbonate in the media being used. (See Table 9.3.)

TABLE 9.2. Selection Markers and Antibiotics

Drug-Resistant Gene	Selection Agent	Vendor
Neomycin-phosphotransferase	G418	Invitrogen
<i>Streptomyces hindustanus</i> bleomycin gene	Zeocin	Invitrogen
Hygromycin phosphotransferase	Hygromycin	Clontech
Puromycin N-acetyl transferase	Puromycin	Clontech
Dihydrofolate reductase	Methotrexate	ATCC ^a

^a ATCC catalog number for vector pSV2-dhfr is 37146 [20].

TABLE 9.3. Empirical Conditions for Attached CHO Culture

Vessel Type	Surface Area (cm ²)	Growth Medium Volume	Cell Dissociating Agent Volume	Approximate Seed Density Total Cells/Flask	Approximate Total Cell Number at Confluence
96-well plate	0.3	200 µl	100 µl	4.5–9 × 10 ³	3–4 × 10 ⁴
48-well plate	0.7	500 µl	100 µl	1–2 × 10 ⁴	0.7–1.4 × 10 ⁴
24-well plate	2	2 ml	0.5 ml	3–6 × 10 ⁴	2–4 × 10 ⁵
12-well plate	4	3 ml	0.5 ml	0.6–1.2 × 10 ⁵	4–8 × 10 ⁵
6-well plate	9.6	5 ml	0.5 ml	1.4–2.8 × 10 ⁵	1–2 × 10 ⁶
60-mm dish	20	5–6 ml	1 ml	3–6 × 10 ⁶	2–4 × 10 ⁵
100-mm dish	60	10–15 ml	1–3 ml	0.9–1.8 × 10 ⁶	0.6–1.2 × 10 ⁶
T-25	25	5–6 ml	0.5–1 ml	3.8–7.5 × 10 ⁵	2.5–5 × 10 ⁶
T-75	75	10–15 ml	1–3 ml	1–2 × 10 ⁶	0.75–1.5 × 10 ⁷
T-175	175	25–30 ml	2–4 ml	2.6–5.2 × 10 ⁶	1.75–3.5 × 10 ⁷
Roller bottle	850	100–200 ml	10–20 ml	1.3–2.6 × 10 ⁷	0.85–1.7 × 10 ⁸
Expanded roller bottle	1700	300–400 ml	25–50 ml	2.5–5 × 10 ⁷	1.7–3.4 × 10 ⁸
10 layer cell factory or cell stacker	~6360	1000–2000 ml	100–200 ml	0.9–1.9 × 10 ⁸	0.64–1.3 × 10 ⁹

Protocol 1: Passaging Anchorage-Dependent CHO Cells CHO cells are normally split two or three times per week. Although CHO cells can be passaged based on predetermined split ratios, counting cell numbers at each passage (e.g., using a hemocytometer) will help achieve target seed density more reliably. It is good practice to observe the cells at each passage, noting the appearance and morphology of the cells, the degree of confluence (for adherent cells in particular), and whether or not contamination has occurred. Attached cells can be passaged using enzymatic or nonenzymatic methods. Enzymatic methods are generally quicker, but, if cell surface receptors are to be preserved or the complete removal of animal derived components is preferred, then one can choose nonenzymatic detachment methods.

Basic reagents: PBS–CMF (phosphate-buffered saline–calcium and magnesium free), Trypsin/EDTA solution (irradiated trypsin or recombinant trypsin is preferred to reduce the risk of contamination by mycoplasma and viruses), and EDTA solution, fresh growth media.

Methods

1. Remove spent growth media using a sterile pipette or decanting into a clean waste container. Rinse flask with PBS–CMF. If dissociation buffer is to be used, a second rinse with PBS–CMF will help ensure quick and complete detachment of the cells.
2. Add 0.01–0.04 ml of trypsin or dissociation buffer per square centimeter of growth area. Gently tilt and rotate the flask to ensure coverage of the trypsin

or dissociation buffer over all of the monolayer. The flask can be placed in the 37°C incubator, but in most cases cells will detach in 5–10 min at room temperature. Detachment can be encouraged by sharp rapping of the flask against the palm of your hand.

3. Once the cells are detached, add fresh growth media to stop trypsinization or to dilute the EDTA. The volume added depends in part on the amount of trypsin/dissociation buffer added and the desired volume from which to subculture. For a T-75 flask, bring the volume in the flask to 10 ml. Pipette the cells in and out of a pipette to break up any clumps.
4. Take a sample and count the cells using a hemocytometer or any cell counting device. The cells may or may not need to be centrifuged, depending on how the cells are to be split or reseeded. If the split ratio is low (1 : 2 or 1 : 3), then centrifugation is recommended. Remove cells to a sterile centrifuge tube and centrifuge for 10 min at 1000 rpm. Cells are split in part depending on growth characteristics (slow or fast) and what purpose the cells will be used for and when. Alternatively, new flasks are seeded at a known cell number. CHO cells are generally seeded at $1.5\text{--}3.0 \times 10^4$ cells/cm². That is, approximately, $1\text{--}2 \times 10^6$ cells for a T-75 flask.

Note: Cells will detach faster if the trypsin or dissociation buffer is prewarmed to 37°C.

Protocol 2: Passaging CHO Cells in Suspension Culture

1. In a BSC take a sample of culture to be passaged and count using a hemocytometer or any cell counting device.
2. Based on the target seed density, pipette appropriate volume of cells into the new vessel and add fresh growth media to the appropriate final volume.
3. New flasks are generally seeded anywhere between 1 and 3×10^5 cells/ml. If cells are fast growing, lower number of cells may be seeded. Higher final cell densities are targeted if cells are slow growing or are transitioning to new conditions or media. If the volume of spent media is greater than a third of the fresh media, then the cells should be centrifuged and resuspended in fresh media. This avoids seeding the cells with a high percentage of media that has been depleted of nutrients and that would also have a high level of toxic/inhibitory metabolites, for example, lactate and ammonia.

9.2.4 Cryopreservation of CHO Cells

Cell banking should be made on a fairly routine basis due to the risk of contamination in cell culture and the risk of expression instability. Cell banks of the untransfected CHO host and posttransfected cells at various stages should be made to ensure that if cells from earlier time points are needed due to contamination or another reason, a cell source is available without having to remake the CHO lineage.

Protocol 3: Freezing CHO Cells *Reagents and apparatus:* Growth medium, dimethyl sulfoxide (DMSO) (tissue culture grade), trypsin or dissociation buffer, small sterilizing filters (e.g., Nalgene 125 ml 0.2 μ m disposable), sterile centrifuge tubes, centrifuge, sterile pipettes, BSC, sterile cryovials.

1. Prepare freezing media. Freezing media is growth media with 10% DMSO added. This is then filter sterilized. The presence of selection agents is not necessary nor is it harmful to the cells during this process. DMSO concentrations in freezing media for cell banking in general vary from 5 to 15%. For CHO cells 10% DMSO works well.
2. It can be helpful to prepare your cryovials before you start counting cells. Label the number of vials expected to be frozen. This number is based on the use of the cell bank. Label the vials with cell line name, notebook number, and date.
3. If the cells to be frozen are attached, follow passaging protocol through step 4, when the cells have detached. If more than one flask of cells is to be used, combine the detached cells in a centrifuge tube. Take a sample for counting. If cells are from suspension cultures, sample all flasks that are to be combined and count the cells.
4. Centrifuge the cells at 1000 g for 5–10 min. Discard the supernatant. Based on the cell count above and the target cell density per vial and the number of vials to be frozen, resuspend the pellet in the appropriate amount of freezing media. An example follows: two T-175 tissue culture flasks are trypsinized and the cells are combined in a sterile centrifuge tube. Cell count shows that there are a total of 5×10^7 cells in the centrifuge tube. If 10 ml of freezing media is added, then ten 10 ml cryovials can be made with cells at 5×10^6 cells/ml/cryovial.
5. Aliquot cells into labeled cryovials.
6. Cells can be frozen using many different protocols. There are expensive controlled cooling apparatuses that do an excellent job of cooling cells at $-1^\circ\text{C}/\text{min}$. Alternatively, simply putting the cells in a styrofoam container (e.g., the type that 15-ml centrifuge come in), covered with paper towels, and put in a -80°C freezer will freeze cells adequately. Once cells are frozen (normally overnight), they can be then moved into a mechanical -135°C freezer or a liquid nitrogen freezer for long-term storage.
7. It is important that at least one vial from each bank be thawed to evaluate cell viability and sterility. If cells thaw at low viability or become contaminated, then a new cell bank should be made.

Note: It is best to freeze CHO cells when they are harvested in log phase, usually 2 or 3 days post-subculture. CHO cells can be frozen from attached or suspension culture and in media with or without fetal bovine serum (FBS). CHO cells can be frozen at a cell density ranging from 1×10^6 cells/ml to 2×10^7 cells/ml depending on available cells and the need for quick expansion postthaw.

TABLE 9.4. Transfection Methods for CHO Cells

Method	References	Considerations
Electroporation	[29]	Special equipment required
Calcium phosphate	[30]	Economical
Lipofection	[31]	Number of different reagents are available commercially
Polymer mediated Polyethylenimine (PEI)	[32]	Economical

9.2.5 Cell Line Construction

A. Transfection Methods for Cell Line Construction A number of non-viral methods have demonstrated suitability for introduction of expression plasmids into the CHO host. A direct comparison of these methods has not been published. Therefore, selection of a particular method may depend on considerations listed in Table 9.4. Any method selected may require optimization for the particular CHO host to be transfected. Points to consider include DNA amount, ratio of carrier to DNA, cell density, and medium composition. Medium components, in particular serum, can adversely impact some of the methods. Stable transfection and selection can be carried out either in attached or suspension format. Attached culture allows the convenient removal of dead cells that are not stably transfected. Transfections of adherent cells can be carried out on culture vessels of all sizes. Amount of DNA and reagents used should be adjusted accordingly based on the surface area of the vessel.

Protocol 4: Liposome-Mediated Transfection *Materials:* CHO cell host, culture medium, Lipofectamine 2000 (Invitrogen) or another commercially available lipid-based reagent.

Method

1. Day prior to transfection, seed cell stock so that the appropriate confluence (>90%) will be achieved at the time of transfection.
2. Determine the appropriate amount of DNA and lipid reagent to be used depending on cell type and culture vessel size (see manufacturers instructions).
3. On the day of transfection, form DNA–lipid complex:
 - a. Combine culture medium and DNA.
 - b. Combine culture medium and lipid reagent and incubate 5 min at room temperature.
 - c. Combine DNA with lipid reagent and incubate for 20 min at room temperature.
4. Apply the DNA–lipid complexes to the cells to be transfected.
5. Return to incubator and allow the transfection to proceed for 6 h.
6. After 6 h, remove the transfection mixture from the cells and replace with growth medium.
7. 24 h post transfection, expand the cells 1 : 10 in a new culture vessel.

8. Add the appropriate selective pressure 24 h later.
9. Cell death should be apparent within the next 3 days.
10. Between 5 and 10 days after the addition of selective pressure, growth of stably transfected cells should become apparent as small colonies form and increase in size in the culture vessel.
11. At this point there are two options:
 - a. Individual colonies can be selected for expansion and productivity evaluation.
 - b. All stably transfected cells can be combined to form a pool of cells expressing the desired molecule.

Notes

1. Steps 2–6 may be specific to lipid reagents manufactured by other companies and may need a minor amount of optimization depending on specific CHO host used. Step 7 and beyond would apply to any of the transfection methods listed in Table 9.1.
2. Cryopreservation shortly after selection provides a backup stock of cells.
3. The total amount of DNA introduced during the transfection should be within the range specified by the protocol. If more than one expression plasmid is introduced, the total amount of DNA must remain the same and be divided among the plasmids to be transfected. The ratio of DNA for each plasmid can vary and should be determined based on the requirements of the experiment (if more expression of one gene is needed, then that gene should make up a larger proportion of the total).

B. Selectable Markers for Selection of Stable Cell Lines To generate stable cell lines, a gene conferring resistance to a culture additive needs to be included in transfections. Table 9.5 lists a subset of resistance markers and the concentration range of the corresponding selection agents commonly used in CHO cells. The appropriate concentration of drug to be used for selection depends on the cell type and the vectors used to express the selectable marker. Very often the concentration is determined experimentally for each cell type/vector combination. The references listed in Table 9.5 describe different mechanisms employed with drug selection. Cells typically need to be actively dividing so that the drugs can effectively kill those unstably transfected cells.

TABLE 9.5. Ranges for Common Selection Agents

Selection Agents	Typical Concentration Range	References
Neomycin/G418	0.4–1 mg/ml	[33]
Zeocin	50–300 µg/ml	[34]
Hygromycin	0.05–1 mg/ml	[35]
Puromycin	1–10 µg/ml	[36]
MTX ^a	0–100 µM MTX	[37]

^aAmplifiable marker—usually requires the use of a DHFR-deficient host.

Protocol 5: Determination of Selective Agent Concentration For successful establishment of a cell line that expresses proteins in abundance, it is necessary to determine the minimum concentration of selection agents required for efficient killing of untransfected cells. It is important to carry out this experiment for a new vector or host.

Method

1. Plate out attached cells to 25–50% confluence in a 6-well plate.
2. Prepare medium containing a range of drug concentrations.
3. After cells have attached, add medium containing a different concentration of drug to each well. Be sure to include one well with no drug.
4. Monitor health of cells for 3–7 days:
 - a. Check the culture confluence and record.
 - b. Split any cultures that become confluent during the monitoring period.
 - c. If cells do not survive this procedure, a lower drug concentration may need to be selected.
5. Choose the minimum concentration at which no cells survive.
6. Test this selected concentration on cells that have been transfected with an expression plasmid harboring a selectable marker—growth of stably transfected cells should be apparent after cell death ceases.

C. Clonal Cell Line Isolation Isolating cell colonies from cell culture dishes can be achieved in several ways with various levels of complexity. Three different protocols for manual operation are described as follows.

Protocol 6.1: Ring Cloning Method

1. Mark the cell colony location from bottom of the cell culture dish.
2. Remove media and wash cells with PBS–CMF.
3. Apply sterile glass column on top of cell colony and seal with autoclaved Vaseline.
4. Detach cells by one drop of trypsin or detach buffer for 1–2 min.
5. Add 50–100 μl of selection media and transfer cells to 24-, 48-, and 96-well plates and continue the drug selection.

Note: This method requires the colonies in the dish to be far enough apart to allow only one colony in a glass column.

Protocol 6.2: Trypsin Filter Paper Method

1. Mark the cell colony location from bottom of the cell culture dish.
2. Remove media and wash cells with PBS–CMF.
3. Apply 3M filter paper disks (2 mm in diameter, previously sterilized and soaked with trypsin solution) directly on top of individual colonies for 2 min.
4. Scrip the colony with the trypsin filter paper and transfer cells to 24-, 48-, and 96-well plates with fresh selection media to continue the selection.

Protocol 6.3: Pipette Tip Method

1. Mark the cell colony location from bottom of the cell culture dish.
2. Remove media and wash cell with PBS–CMF.
3. For each 10-cm dish, add 1 ml of trypsin and monitor cell shape under microscope. When cells become rounded, slowly remove trypsin and add growth medium back to culture dish to cover all the cells.
4. Scrap and aspirate the cells of individual colonies into pipette tips and then transfer them to 24-, 48-, and 96-well plates with fresh selection media to continue the selection.

D. Screening Stable Cell Lines (Expression Analysis) Several methods are commonly used for identifying high expressing stable cell lines, such as enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blot, and dot blot. During expression analysis, it is common to replace the serum-containing selection medium with serum-free medium when cells grow to confluence. Then cells can continue being cultured at 37°C for 24–48 h prior to the media harvest for analysis.

1. ELISA: A powerful method to detect and quantitate protein expressed into the conditioned media (CM). The common enzymes used for ELISA are horseradish peroxidase (HRP), alkaline phosphatase, and β -D-galactosidase. ELISA can be performed in 96-well (or 384-well) polystyrene plates. ELISA requires a pair of target-specific antibodies. For detail protocols please see “Screening hybridoma culture supernatants using ELISA” by Page and Thorp [38].
2. SDS–PAGE: Can be used to determine the purity and relative molecular mass of target protein in CM after initial screening. If cells have robust expression of target protein, Coomassie blue staining should be able to visualize the protein band without having to concentrate the CM. The minimum amount of protein required for Coomassie blue staining is about 0.2 μ g for each lane.
3. Western Blot. After SDS–PAGE separation, Western blot should be preferred if (a) expression level is relatively low or (b) the CM contain serum, which makes Coomassie blue staining very difficult. A specific antibody, which can recognize denatured target protein, is required. The method has been described in detail by Page and Thorpe [39].
4. Dot blot. A convenient alternative to ELISA, dot blot could be used for a quick initial screening. This method is not quantitative and can only be used for serum-free conditioned medium. It can be done in 2–3 h. Usually 5 μ l of CM is needed to spot directly on nitrocellulose membrane. The rest of procedure is the same as Western blot after protein transfer from polyacrylamide gel to nitrocellulose.

9.2.6 Scale-Up of CHO Cells for Recombinant Protein Production

The scale-up of the established CHO cell lines is a critical step for producing sufficient quantities of recombinant protein. In this section, procedures to grow large-scale, attached as well as suspension, CHO cells are described.

A. Adherent Cells This section describes procedures for growing attached CHO cells at scales sufficient to produce protein for research needs. In general, FBS is present in the media used for the growth and attachment of adherent CHO cells. However, FBS does present purification issues. Fortunately, CHO cells can remain adherent and quite healthy for several days after serum is removed from confluent culture. This allows for the production of CM from adherent cells in serum-free conditions. Serum-free expression media can be harvested from the attached culture at 24, 48, or even 96 h and additional serum-free expression media can be added. The best time for harvests and refeeds needs to be tested empirically. Harvest time is based on expression levels and the quality of protein expressed as well as cell health, for example, levels of viability and attachment. It sometimes is better to do more repeated harvests than one extended production phase. Unlike hybridomas, CHO cells should remain as viable as possible during expression periods to ensure consistent protein quality.

Protocol 7: Scale-Up of Adherent Cells for Expression

Method

1. Determine the doubling time of established CHO cell lines by seeding at desired density, grow them under the cloning conditions, harvesting cells at the time of confluence and counting harvested cells.
2. Confirm secretion of the recombinant protein using appropriate analytical methods, such as Western blot, protein capturing, or activity assay.
3. Determine the ultimate scale of expression based on the expression level and the amount of protein required. Then choose the type, size, and number of tissue culture containers required. The commonly used vessels include T-175's, roller bottles, and cell factories.
4. Continue splitting confluent CHO cells in ratios no higher than 1 : 8 until the culture reaches the target size. During this scale-up, always use the media in which the cell line was cloned.
5. When the final subculture reaches confluence, remove the media, rinse the culture once with PBS-CMF, and then add serum-free media for the eventual protein production.
6. Harvest the conditioned media at predetermined time, usually 24, 48, or 72 h after the media switch. Add fresh serum-free media to culture the cells again for the next harvest. A second, third, or even forth serum-free harvest can be obtained based on the state of the cells (i.e., if they are still well attached and healthy looking).

7. Remove cell debris by centrifugation or filtration prior to purification.

Note: A convenient scenario is to seed flasks with cells late in the week (e.g., Thursday or Friday) in growth media and allow the cells to grow to confluence over the weekend. Three to four days postseeding, the growth media can be removed and serum-free media added. The first harvest of serum-free conditioned media is then obtained 24–72 h postmedia switch.

B. Scaling-Up of Suspension Cells for Expression Once CHO cells are adapted to suspension growth in serum-free media, the eventual volume of the harvest is limited simply by the size of the equipment available. The basic strategy for scale-up is diluting/splitting cultures at high density to low density in the culture vessels such as shakers, spinners, or bioreactors of various degree of sophistication. Cells are maintained on their normal split regimen, splitting every 3 or 4 days. Seed densities remain the same during scale-up.

Cells should remain under selective pressure for as long as possible during scale-up. Timing of selection removal is based mostly on cost. Stable CHO cells will continue to secrete protein for many generations after selection is removed. There is generally plenty of time from removal of selection pressure and the end of the production period before expression decreases to unproductive levels.

Once the cells are at the final volume, expression duration can be lengthened and various strategies implemented to increase titers. These strategies can include media concentrate additions, temperature shifts, and/or chemical inducers such as sodium butyrate.

9.3 GENERATION OF STABLE CELL LINES FOR THE PRODUCTION OF REPLICATION-DEFECTIVE VIRAL VACCINES

A number of production issues must be considered for developing a complementing cell line for propagation of replication-defective vectors as vaccine candidates. First, CHO cells, though an excellent source for protein antigen production, may not be a permissive host for many replication-defective vectors. Second, the complementing cell line must supply the missing protein in sufficient quantities to support packaging of abundant virus particles. Finally, some complementing viral proteins may be cytotoxic resulting in cell line instability. One potential solution is to produce stable cell lines that express the complementing protein from an inducible promoter. The authors have used Vero cells (source: ATCC [Manassas, VA], Vero working cell bank) for producing a packaging cell line in which the complementing protein was expressed under the control of a transcriptional sequence regulated by the cellular heat shock response elements. The induction controlled by heat shock (HS) provides additional advantages because gene expression can be turned on by a simple temperature shift, and no chemical compounds are added to the medium to control gene expression. The detailed procedure for production and characterization of such a cell line has been described recently by Witko et al. [40].

Protocol 8: Production of Replication-Defective Vaccine from Complementing Cell Line Vero cell lines can grow as adherent cells in serum containing Dulbecco's Modified Essential Medium (DMEM) and are also adaptable to serum-free conditions. The induction of the protein of interest and thereby production of the replication-defective vaccine is accomplished by using HS treatment of the monolayer.

1. Seed T-flasks or 6-well plates at a cell density that results in ~20% confluence. Transfer flasks to 37°C incubator (5% CO₂/humidified).
2. When cell monolayer is nearly confluent, remove media and add fresh media.
3. For HS treatment, transfer flasks or plates to 43°C incubator (5% CO₂/humidified) for 2–6 h. For each cell line, determine the optimal time and temperature for HS treatment. Be sure to include one flask as no HS treatment control.
4. Transfer flasks to 37°C incubator (5% CO₂/humidified) for cells to recover and to induce protein synthesis.
5. Monitor level of protein induction after HS treatment by any of the following methods:
 - a. Western blot analysis using supernatant for secreted protein or total cell lysates for intracellular or membrane-associated proteins as mentioned in Section 9.2.5.D.3.
 - b. Immunofluorescence (IF) staining assay is a semiqualitative estimation of protein induction and is performed by immuno-staining of cells fixed with methanol/acetone fixative using monoclonal antibodies specific to the target protein.
6. For production of replication-defective virus, cells are infected at a low multiplicity of infection at the time when production of complementing protein is optimal. When cells indicate visible cytopathic effect, the culture media are harvested and saved for estimation of virus yield by virus-specific potency assay [40]. Culturing conditions that show the highest virus yield are identified.

9.4 CONCLUSIONS

Generation of stable mammalian cell lines represents a major advancement for the manufacture and development of vaccines. The use of cell lines has allowed for the efficient generation of proteins offering enhanced productivity, safety, and cost-effectiveness for subunit vaccine production. They also have provided significant support for the utilization of replication-defective viral vaccine platform technology. In addition, recombinant proteins expressed by stable mammalian cell lines also can be used for assay development and basic science research in support of developed vaccines.

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PART 3

CHARACTERIZATION OF IMMUNOGENS

10

SPECTROSCOPY OF VACCINES

C. Russell Middaugh and Sangeeta B. Joshi

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10.1 INTRODUCTION

The analysis of vaccines has traditionally involved measurement of their induction of immunological responses in animal models or in the case of live viral agents their

ability to transfect cells in culture. Although this has the major advantage that results represent properties of the vaccines necessary for their actual effectiveness, both types of assays suffer from a lack of high precision and accuracy as well as low throughput. This is especially problematic during formulation and stability studies where high throughput and precision are of major importance. This situation differs significantly from that of modern recombinant protein-based products where the physical and chemical properties of the therapeutic agents play major roles in the establishment of the identity, structure, and stability of pharmaceuticals such as therapeutic proteins.

The major problem with the use of chemical and physical approaches in the context of vaccines is the much greater complexity of most vaccines and their formulations. Although a number of experimental vaccines consist primarily of a single type of protein or deoxyribonucleic acid (DNA) molecule, the majority of marketed vaccines consist of multiple, identical components in particle form (virus-like particles, or VLPs) or collections of different components such as viruses or carbohydrate conjugates. Thus, any property measured may directly or indirectly reflect multiple contributions. A wide variety of methods can be employed to characterize such systems. These include chromatography, electrophoretic, hydrodynamic, calorimetric, and spectroscopic techniques. In this chapter we confine ourselves to spectroscopic analyses with other approaches described in additional chapters in this volume. These methods are primarily sensitive to the physical properties of macromolecules and their complexes such as conformational changes, aggregation, and adsorptive phenomena and thus will be the primary targets of our discussion here.

Of what use might the spectroscopic methods be to the vaccinologist? These can, of course, be used to define the identity and structural integrity of vaccine components including target antigens. Of equal or greater importance is their use during formulation development, especially during accelerated and real-time stability studies. Some of their applications will be discussed in more detail below. An absolutely critical question is whether the changes detected by spectroscopic (or other physical) measurements can be directly or indirectly correlated with losses of immunogenicity in actual vaccines. Although this is by no means always the case, it does often seem to be at least empirically true to the extent that formulation development can be guided by such analyses. This controversial issue will also be considered below in selected examples for a variety of different vaccine types.

10.2 SPECTROSCOPIC ANALYSES

Most generally, spectroscopy refers to the study of the interaction of energy in a form such as electromagnetic radiation or sound with matter. There are a very large number of such methods. We will be limited here, however, to those that are most readily available to the pharmaceutical/vaccine scientist and provide useful information concerning the structure and behavior of biomolecules. As is often the case, we will consider these methods in terms of their sensitivity to the different orders of structure into which macromolecular systems are usually classified. Primary structure is generally the province of chemically based methods and will therefore not be addressed

here. For secondary structure analyses, we will discuss far-ultraviolet (UV) circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy, as well as Raman methods. Tertiary (three-dimensional) structure will be discussed in terms of UV/Vis absorption spectroscopy, intrinsic and extrinsic fluorescence analyses, as well as near-UV CD. Although the more in-depth subject of another chapter, we will have occasion to refer to light-scattering methods and quaternary structure (association/dissociation and aggregation) related changes. We will then discuss several methods such as fluorescence anisotropy, red-edge excitation, and quenching measurements in addition to ultrasonic techniques to sense alteration in the internal dynamic motions of vaccine components. Finally, we will describe how it is possible to synthesize results from multiple measurements into global pictures of the behavior of vaccine systems using a method known as the empirical-phase diagram (EPD).

The applicability of spectroscopic methods to adjuvant containing formulations, such as those containing aluminum salts, will also be discussed. We will conclude by very briefly considering the future of spectroscopic evaluation of vaccines. We begin our discussion with secondary-structure-sensitive methods.

10.3 CIRCULAR DICHROISM

The most frequently used method to estimate changes in secondary structure of proteins and DNA is far-UV CD. This technique measures the differences in absorption of left- and right-handed circularly polarized light and thus requires optical activity for signals to be produced. Optical activity arises from interactions between electronic and magnetic transition dipole moments in molecules. This interaction produces a helical circulation of charge (optical activity). Thus, optical activity and in turn CD is especially evident in molecules that possess inherent helicity as seen in the α helices of proteins and the DNA double helix. The chromophores of relevance here are the peptide bonds in proteins and the purine and pyrimidine bases in polynucleotides. Analysis of protein CD spectra in terms of individual spectral contributions can be used to quantitatively estimate secondary structure contents of proteins in terms of amounts of α -helical, β -sheet, and disordered regions. We will consider here the use of this method as it can be applied to various types of vaccines. As is customary, we will focus on work from our own laboratory due to its immediate familiarity but will also comment on the studies of others, especially their unique aspects.

The most obvious use of CD involves protein (usually recombinant) based vaccines. In these cases, the spectra will be dominated by the presence of a relatively pure protein, and the application of CD is essentially identical to that for proteins generally. Recent examples of this type include *Clostridium difficile* toxins [1], Region II of the erythrocyte-binding antigen (EBA-175 RII-NG; for a malaria vaccine) [2], a recombinant ricin toxin A-chain vaccine [3], and a recombinant protective antigen (rPA) used as an anthrax vaccine [4]. Far-UV CD spectra of these four proteins are shown in Figure 10.1. The double minima seen near 208 and 222 nm in the *Clostridium*, EBA, and ricin CD spectra indicate a substantial quantity of α helix in each protein. In contrast, the CD spectrum of rPA is dominated by a minimum at approximately

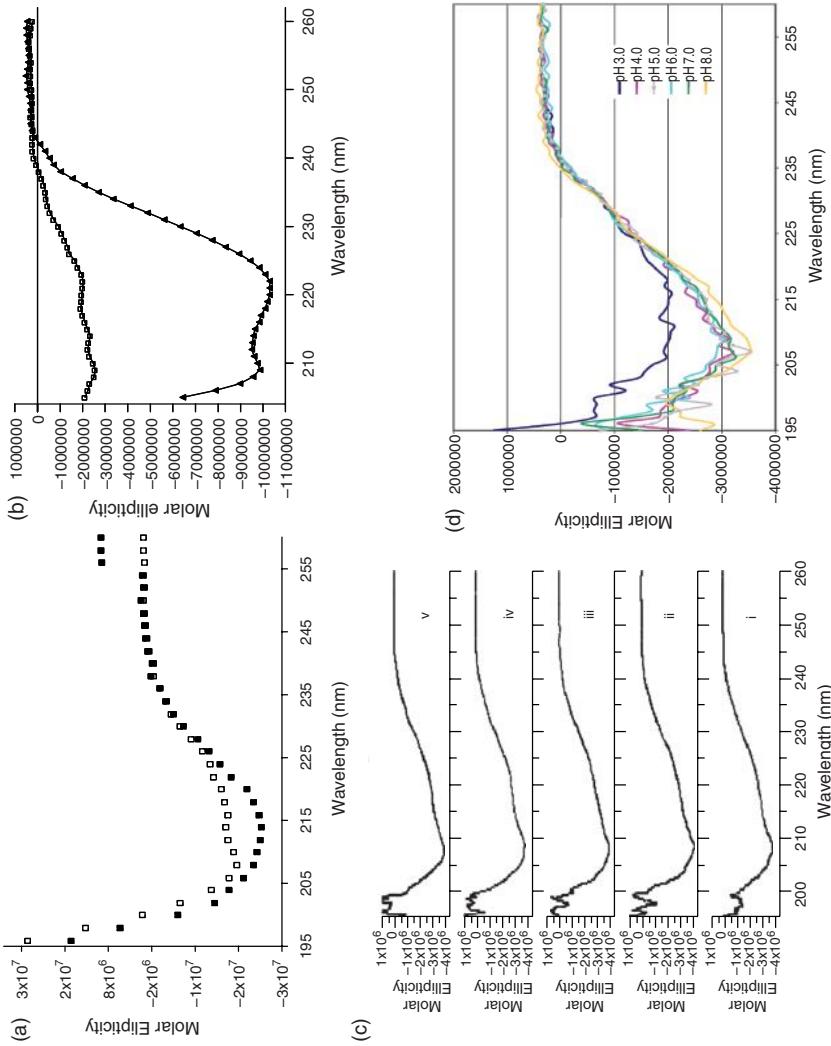


Figure 10.1. (a) CD spectra at 10°C for *C. difficile* toxin B at pH 7.0: before melt (□), after melt (■). (b) CD spectrum at 10°C before (▲) and after (□) thermal denaturation of EBA-175 RII-NG in the presence of 5% sucrose. (c) CD spectra of ricin toxin-A chain at 10°C and pH 4.0 (i), 5.0 (ii), 6.0 (iii), 7.0 (iv), and 8.0 (v). (d) CD spectra of rPA at 10°C. [Reproduced with permission from Wiley-Liss, Inc., the American Pharmaceutical Association (*J. Pharm. Sci.*) and Elsevier, Inc. (*Vaccine*).] (See insert for color representation of this figure.)

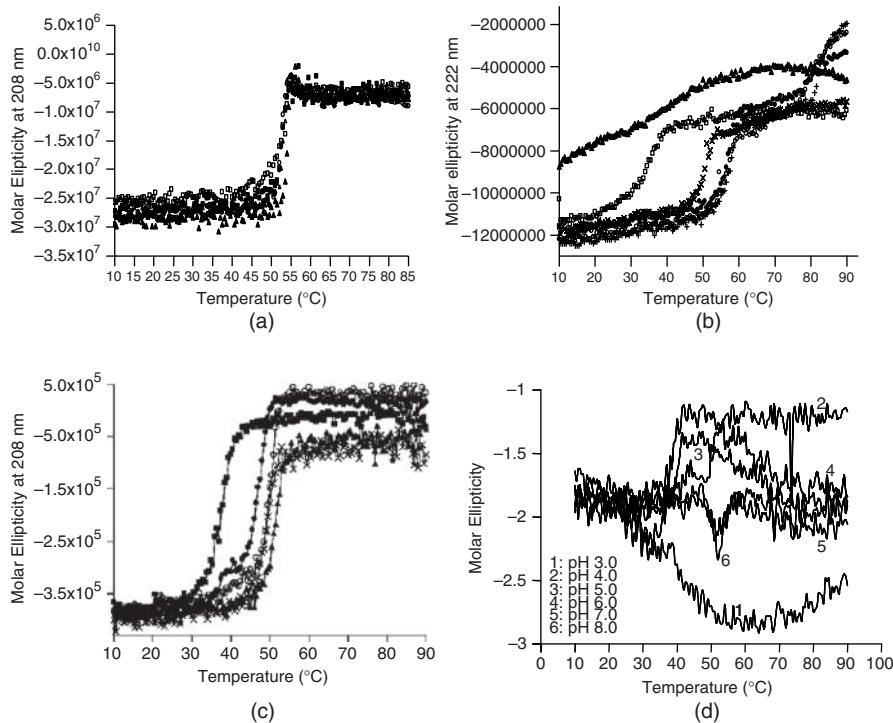


Figure 10.2. (a) CD trace at 208 nm over the temperature range for *C. difficile* toxin A at pH 5.5 (□), 6.0 (■), 6.5 (○), 7.0 (●), 7.5 (△), 8.0 (▲). (b) Thermal melt plots of EBA-175 RII-NG in the presence of 5% sucrose. Molar ellipticity at 222 nm was monitored as a function of temperature for six pH conditions—pH 3.0 (▲), 4.0 (□), 5.0 (x), 6.0 (○), 7.0 (+) and 8.0 (●). (c) Molar ellipticity at 208 nm was monitored for ricin toxin-A chain as a function of temperature for five pH conditions—pH 4.0 (■), 5.0 (x), 6.0 (▲), 7.0 (○) and 8.0 (●). (d) CD thermal melts of rPA monitored at 222 nm. [Reproduced with permission from Wiley–Liss, Inc., the American Pharmaceutical Association (*J. Pharm. Sci.*) and Elsevier, Inc. (*Vaccine*).]

206 nm indicative of a mixture of β structure and α helix. The position of this peak below a 217/218-nm signal typically seen for β structure is consistent with the presence of type II β sheet. In all four cases, spectra were also obtained as a function of temperature and pH (Fig. 10.2). Distinct transitions are seen in all cases, which vary with the two environmental perturbants. Thus, the stability of the secondary structure of these vaccine proteins can be defined by this simple approach. Similarly, VLPs can usually be directly subjected to far-UV CD analyses as has been demonstrated for both the hepatitis B [5] and human papillomavirus vaccines [6]. A more recent example involves VLPs of the Norwalk virus [7, 8]. In this case a minimum is seen at 205 nm with a shoulder at 220 nm. This again suggests a β -II-type protein, which is in agreement with the virus's crystal structure. The pH and temperature dependence of the CD spectra display complex transitions, which have been interpreted in terms of

the domain structure of the proteins [7]. This data has been used in conjunction with other biophysical measurements to prepare more stable formulations of the Norwalk VLP vaccine [8].

The real challenge to the use of far-UV CD in the context of vaccines occurs when the technique is extended to more complex molecular systems such as actual viruses. It is, however, quite possible to make such measurements. This is at least partially possible because molecular entities such as viruses tend to be composed on a weight basis of primarily one or a few components such as coat protein. If structural changes to these components result in changes in immunogenicity, then CD and other spectral measurements can be of significant utility. Note that it is, however, usually necessary to purify the virus before spectral examination. We briefly consider four examples.

Measles virus manifests an α -helical-like far-UV CD spectrum presumably displaying contributions from the N, M, and P proteins [9]. These spectra are both pH and temperature dependent and are very broad in nature as well as distorted by particle aggregation (Fig. 10.3). Respiratory syncitial virus (RSV) also displays an α -helix-like spectrum with broad thermal transitions that are strongly pH dependent [10, 11] (results not illustrated). The CD spectra of adenovirus (Type 2 and 5) are less distinctive, suggesting a mixture of α helix and β sheet but with somewhat sharper thermal transitions [12, 13]. Quite well-resolved CD spectra are also obtained from inactivated viruses such as that used in the hepatitis A vaccine. In this case, the viral ribonucleic acid (RNA) also contributes to the observed CD spectra [14].

Because of the strong far-UV CD of nucleic acids, CD is also quite useful in the study of DNA vaccines, either “naked” or complexed to delivery agents. The most common DNA conformation (the B form) produces positive and negative peaks near 275 and 245 nm, respectively, with a crossover point near 258 nm [15]. Other forms of DNA (e.g., A, C, Z, etc.) possess quite different spectra. When plasmid DNA (the most common form used in vaccines) is complexed to various polycationic delivery agents such as cationic lipid vesicles, polyethyleneimine, polylysine, amino dendrimers, and the like, large changes are seen in the DNA’s spectrum, most usually involving reductions in intensity of the two oppositely signed DNA bands [16, 17]. This has been explained as resulting from significant changes in the interaction between the bases induced by the binding events [18]. Thus, CD spectroscopy has the potential to provide a very sensitive measure of the structure of DNA within these types of vaccines.

10.4 FOURIER TRANSFORM INFRARED SPECTROSCOPY

A second method used to examine secondary structures in proteins and nucleic acids is infrared spectroscopy. This method is always used in a Fourier transform mode today and it is thus referred to by the abbreviation FTIR. This approach analyzes the normal modes of vibrations of covalent bonds (like Raman spectroscopy described below). Changes in dipole moment are necessary for IR absorption intensities to be observed and most commonly involve stretching and bending modes of nonsymmetrically bonded atoms. In the case of proteins, the amide I, II, and III bands near

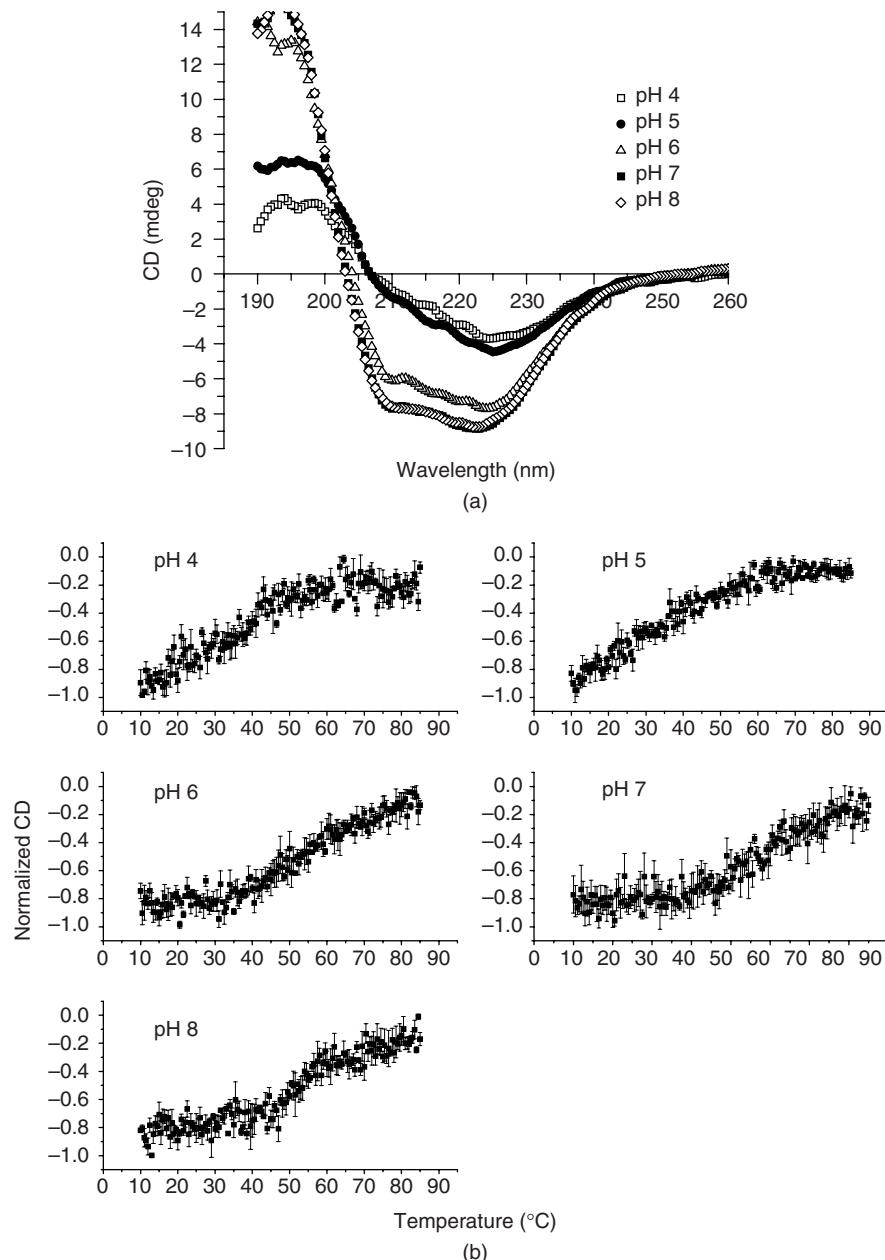


Figure 10.3. (a) Far-UV circular dichroism of measles virus at 10°C. A loss of viral protein secondary structure is exhibited at low pH. (b) CD of measles virus at 222 nm is shown as a function of temperature. Each point represents the mean of three normalized measurements. The error bars represent the standard deviation. [Reproduced with permission from Landes Bioscience, Inc. (*Hum. Vaccine*).]

1700–1600, 1575–1475, and 1300–1220 cm^{-1} due primarily to C=O stretching (80%) and NH stretching (60%) and a combination of C—N stretching (30%) and N—H bending (30%), respectively, are the most commonly employed signals to monitor secondary structure. In contrast, vibrational bands from nucleic acid bases (C=O and N—H stretching vibrations) and phosphate groups as well as OH absorptions from the sugars are most often examined in DNA and RNA. Positions of several of these peaks (after Fourier self-deconvolution or derivative analysis) can be used to estimate secondary structure content. Descriptions of these analytical procedures can be found in a number of reviews [19, 20]. If lipids or carbohydrates are present in vaccines (e.g., Hep B, carbohydrate conjugates, etc.), signals from these components can also often be resolved and used in establishing the molecular identity of the vaccine. Other advantages of the FTIR approach concern its ability to examine samples in virtually any physical state (solid, gel, liquid, etc.) including proteins adsorbed to aluminum salt adjuvants [21] (see below) using alternative sample geometries such as attenuated total reflectance (ATR) and diffuse reflectance (DRIFT) in addition to the more commonly employed transmittance mode. FTIR spectroscopy is also a very versatile technique and can be used in a two-dimensional mode to monitor derivative isotope exchange using the amide II band and employ isotope editing to obtain more detailed structural information. The latter methods are powerful research tools but have not yet been applied to the routine analysis of vaccines, although they will be described further below. We next briefly describe some of the more recent applications of FTIR to vaccines.

Despite its potential widespread utility, FTIR has seen only limited application to vaccines and vaccine-related macromolecules. Here we provide a brief description of some of these applications. The chief use of FTIR over the last 30 years has been to recombinant proteins and the use of FTIR with vaccines reflects this history. Thus, this approach has been employed to characterize a number of potential recombinant protein-based vaccines such as those for ricin A-chain [22], tetanus toxoid [23], human immunodeficiency virus (HIV) gp41 both in solution and adsorbed to aluminum hydroxide [24], and apolipoprotein both in solution and in immune stimulatory complexes (ISCOMs) [25]. The use of FTIR to examine proteins adsorbed to aluminum salt adjuvants is described in detail below and elsewhere [21, 26, 27].

The current situation, however, is that vaccines containing recombinant antigens in monomeric form have not proven very effective, even in the presence of various adjuvants. In contrast, the reassembly of such proteins into spherical particles, especially in the case of viral surface proteins to produce VLPs has been remarkably successful. The two commercially successful VLP-based vaccines for hepatitis B [5] and human papilloma virus (HPV) [28] VLPs have both been analyzed by FTIR with changes in structure easily resolved (Fig. 10.4). In the case of the former, spectra of the VLPs could also be obtained in the solid state while the HPV VLPs could be analyzed in an aggregated form.

FTIR has also been used to probe structural changes in VLPs constructed from the glycoprotein E1 ectodomain of hepatitis C virus for use as a parenteral vaccine [29]. Similarly, conformational changes during the self-assembly of a hepatitis C core protein have been analyzed by FTIR [30]. In this example, a combination of

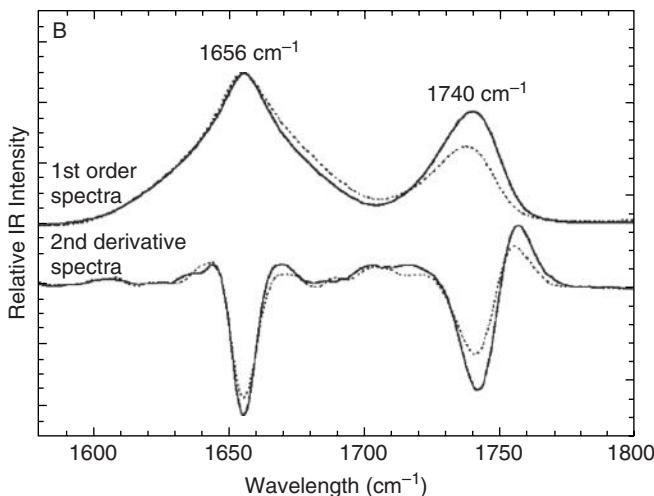


Figure 10.4. FTIR spectra and the second derivatives of the original spectra of HBsAg samples before and after the encapsulation into and release from PLGA microspheres: (solid lines) untreated HBsAg; (dashed lines) HBsAg released from PLGA microspheres. [Reproduced with permission from Wiley-Liss, Inc., and the American Pharmaceutical Association (*J. Pharm. Sci.*).]

H/D exchange and two-dimensional correlation spectroscopy was employed to obtain more internal dynamic information concerning the particles. In a related example, microparticles containing an extracellular antigen used in an oral melanoma vaccine permitted the structure of the antigen to be successfully analyzed [31]. In a final illustration of the use of FTIR for the analysis of vaccines, crosslinked protein crystals of human serum albumin used as a model antigen were successfully analyzed [32].

FTIR has also been successfully used in the analysis of DNA-based vaccines [16, 33]. The focus has primarily been on the DNA component, although in some cases the infrared spectrum of other components within the formulations has been simultaneously monitored. This has been most extensively studied with cationic lipid/DNA complexes [18, 34, 35]. Studies of soluble complexes were also able to detect the direct interaction between the two components, monitor the effect of cholesterol, and note changes in hydration of the complexes [34]. It was also possible to demonstrate that the DNA remained in the B form in contrast to earlier suggestions based on CD measurements that alternative forms might be present [18]. It is important to note that these studies base their analysis on peak shifts (rather than intensity) changes and that multiple measurements were necessary to obtain statistically significant results. More detailed studies of the hydration of solid-state cationic lipid/DNA complexes have also been reported [36]. The flexibility of the approach is well illustrated by further studies of these complexes in which the focus was on the CH₂ asymmetric stretching frequency of the lipids themselves [36]. In this case, it was possible to observe the gel to liquid crystalline phase transition using temperature as a perturbant.

Based on the results described above, it is not surprising that it is also possible to obtain fairly high resolution FTIR spectra of viruses. Although to the best of our knowledge, such spectra have not yet been reported for either live or inactivated viral vaccines, it is clear it should be possible to do so. For example, FTIR studies of the filamentous virus Pf1 [37] and cucumber mosaic virus [38] have been reported. As mentioned above for circular dichroism, such spectra tend to be dominated by one or another of the constituent macromolecules [i.e., the coat protein(s) or the nucleic acid], thus considerably simplifying analysis. The major reason that this approach has yet not been applied to viral vaccines is presumably due to the low concentration of virus in real-world formulations. Nevertheless, it is certainly possible to purify and concentrate viruses of interest and use FTIR in characterization, formulation and stability applications. In a perhaps more extreme but still quite realistic application of FTIR, spectra of intact bacterial cells can be obtained (unpublished results). The utility of such spectra due to their complexity, however, remains to be demonstrated.

10.5 RAMAN SPECTROSCOPY

Raman spectroscopy provides information similar to that of FTIR, although the spectral selection rules are different. In this case, a change in electron polarizability is necessary for a vibrational signal to be seen. At the time of the writing of this review, it seems reasonable to state that this approach has yet to be applied to vaccines to any significant extent. Despite its advantages, its generally low signal-to-noise ratios (necessitating the use of both slower data acquisition times and the use of higher concentrations) and lack of quantitative use of signal intensity changes have generally relegated it to more specialized research uses with macromolecules. Its advantages, however, include lack of strong interfering water vibrations (unlike FTIR) and the presence of well-resolved peaks for aromatic and sulfur-containing side chains as well as amide peptide band peaks similar to those seen in FTIR [39, 40]. The lack of use of Raman spectroscopy in the vaccine world, however, seems poised to change. Recent advances in instrumentation as well as the use of resonance and surface-enhanced effects seem likely to lead to increased application of this powerful approach.

The application of Raman spectroscopy to recombinant protein and VLP-based vaccines is straightforward, and descriptions of such uses are presented in detail elsewhere [39, 40]. Intriguingly, however, increased detailed studies of both viruses and even bacteria suggest that high-resolution vibrational spectra can be obtained that are very sensitive to both structural changes and stability of macromolecular complexes [39, 41]. As with FTIR, it is often possible to see signals from both the protein and nucleic acid components of viruses with the additional advantage that the tertiary structure-sensitive protein side chains as well as purine and pyrimidine bases can often be resolved (Fig. 10.5) (see below also).

Most excitingly, the development of resonance enhancement-based Raman methods offers dramatic increases in sensitivity either through direct excitation of chromophores such as tryptophan or indirectly through the use of metal surfaces such as silver or gold. For example, PEI/DNA complexes have been studied on Ag nanoparticles [42] and viruses [43, 44] as well as bacteriophages, and bacteria themselves

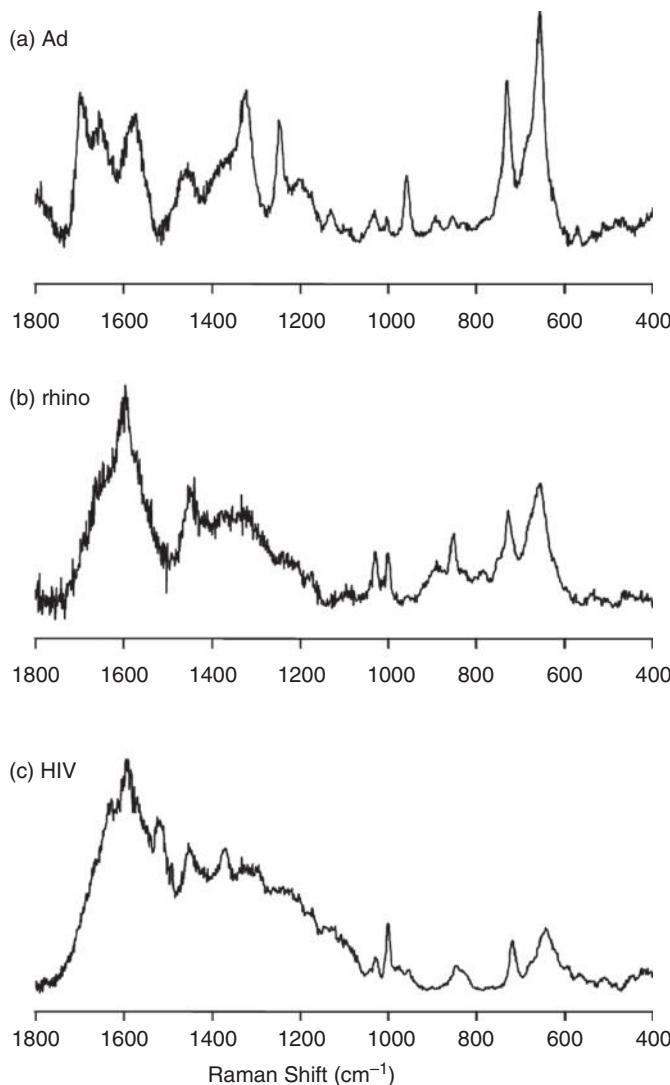


Figure 10.5. SERS spectra of (a) adenovirus (Ad), (b) rhinovirus (rhino), and (c) and HIV viruses. Raman bands can be assigned to chemical constituents such as nucleic acid bases, amino acids, and proteins for all the spectra. The most prominent spectral features observed in the spectra are at 654 cm^{-1} , 730 cm^{-1} , 1247 cm^{-1} , and 1326 cm^{-1} corresponding to guanine, the adenine ring vibration, thymine, and adenine, respectively. The Raman bands between 1580 and 1700 cm^{-1} can be attributed to carbonyl groups on the amino acid side chains and the Amide I vibration while the spectral region near 1000 cm^{-1} has bands due to phenylalanine (1001 and 1030 cm^{-1}). [Reproduced with permission from the American Chemical Society (*None Lett.*).]

[43, 45] have also been successfully characterized by this method. Problems of surface effects need to be resolved, however, since solution and adsorbed species often produce somewhat different Raman spectra. Direct application to vaccines seems in the immediate future. It should also be mentioned that hybrid techniques such as Raman optical activity [46] and vibrational circular dichroism [47, 48] are showing increasing promise, although they have yet to be directly applied to vaccine formulations.

10.6 TERTIARY STRUCTURE

The term “tertiary structure” is usually taken to describe the features of the three-dimensional structure of a macromolecule resulting from a large number of interactions between functional groups that may be quite distant from one another in the primary sequence of amino acids, nucleic acid phosphates, sugars, and bases as well as individual features of lipids and carbohydrates. Here we provide a simple, functional definition: The spectroscopic techniques that we will describe below primarily reflect “changes” in tertiary structure in terms of alterations in the local environments of either specific intrinsic chromophores such as aromatic side chains of proteins or the introduction or loss of binding sites of extrinsic probes. Note that this definition allows us to also examine complex structures such as DNA complexed to delivery vehicles and VLPs as well as intact viruses and bacteria, although the resolution and interpretability of the resultant data may be limited.

10.7 ULTRAVIOLET/VISIBLE ABSORPTION SPECTROSCOPY

Over the last 30 years, the primary use of UV/Vis absorption spectroscopy has been to measure concentrations of macromolecular components such as DNA and protein. Prior to this, these methods had also been used as low-resolution conformationally sensitive analytical tools, but this application was extensively displaced by fluorescence and near-UV-based circular dichroism approaches, which seemed to offer both improved resolution and sensitivity. This has recently changed with the wide availability of diode array spectrometers. These instruments record wavelength intensity data with extremely high precision. This permits high resolution spectra to be obtained, although the diodes are typically spaced at only 0.5- to 2-nm intervals. High-resolution spectra are obtained by interpolating between the data obtained at each wavelength (diode) to the extent that ± 0.01 nm resolved spectra can be obtained under ideal conditions [49]. Such data is typically analyzed in a derivative mode with second- and fourth-order derivatives most commonly employed. Shifts in peak position are the most commonly used experimental parameter, and these are typically obtained as a function of solution variables such as temperature, pH, and ionic strength. Macromolecules can be examined over a wide range of concentrations (e.g., 0.05–400 g/L) by adjusting measurement path length (typically 1 μm to 1 cm). The major problem with this approach is the artifact known as absorption flattening [49], which is due to the shadowing of one particle by another. Due to the particulate nature of many

vaccines (VLPs, viruses, cationic lipid/DNA complexes, etc.), care must be taken in this regard, although minimization of path length can often solve this problem.

High-resolution second-derivative UV absorption spectroscopy has been successfully applied to a number of vaccine candidates. For example, region II of the erythrocyte binding antigen [2], *C. difficile* toxins A and B [1] as well as ricin toxin A-chain [3], all recent vaccine candidates, have been analyzed in some detail by the derivative UV absorption method. In all three cases, thermally and pH-induced transitions are seen in the multiple peaks resolved. An argument can be made that the different location of the three different types of aromatic side chains provide a more detailed description of structural changes than the intrinsic fluorescence approach, which relies almost entirely on the behavior of indole moieties alone. It is proposed that the phenylalanine residues tend to more exclusively sense the highly apolar interior of proteins while the phenolic groups of tyrosine tend to be more interfacially located due to the presence of the polar hydroxide group. Tryptophan residues are found in more diverse environments as seen directly in fluorescence and fluorescence quenching experiments. The same approach seems to work in VLPs as well [7, 8]. So does this approach have any applicability to a more complex particle such as a virus? The answer appears to be yes. In at least two cases, adenovirus [12, 13, 50] and RSV [10, 11], spectral contributions from both proteins and nucleic acid components can be clearly seen (Fig. 10.6). Furthermore, the temperature dependence of the peak positions seen can be directly studied providing stability information about the coat proteins and genetic components of the virus.

High-resolution UV absorption spectra are also available for plasmid-based DNA vaccines as well as potentially RNA-based ones [16]. Perhaps the most common use of UV spectroscopy with nucleic acids is concentration measurements and the

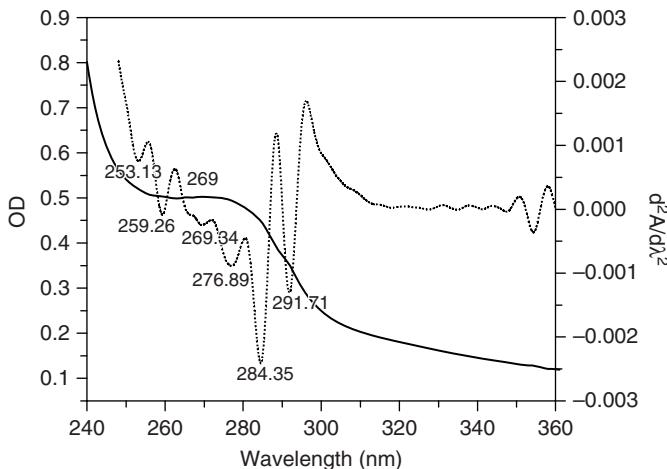


Figure 10.6. Representative UV optical density spectrum of RSV (solid line) and its second derivative (dotted line). Spectrum was obtained at a viral protein concentration of 0.3 mg/mL at 10°C, pH 7. [Reproduced with permission from the American Chemical Society (*Mol. Pharm.*).]

development of melting curves. Analysis of derivative spectra, however, also permit individual DNA base contributions to be at least partially derived, suggesting that this approach may be of future use [51].

10.8 FLUORESCENCE SPECTROSCOPY

Probably the most versatile spectroscopic technique that can be used to examine biological macromolecular-based systems is that of fluorescence [52, 53]. Fluorescence generally refers to the emission of photons from singlet excited states. This emission process is very sensitive to a fluorophore's environment, which results in the energy (wavelength and intensity) of fluorescence emission being very responsive to their immediate environment. Emission from triplet states (phosphorescence) is also a powerful approach [54] but will not be discussed here due to the difficulty of obtaining such spectra under most vaccine formulation conditions. There are a large number of different ways in which fluorescence can be used, but we will restrict ourselves to methods that have either already been applied to vaccines or show some immediate promise in this regard. This will include simple analysis of the wavelength and intensity of fluorescence emission, polarization and quenching of emission, resonance energy transfer, and lifetime measurements. These methods can be applied to either intrinsic chromophores such as the indole rings of Trp residues in proteins or to covalently and noncovalently bound dyes that can be used to label proteins, nucleic acids, and lipid mono- or bilayers. We will consider examples of each in the context of a number of vaccine examples.

The simplest application has been to vaccines containing a homogenous preparation of (usually recombinant) protein molecules. Some examples include rPA vaccine [4], a ricin toxin A-chain vaccine [3], a *C. difficile* toxin vaccine [1, 55], and an EBA-175 RII-NG-based malaria vaccine [2]. The approach has also been successfully applied to VLP-based vaccines such as those for human papillomavirus [6], the hepatitis C envelope glycoprotein E1 ectodomain [29], and Norwalk virus [7, 8] as well as proteins in glycoconjugate vaccines [56]. In such studies, the fluorescence emission spectrum is usually measured as a function of solution variable such as temperature and pH. The position and/or intensity of the tryptophan fluorescence at the wavelength maximum are then plotted as a function of the stress variable. Because absolute fluorescence intensities are usually difficult to define and often less sensitive to environmental changes, the position of the wavelength maximum is often preferred as the output measured. Although simple inspection or derivative analysis is often selected for this purpose, it may be desirable to use a center-of-mass method (Fig. 10.7). Although the latter does not provide a true value of the emission maximum (it is typically shifted 10–15 nm to higher wavelengths), the value obtained often displays superior precision. In the above example, two different types of results are typically seen. The more expected is a red shift as a function of extremes of pH and temperature or other stressed state. Since a red shift in a fluorophore is usually interpreted as an increase in the polarity of its environment, such a result is usually taken as evidence of partial or complete protein unfolding with a shift to 350–355 nm,

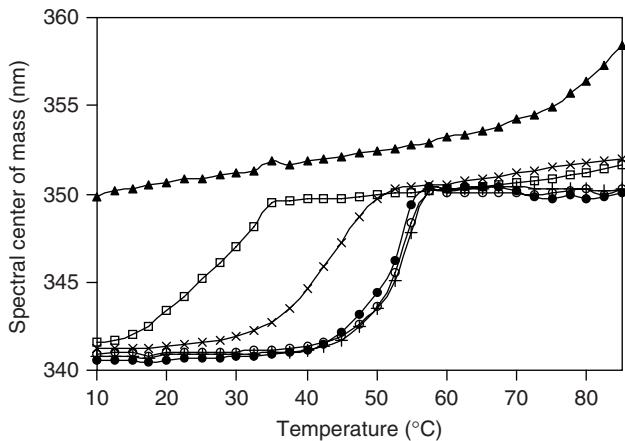


Figure 10.7. Effect of temperature and pH on the intrinsic Trp fluorescence spectral center of mass of EBA-175 RII-NG. The six pH conditions evaluated were pH 3.0 (▲), 4.0 (□), 5.0 (x), 6.0 (○), 7.0 (+), and 8.0 (●). Transitions occurring at higher temperatures indicate more stable conditions for the protein. (The results are shifted +13 nm from actual peak maxima.) [Reproduced with permission from Elsevier, Inc. (*Vaccine*).]

suggesting extensive fluorophore exposure. It is equally common, however, to see a blue shift. This is generally associated with protein or VLP aggregation and presumably reflects the burial of partially exposed indole moieties in the self-associated (aggregated) protein.

Although interpretation of the Trp fluorescence emission becomes much more complex, quite well-resolved fluorescence signals can be resolved from both live and inactivated viruses in solution (Fig. 10.8). Successful application to this type of vaccine candidate includes adenovirus [12, 13, 50], respiratory syncytial virus [10, 11], hepatitis A [14], and measles virus [9]. It should be mentioned that it is also possible to obtain a measurement of light scattering during fluorescence studies. This is simply accomplished by either scanning through the excitation wavelength or preferably by monitoring with a second monochromator located 180° to the fluorescence detector. The latter method is superior since it allows the investigator to independently control the intensity of the scattering signal and subsequent optimization.

All of the above systems can also be probed by the use of extrinsically added dyes. Many proteins and VLPs often display an increase in exposure of apolar sites when they are stressed. A particularly interesting example of this phenomenon involves the formation of what are commonly known as molten-globule states. Thermal induction of such states usually involve the loss of tertiary structure (as monitored by fluorescence, near-UV CD, or absorption measurements) prior to secondary structure loss (as seen by far-UV CD or FTIR studies). These states are of particular interest to pharmaceutical and vaccine scientists since they often provide the origin of degradative aggregation events. Of importance here is that these states often can be detected by the

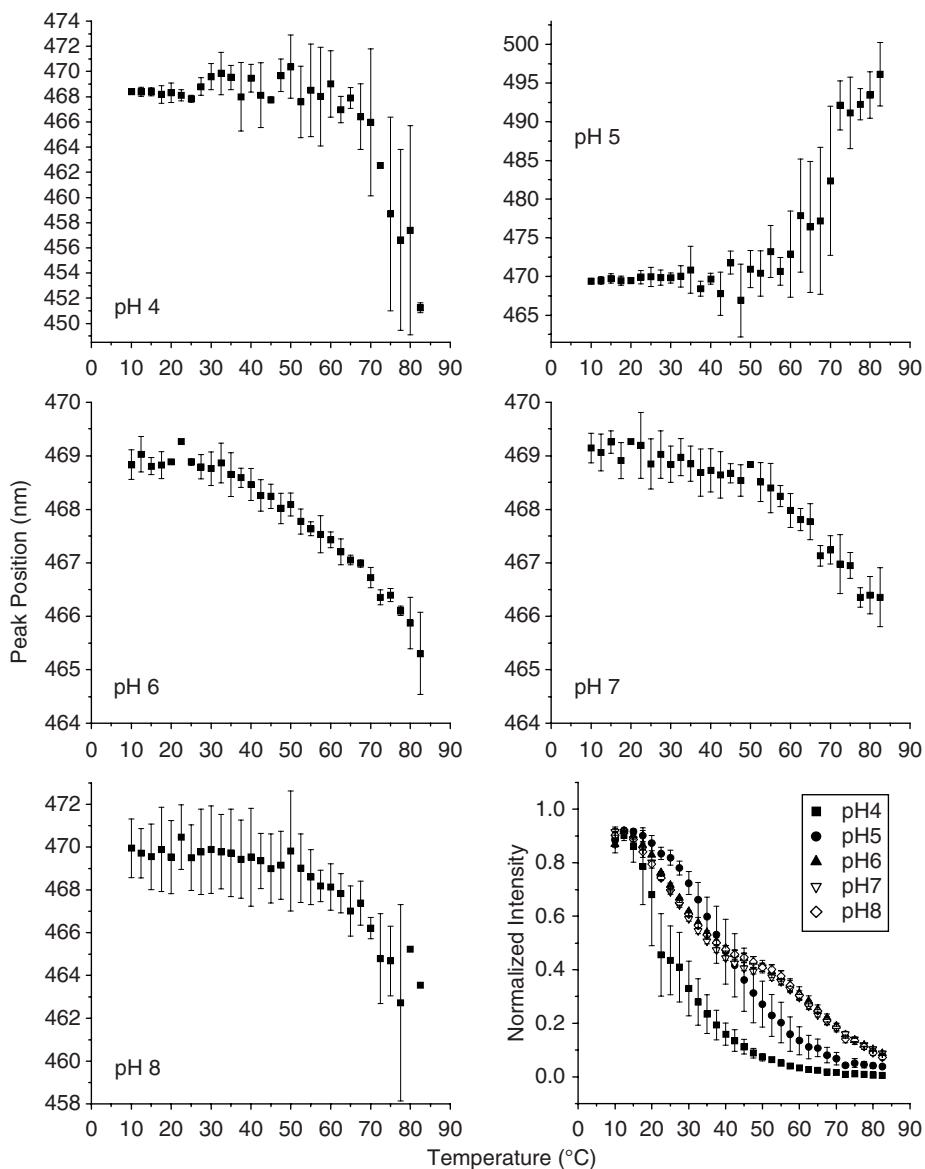


Figure 10.8. Fluorescence of ANS in the presence of measles virus. The average peak position ($n = 3$) is shown as a function of temperature. The lower right panel shows the fluorescence intensity at 485 nm, also as a function of temperature (the mean of three normalized measurements is reported). The error bars represent the standard deviation. [Reproduced with permission from Landes Bioscience, Inc. (*Hum. Vaccine*).]

binding of dyes such as 8-anilino-1-naphthalenesulfonate (ANS) and its dimeric analog (bis-ANS). In fact, numerous examples of this approach can be found in many of the references cited above [2–4, 9, 10]. In general, the appearance of molten globule and other ANS binding states is characterized by an increase in the dye's fluorescence as well as a blue shift due to relief of solvent quenching.

The use of a number of other fluorescent dyes is particularly useful as probes of viral structure. If a virus contains a lipid bilayer, the use of the dye Laurdan is often especially useful. Analysis of spectral changes in this dye has been used to measure the fluidity and stability of membranes. This approach has been successfully applied to both RSV [10] and measles [9] viruses (Fig. 10.9). Another potential use of

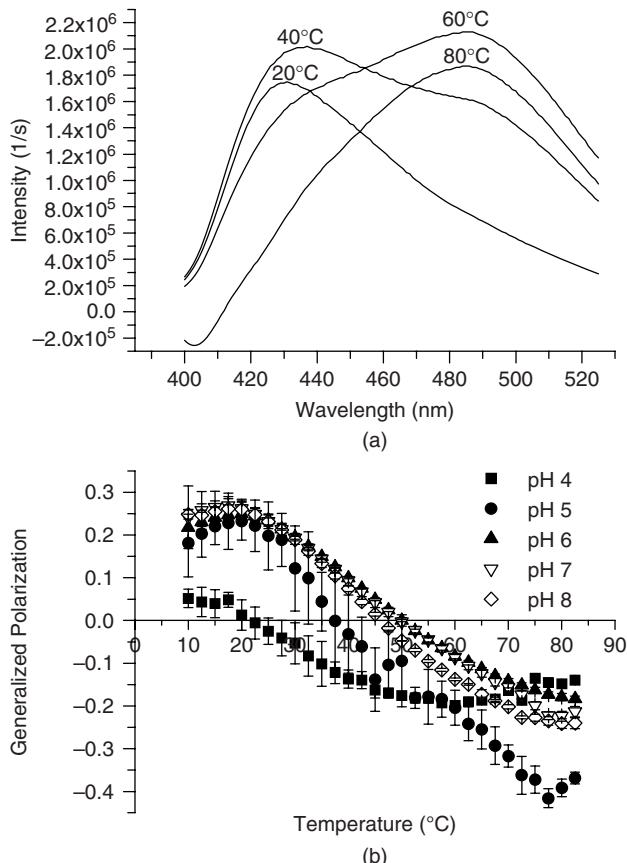


Figure 10.9. Fluorescence of laurdan in the presence of measles virus. Representative spectra taken at pH 7 and various temperatures are shown in (a). Spectra were corrected by subtracting the spectrum at each temperature of a buffer sample containing an equal concentration of dye. Generalized polarization of laurdan fluorescence of MV is shown in (b) as a function of temperature. Each point represents the mean GP of triplicate samples. Error bars represent the standard deviation. [Reproduced with permission from Landes Bioscience, Inc. (*Hum. Vaccine*).]

extrinsically added dyes to vaccines is to use dyes such as Nile Red, Congo Red, and Thioflavin T [57]. These dyes tend to specifically recognize intermolecular β structure, which occurs in amyloid-like structures. In unpublished studies, we have found the latter dyes to be quite useful in the detection of some aggregated viruses of vaccine interest. Various dyes have been used to detect changes in nucleic acids of viruses. For example, the DNA binding dyes, TOTO-1 and propidium iodide, have been used to study changes in the genetic materials of some adenoviruses [12, 13, 50].

Similarly, dyes have been extensively used to structurally characterize DNA plasmids and their complexes for use in vaccines [16]. Most commonly, this involves the use of dyes that become highly fluorescent when they are bound to DNA. When cationic delivery agents such as positively charged lipids, basic peptides, amino dendrimers, polyethyleneimines, or various synthetic block copolymers are added to the labeled DNA, the dyes can be displaced resulting in a decrease in fluorescence (Fig. 10.10) [58]. Much more detailed information can be obtained by the use of fluorescence energy transfer. When the emission peak of a fluorophore (the “donor”) overlaps that of a second fluorescent moiety (the “acceptor”), energy can often be transferred by a nonradiative process from the donor to the acceptor when the donor is excited, resulting in emission from the acceptor. This process is dependent primarily on the distance and relative orientation of the two fluorophores as well as the degree of spectral overlap. As an example of such a study, plasmid DNA was labeled with Hoecht 33285 and cationic lipid with 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine (BODIPY-PE) and the efficiency of energy transfer measured. By varying a variety of compositional and environmental parameters, it was possible to obtain detailed structural information about the complex [59].

The above discussion only hints at the potentially diverse applications of fluorescence-based methods to vaccine systems. Additional techniques such as fluorescence quenching, fluorescence polarization, and lifetime analysis only await application.

10.9 OTHER METHODS TO DETECT CHANGES IN TERTIARY STRUCTURES

In general, intrinsic and extrinsic fluorescence as well as UV absorption spectroscopy are the methods most commonly used to detect changes in vaccine tertiary structures. In the case of recombinant proteins generally, however, several other methods are often used. For example, near-UV CD can be used to detect subtle alterations in the structurally induced optical activity of the aromatic side chains as well as disulfide bonds. Higher concentrations (and/or increased path length) are usually necessary for such studies since the signal is considerably weaker than that seen in the far-UV. Similarly, side-chain signals from the aromatic side chains and disulfides can also be seen in Raman spectra of proteins and used to monitor tertiary structure changes. Furthermore, the near-UV CD spectrum of nucleic acids is also often sensitive to changes in tertiary structures, although as indicated above, interpretation of such

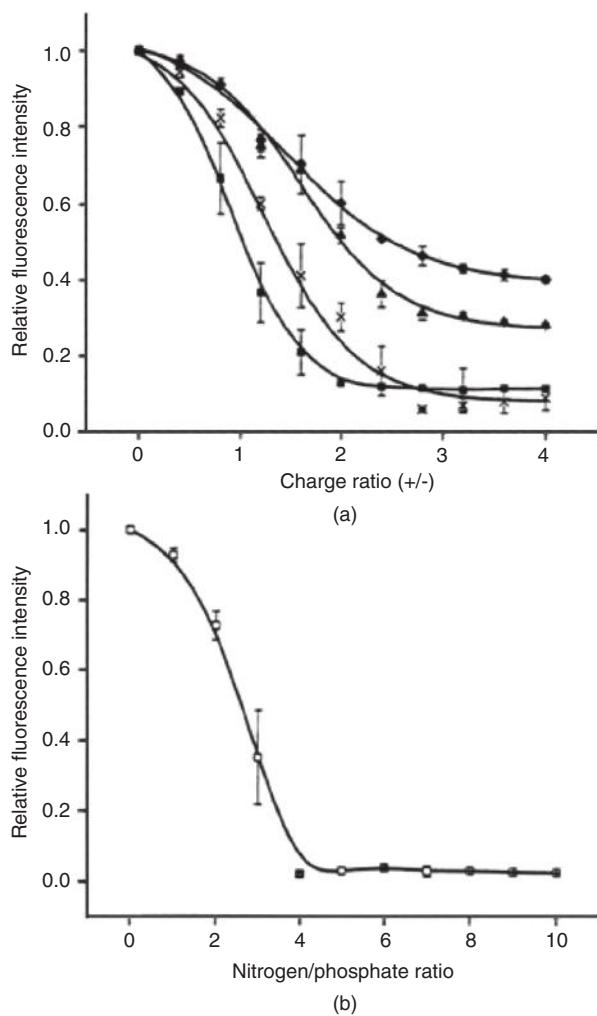


Figure 10.10. Displacement of ETBR by cationic lipids or PEI. DNA was preequilibrated with ETBR at a dye/DNA base-pair ratio of 1:4 in 10 mM Tris pH 7.4. (a) Displacement by lipids. DOTAP (■), DOTAP/DOPE (◆), DOTAP/CHOL (▲), and DDAB (×). (b) Displacement by PEI. Data represent the average and SEM of at least three replicates. Lines represent sigmoidal fits to the data and are meant only as visual guide. [Reproduced with permission from Wiley-Liss, Inc., the American Pharmaceutical Association (*J. Pharm. Sci.*).]

spectra can be quite difficult, although this has been successfully employed in model systems. The presence of structures such as those formed by the terminal repeats in viruses can in principle be seen, although they have not yet been exploited in viral vaccines. Thus, in general, these and other tertiary structure methods have yet to be employed in the analysis of vaccines, although a significant opportunity clearly exists for such applications [25].

10.10 MEASUREMENT OF DYNAMIC ASPECTS OF VACCINE STRUCTURE

The spectroscopic measurements previously discussed all examine time-averaged views of molecular structure and thus provide a relatively static view of vaccine macromolecular components. It is now well accepted, however, that more dynamic behavior also contributes in important ways to the structure and stability of such systems. [60]. The motions involved in such behavior range from small internal fluctuations around individual bonds and highly localized motions to more global behavior such as relative movements of elements of secondary structure to time-dependent changes in domain orientations. Such motions have traditionally been probed by a variety of methods such as proton isotope exchange using mass spectrometry or size exclusion spectroscopy and nuclear magnetic resonance (NMR) methods as well as procedures such as pressure perturbation differential scanning calorimetry (DSC) and molecular dynamics simulations. In addition, a number of spectroscopic methods that can be directly applied to vaccines in many of their forms are also available. Although vaccine “dynamics” have yet to be productively explored, it seems highly likely that this will soon change. Thus, we provide here a few brief examples of such methods in anticipation of future work. We note especially a potentially quite important relationship between the dynamics of proteins and their complexes (e.g., VLPs, viruses) as well as nucleic acids and their stability, which may well prove to be important to the design of more stable formulations. We chose four representative examples of relevant methods based on instrument availability and interpretability of potential results, although other techniques may prove to be helpful as well.

An emerging technique that provides unique information about the dynamic behavior of macromolecules is that of ultrasonic spectroscopy. In this method, the velocity and attenuation of sound waves through a solution of macromolecules is used in combination with precision density measurements to calculate the adiabatic compressibility of the molecule. Thus, internal motions and their coupling to solvent as well as effects of labile cavities in macromolecular interiors may be sensitive to such measurements. Furthermore, compressibility is directly related to fluctuations in volume, an immediate manifestation of dynamic behavior. The instrumentation to perform such measurements has recently become commercially available, suggesting applications to vaccines in the near future. This approach has been successfully applied to proteins including immunoglobulins [61] and should therefore be ready for such applications.

A second spectral method to probe protein dynamics involves the measurement of red-edge excitation shifts. Although it is commonly thought that emission spectra are independent of excitation wavelength, this is often not the case. Fluorescence emission spectra are generally sensitive to solvent reorientation around the fluorophore. When fluorophores are held in relative immobile (highly viscous) environments, solvent reorientation tends to be restricted. This causes a red shift in emission from fluorophores that absorb lower energy photons. In contrast, red-edge shifts are decreased in situations where solvent relaxation is more permissible. In the case of protein indole

side chains, red-edge shifts tend to range from 0 to 15 nm, presumably reflecting differences in the dynamic behavior of these side chains [62–64]. An advantage of this method is its simplicity. One merely needs to vary the excitation wavelength and collect conventional emission spectra. The major disadvantage is an inability to rigorously interpret the resultant data, especially in multityptophan-containing proteins. To the best of our knowledge, this approach has not yet been applied to vaccines, although it has been explored with a number of recombinant proteins [60, 61]. It would be certainly worthwhile to investigate its use in the context of vaccine formulations and their stability.

Another method used to characterize protein dynamics and flexibility is the study of the exchange of peptide backbone amide protons with deuterium. Probably the most informative method used in such analysis is NMR since the exchange rates of individual protons can often be quantified. The often large size of vaccine antigens (e.g., VLPs, viral particles, bacterial cells) clearly limits the application of this approach to the area of interest to us (although see below). Nonspectroscopic methods are also available (e.g., mass spectrometry), but these are not the subject of this review [65]. Infrared spectroscopy (FTIR), however, does offer a convenient method to conduct such analysis. In its simplest form, one can simply monitor changes in the intensity of the amide II band, which is dominated by the presence of peptide NH vibrations and is consequently highly sensitive to isotope exchange. More interestingly, two-dimensional (2D) correlation and heterocorrelation FTIR spectroscopy can be used to analyze specific changes in spectral variables produced by application of external perturbations. This permits a variety of dynamics-related information to be obtained. Although yet to be applied to vaccines, this approach has yielded a quite detailed picture of the exchange rates of 150 kDa IgG molecules [66], suggesting that applications to vaccines are quite feasible.

A fourth spectral approach employs the loss of polarization of fluorescence photons. Such analysis can involve either steady-state or time-resolved measurements with the latter generally preferred. The modern approach of time-correlated single-photon counting (TCSPC) fluorescence anisotropy permits rotational correlation times of both entire macromolecules as well as side-chain and domain entities to be obtained. In the case of the former for proteins, this usually involves analysis of Trp residues. The short lifetime of Trp fluorescence, however, permits only rapid motions to be analyzed. The introduction of extrinsically added long-lifetime (10–100 ns) dyes, which attach either covalently or noncovalently to (preferably specific) sites on macromolecules, may permit slower motions to be detected. Similarly, the addition of long-lifetime fluorescence probes to nucleic acids or carbohydrates or their complexes has the potential to permit analyses of larger scale motion that may be relevant to macromolecular stability. Again, such measurements have yet to be applied to vaccines, but their successful use with IgG molecules [61] suggests that such applications will soon be forthcoming.

10.11 ANALYSIS OF SPECTRAL DATA

In the case of most spectral analysis, especially for highly purified targets such as recombinant proteins or nucleic acids, simple measurements of peak positions and/or

intensity are usually performed as a function of stress variables (temperature, pH, ionic strength, agitation, freeze–thaw cycles, etc.). Because most of the methods employed (e.g., CD, fluorescence, FTIR, etc.) are fairly low resolution, one is usually left with only a vague picture of the relationship between structural changes as induced by various environmental perturbations and stability. Recently, however, a new approach has been developed that combines a variety of different methods into a more global picture known as an empirical phase diagram (EPD) [2, 3, 67, 68]. In this method, the macromolecular system being examined is represented as a vector in a highly dimensioned measurement and environmental variable space. Thus, any combination of experimental variables such as temperature and pH is associated with normalized values of experimental measurements. These variables are combined into single vectors that reflect the physical state of the macromolecule under the indicated physical conditions with the dimension of the vector equal to the number of variables in a data set. All of these vectors are combined into a density matrix, and the eigenvectors and eigenvalues of the vectors are then assigned a color based on an RGB (red–green–blue) color scheme. The result is a color map that displays differently colored regions that represent different physical states of the target macromolecular system as a function of chosen environmental (stress) alterations. Most of the measurements that have employed the EPD approach are spectroscopic in nature with CD, intrinsic, and extrinsic fluorescence and light scattering most frequently used. Thus, changes in secondary, tertiary, and quaternary structure can often all be seen in the pattern of color in an EPD. It should be noted that other techniques such as differential scanning calorimetry, size exclusion chromatography, and isoelectric focusing can also be included in EPDs (and frequently are), but the spectroscopic techniques that are the subject of this chapter are the most commonly used. Perhaps surprisingly, this approach also seems to work well for more complex systems, including VLPs, viruses, bacteria, and nucleic acid complexes. Examples of the vaccine-related EPDs and protein pharmaceuticals are shown in Figure 10.11. All display distinct regions of color that can be associated with various physical states of the vaccines. The real power of the approach resides in the presence of the apparent phase boundaries. It should be emphasized that EPDs are not “thermodynamic” phase diagrams (i.e., no equilibrium is implied across the apparent phase boundaries) but rather merely provide a visual description of the effect of various stress variables upon structural aspects of the subject system. Therefore, the apparent phase boundaries are simply descriptions of the conditions under which structural change is induced. They can be directly used, however, to identify conditions with which to develop high-throughput screening assays with which to test compound libraries for the presence of agents (potential excipients) that can be used to expand the apparent phase boundaries resulting in more stable vaccine formulations (Fig. 10.12).

10.12 SPECTROSCOPY IN THE PRESENCE OF ADJUVANTS

If the presence of adjuvants does not interfere with the acquisition of spectral data (e.g., the absorbance of the adjuvant does not interfere with that of the antigen in crucial spectral regions), then spectroscopic studies may well be straightforward. Even

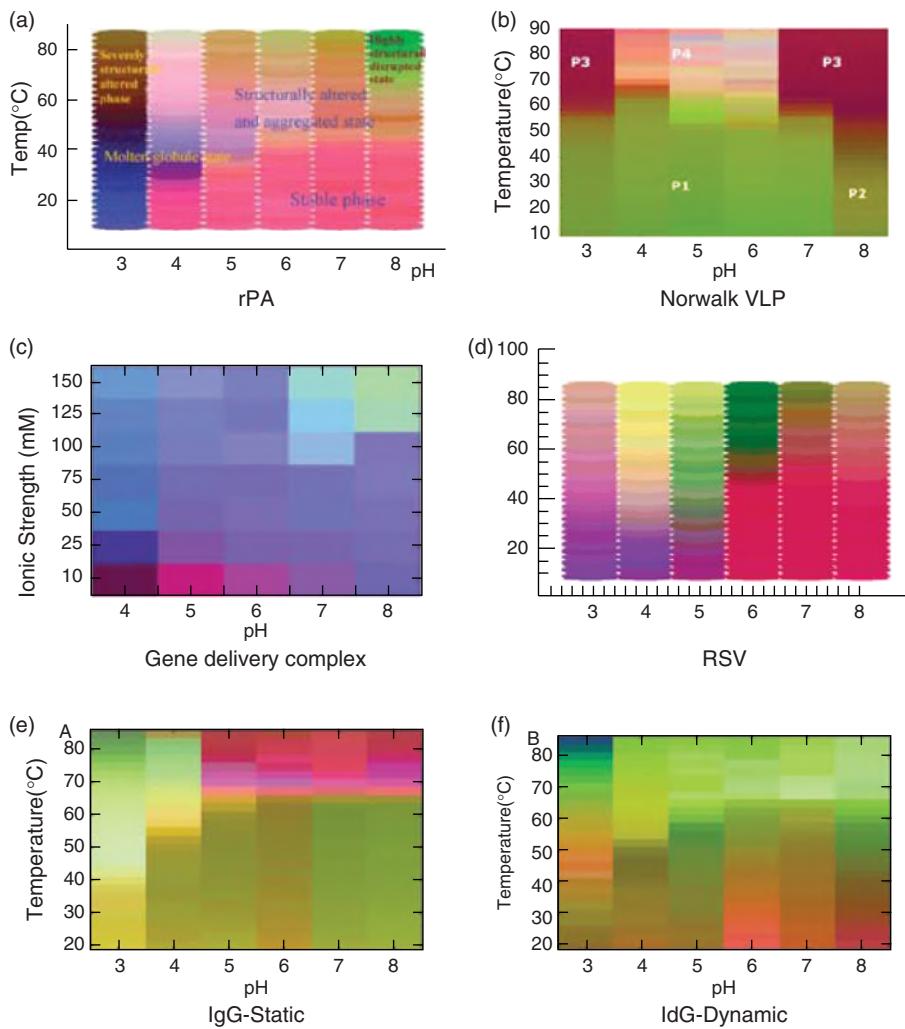


Figure 10.11. EPDs of various vaccine candidates and protein pharmaceuticals using static (a)-(e) and dynamic (f) spectroscopic techniques (see text for details). Blocks of continuous color represent uniform phases, conditions under which the raw data-derived vectors behave similarly. (a) Recombinant protective antigen, (b) Norwalk VLPs, (c) gene delivery complex (DNA and DOTAP/DOPE), (d) respiratory syncytial virus, IgG, (e, f). [Reproduced with permission from Wiley-Liss, Inc., and the American Pharmaceutical Association (*J. Pharm. Sci.*), American Chemical Society (*Mol. Pharm.*), and the American Society for Biochemistry and Molecular Biology (*J. Biol. Chem.*).] (See insert for color representation of this figure.)

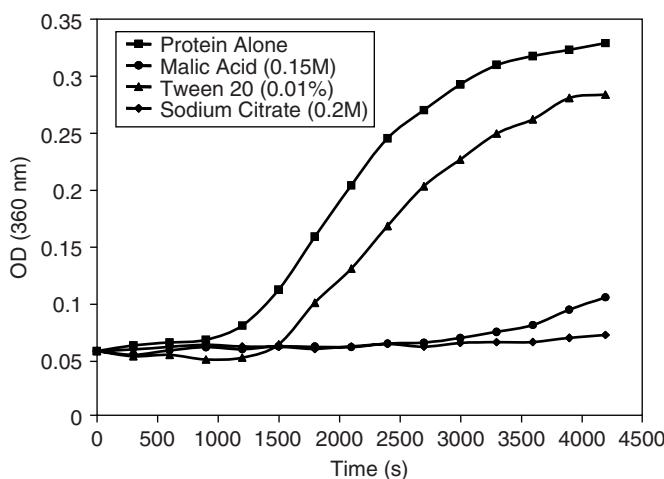


Figure 10.12. Aggregation of rPA in the presence and absence of excipients (0.15 M malic acid, 0.0 1% Tween 20, and 0.2% sodium citrate). The conditions for the screening assay were identified from the EPD of rPA. [Reproduced with permission from Wiley–Liss, Inc., and the American Pharmaceutical Association (*J. Pharm. Sci.*).]

if such interference is present, it may be possible to deconvolute the mixed spectral signals and still conduct an informative analysis. This is most assuredly not the case, however, for the most commonly employed adjuvants, the aluminum salts and various emulsions, in which significant optical opacity is often present. Methods are available, however, that permit at least a partial resolution of this problem. This is best illustrated by consideration of the aluminum salts. Both aluminum hydroxide (Alhydrogel) and aluminum phosphaste (Adjuphos) have been employed as adjuvants, with their different physical characteristics (primarily charge) used as a basis for adjuvant selection. In general, negatively charged antigens are best adsorbed to positively charged aluminum hydroxide and positively charged molecules to negatively charged aluminum phosphate, although many exceptions do exist due to the potentially complex surface interactions that can occur [69]. Protein is adsorbed onto their surface at a level of 0.1–1 mg protein/mg aluminum and the resultant suspension used as the vaccine. The adsorption process itself can be quantitatively followed by isothermal titration calorimetry (ITC), while the stability of the adsorbed protein directly monitored by DSC [21], two nonspectroscopic techniques. The simplest approach to obtain such isotherms, however, is to employ a simple subtractive technique in which one adds increasing amounts of protein to a constant amount of adjuvant and removes the bound material by centrifugation. Protein in the supernatant is then easily detected by UV absorption spectroscopy and the amount of bound antigen calculated by subtraction. Both fluorescence and vibrational spectroscopic methods can, however, be directly applied in these circumstances. In the case of FTIR, a slurry of antigen/aluminum salt complex can be placed directly on an ATR crystal and spectra obtained [21]. Similar studies have been performed in a transmittance mode using thin films of

antigen/adjuvant complex [26]. Although the strength of the resultant signal can be fairly weak, this approach has worked for several antigens [2, 3, 21, 26]. In some cases, CD spectra of protein adsorbed on aluminum salts may be possible, but differential scattering and adsorption flattening make such studies difficult at best. FTIR spectra of bound protein can be obtained as a function of temperature, permitting a direct evaluation of the accelerated stability of proteins adsorbed onto the adjuvant surface. It should be possible to perform similar studies with Raman spectroscopy, but to the best of our knowledge, this has yet to be reported with vaccine formulations.

A second method employs intrinsic fluorescence. Although it is sometimes possible to use conventional right-angle geometry in such studies, front-face fluorescence (FFF), which looks at emission off the surface of the sample, is often employed. This usually permits the acquisition of fluorescence spectra from even the most opaque suspensions, albeit at the cost of linearity of the observed fluorescence. The FFF approach has been successfully employed with a number of aluminum salt adjuvanted vaccines including EBA-175 RII-NG, *C. difficile* toxins, and ricin toxin-A chain [2, 3, 55]. Temperature can again be used to probe accelerated stability, although the potential of temperature-induced release of protein from the adjuvant surface must be carefully monitored. Using such methods, it has been possible to study the effect of stabilizers on adsorbed antigens. In general, it has been found that adsorption of proteins to aluminum salt adjuvants often results in their destabilization [21, 69]. Nevertheless, it was found that stabilizers that were effective in solution were also able to stabilize the proteins on adjuvant surfaces, although to a lesser degree than solution state antigens [69]. Studies of the dynamic properties of proteins adsorbed to aluminum salt adjuvants using the methods described above have yet to be performed, but recent studies demonstrating that mass-spectrometry-based studies of this type are possible [70, 71] suggest that such analyses should not be long in coming. It seems likely that a better understanding of the effect of surface adsorption on protein dynamics should be especially useful in improving our understanding of the effect of surface interactions on protein stability.

Both water-in-oil emulsions [72] and proteins incorporated in Immune Stimulations Complex (ISCOMs), a potent adjuvant [73], have also been successfully examined by spectroscopic method such as FTIR, CD, and fluorescence suggesting continued advancement in these approaches.

10.13 NEW SPECTROSCOPIC APPROACHES

New spectroscopic approaches are always being developed, but if current trends continue, it seems likely it will be some time before they find their way into the vaccine world. This need not be the case, however, and it would seem that only a change in attitude is necessary for this not to be the case. What initially needs to be acknowledged is that physical and chemical changes in vaccine substances have a role to play in the design and development of future vaccines. While immunogenicity studies will no doubt continue to be necessary to ensure the safety and efficacy of such vaccines, the elucidation of the physical and chemical origin of stability changes can play a

major role in vaccine development. We indicate one such approach above (EPDs) that involves combining experiment results to obtain a more multidimensional picture of vaccine behavior.

10.14 CONCLUSIONS

Another future advance in the spectroscopic analysis of vaccines may well lie in the extension of modern NMR techniques into such work. Current major limitations of NMR into studies of the kind of macromolecules and molecular complexes seen in vaccines include their size, which tends to broaden out resonances of interest and the need to label atoms of interest with NMR active nuclei. New labeling techniques, however, are becoming available that may permit much larger molecular entities to be characterized [73, 74]. Of more immediate interest is the demonstration that if one uses a simple comparative approach employing overlaying of 2D ¹H, ¹⁵N HSQC correlation spectra, conformations of proteins can be compared at very high resolution [75]. The application of this or related methods should permit very small changes in the structure of vaccine components to be detected. This should, in turn, permit the creation of more stable vaccines. It seems safe to say that the application of spectroscopic methods to vaccines has barely been touched and consequently awaits immediate further developments of significant importance to the vaccine industry.

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11

BIOPHYSICAL CHARACTERIZATION OF PROTEIN ANTIGENS WITHIN VACCINE FORMULATIONS

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11.1 INTRODUCTION

Characterization of protein antigens is a challenging task and requires a unique case-by-case approach. Proteins are large molecules with complex and varied structures, sensitive to conditions such as pH and temperature, and tend to lose their higher order structure if these conditions are suboptimal. Proteins also have a tendency to form complexes, aggregates, and undergo posttranslational modifications such as glycosylation, which affects their properties. The problem is amplified when more than one protein is involved in a formulation as it leads to protein–protein interactions. Driving causes of aggregation are nonnative conformations and hydrophobic interactions [1]. Aggregation is a multistage process involving intermediates and could be reversible or irreversible. It may be caused due to steric, electrostatic interactions, or solvation [2]

and manifests itself in gel, precipitate, or fibrous form [3]. Using the correct predictive tool to characterize aggregation is important to identify the causes of aggregation. For instance, differential scanning calorimetry (DSC) could be a useful tool for evaluating aggregation arising due to conformational instability while solution storage modulus, G' , measured with a rheometer, is a good tool to predict protein–protein interactions in colloidal aggregation [3, 4]. Deamidation (Asp, Gly), oxidation (methionine), and clipping (Asp, Pro) are chemical degradation pathways for proteins, and an organic mobile phase in size exclusion chromatography (SEC) can help identify these clipping species [5]. Understanding the path of aggregation will help a formulation scientist choose the right type of excipients to obtain a stable formulation. Amino acids, such as arginine, a neutral crowder, could be used as excipients to reduce the free energy of protein–protein interactions in colloidal aggregation [6].

The capabilities and constraints of a biophysical technique would propel the characterization process of protein formulations. Online Static light scattering with SEC(SLS/SEC) measures molecular weight, radius of gyration, and intrinsic viscosity while Raman spectroscopy provides chemical identification of particulates [7, 8]. Fluorescence, circular dichroism (CD) spectroscopy, and Fourier transfer infrared (FTIR) spectroscopy are commonly used to monitor secondary conformational structures [3, 9, 10]. Surface plasmon resonance (SPR) transfer, asymmetric field flow fractionation (aFFF), and electron microscopy are other biophysical tools used in characterizing protein formulations [11].

Identifying and monitoring conformational and colloidal stability of proteins in formulations using biophysical characterization techniques is critical to preparing a robust formulation using the right excipients and process parameters/conditions. These techniques would target to assess the excipients and process parameters. Monitoring of crystallization in freeze concentrates using X-ray diffractometry (XRD), citrification using DSC, and evaluation of excipients such as polysorbate 80 on crystallization would be some examples [4]. The right choice of sugars as lyoprotectants is vital to obtain an elegant cake and prevent aggregation. Tools such as FTIR serve as good indicators for selection of an optimum ratio of sugar and protein to obtain stability [12, 13].

In the Formulation and Delivery group at Novartis, we have investigated the use of several tools to determine the properties of complex protein vaccine formulations. These analytical assays are designed to evaluate the nature of an antigen after its processing into a formulation. These assays can then be used as stability-indicating tools. Some of the methods and their applications used by our group and others in the field are discussed briefly in this chapter. Details on mechanisms of techniques are outside the scope of this chapter.

11.2 CHROMATOGRAPHY-BASED METHODS

11.2.1 Reverse-Phase Chromatography

The most common vaccine proteins are the trivalent influenza antigens. The large and complex hemagglutinin (HA) protein component determines the potency of flu

antigens. This HA component is classically quantified by single radial immunodiffusion (SRID) assay. The SRID assay is a labor-oriented and time-consuming assay with low sensitivity. With the flu antigens varying every season due to new emerging strains, a quick and easy assay to quantify the flu proteins is extremely desirable. Quantification of HA using reverse-phase high-performance liquid chromatography (RP-HPLC) has been performed by our group as well as other researchers [11,14]. Using the varying hydrophobic binding interaction between the HA protein and other components in the formulation with the hydrophobic stationary phase, separation was achieved and the antigen quantified (data not shown). A strong correlation was found with the traditional SRID assay.

In another study, our group evaluated the effect of lyophilization process on a formulation comprised of three proteins. RP-HPLC was performed on the formulation before and after lyophilization using a linear gradient elution method. In Figure 11.1, the top chromatogram is a sample run of formulation before lyophilization, and the bottom chromatogram indicates the presence of additional peaks in the formulation postlyophilization.

11.2.2 Size Exclusion Chromatography

Size exclusion chromatography is a commonly used chromatographic tool to monitor aggregates in proteins and protein formulations and has been discussed in the literature extensively [7, 15]. In a study conducted to monitor the stability of three proteins in a lyophilized formulation, we evaluation columns of different particle sizes and pore sizes using an isocratic mobile phase. The effect of increase in ionic strength of mobile phase was also studied. Samples were run before and after lyophilization and aggregates monitored. As seen in Figure 11.2, good separation of peaks of proteins and aggregates was not achieved using TOSOH G3000 column and PBS as an isocratic mobile phase. Changing the column to TOSOH G4000 (Fig. 11.2b) and increasing the ionic strength of the mobile phase allowed for finer detail analysis of the aggregate composition. Figure 11.2a shows SEC chromatograms evaluating protein stability pre- and postlyophilization using G3000 column and PBS as the mobile phase. The chromatogram on the top was done prior to lyophilization, and the chromatogram on the bottom indicates the presence of aggregates/fragments from the proteins postlyophilization. In Figure 11.2b, both chromatograms were obtained using TOSOH G4000 column with a higher ionic strength. Top and bottom chromatograms were before and after lyophilization.

As illustrated in this study as well as many more in the literature, care must be taken to choose the right column as well as composition of the mobile phase to elute aggregates and obtain good resolution of the proteins. SEC tools used in isolation to monitor aggregates would not give a complete picture as there could be a dilution of aggregates during sample runs, potential column interactions and larger aggregates may be lost in void peaks or guard columns, which brings us to the next section that discusses static and dynamic light-scattering methods.

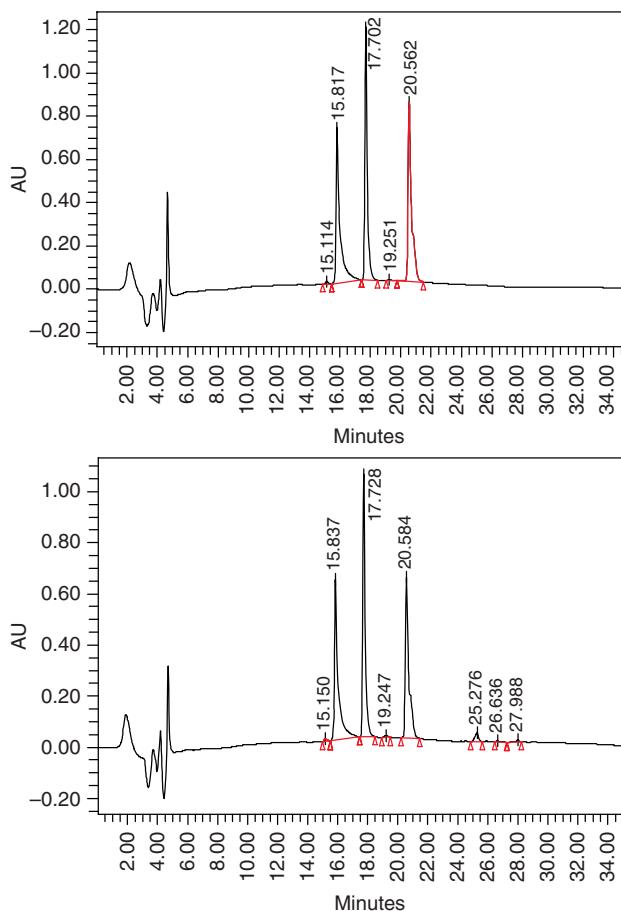


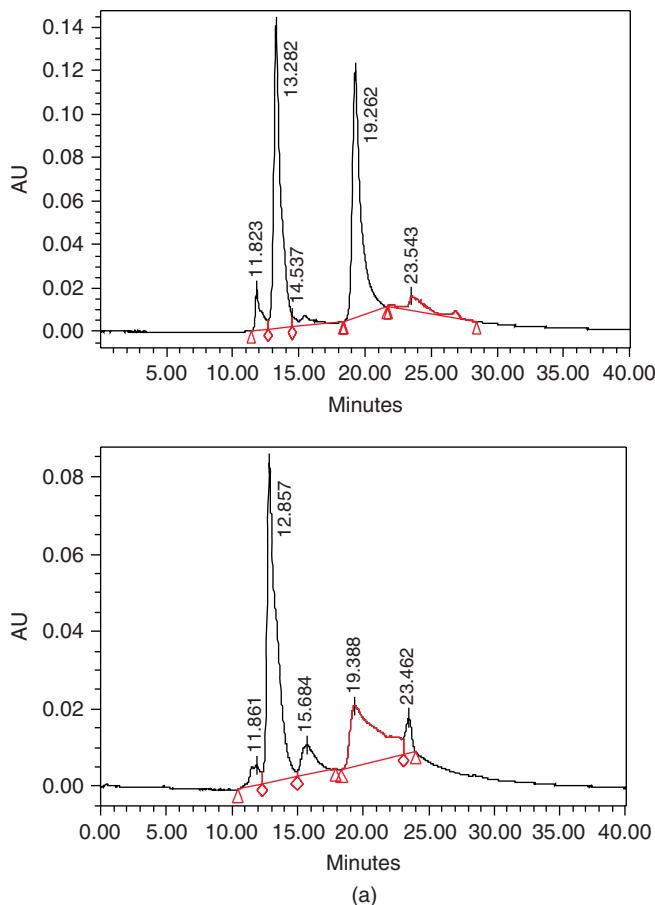
Figure 11.1. Illustrates effect of lyophilization process on a formulation comprising of three proteins. RP-HPLC was performed on the formulation before and after lyophilization. The chromatogram on the top represents proteins before lyophilization and the chromatogram on the bottom indicates presence of additional peaks from the proteins postlyophilization.

11.3 LIGHT-BASED METHODS

11.3.1 Static Light Scattering and Dynamic Light Scattering

The combined biophysical techniques of static (SLS) and dynamic light scattering (DLS) have illuminated the aggregated states of proteins in formulations. DLS provides the hydrodynamic radius of aggregates while SLS yields size of aggregates. As discussed earlier, SEC is a very good tool to monitor aggregates. However, aggregates are diluted during the separation process in SEC. DLS can be performed on a sample without dilution and aggregates monitored. Also, larger aggregates that escape size exclusion chromatography can be visualized with these light-scattering techniques.

Middaugh et al. evaluated formulation excipients in an attenuated live measles virus (MV), a thermolabile protein, to stabilize the temperature-induced aggregation and to protect the viral protein [10]. They showed that SLS and DLS could be used to screen excipients and conditions, such as temperature and pH, to stabilize MV protein. The limitation of the DLS method is that it can only measure aggregates below 1 μm . This limitation can be overcome by using SLS for monitoring aggregates greater than 1 μm in size. Thus, a combination of biophysical techniques such as SLS, DLS, and SEC are useful to monitor aggregates in protein formulations.



(a)

Figure 11.2. (a) SEC for evaluating stability of proteins pre- and postlyophilization using G3000 column and PBS as mobile phase. The chromatogram on the top was done prior to lyophilization and the chromatogram on the bottom indicates presence of aggregates/fragments from the proteins post-lyophilization (b) Top chromatogram was obtained using G4000 column before lyophilization and bottom chromatogram was after lyophilization using G4000 column. Ionic strength of mobile phase was increased and identical in both runs.

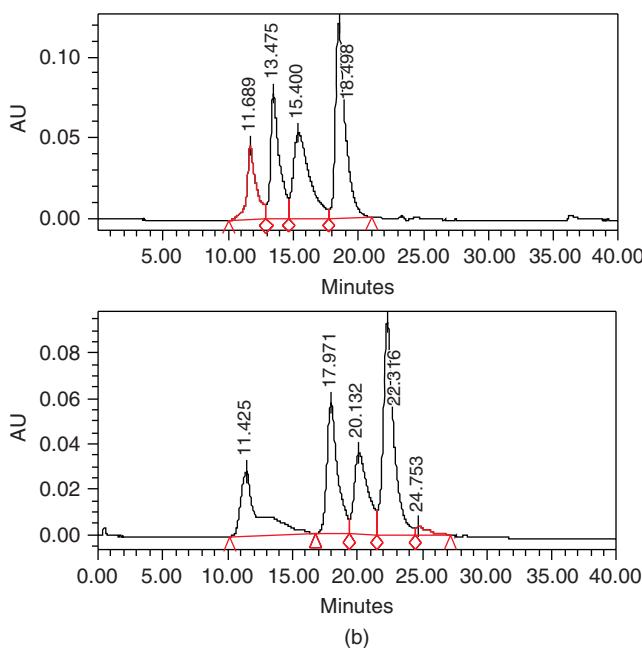


Figure 11.2. (Continued)

11.3.2 Circular Dichroism

Circular dichroism (CD) spectroscopy is useful in determining the secondary and tertiary conformational structure of a protein. Structural asymmetry of the proteins results in differences in absorption of left-handed polarized light versus right-handed polarized light, and the difference in absorption is measured with this tool. The CD spectrum obtained is used as a reference and structural compositions of alpha helices, beta sheets and random coils can be quantified using softwares such as CD Pro. The reference spectrum is compared with spectra of optimized formulations obtained by varying process variables and excipients. CD spectroscopy can be used in stability studies of optimized formulation to evaluate the product's conformational stability. The effect of stress conditions such as temperature, pH, and freeze–thaw on conformational stability of influenza proteins using CD was studied by Luyks et al. [16]. We have used CD spectroscopy as a tool to monitor the secondary structure of our protein formulations for effects of lyophilization, processing conditions, excipients, and in stability studies. Figure 11.3a depicts loss in the secondary structure of a protein after 4 weeks at 37°C, and Figure 11.3b shows the effect of lyophilization in a protein formulation.

11.3.3 Fluorescence Spectroscopy

Fluorescence spectroscopy is a simple and easy tool to monitor conformational changes of protein formulations by tracking fluorescence decay. Indicating factors for this

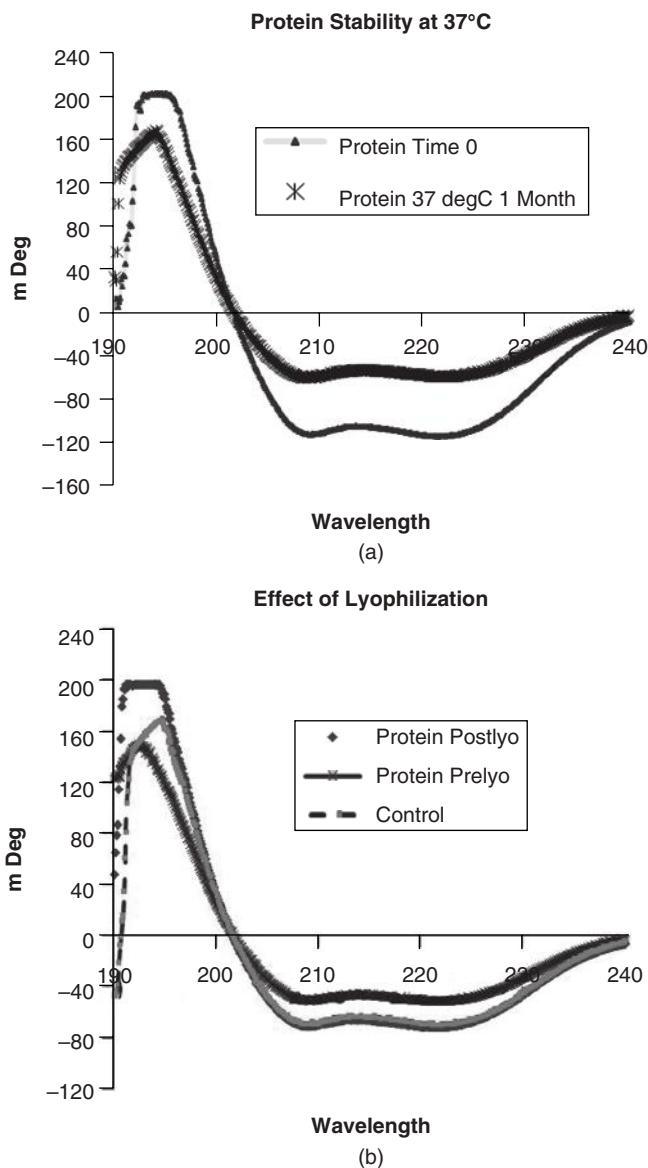


Figure 11.3. (a) Loss or change in secondary structure resulted in differences to far ultraviolet -CD spectra comprising of α helices, β sheets, and random coils (b) Lyophilization process stabilized secondary conformational structure of protein.

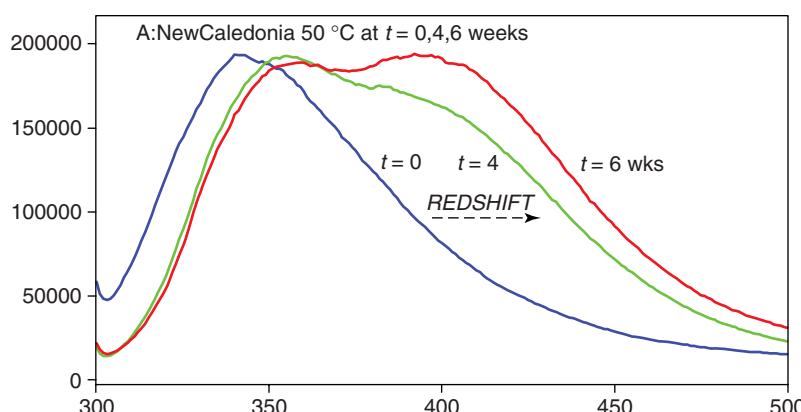


Figure 11.4. Red shifts in emission maxima over time at 50°C indicating protein unfolding.

decay are shifts in emission maxima and change in fluorescence intensity that occurs due to exposure of fluorescent amino acid residues from their native environment on protein unfolding. This can be seen in Figure 11.4, which depicts shifts in emission maxima and is indicative of protein unfolding from its native state at 50°C. Luyks et al. also used fluorescence spectroscopy to evaluate the effect of stress conditions such as temperature, pH, and freeze–thaw on conformational stability of influenza proteins [16].

11.3.4 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is yet another efficient biophysical technique to evaluate a protein's secondary structure. It can be employed to track changes during processing of a formulation and monitor the stability of a formulation. Changes in infrared spectral bands of amide I and amide II occur due to stretching of the C=O bond and bending of N—H bond of the protein, which contribute to the secondary conformational structure. FTIR spectra in Figure 11.5 shows the percentage of the α helix, the β sheet, the β turn, and the random coil in a liquid protein formulation.

Von Hoff et al. studied the secondary conformational structure of 16 proteins using FTIR and quantified the transition of α helix to β sheet [9]. Their study described it to be an objective way to determine damage to secondary structure of proteins. A strong correlation of data was reported with data obtained from FTIR and CD spectroscopy in their study [7]. A study was conducted at Amgen by Prestrelski et al. evaluating pH conditions and stabilizers for IL-2 protein, a protein that is sensitive to degradation during lyophilization [12]. Utilizing FTIR as a biophysical tool to monitor the secondary structure of the protein, they arrived at the right excipients that had the capacity to substitute for water during lyophilization process.

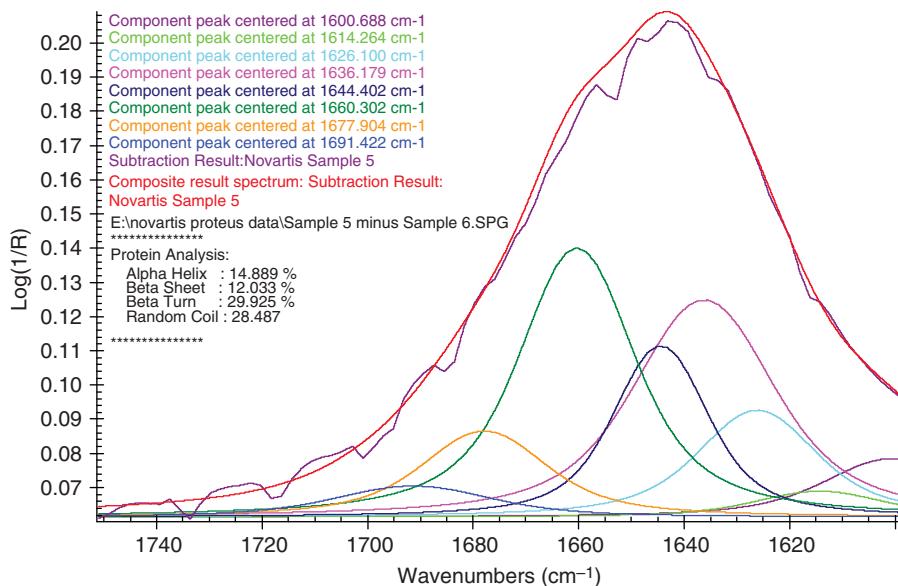


Figure 11.5. FTIR depicting percentage of α helix, β sheet, β turn and random coil in a liquid protein formulation. (See *insert* for color representation of this figure.)

11.4 DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is an effective method to monitor the thermal stability of protein formulations. DSC is one of the first tools utilized for the selection of excipients in a formulation. Optimization of excipients can be done by evaluating their effect on T_m of the protein in question. Liao et al. have shown the effect of lyophilization excipients on protein formulations using DSC [4]. DSC heating curves were used to simulate different annealing conditions during lyophilization of protein with excipients, along with relevant controls. The onset of crystallization temperatures was determined and this data then evaluated with that obtained from X-ray powder diffractometry, a tool that will be discussed in the following section. The study underlined the importance of developing a lyophilization cycle in the presence of proteins rather than develop a placebo cycle, as some proteins, depending on their concentration, do have an impact on the lyoprotectants and, thus, on the stability of the formulation [4].

11.5 X-RAY POWDER DIFFRACTOMETRY

As discussed in the preceding section, X-ray powder diffractometry (XRD) is a critical tool to monitor crystallization states in a protein formulation. Using XRD in conjunction with DSC, Liao et al. have shown the importance of using this tool during

different stages of lyophilization to monitor the states of mannitol [4]. Monitoring the phase transitions with XRD and identifying critical process parameters helped the group in developing a robust formulation,

11.6 SUMMARY

Formulation optimization and characterization is carried out for various projects based on need and applications. Some protein antigens are more stable than others and can be easily formulated into stable formulations. Others may require extensive evaluation of excipients, stabilizers, and buffers and sound characterization techniques are necessary to elucidate such formulations. Obtaining data and results from biophysical techniques to characterize the formulations are quicker, less cumbersome, and more accurate when compared to immunological assays. When it comes to developing a formulation for pandemics such as swine flu, where time is of the essence, biophysical techniques would be a better option when compared to an SRID assay. As can be seen from the sections above, a conjunction of biophysical tools is required to elucidate the complex protein antigens in the formulation. There are yet many biophysical techniques such as analytical ultracentrifugation, surface plasmon resonance (SPR) transfer, asymmetric field flow fractionation (aFFF), electron microscopy (SEM and TEM), analytical ultra centrifugation (AUC), and many more that are used in characterizing proteins in a vaccine formulation that have not been reviewed here and are beyond the scope of this chapter.

Acknowledgments

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PART 4

FORMULATION OPTIMIZATION
AND STABILITY EVALUATION

12

STRUCTURAL CHARACTERISTICS PREDICT THE STABILITY OF HIV

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Indresh K. Srivastava

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12.1 INTRODUCTION

A good vaccine candidate is designed to present antigens in a conformation that allows the body to elicit a potent, robust, and functional immune response. Thus, preservation of key structural determinants during storage of vaccine is important for its effectiveness. The vaccines can be destabilized in numerous ways, such as

hydrolysis, oxidation, or noncovalent aggregation. These processes may alter the three-dimensional structure of the vaccine, therefore, affecting its stability and potency [1]. Indeed, even candidate vaccines composed of short peptides were unsuccessful in phase III clinical trials [2]. Even conventional vaccines such as killed and inactivated pathogens and subunit vaccines are sensitive to thermal instability resulting in shortened shelf life [3–7]. Therefore, developing assays that may provide the information on the structure and conformation of the immunogen will be of help not only in determining the stability of vaccines but also for developing strategies to enhance the stability of vaccines.

There are currently 61 vaccines licensed for immunization and distribution in the United States [Food and Drug Administration (FDA), 2007]. Effective vaccines are designed to present antigens in the correct conformation to the immune system in order to elicit strong, stable, and functional immune responses. Therefore, the stability of the immunogen, which in most of the cases are protein, influences shelf life and storage conditions, which is one of the crucial elements for the success of either preventive or therapeutic vaccines. For that reason it is important to understand the changes in the epitope structure and antibody recognition that such molecules undergo in response to environmental changes. Until now quality controls of the stability of protein vaccines are limited to conventional assays such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), high-performance liquid chromatography (HPLC), and immunoprobining, which are not sufficient to evaluate all the important stability parameters. Therefore, there is a need to develop state-of-the-art analytical techniques to better evaluate the stability of vaccines.

Developing a vaccine against the acquired immunodeficiency syndrome (AIDS) is a challenging task. One of the primary obstacles in developing an effective vaccine is the ability of vaccine to induce broadly reactive neutralizing antibodies against heterologous human immunodeficiency virus (HIV-1) primary isolates. Structural analysis of HIV Env sheds some light on its lack of ability to induce broadly neutralizing responses. For example, the Env protein is highly variable among different isolates and has five hypervariable loops. These loops are the most accessible part of the protein and hence the primary targets for inducing strain-specific antibodies [7, 8]. In addition, the extensive glycosylation may affect the ability of HIV Env in generating broadly neutralizing antibody responses, which in some strains equals half of the mass of the total protein [9]. It has been proposed that extensive glycosylation masks the critical parts of the molecule and, therefore, has been termed appropriately as a glycan shield [10]. Furthermore, historically the focus for the vaccine development has been on the monomeric form of the Env protein instead of the native trimeric form [11, 12].

One of the strategies for designing a better immunogen is to mimic the functional HIV envelope glycoprotein, which forms a heterodimeric trimer complex of gp41 and gp120. Previous studies have shown that the oligomeric state of the HIV-1 Env protein may influence its antigenic conformation and exposure of relevant functional epitopes [13, 14]. Furthermore, the envelope glycoprotein trimer was shown to bind mainly to neutralizing monoclonal antibodies (MAbs), while the monomeric gp120 also binds to nonneutralizing MAbs [13, 15–18]. An additional strategy for enhancing the immunogenicity of Env is to expose the conserved regions of the protein. One

of the approaches to accomplish this task is to delete the variable loops that are not essential for the virus infectivity (like V1/V2) [19, 20]. However, it remains to be seen if these loops are involved in stabilizing the Env structure. A V2 deletion in oligomeric gp140 (a trimeric form of the Env protein) from strain SF162 induced antibodies that neutralized five of seven primary isolates [17]. Moreover, immunization of Rhesus macaques using a DNA (deoxyribonucleic acid) prime and protein boost regimen followed by a challenge with pathogenic SHIVSF162P4 conferred partial protection to immunized macaques compared to naïve animals [21].

The Env glycoprotein contains several conserved functional domains that are targeted by neutralizing mAbs. One such domain is the CD4 receptor binding site (CD4BS) in gp120. CD4BS is the target of mAb b12, the most potent and well-characterized neutralizing antibody against CD4BS [22]. This domain undergoes a series of conformational changes during entry of the virion into the host cell [23, 24]. The high-resolution crystal structure of gp120 bound to CD4 has provided very detailed information about the CD4 binding domain [23, 24], which is composed of a hydrophobic pocket that binds to the Phe43 from CD4. Recently, the crystal structure of b12 was solved in complex with gp120 by Zhou and co-workers [25], providing a detailed description of the b12 epitope at atomic level resolution. Therefore, monitoring the changes in the affinity of CD4 and b12 with the Env protein could be an indicator of the stability of an HIV vaccine. In addition, the binding of 17b mAb that targets epitopes in CD4 induced conformation of gp120 (CD4i) and neutralizes sensitive primary isolates [26], which may also be an important reagent for predicting the structural flexibility of HIV Env.

In this proof of concept study, we determined the stability of two different Env glycoproteins derived from SF162 (o-gp140 and o-gp140 Δ V2) using a combination of biophysical assays including circular dichroism (CD), surface plasmon resonance (SPR), differential scanning calorimetry (DSC), and three-angle light scattering (LS) coupled with refractive index (RI), and viscometer. The data suggest that Env trimers are stable over a wide temperature range as measured by CD. Similarly, Env proteins were stable for six thermal cycles, as determined by DSC. However, using SPR and LS, we demonstrated that the binding of these proteins to CD4 and to mAbs b12 and 17b was destabilized following the exposure to higher temperatures. This could be due to either the loss of the epitopes or blockade of the sites caused by aggregated state of the protein. These results demonstrate ways to probe the fine structure of the proteins that may be more sensitive indicators of vaccine stability and integrity.

12.2 RESULTS

To study the structural stability of the HIV vaccine, we used two different recombinant Env protein based vaccines derived from subtype B primary isolate SF162: a trimeric gp120-gp41 fusion protein denoted as gp140, and gp140 containing a 30-amino-acid deletion in the V2 loop. This latter protein has a more exposed co-receptor binding site and is termed as gp140 Δ V2 [19, 20] (Fig 12.1). To facilitate correct folding and full glycosylation, both constructs were expressed in CHO cells. The biophysical

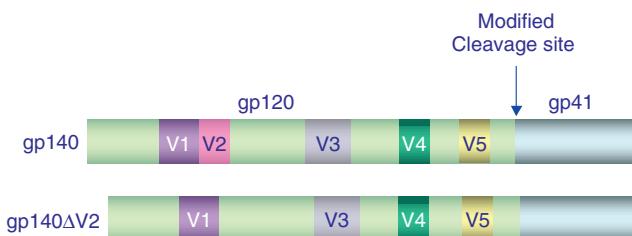


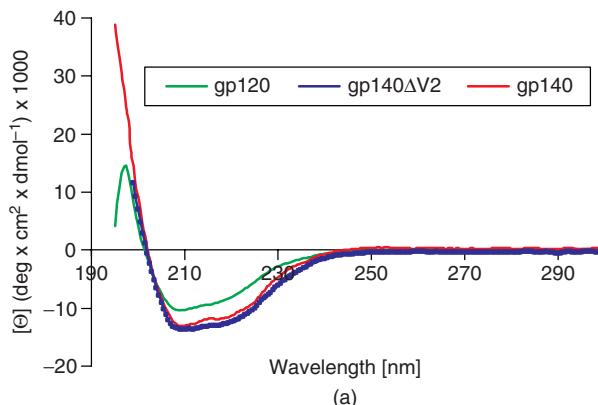
Figure 12.1. SF162 envelope glycoprotein constructs used in this study. The V2 loop is deleted from the Δ V2 constructs. gp41, lacking the transmembrane domain and the intracellular domain, was added to the gp140 constructs. In order to create the trimer form of the protein, the protease cleavage sites were modified as described in Srivastava et al. [27]. (See insert for color representation of this figure.)

and immunogenic properties of these constructs have been described in detail elsewhere [14, 27, 28]. The proteins used in this study were purified to >90% purity and homogeneity as described elsewhere in detail [28].

To assess the relative proportion of different secondary structure elements into the two protein vaccines, we determined the CD spectra for o-gp140 and o-gp140DV2 proteins at 25°C. Analysis of the spectra using the CDNN software [29] demonstrated that both these proteins have approximately 38% helix, 15% β sheet, and 29% random coil (Fig. 12.2). The increase in helical content observed for the two trimeric versus monomeric Env proteins could be attributed to the high helical content of gp41 or to the oligomerization state of Env ecto domain. We then determined the melting curves for both these proteins in a temperature range from 5 to 90°C at 222 nm (measurements taken at 5° intervals). As shown in Figure 12.3, only minor changes were observed in the secondary structure of these proteins over this wide temperature range. It was quite surprising that even at the highest temperature range we did not observe any major unfolding for either protein (Figure 12.3) since proteins generally tend to largely unfold at higher temperatures and are therefore less stable. In order to better understand if the thermal stability for the Env trimer was due to gp41 or due to the trimeric conformation or both, we also studied the gp120 monomeric glycoprotein in CD (data not shown). Minor changes in the secondary structure for gp120 were observed after heating the protein to >700°C as indicated by a slight shift in the minimum point from 208 to 206 nm. However, these differences are rather subtle to draw a conclusion.

We have performed DSC analysis of Env proteins \pm V2 loops to determine the midpoint transition temperatures (T_m). As shown in Figure 12.4, both trimers irrespective of the presence or absence of V2 loop have one major transition consistent with the proteins being homogenous. Since the C_p values are quite low (0.025 Kcal/mol/°C), it is possible that we are observing changes in the tertiary structure of Env trimer, while secondary structure remains stable. This is consistent to the data observed in CD where minor changes were observed in the secondary structure over a wide temperature range (5–90°C).

To further analyze the differences between the conformational state of Env proteins before and after exposure to different temperatures, size exclusion



(a)

Figure 12.2. Secondary structure analysis of Env proteins treated at 25°C (overlay of the circular dichroism spectra). (a) CD spectra of gp120, gp140, and gp140 Δ V2 measured at the same condition (see material and method). (b) Summary of the fraction of different secondary structure elements in each Env protein. (See insert for color representation of this figure.)

chromatography (SEC)-HPLC analysis was performed (Fig. 12.5a). Preliminary analysis suggests that a large proportion of protein was aggregated at higher temperature. However, the level and proportion of aggregation could not be clearly determined. Therefore, we performed triple angle light scattering (TALS)-refractive index (RI)- viscosity (Vis) analysis and determined the molecular mass and hydrodynamic radius (R_h) of gp140 (data not shown) and gp140 Δ V2 before and after heat treatment. The R_h value of the o-gp140 Δ V2 under native conditions (prior to heating) was found to be 7.3 nm. However, there was a significant increase in R_h value (from 7.3 to 9.1 nm) o-gp140 Δ V2 after exposing it to higher temperatures. As similar profile was observed for o-gp140 before and after heating to 90°C (9.0–10 nm). These data suggest that Env trimers tend to aggregate after exposure to the higher temperature. The changes observed in the light-scattering profile and the hydrodynamic radius suggest that irreversible structural changes occurred in the Env proteins as a result of exposing these proteins to higher temperatures (Fig. 12.5b).

To further analyze the structural integrity, binding of sCD4 and neutralizing mAbs was measured before and after exposing them to three different temperatures (60, 75, and 90°C). The binding of the Env glycoproteins to sCD4 in SEC-HPLC using fluorescently labeled sCD4 (CD4-fluorescein isothiocyanate) [27] demonstrated that sCD4 binding of these proteins was markedly affected by temperature treatment, and significant loss of sCD4 binding was observed for both gp140 and gp140 Δ V2 after heating them to 90°C (Fig. 12.6d). To obtain quantitative data on the magnitude of binding of each vaccine to sCD4 before and after exposing them to higher temperatures, SPR analysis was performed. In this assay different Env proteins before and after temperature exposure were flowed over sCD4 immobilized on a Biacore biosensor chip (Biacore AB, Uppsala, Sweden). Before the temperature treatment, both the proteins had nanomolar affinity for CD4 consistent to the previously published data

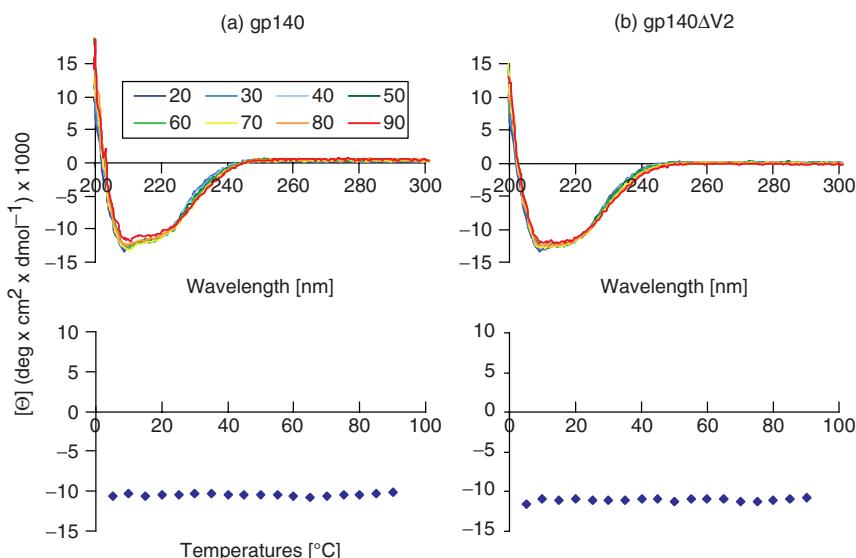


Figure 12.3. Circular dichroism spectroscopic analysis of Env proteins treated at different temperatures: (a) gp140 and (b) gp140 Δ V2. The upper panel shows the spectra measured of each protein at 199–300 nm wavelengths from 5 to –90°C. The lower panel in all shows the molar ellipticity at 222 nm from 5 to 90°C.

[14]. However, magnitude of binding of these proteins to sCD4 gradually decreased after exposing them to 60 and 75°C, and was totally abolished after exposing the proteins to 90°C (Figs. 12.6a and 12.6b). The relative change in binding of Envs to sCD4 upon heating is summarized in Figure 12.6c. It seems that structurally Env trimers most likely represent two different conformations of the Env protein, one where the CD4 binding site is damaged/altered, (exposing the protein to temperature higher than 63°C where we have observed changes in the tertiary structure) and the other where the CD4 binding site is intact (below 63°C). As a result we observed a reduction in the magnitude of sCD4 binding (due to the molecules that lost the CD4 binding site), but no change in association or dissociation rates for the molecules where CD4 binding site was not perturbed. Treatment of these proteins to extreme temperatures such as 90°C perturbed the CD4 binding site in all the molecules; therefore, there was no binding to sCD4. These data suggest that irreversible structural changes take place in tertiary structure of both o-gp140 and o-gp140 Δ V2 vaccine as a result of exposure to high temperatures (90°C) that affects CD4 binding. Furthermore, it seems that compared to Env trimers the CD4 binding site is relatively more stable in the Env monomer because the monomer retained 34% of its CD4 binding ability after even heating the protein to 90°C (Fig. 12.7). However, due to the limited amount of data it will not be possible to conclude that monomer is more stable compared to trimer. Therefore, systematic evaluation of monomer and trimer derived from different isolates will need to be performed to validate these results.

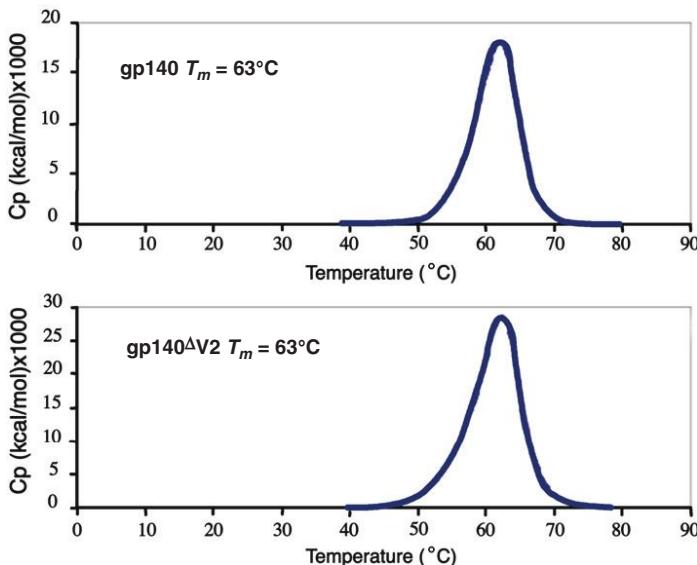


Figure 12.4. Differential scanning calorimetric analysis of proteins. The HIV Env trimers with and without V2 loop were analyzed using DSC. The midpoint transition temperature (T_m) is indicated.

To further characterize fine structural changes in these proteins, the affinity of two mabs—b12 which binds to conserved CD4 binding site on Env [30, 31] and 17b (CD4 inducible site) [26]—were studied. The binding affinity of unheated Env proteins was in the nanomolar range similar to earlier reports [14, 22]. Heating both intact and Δ V2 trimer to lower temperatures (60 and 75°C) resulted in a partial loss of b12 binding (Fig. 12.8). However, after exposing these vaccines to 90°C , both gp140 and gp140 Δ V2 completely lost their ability to bind to b12 (Fig. 12.8). This is consistent with the CD4 binding data. Interestingly, gp120 partially retained (36%) its ability to bind to b12 compared to trimers (Fig. 12.8). Next we tested the binding of these proteins to 17b in the presence and absence of sCD4. The data suggest that 17b recognition by Env trimers was also abolished after heating the proteins to 90°C (Fig. 12.9). Once again consistent to the previous data with sCD4, and b12, it appears that gp120 was able to retain some of its binding to 17b even after heating the protein to 90°C (Fig. 12.9).

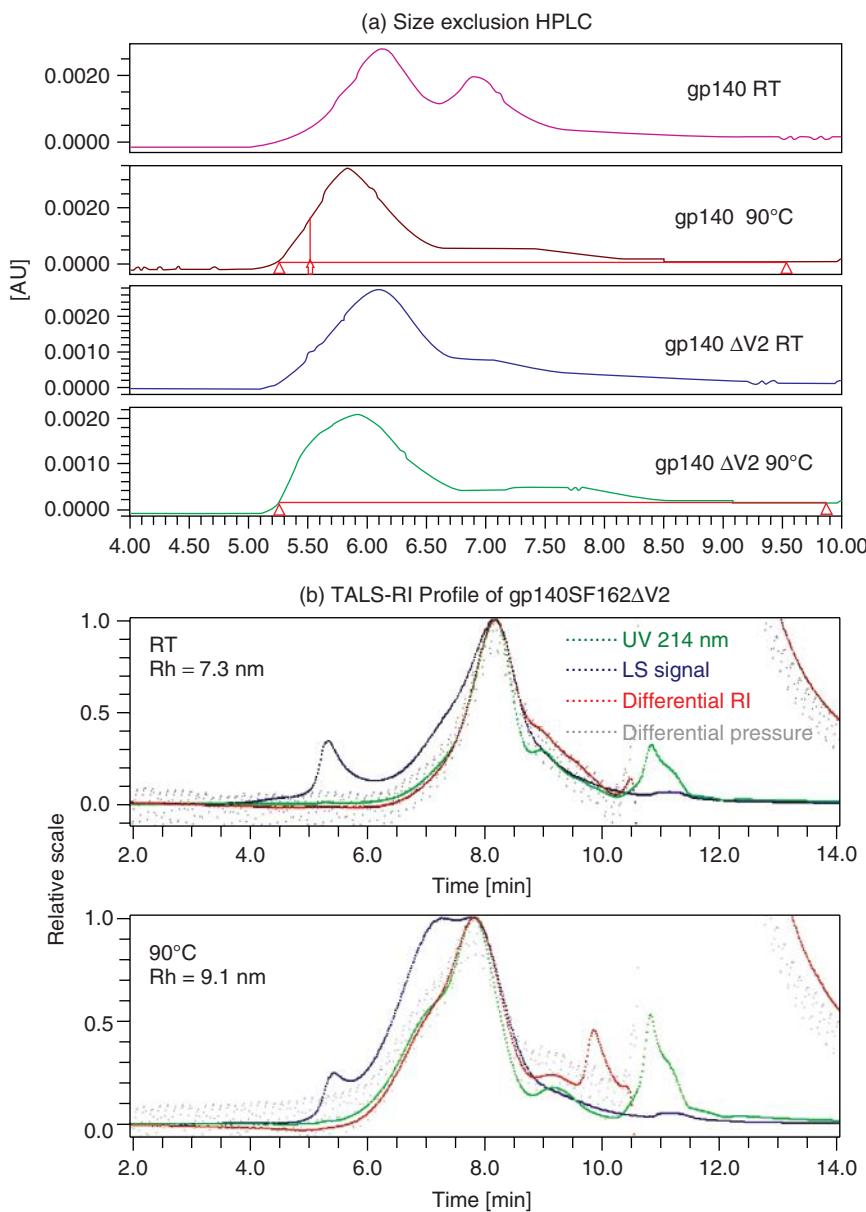


Figure 12.5. Size exclusion and light-scattering profiles of Env proteins before and after heating to 90°C: (a) Ultraviolet (UV) absorption of gp140 and gp140 Δ V2 before and after heating to 90°C and (b) light-scattering (LS) analysis of gp140 Δ V2 before (RT upper panel) and after heating (90°C—lower panel). The green chromatogram corresponds to the UV signal at 214 nm. The blue chromatogram corresponds to light-scattering signal. The red chromatogram corresponds to differential reflective index signal and the gray chromatogram corresponds to the differential pressure.

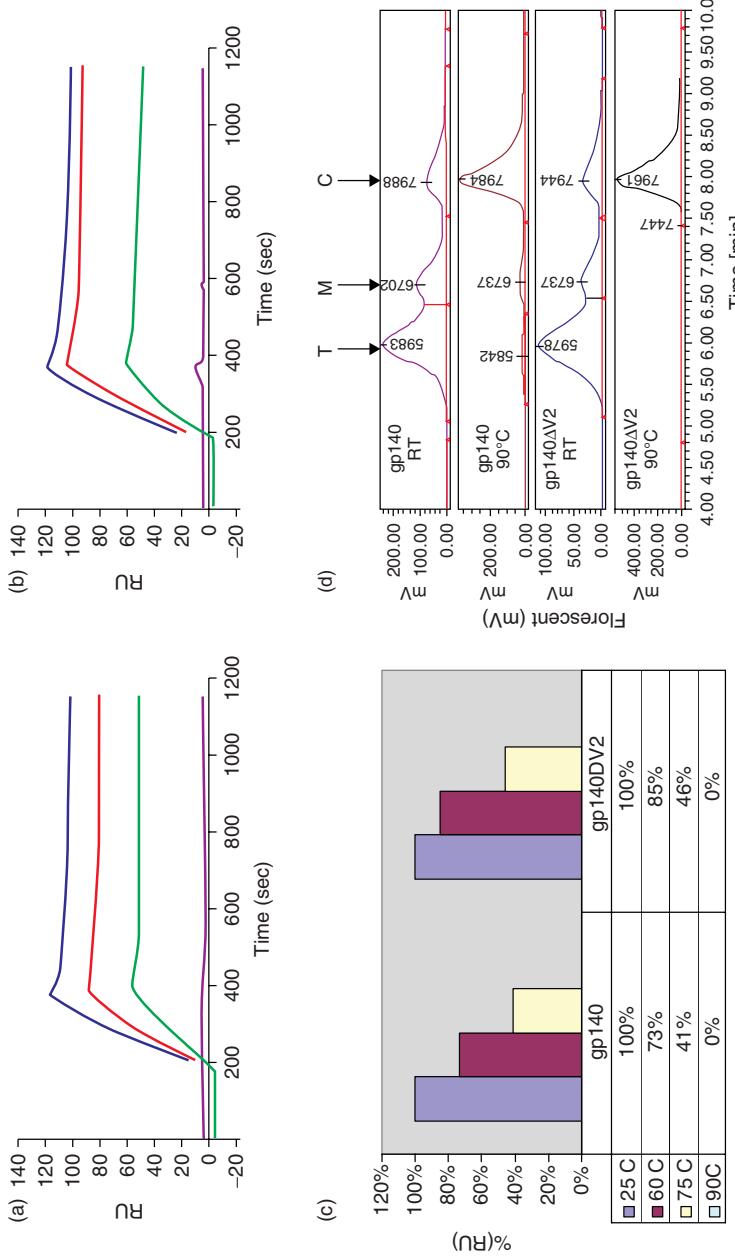


Figure 12.6 CD4 binding to Env proteins before and after heat treatment. SPR analysis of (a) gp140 and (b) gp140ΔV2 to CD4. All measurements were taken using 2000 RU of four domains CD4 coupled to a CM3 sensor chip. The concentration ranges for HIV Env proteins were adjusted to 200 mM. All four proteins were heated to four temperatures. The following colors of chromatograms represent different binding of proteins previously exposed to different temperatures: blue 25°C, red 60°C, green 75°C, pink 90°C. (c) Summary of the relative binding of the Env proteins to CD4. The relative binding was calculated by the highest response (RU) of each temperature divided by RU at room temperature (RT). (d) CD4 binding of the Env proteins using HPLC-based assay with fluoresceinated CD4. Retention time of the gp140 trimer, the gp140 monomer, and residual CD4 are labeled in T (trimer), M (trimer), and C (monomer). (See insert for color representation of this figure.)

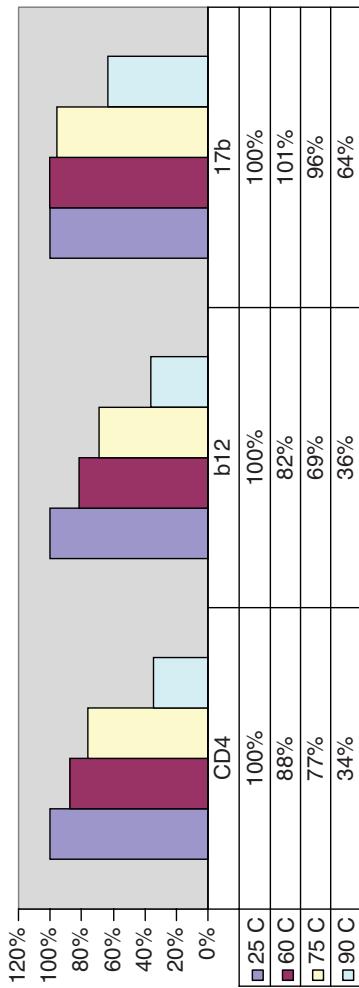


Figure 12.7. Binding of gp120 monomer to different ligands after heat treatment. Summary of the relative binding of the gp120 to CD4, b12, and 17 mAbs. The relative binding was calculated by the highest response (RU) of each temperature divided by RU at room temperature divided by RU at 25°C.

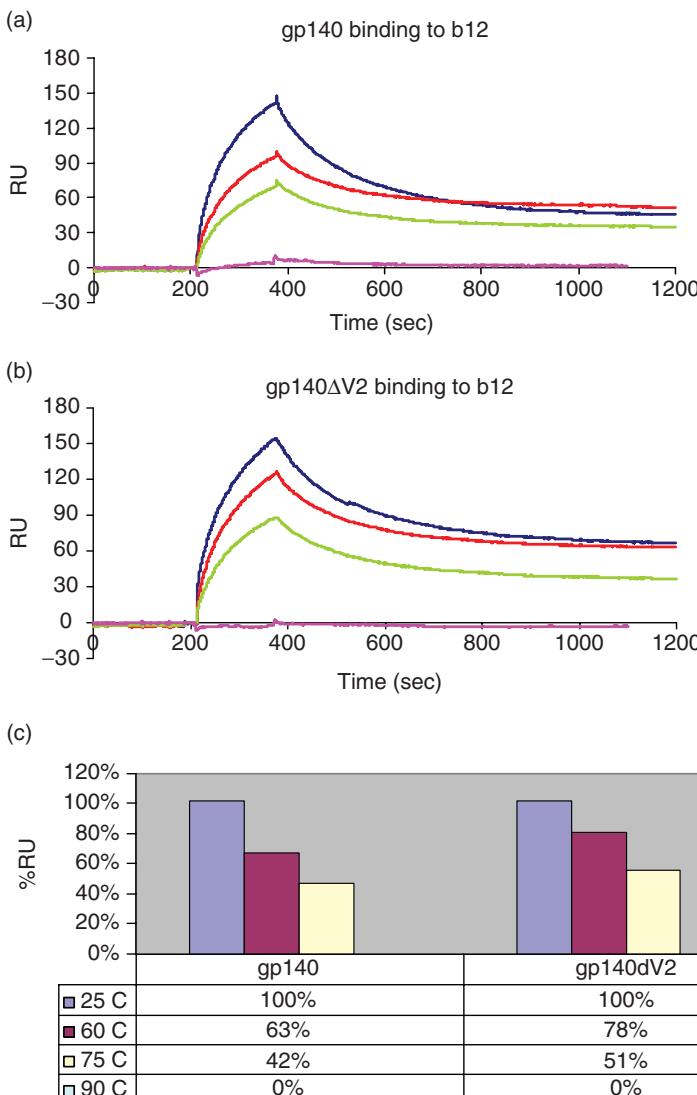


Figure 12.8. Binding of Env proteins to mAb b12 after heat treatment. SPR analysis of (a) gp140 and (b) gp140 Δ V2 to b12 mAb. All measurements were taken using 2000 RU of b12 coupled to a CM3 sensor chip. The concentration ranges for HIV Env proteins were adjusted to 200 mM. All four proteins were heated to four temperatures. The following colors of chromatograms represent different binding in different temperatures: blue 25°C, red 60°C, green 75°C, and 90°C pink.(c) Summery of the relative binding of the Env proteins to b12 mAb. The relative binding was calculated by the highest response (RU). (See insert for color representation of this figure.)

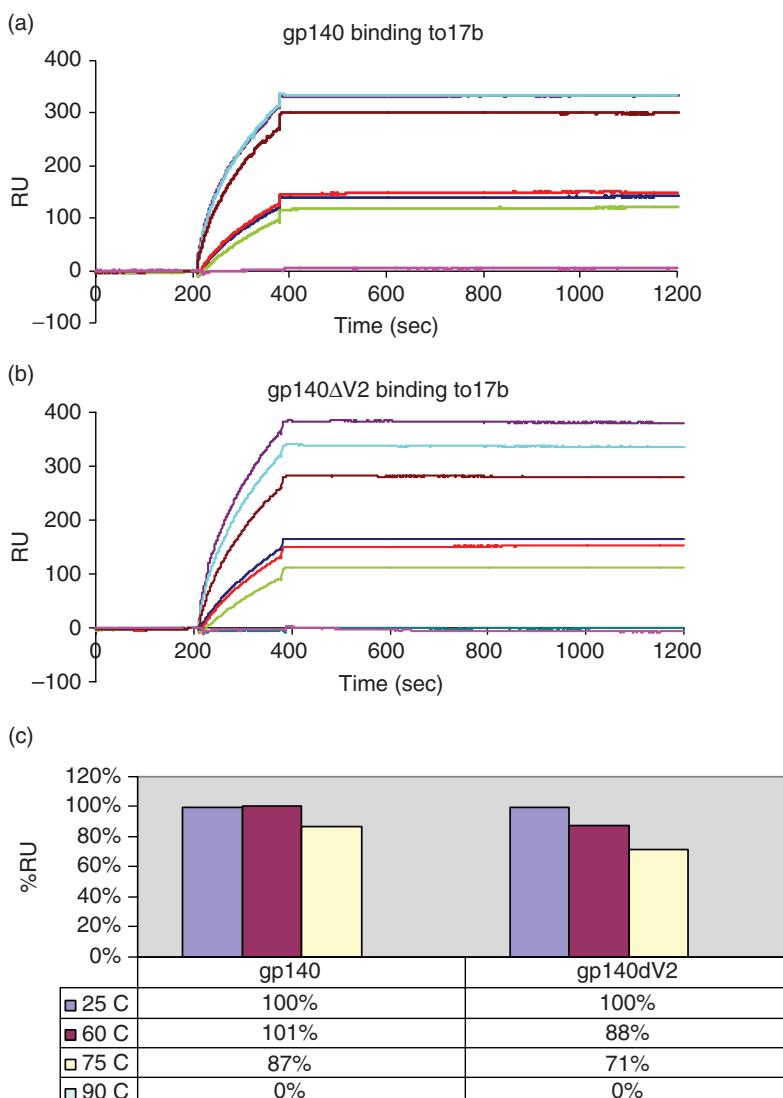


Figure 12.9. Binding of Env proteins to mAb 17b after heat treatment. SPR analysis on (a) gp140 and (b) gp140 Δ V2. The proteins were heated to four temperatures. The following colors of chromatograms represent different binding in different temperatures: blue 25°C, red 60°C, green 75°C, and pink 90°C. 17b binding to the Env proteins was detected also adding CD4 to stabilize the CD4 inducible conformation. Env+CD4 chromatographs are colored in: purple 25°C, light blue 60°C, brown 75°C, and black 90°C. (c) Summary of the relative binding of the ENVs proteins to 17b+CD4. The relative binding was calculated by the highest response (RU). (See insert for color representation of this figure.)

12.3 DISCUSSION

To evaluate various aspects of protein vaccine stability different biophysical techniques were evaluated. Surprisingly, the overall secondary structure of two Env protein vaccines is highly thermostable, even up to 900°C. However, using SPR analysis, we demonstrated that the magnitude of binding of these proteins to different ligands such as CD4 and mAbs b12 and 17b are adversely affected as a result of exposing these proteins to higher temperatures. In particular, the loss in magnitude of binding is significantly higher when these proteins were exposed to extremely high temperatures (900°C) compared to moderately higher temperatures (600°C). These results indicate that the CD4BS and CD4-inducible domains are irreversibly modified at elevated temperatures. These observations suggest that additional structural tools can be used to investigate the dynamic behavior of protein vaccines, which may provide predictors of vaccine stability. Furthermore, these assays could also be used as structural tools for designing better and more potent immunogens.

The gp120 monomer appears to be relatively more thermal resistant compared to the Env trimers (o-gp140 and o-gp140ΔV2), as gp120 had a relatively slower loss of CD4 binding and retained a residual binding of 34% even after heating at 90°C. Previously, we have shown that the hydrodynamic radius (R_h) of the purified trimer gp140ΔV2 SF162 is less than three times larger than that of gp120ΔV2 SF162 (7.27 versus 4.28 nm), suggesting a more compact arrangement of the trimer relative to the monomer [28]. Furthermore, the compact folding of trimer is neither strain nor subtype dependent because we have demonstrated a more compact folding of trimer derived from another subtype B strain, that is, HIV-1US4 [27] and also for subtype C isolate TV1. These results suggest that loose folding of gp120 may allow it to have a better ability to refold and reconfigure in response to heating and cooling as compared to trimer. This observation is further supported by high-resolution structure information demonstrating a large conformational flexibility of gp120 [32–34] compared to gp140 [14]. Therefore, the increased flexibility of gp120 may be responsible, at least in part, to its thermal stability.

In order to follow the structural changes in Env proteins following heat treatment we focused on the binding of two mAbs: b12 (binds to the CD4 binding site on Env) and 17b (binds to the CD4 inducible site). Although the high-resolution structures of both b12 and CD4 bound to gp120 [25] were solved and revealed important information about the binding sites, however, due to technical challenges this structure lacks the V2 loop. Therefore, structural information about the role of the V2 loop in binding and stabilizing the interaction with these mAbs remains poorly understood at the structural level. Both CD4 and the b12 epitopes in gp140 ± V2 proteins were affected as a result of exposure to higher temperature. This was expected since b12 and CD4 binding sites have considerable overlap. However, compared to o-gp140, CD4 and b12 binding to gp140ΔV2 were relatively less affected as a result of heat treatment. After heating both the proteins to 60°C the relative binding of o-gp140 to CD4 and b12 was 73 and 63%, respectively, and binding of CD4 and b12 to o-gp140ΔV2 were 85 and 78%, respectively.

The CD4-indicible epitope recognized by mAb 17b was evaluated in CD4 bound conformation of the Env-based vaccines using soluble CD4. Overall the changes in affinity as a result of heating were more modest for 17b than was observed for CD4 and b12. The 17b epitope is located across the base of the antiparallel, four-stranded β -sheet (β 2– β 3 and β 20– β 21) where the V1 and V2 are located between β 2 and β 3 [23]. The bridging sheet that links the inner and outer domains of gp120 is formed upon binding to CD4. This epitope region is flexible and undergoes a major conformational change, which could explain its superior thermostability compared to CD4/b12 binding of the Env proteins. In the context of the intact monomer (Fig. 12.7) and trimer (Fig. 12.9), the binding of 17b was less affected by exposing these proteins to higher temperatures compared to the V2 loop that deleted monomer and trimer (Fig. 12.9). These data suggest that deletion of the V2 loop has a larger impact on 17b binding compared to the oligomeric state of the protein. This observation also suggests that deletion of the V2 loop further exposes the 17b epitope, therefore upon heating the epitopes are more affected in the V2 loop deleted protein compared to intact proteins where the V2 loop may somewhat shield the epitope.

It was quite interesting to observe that the secondary structure of Env proteins \pm V2 loop is quite stable because we did not observe any change in the CD spectra even after heating the proteins to 900°C (Fig. 12.3). However, using SPR (Figs. 7–9) and SEC-TALS-RI-Vis (Fig. 12.6d), we demonstrated that binding of Env proteins exposed to different temperatures to CD4 and to mAbs b12 and 17b was affected. The monomeric forms tended to be more efficient in maintaining the CD4 binding site intact, whereas the natural constructs were more stable compared to the V2-loop-deleted structures for maintaining the 17b binding sites. Our results demonstrate that despite the high thermostability of the Env glycoprotein, at least some of its functional domains are sensitive to heat. This observation may have implications on understanding the long-term stability of future HIV and other vaccines.

The set of analytical techniques introduce here could be used for protein-based candidate vaccines as a sensitive means to evaluate stability. This set includes circular dichroism (CD) for overall characterization of secondary structure changes, DSC for understanding changes in heat capacity and tertiary structure; SPR for measuring specific affinity and kinetics of binding to antibodies and ligands as a result of changes in secondary and tertiary structures; and SEC to detect changes in aggregation status. While these techniques were used here to characterize functional changes in response to heating, they could also be used for measuring changes after any other treatment that vaccines may undergo such as lyophilization, oxidation, and exposure to light. Therefore, these techniques may be useful in the development of new protein-subunit-based vaccines.

12.4 MATERIALS AND METHODS

Preparation of the HIV Envelope Proteins The CHO cell clones producing the Env proteins of interest were bioreactor adapted in low serum containing media as described in details elsewhere [27, 28]. All the envelope proteins were purified as described using a combination of lectin capture and ion exchange

and size exclusion columns [28]. All the proteins were heated for 15 min at a given temperature. Briefly, the heat block is filled with water, set at a desired temperature. After reaching at the set temperature, Env protein sample is subjected to heat treatment for 15 min at that temperature.

12.5 CIRCULAR DICHROISM

Circular dichroism (CD) spectra of all samples were recorded on an AVIV model 410 CD instrument (AVIV Associates, Lakewood, NJ). The ellipticity was measured at 0.5-nm intervals and three measurements were averaged for each temperature. Protein concentration was adjusted to 0.15 mg/ml. To follow the melting of the peptide conformation the ellipticity was measured at 220 nm over the temperature range 5°C–90°C with 5° intervals. The protein concentration was determined using BCA assay (Pierce). CD intensities were normalized as mean residue ellipticities (degree × cm²/dmol). Secondary structure analysis was carried on using the CDNN software [29].

12.6 HPLC CD4 BINDING ASSAY

To determine the capability of purified envelope proteins to bind CD4, we used an HPLC-based assay with fluorescinated CD4. Purified sCD4 was labeled with amine-reactive succinimidyl esters of carboxyfluorescein according to the instructions provided by the manufacturer (Molecular Probes, Inc., Eugene, OR). Approximately 1 μg of purified HIV Env gp120 was mixed with 0.33 μg of fluorescinated CD4 in a reaction volume of 60 μl using 2 × PBS at pH 7.4. After 15-min incubation at room temperature, 50 μl of this sample was injected onto a BioSil SEC-250 (Bio-Rad Laboratories) gel filtration high pressure liquid chromatography (HPLC) column using an Alliance 2690 HPLC system (Waters Corporation, Milford, CA). Samples were run in 20 mM NaH₂PO₄-2 mM Na₂HPO₄-400 mM ammonium sulfate buffer (pH 6.0) at a flow rate of 1 ml/min. The fluorescence profile was monitored at 490 nm using a 996 fluorescence detector and the Millennium software package (Waters Corporation).

12.7 DIFFERENTIAL SCANNING CALORIMETRIC ANALYSIS OF PROTEINS

The DSC analysis of the different vaccine samples was performed using the Capillary DSC instrument (MicroCal, Inc., MA) following the manufacturer's recommendations. Briefly, the samples were analyzed between the temperatures of 20–80°, at concentration of 150–200 g/ml, filtering period of 10 s, and at a scan rate of 70°C/h. The prescan thermostat was set at 10°. In all the experiments we used PBS as a blank. The data was analyzed using the Origin 7 software (MicroCal, Inc.) by subtracting the reference scan from the experimental scan, and the sample concentration was normalized. The baseline was corrected, and the data was fitted using a non-2-state data-fitting model. We allowed cursor initialization of each transition and did nonlinear curve fitting.

12.8 HYDRODYNAMIC RADIUS MEASUREMENT

The hydrodynamic radius (R_h) of gp140 and gp140 Δ V2 were determined using a triple detector MALS light scattering online with Viscostar, differential viscometer to measure the intrinsic viscosity and Optilab rEX Refractive index detector (Wyatt Technology Corp. Santa Barbara, CA) in conjunction with a gel filtration HPLC column (Bio Sil SEC-400; Bio-Rad Laboratories) using the Alliance 2690 HPLC system (Waters Corporation). Approximately 70 μ g of the purified protein o-gp140SF162 Δ V2 at room temperature and after heating to 90°C was analyzed in 20 mM NaH₂PO₄, 2 mM Na₂HPO₄, 400 mM ammonium sulfate buffer (pH 6.0) at the flow rate of 1 ml/min. To analyze the data ASTRA 5 software was utilized using 0.184 ml/g dc/dn value.

12.9 SURFACE PLASMON RESONANCE ASSAY

Surface plasmon resonance assays were performed using a BIACORE 3000 optical biosensor system (Biacore) with simultaneous monitoring of relevant flow cells. The gp120, gp140, and gp140 Δ V2 glycoproteins from SF162 stain were heated to 60, 75, and 90°C, respectively, and cooled back to room temperature prior the SPR assay. To perform the kinetic study of the binding of the Env to sCD4, sCD4 was immobilized using carbodiimide coupling onto a low charge CM3 sensor chip to attain a response of 2000 RU. Using phosphate buffered saline (PBS) (pH 7.4) with 0.05% Tween 20, association was assessed by passing subtype the Envs over the CD4 in different flow cells at a flow rate of 20–30 μ l/min. The concentrations of all four vaccines were adjusted to 0.2 mM and bovine serum albumin (BSA) was used as negative control. To perform the binding of HIV Env to different mAbs such as b12 and 17b, these antibodies were immobilized onto a CM5 sensor chip using amine coupling reaction to attain approximately 1000 RUs, and the assay was performed under the experimental conditions described as above. The relative binding was calculated by the highest response (RU) of each temperature divided by RU at 25°C.

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13

SELECTION OF OPTIMAL ADJUVANTS AND PRODUCT FACTORS THAT AFFECT VACCINE IMMUNOGENICITY

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13.1 INTRODUCTION

Human vaccines have now been used for over two centuries since the first vaccination trial for cowpox by Edward Jenner. They have been playing a key role in preventing or controlling the occurrence and spreading of numerous deadly diseases. It is estimated that universal influenza vaccination would save 250,000–500,000 annual deaths worldwide [1]. Economically, vaccines reduced significantly overall health-care costs—a worldwide challenge. The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) has saved \$10,400 per life-year health-care cost on invasive pneumococcal diseases since 2000 [2]. Through prevention or treatment of many conditions such as allergic reactions, autoimmune disorders, and lifestyle-related conditions, vaccines are dramatically improving our quality of life. Despite the long

vaccine history and success, design and development of efficacious and safe vaccines has been traditionally semiempirical, even though novel methods are being developed such as reverse vaccinology [3]. Partly because of this, many infectious and/or diseases are still poorly controlled, such as human immunodeficiency virus (HIV), malaria, pandemic flu, and various types of cancers. Various strategies have been investigated for the improvement of vaccine efficacy. Among these, use of a vaccine adjuvant(s) has been a top choice with successes. Until recently, however, only one type of adjuvant—aluminum salts—has been used/approved in the United States, even though a variety of adjuvants have been evaluated in the past few decades. Therefore, development or identification of effective or novel vaccine adjuvants is urgent and remains to be an area of extensive investigation [4–6]. This review summarizes the key adjuvant or adjuvant systems that have been evaluated in recent years and product-related factors that affect the vaccine immunogenicity/efficacy with an intention to facilitate more efficient development of vaccine products to combat human diseases.

13.2 VACCINE-INDUCED IMMUNOLOGICAL RESPONSES

Many diseases result from inadequate response from the innate immune systems. Vaccines are used to increase the immunological response for presentation or treatment of human diseases. Many types of vaccines have been designed to initiate the desired immune response with minimal safety issues. Table 13.1 lists the major types of vaccines based on different classification categories.

13.2.1 Major Cells and Receptors in an Immune Response

The natural immune system involves many types of cells in an immune response, including dendritic cells, macrophages, mast cells, eosinophils, neutrophils, B lymphocytes, and T lymphocytes. A key player in the human immune response is the dendritic

TABLE 13.1. Common Types of Human Vaccines

Classification	Type of Vaccines	Examples	References
Antigen type	Whole cell	<i>Leishmania amazonensis</i>	437
	Virus		
	Bacteria	<i>Shigella flexneri 2a</i>	438
	Protein	Smallpox proteins	439
	Peptide	Amyloid- β	440
	DNA	Malaria	371
Antigen treatment	Attenuated live	<i>Shigella flexneri 2a</i>	438
	Inactivated	Avian influenza (H5N1)	361
	Conjugated	Polysaccharide conjugate	3
Purpose	Prophylactic	Flu	133
	Therapeutic	Cancer	339
Administration	Injectible	Flu	269
	Mucosal	Flu	441

cells (DCs) [7, 8]. They sample/acquire and present vaccine antigens administered and, thus, are also called antigen-presenting cells (APCs). DCs are considered the most effective APCs among several cell types [9]. Activated DCs drain into the local lymph node, where they present the antigen and direct the differentiation of T helper (Th) cells into different effector cells, which lead to recognition of B- and T-cell epitopes. While B-cell epitopes are usually conformational, T-cell epitopes are typically limited to human leukocyte antigen (HLA) antigens [10].

There are a few types of receptors involved in an immunological response. The innate immune system has special pattern recognition receptors (PRRs), which can recognize conserved microbial structures—pathogen-associated molecular patterns (PAMPs) [11]. PRRs enable recognition of self and foreign antigens. A major category of such receptors are Toll-like receptors (TLR1–9) for capturing pathogens and activating DCs. They play a crucial role in pathogen recognition and the induction of immune response. They are widely expressed in cells of the immune system, epithelial cells, endothelium, cardiomyocytes, and adipocytes for recognition of pathogens [11] and could be utilized potentially as activation pathways for the same or different adjuvants [6, 12].

Stimulation of TLRs may lead to several events—stimulated synthesis of antimicrobial substances and pro-inflammatory cytokines (Tumor necrosis factor TNF- α , interleukin IL-1, IL-6, IL-8, IL-12, etc.), activation of dendritic cell maturation (increased expression of co-stimulatory molecules such as CD40, CD80, and CD86 and major histocompatibility complex (MHC) antigens), and more effective antigen presentation. Upon activation, TLRs could also influence directly or indirectly the function of T cells such as CD4+, CD25+, and T regulatory cells (Tregs). Therefore, failure to trigger this activation mechanism could lead to failure of a vaccine such as the respiratory syncytial virus vaccine in 1966 (Newswise.com, Dec-15, 2008).

13.2.2 Th1 vs. Th2 Type

Dendritic cells present antigen peptides as a peptide/MHC class II protein complex, which binds to the T cell receptors (TcRs) on T helper cells for activation of T helper cells—activation of the major biochemical pathways in the cytosol of the T helper cells (signal 1). Interaction between CD28 on T helper cells and CD80 or CD86 on DCs activates the second independent biochemical pathway (signal 2). Activation of the two pathways within T helper cells leads to the self-cell proliferation by releasing IL-2 (T-cell growth factor). After many generations, the T helper cells differentiate into effector T helper cells, memory T helper cells, and suppressor T helper cells.

The proliferating T helper cells differentiate into two major subtypes: Th1 and Th2 cells. DCs are critical in controlling the direction of T helper cells' differentiation through secretion of certain cytokines [13]. Secretion of interferon (IFN- γ), IL-2, and IL12 promote Th1 differentiation, leading to secretion of IFN- γ , IL-2, and IL12, proliferation of cytotoxic CD8+ T cells, macrophage activation, and production of TNF- γ , TNF- β , and IgG2a. Secretion of IL-1 β and IL-18 promote Th2 differentiation, leading to secretion of IL-4, IL-5, IL-6, IL-10, IL-13, and stimulation of B-cell proliferation, and subsequent Ab production, with a typical initial phase of

IgM production, followed by more specific IgG conversion with a few days to weeks [14]. Enhancement of antigen-specific antibody production is a critical postvaccination event, as the natural antibodies of the innate immune system is often ineffective due to their low affinity and non-anamnesia in an immune response [15]. Secretion of host IFN- γ is not required to initiate a Th1 immune response [16]. Additional T cells can be involved such as Th17, which produces IL-17.

Vaccine-induced immune responses can be generally divided into these two different biased effects: Th1 vs. Th2 types. Many factors can contribute to the type of specific immune response of a vaccine, including the types of antigens, adjuvants, and the like. Even the intensity of an immune response can make a difference. A weak immune response is generally Th2 biased and, therefore, increasing the intensity of response may lead to a Th1 response [10]. The Th1 response to an intramuscular flu vaccine (Fluarix) increased when the dose was increased from one-tenth of a full dose to a full dose, with a relatively similar Th2 response [17].

It is generally believed that activation of Th1 and Th2 effector cells is effective against intracellular and extracellular pathogens, respectively [7]. However, the actual protection offered by these two pathways is still not so clear-cut. For example, it is expected that Th2 immune responses are required for protection against extracellular bacteria, such as *Helicobacter pylori*, but only a Th1-promoting vaccine (*H. pylori* sonicate + CpG oligonucleotide) showed protection in mice [18]. Similar results were obtained where a strong Th2-inducing protein vaccine was not as effective in the protection of mice from virus challenge [19].

13.3 ALUMINUM SALTS

13.3.1 Introduction

Aluminum salts have been used extensively as adjuvants both in veterinary and human vaccines [20, 21]. These salts include aluminum hydroxide [actually aluminum oxyhydroxide AlO(OH)], aluminum phosphate [actually aluminum hydroxyphosphate, Al(OH) x (PO₄) y], and alum [KAl(SO₄)₂]. Alum is often used in the literature exchangeably with the first two aluminum salts [22]. The major characteristics of the two major salt types are listed Table 13.2. In addition, the rate of acid neutralization was shown to be a better parameter for characterizing time-induced changes in physical properties of these salts compared to adsorptive capacity [23].

The U.S. regulatory limit for aluminum in biological products (including vaccines) is 0.85 mg/dose and 0.125 mg/dose in Europe. The amount of aluminum in most vaccines is generally below this limit. Nevertheless, the recent investigation on the cause of Gulf War syndrome links subcutaneous administration of two equivalent-to-human doses of aluminum hydroxide to neuron toxicities in mice [24]. Therefore, a minimal amount of such salts is recommended for use as vaccine adjuvants.

13.3.2 Mechanisms of Action

Aluminum salts effectively enhance the immunogenicity of many types of vaccine antigens, including proteins [25], viruses [26], and the like. Their mechanism of action

TABLE 13.2. Major Characteristics of Aluminum Hydroxide and Aluminum Phosphate

Aluminum Salts	PZC	Size	Solubility	Other Properties	PK Properties	References
Aluminum hydroxide $\text{Al}(\text{OH})$	11.4	Primary particles, fibers, $4.5 \times 2.2 \times 10 \text{ nm}$ Aggregates: 1–20 μg in diameter	Well-shaped with $\text{pH} < 1 \mu\text{g/ml}$ at $\text{pH } 5\text{--}9$; crystalline	Surface area of primary particles: about 500 m^2/g	17% absorption in 28 days following IM injection in rabbits; mainly distributed in kidney and spleen	28; 55, 442
Aluminum phosphate $\text{Al}(\text{OH})_x(\text{PO}_4)_y$	9.6–4.0; 5.5 (prepared at $\text{pH } 3$) 4.2 (prepared at $\text{pH } 7.5$)	Primary particles, plates around 50 nm; Bell-shaped with pH; octahe- drally/tetrahedrally coordinated aluminum	Well-shaped with $\text{pH} < 5 \mu\text{g/ml}$ at $\text{pH } 5\text{--}6.5$; amorphous	Density 2.05 g/ml at pH 3 to 2.15 g/ml at pH 7	70% absorption in 28 days following IM injection in rabbits; mainly distributed in kidney and spleen	28; 54, 443; 55; 444; 442

for the stimulation of the immune system remains a subject of continuous investigation [21, 22, 27]. Several mechanisms have been demonstrated, including recruitment of dendritic cells, retention of antigens, more efficient uptake of antigens by dendritic cells at the injection sites, more effective activation of antigen-specific T cells, and complement activation and induction of necrosis at the injection site [28–32]. Aluminum salts have been shown to enhance activation of antigen-specific T cells by mouse dendritic cells through IL-2 production [31], stimulate release of IL-1 β and IL-18 from mouse dendritic cells via caspase-1 activation [31], and protein antigen process and presentations by DCs [33]. More recently, several studies showed simultaneously that aluminum stimulates immune response to an antigen through the nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs; cytosolic), specifically NALP3 (Nacht Domain-, Leucine-Rich Repeat-, and PYD-containing protein 3). Aluminum adjuvants activate an intracellular innate immune response system, called the NALP3 inflammasome (also known as cryopyrin, CIAS1, or NLRP3) [34] and via NALP3, induce secretion of mature IL-1 β , IL-18, and IL-33 in human and mouse macrophages [35]. Other particulate adjuvants, such as QuilA and chitosan, induce similar inflammasome activation, suggesting that activation of the NALP3 inflammasome may be a common mechanism of action for particulate adjuvants [35]. However, aluminum-induced release of IL-1 β via the NLRP3 inflammasome is dispensable for aluminum-mediated adjuvant activity in mice [22]. Therefore, any activators of NLRP3 may be potentially effective adjuvants.

The available data both in vitro and in vivo suggest that aluminum salts generally induce a Th2 response [31, 36]. Th2 differentiation in mouse DCs was apparently directed by the specific secretion of IL-1 β and IL-18 and lack of IL-12 secretion (IL-12 directs differentiation of CD4 T cells to Th1 cells) [31]. The Th2 response of alum is not purely a particulate-related consequence, as incubation of conjugated ovalbumin to polystyrene beads (48 nm) induced substantial Th1 responses with moderate Th2 responses in sheep [37]. Generally, the Th2 effect is dependent on the amount and type of aluminum salts used in the formulation [31]. Differential effects have been seen between 500 and 158 μ g of aluminum (Alhydrogel) with 25 μ g of *Bacillus anthracis* recombinant protective antigen (rPA) in inducing neutralizing antibodies [38].

On the other hand, aluminum salt may not always induce additional immunogenicity enhancement. Aluminum hydroxide at 0.6 mg in 0.5 ml did not show any significant effect in inducing serum anti-HA antibody formation after immunization of healthy elderly adults with 7.5, 15, or 45 μ g hemagglutinin (HA) by intramuscular injection (IM) [39]. Aluminum hydroxide can even suppress the immune response of vaccines in some cases [28, 40].

13.3.3 Antigen Adsorption

The large surface area of aluminum salts is ready for antigen adsorption (Table 13.2). There are basically two major adsorption mechanisms of antigens on the surface of aluminum salt—electrostatic attraction and ligand exchange [41, 42]. When the antigen and aluminum salts carry the same type of charge (repulsive force), antigen absorption is likely through ligand exchange [43, 44]. Other mechanisms of adsorption were also proposed, including hydrophobic interactions [43] and non-charge-associated

surface interactions such as monosaccharides on the surface of aluminum oxide at the point of zero charge (PZC) (pH 9.0) [45]. These different mechanisms of adsorption may coexist for a single antigen (30).

The adsorption of proteins to aluminum salts is generally very fast. The adsorption of several antigens— α -casein, bovine serum albumin (BSA), myoglobin, and recombinant protective antigen (rPA)—to aluminum hydroxide took within one minute [46]. So is the adsorption of three recombinant botulinum neurotoxin antigens to aluminum hydroxide [41]. This may take a little longer if ligand exchange and/or phosphorylation of aluminum hydroxide (in a phosphate buffer) takes place [47].

Two parameters are often used to describe antigen adsorption—adsorption capacity (or maximum adsorption) and adsorption coefficient (the binding strength). The adsorption capacity seems to be maximized to a monolayer coverage. This is certainly the case for ligand-exchange-dominant adsorption of a monoclonal antibody on aluminum hydroxide [42] and hepatitis B surface antigen (HBsAg) on aluminum hydroxide [48]. For HBsAg the monolayer coverage is at 1.7 mg/mg Al [48]. Similar levels of protein antigen adsorption were also reported for several other proteins. The adsorptive capacity of lysozyme, ovalbumin, and BSA at pH 7.4 is 1.4 (AdjuPhos), 1.6 (Alhydrogel), and 2.2 (Alhydrogel) mg/mg Al, respectively [49]. On the other hand, the adsorptive capacity of proteins can vary significantly (>10 times), depending on the source, preparation method, and age of the aluminum salts [46, 47, 50, 51]. If the protein and aluminum salts carry the same charge, a significantly lower amount of antigen can be adsorbed. The maximum adsorption of endotoxin (pI about 2) at pH 7.4 and 25°C is only 3 μ g/mg Al on aluminum phosphate [50]. The amount adsorbed under such conditions would heavily depend on the number of phosphorylation sites on the antigen [30, 52].

The binding strength of antigen can be evaluated based on its resistance to desorption in an elution buffer or in an interstitial fluid [48]. The adsorption coefficients often correlate with the adsorption capacities [47, 50] and can be significantly different in different studies [53]. Generally, ligand exchange-based adsorption offers more binding strength than that from electrostatic attraction. The adsorption of HBsAg was strongly adsorbed on aluminum hydroxide with an adsorptive coefficient of 6.0 ml/ μ g due to the presence of phospholipids for ligand exchange [48]. The elution of ovalbumin from aluminum hydroxide adjuvant upon exposure to interstitial fluid was inversely related to the degree of phosphorylation of the ovalbumin [30].

13.3.4 Factors Affecting Antigen Adsorption

The rate of antigen adsorption and adsorption capacity on aluminum salts depend on many factors. The most critical one is arguably the solution pH, as this will determine charged states of antigen and aluminum salts and, hence, the maximum adsorption. The influence of pH on antigen adsorption can be complex because of the presence of multiple adsorption mechanisms, pH-dependent stability of antigens, and pH-dependent solubility of both antigens and aluminum salts [42]. Different parabolic pH relationships were reported on lysozyme adsorption on aluminum phosphate (minimum at pH 4) [54] and ovalbumin (pI = 4.7) adsorption on aluminum hydroxide (maximum

at pH 4.3–6.2) [55]. Maximum adsorption can be found even in a pH range, where antigens and aluminum salts carry the same charges [42].

Other solution factors can also play a significant role. Presence of phosphate ions in a solution can significantly reduce both the adsorption capacity and coefficient of antigens on aluminum hydroxide due to a variable degree of phosphorylation [44, 47]. The adsorption capacity of a monoclonal antibody at pH 7.4 decreased from 1.5 mg/mg Al in water to 0.14 mg/mg Al in the presence of 100 mM phosphate [42]. The ionic strength of the solution may change the adsorption capacity of an antigen based on electrostatic interactions [42] but should not have a significant effect on ligand exchange-based antigen adsorption [48]. Presence of other proteins, excipients, and/or multivalent anions (such as α -hydroxy carboxylic acid, citric acid, lactic acid, and malic acid) would also influence antigen adsorption. The adsorption capacity of a monoclonal antibody at pH 7.4 decreased from 1.5 mg/mg Al in water to 1.1, 0.88, and 0.83 mg/mg Al, respectively, at 5°C, room temperature, and 37°C in simulated interstitial fluid (25 mg/ml BSA; 2.7 mM citrate, 5 mM phosphate, 154 mM NaCl) [42]. Several excipients have been shown to have a negative effect on antigen adsorption, including ethylenediaminetetra acetic acid (EDTA) on the adsorptive capacity of recombinant protective antigen (pI 5.6) on aluminum hydroxide [47], trehalose, or a combination of trehalose and Tween 20 on the total adsorption capacity of trivalent protein antigens on Alhydrogel [52], and sucrose on the adsorption capacity of all three recombinant botulinum neurotoxin antigens [41]. The negative effect of neutral molecules could be due to their stabilizing effect on antigens, preventing antigens from effective hydrophobic interactions with the aluminum surface.

13.3.5 Degree and Strength of Adsorption vs. Immunogenicity

It has been a general belief that antigens need to be adsorbed on aluminum salts for optimal immunogenicity effect. However, the exact relationship between the degree of antigen adsorption and *in vivo* immunogenicity is not thoroughly understood and inconsistencies do exist. Subcutaneous administration of lysozyme/aluminum hydroxide mixtures with different degrees of adsorption of 3, 35, or 85% led to generation of the same level of antilysozyme antibody titers in rabbits [25]. One study shows that administration of nonadsorbed protein antigens (dephosphorylated α -casein, dephosphorylated ovalbumin, or lysozyme) with aluminum phosphate induced levels of antibody titers that were similar to that for antigens adsorbed on aluminum phosphate in mice [56]. Using recombinant N terminus of Als3p (rAls3p-N) as a model antigen, Lin et al. [57] found that more rAls3p-N was bound on aluminum hydroxide in saline than in phosphate-buffered saline (PBS), but the immunogenicity and efficacy were superior with antigens in PBS.

Indeed, several studies showed that a tight binding between antigens and aluminum salts would inhibit immunogenicity. Using α -casein and dephosphorylated α -casein as model antigens and nontreated/phosphate-treated aluminum hydroxide as adjuvants, it was shown that the geometric mean antibody titer in mice was inversely related to the adsorptive coefficient of antigens [58]. Similar results were obtained with HBsAg as a model antigen with aluminum hydroxide [53] or with aluminum

hydroxyphosphate sulfate in mice [59]. These results clearly support that the immune response could negatively correlate with the degree of adsorption of proteins on aluminum adjuvants [42].

Can binding strength of an antigen in interstitial fluid predict immunogenicity better, as this fluid mimics the *in vivo* tissue environment? Again, results from these studies were not completely consistent. While the degree of lysozyme adsorption on aluminum hydroxide in sheep interstitial fluid correlated with the immune response (formation of anti-lysozyme antibodies) after subcutaneous administration [25], tight binding of antigens (nonrelease in interstitial fluid) has generated conflicting results—negative for endotoxin vaccine with aluminum hydroxide in rats [50] but positive for α -casein with aluminum hydroxide in mice [29]. In certain cases, enhancement of immunogenicity is achieved even when the antigens are not adsorbed on aluminum salts [56]. Therefore, the binding strength of antigen in interstitial fluid does not predict immunogenicity.

13.3.6 Storage and Process Stability of Aluminum Salts

Storage Stability The storage stability of aluminum salts has not been studied extensively because of its long history of use, and lack of accurate methods for monitoring its stability, such as particle size. These salts appear to be stable under normal storage conditions but subtle changes may occur. Aging of aluminum phosphate prepared under uncontrolled pH conditions resulted in a drop in pH (as much as 0.9 unit at P/Al ratio of 0.25) in 3 months at room temperature [54].

Thermal Stability Thermal treatment of aluminum hydroxide at 80°C for 24 h did not affect the adsorption capacity of ovalbumin (pI 4.7) [51]. Higher temperature causes changes. Aluminum phosphate remained amorphous when autoclaved for 30 or 60 min at 121°C but deprotonation and dehydration reactions in this process reduced the lysozyme adsorption capacity, rate of acid neutralization at pH 2.5, and point of zero charge [60]. Autoclaving aluminum hydroxide adjuvant increased the degree of crystallinity with the same deprotonation/dehydration reactions, which also reduced the protein adsorption capacity and viscosity [51, 60].

Freeze–Thaw Stability The aluminum salts do not tolerate the freeze–thaw process well, as freezing causes irreversible coagulation [28]. Freeze-thawing of 0.2% Alhydrogel at pH 4.0 (to –40°C) caused particle aggregation, which is inversely related to the cooling or thawing rate [61]. Freeze-thawing of aluminum hydroxycarbonate gel caused coagulation, leading to the formation of visible aggregates without changing the point of zero charge [62]. Single or repeated freezing of alum-containing hepatitis B vaccine at temperatures of –10°C or lower resulted in aggregation of the adjuvant–antigen particles, which exacerbated with duration of freezing, lower temperature, and the number of freezing episodes [63]. The rate of freezing was inversely related to the aggregate size (aluminum hydroxycarbonate gel) [62].

The freezing-induced aggregation of aluminum salts could be reversed by ultrasonic treatment or homogenization [62]. Use of proper formulation excipients

could potentially inhibit the freezing-induced particle aggregation. The freezing-induced particle aggregation in a hepatitis B vaccine could be prevented by including poly(ethylene glycol) (PEG) 300, propylene glycol, or glycerol [64]. At least 10% propylene glycol appeared to be needed for complete protection from freezing-induced particle aggregation [63]. The protective effect seems attributable to their general lyoprotective effect rather than reduction in freezing temperature. Other excipients are also found to be effective in inhibiting freezing-reduced aggregation, such as adsorbable polymers or surface-active agents [62] and trehalose [61].

Freeze-Drying Stability The additional drying process after freezing also leads to aggregation of aluminum salts. It has been shown that freeze-drying of 0.2% Alhydrogel caused particle aggregation with bigger median diameter than that caused by the freeze-thawing process alone [61]. Lyophilization of a model antigen, bovine intestinal alkaline phosphatase adsorbed on aluminum hydroxide, induced adjuvant aggregation and reduced the antigen's enzymatic activity (up to 50% drop in activity) [65]. As for the freezing process, addition of adequate amount of excipients such as trehalose could potentially minimize or prevent freeze-drying-induced aggregation [61].

The effect of process-induced aggregation of aluminum salts on immunogenicity may depend on the degree of aggregation and type/level of antigen. While lyophilization-induced particle aggregation (2–16 μm mean particle diameter) did not translate into any change in immunogenicity of alkaline phosphatase in terms of antiantigen titers (IgG1) in mice [65], freezing-induced aggregation of aluminum salt reduced significantly the immunogenicity of hepatitis B vaccine in mice [63, 64].

13.3.7 Stability of Antigen with Aluminum Salts

The stability of antigen in the presence of aluminum salts has not been studied extensively, partly due to the interference of aluminum salts with different analytical assays. Certainly, the tertiary structures of antigens can change to a variable degree upon adsorption to aluminum salts. This is the case for all three protein antigens, lysozyme, ovalbumin, and bovine serum albumin with reduced T_m 's upon adsorption onto aluminum salts [49]. Of course, change in antigen structure or aggregation state may or may not translate into different effect in immunogenicity. In fact, the perturbed tertiary structure could possibly facilitate the presentation of antigens and thus contribute to the adjuvant activity of the aluminum salts [49].

Because of the interference of aluminum salts, antigens are sometimes analyzed after dissociation. However, dissociation may reverse or further alter the structural changes caused by adsorption. The adsorption of BSA and multiple antigens (for group A *Streptococcus*; GrAS) on aluminum hydroxide were easily dissociable with a 48-h treatment at 4°C with 0.85% phosphoric acid plus 4 M GdnHCl, and the eluted antigens are structurally and functionally intact as judged relative to both treated and untreated antigen controls by circular dichroism, 4,4'-bis(1-anilinonaphthalene 8-sulfonate) (bis-ANS) binding, and antigenicity determined by enzyme-linked immunosorbent assay (ELISA) [66]. In such cases, the adsorption-induced structural changes can only be studied without the dissociation process.

During storage, a few examples show that antigen stability was reduced with aluminum salts. It was found that BSA formulated with aluminum hydroxide showed more dimer formation ($31 \pm 5\%$) relative to the elution control (9% dimer) upon storage [66]. The oxidation and deamidation of aluminum-adjuvanted rBoNT(Ec) was faster than that without adjuvant both at 4 and 30°C and could not be improved by addition of 7.5% sucrose or combination of sucrose and 0.01% Tween 20 [52]. One cause for the protein instability is the different microenvironment pHs on the aluminum particle surface. It has been shown that the surface of aluminum hydroxide is about 2 pH units higher than the bulk pH due to the accumulation of hydroxyls on the particle surface [67]. Therefore, for a pH-sensitive antigen, the bulk pH needs to be adjusted lower than its optimal value to maximize the stability in the microenvironment of the adjuvant. Addition of formulation excipients can also be effective, such as 20% propylene glycol in an aluminum-hydroxide-adjuvanted hepatitis B vaccine (HBsAg) [68].

Process-induced aggregation of aluminum salts generally exacerbates protein instability. Single or repeated freezing of aluminum-containing hepatitis B vaccine at temperatures of -10°C or lower resulted in structural damage of the antigen, probably due to the aggregation of particles [63, 64]. Lyophilization of bovine intestinal alkaline phosphatase, adsorbed on aluminum hydroxide, reduced the phosphatase enzymatic activity up to 50% [65]. Such a process could potentially reduce the *in vivo* immunogenicity [69].

13.4 NONALUMINUM ADJUVANTS

Because of the limited effect of aluminum salts, especially when used with recombinant proteins, nonaluminum adjuvants have been searched and tried extensively for the past 20 years [8, 70, 71]. Novel adjuvants are greatly desired with more potency, more balanced immune response, and less side effect/reactogenicity [70, 71]. Although not proven in the United States, some of these novel adjuvants have been on the European market for some time (Table 13.3). In general, such adjuvants work through several mechanisms—antigen presentation, adjuvant–antigen complex/depot, enhanced antigen delivery to/into DCs, recruitment of immune cells, and immunomodulation.

13.4.1 Oligonucleotides

Oligonucleotides (ODNs) are widely studied as effective vaccine adjuvants. A major category of ODNs is the unmethylated CpG ODNs resembling bacterial deoxyribonucleic acid (DNA) structure. A series of review articles were recently published addressing CpGs as stand-alone or secondary immunotherapeutic agent [72], approaches for enhancement of immunostimulating effect of CpGs [73], microparticle-mediated enhancement of immunostimulating effect of CpGs [74, 75], dichotomous effects of CpG as a cancer vaccine adjuvant [76], use of various methods [77] or lipids [78] for improvement of CpG stability and delivery, and use of CpG–antigen conjugates for improvement of vaccine delivery and immunogenicity [79]. Non-CpG ODNs include 5'-TC dinucleotide structure with a thymidine-rich sequence [80].

TABLE 13.3. Modern Vaccine Adjuvants

Adjuvants	Adjuvant Composition	Representative Products	Indication
AS03	Squalene-based oil-in-water emulsions	Pandemrix	Pandemic flu
AS04	Alum + MPL	Fendrix Cervarix	HBV HPV
MF59	Squalene-based oil-in-water emulsions	Fluad	Seasonal flu
Virosomes (150 nm)	Phosphatidylcholine bilayer liposomes	Inflexal V	Seasonal flu

Oligonucleotides can be effective immunomodulators through activation of TLR9 to initiate immune response [72]. TLR9 is localized both intracellularly (endosomes of myeloid cells) and on the surface of epithelial cells [81]. TLR9 agonists directly induce the activation and maturation of dendritic cells and enhance differentiation of B cells into antibody-secreting plasma cells [72]. Since TLR9 signaling is not absolutely required in mice [82], other mechanisms of action could also be responsible for their immune enhancement, such as up-regulation of gene expression in mice [83] and formation of antigen–adjuvant complexes [84, 85]. Combined use of vaccines and immunostimulants is emerging as one of the innovative approaches in adjuvant development [86].

The CpG ODNs can be further classified into several categories (A, B, and C class) based on their relative activity on B cell and natural killer (NK) cell activation and cytokine production [87, 88]. All classes can be potent Th1 adjuvants [87, 88]. The Th1 polarization was demonstrated in vitro by treatment of bone-marrow-derived dendritic cells [89]. Enhanced in vivo immunogenicity (Th1) response was also demonstrated for a variety of antigens in mice, such as whole homogenate (WH) of *Trypanosoma cruzi* antigens [90], cystein proteinase [91], keyhole limpet hemocyanin (KLH)-conjugated peptides [92], and inactivated poliovirus vaccine [93]. The Non-CpG ODNs can induce activation of hB cells, but lacked significant stimulation of Th1-like cytokines/chemokines as CpG ODNs do [94].

Use of CpGs often generates a more balanced immune response. For example, use of CpG 2007 (22-mer) not only enhanced antigen (hen egg lysozyme)-specific humoral responses, but also induced long-lasting cell-mediated immune response against the model antigen (HEL) in calves after SC administration [84]. Similar examples of antigens include OVA by CpG 1826 (20-mer) [85], HBsAg by CpG 7909 (a 24-mer B-class CpG) [95], hepatitis C virus by three classes of CpGs [88], inactivated gp120-depleted HIV-1 immunogen by CpG ODN 2006 [96], and diphtheria–tetanus–pertussis (DPT) vaccine [97]. A balanced effect can make a vaccine effective against challenging disease such as tuberculosis [98]. A balanced immunogenicity effect can be also obtained with a DNA vaccine administered with CpG-enriched plasmids (5–20 CpG copies) in BALB/c mice [99].

CpG is quite effective in comparison with other adjuvants. IM injection of a whole homogenate (WH) of *T. cruzi* (parasite) antigens adjuvanted with 100 µg CpG

generated significantly higher serum antibody titers than with 625 µg alum in mice [90]. With a recombinant fusion protein (ovalbumin-LHRH) as an antigen CpG ODN 2006 in water-in-oil emulsion as an adjuvant was found to be equally potent to complete Freund's adjuvant (CFA) in immunization of heifers [100] and more effective than modified CFA (*Mycobacterium butyricum* instead of *Mycobacterium tuberculosis*) in immunization of rats [69]. Three shots of CpG BW006 (23-mer)-adjuvanted rabies virus vaccine offered equal protection against rabies virus challenge as with five shots of aluminum-adjuvanted rabies vaccine in mice [26]. Because the effect of CpG is clearly dose dependent in several studies [90, 101], reducing the dose of CpG or antigen could make it less effective. It was found that subcutaneous administration of γ -irradiation inactivated Venezuelan equine encephalitis virus (VEEV) vaccine (gV3526) with 20 µg of CpG 2395 induced less immunogenicity than that with 1 mg of aluminum hydroxide in mice [102]. CpG 2006 at 10 µg was less effective than 150 µg of aluminum hydroxide in enhancing the neutralizing titer after intramuscular injection of 0.625 µg Sabin strain of poliovirus type 2 in mice [93].

For aluminum-based vaccines, addition of CpG may further improve the immune response. Such examples include BioThrax (a licensed anthrax vaccine) in mice (intraperitoneally (IP) or subcutaneously (SC) and guinea pigs [103], hepatitis B virus vaccine (Engerix-B) in chimpanzees [104], poliovirus vaccine in mice [93], and *Plasmodium falciparum* Apical Membrane Antigen 1 vaccine in humans [105]. This also seems to be the case for non-CpG ODN, such as IMT504 (24-mer) with aluminum-based recombinant HBsAg in monkeys [94]. When CpGs are used with aluminum-based vaccines, the immunostimulatory effect of CpG may depend on the relative association of CpG and antigen to the aluminum adjuvant [106]. A large percentage of CpG 7909 (60%) at 1 mg/ml can be adsorbed easily onto Alhydrogel (1.6 mg/ml Al₂O₃) in 10 mM Tris buffer [106]. Dose-dependent CPG 7909 binding to AMA1-C1/Alhydrogel was bell shaped with the highest antibody response in mice at a CPG 7909 concentration corresponding to saturated binding to Alhydrogel, while unbound CpG 7909 was found ineffective in enhancing antibody response [107]. In contrast, changing the percentage of bound CpG 7909 on Alhydrogel with different buffer systems did not change the peak level of antibody formation in a phase I trial of a malaria vaccine [108].

CpG ODNs could also improve the immune response of mucosal vaccines, such as those applied in vagina and gastro intestinal (GI) tract [109]. CpG was shown to promote a strong antigen-specific Th1-like immune response in the mucosa and local lymph nodes after mucosal application with glycoprotein D of herpes simplex virus type 2 (HSV-2) and protection against mucosal viral challenge in mice [110]. Intranasal administration seems to be especially effective in inducing both systemic and mucosal immune responses [111]. Because of this, intranasal administration of CpG ODN in both murine leishmaniasis and toxoplasmosis models in mice resulted in comparable results against challenge as that after subcutaneous administration [112]. In comparison, oral delivery of CpG ODN has not generated consistent results. While oral delivery of CpG ODN (20-mer) with purified HBsAg or tetanus toxoid (TT) in mice augmented both mucosal (IgA in lung, vaginal, or gut washes, feces, and saliva) and systemic immune responses (IgG in plasma, CTL, T-cell proliferation)

[113], oral uptake of uncoupled CpG ODN resulted in a complete failure of treatment against murine leishmaniasis and toxoplasmosis infection in mice presumably due to CpG degradation [112]. To overcome the stability problem, Wang et al. [114] designed “second-generation” immunomodulatory oligonucleotides including: CpR, YpG, or R'pG ($R = 2'$ -deoxy-7-deazaguanosine, $Y = 2'$ -deoxy-5-hydroxy-cytidine, and $R' = 1-[2'$ -deoxy-beta-*d*-ribofuranosyl]-2-oxo-7-deaza-8-methyl-purine). Indeed, these were significantly more stable than CpG DNA following oral administration and induced stronger local (IgA) and systemic (serum IgG2a) immune responses to ovalbumin (model antigen) than CpG DNA in mice.

On the other hand, use of CpG is not always beneficial. The humoral response to intramuscular immunization with Fluarix in healthy volunteers were not significantly enhanced by inclusion of 1 mg CpG 7909 in healthy volunteers, although a positive effect was seen at a 1/10th dose of Fluarix [17]. A CpG ODN (20-mer) was found ineffective in enhancing the antibody titer (IgG2a) of urea-solubilized p55 antigen (from HIV-1) in mice, although positive adjuvant effect was seen when it was formulated in an emulsion or when p55 was bound to polylactide-*co*-glycolide microparticles [115]. Addition of CpG 1826 did not lead to additional immune enhancement for a peptide vaccine (complexed with an immune-enhancement double-stranded RNA (dsRNA) adjuvant—pI:C/E749-57) in mice [116] and for a montanide ISA720-adjuvanted opossum vaccine in rats [117]. While co-administration of CpG 1826 with a respiratory syncytial virus vaccine increased the efficacy of the vaccine, co-administration during primary infection actually enhanced the severity of the disease in mice [118].

The neutral or negative effect of CpG could be potentially due to its inherent property. All classes of CpGs were able to induce formation of IL-10 in healthy and hepatitis C virus (HCV) peripheral blood mononuclear cells (PBMC), which is proposed to promote formation of regulatory T cells (Treg), leading to inhibition of Th1-type T-cell responses [88]. Their effect on antigen integrity and the effect of other formulation excipients can also be partially responsible. CpG caused dissociation of antigen from Alhydrogel in the presence of phosphate, and formulation excipients strongly affected CpG 7909 adsorption on Alhydrogel [106]. Recent studies showed that the effect of CpG 7909 is also related to physical training in rats [119] and the immune status of the subjects [120]. Clinically, CpG is well tolerated in general but more frequent injection site pains and headaches are observed at a high dose in healthy volunteers [17, 101].

13.4.2 Emulsions

Traditionally, there are two types of emulsions for pharmaceutical applications—water in oil (w/o) or oil in water (o/w). Both types have been tried as vaccine adjuvants. Complete Freund's adjuvant (CFA) is a historically tested water-in-oil emulsion containing killed bacteria [121]. It has been proven to be a very effective adjuvant and generates balanced immune response [91]. The humoral immunogenicity enhancement of CFA is more effective than aluminum salts for a 42-amino-acid amyloid-beta peptide antigen [122] and for cystein proteinase antigen in mice [91]. However, severe toxicities have been observed even at a reduced dose, such as weight loss, leukocytosis, abdominal

adhesions, granulomatous peritonitis, and disrupted hyalimized myofibers in mice [91, 122]. Other animal toxicities include skin lesions in rats and arthritis in dogs [69].

Because of the high toxicities, incomplete Freunds adjuvant (IFA) has been developed. With less toxicities, this adjuvant is less potent in mice [122]. In addition, the water-in-oil emulsions were highly viscous and not stable [121]. Other water-in-oil emulsions were then developed, including Montanide ISA 51, which contains mineral oil and mannide mololeate as a surfactant. This adjuvant can generate similar quality and intensity of immunogenicity to aluminum hydroxide but side reactions are not desirable, including granuloma, local pain, tenderness, and erythema [121]. Montanide ISA 720 is another one (containing squalene, a metabolisable oil), which was shown to increase the humoral response to a malaria vaccine candidate in rhesus macaques and more potent than Alhydrogel [123]. A dose-escalating phase 1 trial of a vaccine containing recombinant *P. falciparum* apical membrane antigen 1 (AMA1) formulated in Montanide ISA 720 did not show any vaccine-related serious adverse events [124].

In comparison, oil-in-water emulsions seem to be safer than water-in-oil emulsions. The representative one is MF59, consisting of 4.3% metabolizable oil squalene from shark liver, 0.5% polysorbate 80, 0.5% sorbitan triolate, and 10 mM sodium citrate with a size of 160 nm [32, 36, 125]. It has been used in an influenza vaccine (Fluad) with good safety in more than 20 countries since 1997 [126, 127]. Indeed, MF59 does not seem to induce significant side effects compared with vaccines without MF59 in healthy adults [128]. Although antisqualene antibodies were frequently detectable at very low titers in the sera of naïve subjects, combined use of MF59 with a subunit influenza vaccine neither induced antisqualene antibodies nor enhanced preexisting antisqualene antibody titers [129]. However, MF59 has been shown to increase frequency of injection site pain in humans [130] and induced additional side effects (both systemic and local) in pigs [131, 132].

MF59 has been shown to initiate greater, longer-lasting, and broader immune responses than a nonadjuvanted split flu vaccine in healthy young children [133, 134] and in adults [130]. As an adjuvant for flu vaccine, it is more potent than aluminum-based adjuvants in terms of both antibody and T-cell responses [126]. Therefore, MF59-adjuvanted vaccines can be more effective than commercial product, such as hepatitis B virus (HBV) vaccine (containing recombinant PreS2 and S antigens) in healthy adult subjects [128]. However, the species- and antigen-dependent variation in immunogenicity enhancement by MF59 appears to be a deficiency of this adjuvant system [10].

The immune enhancement mechanism for MF59 has not been thoroughly investigated [27]. It has been proposed that MF59 recruits APCs to the injection site, enhance uptake of antigens into APCs, and activate innate immunity without activating TLR pathways [27, 125]. Although MF59 may be cleared independently from soluble antigens after intramuscular injection [135], its efficacy as adjuvant is likely attributable partly to its depot effect [121]. The individual components of the emulsion do not seem to be special, as replacement of the oil and surfactant maintains satisfactory immunological properties (antibody response to an antigen) in mice [136].

Other MF59-like oil-in-water emulsions have also been developed. Such a squalene-based emulsion system (with 5% squalene, 4% Poloxamer 105, and 2% Abil-Care as emulsifier) was mixed with inactivated virus suspension (rabies or

porcine parvovirus) and increased the immunogenic activity of nonpotentiated rabies vaccine approximately 1.8-fold, more than the effect of aluminum hydroxide [137]. CoVaccineHTTM, a submicron emulsion of squalene-in-water emulsion containing sucrose fatty acid sulfate esters, has been shown to increase the antibody responses against the homologous and an antigenically distinct heterologous influenza A/H5N1 strain for a whole inactivated influenza A/H5N1 virus vaccine in mice through TLR4 signaling [138]. AF03, another oil-in-water emulsion adjuvant, was found to induce stronger antibody responses to a pandemic influenza vaccine (at 0.3 µg HA) than nonadjuvanted vaccine in both naïve and seasonal flu-primed mice [139].

A frequently studied series of oil-in-water emulsion adjuvant systems are the AS series. A representative of this series is the AS03, which is a 10% oil-in-water emulsion-based adjuvant system comprising squalene, α -tocopherol, and emulsifying agent Tween 80 [140–142]. AS03 has been used in the product Prepondrix [H5N1 vaccine from GlaxoSmithKline (GSK)] in the European Union (EU) for a few years. This adjuvant (AS03A) has been shown to enhance the initial priming effect of pandemic influenza vaccination and promote a rapid humoral response to single boosting dose with a heterologous strain, therefore, not only reducing the dose of the antigen (up to 24 times) but also offering better cross protection against drifted strains in young, elderly, or Asian subjects [143, 144]. In a different study, administration of AS03A-adjuvanted inactivated split-virion H1N1 pandemic vaccines generated same level of immunogenicity as unadjuvanted vaccine at a fourfold higher dose in adults [145].

13.4.3 Iscomatrix

The immunostimulating complexes (ISCOMs) are antigen-containing particulate systems while iscomatrix systems are antigen-free, structurally similar systems [146–148]. Its first description was reported more than 2 decades ago as a novel structure for antigenic presentation of membrane proteins with potent immunomodulatory capability. An iscomatrix system consists of a Quil A-based saponin mixture (see discussion of QS21 below) combined with cholesterol [147]. This system enhances immunogenicity through several mechanisms, including recruitment and activation of APCs, extension of antigen presentation in the draining lymph node, enhancement of CD8 cross presentation, and induction of IFN- γ and IL-6 [32, 149]. Association of antigen with iscomatrix seems necessary for the optimal induction of cytotoxic T lymphocyte (CTL) responses [150].

Preclinical and clinical studies have shown that the iscomatrix adjuvant promotes both humoral and cellular immune responses due to the powerful immunomodulatory capability of saponin [147, 148]. Subcutaneous injection of iscomatrix-adjuvanted 4 dengue virus envelope proteins (10 µg) resulted in adequate protection in both mouse and monkey challenge models [151]. Such an immune enhancement effect of iscomatrix (50 µg) on recombinant HIV gp120 vaccine can be significantly greater than that of aluminum hydroxide [152]. Similarly, immunization of patients with a mixture of HPV16 E6E7 fusion protein and iscomatrix adjuvant-induced antigen-specific cell-mediated immunity in terms of antibody formation, delayed type hypersensitivity, in vitro cytokine release, and CD8 T cell responses [153]. To mitigate the potential safety

issues related to iscomatrix, Matrix M, the particles made of two selected and purified fractions of saponin, was developed and found to be effective to initiate strong, immediate, and long-term humoral immune response for influenza H5N1 vaccine with a balanced Th1/Th2 cytokine profile and high cross-reactivity against drifted H5N1 viruses in mice [154].

The lipophilic nature of iscomatrix also makes it an effective mucosal adjuvant. It was shown to induce more effective pulmonary protection (10- to 100-fold dose sparing) against viral challenge when it is used intranasally with split influenza vaccines in mice [155]. Deep pulmonary delivery of several iscomatrix-based vaccines has been shown to induce antigen-specific mucosal and systemic immunity [156, 157]. It was also shown to induce local and systemic immune responses against orally delivered protein antigens, partly due to the enhancement of antigen absorption in mice [149].

13.4.4 Liposomes/Proteoliposome/Virosomes

Liposomes are promising vaccine adjuvants, as they can be made of a variety of lipids and other additives [5, 158–160]. Liposomes have been shown to up-regulate several chemokine genes, including CCL2, CCL3, and CCL4, in dendritic cells (DC) [161]. Liposomes can deliver encapsulated antigen into cytosol of the antigen-presenting cells for both cell-mediated as well as humoral immune responses [162, 163]. It is believed that uptake of liposomes is generally through a passive phagocytic or endocytic process, not by fusing with cellular membranes [158]. Charged liposomes can bind to antigen readily and enhance the uptake of antigen and the efficiency of antigen presentation [164]. Liposome–antigen complexes could induce significantly higher cellular immune responses than antigen carried by aluminum hydroxide after subcutaneous administration in mice [165]. Other additives, such as vitamin E, can be included to improve the adjuvant effect (novasomes) [166].

Liposomes were shown to be effective in inducing different types and levels of immune response for a variety of vaccines/antigens, such as cytosolic proteins (sAg) of *Plasmodium yoelii nigeriensis* [162] and tuberculosis vaccine candidate Ag85B-ESAT-6 [167]. Liposomes can generate more efficient immune responses [168] and less side effects (limited IgE levels) than aluminum-adjuvanted vaccines [163].

The type and degree of immunogenicity enhancement effect depends on liposome's composition, size, and the type of antigens [159]. Liposomes made of *Saccharomyces cerevisiae* membrane lipids are more effective than egg PC liposomes in inducing IgG2a, IFN- γ , and IL-4 cytokine [162]. Cationic lipid vesicles (comprising a cationic cholesterol derivative, DC-Chol) bind strongly split influenza vaccine antigens and induced robust anti-influenza immune responses while neutral cholesterol/DOPC liposomes displayed virtually no stable antigen binding and no adjuvant effect in mice [169]. Saturated phospholipids are more effective than unsaturated phospholipids in the enhancement of allergen-specific IgG response upon immunization in mice [163]. Increasing the amount of fusogenic lipids (dioleoyl phosphatidylethanolamine) could further enhance the Th1 response to model ovalbumin antigen in mice [170]. Bile salt-incorporated lipid vesicles (bilosomes) of 980 nm in size containing influenza A antigen generated significantly more Th1-biased response than vesicles of 250 nm

in mice [171]. Vaxfectin, a cationic lipid-based adjuvant, was shown to increase the immune response for a seasonal influenza vaccine with a ≥ 10 -fold dose sparing effect in mice [172, 173]. Vaxfectin at a lower dose (30 μg) did not enhance antibody response but increased the number of IFN- γ secreting T cells by up to 18-fold. This suggests that a preferable Th1 or Th2 response can be selected simply by varying the ratio of adjuvant to antigen [172, 173]. Components in liposomes such as lipid A [174] or an immunomodulator α,α' -trehalose 6, 6'-dibehenate (TDB) can also be added or varied to achieve the same purpose [160].

More complexed lipid-based vesicles were also developed as potential adjuvants, including proteoliposomes (PL), cochleate structures (CS), and virosomes. Proteoliposomes in different sizes contains bacterial membrane components (e.g., the outer membrane of *Neisseria meningitidis* B), including lipopolysaccharides (LPS), phospholipids, and traces of bacterial DNA to improve immunogenicity [175, 176]. Cochleate structures (AFCo1) are made through interaction of divalent cations with anionic lipids in proteoliposomes. Virosomes mimic the structure of a virus. Proteoliposomes can up-regulate MHC-II, CD40, CD80, and CD86 expression and production of TNF- α and IL-12 (p70) in dendritic cells [177]. They were found to be effective in inducing a Th1-type immune response (production of IgG2a and IFN- γ) to allergens even in the presence of alum in mice [178]. Proteoliposomes can also be used as effective mucosal adjuvants. Intranasal administration of proteoliposomes containing LPS into mice led to high anti-LPS IgG titers [179]. Similarly, intranasal administration of proteoliposomes (AFPL1; 70 nm in size) containing glycoprotein D (gD) of herpes simplex virus type 2 (HSV-2) induced gD-specific IgG antibody formation, leading to partial protection against genital herpes infection in mice [175]. In comparison, intranasal administration of AFPL1-derived cochleate structures (AFCo1) containing the same protein elicited a complete protection in the study [175]. Indeed, the cochleate structures can be more potent than aluminum salts in inducing high levels of both IgG1 and IgG2a for a variety of pathogen-derived antigens in mice [180]. Virosomes have been successfully used in three commercial products [181]. The representative product is Inflexal V, a virosomal adjuvanted influenza vaccine, which is made of phosphatidylcholine bilayer liposomes, containing neuraminidase and hemagglutinin. By mimicking natural infection, the vaccine has shown good efficacy for all age groups [182]. The entire virosome has a diameter of 150 nm and have been proven safe with mostly mild to moderate symptoms resolvable within a few days [183].

The effect of liposomes on DNA vaccines has been inconsistent. Intramuscular injection of Vaxfectin-adjuvanted vaccines containing five *P. falciparum* protein-encoding plasmids enhanced both antibody and cellular immune responses to each component of the multiantigen vaccine with no apparent antigenic competition in mice [184]. In human trials, intramuscular injections of Vaxfectin-adjuvanted H5 hemagglutinin-encoding DNA vaccine led to fourfold rises in hemagglutination inhibition (HI) titers in 47–67% of subjects [185]. However, $\leq 20\%$ of subjects showed such a response after immunization with a trivalent Vaxfectin-adjuvanted DNA vaccine [185]. While liposomes, made of phosphatidyl choline (PC), dioleoyl phosphatidyl ethanolamine (DOPE), and dioleyoxy trimethyl ammonium propane (DOTAP) in a molar ratio of 4:2:1, were shown to be good adjuvant for DNA vaccination against

hepatitis infection in Rhesus monkeys [186], subcutaneous administration of hepatitis E virus neutralizing epitope-encoding or hepatitis B virus surface antigen-encoding DNAs entrapped in liposomes consisting of the same types of lipids did not elicit antibody response in mice [187]. In fact, lipid–DNA complexes actually appear to inhibit the expression of the complexed DNA compared with naked DNA after IM injection [158].

13.4.5 Polymeric Particulates

Polymeric particulates can be used as effective adjuvants [188]. Various polymers have been demonstrated to have such properties, such as polylactic acid (PLA), poly(lactide-co-glycolide) acid (PLGA) [189], chitosan [190], poly(γ -glutamic acid) (γ -PGA) [191], polyphosphazene [192], starch [193, 194], and polystyrene [37]. They enhance immunogenicity through several mechanisms, including enhancement of antigen uptake by dendritic cells (in vitro) [191], maturation of dendritic cells [191], promotion of proliferation of antigen-specific T cells [191], stimulation of both B and T lymphocytes [190], facilitation of activation of dendritic cells [195], and controlled release of antigen by particles [196]. Uptake of such microparticulate adjuvants by DCs can activate the NALP3 inflammasome, promoting innate and antigen-specific cellular immunity [75].

A widely studied polymer is PLA or PLGA. Antigens can either be adsorbed on the surface or encapsulated inside the polymeric particles. Intramuscular administration of adsorbed antigen—*N. meningitidis* serotype B (Men B) (99% adsorption efficiency) on PLGA particulates with CpG significantly increased the anti-Men B serum antibody titers and serum bactericidal titer in mice [189]. The immunogenicity enhancement can be comparable to that by traditional aluminum-hydroxide-adjuvanted vaccine [197] or even better than that by more advanced adjuvant such as Montanide ISA 720 [198]. Using poly(lactide-co-glycolide) as a model polymer and Men B as a model antigen, encapsulating CpG within PLG microparticles induced statistically significant higher antibody, bactericidal activity, and T-cell responses when compared to the soluble form of CpG [189].

Another widely studied polymer is chitosan. Addition of 0.5% of a chitosan derivative to an inactivated influenza vaccine resulted in a 4- or 6- to 10-fold increase in antibody titers after intramuscular injection of one- or two doses in mice, respectively [199]. Their effect of immune enhancement can be better than the traditional aluminum adjuvants. Intramuscular administration of chitosan nanoparticle (160–200 nm)-encapsulated recombinant hepatitis B surface antigen (rHBsAg; 10 μ g) induced a 9-fold higher anti-HBsAg IgG level compared to the conventional alum-adsorbed vaccine in mice [196]. Subcutaneous immunization of mice with HIV-1 p24 encapsulated in γ -PGA nanoparticles enhanced production of antigen-specific serum antibodies equivalent to that of the potent CFA [191]. Mannosylation of chitosan can further enhance the adjuvant activity of chitosan, as demonstrated both in vitro experiments and in vivo intranasal immunization in mice [200].

The degree and types of immunogenicity enhancement may be dependent on the types of polymers and their preparations. While immunization with zinc-chitosan

particles bound to histidine-tagged recombinant protein antigen promoted an IgG1 response in mice, subcutaneous immunization with a simple mixture of an influenza hemagglutinin (HA) vaccine and amphiphilic poly(γ -glutamic acid)-graft-l-phenylalanine copolymers (γ -PGA-NPs) led to a more balanced immune response than aluminum-adjuvanted vaccines in mice [201]. Some polymer particulates exert their immune enhancement activity upon conjugation with antigens, such as starch microparticles for protein antigens in mice [193, 194], and polystyrene beads for protein antigens in sheep [37].

The physical properties of particulates such as size and charge can have a significant effect on the immunogenicity. While no significant difference was found in the immune response (serum bactericidal activity and antibody titers) after intramuscular administration of MenB antigen adsorbed on PLGA nanoparticles (110 nm) or microparticles (800–900 nm) in mice [197], PLGA–CTAB (cetyl trimethylammonium bromide) particles of 0.3 μm were more effective than larger particles (1 and 30 μm) in the elicitation of transgene-specific serum IgG responses [202]. The greater effect with smaller particles could be related to two factors—higher adsorption capacity of smaller particles and greater efficiency of particle uptake by dendritic cells. It has been shown that the antigens adsorbed on aluminum particles of 3 μm are internalized more effectively than those of 17 μm [30]. Charged nanoparticles could present antigens effectively through electrostatic interactions with antigens. Immunization of HIV-1 Tat (1–72) protein (1 μg antigen dose) coated on anionic nanoparticles generated higher antibody titers than alum-adjuvanted vaccine [203]. Polyphosphazene (ion crosslinkable water-soluble polymers) has been shown to have a synergistic effect on enhancement of the secretion of cytokines in vitro when used with CpG and indolicidin, likely due to formation of antigen–adjuvant complexes [84]. Cationic block copolymers and DNA can easily form complex structures via electrostatic interaction and were shown to elicit broad and long-lasting antigen-specific humoral and cellular responses after intramuscular administration in mice [204].

Polymeric particles are also effective mucosal adjuvants. Examples include oral administration of PLGA microsphere-encapsulated concentrated rabies virus (CRV) in mice [205] or polyacryl starch microparticle-conjugated diphtheria toxin in mice [206]. In contrast, no immune response was seen after intranasal immunization with a mixture of γ -PGA-NPs and influenza virus hemagglutinin (HA) in mice [207]. The oral uptake of particulate antigens is presumably through the Peyer's patches in the GI tract [194].

13.4.6 Saponins and QS21

Saponins are natural glycosides of steroids or triterpenes. They possess certain adjuvant activities due to their structural features of sugar chain(s), its length, and hydrophilic and lipophilic properties [208]. Therefore, their adjuvant activity can be further modified by structural modifications [209]. The widely used saponin-based adjuvants are Quil A, isolated from the bark of *Quillaja saponaria* (QS) Molina, and QS21, a more purified form, is the 21st of 22 fractions in Reversed Phase-High Performance Liquid Chromatographic (RP-HPLC) of semipurified QS bark extracts [210].

These adjuvants have been evaluated in numerous preclinical and clinical trials [211]. They prove to be very effective in enhancing the immunogenicity of various vaccines, including A β (1–15) in mice [212], foot-and-mouth disease virus (FMDV) antigen in mice [213], L1 and A33 proteins in mice [214], vaccinia virus proteins in monkeys [214], parasitic antigens in sheep [215], and keyhole limpet hemocyanin (KLH) conjugate antigens [210, 216]. The effect is often toward a Th1-type response in comparison with aluminum-based vaccine such as A β (1–15), PADRE-A β (1–15)-MAP [212], L1 and A33 proteins in mice [214], and parasitic antigen (*Fasciola hepatica*) in sheep [215].

Quil A or QS21 is often much more effective than aluminum salts in immunogenicity enhancement for many antigens, such as A β (1–15) in mice [212], and SPf66 (a synthetic malaria peptide vaccine) [217]. Exceptions do exist. QS21 did not exert any significant effect on either binding or neutralizing antibody titers after IM immunization of gp120 HIV-1(MN) protein (rsgp120) at doses of 100, 300, and 600 μ g, even though it is effective at a lower antigen dose [218]. Similarly, QS21 adjuvant was not effective in enhancing the immunogenicity of an inactivated influenza vaccine in healthy young adults, in terms of serum titers, T-cell cytotoxicity, and IFN- γ levels [219].

A key issue of saponins is their induction of red blood cell hemolysis, and they are painful to inject with high local reactogenicity [32, 211]. The more purified form QS21 is much better tolerated but has also been associated with some side effects, preventing its use as an effective adjuvant. These side effects include moderate to severe pain associated with a recombinant HIV protein vaccine [218], site pain, and post-vaccination myalgias associated with inactivated influenza vaccine in healthy young adults [219], malaise, headache, fever, and nausea [218]. Two out of 89 subjects developed severe vaccine allergy following the third dose of 1/3 QS21/SPf66 formulations (a synthetic malaria peptide vaccine) [217].

Because of these potential toxicities, alternative sources or structural modifications of saponins were then sought. Platycodin D (PD), a saponin from the root of *Platycodon grandiflorum* without the acyl domain, is less hemolytic, very stable in aqueous solution, and enhanced the immunogenicity of HBsAg in mice [220]. This led to deacylation of QS21, but the deacylated QS21 was less effective as an adjuvant for ovalbumin in inducing IgG1 responses and inactive in inducing IgG2a or CTL responses at any doses in mice [221]. A semisynthetic saponin (GPI-0100) was found to enhance the immunogenicity of a single tandem fusion protein in mice [222] and enhance antibody titers against the glycolipid Globo H and the glycosylated mucin MUC2 with only occasional grade II local toxicity at a dose of 5000 μ g in cancer patients [223]. Less hemolytic saponins were also found in Chinese herbs [224]. Some are effective, such as *Achyranthes bidentata* (herb) saponins [225], *Glycyrrhiza uralensis* (herb) saponins (GLS) [226], *Bupleurum chinense* (herb) saponins [227], and *Panax ginseng* (root) saponins [228].

13.4.7 Virus-like Particles

Use of virus-like particles (VLPs) in vaccines has been widely studied [229]. VLPs are effective vaccines against the corresponding virus such as influenza VLPs [166].

VLPs possess key immunologic features of viruses—repetitive surfaces, particulate structures and induction of innate immunity through activation of pathogen-associated molecular pattern recognition receptors. Therefore, they can also facilitate presentation of a foreign antigen or hapten to the immune system through genetic fusion or chemical conjugation.

Most VLP conjugate vaccines seem to be effective. Examples include CCR5 peptide–VLP conjugates for HIV [230], M2 peptide–VLP conjugate for influenza [231], and nicotine–VLP conjugates via succinimide linkers for nicotine addiction [232]. It should be noted that VLPs may not be very stable in an aqueous solution and could be responsible for lower than expected immunogenicity [233]. Conjugation of VLPs with haptens may improve the stability of VLPs [231]. VLPs can be formed in vivo through DNA vaccination, which can be efficient immunogens for inducing cellular immune responses [234].

Virus replicon particles (VRP) can also be used as an effective systemic, cellular, and mucosal adjuvant. Intramuscular delivery of equine encephalitis VRP-conjugated OVA induced dose-dependent immune response in mice [235]. Alphavirus replicon particle (RP) containing H3N2 flu genes have been shown to be effective in inducing high antibody titers to the influenza HA protein in pigs [236].

13.4.8 Carrier Proteins

Many small-molecule drugs or even protein antigens cannot initiate any or adequate immune response. They can be conjugated or fused to an effective protein antigen to generate adequate immune response. Such carrier proteins have been recently reviewed, such as keyhole limpet hemocyanin (KLH) and bacterial proteins [237]. KLH belongs to the largest oxygen-transporting proteins in nature, and the glycoprotein moiety is critical for the antigenicity of the molecule. Yet, use of KLH conjugate alone may or may not initiate adequate immune response and another adjuvant often has to be used. Such combinations include KLH conjugates with QS21 for cancer antigen (GD3; ganglioside) [210], with Freund's adjuvant for influenza virus A M2 peptide [238], and with other adjuvants for cancer antigens (MUC1 and GD3) [209, 216]. The structure of linkers between hapten/antigen and KLH carrier protein may have a significant effect on the immunogenicity and selectivity of the conjugated vaccine [239].

Conjugation or fusion of a hapten or antigen with a bacterial or parasitic protein is frequently used. A class of such proteins is the bacterial heat shock proteins (HSPs). Success examples include HSP70 conjugates with viral MHC class-I-restricted epitope (for herpes simplex virus type-1) [240], and HSP65 conjugates with a protein containing linear repeats of the gonadotropin-releasing hormone (GnRH3), the hinge region of human IgG1 (hinge), or a T-helper epitope from the measles virus protein (MVP) [241]. The immunogenicity enhancement can be comparable to that in the presence of Freund's adjuvant. Because HSPs can effectively induce partial maturation of DCs in vitro [242], such proteins, as free forms, can also enhance immune responses of antigens, but the effect may not be as effective as other adjuvants such as CFA or LPS in mice [242]. Plasmid expressing HSPs could achieve the same effect [243].

Other bacterial proteins can also be effective. Fusion of the *Brucella* spp. lumazine synthase (BLS), a highly immunogenic (decameric) protein, with a model virus protein

domain [C486 bovine rotavirus VP8 core protein (VP8d)] led to almost 100% protection against homologous challenge with C486 bovine rotavirus [244]. Conjugation of synthetic peptides of influenza virus A M2 extracellular domain with *N. meningitidis* outer membrane protein complex (OMPC) was able to confer protection against lethal challenge of either H1N1 or H3N1 virus in the presence of Freund's adjuvants in mice [238].

13.4.9 Toxins

Toxins represent one of the most potent adjuvants that have been tested. Different toxins may have different degrees of effect. Zonula occludens toxin (a bacterial enterotoxin; 45-kD single polypeptide chain) is highly efficacious when compared to the mucosal adjuvant *Escherichia coli* heat-labile enterotoxin (LT) [245]. However, two major issues prevent them from use as vaccine adjuvants—their toxicity and the residual bacterial endotoxins. Options to bypass their toxicities include use of subunits of toxins, toxoids, or structurally modified versions [246]. As anticipated, the subunits may not be as effective [247].

Conjugation of these less effective toxin subunits or toxoids to antigens can potentially restore the efficiency in immunogenicity enhancement. Successful examples include fusion of subunit B of LT to a viral protein [248], conjugation of toxoids to capsular polysaccharides of many invasive bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, meningococci, pneumococci [3, 249, 250], and conjugation of LT subunits with CpG [109, 251]. It should be noted that the bacterial toxins can initiate a strong humoral immune response, which may overshadow the response to the conjugate antigen [8]. Adjuvants could also potentially enhance antibody formation against both the conjugate antigen and the carrier protein [216].

Bacterial toxins are considered the most potent mucosal adjuvants such as heat-labile enterotoxin and cholera toxin [36]. Intranasal delivery of ovalbumin with a recombinant bacterial enterotoxin *Zonula occludens* toxin (Zot; 45-kDa single polypeptide chain) induced high Ag-specific serum IgG titers over a year and is effective also through other mucosal routes in mice [245]. It can increase reversibly the intestinal mucosa permeability by affecting the structure of tight junctions. Several mutant heat-labile enterotoxins were found to be effective as adjuvants for nasal administration of deglycosylated chain A ricin (DGCA) [252], or influenza vaccine [253]. Cholera toxin B subunit (CT-B) has been shown to increase significantly the levels of antigen-specific serum IgG antibody titers after intranasal administration of DPT combination vaccine [254] and induced both anti-HA IgA and IgG in the airway and serum upon intranasal immunization of mice with influenza HA [255]. Vaginal administration of inactivated caprine herpes virus 1 vaccine with an enzymatically inactive mutant of the heat-labile enterotoxin of *E. coli*, LTK63 induced high levels of secretory IgA [256].

13.4.10 Lipopolysaccharides/Polysaccharides

Lipopolysaccharides (LPS) have been found to be effective adjuvants in many studies. Mechanistically, LPS activates TLR4 and directs DCs for a Th1 response [13, 31]. LPS

can facilitate internalization of particles by dendritic cells [257], stimulate significantly expression of CD40, CD80, CD86, and CD275 in dendritic cells and also stimulate release of IL-6, IL-12p40, and IL-12p70 [31]. Generation of a Th1 response can also be induced by LPS fused with calreticulin/peptide complex in mice [258]. However, bacterial polysaccharides are T cell-independent immunogens and if bound to proteins, both T- and B-cell arms of immune response can be engaged [237].

The potential toxicities of these compounds, especially those from Gram-negative bacteria, are the barrier for human use [259]. The purified *N. meningitidis* serogroup B lipopolysaccharide has high endotoxic activity but can be detoxified through structural modifications. LPS analog LpxL1 from *N. meningitidis* is nontoxic, and incorporation of this analog LpxL1 in influenza H5N1 virosomes induced significantly enhanced H5N1-specific total IgG titers as compared to nonadjuvanted virosomes in mice [260]. Conjugation with tetanus toxoid (TT) reduced the endotoxic activity of LPS by 2400 times, and the conjugated LPS-TT elicited higher anti-TT IgG2a and IgG1 levels than unconjugated TT in mice [261]. In addition, high levels of anti-LPS IgG and IgG subclasses were also detected in sera.

Many nonmicrobial polysaccharides have been shown to potentiate immune response, such as water-soluble polysaccharide (PAP) from the mycelium of *Polyporus albicans* (Chinese edible fungus) for subcutaneous immunization of mice with 0.1 mg ovalbumin [262], BOS 2000 (polysaccharides from *Boswellia serrata*) for hepatitis B in mice [263], lentinan [a (1–3)- β -D-glucan from the mushroom *Lentinus edodes*] or its sulfated form for Newcastle disease (ND) vaccine [264]. Their effects can be more effective than alum-based vaccine [263]. Intradermal administration of BSA conjugated to microparticulate β -glucan (MG) from the yeast *S. cerevisiae* enhanced the primary IgG antibody response to BSA in a manner comparable to the prototypic complete Freund's adjuvant in mice [265]. γ -Inulin, a human polysaccharide, was found to provide equal immunogenicity enhancement for HBsAg as FCA in mice without the toxicities [259].

13.4.11 Monophosphoryl Lipid A/Lipids

Monophosphoryl lipid A (MPL or MPLA) is a detoxified (chemically modified) form of the endotoxin lipopolysaccharide (from *Salmonella minnesota*). Its properties and applications have been reviewed recently [266]. It exerts its action through Toll-like receptor 4. OM-174, a lipid A analog when injected intravenously or subcutaneously in mice, induces the migration of DCs from the periphery to the T-cell areas of lymphoid organs, and their maturation into cells expressing high levels of MHC class II and co-stimulatory molecules, with its potency close to that of *E. coli* lipopolysaccharide (LPS) [267].

Both Th1- or Th2-biased response was observed with MPL-adjuvanted vaccines. Administration of the full-length recombinant toxoplasma GRA2 or GRA6 protein antigen in combination with MPL adjuvant led to high ratios of specific IgG2a/IgG1 (Th1) response in mice [268]. Similar Th1 effects were also observed with Fluzone adjuvanted with synthesized lipid A (6 acyl chains with a single phosphorylation site) in mice [269] and *Leishmania* sterol 24-c-methyltransferase antigen with MPL-SE [270]. In contrast, subcutaneous immunization of MPL-adjuvanted ovalbumin or

glutaraldehyde-modified ragweed pollen extract enhanced IgG1 titer (barely increased IgG2a) in mice [271].

MPL is currently a component of a licensed cancer vaccine, Melacine (Corixa Inc., Schering Plough). Nevertheless, its potential toxicity led to synthesis of MPL analogs. Three such analogs, synthesized by different substitutions at 3-O-position of the reducing sugar, were all found as effective as the natural compound in inducing antigen-specific T-cell proliferation and interferon- γ production with a liposome vaccine [272].

Many lipids have been shown to have immunomodulating effect. Lysophosphatidylcholine (LPC) mixed with various antigens induced cytotoxic T-cell responses and production of antigen-specific antibodies with an efficiency similar to alum in mice [273]. Immunization of mice with an HBV vaccine with various doses of β -glucosylceramide (β -GC), β -lactosylceramide (β -LC), or a combination of both augmented both the anti-HBV titers and the percentage of mice exhibiting high titers [274]. A similar lipid, α -galactosylceramide, increased the immune response after repeated intranasal or oral delivery of HIV peptide antigens [275]. This compound was able to stimulate repeatedly NKT cells without inducing anergy of NKT cells [276]. Synthetic alkylglycerol analogs in a mixture with ovalbumin have been shown to increase anti-Ova IgG antibody production in sera of immunized mice and the relative levels of IgG1 and IgG2a depended on the carbon chain length [277].

Mucosal applications of lipids can also be effective. Several studies have shown that intranasal delivery of an HIV-1 genetic vaccine (plasmids) in the presence of a cationic lipid adjuvant, the Eurocine N3, resulted in vaginal and rectal IgA responses as well as systemic humoral and cellular responses in mice [278, 279]. Initial immunization can be boosted intranasally with a gp41 peptide in an anionic L3 adjuvant for a lower dose of DNA [278].

13.4.12 Cytokines

Interferons Type I interferons (IFN- α , IFN- β , and IFN- ω) are cytokines with multiple biological activities in innate immunity, in dendritic cell maturation/differentiation, and so forth. IM vaccination of mice with a flu vaccine with IFN- α generated clear-cut IgG production (IgG2a rather than IgG1 and also IgA) and complete survival to infection with flu virus [280]. In a similar study, intramuscular injection of flu vaccine Vaxigrip ad-mixed with IFN- α markedly increased the serum levels of all four classes of flu-specific IgGs (IgG, IgG1, IgG2a, and IgA) in a dose-dependent manner in mice [281]. Co-administration of interferon- α 2b (Intron A) with hepatitis B vaccine (Egerix B) by IM initiated earlier and higher seroprotection with improved Th1 response in hemodialysis patients [282]. Plasmid encoding IFN- α can also be an effective adjuvant for protein antigens [283].

IFNs can be effective in mucosal vaccinations. A single intranasal administration of IFN $\alpha\beta$ -adjuvanted vaccine resulted in a full protection of 100% of mice against virus challenge while vaccine alone was only partially effective (40%) [284]. Results also showed that type I IFN induced a significant increase in antibody titers in all IgG subclasses with particular effect on IgG2a and IgA. However, the efficacy

seen in animals has not translated into efficacy in humans. Addition of IFN- α to a trivalent flu vaccine did not change significantly the serum-neutralizing antibody response nor the IgA antibody response in respiratory secretions of humans after nasal administration [285].

Granulocyte-Macrophage Colony-Stimulating Factor Granulocyte-macrophage colony-stimulating factor (GM-CSF) can attract and stimulate macrophages, leading to increased antigen presentation [286]. It increased the rate of immune response (earlier seroconversion) and anti-HB titers in human subjects relative to hepatitis B virus vaccination alone [287]. Administration of plasmids encoding GM-CSF can also be effective for DNA vaccines, such as HIV-1 Gag DNA vaccine [288–290] and murine vaccinia env pDNA vaccine [289, 290].

However, some results from human trials have not been consistent. In some studies, GM-CSF appeared to have minimal effect or even a suppressive effect, apparently related to the dose level and frequency [286]. In a recent clinical trial, use of GM-CSF as adjuvant in a novel multiepitope peptide vaccine was minimally immunogenic [6 of 80 volunteers] in developing transient HIV-specific responses [291]. In addition, the vaccine induced formation of anti-GM-CSF antibody in the majority of GM-CSF recipients [291]. In another example, subcutaneous administration of GM-CSF as an adjuvant for a TT-adjuvanted influenza or hepatitis A vaccine did not augment the antibody responses in normal volunteers relative to vaccination alone [292]. In fact, subjects who received GM-CSF had statistically significant lower increases in anti-tetanus antibodies.

Interleukins Several interleukins have been tried as vaccine adjuvants and proven to be effective. IL-12 has been extensively tested for its adjuvant activity not only systemically but mucosally [293]. Its effect seems due at least partially to its inflammatory properties. SC injection of recombinant human IL-12 (rhIL-12) in patients with renal cell cancer induced dose-dependent systemic activation of multiple inflammatory mediator systems [294]. IL-12 promotes differentiation of CD4+ cells toward a Th1 immune response. Increasing the dose of rhIL-12 in a human cytomegalovirus (CMV) vaccine gradually increased the peak anti-CMV gB IgG titers and CMV viral lysate-specific CD4+ T-cell proliferation [295]. DNA expressing IL-12 was also shown to be an effective adjuvant for pneumonic plague after both systemic or nasal administration in mice [296, 297] and for SIVmac239 gag pDNA vaccine in Rhesus macaques [298].

However, use of IL-12 as an adjuvant should be limited to severe diseases as it increases the incidence of local and systemic side effects. Such side effects include injection site pain, fever, headache, myalgia, general pain, chills, and increased cough side effects observed when used concurrently with a pneumococcal polysaccharide vaccine in healthy volunteers [299]. Therefore, the amount of IL-12 as an adjuvant was suggested not to exceed a dose of 0.1 $\mu\text{g}/\text{kg}$, in order to avoid severe systemic inflammatory responses [294].

IL-15 is a powerful immune stimulatory cytokine with a wide range of biological activities. It plays critical roles in the activation, proliferation and differentiation

of CD8+ T cells and NK cells. SC vaccination of mice with a multivalent vaccine with IL-15 induced comparable CD4+ T cell and greater CD8+ T cell and antibody responses against *M. tuberculosis* compared with the standard BCG vaccine [300]. Similarly, plasmids encoding IL-15 could also be effective for a variety of vaccines tested in mice, such as a tumor antigen-derived peptide vaccine [301], HIV-1 DermaVir vaccine formulated with HIV-1 Gag plasmid (more potent than IL-7) [302], and flaviviruse DNA vaccine [303].

Metronomic low dose of IL-2 as a biological adjuvant has been applied in combination with a recombinant vaccinia virus vaccine encoding prostate-specific antigen (PSA) can induce prostate-specific immune responses with markedly reduced toxicities [304].

13.4.13 Specific Peptides/Proteins

Generally, peptides are small enough and will not significantly change the immunogenicity of an antigen. However, peptides of special structures do have such an effect. Among all peptides studied, IC31 is the most widely reported. Actually, it is a vehicle made of both cationic antimicrobial peptide KLKL(5)KLK and ODN. It generally promotes efficient Th1 responses [85]. This adjuvant was found to increase the immunogenicity of a mycobacterial vaccine antigen and offered a comparable protective efficacy to the standard BCG vaccine in mice [305]. In a different study, IC31 was shown to increase the HI titers and induction of IFN- γ producing CD4+ T cells after single vaccination with influenza vaccine in both young and aged mice [306].

Several other small peptides were found to be effective as adjuvants. Co-administration of WKYMVm (a synthetic peptide) with HIV, HBV, or influenza DNA vaccines selectively enhanced the vaccine-induced CD8(+) T-cell responses in a dose-dependent manner in mice [307]. Another one is complement component C5a-YSFKPMLaR (EP54). B- and T-cell epitopes attached to EP54 (0.2 μ M) were easily internalized by human DCs, which induced activation of genes specific for the Th1 and Th2 cytokines [308]. Another complement component C5a65-74 (EP67) was also shown to induce Th1 (inflammatory) cytokines from C5a receptor-bearing antigen-presenting cells and immunization of EP67-ovalbumin resulted in higher OVA-specific antibody titers (both IgG1 and IgGa and IgG2b) in mice [309].

Host defense peptides (HDPs) are small and positively charged peptides. Indolicidin (a bovine HDP; cationic; 13-mer), CpG1826 (20-mer), and polyphosphazene can form a complex, enhancing antibody formation and cell-mediated response in mice [85]. Substitution of the proline residues in indolicidin with arginine increased the synergistic adjuvant effect of the peptide. Similarly, a synthetic cationic defense peptide (HH2; 12-mer) forms a complex with CpG 10101 and significantly increases the immunogenicity of pertussis toxoid in terms of toxoid-specific antibody formation (both IgG and IgA) relative to toxoid alone in mice [310]. A modified (Arg and IsoLeu replaced) HDP-Bac2A (11 aa) was also shown to increase the immune response (Th2) to subcutaneous OVA and enhance the adjuvant activity of CpG to OVA in mice [311]. A hypothesis for the improved adjuvant activity of the modified peptides is the enhanced stability.

Peptides derived from microorganisms can be used as effective adjuvants, such as muramyl glycopeptides, analogs of muramyl dipeptide (MDP), glucosaminyl-muramyl dipeptide (GMDP), and desmuramyl peptides. Changes in both the sugar and the peptide structures can improve the immunostimulating and adjuvant activity and suppress adverse side effects [312]. Co-administration of GK1 (19-mer from *Taenia crassiceps cysticerci*) with an influenza vaccine increased levels of anti-influenza antibodies (higher IgG levels than that with aluminum hydroxide), reduced the local inflammation that accompanied influenza vaccination itself, and favored virus clearance after infection in mice [313].

CEL-1000 (18-mer) is an analog of peptide G (a peptide from human MHC-II β chain, aa 135–149), which is known to enhance immune responses of other immunogenic peptides. They can be conjugated to HIV (HGP-30) and malaria peptides as potential vaccines. SC administration of the conjugate CEL-1000-HGP-30 led to a 4- to 10-fold higher titer antibody response than seen with several other peptide conjugates (such as KLH) in mice [314]. Improved adjuvant activity of CEL-1000 for the peptide conjugates was also demonstrated by a shift in the antibody isotypes toward a Th1 response (IgG2a). CEL-1000 did not induce detectable self-directed or cross-reactive antibodies [314].

These peptides can also be used as mucosal vaccine adjuvants. Macrophage-activating lipopeptide (MALP; a TLR2/6 agonist) has been proven to be an effective adjuvant in several studies. Intranasal vaccination with HIV-1 matrix protein p17 and immunomodulator MALP-2 (a synthetic derivative of the MALP) stimulated strong humoral and cellular immune responses both at systemic and mucosal levels [315]. Similar results were reported in heterologous prime/boost vaccination—intradermal priming with the HIV-1 Tat protein and intranasal boosting with the Tat protein co-administered with the mucosal adjuvant MALP-2 in mice [316]. The pegylated form of MALP-2 was also effective in inducing strong humoral and cellular immune responses after intranasal vaccination in mice [317].

Lactoferrin is a natural immune modulator and has been shown to decrease pro-inflammatory cytokines and chemokines, increase regulatory cytokines, enhance the ability of BCG-infected bone marrow-derived dendritic cells (BMDCs) to respond to IFN- γ activation through up-regulation of DC maturation markers, increase IFN- γ production, promote generation of antigen-specific T cells, and the like. [318, 319]. Indeed, it was shown that co-administration of lactoferrin with a BCG vaccine increased host protection against *M. tuberculosis* (MTB) infection in terms of organ bacterial load, lung histopathology, and significant reduction in tissue colony-forming units (CFUs) compared with BCG alone in mice [320]. In addition, it was shown that the sialylated form of lactoferrin was more effective in increasing the immune response in terms of IFN- γ , IL-6, and IL-12 production in mice and in protecting the animals against infection in challenge studies [321].

CD40 is a co-stimulatory receptor on B lymphocytes. Agonistic antibodies against CD40 have great potential as immunological adjuvants to induce strong antibody responses against conjugated antigen [322, 323]. It is believed that the adjuvant effect is mediated at least in part through enhanced antigen presentation by specific B cells [323]. Such immune enhancement activity of CD40 antibody is similar to that of

CD40 ligand, which has been shown to enhance cellular immune activity in mice [324]. Conjugation of anti-CD-40 to antigen (HSV glycoprotein D) enhanced the immunogenicity of the antigen [325]. Similarly, chemokine (C-C motif) receptor 5 (CCR5) agonists could also be used as potential adjuvants [326].

Another similar concept is the use of immune complex to enhance the immunogenicity of antigens. It has been shown that interaction of anti-CD4-binding site (CD4bs) Abs with HIV-1 gp120 induces conformation changes that lead to enhanced antigenicity and immunogenicity of neutralizing epitopes in the V3 loop and C1 regions of gp120 of several subtypes and the immune complexes as immunogens induced serum Abs to gp120 and V3 at significantly higher titers than those induced by the respective uncomplexed gp120s [327]. It is possible that such an immunogenicity enhancement is due to increased rigidity of the protein antigen. Such a rigidity concept may explain why administration of plasmids encoding aggregation-prone 103Q-GFP (green fluorescent protein) generated significantly higher anti-GFP antibody titer than plasmid encoding control or soluble 25Q-GFP [328].

13.4.14 RNA-like Compounds

Foreign RNAs and RNA-like compounds can initiate immune responses. dsRNA in the form of synthetic poly(I:C) (polyinosine–polycytidylic acid) is such an example. The immune stimulatory effect of poly(I:C) is likely through activation of TLR3 [329, 330]. Stimulation of bone-marrow-derived murine dendritic cell populations with poly(I:C) results in Th1-polarized maturation of dendritic cells in mice [89]. Indeed, poly(I:C) treatment in mice was associated with a rapid induction of inflammatory cytokines in the serum, including IL-6, IL-10, MCP-1, TNF- α , IFN- α , and IFN- γ and selective increases in the numbers of NK [NK1.1(+)]CD11b(+) cells [329].

The immunostimulation of poly(I:C) can be more effective than other adjuvants. Poly(I:C) complex with a peptide epitope—E749-57 (derived from the HPV 16 E7 protein)—was shown to induce strong E749-57-specific CTL responses, leading to significant regressions of model human cervical cancer tumors—an effect much better than E749-57 conjugated with CpG1826 in mice [116]. Similarly, inclusion of poly(I:C) (100 μ g) in a Montanide-ISA51/GP33 (50 μ g, peptide 33–41 of lymphocytic choriomeningitis virus (LCMV) glycoprotein) vaccine significantly enhanced the proliferation of antigen-specific CD8+ T cells, greater than CpG 1826 [50 μ g]-adjuvanted vaccine after SC administration in mice [330].

Recently, another similar adjuvant, polyI:polyC12U (Ampligen), a Toll-like receptor 3 agonist, has been shown to be effective as a mucosal adjuvant for intranasal H5N1 influenza vaccination (formalin-inactivated A/Vietnam/1194/2004 strain), and protected mice against both homologous and heterologous viral challenges [331]. The intranasal administration proves to be more effective than SC injection [331].

13.4.15 Surfactants

Surfactants can be used as potential adjuvants, such as fatty acid derivatives, dimethyl dioctadecyl ammonium bromide (DDAB), and poloxamers. Mannide monooleates induced both IgG1 and IgG2a antibody responses in mice in a dispersed form without

base oil [332]. On the other hand, injection of certain surfactants can produce local irritation reaction [333]. Therefore, such surfactants are usually used in an emulsion. CoVaccine HT is a microemulsion of squalene-in-water (squalene droplets of 130 nm) containing the key sucrose derivatives with seven lauric acid esters and one sulfate ester immobilized on the oil droplets. This adjuvant system enhanced both humoral and cell-mediated responses against a wide range of antigens, for example, inactivated viruses, bacterial subunits, recombinant proteins, virus-like particles, and peptide–protein conjugates in large nonrodent animal models and showed significantly lower reactogenicity than simple mineral oil emulsions (w/o or o/w) [334].

Other surfactant-like compounds may have similar effects. Addition of a nonionic surfactant-like block copolymer CRL1005 (95 : 5 = polyoxypropylene: polyoxyethylene) to an inactivated whole influenza virus vaccine significantly enhanced virus-specific IgG and hemagglutination-inhibition (HI) antibody responses in mice following SC vaccination [335]. Addition of a cationic surfactant-like preservative, benzalkonium chloride (BAK), in a plasmid DNA vaccine formulation (HIV-1 gag) containing a nonionic triblock copolymer adjuvant (CRL1005) leads to an enhancement in the gag-specific cellular immune response (increased IFN- γ production) [336].

Surfactant adjuvants have also been used in mucosal vaccines. Nasal administration of antigens diphtheria toxoid, tetanus toxoid, and BBG2Na (recombinant fragment of the G protein of respiratory syncytial virus) with dimethyldioctadecylammonium bromide (DDA) induced both mucosal and systemic immune responses [337]. DDA was found equally effective as Adju-Phos when used with BBG2Na for protection against viral challenge in mice and cotton rats [337, 338]. Intranasal administration of influenza HA vaccine with Surfacten, a modified pulmonary surfactant, induced higher protective mucosal immunity in the airway without inducing a systemic response in mice [255].

13.4.16 Other Bacterial Components

Bacterial components represent natural foreign antigens. Heat-killed *Mycobacterium vaccae* preparation is an effective adjuvant in the treatment of lung adenocarcinoma and renal cell cancer through selective enhancement of Th1 and down-regulation of Th2 T-cell activity [339]. The *M. vaccae*-induced cancer cell apoptosis, an innate immune response, is the result of a broad spectrum of interactions between pathogen-associated molecular patterns (PAMP) and pattern recognition receptors (PRR) [339]. Similarly, conjugation of M2 peptide to *N. meningitidis* outer membrane complex effectively increased the immunogenicity of this weak antigen, and the effect was superior to M2 peptide expressed on the surface of HBVc antigen in mice [233].

Peptidoglycans Peptidoglycans are of bacterial origin and have certain immunostimulant activity. These compounds activate cells primarily via the cytosolic NLR family member NOD2 and therefore lead to enhancement of antibody production [340]. Peptidoglycan monomer (PGM), a natural compound of bacterial origin (*Brevibacterium divaricatum*), is a nontoxic, nonpyrogenic, water-soluble immunostimulator. Immunization of mice (twice) with OVA+PGM induced

significantly higher anti-OVA IgG levels than OVA alone [341, 342]. In comparison, the individual PGM components—the pentapeptide or the disaccharide, are not effective [341]. Addition of peptidoglycan monomer into OVA-containing liposomes switched the response from Th1 to Th2 type after SC injection in mice [170].

Other peptidoglycan fragments, known as muramyl peptides, are also effective. A series of di-, tetrasaccharide peptides and their stearoyl derivatives were found to activate NF- κ B pathway through NOD2 [343]. While muramyl peptides preferentially stimulate IgG1 production, the tetrasaccharide containing muramyl peptide induces additional production of IgG2b subclasses [343]. However, MDP, a component of the peptidoglycan polymer, has minimal adjuvant properties for antibody production compared to the TLR agonist lipopolysaccharide under a variety of immunization conditions [340].

cdiGMP Cyclic dimeric guanosine monophosphate (cdiGMP) is a bacterial intracellular signaling molecule capable of stimulating protective innate immunity against various bacterial infections [344]. Its application as a systemic or mucosal vaccine adjuvant has been recently reviewed [345]. Subcutaneous co-administration of β -galactosidase (β -Gal) and bis-(3',5')-cdiGMP elicited strong cellular immune responses, characterized by a balanced Th1/Th2 pattern and significantly higher antigen-specific serum IgG titers than β -Gal alone in mice [346]. In a different study, it was found that intraperitoneal co-administration of cdiGMP with pneumolysin toxoid (PdB) or pneumococcal surface protein A (PspA) resulted in significantly higher antigen-specific antibody titers and increased survival of mice, compared to alum adjuvant [344].

Spores The negatively charged and hydrophobic surface of bacterial spores can adsorb protein antigens for enhanced immunogenicity [347]. It was found that mice immunized intranasally or intragastrically can be protected against challenge with different kinds of toxins [347]. Killed or inactivated spores appear equally effective as live spores in these studies. Intranasal administration of two model antigens, tetanus toxoid fragment C (TT) and ovalbumin (OVA), with *Bacillus subtilis* spores (safe and fully tolerated by ingestion in humans) increased T-cell response and specific IgA both in the local respiratory and distal vaginal mucosa, as well as increased antigen-specific IgG antibody in draining lymph node and blood in mice [348].

Flagellin IM administration of influenza virus epitope peptides carried by recombinant flagellin (originated from nonvirulent salmonella) induced both humoral and cellular responses and conferred some protection against lethal challenge (H5N1) [349]. The intensive response to flagellin is mediated by Toll-like receptor 5, linking innate and adaptive immunity [13]. Because of this, a powerful vaccine can be created by fusion of flagellin with an antigen [350].

13.4.17 Miscellaneous Compounds as Adjuvants

Many other compounds with or without proper delivery vehicles can provide superior immunogenicity. Topical application of Imiquimod (5% cream) over the site

of subcutaneous injection with ovalbumin-enhanced anti-OVA antibody responses 100-fold and markedly increased cellular responses (shifted toward a Th1 type) in mice when compared to the ovalbumin alone [351]. RLJ-NE-299A, a mixture of iridoid glycosides from the plant *Picrorhiza kurroa*, was found to increase the level of both immunoglobulins IgG2a (Th1) and IgG1 (Th2) subtypes following immunization of mice with HBsAg [352]. Tomatine, a glycoalkaloid (rod-like structures under microscope), was shown to be capable of stimulating potent antigen-specific humoral and cellular immune responses leading to protection against malaria, *Francisella tularensis*, and regression of experimental tumors [353]. Other compounds as potential adjuvants include antiviral drugs such as Ribavirin [354], the active form of vitamin D₃ (1,25(OH)2D3) [355, 356], calcium phosphate [259, 357], many herbs such as *Astragalus membranaceus* and *Scutellaria baicalensis* [358, 359], and tumor cell components [360].

13.5 OTHER PRODUCT-RELATED FACTORS

There are many drug product-related factors that affect the immunogenicity of vaccines. These include the dose and treatment of antigen, antigen type and combinations, DNA vaccination, adjuvant combinations, formulation/stability factors, routes of parenteral administration, parenteral vs. mucosal vaccination, alternative vaccine delivery methods, and vaccination regime.

13.5.1 Dose and Treatment of Antigen

The degree of immunogenicity response of vaccines clearly depends on the dose of antigens. This has been shown to be the case for IM injection of inactivated, subvirion flu vaccine (HA) in mice [361] and in healthy young subjects [362].

Treatment of antigens by different methods may lead to different outcome in immunogenicity. The rabies virus vaccines inactivated by ionizing radiation was equal or superior to that inactivated by β -propiolactone in its ability to protect mice from rabies infection [363]. Atomization of a nasal adenovirus-based vaccine caused aggregation of virus and drop of virus titers [364]. As mentioned before, freeze–thaw of aluminum-adjuvanted vaccines can result in structural damage of the antigen and potentially reduce the in vivo immunogenicity [63, 64, 69]. Lyophilization of bovine intestinal alkaline phosphatase, adsorbed on aluminum hydroxide, reduced the phosphatase enzymatic activity up to 50% [65]. Spray-drying of a measles virus vaccine affects the virus recovery and storage stability of the vaccine, depending on the starting concentrations [365].

13.5.2 Type and Combination of Antigens

The type of antigens affects the type and degree of immunogenicity significantly. Protein antigens typically induce a predominant Th2 response in *Balb/c* mice [214]. In comparison, LPS activates TLR4 and direct DCs for Th1 responses via IL-12p70

production, while the TLR2 agonist, Pam3cys, and schistosome egg Ags barely induce IL-12p70 production and yield a Th2-biased response [13].

Multiple antigens are often used in a single vaccine to protect against multiple strains or types of organisms. While successes were seen in many trials such as liposome-based vaccines for combined hepatitis E and hepatitis B vaccines [187], some studies have shown variations in responses. Subcutaneous administration of both human serum albumin (HSA)- and cholera toxin B subunit-conjugated starch particles reduced the subclass ratio (IgG1/IgG2a+IgG2b) of the single HSA antigen vaccine, suggesting dependence of Th1/Th2 balance on the inherent properties of the multiple antigens in the microparticles [194]. Administration of three malaria antigens in a single vaccine with an adjuvant system (AS02A) resulted in reduction of one antigen-specific antibody response due to presence of the other two antigens in monkeys [366]. Such an immune interference was also evident when these antigens were administered concurrently at separate sites [366].

13.5.3 DNA Vaccination

DNA vaccines have been tested extensively both for prophylactic or therapeutic purposes. Different aspects in DNA vaccination have recently been reviewed, including mechanisms of immune response for DNA vaccination [9], strategies for developing and optimizing gene-based vaccines [367–369], DNA vaccine delivery [158, 370], and the development of DNA vaccines for treating malaria [371] or tumors [372]. The reasons for development of DNA vaccines are (1) to achieve greater efficacy than protein vaccines and (2) to meet timely the massive market demand. It is believed that a few hundred nanograms of protein, expressed de novo in the host cells, can initiate formation of high-affinity antibodies equivalent to that initiated by a purified recombinant protein at 100–1000 times higher dose [9].

To date, the success of DNA vaccines has been limited to small-animal models at relatively large doses [368]. The success is often dependent on a boosting dose of vaccine of recombinant viral vector or protein [373]. Often, more than one booting dose may be needed [374]. Recent studies suggest that co-administration of a DNA vaccine with its cognate coded protein antigen could lead to stronger immune responses for hepatic C in mice [374] and for flea allergy dermatitis in cats [375].

13.5.4 Combinations of Adjuvants

As stated, different types of adjuvants may have different mechanisms of enhancing immunity. Therefore, combining two or more adjuvants may further enhance the degree of immune response due to multiple mechanisms of actions. In several studies, combination of aluminum salts and deacylated MPL was shown to induce a robust and persistent immune response with HPV-16/18 protein VLP vaccine in women (Cervarix, GSK Biologicals) [376] and more effective with a hepatitis B vaccine than the commercial product Engerix-B in humans [377]. Another reason for combing adjuvants is to change the type of immune response. For example, combining emulsion and

a synthetic lipid A in Fluzone vaccine significantly and synergistically increased Th1-type response in mice [269]. Use of CpG often change a Th2-type immune response of aluminum-based adjuvants to a Th1-type immune response [94, 97, 214].

A variety of adjuvant combinations have been tried. Liposomes can easily accommodate another adjuvant for enhanced immunogenicity, such as TLR7 ligand—3M-019 [378], Quil-A [379], and CpG ODNs [117, 380, 381]. Liposomes have been shown to enhance the uptake of CpG ODN (16-mer) by immune cells in spleen and lymph nodes after SC injection in mice [381]. CpGs in combination with aluminum adjuvants have been shown to enhance further the immunogenicity of many different types of antigens relative to aluminum alone, such as extracellular domain of influenza H5N1 virus (M2e) [382]. Other combinations include MPA/trehalose dicorynomycolate for a protein vaccine[383], chitosan/LTK63 (a nontoxic *E. coli* enterotoxin mutant) for DT-conjugated group C meningococcal polysaccharide antigen [384], chitosan/muramyl di-peptide (MDP) for *H. pylori* urease [202], PLG microparticles/MF59 for recombinant protein antigens [385], PLGA particles/LPS for West Nile encephalitis [257], proteosome/LPS for influenza virus [386], QS-21 combinations with a variety of adjuvants for KLH-conjugated peptide vaccine (209), and FML (a glycoprotein complex) combinations with a variety of adjuvants for visceral leishmaniasis (VL) [387]. More complex adjuvant systems (≥ 3 adjuvant components) have also been tried. These include GM-CSF/IL-2/emulsion for HPV16 E7 peptide antigen [388], liposome/polycation/DNA (LPD) particles for HPV 16 E7 protein antigen [389], CpG (1826)/indolicidin/polyphosphazene for OVA [85], and QS21/GM-CSF/MPL/emulsion system for KLH-conjugated peptide antigens [216]. Although some of these combinations have been compared, the results are difficult to interpret as their doses and preparations are different [209, 387].

A widely investigated adjuvant combination containing ≥ 2 adjuvant components is the adjuvant systems (AS) series of adjuvants, including AS01B (liposomes containing deacylated MPL and QS21) [390], AS02A (an oil-in-water emulsion containing deacylated MPL and QS21) [391], AS05 (liposomes containing aluminum hydroxide, deacylated MPL, and QS21 in a weight ratio of 10:1:1), and AS06 (containing equal amount of CpG ODN 1826 and aluminum hydroxide) [392]. These systems seem to be effective in enhancing both humoral and cellular immunogenicity of different types of antigens in human subjects [391, 393]. In a comparative study, AS01B offered the best enhancement in cellular immunity for a malaria vaccine relative to AS02A, AS05, and AS06 both in Rhesus macaques [392] and human subjects [394]. The superiority of AS0101B was confirmed in a recent study with recombinant hepatitis B surface antigen in terms of cellular immune responses among several AS series of adjuvants and all the AS adjuvants (MPL/QS21) are more effective than CpG (dosed at 0.5 mg) in healthy adults [395]. These studies clearly indicate that liposomes are superior to emulsions, suggesting that more rigid particulates might be more immunogenic. Although the side effects associated with these AS systems seem to be acceptable, consistent preparation of these multicomponent systems could be a challenge.

It should be noted that combinations of adjuvants may further enhance the side effects of individual adjuvants and potentially lead to greater and perhaps different types of toxicities from those generated by individual adjuvants. Infinite enhancement

of vaccine immunity may not be the best choice for developing vaccines against certain viruses. This is because vaccines can actually enhance the susceptibility of the host to virus infection, such as certain flavi-, corona-, paramyxo-, immunodeficiency-, and lentivirus vaccines, likely due to antibody-dependent enhancement of viral entry [396]. Due to the complication of characterizing multicomponent adjuvant systems, a single adjuvant should be the choice in a vaccine unless it is not effective. Future novel adjuvants would ideally offer earlier, robust, and durable immunity with less adverse events.

13.5.5 Vaccine Formulation/Stability

Many vaccines are not stable enough during preparation and/or storage, affecting their efficacies [233, 365, 397, 398]. In such cases, these vaccines have to be stored frozen or cold or dried for long-term storage and transportation to many parts of the world. A stringent requirement of cold-chain shipping may make the vaccines unavailable to certain countries due to limited transportation systems. Therefore, development of room-temperature-stable vaccines is highly desirable.

A key approach in the development of stable vaccine formulations is the identification of the right formulation pH. Formulation pH has been shown not only to affect the stability of antigens but also its interaction with adjuvants, affecting the immunogenicity. The solution pH is critical to maintain the efficacy of protein-conjugated polysaccharide vaccines, as generation of free polysaccharides through pH-dependent hydrolysis may render the vaccine ineffective [250]. The formulation pH also controls the hydrolysis of the linker for VLP-nicotine conjugate vaccine [232].

Another simple method in stabilizing a vaccine formulation is the use of formulation excipients. Several formulation excipients—surfactants, sugars, polymers, and divalent cations—influence the recovery and storage stability of the measles virus vaccines [365]. Freeze—thaw-induced aggregation of VLP conjugate vaccines can be inhibited in the presence of Tween 20 (0.005%) and low ionic strength (30–150 mM NaCl) [231]. More drastic approaches can also be taken to improve the formulation stability. Conjugating human papillomavirus VLPs with a peptide variant of the influenza type A M2 protein make the VLPs more resistant to heat-induced aggregation [231].

If a vaccine is not stable through pH adjustment and use of formulation excipients, a powdered vaccine may have to be developed. Lyophilization is traditionally the drying method for protein products. The lyophilized recombinant ricin A chain vaccine (RiVax) could be stored at least a year at room temperature with the same efficacy in mice, while the liquid form is not stable [398]. The lyophilization process, however, can potentially affect the integrity of a vaccine. While lyophilized emulsion-based malaria vaccine was equally safe and efficacious compared to a liquid formulation in humans [399], lyophilization induced significant damage to a measles virus vaccine [365]. Proper selection of formulation excipients such as sucrose/trehalose and Tween 80 may be necessary in stabilizing unstable vaccines [231, 398]. Alternative drying methods could also be investigated. Among different drying methods examined, foam drying of the measles virus vaccine led to the most loss of measles virus titers relative to spray-drying or freeze-drying (lyophilization) but foam-dried virus vaccines

maintained the best storage stability at 37°C than vaccines prepared by the other two methods [365]. Among the three methods, spray-drying offered a more balanced benefit.

It should be noted that vaccine formulation excipients or impurities may have a significant effect on the immunogenicity of a vaccine. It was shown that presence of 0.2% formaldehyde in aluminum (500 µg)-adjuvanted recombinant protective antigen vaccine induced significantly higher anti-rPA IgG titers ($p < 0.0001$) and toxin neutralizing antibody titers than the control vaccine in rabbits [38]. Vitamin D₃ can promote antigen presentation in subcutaneous tissues for both mucosal and systemic immune responses [356]. Therefore, antigens need to be characterized in the presence of formulation excipients and/or impurity. Since the adjuvant often interferes with the analytical assays, antigens often have to be separated from adjuvant before analysis can be done. For aluminum-based vaccines, protein desorption can be done in a low-pH succinate buffer [52] or simply outside a certain pH range [55]. In certain cases, protein denaturants may have to be used to dissociate proteins from aluminum-based adjuvant. Bovine serum albumin and a multiantigen vaccine for group A Streptococcus were eluted with a 48-h treatment at 4°C with 0.85% H₃PO₄ plus 4M guanidine HCl [66]. Of course, the denatured proteins need to be renatured by desalting for antigen integrity analysis. Structural modification of the protein sequence can be determined by different digestion methods [400].

13.5.6 Administration Routes

There are several possible administration routes for vaccines. The type and extent of immunogenicity of vaccines may depend strongly on the route of administration. It is generally believed that intravenous injection would lead to the least immune response relative to other common injection routes, such as intradermal (ID), subcutaneous (SC), and intramuscular (IM) injection [401, 402].

Among the non intravenous (IV) routes, ID injection seems to initiate equal or greater immune response than SC or IM injections, presumably because the dermis contains more dendritic cells [403]. While ID administration of whole avian influenza H9N2 viruses (5 or 15 µg hemagglutinin) induced the same level of antihemagglutinin titers as IM administration in humans [404], ID administration of a trivalent influenza vaccine at 3 µg of hemagglutinin antigen or virosomal adjuvanted hemagglutinin antigen (Inflexal V) generated similar immune response as that by IM injection of a higher dose ($> 2\times$) of antigen in infants [405] and healthy adults [406]. Similarly, ID administration generated more potent immune response than SC administration for a HCV peptide vaccine IC41 in healthy subjects [407] and for 2 peptide antigens encapsulated in PLGA microparticles in mice [198].

Between SC and IM injections, the immune responses varied. SC immunization of starch microparticle-adjuvanted human serum albumin induced a stronger Th2 response than IM immunization in mice [193]. The immunogenicity of β-interferon induced by SC injection was significantly higher than that by IM injection [408]. In contrast, using a γ-irradiation inactivated Venezuelan equine encephalitis virus gV3526 as a model antigen in the presence of CpG or aluminum hydroxide, IM administration of gV3526 at 0.04 µg/dose initiated an equally effective immune response to

SC administration of 0.2 µg/dose in mice [102]. It was also noted that IM injection of pertussis vaccine in the thigh caused greater immune response than in the buttock [409].

The routes of administration can have a significant effect on immunogenicity, depending on the type of adjuvants used and its method of use. Complete Freund's adjuvant (CFA) is often more effective for potentiating immune responses in mice when administered intraperitoneally (IP) than subcutaneously [122]. CpG adjuvants are believed to be better administrated by IM than SC [10]. It was noted that IFN- α markedly increased the serum levels of all four classes of flu-specific IgGs (IgG, IgG1, IgG2a, and IgA) after IM injection of its mixture with a flu vaccine in mice but IP injection of the same amount of IFN- α at a site distant to the site of flu vaccination actually inhibited the antibody response relative to the vaccine alone by IM [281]. The possible reason was thought due to trafficking of IFN-activated APCs away from the site of flu vaccination [281].

13.5.7 Parenteral vs. Mucosal Vaccination

Most pathogens enter the host via mucosal membranes of the respiratory, digestive, and genital tracts, but most vaccines have been administered through parenteral route. The main reason for injection is that mucosal membranes do not initiate adequate systemic immune response. In addition, enzymes present in the mucosal tissues can easily degrade these antigens. Therefore, significant efforts have been dedicated in the development of effective mucosal vaccines (oral, nasal, pulmonary, virginal) in combination with various kinds of mucosal adjuvants [410, 411]. Development of such vaccines is important as such a method of immunization would be much more suitable for mass vaccinations and may allow adjustment of the type of immune response through selection of different mucosal administration routes [412].

Because of the weak immune response of mucosal vaccines, use of a vaccine adjuvant is generally required to initiate a proper level of immune response. Most vaccine adjuvants discussed above have been tried in different mucosal vaccines [413]. These adjuvants may have different selective activities. While intranasal administration of cholera toxin-adjuvanted H1N1 virus vaccine generated both mucosal IgA (nasal wash) and serum IgG antibody in young and aged mice [414], nasal administration of a similar vaccine with cholera toxin B subunit as adjuvant failed to induce influenza-specific immune responses in both young adult and aged mice [415]. Therefore, proper choice of adjuvants is a key to improve the immunogenicity of mucosal vaccines. One of the effective mucosal adjuvants is toxins, such as CT [414, 416] or LTs [252]. These mucosal adjuvants seem to be able to promote movement of dendritic cells from the skin to Peyer's patches [355]. Lipids [416] and bile salts [171] are also quite effective for oral vaccines because of their potential effect on membranes.

It is evident that mucosal vaccines can be equally or even more effective than parenteral vaccines. Pulmonary immunization of rats with VLP-based vaccines targeting the two domains of HIV co-receptor CCR5, induced high IgG and IgA titers in the serum as that after IM injection, but only aerosol vaccination induced formation of IgA antibodies at local mucosal sites [230]. Similarly, significant rise in IgA

and IgG antibodies in the lower airways was noted only after nasal vaccination (not by IM administration) of a fusion protein vaccine [413] or an inactivated influenza virus vaccine [417]. Therefore, intranasal vaccination offered at least equal protection as that by IM injection for an anthrax vaccine in a rabbit model [418], and more protection as that by SC injection for a H5N1 or seasonal flu vaccine in the presence of adjuvant, polyI:polyC12U (Ampligen), a Toll-like receptor 3 agonist in mice [331]. Similarly, oral administration of bile-salt-incorporated lipid vesicles (bilosomes) containing influenza A antigen actually generated significantly higher antibody titers than IM injection in ferrets [171] and because of the systemic effect, oral administration of the chlamydial major outer membrane protein (MOMP) antigen formulated in lipids or combination of both cholera toxin and CpG protected mice against genital tract chlamydial infection [416]. A recent study has shown that sublingual administration of human papillomavirus 16 L1(HPV16L1) protein vaccine produced the most effective mucosal secretory IgA (sIgA) and serum IgG responses among several mucosal and parenteral administration, including intranasal, intravaginal, transdermal, and transdermal in mice [419].

13.5.8 Alternative Vaccine Delivery Methods

The efficacy of a vaccine can be strongly dependent on the delivery method. Several types of alternative delivery methods are listed here.

Microneedle Delivery Theoretically, use of microneedles could be more effective than a single-needle injection, as the antigens might be more evenly distributed after injection. Several studies demonstrated that microneedle-based intradermal administration of vaccines can achieve the same immune response as that by IM injection of a much higher dose, such as influenza vaccine [420, 421] and aluminum-based recombinant protective antigen vaccine [422]. Since intradermal injections frequently outperform intramuscular injection, the real advantage of microneedle injection still needs to be confirmed. It was noted that microneedle-based delivery may cause more frequent local reactions (mild and transient) [421].

Needle-Free Injection/Delivery The fear of needle injection especially among children leads to the development of needle-free injection technologies, even though such injections can actually cause more pain than traditional needle and syringe injections [423]. Due to the potential splash-back contamination issue, single-dose injector devices are preferred [424]. It was noted that the jet injector devices are associated with higher levels of pain and more local reactions than that by IM immunization [424].

Another needle-free delivery method is transcutaneous immunization (TCI) for vaccination [425]. Such a delivery method relies on good permeability of antigens and, therefore, lipid-based formulation would be expectedly beneficial. For example, transcutaneous application of lipid C (lipid oral vaccine delivery system) vaccine formulation containing chlamydial major outer membrane protein antigens led to partial protection against live challenge in mice [426]. Similarly, transcutaneous administration of lipid C-based vaccine containing *Helicobacter sonicate* significantly

reduces the gastric bacterial burden in mice following gastric challenge with live *H. pylori* [427].

Using tetanus toxoid as a model vaccine, it was shown that low-frequency ultrasound (as a potent physical adjuvant) could enhance delivery of tetanus toxoid into the skin without any help of actual adjuvant [428]. In a recent report, it was demonstrated that tattoo administration of HPV peptide vaccines in the absence or presence of CpG ODN1826 induced more antipeptide antibody response than subcutaneous administration in mice [92].

DNA Vaccine Delivery Although DNA vaccines have been tested for a long time, no effective vaccines have been developed for human use. One key challenge is the low efficiency of DNA expression, mainly due to limited entry of DNA into the host cells and limited duration of expression. Therefore, ex vivo (tissue removal) has been tried to improve the DNA delivery [429]. Alternative delivery methods include use of DNA–lipid complexes/particles, such as dehydration rehydration vesicle (liposomes), lipoplexes (liposome + cationic lipids + DNA), virosomes (nucleic-acid-free viral particles) [370], electroporation-mediated delivery [372, 430], tattooing-mediated delivery [92, 431, 432], jet injection [433], and DNA microparticle bombardment (biolistics) [9, 434, 435]. With advanced gene gun delivery devices, dose-dependent immunogenicity of DNA flu vaccines was arguably demonstrated in humans [436]. The future of these DNA delivery alternatives remains to be seen.

13.6 SUMMARY

Development of human vaccines has been largely semiempirical. This is because there are many known and unknown factors that can dramatically change the type and degree of immune responses. On the top of a list of factors is the vaccine adjuvant. A vaccine adjuvant and adjuvant systems generally lead to a much improved immunogenicity and have been an extensive area of research and development. Although many adjuvants have been tested, few have been successfully commercialized in human vaccines, partly due to our poor understanding of their immune enhancement mechanisms and related side effects. Therefore, it has been generally recommended that a single adjuvant should be used unless it is not effective, that is, keep it simple and stupid (KISS).

In addition to adjuvants, there are many other product-related factors that influence the immunogenicity of vaccines. These include the dose and treatment of antigen, antigen type and combinations, DNA vs. protein vaccination, adjuvant combinations, formulation/stability factors, routes of parenteral administration, parenteral vs. mucosal vaccination, alternative vaccine delivery methods, and vaccination regime. All these factors need to be considered during vaccine development.

The human society is still facing many uncurable deadly diseases today. Given the high cost of drug product development, the high pressure of reducing health-care cost, and the public desire for a better quality of life, the development of both prophylactic and therapeutic vaccines would remain a focus of many pharmaceutical companies.

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LYOPHILIZATION AND STABILIZATION OF VACCINES

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14.1 INTRODUCTION

Vaccines are a growing and vital component in the effective implementation of best practices in public health policy [1–4]. Having established themselves historically as a crucial medical intervention against pathogens that threaten infants at the vulnerable beginning stage of life [5, 6], vaccines are now being recognized for their value well beyond immunization of infants against childhood diseases, with several significant health applications such as a first-line treatment against recurrent pandemic infections such as influenza [7, 8], outbreaks of other communicable diseases [9, 10], creation of stable vaccine stockpiles [11–13], effective medical interventions targeting emerging

pathogens and infections that challenge the developing world in addition to bioterrorism agents that may be targeted at first-world countries [14–16], and formulations for needless delivery such as aerosols [17–19].

Due to the expanding role of vaccines as an effective medical intervention in public health, there is an elevated importance to solve technological hurdles that will allow the creation of significant stockpiles of stable and effective vaccines, to be able to ship them rapidly to remote locations around the world with less onerous cold chain requirements, to be confident of their potency and stability when subjected to wide ranges of physical and biochemical stresses over extended periods of time needed for shipment and storage, and to be easily reconstituted and administered to patient populations around the world [20–24]. Lyophilization is a processing method that has shown considerable success in achieving enhanced stability for complex biological molecules such as therapeutic proteins [25–27] and even live and attenuated pathogens found in many vaccines [28–30], and can provide a cost-effective path to immunization in resource-poor countries that may have the greatest need for vaccinations [31–34].

14.2 LYOPHILIZATION PROCESS

Lyophilization is undertaken to expand the stability or shelf life of a given vaccine formulation through controlled removal of aqueous solvent under reduced temperatures with substitution of suitable molecular species that through specific interactions protect the key structural and functional properties of the formulation's biologically active ingredients [35–37]. Other methods of dehydrating samples such as spray drying [38, 39], supercritical fluid processing [40], *Xerovac*, and other nonfreezing evaporative drying methods [41, 42] are also being developed. Since lyophilization is a complex process that adds time and cost to the production of biologics, it is generally undertaken only when the benefits such as exceptional performance criteria for shipping, storage, preservation of activity, and ease of administration or reconstitution outweigh the additional time and cost of lyophilization cycle development. As a consequence, about 25% of biotechnology products are currently being produced in the lyophilized state [9].

Vaccines are a class of biotechnology product that generally possesses significant thermal instability [43–47]. Empirical evidence of loss in vaccine potency during transportation and storage has been recognized in several programs such as the Expanded Programme on Immunization (EPI) for vaccines such as Bacille Calmette-Guerin (BCG), a vaccine against tuberculosis, diphtheria, tetanus, pertussis (a combination vaccine) (DTP), oral polio vaccine (OPV) and measles [48, 49]. This led to strict Centers for Disease Control (CDC) and World Health Organization (WHO) guidelines on recommendations for storage and handling of vaccines to retain potency [50, 51] involving narrow temperature ranges (2–8°C) with restrictions not to expose to freezing conditions or elevated temperatures, or excessive light, or thaws; and instructions to use within 24 h of reconstitution, or discard after a few days are called for numerous vaccines. Due to such restrictions it is estimated that approximately 50% of current vaccines produced are wasted [52].

Both the formulation composition and freeze-drying process require simultaneous optimization in order to produce a stable product [53–55]. The lyophilization process

involves loading vials filled with the appropriate formulation onto a temperature-controlled shelf inside a variable pressure chamber. The sample is then slowly frozen, concentrating the water-soluble components, and vacuum pumps activated to reduce the chamber pressure to $\sim 10\text{--}100\ \mu\text{m}$. Heat is then applied to provide the energy required for sublimation of the ice (bulk water) in the primary drying step, and efficient heat transfer through the shelf and mass transfer from the pores and channels in the formulation structure is required [53, 56]. The temperature is then raised and additional heat supplied to lower the amount of water present from about 20 to 1% w/w in the secondary drying (water desorption) step. The final product is a dried cake consisting of a bulk, amorphous phase of a glassy matrix of sugars such as sucrose or trehalose or polyols such as mannitol.

The lyophilized cake will have a characteristic glass transition temperature (T_g') that signifies a point where mobility of components in the matrix and free volumes available can lead to collapse of the glassy, amorphous structure. Low-molecular-weight components such as salts and buffers tend to lower the T_g' value, while higher molecular weight components such as polysaccharides, polymers, and proteins will raise the T_g' [54]. It is desirable to have a T_g' higher than temperatures that the vaccine formulation will be exposed to during its period of shipment and storage. The amount of residual water present in the freeze-dried lyophilized cake will dramatically change the T_g' and can adversely impact stability and potency of the biological molecules present [54, 55, 57–65] and is a critical quality attribute of the lyophilized product.

Stabilization of the biomolecules present typically occurs through the mechanism of substituting the interactions normally occurring in the aqueous state (with defined pH, ionic strengths, and other species present) with molecular replacements such as sugars, surfactants, and polymers. These excipients create high-viscosity chemical microenvironments that can protect molecules such as proteins from denaturing or aggregating and help preserve the structural integrity needed for activity, including formulations of live and attenuated organisms [66–71]. Significant research is occurring to identify excipients that can impart protection from the stresses of freezing and dehydration over the temperature ranges and pressures seen during the lyophilization process and subsequent shipping and storage [72–76].

14.3 EXAMPLES OF PROGRESS IN CREATING LYOPHILIZED VACCINES

Vaccines commonly used in immunization programs display a relatively wide range of stability. For example, tetanus and diphtheria toxoids as monovalent vaccines adsorbed on alum salts are stable for over 2 weeks at 45°C , while the oral poliomyelitis vaccine will lose over 50% of its potency in one day at 41°C and lose satisfactory titer after 1–3 h at 50°C [51]. For vaccines that are being used almost exclusively in tropical regions such as yellow fever, the advantages of developing formulations and lyophilization cycles to confer greater stability at elevated temperatures has been appreciated for several decades [77–79]. The solution of careful vaccine vial monitoring to guarantee the fidelity of the cold chain has been endorsed by the WHO [80]. It was found that including stabilizing

excipients such as sugars, amino acids, and divalent cations in mixture of lactose (4%), sorbitol (2%), histidine (0.01 M), alanine (0.01 M) in phosphate buffered saline with Ca^{2+} and Mg^{2+} would meet the 1987 WHO guidelines that each lot of vaccine meet the following stability test: maintenance of potency (>1000 mouse i.c. $\text{LD}_{50}/\text{human dose}$) (potency as measured by greater than one thousand times the mouse intracerebral dose to cause 50% death, divided by human dose; With no more than a tenfold decrease in antibody titers after 14 days at 37 Centigrade) with mean loss of titer $<1.0 \log 10$ after being held at 37°C for 14 days. Formulation and process improvements have led to greater stability in the 17D yellow fever vaccine [36–38].

Measles is a similar case of a live vaccine that requires both lyophilization and cold chain to retain potency [81–83]. The recognition that stability would play an important role in the production, distribution, and administration of an effective vaccine led to empirical studies that identified rapid methods of testing and screening formulations and lyophilization processes [84–86]. Stabilization has been achieved primarily through empirical screening rather than systematic approaches due to the complex biochemical nature of the virion, but it was found that the addition of MgCl_2 would significantly increase titers following stressed stability testing [87, 88]. Additional stabilizers such as sorbitol, sucrose, lactose, sodium glutamate, and gelatin have been shown to increase stability [89, 90]. Since measles was identified as a key cause of 873,000 deaths in 1999 [91, 92], expanded immunization programs were able to utilize heat-stable strains compatible with lyophilization and improved surveillance and monitoring to help bring about the reduction of this deadly disease in needy countries such as Nigeria and Cameroon by approximately 60% [93, 94]. The current goal of the WHO–UNICEF (World Health Organization and United Nations Children’s Fund) Global Immunization Vision and Strategy for 2006–2015 is to reduce measles deaths by 90% of their 2000 levels [95].

Varicella-zoster virus is another labile, enveloped pathogen but with double-stranded DNA encapsulated in an icosahedral nucleocapsid. A stable, attenuated Oka strain was created and selected for future vaccine development [96–98]. The integrity of the varicella envelope and biochemical contents were correlated to activity/infectivity [99] and formulations utilizing typical excipients such as glutamate, gelatin, and albumin were subsequently put into a lyophilization process, but the final product required storage at –20°C to guarantee potency [100–102]. Reformulation efforts have led to improving the earlier storage conditions at –20°C to a vaccine that is stable at 2–8°C for 2 years [103]. Additional formulation efforts to produce a stable varicella vaccine without animal-derived components have also shown promise to address future regulatory concerns [104].

Bacillus Calmette-Guérin (BCG) vaccine against tuberculosis is the oldest bacterial vaccine that has been administered to over 4 billion people around the world, with the exception of the United States and the Netherlands [105]. The WHO has recommended the use of lyophilization to stabilize the BCG seed lots in addition to the vaccine itself since the biological components of mycobacterium have severe stability problems in a liquid formulation [106]. Excipients such as sugars and amino acids have been successfully formulated to stabilize and allow proper reconstitution of the BCG vaccine that have shown culturable particles stored at 37°C for 28 days

to grow within 20% of cultures stored at 4°C [107]. Conditions for creating a stable, frozen formulation have been developed but do not lead to a practical vaccine for administration the way a lyophilized product can be easily reconstituted [108].

14.4 CURRENT RESEARCH AND FUTURE TRENDS IN VACCINE LYOPHILIZATION AND STABILIZATION

Vaccines can be classified into the biological categories of live and inactivated/killed (whole agent) or subunit purified and recombinant (including conjugates). The latter category offers a higher level of safety assurance due to greater purity and absence of a need for an inactivation process and represents new opportunities for vaccine stabilization due to the possibilities of detailed characterization and biochemical definition of the formulated product. Research efforts for the former category (live and inactivated organisms) can show further improvement in stabilization through the similar, empirical approaches historically employed such as excipient screening with accelerated stability testing, while the latter category (purified and recombinantly expressed vaccines) will benefit from rational and systematic approaches that utilize modern analytical, biophysical, and structural characterization [109, 110].

Stabilizing agents for live vaccines continue to be discovered empirically through screening processes. For example, it has been found that heavy water (D_2O) stabilizes live poliovirus (such as Sabin type 3) alone or additively with the addition of magnesium chloride [111, 112], though the precise mechanism of this additional stability is not completely known. Polio is the only live vaccine that may be formulated in a liquid state with acceptable stability. Live viruses lose their infectivity from at least two mechanisms: protein degradation often following capsid denaturation and viral genome [e.g. ribonucleic acid (RNA)] degradation [113]. Molecules that stabilize the protein capsid such as pirodavir may fail to shield the genome from damage, creating a multidimensional challenge to guarantee potency [114]. Biological extracts from “extremophiles,” organisms that live and thrive under extreme thermal and chemical conditions, have also been tested as stabilizers [115]. It has been suggested that since the correlates of stability at elevated temperatures are so difficult to ascertain and the rewards and protections for such research so limited, the probable answer to such difficulties will likely be solved by improvements in the cold chain [116]. Despite such characterization challenges, efforts have shown that biophysical properties measurable by light scattering and spectroscopic data can show correlations to biological activity for live measles vaccines [117], and alternative processing methods such as spray-drying may also increase stability [118]. Alternative drying methods such as spray–freeze drying, air drying, and freeze-drying can minimize unfavorable events such as aggregation for delivery systems including aluminum salts [119].

If an effective recombinant or subunit vaccine can be produced, then many of these “insurmountable” challenges such as characterizing the complex nature of a complete virus or bacteria are less of a problem with only empirical solutions. For example, efforts to stabilize the Malaria EBA-175 RII-NG antigen through elucidating its phase diagram over pH and temperature process parameters and high-throughput

screening of excipients and delivery systems represents a rational, systematic approach in the development of stable vaccine formulations [120]. In principle, this approach can be extended to complex formulations with delivery systems including aluminum salts [121], liposomes [122], and biodegradable particulate delivery systems [123, 124] including nanoparticles to deliver deoxyribonucleic acid (DNA) plasmids and protein antigens [125]. Fundamental structure–activity relationships for DNA and protein biomolecules may be established using modern microspectral, thermodynamic, and biophysical methods that can allow the progress of both composition (formulation) and process (lyophilization) toward creating stable, potent vaccine formulations [126–129].

14.5 CONCLUSIONS

Vaccines demonstrate exceptional thermal instability. Even short temporal excursions both above and below a narrow temperature range (2–8°C) can lead to an unacceptable loss in potency; thus a robust cold chain (consistent refrigeration) is required to avoid product failure. For many developing countries and tropical climates where diseases are endemic, the reliability of a cold chain is not guaranteed. Improvements in cold-chain monitoring have helped identify when problems occur and vaccine lots should be discarded. A more universal solution that can ensure vaccine effectiveness, permit less onerous restrictions on the transportation, storage, shelf-life, and administration of vaccine to promote the establishment of stockpiles and expansion of coverage and reduction of cold-chain-associated costs is stabilization through formulation and lyophilization. Historically the production of a stabilized, freeze-dried product was necessary to provide live vaccines that were stable enough to extend immunization coverage to people in all locations and environments of the world. Current research in purified and recombinant vaccines comprised of proteins, polysaccharides, conjugates, and DNA has opened up another possibility of characterizing biomolecular structure and disposition and how it correlates to activity with modern analytical methods. Through combining classical formulation and process development efforts with insightful analytical tools, it may be possible to rationally and systematically create vaccines with sufficient thermal and chemical stability to satisfy the global health burden of effectively combating both existing and emerging infectious diseases.

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15

EFFECT OF BUFFERS AND STABILIZERS ON VACCINE STABILITY AND EFFICACY

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15.1 MECHANISMS OF STABILIZATION OF VACCINES BY BUFFERS/STABILIZERS

15.1.1 Decreased Adsorption/Aggregation/Denaturation

The stabilization of vaccines by surfactants may be related to their surface activity. Surfactants tend to accumulate on the interfaces rather than in bulk solution at or above their critical micelle concentration (CMC). Hence in the presence of surfactants,

vaccine adsorption at interfaces is significantly reduced [1]. It had been proposed that surfactants bind to the hydrophobic regions of the proteins and render them less surface active [2]. Several surfactants such as polysorbate 20, polysorbate 80, and brij 35 have been shown to stabilize vaccine formulations via this mechanism [3]. Polyol osmolytes such as glycerol has been shown to stabilize the antigen of *Bacillus anthracis* against thermal denaturation [4]. Polyol osmolytes are usually produced in insects, microorganisms, and plants in response to denaturing stresses [4]. The mechanism of stabilization is via preferential exclusion from the protein surface, which results in preferential hydration of the proteins [4]. Lactalbumin hydrolysate is an effective inhibitor of thermally induced aggregation/denaturation of live-attenuated peste des petits ruminants (PPR) vaccine [5].

15.1.2 Decreased Deamidation

Deamidation is the hydrolysis of amide side chains of amino acids in peptides and proteins [6]. It occurs mainly at Asn side chains and at a much slower rate at Gln residues. Deamidation reaction occurs via nucleophilic attack on side-chain carbonyl carbons of Asn and Gln to yield corresponding aspartate (Asu) and (Glu) residues, respectively [7]. Temperature, pH, and protein conformation have profound effects on deamidation reactions [8]. Spontaneous deamidation of *B. anthracis* protective antigen (PA) resulted in thermal instability, aggregation, and loss of protein function (reduced cytotoxicity) upon storage at 37°C for 48 h in a pH 8.0 buffer [8]. The deamidation rate of the PA was reduced at a lower temperature (2–8°C) and a pH 7.4 buffer. The control of pH with appropriate buffers at pH of optimum stability is an effective method of attenuating deamidation degradation pathways [6, 9].

15.1.3 Decreased Free-Radical Oxidation

Free-radical oxidation reaction is one of the major degradation pathways for protein and vaccine formulations. Several factors that influence the rate of oxidation reaction have been identified [10]. These include temperature, metal ions, and the presence of hydroxy free radicals and molecular oxygen. The effect of free-radical oxidation inhibitors as well as known metal chelators on the stability of adenovirus-based vaccines was studied [11]. It was found that hydroxy free-radical scavengers/oxidation inhibitors (ethanol, ethanolamine, L-histidine) and metal chelators [ethylenediaminetetraacetic acid (EDTA) and citrate] were effective stabilizers of adenovirus-based formulations [11].

15.1.4 Increased Viral Capsid Protein Binding

Different inactivation mechanisms have been reported for the loss of viral infectivity of oral polio vaccine upon moderate heating [12]. These include (1) viral capsid protein degradation and (2) viral ribonucleic acid (RNA) degradation. It has been shown that viral capsid protein denaturation could be prevented by the addition of pirodavir, an antiviral molecule and magnesium chloride [13]. Pirodavir binds to capsid protein VP1

and prevents uncoating of the viron and stabilizes viral capsid [12]. The stabilization of the viral capsid protein after drug binding was entropic rather than enthalpic in origin since the slopes of the arrhenius plots for the virus and drug–virus complex were identical [13].

15.1.5 Binding to Specific and/or Multiple Sites on Vaccine

Certain salts such as magnesium chloride have been reported to stabilize vaccines against thermal degradation [14–16]. It has been shown that magnesium chloride binds to capsid protein VP1 of the oral polio vaccine and stabilizes it against thermal degradation [17].

15.1.6 Cryoprotection/Lyoprotection

Vaccines undergo conformational changes following freeze–thaw cycling [18, 19] and freeze-drying [19–22]. It is well known that carbohydrates such as sucrose, trehalose, and dextran prevent the damaging effects of freezing and drying stresses [19–22]. In the dried state, vaccines are embedded in the amorphous glass matrix. The stabilization effect of these sugar glasses occurs via reduction of diffusion and molecular mobility, which in turn decrease the aggregation and degradation of dried material. In addition, during the freeze-drying process, the water molecule that hydrogen bonds with vaccines is replaced by hydroxy groups of the carbohydrate, thus maintaining structural integrity of the vaccine or proteins [20].

15.1.7 Formation of Stabilizing Hydrogen Bonds

The effect of urea on the stability of several types of vaccines (measles, mumps, and rubella) was evaluated [23, 24]. The lyophilized vaccines were not adequately stabilized in the presence of commonly used lyoprotectants. However, the addition of urea and derivatives significantly improved the stability of the lyophilized vaccines. It was postulated that the stabilizing effect of urea was due to its stabilizing hydrogen bonding in the lyophilized matrix [23, 24].

15.1.8 Osmoprotection

Glycine and its methyl derivatives prevent the thermal inactivation of protective antigen of *B. anthracis* by osmoprotection mechanism [25]. Glycine and derivatives are osmolytes that are found in organisms during stressful conditions and that stabilize proteins both in vitro and in vivo. Osmolytes such as glycine are known to stabilize proteins by preferential exclusion from the native protein surface and hydration of the protein molecule [25]. However, its methyl derivative, which is more hydrophobic, is preferentially excluded from native protein surface and less excluded from the denatured protein surface. This increased affinity (binding) of the methyl derivatives to the denatured protein surface pushes the equilibrium from native to denatured state, thus explaining why the methyl derivatives are less effective in vaccine stabilizers.

15.1.9 Thermostabilization

Deuterium oxide enhanced the thermostability of poliovirus and yellow fever 17D vaccine virus strain [26]. This may be attributed to the greater strength of deuterium, as opposed to hydrogen bonds, in protecting proteins and vaccines against denaturation. Furthermore, it had been shown that deuterium oxide prevented the degradation of viral RNA in the capsid of oral polio vaccine [12]. Disulfide bond formation in recombinant protein vaccine candidate enhanced the thermostability profile via the formation of dimer and trimer viral capsid proteins [27]. It had been shown that disulfide bonds stabilize JC virus capsid structure by preventing calcium from chelation [28]. Calcium ions play an important role in viral assembly [29].

15.1.10 Crosslinking of Reactive Sites on Protein Subunits

Formaldehyde treatment of the Q strain of the cucumber mosaic virus stabilized the capsid structure and enhanced the immunogenicity in mice [30]. The mechanism of stabilization was postulated to be via crosslinking of reactive sites on adjacent subunits of protein shells [30]. Formaldehyde also stabilized the pertussis vaccine without affecting immunoreactivity via the same mechanism [31].

The mechanisms of stabilization of vaccines by buffers and stabilizers are summarized in Table 15.1. below.

TABLE 15.1. Mechanisms of Stabilization of Vaccines by Buffers/Stabilizers

Proposed Mechanism(s)	Examples of Buffers/Stabilizers	References
Decreased adsorption/aggregation/denaturation	Surfactants: polysorbate 80, polysorbate 20, brij 35 Polyalcohol osmolytes: glycerol Proteins: lactoalbumin hydrolysate	1–3 4 5
Decreased deamidation	Citrate buffers	6, 9
Decreased free-radical oxidation	EDTA, ethanol, triethanolamine, L-histidine	10, 11
Increased viral capsid binding-inhibition of thermal denaturation	Pirondavir	12–14
Binding to specific and multiple sites on vaccine	Divalent cations, e.g., magnesium chloride	15–17
Cryoprotection/decreased freeze-thaw-induced inactivation	Sucrose, sorbitol, glycerol	18
Lyoprotection/decreased freeze-dry-induced inactivation	Sucrose, trehalose, dextran	18–22
Formation of stabilizing hydrogen bonds donors	Urea	23, 24
Osmoprotection	Glycine and methyl derivatives	25
Thermostabilization	Diamide, deuterium oxide	26–29
Cross-linking of reactive sites on protein subunits	Formaldehyde	30, 31

15.2 EFFECT OF EXCIPIENTS ON VACCINE EFFICACY

15.2.1 Buffer Effects

An exhaustive review of the effect of buffers on protein conformational stability is available ([32], and references therein). Protein antigen conformational stability is inextricably linked to antigen presentation (see 15.2.2) and, consequently, to the elicitation of an effective and robust immune response. Therefore, the profound effects that buffers exert on the tertiary and quaternary structure of protein antigens implicate them as key excipients in vaccines. Many of the buffer effects on antigen conformational stability continue to persist even *in vivo* (after subcutaneous or intramuscular vaccine injection) because—unlike intravenous administration—they are not rapidly diluted.

15.2.2 Antigen Conformation

Because a protein needs to be unfolded for its major histocompatibility complex (MHC) specific sequence to adopt a competent binding conformation, unfolding of antigens in antigen presenting cells (APCs) is an indispensable event that protein antigens must undergo in order to be presented to helper T cells. Therefore, efficiency of antigen processing depends on a complex interplay between APC processing capacity and those characteristics of the antigen that control its unfolding capacity [33].

Noncovalent type of stabilizing interactions such as hydrophobic interactions, van der Waals interactions, hydrogen bonds, and electrostatic interactions, which contribute to the stabilization of the tertiary and quaternary structure of a protein antigen, control its presentation efficacy [34]. Such non-covalent-based interaction-based stability of antigens has been shown to control processing in APCs, T-cell stimulation, and the kinetics of expression of T-cell determinants.

Consequently, the effects of excipients on the physicochemical shelf-life stability and on the immunogenicity of the vaccine may be inextricably linked. As an example, both the degree of adsorption of the antigen to the adjuvant (which determines immunogenicity) as well as the thermal stability of the antigen are influenced by the type of buffer used in the formulation [35]. It has been suggested that the structural destabilization observed for protein antigens adsorbed on the surface of aluminum salt adjuvants makes them more susceptible to proteolytic processing by the immune system, resulting in enhanced antigen presentation [36]. As an example, buffer-mediated disaggregation of viral particles in Rotateq vaccine may confer enhanced infectivity in addition to thermostability and ease of manufacture [37].

Capsid-binding antiviral drugs confer greater thermal stability to capsid proteins by increasing their conformational entropy, consequently, decreasing the entropy gain that can be achieved by conformational transition to a structure that facilitates cell entry [38]. The reverse situation is desirable for intentionally administered replication incompetent virus-like particles (VLPs), where cell entry may be advantageous to elicit a robust immunogenic response. Proton transfer between buffers and (catalytic or allosteric) protein sites is known to be associated with partial conformational changes in the protein structure that is cooperative within considerable but localized

regions of the protein domain ([32] and references therein). It follows that the ease with which VLP vaccines enter cells can hence be modulated by the choice of buffer in the formulation, much like their morphology can be affected by the type of buffers used in their *ex vivo*, extracellular assembly [39].

15.2.3 Adsorption to Adjuvant

The chemical stability of adsorbed antigens that degrade by a pH-dependent mechanism can be either faster or slower than expected depending on the surface charge of the adjuvant and the pH stability profile of the antigen. Antigens that have been reported to be sensitive to pH changes in terms of immunogenicity/stability include foot and mouth disease, tetanus toxoid, influenza A, and *Mycoplasma hyopneumoniae*. The type of buffer has the capacity to modify the surface charge of the stern layer/diffuse double layer (the so-called zeta potential) of the adjuvant due to its inherent ionization potential [40]. This represents one possible mechanism by which the type of buffer can influence the stability/immunogenicity of the adsorbed antigen.

15.2.4 Osmolality

With regard to buffers used for the cholera vaccine as they now exist, some have been found to result in diarrhea among some of the persons taking the vaccine. Although not completely understood, it is believed that the diarrhea is due to a high osmolality of the buffer acting as an osmotic laxative. Although the diarrhea observed may be termed a side effect, it may also lessen immunogenicity if the vaccine antigens are swept out of the intestine before they have a chance to contact the antigen sampling cells. Lactose has also been found in some buffers, which contributes to diarrhea in lactase-deficient persons even in low concentrations. Buffer containing carbohydrates such as rice syrup solids (Ceravax) and sucrose (Rotateq buffer) may increase antigenicity by increasing the rate of absorption of the antigen once it enters the intestine, much like an oral rehydration solution [41].

15.2.5 Influence on Antigen Delivery System

The choice of buffer may influence the structural configuration of the phospholipids in the formulation. Because phospholipids are often used as carriers or complexing agents such as in liposomes or lipoplexes for enhancement of transfection, their structural characteristics play an important role in transfection efficiency. For example, the reason for the superior transfection efficiency of a lipoplex that can assume the hexagonal morphology (in the presence of a suitable buffer) has been suggested to be related to an easier endosomal escape of the transfected gene. The transfection efficiency of cationic polymeric gene delivery vectors was shown to be correlated to the buffer capacity because extensive protonation of the polymer at the decreased endosomal pH induced osmolytic and conformational and/or electrostatic changes that facilitated endosome disruption [42].

15.3 SURFACTANT EFFECTS

Controlled sustained antigen release is an important factor in the generation of an effective immune response and high titers of antibodies. Surfactants are able not only to modulate antigen release by virtue of their hydrophilic-lipophilic balance (HLB), or by the phenomenon of reverse thermogelation (such as that exhibited by pluronic F127), whereby the formulation undergoes a phase transition from liquid to gel upon reaching physiological temperatures, but also to affect antigen stability by modification of their secondary and tertiary structures. Furthermore, surfactants such as glyceryl monolaurate may modulate T-cell proliferation by affecting the cell signaling pathways leading to the regulation of IL-2 production. Pluronics also induce transcriptional activation of gene expression by activating the *P53* and *NFkB* pathways [43].

15.4 ANTIOXIDANT EFFECTS

Although currently marketed vaccines do not contain antioxidants per se, emerging technologies that can stabilize antigen in liquid vaccine formulations at room temperature may necessitate the addition of antioxidants.

Many intracellular pathogens, most notably the human immunodeficiency virus (HIV) and the hepatitis B virus use host glycosylation enzymes to extensively glycosylate their surface antigens. This property is responsible, in part, for the attenuated host immune response to infection. Inhibition of cellular glycosylation enzymes leads to the misglycosylation and subsequent misfolding of the matrix proteins of such viruses. Host cell proteins are also affected, but these cells can identify and eliminate the defective proteins, whereas the virus cannot. The resulting defective viral particles are unable to initiate another round of replication. Among inhibitors of glycosidases are molecules such as mesquitol analogs, polycyclitols, and salacinol, all of which coincidentally also possess antioxidant and/or free-radical scavenging ability [44]. The inhibitory potencies of these compounds can be attributed—in part—to the number of phenolic hydroxyl substituents. Antioxidants can, therefore, modulate the glycosylation of viruses and, consequently, the robustness of the host immune response.

Antioxidants such as calcitriol promote the induction of mucosal immunity when incorporated into vaccine formulations. Antioxidants may also modulate granulocyte macrophage colony-stimulating factor (GM-CSF) release.

Because vitamin E has structural similarity to the thiazolidine, troglitazone, a powerful PPAR γ (peroxisome proliferator-activated receptor) ligand, it has the ability to elevate PPAR γ mRNA and protein in colon cancer cell lines [45]. PPAR γ , in turn, negatively regulates SOCS-2 (suppression of cytokine signaling) gene expression [46]. Enhancing immune responses by removing (SOCS) inhibition by single interfering ribonucleic acid (siRNA) results in a greater adjuvant and antiviral response (particularly against HIV) that improves vaccine-induced immune responses [47]. Serum levels of vitamin E have been shown to positively regulate the immune response to influenza vaccine both in healthy adults and in the elderly [48].

15.5 PRESERVATIVE EFFECTS

Quaternary ammonium salts usually manifest with an antibacterial activity and hence should (and are) excluded from whole-cell attenuated bacterial vaccines. On the other hand, such compounds have been evaluated as adjuvants for deoxyribonucleic acid (DNA) vaccines, for example, dimethyldioctadecylammonium bromide (DDA).

15.6 SUGARS

15.6.1 Schiff Base Formation

Transient and reversible Schiff base formation between specialized carbonyls present on the surface of APCs and amines present on T-cell surfaces (oxidative mitogenesis) is a recognized mechanism to initiate T-cell activation [49]. This phenomenon is exploited by the exogenous adjuvants QS-21 [50] and Tucaresol to provide a co-stimulatory signal to CD4+ T helper (Th) cells. Monosaccharides that exist predominantly in the cyclic configuration are nevertheless potential donors of carbonyl groups in their acyclic configuration. At sufficiently high concentrations, the Schiff base forming capacity of these groups may inhibit the inductive interaction between APC and T cells and thus inhibit vaccine efficacy [51]. On the other hand, inclusion of a sugar such as mannose, whose receptor is abundantly expressed on the surface of APCs, may serve to increase vaccine immunotropism.

The presence of formaldehyde in vaccines is either intentional, as a preservative (Biothrax), or a nonintentional, viral inactivating agent (Havrix) while the presence of gelatin as a stabilizer, deagglomerator, or cryoprotectant is encountered not infrequently. The presence of carbonyl groups in these compounds may serve to initiate T-cell activation by the phenomenon of oxidative mitogenesis mentioned above.

15.6.2 1–3 Glycans

Many fungi produce $\beta-(1 \rightarrow 3)$ -D-glucans that can activate innate immune systems when they are administered into vertebrates. Among others, curdlan, lentinan, and schizophyllan have already been used in tumor immunotherapy. Zymosan enhances the immune response to DNA vaccine for HIV-1 through the activation of the complement system [52]. Although the immunogenicity-enhancing effects of 1 \rightarrow 3 glycans have been reported predominantly for covalently attached sugars (to antigen), physical complexation, such as that of zymosan to CpG DNA [53] and co-administration, such as the synthetic unit of lentinan with hepatitis B surface antigen [54], has also been reported to be synergistic.

There is no record of the above-mentioned 1–3 glucans being used as osmoprotectants during lyophilization of vaccines. Since they are polymers of monosaccharides, these sugars should act as “water replacers” to preserve the conformation of protein antigens during freeze-drying, as well as to potentiate the immunogenicity of the antigen upon administration.

15.7 AMINO AND NUCLEIC ACIDS

15.7.1 Arginine

The uptake of antigen-containing apoptotic vesicles released by infected APCs represents one mechanism by which *Mycobacterium tuberculosis* antigens can enter MHC class I cross-processing pathways [55]. MHC class-I-restricted CD8+ T cells are necessary to mount an immune response against *M. tuberculosis* [55]. However, superoxide dismutase (SOD) secretion by the currently utilized *Mycobacterium bovis* in the Bacillus Calmette-Guerin (BCG) vaccine may attenuate antigen-presenting cells (APC) apoptosis and hence the cross-priming response [56]. Inclusion of certain amino acids such as the aggregation suppressor [57] and NO precursor, L-arginine, has been shown to enhance the immune response [58, 59], probably in part due to the increased production of reactive oxygen species (ROS) which may overwhelm the neutralizing capacity of SOD, thus allowing the resumption of apoptosis.

15.7.2 Histidine

Presentation of soluble viral antigens to specific CD8+ T-cell clones by dendritic cells (DCs) is greatly improved when DCs are pulsed with the soluble antigen in the presence of chloroquine, which prevents endosome acidification, thereby reducing endosomal proteolytic activity, and seems to promote the transfer of endocytosed material into the cytosol.

pH-sensitive, cationic transfection lipids containing lysosomotropic imidazole head groups are lysosomotropic because the weakly basic imidazole head group, with its pK_a being within the acidity range of endosome lumens (pH 5.5–6.5), acts as a proton sponge while inside the endosome compartments. This so-called endosomal buffering is believed not only to inhibit the degradative enzymes (which perform optimally within the acidic pH range of the endosome–lysosome compartments) but also to induce stronger electrostatic repulsions among the protonated imidazole head groups of the cationic liposomes, leading to osmotic swelling and eventual endosomal bursting due to water entry.

Histidine is an amino acid with an imidazole side chain and a pK_a of 6.0. This property makes it an ideal endosome buffering agent and, consequently, as an agent to increase antigen cross presentation.

15.7.3 Guanosine

Guanosine has been patented as an excipient in cisplatin formulations in order to reduce the latter's toxicity [60], and its polyphosphate derivatives have been patented as adjuvants [61]. The small antiviral compound, imiquimod, which is an imidazoquinoline amine analog of guanosine, is a known ligand for TLR7. Poly-guanosine strings have been reported to improve cellular uptake and immunostimulatory activity of phosphodiester CpG oligonucleotides [62]. It has been proposed that the reactive electrophilic oxidant products of phagocytic white blood cells react with pathogen-derived nucleic acid components to produce oxidized guanosines. These, in turn, activate the innate immune system via TLR [63].

15.8 CHELATING AGENT

Immunostimulatory complexes (ISCOM) devoid of antigens (ISCOMATRIX), which incorporated a chelating iminodiacetic acid, separated from a diacyl C16 chain by a triethylene glycol spacer, could induce a cytotoxic T lymphocyte (CTL) response at an antigen dose approximately 10-fold lower than ISCOM that did not have such a chelating agent so incorporated [64]. Some of the excipient effects listed above are more relevant to “whole-body” administration, rather than administration as a vaccine component. For example, the greater robustness of immune responses to vaccines in the developed world can be attributed, in part, to better nutritional levels of antioxidants and vitamins. It is, however, reasonable to assume that the robustness of the immune response is also proportional to the magnitude and intensity of priming (by the vaccine formulation). To that extent, vaccine excipients are expected to modulate vaccine efficacy in addition to their better recognized function as stabilizers, cryo/thermo or osmoprotectants, or otherwise enhancing the storage shelf life of the formulated antigen.

It has been demonstrated that heavy water (D_2O) contributes to the thermostabilization of certain strains of the polio and yellow fever viral vaccines by blocking the nuclease activity of the viral polymerase molecule(s) carried by the virion. This results in the stabilization of intracapsid or RNA–capsid hydrogen bonding structures [65, 66].

Vaccine particles formulated with noncrystalline sugars and suspended in non-aqueous vehicles frequently settle out and are difficult to redisperse prior to administration. It is possible to form these vaccine–sugar particles as spheres enclosing a trapped gas so that the density of these particles is reduced to approximate that of the suspending oil. The components of such a formulation may include particles composed of biological material, a glass forming sugar, monosodium glutamate, and ammonium bicarbonate (carbon dioxide) suspended in a mixture of medium-chain triglycerides [67].

15.9 POLYMERIC ZWITTERIONIC BUFFERS

The ability of zwitterionic polysaccharides (ZPS) to elicit potent CD4+ T-cell responses depends on the presence of both positively charged and negatively charged groups on each repeating unit of the polysaccharide. It has been speculated that a high density of charge on the ZPS molecules promotes nonspecific promiscuous binding to Human Leukocyte Antigen DR subregion (HLA-DR) molecules on the APC surface [68]. It follows that (as yet nonexistent) polymers of ampholytic buffers, namely, (1) (polymers of) aminoalkylsulfonate zwitterions (Good's buffers), (2) (polymers of) bile salt derivatives containing sulfobetaine moieties (CHAPS, CHAPSO) (both shown in Fig. 15.1), and (3) zwitterionic polyelectrolytes (polyoxidonium) may turn out to be efficient CMI inducers. Although the polymers of molecules in (1) or (2) do not yet appear to exist in the literature, (3) has been used clinically in the Russian Federation as an immunostimulant in the Grippol vaccine. The hypothesized immunostimulant property of these (hypothetical) polymeric buffers may be increased

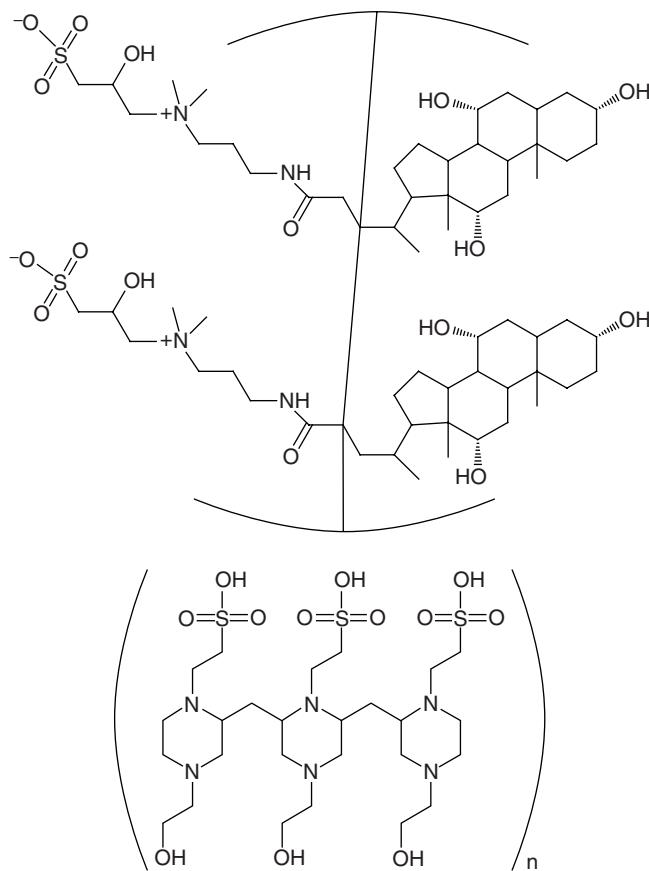


Figure 15.1. (Hypothetical) Polymers of HEPES and CHAPSO buffers.

by conjugation to VLPs. [69]. Because these hypothesized polymers are already used as excipients in their monomeric form, and because there is already a precedent for immunostimulatory properties of similar polymers, the synthesis of such polymers may be worthy of perusal.

15.10 ABBREVIATIONS

TPGS	tocopherol polyethylene glycol succinate
GM-CSF	granulocyte macrophage colony-stimulating factor
SOCS	suppression of cytokine signaling
PPAR	peroxisome proliferator-activated receptor
MHC	major histocompatibility complex
APC	antigen-presenting cell
NO	nitric oxide

SOD	superoxide dismutase
DC	dendritic cell
siRNA	single interfering RNA
TLR	Toll-like receptor
VLP	virus-like particle
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CMI	cell-mediated immunity

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PART 5

CLINICAL AND
MANUFACTURING ISSUES

16

SELECTION OF FINAL PRODUCT CONTAINERS

Luis Baez and Adrian Distler

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16.1 INTRODUCTION

The aim in selecting an appropriate container for a biologic is to deliver a product that is safe, stable, and effective, while being amenable to cost-effective manufacturing, flexibility for clinical trials, and rapid commercialization. Many considerations go into appropriate selection from product compatibility, vendor selection and qualification, identification of critical quality attributes, and adherence to regulatory guidance. Often market competition or potential market position can factor in the selection and weigh as significantly as technical factors in the selection process.

The selection of a final product container may seem rather simple when the decision is guided by convention. This may occur when the selection of a new product container and closure system defaults to containers already in use for marketed products. Although simple in approach at first, this strategy can result costly and unsuccessful if the product and manufacturing process requirements are not met by the container selection. Prudent product studies and knowledge of clinical protocols along with understanding of eventual commercial application is needed. A flawed selection may result in loss of product stability and potency, a diminished shelf life, inappropriate dosing, issues with patient compliance or comfort, loss of market share, as well as even resulting in safety concerns. Inappropriate container selection can result in adulteration of or may result in the contamination of drug product.

Innovation in delivery systems, processing technologies, new choices in materials of construction, and regulatory guidance must be considered together with patient and health-care provider preferences in mind. The specific critical quality attributes of the drug substance and drug product, the manufacturing process and the product life cycle will also add complexity to a network of interrelationships centered at providing patients with effective and safe solutions to specific health-care needs.

With the increasing variety and complexity of choices available for the selection of containers for parenteral biologic products, it is a necessity to seek solid guidance on this important selection. This discussion will center on the interrelationships that need consideration when selecting a final product container for a parenteral product. Aspects include design, product quality attributes, developmental studies, process quality, suppliers, and container closure integrity (CCI). We will also review a number of considerations in alignment with current industry guidance and regulatory expectations to further emphasize the importance associated to the selection of a final product container.

16.2 CONTAINER SYSTEMS

There exists a wide variety of options when selecting a container system. Among the more prevalent options in platforms include vials, prefilled syringes, ampoules, and cartridges for devices. Vials and syringes are among the most popular container systems for parenteral products.

Traditional vial configurations comprise a type I borosilicate glass vial, an elastomeric closure or stopper, and a seal component, usually an aluminum seal with plastic flip cap. All of which are available in a range of sizes to accommodate various dosages. Variations in vial and stopper design will respond to product requirements such as lyophilized versus liquid product. For example, the vial design could incorporate a blowback feature for raised positioning of the complementary stopper feature during the lyophilization cycle. Vial designs with no blowback are usually used for liquid product applications where the stopper will not require a raised position. The stopper also will incorporate features in response to product and manufacturing process requirements. These could span from design features specific to liquid or lyo applications, barrier and lubricant coatings, or films to reduce interaction with the product

and improve movement and migration on filling equipment. Improved features can also be found in newer rubber formulations and materials of construction that provide lower glass transition properties in response to extreme temperatures anticipated as part of storage and distribution. Other variations stemming from the integration into the manufacturing process such as dimensional thickness, vial shoulder or bottom roundness, weight or resistance to thermal shock, for example, should be considered as well. Plastic vial alternatives are also emerging with comparable performance to glass. We will expand on these alternatives later in the discussion.

Prefilled syringe images, on the other hand, will comprise a glass or plastic barrel, elastomeric plunger (or piston) and tip cap, and a plastic plunger rod. Typically, a medical-grade lubricant is needed on the inner surface to assure proper plunger placement and motion during dose delivery. The most conventional lubricant option for glass is silicone oil, however, alternate lubricants as well as options between atomized or baked silicone are also available. Some newer syringes eliminate the need for silicone altogether through the use of novel plunger coating or surface materials. Syringes also offer prefilled container options that will add components such as staked needles and needle shields or Luer-lock adaptors. Prefilled syringe containers are a well-documented example where demand has consistently increased over conventional vial containers due to a number of advantages such as ease of dose administration, reduced handling of the product before administration, and reduced risk for dosing errors when compared with transferring liquid from a vial into a syringe [1]. From the product manufacturing perspective, prefilled syringes allow reduction in product overfill requirements (overage) and the resulting product waste associated with liquid transfer from vials. When transferring product from vial to a disposable syringe, usually not all of the product can be removed from the vial. This hold up volume must be accounted for when filling the product to assure the deliverable dose can meet the label claim on the unit. Component preparation costs can also be reduced with precleaned and sterilized syringes providing a ready-to-fill container with minimal container preparation requirements and reducing unit operations complexity by elimination of washing, siliconization, and sterilization operations at the fill manufacturing site. Syringes are also increasingly preferred for their ease of use and increasing versatility including use in autoinjector devices and even in applications for lyophilized product with dual-chamber syringes. However, additional consideration is needed when weighting the advantages and disadvantages of a prefilled syringe configuration against the more traditional ampoule or glass vial options. Complexity in product–container interactions, such as surface-to-volume ratio or constant contact of product and elastomeric components, for example, will increase in the prefilled syringes. Interaction between the drug product and the stopper material if uncoated as well as silicone oil used for lubrication of conventional glass syringe barrels will require demonstrated compatibility. Additional concerns include potential trace metals and adhesive leachables from the fabrication of staked needle syringes. Examples of interactions between product formulation and rubber components with potential impact to product quality have been previously documented in the literature, thus raising awareness of these important considerations [2, 3]. As stated before, the multiplicity of factors and associated risks will require careful assessment through product

compatibility and stability studies to assure critical quality attributes of the product are maintained.

16.3 GUIDANCE AND RECOMMENDATIONS FROM INDUSTRY AND REGULATORY AGENCIES

Understanding the regulatory landscape when selecting a product container can help assure compliance with applicable regulations and cGMPs. These regulations lay out the expectations in developing and manufacturing a parenteral product and compliance obligations related to approval and postapproval use. These regulations cover aspects ranging from quality testing to manufacturing and beyond.

Supporting regulation for components, containers, closures, and related aspects of manufacturing can be found in the Food and Drug Administration (FDA) Title 21—Food and Drugs, of the Code of Federal Regulations, Part 210—Current Good Manufacturing Practice in Manufacturing, Processing, Packaging, or Holding of Drugs; General and Part 211—Current Good Manufacturing Practice for Finished Pharmaceuticals. The FDA Guidance for Container Closure Systems for Packaging of Human Drugs and Biologics [4] provides general principles on the information on packaging materials that will be required to support a product application and outlines the general considerations for suitability for the intended use, including product protection provided by the container closure system, product compatibility, safety, and performance throughout the product life cycle. Physical characteristics and chemical composition (extractables/leachables) are also emphasized as important requirements to assure compliance to product safety and quality. These aspects are also summarized in the Center for Biologics Evaluation and Research (CBER) 1999 Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for Vaccine or Related Product Guideline [5] and are discussed in an applied perspective to manufacturing processes in the 2004 Aseptic Processing [6] guidance where component preparation, processing, and inspection in the finished form are discussed. The General Chapter <1207> of the U.S. Pharmacopeia on Sterile Product Packaging summarizes recommendations addressing package and container closure integrity throughout the product life cycle and within three distinct phases defined as initial development, routine manufacturing, and shelf-life stability [7]. The chapter provides useful recommendations on the different test conditions to consider, package and/or container conditioning for testing of real life or worst-case conditions, physical and microbial test applications, and selection of evaluation methods.

Most recently, the 2008 guidance on Container and Closure System Integrity Testing in lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Product adds detail to the expectations for sterility testing and the demonstration of microbial integrity of a container closure system [8]. The guidance emphasizes the advantages of container and closure system integrity testing over sterility testing applied to product stability protocols with allowance for physical, chemical, or microbial-based methods. Proper validation of the selected method is pivotal to

this recommendation in order to support method compatibility with the product and acceptable detection capability to demonstrate a breach in container and closure system integrity. This last example illustrates the evolving regulatory environment and the continuing need to develop mature quality and compliant systems in support of container and closure selection.

Additional guidance can also be found in regulatory documentation such as the Container Closure System section of the International Conference on Harmonization (ICH) Q8 Pharmaceutical Development Guideline [9] and the European Medicines Agency (EMEA) guidance on Plastic Immediate Packaging Materials [10]. Both documents emphasize the requirement to provide a rationale and justification for the container selection and demonstration of its feasibility, safety, and performance throughout the product life cycle. Integration into the quality by design (QbD) framework in the 2006 FDA guidance on Quality Systems Approach to Pharmaceutical cGMP Regulations [11], the ICH Q8 Annex on Pharmaceutical Development [12], ICH Q9 on Quality Risk Management [13], and the ICH Q10 Draft Consensus Guideline on Pharmaceutical Quality System [14] adds emphasis to the science-based and risk-based approaches required to assure consistent drug product quality and set new paradigms in development of target product profiles and creation of a product design space that can be applied to the selection of primary container systems for finished drug or biological products. Additional guidance on component test matrices and standard test methods can be found under the Bio-Process Systems Alliance (BPSA) Guidelines and Standards Committee. The BPSA focuses on best practices for quality testing of materials and functionality of single-use components or systems for the biopharmaceutical industry [15].

16.4 CONTAINER DESIGN AND SELECTION

The design and selection of a container and closure system integrates the identified requirements of the active pharmaceutical ingredient (API) or biological excipients and formulated product with the manufacturing process requirements and the product life cycle. Intercalated between the container components (inputs) and the finished product (output), we will find a number of areas and/or processes that will require careful consideration in order to (1) define the critical-to-quality input variables or parameters and (2) outline the control strategies that will demonstrate control of the critical variables identified. Altogether, the integration of all involved key areas/processes will require development of comprehensive process knowledge with clear understanding on specifications and associated variability. This integration will vary throughout container platform selections and at the same time will have some commonalities in the fundamental expectations from regulatory agencies on the assurance of product safety, identity, strength, purity, and quality.

Borrowing from QbD concepts, the design and selection stage is expected to be accomplished by the systematic screening of potential container alternatives. Upfront criteria and specification targets with range limits are set to define the target container profile. This task will consider the requirements in physicochemical and biological

compatibility as well as the functional performance requirements. The physicochemical and biological requirements are defined under Pharmacopeia requirements, whereas functional aspects will comprise user-defined requirements, including integration with the drug or biological product manufacturing process. The user-defined requirements should look at the container design and selection as the total journey through the product life cycle.

The journey will start with the upstream processes in component fabrication and associated raw materials. Assurance of demonstrated control by a vendor over the production of components and handling of raw material is just as critical as the production of the parenteral product. Change control processes for any material vendors, compounding, formulation, or forming processes as well as tooling and molds are essential. From this point, the container components will integrate into the pharmaceutical or biopharmaceutical manufacturing process by way of incoming inspection in compliance with predefined acceptance quality levels agreed to by the supplier and the drug or biological product manufacturer within the context of a quality agreement. For example, specific defect rates and acceptability must be established based on initial criticality assessment. These range from physical dimensional attributes to cosmetic imperfections. In addition, sterility of incoming components and packaging control need consideration as a range of container components come presterilized direct from the manufacturer and ready for product filling. Next, the components are received and tested against raw material specifications using reject quality limits (RQL)-based sampling programs. After release, the container components will move through the conditioning and preparation stages and will continue through the product manufacturing process into the filling and finish processes. This stage initiates the interaction of the container and closure system with the product that will continue throughout the product life cycle.

Container selection will need to include the ability to use the container on a given manufacturing filling line. Without a correct match there is the potential for the lengthy and costly process of retrofitting a production line, which may include from simple changes to parts to the introduction of new equipment altogether. It is important to also consider the potential for future technology transfers to additional sites or facilities as a result of manufacturing capacity expansions or transfer to contract manufacturing sites as these may add complexity to technology transfer and supply chain requirements, comparability demonstration, as well as validation and regulatory approval.

The subsequent phases within the product manufacturing process will move the container through final inspection, labeling and packaging, storage, shipping, and distribution. After this sequence the finished product will finally reach the end user, and conclusion of the container life cycle is reached with the final disposal. This integration of container design, functional requirements, and maintenance of product quality attributes throughout the product life cycle will need to be addressed in the context of the QbD framework by defining the ranges or tolerances applicable to the container and product manufacturing processes and the functional requirements that will consistently achieve product quality. The combination of inputs from the container system, the product manufacturing parameters, and product quality attributes will define the

design space supporting the specific quality characteristics of the drug or biological product.

Design and selection of a container system should consider the current state of container platforms available as well as future container platforms that may bring additional advantages. In many instances the container selection can be driven by the current or existing marketed containers due to demonstrated satisfactory performance and added timeline pressures in preclinical and clinical product development. Understandably, this approach may prove advantageous for later generations of a product or when products with similar properties or requirements are developed. However, in some instances the selection of a container image does not always include a comprehensive account of the current status or challenges that the current market image may be encountering. Also, in numerous instances personnel closer to the current performance of the container system such as the manufacturing and technical support areas, may not be integrated with the selection and decision process, mostly residing in early or precommercial product development. It is advisable that this selection not be based solely on an existing approved market container but should include a comprehensive evaluation of the container system within the context of current regulatory scrutiny and expectations for product life-cycle performance. Ultimately, the demonstration of suitability for the intended use is required as part of the application for the drug or biologic product per regulatory guidance.

Going beyond the need for integration among the precommercial product development and technical and manufacturing support, we can find a number of areas where valuable input can be found. Health-care providers as well as patients can provide insight through the use of focus groups, into aspects of preferred container or delivery options such as ready-to-use containers as in the case or prefilled syringes for parenteral administration, plastic ampoules as in the case of pediatric oral vaccines, and assisted delivery options in the case of autoinjector technologies. Market analysis also contributes valuable information on user preferences and plays an important role in the continuous improvement and development of new technologies as well as challenges faced with product globalization. These collective considerations can then be matched to the specific product attributes to further refine the container choices available for consideration. Examples of key points to consider will be the dosing regime intended for the product, as this will impact container selection from a single or multidose use, coring and reseal properties of elastomeric components of the container as well as applicable disposable solutions suited for single-use applications whether self-administered or by a health-care professional. Product stability requirements will dictate formulation composition including whether a liquid or lyophilized form will be required. These will in turn define the container component design and associated requirements that will achieve optimum performance through the manufacturing process and product life cycle. Stricter stability requirements impacting headspace atmosphere composition, for example, will require tighter permeation requirements on a container and closure materials of construction and design in order to assure potency, purity, and overall quality. The product formulation and stability requirements will also have an impact on the selection of container components as variables

stemming from the contact or interaction of the container components, and the product can impact product stability, thus defining and/or narrowing the options available for selection.

An additional point for consideration is the collection and analysis of information regarding developments in the container marketplace. These collection and analysis initiatives fulfill a valuable function gathering input and information on existing and emerging container platforms while at the same time tracking trends in the industry as well as competitive products. Benefits are far reaching, including continuous improvement and optimization of existing product life cycles and competitive advantage whether on marketed products or during the pipeline stage.

16.5 CRITICAL TO QUALITY ATTRIBUTES OF THE PRODUCT

Together with the customer requirements, available container options, and pertinent regulatory requirements, the product formulation will require evaluation for compatibility that will include life-cycle stability throughout applicable product strengths and container sizes. Relevant aspects to consider include solvent system requirements, compatibility with container components throughout the intended life cycle, light sensitivity, headspace atmosphere requirements, trace metal sensitivity, pH, temperature range requirements for stability, susceptibility to oxidative degradation or inactivation, and impact from transportation and distribution, among some examples. It is here where consideration of container platforms such as vials versus syringes or plastic versus glass must be carefully evaluated against our full understanding of the product properties and critical quality attributes in order to meet performance requirements for the container system, the product, and ultimately the patient or consumer.

Next, let's examine how our understanding of product-specific requirements can inform the understanding of product stability. For a biologic there are many pathways to loss of stability and product integrity. For each specific product these pathways should be understood, have qualified analytical methods that can detect them, and appropriate limits or acceptance criteria should be established based on safety studies during clinical trials. These pathways can either be physical or chemical in nature. Physical degradation can be observed in such phenomena as aggregation when individual proteins or peptides associate due to surface interactions. Chemical degradation can result in methionine oxidation induced by free radicals or oxygen or in clipping where the amino acid chain backbone is cleaved. Knowledge of such stability concerns can play a key role in the input of container selection. If we consider sensitivity to oxidation in the design and selection of a final container, for example, we will apply special consideration to gas permeability for the container and closure system, intended storage time and temperature, as well as extractables and leachables for the container system. Although our scope is applied to the selection of a final container, product quality considerations for this example will also span upstream processing and bulk storage to fully assess the potential impact to the finished product due to oxygen permeability through plastic containers (rigid or flexible containers) used throughout processing, storage, and formulated form however, upstream processing will not be

discussed here. Aside from the container permeability, we will look for other container interactions that may have a similar impact to the product by way of leachables. This consideration should not be limited just to the container materials of construction but should also consider leachates from container label adhesives, trace chemicals or metals associated to component manufacturing processes such as staked needle adhesion or syringe barrel formation. Examples of antibody proteins susceptibility to trace metals, specifically oxidized tungsten residue, have been documented in the literature [16]. In the case referenced, the tungsten oxide on the glass surface originated from the oxidation of tungsten pins used to form the needle channel during barrel tip formation, which was associated to aggregation of a protein product. Other interactions such as silicone-oil-induced aggregation of proteins have also been reported [17]. All these cases further emphasize the need for an understanding of the critical quality attributes of the product as well as the constant vigilance for variables with potential detrimental impact to the finished product when a container and closure system is selected. As mentioned earlier, it is also important to keep abreast of alternatives that may offer solutions to potential problems. In this latter case, feasible alternatives can be offered in baked-silicone, alternate lubricants, coating films, or deposition technologies as replacements for conventional sprayed silicone oil with added advantages in lower silicone content applied while delivering comparable lubricity. Alternative solutions can also be found in plastic syringe platforms where combinations of fluoropolymer-coated plungers offer silicone-free possibilities; however, additional product requirements and manufacturing process infrastructure changes need to be considered. The multiplicity of factors to consider is a constant reminder of the need to understand not only the product-specific requirements but also the component supplier process and associated raw materials in order to effectively avoid or remediate any potential adverse impact to the finished product and ultimately to the patient.

As mentioned earlier, the final container design and selection phases require some extent of prescreening that can be accomplished based on physicochemical characterization and biological compatibility data as well as supplier functional data that may/may not comprise the user-defined requirements. Specific details on product susceptibility such as trace metals, aggregation, oxidation, or other, if already known or characterized, can be addressed with the supplier by understanding the container or component manufacturing process. Alternatively, a preliminary product stability assessment with the prospective container may be conducted before a formal decision to qualify the container is made.

An additional point for consideration before final selection and start of qualification efforts is to consider whether the container(s) selection will result in a single or multiple-sourced situation and what are the potential implications to product manufacturing from a supply chain risk mitigation perspective. Qualification of a single or multiple suppliers will require a series of activities comprising facility and systems audits, establishment of quality, supply and change control agreements in order to assure container components fabrication, cleaning, sterilization and distribution processes are defined, controlled and mutually understood. Even though the complexity of multiple sourced components allows for effective risk mitigation from the supply chain perspective and could provide for better pricing due to competition, it also adds to the

initial costs and complexity of additional product stability studies, added complexity in manufacturing documentation and potential for added complexity in manufacturing process integration spanning from component processing, handling, and up to final inspection. Subtleties as far as automated inspection requirements can be envisioned in the event of multiple-sourced components. Whether flexibility of multiple sourced operations can provide economical advantages requires careful consideration of the quality needs, costs, and risk mitigation requirements. Also, the need for speed to pivotal clinical trials may need to be balanced against the complexities of second sourcing initiatives. An option to consider will be to examine sourcing alternatives after critical milestones are met in the product development process. Finally, it is recommended that consideration be given to how changes of raw materials and how the supplier implements change control and notification so that understanding how changes will be authorized and communicated is clearly and mutually understood. This level of mutual communication will allow for opportunities and time to address any needed changes to the validated container platform thus assuring the identity, safety, potency, purity and quality of the finished product.

16.6 PRESCREENING OR DEVELOPMENTAL STUDIES

Prescreening or developmental studies may be considered to aid with container selection and initiation of component qualification activities. These studies will support suitability of the selected containers and will assist in defining acceptance criteria for upcoming qualification and/or validation activities. Screening procedures may include pharmacopeia testing or assessments of functionality aspects. Studies may also include functional testing such as glide force and break-loose extrusion testing for syringes, multiple puncture tests for elastomers, drop tests, or deliverable volume measurements. Extreme process conditions as well as container and closure preconditioning requirements such as cleaning, sterilization, storage time and/or temperature, transport, distribution, and expiry terms applicable and specific to the process and/or product should be considered as part of this preliminary or abbreviated evaluation. For example, freeze–thaw cycling studies would be important for a product that is to be stored frozen or lyophilized. Container failure can occur depending on size, volume, product formulation characteristics, and temperature conditions. The container must carefully be matched for fit with respect to formulation. Crystallization of sugars upon freeze or temperature cycling under specific conditions can induce stress leading to failure [18, 19]. Evaluation of extreme process conditions can also aid when critical product or process requirements can influence disqualification of prospective container candidate(s) and help define the container and closure design space within which product and associated processes will operate. Product testing can be performed through stability studies to understand how the established product quality attributes are maintained over time. These studies may be chosen to be incorporated with product formulation activities. Studies can also help narrow selection decisions and may be particularly useful when identifying product impact using different types of stoppers or stopper coatings, for example, or other variations of the container system. Beyond stability studies, evaluation of process conditions should be undertaken to

define the appropriate design space within which the limits of process parameters are identified to mitigate risk or harm to the product. Product in a given container can be exposed to various light conditions, temperature cycle, and transportation conditions to mimic production or supply chain conditions. The results will help to define acceptable ranges of operation and reveal interactions between critical process parameters and component specifications.

In addition to functionality and product contact studies, extractables and leachables testing are an important consideration in selecting an appropriate container. Typically extractables studies comprise of testing water, a product placebo (delivery vehicle), or a selection of model solvents to accelerated time and temperature conditions in the container of choice. The water, placebo, or model solvent is then chemically tested for compounds that have been extracted out of the container and closure systems. These compounds need to be identified and assessed for safety and product impact concerns. Testing for leachables involves assaying the container closure system with product over time to identify compounds that may leach from the system under intended storage conditions. Again, the compounds need to be identified to assess safety and product impact. Variations in product formulation such as pH can readily change the extractable profile.

As mentioned earlier, the consideration of existing and already qualified container platforms should assess product and process-specific requirements in order to ensure that critical to quality attributes are met prior to a container implementation decision. A straightforward component specification, as in a coefficient of thermal expansion for a type I borosilicate vial, when not specified in the internal component specifications and/or supplier agreement, could become a critical item to a freezing process modification, thus increasing the potential for vial breakage. Consideration of prescreening or developmental studies will be regarded as a recommended but discretionary measure based on the product formulation and process knowledge and understanding of the available container options. Qualification activities for an existing container platform will require an assessment of the application to determine appropriate studies where needed.

16.7 QUALITY THROUGHOUT THE PROCESS

Product globalization has played a pivotal role in the increased awareness in the distribution and supply chain requirements as well as increased demand for cosmetic appearance quality and the resulting emphasis on inspection for both incoming and final product. Product distribution requirements have also seen increased emphasis in areas of supply chain security, brand security (antitampering, anticounterfeiting measures, and authenticity testing), as well as cold-chain management for products requiring storage under controlled temperature conditions. Guidelines on the identification and classification of container imperfections for tubular and molded glass and on cold-chain distribution can also be reviewed in the recent technical reports of the Parenteral Drug Association (PDA) [20, 21].

Regulatory expectations are also increasingly focusing on the need to better understand defect definitions and severity classifications applied to the impact on the

container closure system performance. Additional recommendations or expectations on the generation of defect libraries for training/qualification purposes, generation of standards for validation of inspection systems, and support of product investigations are increasingly noted in discussion forums among regulatory agencies and pharmaceutical and biopharmaceutical sectors. This increased scrutiny further emphasizes the need for a thorough understanding of the variability inherent in component manufacturing processes and resulting component quality that a supplier can provide. This understanding will in turn lead to the alignment and agreement on acceptable quality levels and optimization of both supplier and product manufacturing processes toward defect reduction and elimination. This latter approach is becoming more prevalent as pharmaceutical and biopharmaceutical manufacturing strive toward reduction in reject and scrap rates, lean manufacturing, and optimization of product manufacturing. This stricter requirement to flawless execution and defect reduction to parts per million levels will further expand defect reduction and assurance of quality supply to raw material and component suppliers where upstream control measures can be effectively applied. More emphasis is being placed on 100% upfront inspection of components with validated inspections systems to eliminate defects prior to filling costly product in addition to the typical postproduction inspection. Vendors may include several levels of inspection that detect a higher level of cosmetic defects depending on customer requirements.

16.8 CONTAINER SYSTEM COMPONENTS, SUPPLIERS, AND THE RAW MATERIAL NETWORK

Component suppliers face important challenges in quality optimization among raw material suppliers as a result of the evolution on regulatory guidance, implementation of QbD practices, leaner manufacturing trends, and increasing demand for single-use disposable technologies. Application of the recommendations discussed earlier will provide the component supplier with a clear understanding of how to deliver consistent high-quality components with effective risk mitigation measures built into their supply networks and associated processes. An example of this interrelationship can be found in tubular glass quality applied to formation of syringe barrels and parenteral vials. Mitigation of tubing cane defects such as refractory or undispersed material, hairline defects, dimensional exactness, and others at this upstream stage will have profound impact on the cosmetic quality of the formed containers. This will in turn impact associated inspection processes whether incoming or final and ultimately finished product discard rates due to cosmetic defects. An example of risk mitigation practices through better understanding of the upstream process can be presented in a cosmetic defect reduction collaboration between a drug product manufacturer and the container supplier. In this particular case the involvement of the tubing cane supplier provided better understanding on the rate of tubing cane defects originating from the tubing cane forming operation. The engagement of the upstream processes and raw material supplier resulted in increased awareness from the tubing cane and container suppliers, insight into the emergence of refractory defects as a result of aging

on the tubing cane-forming ovens, optimized monitoring of maintenance cycles for cane-forming operation, and implementation of inspection equipment for monitoring of tubing cane quality. This insight allowed better understanding between component and raw material suppliers on the impact of tubing defects on finished containers and opened opportunities for optimization on tubing cosmetic quality based on closer monitoring of tubing cane formation and maintenance at the tubing cane-forming stage. An additional consideration stemming from the need for controlled requirements on cosmetic and dimensional component quality resides on their impact on visual inspection methods whether automated or manual. Validation of these systems depends on a clear understanding of defect classifications and definitions and controlled component manufacturing in order to avoid sporadic and/or uncontrolled emergence of component defects. Furthermore, the understanding of this entire process by all segments involved is important as defect detection at the finished product stage, whether automated or visual, will bring a higher level of scrutiny at times aided by the presence of the product, as in the case of liquid products where magnification of defects can occur or with lyophilized product where greater detection of color and texture differences can be attained. Most important, defect detection at this finished product stage will be more costly compared to controlling quality upstream in the container component fabrication stage.

All together, stricter requirements in glass quality and defect reduction in the parenteral vial and syringe container platforms have made their impact felt on improvements from tubing cane formation as well as better controlled glass forming processes and inspection technologies, all aimed at providing superior quality to the end user. Assurance of quality glass components whether a vial, syringe, or other will require evaluation of the supplier production process with emphasis on quality systems understanding together with defined physicochemical and cosmetic characteristics critical to product quality and process performance.

Parenteral platforms based on vial containers or prefilled syringes bring us to the world of glass properties and glass forming. Type I borosilicate glass constitutes the standard material of choice, although newer alternatives in olefin-based plastic platforms are also available but not as widely applied. Experience through numerous drug and biological applications have taught us the importance of surface inertness and the role it plays in shelf-life stability. Reports in the literature on the surface inertness of glass and associated problems with pH changes, surface delamination, and adsorption have brought to light the importance of the glass forming process, temperature control during formation and annealing, and the resulting role surface chemistry plays in the container life-cycle performance. Surface treatments have applied ammonium sulfate as a control measure for surface alkalinity and delamination; however, significant improvements in forming process controls and surface coating technologies are providing glass containers with superior purity and inertness and improved product stability.

Container design specifications when not integrated into manufacturing performance can manifest in a number of problem scenarios. Let's take an example on a glass vial where component specifications such as bottom height, heel radius, and vial weight variability are considered. The potential impact to manufacturing process

performance can be felt through vial migration along conveyor paths and transfer plates through accumulator tables, washing and depyrogenation tunnels, and other segments along the filling, capping, and inspection lines. Component performance should always be evaluated in the intended manufacturing process to assure optimum performance through parameters such as line speeds, line discards, and total line interruptions or stoppages due to component-related issues. Examples applicable to syringes can be offered for component features such as flange thickness dimension. Problems associated with this component feature could manifest in cracked or broken flanges when design specifications are not optimized or show high variability. This deficiency could be amplified when applied to assembly in autoinjector devices, where a flange thickness design and specification could potentially constitute a critical attribute for autoinjector operation. An example specifically applied to lyophilized product containers can be offered here to demonstrate how impact can be felt as far as the final product quality. Vial manufacturers typically offer lyophilization vials that will include a blowback feature, designed with the purpose of positioning the lyophilization stopper in a raised position through the freeze-drying process. This vial feature located in the upper region of the inside wall of the vial neck and is created by the interaction of a metal plug during the vial opening forming process. This particular interaction is known to result in the generation of toolmarks as well as variability in blowback dimensions. A viable way to eliminate these potential defects with vial fabrication can be offered by a re-design on the stopper component, where a raised stopper positioning could be attained without the need for a blowback feature, thus reducing the required manipulations during the vial opening forming process. Stopper features such as single opening or “igloo” design with longer plug dimensions and positioning knobs for stopper support on the horizontal plane of the vial flange will provide a suitable alternative to a vial with no blowback feature in a lyophilization application. This in turn will reduce and/or eliminate potential defects due to toolmarks and blowback variability while providing a uniform vertical plane on the vial opening inner wall and increased surface contact between the stopper and the glass surface thus supporting container closure integrity. Potential consolidation of vial specifications can also be envisioned here if a common vial design for liquid and lyophilized products is anticipated.

It is also important to acknowledge how the continuous striving for improvement in component quality has propelled the emergence of polymer alternatives for syringe and vial containers beyond conventional borosilicate glass. The potential advantages of this platform deserve consideration when designing or selecting a primary container for a biological or drug application. Most notably, cyclic olefin polymers are emerging as an alternative to borosilicate glass containers. These polymer containers, currently available from several parenteral component manufacturers, provide superior dimensional control and glass-like transparency essential for final product inspection, while eliminating a number of cosmetic defects inherent to glass forming and manufacturing of glass containers. Chemical resistance and barrier properties are two important aspects to evaluate when polymer containers are considered as alternatives to conventional borosilicate glass. These characteristics will also impart additional requirements to qualification and product stability testing of plastic containers as aspects of barrier

and permeability properties, polymer and additive leachables, and label solvent and ink migration, normally not considered with more inert borosilicate glass containers, will now require consideration with the plastic alternatives.

Next, we will consider the elastomeric components of the primary container system, whether in a vial stopper or a plunger and needle shield syringe system. Selection of an elastomer formulation will require evaluation of product compatibility and life-cycle performance early in product development. Elastomer manufacturers offer a number of formulations with cleaner chemical composition, free of latex or natural rubber and with reduced levels of extractables and leachables. Newer synthetic formulas are also providing better temperature range performance with lower glass transition temperatures, which assures optimum closure performance when exposed to low temperatures during shipping and distribution. Newer formulations are also providing improved chemical inertness, lower absorption of water, and lower gas permeation, which improves quality assurance over the product life cycle. At the same time, lubricity has notably improved with newer elastomer formulas, which translates into less stopper clumping and improved movement throughout the product filling process. Syringe performance in plunger break and gliding force can also be improved with appropriate elastomer selection. Developments in elastomer formulas and coating films have also opened new alternatives where siliconization of syringe barrels is no longer necessary. Fluorocarbon film coatings can also reduce the interaction between product and the elastomer, thus reducing the risk for leachables over the product life cycle, improve sorption resistance and lubricity, and even contribute to the elimination of particulate or interaction with undispersed elastomer material from stopper or plunger surfaces. All these factors considered during design and selection will influence aspects from component performance in the manufacturing operation, product quality impact in container closure integrity, leachables, extractables, product purity, safety potency and container, and product life-cycle performance.

In alignment with the recommendations discussed above, the integration of supplier and customer needs is imperative to assure consistent quality on the finished product. Additional aspects of elastomers that merit attention reside in the quality systems built in the elastomer manufacturing process. Here an in-depth understanding of the formulation and manufacturing process together with continuous and open communication, coordination, and adequate change control practices between supplier and product manufacturer are required to deliver quality product consistently. Aspects of component fabrication such as design and construction of component molding tools can ultimately impact dimensional tolerances or cosmetic quality in formed components with consequences felt from incoming component inspection to final container performance and product stability. Dimensional tolerances and variability in component fabrication process constitute a set of conditions where limited attention is focused and where impact as far reaching as product stability and integrity can be envisioned. The normal decay of molding tools as the result of normal operation has brought to light the need for constant surveillance of closure integrity and the emergence of testing technologies aimed at fast, cost-effective, and nondestructive container integrity testing where component blemishes or defects could end up impacting product quality.

This topic will be revisited in more detail later in this discussion. Other areas with far-reaching impact can be found when cleaning molding tools, mold release agents, and handling of process materials during forming, cutting, and trimming, where findings of embedded foreign material in the finished component are possible. Regular maintenance and monitoring of molding tools are also aspects of component variability with consequences impacting container closure integrity and product stability. Even though a mold cavity may not deteriorate to the point of generating a component that will allow microbial or particulate ingress, the gradual decay and resulting blemishes on the molded component may allow for molecular exchange of gases that may be of impact to a product maintained under pressure or a specific headspace atmosphere. This example also highlights the shared responsibility on the product manufacturer to monitor such critical parameters as a safeguard against potential component defects with critical impact to the finished product, especially in cases of multicavity molds. Compounding and formulation processes for elastomers can impact compliance with compendia requirements, leachables, extractables, and potential product stability. For these reasons, changes associated with component fabrication processes and associated raw materials must also be clearly controlled and communicated in order to assure no impact to the product and final user is imparted.

16.9 CONTAINER DEVELOPMENT AND INTEGRITY EVALUATION

The demonstration of container closure integrity will be an essential part of the container qualification as it will constitute the foundation that will assure the product maintains its identity, strength, quality, sterility, and purity throughout the life cycle. Product globalization will also bring new challenges to container design, development, and qualification, imposed by requirements in shipping, distribution, and associated product stability. Innovations in product formulation such as stabilizer excipients, lyophilization technology, and improvements in cold-chain distribution of products have all targeted the preservation of product stability. At the same time these improvements have translated into container and closure requirements such as materials of construction with higher tolerances to extreme cold temperatures during distribution, improved water vapor and gas transmission rates, use of modified headspace atmospheres to protect from product deterioration, and use of surface coating technologies in order to again minimize interaction between container components and product. The innovation in approaches to safeguard product stability in turn has propelled the evolution of testing technologies for container closure integrity. During earlier times, the demonstration of container closure integrity relied on the verification by sterility testing initially and at product expiry. This position, supported by regulatory agencies, will eventually evolve to a recommendation for demonstrated container closure integrity by initial validation of microbial integrity. The latter requirement led to a significant growth rate in the development of microbial-based test methods for qualification of initial container closure integrity while still allowing for sterility testing at product release and expiry. Most recently, the 2008 Guidance for Container and Closure System Integrity Testing in lieu of Sterility Testing [8] addressed the suitability

of sterility testing at the initial or product release and final or expiry time points. The guidance pointed to the limitations on the use of sterility testing to demonstrate “the continued capability of containers to maintain sterility.” To this end, the guideline allows the consideration of physical as well as microbial-based methods as alternatives to sterility testing as part of the stability protocol. In anticipation of the regulatory guidance and understanding the limitations of the sterility test, in providing an assessment of performance of a container and closure system, the industry sector has been working on a diversified approach to container and closure integrity testing. In accordance with regulatory expectations, the development of methods and approaches to container and closure integrity testing will require a scientifically sound approach to method validation.

The General Chapter of the United States Pharmacopeia (USP) on Sterile Product Packaging provides useful perspective into package development and integrity evaluation in alignment with industry trends [7]. The chapter discusses how product package integrity testing ensures that product attributes including sterility are maintained throughout the intended product life cycle. The differentiation of three phases for integrity evaluation of sterile packaging as development, routine manufacturing, and stability brings together a number of considerations discussed previously. Integrity evaluation during the development phase addresses the user and product-specific functional aspects with the product manufacturing process. During this initial phase of the package or container development, microbial and/or physical test methods are evaluated with consideration to container integrity boundaries imposed by the functional and manufacturing requirements. As part of these requirements, it is important that the container system preparation and conditioning as well as the impact of shipping and distribution operations be considered to assure development of a robust container or package system. Consideration to the inherent variability in multiple manufacturing sites or the use of contract manufacturers need to be considered here since implications to method validation and transfer need to be addressed beforehand. Once the method(s) is identified it will need to support process control measurements in routine manufacturing as confirmation of acceptable package or container performance. Here the consideration of a physical test method will be favored over a microbial-based alternative since the former will offer a nondestructive alternative with faster test time and more economical performance.

The evolution of container or package integrity test methods has seen the application of microbial-based methods such as liquid immersion and aerosol challenge methods using a number of bacterial cells and/or bacterial spore forms. Even though microbial-based methods are acceptable for initial container closure integrity and are even regarded as acceptable for testing during shelf life and expiry as part of product stability protocols [8], the approach will require justification for the use of media-filled units and regulatory approval. Further limitations with microbial-based methods arise from in-process requirements with testing of sensitive products during manufacturing. Most applicable examples can be found with products requiring modified headspace atmospheres in order to protect the product from degradation. Here the consensus among regulatory agencies, USP, and industry experts is that physical test methods can provide alternatives that offer faster test time, nondestructive and 100% testing

of product samples, lower costs when applied in routine testing, and less variability among users [22]. Recommendations in the selection of a physical method emphasize the need to demonstrate a correlation to microbial-based testing and comparable or superior sensitivity to the microbial-based method. This evaluation will also determine at what physical measure the container system will fail to preclude microbial ingress, thus demarcating the pass/fail method criterion. The minimum expectations for package or container system integrity should include conformance to the physical test method acceptance criteria as well as conformance to visual and dimensional inspection criteria. The life cycle of the finished dosage form is expected to maintain quality within a predefined set of attributes that will assure a sustained level of performance through expiry. Performance of the container and closure system must take into consideration shipping and distribution requirements and the temperature sensitivity of the product. Container and closure performance must account for potential extremes in temperature and the combined impact with ambient pressure variations and exposure time in order to assure robustness of the container system and ultimately product quality.

Finally, integrity testing of a package or container system through the developmental, manufacturing, and stability phases brings the requirement to establish a performance baseline for the package or container system within defined process conditions. This performance baseline will encompass the inherent variability in the manufacturing of the container components, the process variability associated with the manufacturing of the biological or drug product, shipping and distribution variability, and container system performance throughout expiry as the sum of all the process variables combined, thus defining the container system design space. Revalidation should be evaluated when modifications are made to the container and closure system, associated components, or process (es) that provide assurance of package or container integrity.

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17

FROM THE LAB TO THE CLINIC: FILING A PHASE I IND FOR AN INVESTIGATIONAL VACCINE

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17.1 INTRODUCTION

The purpose of this chapter is to provide general operational guidance for the filing of a phase I investigational new drug (IND) to initiate clinical trials for a vaccine in the United States. This information will be well known to experienced regulatory affairs

professionals and is intended to provide an overview and reference for those new to clinical vaccine development and regulatory affairs.

Within the United States, the responsibility for regulating vaccines, including regulation of clinical trials of investigational new vaccines, is the responsibility of the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA). The authority for regulation of vaccines is provided by the Public Health Service Act and sections of the Federal Food Drug and Cosmetic Act.

Filings for vaccines intended to prevent or treat infectious diseases are reviewed by the Office of Vaccine Research and Review (OVRR) within CBER. Vaccines for other indications, including therapeutic cancer vaccines, are reviewed by the Office of Cellular, Tissue and Gene Therapies (OCTGT) within CBER even if the vaccine construct itself is not gene based. The staff of the former Office of Therapeutics Research and Review of CBER, which had reviewed therapeutic proteins and monoclonal antibodies as well as therapeutic vaccines for noninfectious disease indications, was moved to the Center for Drug Evaluation and Research (CDER) in October 2003. However, responsibility for review of therapeutic vaccines was retained by CBER and has been assigned to OCTGT.

17.2 THE PRE-IND MEETING

Frequently, companies, institutions, or academic investigators (“sponsors” in FDA terminology) will want to review their plans for filing their IND with the FDA. The usual purpose of this meeting is to obtain concurrence from the FDA that the sponsor’s preclinical, toxicology, chemistry, manufacturing, and controls (CMC), and clinical study plans for the phase I trial are appropriate and to obtain guidance from the FDA especially in the event that the agency does not concur with the sponsor’s proposals. By obtaining early guidance, the likelihood of incurring delays resulting from FDA placing the IND on “clinical hold” after receipt and review of the application can be reduced. The FDA benefits from the broad experience of reviewing and monitoring the applications from many Sponsors over long periods of time and will use its judgment and experience in providing guidance to sponsors, especially related to potential safety concerns.

The procedures to follow to request a pre-IND meeting with the FDA are specified in Guidance for Industry, Formal Meetings with Sponsors and Applicants for PDUFA Products (February 2000) [1]. Guidance specific to manufacturing and testing issues (chemistry, manufacturing, and controls) for IND meetings is contained in the FDA’s Guidance for Industry, IND Meetings for Human Drug and Biologics (May 2001) [2]. Further information is available from CBER’s Manual of Standard Operating Procedures and Policies, Scheduling and Conduct of Regulatory Review Meetings with Sponsors and Applicants, SOPP 8101.1 (May 18, 2007) [3].

A pre-IND meeting will generally be held within 60 days of the written request to the agency as a “Type B” meeting, following the FDA’s meeting request classification. Generally, the FDA will ask that sponsors consolidate their various questions about vaccine development spanning preclinical development (e.g., laboratory and/or animal

proof of concept studies), pharmacology and toxicology, chemistry, manufacturing, and controls (CMC), and clinical development into a single meeting. Typically, in vaccine development, specific FDA guidance or agency concurrence on safety assessment (pharmacology/toxicology) is the first item that needs to be resolved, and the timing for the pre-IND meeting may hinge on the timing to initiate these studies. It may be possible to hold an early or separate meeting on preclinical development (such as proof of concept animal studies) and safety assessment protocols prior to the pre-IND meeting, often informally referred to as a “pre-pre-IND meeting,” but the willingness of the agency to grant such a meeting will depend on the nature of the program (e.g., how unique or different is this program from other programs), the extent of prior guidance already provided to the sponsor, and agency workload to accommodate such a request.

Meetings should be requested in writing to the appropriate division director (i.e., for vaccines: Office of Vaccine Research and Review or Office of Cell, Tissue and Gene Therapies). Typically, the request can be submitted by fax. Prior to submission, the sponsor should contact the review division by telephone to determine to whom the fax should be directed and to arrange for confirmation of the receipt of the letter and to establish the number of copies of the background document to be provided.

17.3 REQUEST LETTER FOR A PRE-IND MEETING

For a pre-IND meeting the request letter should specify the following items [1]:

1. Name of the product.
2. Chemical name and structure. (For vaccines, a description of what the vaccine contains is often useful, for example: recombinant protein XYZ adsorbed onto adjuvant ABC.)
3. Proposed clinical indication(s). It is often appropriate to indicate the condition and whether the vaccine is intended to be for prevention, treatment, or both.
4. Type of meeting being requested (e.g., Type B for a pre-IND meeting).
5. Statement of the purpose of the meeting.
6. A statement of the desired outcome of the meeting.
7. A preliminary agenda, including estimated amounts of time for each agenda item. (Typically, a pre-IND meeting is granted for 60–90 min.)
8. A draft list of specific questions, grouped by discipline. These draft questions will enable the FDA to begin to plan for the meeting and determine which disciplines will need to be involved. The draft list of questions may be refined or expanded once the final questions are submitted in the background package but should be as comprehensive as possible at the time of the meeting request.
9. A list of individuals and their titles who will participate in the meeting from the sponsor. (This list may be revised with the final background package.)
10. A list of the agency staff that the sponsor would like to have attend. If the sponsor does not know the names of the specific reviewers, the sponsor can

simply specify the discipline (e.g., “a clinical reviewer”). For combination products (e.g., for vaccines that utilize a unique delivery device for administration), sponsors can request participation from other centers within the FDA as well (for the example given, the Center for Devices and Radiological Health, CDRH).

11. The date that the supporting information (“pre-IND meeting package”) will be provided. For a Type B meeting, the final supporting information needs to be submitted at least 4 weeks before the meeting or the agency may cancel the meeting.
12. Suggested dates and times for the meeting.

17.4 PRE-IND MEETING PACKAGE (BACKGROUND PACKAGE)

The pre-IND meeting package needs to be submitted to the agency at least 4 weeks before the scheduled meeting. Failure to submit the package may result in the agency’s cancellation of the meeting.

The elements of this package are the following, per the FDA’s guidelines [1]:

1. Product name.
2. Chemical name and structure.
3. Proposed indication(s).
4. Dosage form, route of administration, and dosing regimen (frequency and duration).
5. Brief statement of the purpose of the meeting.
6. A list of specific objectives/outcomes of the meeting.
7. Proposed agenda, estimated amount of time per agenda item, designated speaker(s).
8. List of specific questions grouped by discipline.
9. Clinical data summary. If data exist from other sponsors using the same vaccine and have been published in the literature, or if studies have been conducted by the sponsor outside the United States, the results should be summarized and referenced. A draft clinical protocol or outline of the proposed protocol to be conducted should be provided. A complete or final protocol generally need not be provided, but it must be understood that the ability of CBER to provide meaningful guidance and feedback on the proposed clinical study may be impacted by the degree of detail provided by the sponsor, at least to some extent.
10. Preclinical data summary. If any safety assessment (toxicology) studies conducted under good laboratory practices (GLP) have been conducted or are in progress, they should be described. Any non-GLP animal studies or in vitro laboratory studies that describe or define the potential activity, potency, or safety of the proposed vaccine candidate or that serve to demonstrate “nonclinical

“proof of concept” should also be included. Information should be provided about whether the same vaccine lot will be used for the toxicology studies and the clinical study. If they are not the same lot, information should be provided on any manufacturing, testing, or analytical differences between the toxicology and clinical batches, and justification should be provided on applicability of the toxicology studies with the vaccine lot used to the proposed human clinical trial with its intended clinical lot. The design of the toxicology study should support the intended human clinical exposure, as described further below.

11. Chemistry, manufacturing, and controls information. This section should outline the basic manufacturing process for the proposed vaccine, including critical in-process tests, as well as quality control tests to be conducted at key stages in the process as well as on the final product. The intended acceptance criteria (specifications) should be provided, if known. An outline of the stability plan for key intermediates and the final product should be provided.

If vaccine lots produced under good manufacturing practices (GMP) have been produced already, a certificate of analysis or a data table listing the tests performed and results obtained should be provided. If non-GLP research and development (R&D) batches have been produced to support proof of concept studies or pharmacology/toxicology studies, or if the R&D batches serve as pilot lots to guide future GMP manufacturing, testing results of these lots should also be provided. An explanation of any changes introduced or planned to be introduced with the production of further lots should be provided.

Special information needs to be provided for proteins produced using mammalian cell lines concerning plans for testing and characterization of cell banks, including adventitious agents testing and removal or inactivation of adventitious agents (see Guidance for Industry, Characterization and Qualification of Cell Substrates and other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Disease, September, 2006), and testing for potential immunogenicity of the product impurities [4]. Information should also be provided concerning the use of any materials from animal sources (i.e., consideration of adventitious agents and transmissible spongiform encephalopathy), for any novel excipients used, dosage forms, and novel drug delivery systems.

17.5 CONDUCT OF THE PRE-IND MEETING

The sponsor can request that the pre-IND meeting be held “in person” with CBER. Almost always, however, CBER will request that the meeting be held as a telephone conference call for efficiency. Data or topics not presented in the final background package generally will not be reviewed or discussed by CBER at the pre-IND meeting, unless specific agreement has been reached with CBER to do so prior to the meeting.

Prior to the meeting (typically the day before the meeting or the day of the meeting), CBER may fax the sponsor written responses to each of the questions posed and may provide additional guidance or comments on matters not specifically

identified by the sponsor that are of particular importance to the agency. CBER may ask the Sponsor to determine whether the provided responses are sufficiently clear that no teleconference is still needed, or whether some (or all) of the questions warrant discussion at the teleconference. The sponsor may elect to discuss some or all of the questions and responses with the agency to better understand the CBER's rationale and to provide the sponsor's perspective in the event of disagreement. Once conducted, the FDA will generally issue minutes within 30 days of the meeting. The sponsor can also submit minutes of the meeting to the FDA. These sponsor minutes can be useful in assuring that both the sponsor and the FDA have a common understanding of the outcome of the meeting. Any significant disagreements should be discussed with the FDA in a follow-up discussion.

17.6 FILING THE IND

The requirements for conducting a human clinical trial with an IND (which includes investigational vaccines) in the United States are specified in the Code of Federal Regulations (CFR). These requirements include the filing of an (IND) application as specified by 21 CFR 312, with the content and format of the IND described in detailed in 21 CFR 312.23. The FDA's primary objectives when reviewing an IND in all phases of study are to "assure the safety and rights of subjects" (21 CFR 312.22) and in later phases of development (i.e., in phases 2 and 3) to help assure the quality of the evaluation to permit determination of effectiveness and safety as well.

All vaccine INDs should be submitted in triplicate to:

Center for Biologics Evaluation and Research
HFM-99 Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

The IND should be addressed to the attention of the office director (Office of Vaccine Research and Review or Office of Cellular, Tissue and Gene Therapies).

A signed Investigational New Drug Application form (Form FDA 1571) must accompany *The IND Submission*. This form outlines the submission requirements of the IND. By signing, the sponsor or sponsor's authorized representative agrees not to begin clinical trials until 30 days after FDA's receipt of the IND unless other notification is received from the FDA that such studies may begin earlier; agrees not to begin or continue clinical studies if placed on clinical hold by the FDA; acknowledges that an Institutional Review Board (IRB) that complies with the requirements set forth in 21 CFR 56 will be responsible for all clinical studies conducted; and acknowledges that the investigation will be conducted in accordance with all other applicable regulatory requirements.

General guidance for completing the IND application form (Form FDA 1571), including a link to useful other references, is available online from the FDA at

<http://www.fda.gov/cber/ind/ind.htm>. Other guidance from CBER on INDs can be found at <http://www.fda.gov/cber/ind/indpubs.htm>.

The IND may be submitted in paper or electronic format. Guidance from CBER on submitting the IND in electronic format is available in *Guidance for Industry, Providing Regulatory Submissions to CBER in Electronic Format—Investigational New Drug Applications (INDs)* (March 2002) [5].

In either medium, the content of the IND is as follows (for reference, see 21 CFR 312.23(a) and Form FDA 1571):

1. Cover sheet (Form FDA 1571).
2. Table of contents.
3. Introductory statement.
4. General investigational plan.
5. Investigator's brochure.
6. Clinical study protocol(s), investigator data, (clinical) facilities data, and Institutional Review Board (IRB) data. The investigator data, facilities data, and IRB information are generally supplied by completing Form FDA 1572 Statement of Investigator and submitting the curriculum vitae (CVs) of the investigator and subinvestigators.
7. Chemistry, manufacturing, and control information and environmental assessment or claim for exclusion.
8. Pharmacology and toxicology data.
9. Previous human experience.
10. Additional information.

The general content and organization of each of the IND sections is described below.

Introductory Statement The introductory statement should provide a brief overview, giving the name of the vaccine and active ingredients (such as adjuvants), pharmacological class (e.g., “live attenuated vaccine”), composition and structure, dosage form, and intended route of administration.

17.7 GENERAL INVESTIGATIONAL PLAN

The general investigational plan (GIP) should provide the broad objectives and planned duration of the proposed clinical investigations to the extent this is known at the beginning of the program. The GIP generally does not need to be an extensive document but should lay out the intent of the proposed first-in-man (FIM) study(ies) and, if known, should provide some general information on the direction of future clinical development envisioned. If the vaccine has been studied outside the United States, information on previous human experience should be provided. If the sponsor has studied a highly related vaccine construct, or if a similar vaccine has been studied by others and reported in the literature or is otherwise known to the sponsor, this

information should also be summarized, especially as it relates to the safety of the proposed clinical trial. IND numbers for related vaccines submitted by the sponsor should be referenced. If the vaccine was withdrawn from clinical studies or from marketing for any reasons related to safety or effectiveness, this information should be disclosed and the reasons provided.

17.8 INVESTIGATOR'S BROCHURE

The investigator's brochure (IB), sometimes referred to as the clinical investigator's brochure (CIB), must be provided in the IND. According to 21 CFR 312.23(a), the IB should include: information about the active component [which would include adjuvant(s), if any] and formulation; a summary of pharmacological and toxicological effects in animals, and if known in humans; a summary of the pharmacokinetics (PK) and disposition in animals, and if known, in humans (see next paragraph); a summary of previous human experience; a description of possible risks or side effects anticipated based on existing experience and experience with related vaccines; and precautions or special monitoring to be undertaken as part of the planned investigation.

For vaccines, measurement of immune response(s) is often provided rather than PK as would be evaluated for a traditional small-molecule drug. Disposition is frequently not studied for a vaccine unless novel adjuvants or excipients are used, or unless DNA (deoxyribonucleic acid) or viral vectors are used, for which biodistribution and genetic integration studies may be needed.

Further guidance on the content and format of an IB is provided by the International Conference on Harmonization (ICH), Guideline E6, Good Clinical Practice: Consolidated Guideline (section 7) [6]. ICH is a tripartite harmonization initiative of the FDA, the European Union and Japanese regulatory authorities.

Clinical Study Protocol(s), Investigator Data, Facilities Data, and Institutional Review Board (IRB) Data Elements of the clinical protocol required for submission in the IND are provided in 21 CFR 312.23a(6). The CFR requirements for the protocol and information to be contained in this section of the IND include the following:

1. The purpose and objectives of the study.
2. Name, address, and statement of the qualifications of the investigator and name/address of each subinvestigator; name and address of the research facilities used; name and address of each reviewing IRB. This information is generally supplied to CBER in the IND with FDA Form 1572 and accompanied by the CVs of the investigators and subinvestigators.
3. Patient section criteria (inclusion/exclusion criteria) and estimate of the number of patients to be studied.
4. Study design, including control groups and methods to minimize bias on the part of subjects, investigators, analysts.
5. Methods for determining dose, planned maximum dose, duration of patient exposure.

6. Description of the observations and measurements to fulfill the objectives of the study.
7. Description of the clinical procedures, laboratory tests and other measures to minimize patient risk.

Guidance is also provided in ICH E6 (section 6), which provides greater detail and granularity on the required elements of the clinical protocol [6].

While not specified in the CFR, many sponsors provide the sample Informed Consent Form that will be provided to the IRBs in this section of the IND for review by CBER. This document may be requested by CBER if not supplied in the submitted IND.

CBER has recently published a Guidance for Industry, Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials (September 2007) as a means of standardizing the assessment of the severity of clinical and laboratory adverse events in vaccine clinical trials [7]. This document should be considered carefully in the preparation of the clinical protocol. Any planned deviation from the guidance should be discussed with CBER during the pre-IND meeting or through other communications.

17.9 CHEMISTRY, MANUFACTURING, AND CONTROL (CMC) INFORMATION

The FDA acknowledges that the amount of information and detail in the CMC section of the IND necessary to permit adequate assessment of the “quality, purity and strength of the investigational drug” varies according to the phase of development [21 CFR 312.23(a)(7)(i)]. In assessing the amount of information and detail required for a Phase I IND, FDA places an emphasis on assuring patient safety and considers factors including “novelty of the drug, the extent to which it has been studied previously, the known or suspected risks” [21 CFR 312.22(b)].

Per 21 CFR 312.23(a)(7)(iv), the CMC section of the IND is to contain:

1. Drug substance description. This section should include a description of the physical, chemical, or biological characteristics of the vaccine, the name and address of the manufacturer, the method of preparation, acceptable limits, and analytical methods to assure identity, strength, quality, and purity of the drug substance, and information to assure stability during the toxicology studies and planned human clinical trials. References to the United States Pharmacopoeia-National Formulary (USP-NF) (for raw materials and excipients) should be given when materials used comply with these standards. While not specified in the CFR, generally flowcharts and tables are useful in this section of the IND to show the manufacturing process (production of the antigen or other components through fermentation, cell culture or other means, purification processes, etc.), acceptable ranges, and points at which samples are removed for quality control (QC) testing and in-process evaluation. Tables showing the intended stability program, indicating the analytical methods, specifications or expected

values, and time points are also useful here. As appropriate to the expression and production systems employed, control of critical raw materials, cell bank characterization, and viral safety assessment will be critical major features of this section of the IND [see ICH Guidances below], as these aspects of production can directly affect the quality and safety of the investigational vaccine.

2. Drug product description, with a list of all components used including back-up sources/suppliers and in-process materials and excipients, quantitative composition of the final drug product including expected ranges, name, and address of the drug product manufacturer, description of the manufacturing and packaging operations, analytical methods and acceptable limits to assure identity, strength quality, and purity of the drug product. References to the USP-NF (for raw materials, etc.) should be given when materials used comply with these standards. As before, flowcharts and tables may be useful in presenting the information required in this section. Detail on sterilization or aseptic processing of the vaccine and sterilization of packaging components, and on analytical testing including stability assessment are appropriate, as these aspects can directly affect the quality and safety of the investigational vaccine.

3. Placebo description, including the composition, manufacture, and control (tests, specifications, etc.) for any placebo used in the clinical trial.

4. Labeling of the investigational vaccine, including a copy of the vial labels and labeling provided to all investigators. FDA's use of the word "labeling" extends beyond the actual "vial or box labels" to include printed documents such as the prescribing information ("package insert") for an approved vaccine, and in the case of an investigational vaccine, the investigator's brochure [described above]. The requirements for the vial label (or other immediate package label) for an investigation product are described in 21 CFR 312.6 and must include the statement, "Caution: New Drug—Limited by Federal (or United States) law to investigational use."

5. Environmental assessment or claim for categorical exemption under 21 CFR 25.30 or 21 CFR 25.31. IND applications are generally exempt from the requirement of an environmental assessment under the President's Reinventing Government (REGO) initiatives of 1995. The sponsor needs to state that the application is exempt from environmental assessment as an IND application and that no extraordinary circumstances exist, as described in FDA's Guidance for Industry, Environmental Assessment of Human Drug and Biologics Applications (July 1998) [8].

In addition to general information concerning CMC considerations for development of investigational products available from ICH presented in the "Quality" category, more specific guidances related to biotechnology-derived products have also been published by ICH:

ICH Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origins [9]

- ICH Q5B: Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products [10]
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biotechnology Products [11]
- ICH Q5D: Derivation and Characterization of Cell Substrates Used for the Production of Biotechnological/Biological Products [12]
- ICH Q5E: Comparability of Biotechnological/Biological Products Subject to Changes in the Manufacturing Process [13]

As previously indicated, these features of biotechnology products are critical to assuring the quality and safety of cell-culture-based investigational vaccines and will be a major focus on CBER's review of the CMC section of the IND.

17.10 PHARMACOLOGY AND TOXICOLOGY INFORMATION

Pharmacology and toxicology testing for vaccines frequently differs from the standard test batteries performed for typical small-molecule pharmaceutical drugs due to the biological composition of the vaccine components (typically protein, polysaccharide, and/or nucleic acid) and the known metabolic fate of these classes of molecules. Synthetic adjuvant compounds, however, may require more standard assessment of toxicity, metabolism, and elimination. The fate (biodistribution, integration) of “gene-based vaccines” such as plasmids and viral vectors will also require special consideration and potential study in pharmacology/toxicology assessment.

The World Health Organization (WHO) Guidance on Nonclinical Evaluation of Vaccines (November 2003) serves as global guide to harmonize nonclinical safety evaluation for preventative infectious disease vaccines [14] and is generally supported by CBER [15].

In general, for vaccine toxicology studies, a single relevant animal species is considered sufficient for IND studies. The investigational vaccine should be immunogenic in the animal species proposed, and, optimally, any adjuvant in the vaccine formulation will also exert an adjuvant effect in the animal species as well, to permit detection of any immunologically mediated toxicity and to permit assessment of the extent and quality of the immune response. Also ideally, the animal model for toxicology studies will also be permissive to the pathogen that is targeted (or other disease state for therapeutic vaccines). Both local and systemic effects should be evaluated [15].

Dosing of the test vaccine in animals during toxicology testing is generally expected to include a full human dose, unless it can be demonstrated that this is not reasonably feasible. The number of doses administered is generally “ $N + 1$ ” (i.e., the number of doses intended to be administered to humans plus 1) for infectious disease vaccine reviewed within office of Vaccine Research and Review, while for therapeutic vaccines reviewed within the Office of Cellular, Tissue and Gene Therapies, the number of doses administered only needs to match the number intended to be administered in humans, as the benefit–risk balance is generally viewed to differ in the two settings. Generally, the duration between doses does not need to mimic

the intended clinical application; however, adequate time to permit an immunological response to develop is necessary. The route of administration should generally match the intended clinical application, and any differences in production method or analytical results between the article used in the toxicology studies and the intended clinical study should be justified [15].

CBER has issued Guidance for Considerations for Reproductive Toxicity Studies for Preventive Vaccines for Infectious Disease Indications (August 2000) for vaccines indicated for females of childbearing potential and pregnant individuals [16]. The required timing for conducting reproductive toxicity studies during clinical development is described. Data from reproductive studies for vaccines specifically developed for immunization of pregnant women must be submitted to the IND prior to enrollment of pregnant women in clinical trials. For vaccines indicated for women of childbearing potential, such women may be included in clinical trials prior to the conduct of reproductive toxicity studies provided that appropriate precautions are taken, including pregnancy testing and birth control. The results of the reproductive toxicity studies must be included in the Biologics License Application (BLA) if not submitted earlier.

Previous Human Experience Detailed information of any previous human experience of the vaccine known to the sponsor should be provided relating to safety or effectiveness. The information should include any investigations conducted outside the United States or any marketing experience from outside the United States. Any published material should be provided. A list of countries where the vaccine has either been marketed or withdrawn should be provided.

17.11 OTHER INFORMATION

A new requirement for Certification of Compliance with the requirements of the ClinicalTrials.gov Data Bank has recently been issued and can be satisfied by completion of Form 3674 available on the CBER web page (<http://www.fda.gov/cber/ind/ind.htm>). The ClinicalTrials.gov database is intended to capture information on clinical trials with efficacy endpoints to promote public awareness and to facilitate public access to clinical trials. Phase I trials generally do not have efficacy endpoints and are not large enough to permit demonstration of efficacy. However, completion of Form 3674 is still necessary with submission of the initial IND.

For gene-based therapeutic vaccines regulated by the CBER Office of Cellular, Tissue and Gene Therapies, responses to questions posed in a March 6, 2000, letter to IND holders is requested by the agency and should be included in the IND filing. The questions are posted on the FDA web page (<http://www.fda.gov/CBER/ltr/gt030600.htm>).

17.12 AFTER FILING THE IND

Once an IND has been filed, the sponsor may begin clinical studies 30 days after the FDA's receipt of the document unless the FDA informs the sponsor that the trial is

being placed on clinical hold and may not proceed. Clinical holds are described in 21 CFR 312.42. Reasons that clinical holds may be placed on a phase I trial prior to or after initiation of clinical studies, per regulations, include: unreasonable and significant risk of illness or injury; the clinical investigators named in the IND are not qualified; the investigator brochure is misleading, erroneous, or incomplete; the IND does not contain sufficient information to assess risks to subjects; and inappropriate gender exclusion. An IND study at any stage may be placed on clinical hold by the FDA if it is not designed to be adequate and well controlled. The FDA will attempt to resolve deficiencies associated with the initial IND filing, if possible, prior to the conclusion of the 30-day review period, typically by contacting the sponsor by telephone. Once a study has been placed on clinical hold, the study may only initiate when the FDA has notified the sponsor that the study may begin. The FDA will notify the sponsor in writing of the reasons for placing the trial on clinical hold within 30 days of placing the hold order. Meetings with the FDA may be held to resolve the clinical hold, as per FDA's Guidance for Industry, Formal Meetings with Sponsors and Applicants for PDUFA Products (February 2000) [1].

Once the IND is in place, additional documentation is required to be submitted to CBER periodically. These updates include: protocol amendments (21 CFR 312.30), information amendments (21 CFR 312.31), IND safety reports (312.32), and annual reports (21 CFR 312.33). All documentation submitted to CBER regarding an IND must be accompanied by Form FDA 1571 and be sequentially numbered. These submissions are required to maintain the IND in active status and serve to keep the FDA informed of the progress of the clinical evaluation and of any serious and unexpected safety events, as well as keeping the FDA informed of changes to the plans submitted in the original IND filing.

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