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HISTORY OF VACCINE PROCESS DEVELOPMENT

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1.1 INTRODUCTION

The goal of vaccine process development is to develop a manufacturing process that can consistently produce a vaccine that is safe and efficacious. During vaccine discovery, the etiologic agent is identified, the immunogen, adjuvant (if applicable), and administration regimens are developed in animal models such that the vaccine candidate produces a prophylactic immune response that is safe and effective. A requirement of the manufacturing process is to preserve the immunological properties innate to the molecular/biological architecture defined in vaccine discovery and enable production of the vaccine in increasingly larger quantities for use in human clinical studies and later commercial supplies. These activities of vaccine discovery and process development must be well integrated, require collaborative efforts and iterative refinements. The safety and efficacy of the vaccine gets proven though phases of clinical studies with increasing number of subjects. The final process developed and used to produce the vaccine for pivotal clinical trials becomes the manufacturing process which is licensed by regulatory authorities for full-scale production to supply the market.

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Unlike for many other pharmaceutical drugs, the manufacturing process used to produce the vaccine is still frequently tied to the definition of the product. While many modern vaccines are highly purified biomolecules, others are complex preparations, such as live viral vaccines or multivalent conjugate vaccines, consisting of the antigen, trace levels of cellular and process residuals, excipients, as well as adjuvants. For some types of vaccines the "product-is-the-process" interdependence can be greatly alleviated by modern process and analytical technology. This approach is built upon much greater scientific understanding of the process and product characteristics which allows greater process control and performance.

Generally speaking, the immunogen is generated via a cultivation process (also referred to as *the upstream process*) and is characterized by an appropriate choice of cell substrate, growth media and a fermentation or cell culture conditions that reproducibly produce the antigen in large quantities (note that in the rest of the chapter, the word *antigen* is used as a synonym for immunogen). The vaccine purification process (also referred to as *the downstream process*) maybe designed to remove host cell impurities, as well as process additives and yields a bulk vaccine (drug substance). The bulk vaccine is converted into a final vaccine product (drug product) in the formulation, fill, and finish processes. Through this stage, the vaccine is formulated into a final composition that imparts long-term stability, whether in liquid or lyophilized form, and then presented in an appropriate final container, such as vials or prefilled syringes. An adjuvant may or may not be used as part of the drug product depending on the type of the vaccine.

This chapter outlines the history of vaccine manufacturing from a bioprocess development perspective. With the maturation of the biotechnology industry, vaccine manufacturing has evolved significantly over the years. An understanding of the evolution of vaccine manufacturing processes can be instructive in the development of future generations of vaccines. There have been previous reviews on various aspects of vaccine bioprocessing. An excellent review on viral vaccine production is presented by Aunins (2000, 2009). Other viral vaccine production reviews have been written by Shevitz et al. (1990), Ellis (2001), Bailey (2007), and Genzel and Reichl (2007a; 2007b), which also include production of viral vectors. Bacterial vaccine production has been reviewed by Liljeqvist and Stahl (1999a; 1999b) and Ellis (2001). Broader reviews of vaccine bioprocessing include those by Aunins et al., (2010), Dekleva (1999a; 1999b), and Josefsberg and Buckland (2012). This chapter encompasses a broad range of vaccine bioprocesses including whole-viral and -bacterial vaccines, as well as subunit and conjugate vaccines. This chapter is focused on drug substance processes; the area of drug product manufacturing processes, including the topic of adjuvants, is quite rich in its own right, but is beyond the scope of this chapter.

1.2 VACCINES BIOPROCESS EVOLUTION

Vaccines and vaccine candidates have been directed at infectious (bacteria, viruses, and fungi), parasitic, and non-infectious diseases, such as cancer and Alzheimer's

disease. They can be largely classified as either live, attenuated, inactivated (or killed), or subunit. Production can be in the native organism or in a heterologous host. Recombinant vaccines have been in the form of protein subunit vaccines and modern live viral vaccines (Nkolola and Hanke, 2004; Polo and Dubensky, 2002; Ellis; 2003). Genetic and peptide vaccines are some other categories, which could be considered subcategories of the aforementioned broad categories. Genetic vaccines are those where the immunogen is delivered in the form of a gene via a naked DNA or a viral vector. The evolution in vaccinology has taken vaccines from complex preparation of undefined contents to whole organisms to highly purified whole organisms and subunit components. This evolution of vaccines is directly related to the development of bioprocess technologies. At the outset, identification of the etiologic agent requires bioprocessing, albeit at a much smaller scale and without the worry of scalability or manufacturability. Although the long-held goal of vaccine innovation would not include any whole organisms (live, attenuated, or inactivated) it is not currently possible nor necessary, particularly in the case of some viral vaccines. Further simplification via reverse vaccinology (Hilleman, 2002; Rappuoli, 2007) leading to genetic or peptide-based vaccines is certainly an attractive goal from the bioprocess technology perspective. If these approaches can be established, they will represent a powerful step change not only in vaccinology, but also in the ease of vaccine bioprocessing because they can consistently be based on well-defined platform technologies.

Although contemporary vaccine history is known to start with Edward Jenner, who developed the small pox vaccine in 1796 using the pus of patients with cowpox with a predecessor, variolation, was known to have been practiced much earlier in China, India, Turkey, Persia, and Africa (Behbehani, 1893). Nevertheless, Jenner is credited for initiating this safer approach in vaccine development, as well as coining the term *vaccine* from *vacca* (Latin for cow). This early history has been reviewed quite extensively (e.g., Galambos, 1999; Hilleman, 2000; Plotkin and Plotkin, 1999; Lederberg, 2000; Plotkin, 2009). Since the days of Jenner, vaccinology has proven to be a tremendous benefit to all mankind. In particular, vaccines have had a significant impact on increasing life expectancy since dawn of the twentieth century. With modern molecular techniques, the molecular architecture of the etiologic agent and the antigen continues to be better defined, and consequently, vaccine manufacturing processes are more capable of producing better defined antigens, in many cases rivaling the production of therapeutic proteins where the concept of a "well-characterized" biologic is well established.

1.3 LIVE ATTENUATED AND INACTIVATED VIRUS VACCINES

The earliest vaccines were live attenuated organisms – e.g. smallpox vaccine by Jenner and rabies vaccine by Pasteur. Live vaccines have complex upstream cultivation processes and undergo minimal downstream processing. Because they are live, the degree of attenuation and genetic stability is particularly important, as it relates to the reversal of virulence. Furthermore, the choice of the host can have an impact on vaccine safety and reactogenicity because of potential host cell residuals, growth

media components, as well as the potential for adventitious agents. The production system, in the case of Jenner's smallpox vaccine was patients with cowpox. Pasteur used rabbits as the bioreactor to produce the immunogen for the rabies vaccine. This type of *in vivo* production is still in use for the production of a Japanese encephalitis (JE) vaccine. JE-VAX®, licensed in 1954 in Japan and in 1992 in the United States, was derived from mouse brain (in this case, the vaccine is administered as an inactivated virus) and was being supplied in the United States until it was discontinued in 2007. Other mouse-brain-derived JE vaccines are still being manufactured in South Korea, Taiwan, Thailand, and Vietnam but are slowly being replaced by cell-culture derived vaccines. (www.path.org, JE vaccines at a glance; Zanin et al., 2003). *In vivo* production continues to be widely used in veterinary vaccines (Aunins, 2000).

In 1931, Ernest Goodpasture discovered that a hen's egg was an ideal sterile production system for fowl pox virus, and a whole new, enduring bioreactor system was born (Woodruff and Goodpasture, 1931). This led to the licensure of the first influenza vaccine in 1945 (Salk and Francis, 1946). Also, in the 1930s, a yellow fever vaccine based on the 17D strain was developed (Vainio and Cutts, 1998). *In ovo* production is still widely practiced today for the production of both of these vaccines. Scale-up of vaccine production is accomplished by scale-out, that is, simply increasing the number of eggs used with automation to facilitate processing (Hickling and D'Hondt, 2006). At 1–3 flu vaccine doses yielded per egg (Blyden and Watler, 2010), millions of eggs need to be processed each year. *In vivo* or *in ovo* cultivation of viruses is not optimally suited for the industrialization of vaccine production (although it has been practiced for a very long time owing to the slow implementation of alternatives; to be discussed in the following sections) because of the need for sufficient quantities of controlled live animals (e.g., pathogen-free) at the very outset, difficulties of process control during production and inability to scale-up, as well as the very long cycle time for vaccine production.

A major breakthrough in the use of *in vitro* cultivation was the success in propagation of polio virus by Enders (Enders et al., 1949) in primary cells. The cell substrate used was non-neural human cells. Soon thereafter, the inactivated polio Salk vaccine was licensed and produced in primary monkey kidney cells related to the now widely used Vero continuous cell line (Barrett et al., 2009). The significance of this breakthrough is still playing out, with viral vaccines previously prepared in animals or eggs being transitioned to more industrially suitable cell-culture-based production systems. The advantages of this transition are many, including ease of scale-up, rapid response to potential pandemics, as well as a way to address the problem of egg-related allergies. In addition, cell culture allows for the direct monitoring and control of the cell and virus culturing processes. The choice of cell substrates is one of the most critical factors in the manufacture of viral vaccines. This is a topic that has garnered a lot of attention in the literature (Hayflick, 2001; Aunins, 2009; Lubiniecki and Petricciani, 2001; Petricciani and Sheets, 2008) and is of continued interest. Workshops held by the World Health Organization (http://www.who.int) and the International Association of Biological Standardization (http://www.iabs.org) continue to advance the field, particularly in the establishment of standards.

This transition from *in vivo* to *in vitro* production came about with the use of primary cells, where cells from specific organ of an animal was used for virus propagation. Primary monkey kidney cells were used by polio vaccine manufacturers but later discontinued in the United States and Europe. Vaccines still produced in primary cells include measles, mumps, and rabies (e.g., chick embryo fibroblasts for M-M-R® II and RabAvert®). However, they suffer from many of the same drawbacks as *in vivo* and *in ovo* productions, such as the need to maintain captive herds (Aunins, 2000). Hence, the field transitioned to the use of human diploid cells. There are two popular cell lines—WI-38 developed in the Wistar Institute in the United States and subsequently MRC-5 in the United Kingdom, both derived from human lung cell from distinct sources. Cell banking was initiated with this transition and is now a standard feature of any biomanufacturing activity. Cell banking allows the use of a consistent, stable, and well-tested substrate for each batch of vaccine production. These cells are used for the production of rubella, varicella, hepatitis A, polio, and rabies (e.g., WI-38 for rubella in M-M-R II, MRC-5 for Varivax®, Vaqta®, Havrix®, Imovax®, Poliovax®; FRhL-2 for Rotashield®, a rotavirus vaccine from Wyeth, now withdrawn, after reports of intussusception).

Human diploid cell lines have had a long and excellent safety record. However, they do suffer from the limitation of senescence, the need for surface adherence and requirement for bovine serum during cell culture. Senescence is the ceasing of cell division after a certain number of cell divisions and is characterized by the Hayflick limit (Hayflick and Moorhead, 1961). Continuous cell lines overcome many of the disadvantages presented by human diploid cells. Being theoretically immortal, they differ from human diploid cell lines primarily in their capacity to replicate. Prominent examples include Vero cells from African Green Monkey and Madin-Darby canine kidney (MDCK) cells. The use of continuous cell lines drove the need for an explicit purification target on the final product residual DNA level to address potential safety concerns related to oncogenicity of the host cell DNA or infectivity by adventitious agent(s). This level has evolved over time as the assessment of oncogenicity and adventitious agent risk has been better understood and is now recommended to be less than 10 ng host cell DNA/dose (WHO, 2010). The exact level may need to be adjusted if cells with specific concerns (e.g., tumorigenic phenotype) are used. In addition, mitigation of the aforementioned risks also calls for reducing the length of DNA to less than that of a functional gene, which is thought to be approximately 200 base pairs (FDA, 2010). Continuous cell lines are used for flu, JE, rotavirus, and polio vaccines (e.g., Vero for Ixiaro®, Imojev®, Ipol® and Rotateq®, Rotarix®; MDCK for Optaflu®, Celtura®, Flucelvax®).

The aforementioned cell lines require adherent surfaces for growth. As such, bioreactor systems such as roller bottles, T-flasks, cell cubes, and cell factories are widely used for the production of vaccines at the industrial scale. However, these suffer from many limitations driven primarily by the need to scale-up by increasing the surface area (Ozturk, 2006). The use of microcarriers provides a more scalable solution for vaccine production in attachment-dependent cell lines. This technology is employed for the production of polio and rabies vaccines, primarily with Vero cells. Vero cells have also been adapted to serum-free media (e.g., Gould et al., 1999; Butler et al.,

2000; Merten, 2002; Rourou et al., 2007; Bergener and Butler, 2006; Rourou et al., 2009; Toriniwa and Komiya, 2008; Chen and Chen, 2009) and ultimately for suspension (Paillet et al., 2009). Scalable microcarrier technology is now the convention for newer vaccines in development using adherent cell lines such as the one for chikungunya (Tiwari et al., 2009). Similar advances have been made with MDCK cells (Genzel et al., 2006a; Genzel et al., 2006b; Chu et al., 2009). Corresponding changes to downstream processing have also been made to match the advances to upstream processing, such as the use of more conventional harvest technologies along with column chromatography to achieve a high level of purification (Wolff and Reichl, 2008).

The evolution in the commercial production for flu and JE vaccines nicely illustrates the advances in upstream bioprocess technology for viral vaccines. Starting with *in vivo* or *in ovo* production, these vaccines have transitioned to continuous cell lines in adherent culture and finally to fully suspension cultures with the recent licensure of Optaflu®, Celtura®, and Flucelvax® in MDCK cells (Genzel and Reichl, 2007a; Genzel and Reichl, 2007b; Doroshenko and Halperin, 2009; Genzel and Reichl, 2009). A cell-culture-based JE vaccine is now approved (Ixiaro®) in the United States as is a serum-free cell-culture-based JE vaccine (Imojev®) in Thailand and Australia (Zanin et al., 2003). The scale of flu vaccine manufacture has reached multiple thousands of liters. As examples, Baxter has a 6000 l facility in the Czech Republic for production of flu vaccine in Vero cells on microcarriers (capacity of 20 MM doses/year) (Kistner, 2005), Novartis has a 2500 l scale facility in Marburg, Germany for flu in MDCK cells in suspension, and a 5000 l scale facility (reported to produce 150 MM doses/year) in Holly Springs, United States. The scale of these operations rival those used for animal viral vaccine production. A recent review on the scale-up of viral vaccine production has been published (Whitford and Fairbank, 2011).

Recombinant or designer cell lines such as PER.C6®, AGE1.CR®, CAP®, and EB66® are now being used in the development of various vaccines (Fallaux et al., 1998; Altaras et al., 2005; Jordan et al., 2009; Olivier et al., 2010; Tintrup, 2011). In these cases, normal human or animal cells are transformed by viral or cellular oncogenes or by immortalizing cellular genes, to render them practically immortal. These cell lines are also amenable to serum-free suspension cell culture. No vaccine has been licensed to date in these cell lines.

1.4 LIVE OR WHOLE-KILLED BACTERIAL VACCINES

Although currently less prevalent compared to the live viral vaccines, there is still a rich history of live bacterial vaccines, and it may be experiencing renewed interest (Lindberg, 1995, Detmer and Glenting, 2006). The use of live attenuated bacteria started with Louis Pasteur's discovery of attenuation and immunogenicity of a chicken cholera culture in 1879 (Plotkin and Plotkin, 1999). The Bacille Calmette-Guerin (BCG) vaccine for tuberculosis, as well as vaccines for typhoid and plague, were developed around the same time (Plotkin and Plotkin, 1999). Live and

whole-killed bacteria have comparatively the simplest manufacturing bioprocesses of all vaccines. Production generally involves cultivation of the bacteria, harvesting, and then inactivation if applicable, followed sometimes by lyophilization. Akin to cell culture technology, microbial fermentation technology has evolved significantly over time. Microbial cultivation technology has evolved from static cultures using complex medium with animal-derived components in bottles to the contemporary use of fed-batch fermentation with chemically defined medium in stirred tank fermentors using aeration for aerobic cultures with state-of-the-art process monitoring and control (Aunins et al., 2010). The literature is rich – with historical aspects (Scott, 2004; El-Mansi et al., 2007; Junker, 2005; Shiloach and Fass, 2005) as well as advances in microbial fermentation technology (Junker, 2004; Choi et al., 2005; Schmidt, 2005; Hewitt and Nienow, 2007).

Live bacterial vaccines are of special significance in bacterial enteric diseases, where the live bacteria mimic the route of infection and provide immunity. Cholera and typhoid fever caused by enteric pathogens *Vibrio cholerae* and *Salmonella typhi*, respectively, are key examples (Dietrich et al., 2008). Cholera vaccines have been produced in either live or whole-killed forms. Oral live cholera vaccines contain the *V. cholerae* bacteria attenuated by removal of genes encoding subunits of the cholera enterotoxin (Chaudhuri and Chatterjee, 2009). Dukoral® made by Crucell in the SBL Vaccin AB facility in Solna, Sweden, is a multivalent oral cholera vaccine consisting of three different strains of *V. cholera*. The bacteria are inactivated by heat and/or formalin treatment, and then combined with a recombinantly produced and purified cholera toxin B (which is really a strain of *V. cholerae* lacking the toxin A gene). The production of this vaccine is by traditional microbial fermentation in a stirred tank (EMEA, 2005) and leverages the advances made in classical fermentation processes, such as those for *Escherichia coli* (de Mare et al., 2003). Shanchol™ from Shanta Biotechnics (now a division of Sanofi-Pasteur) is another vaccine for cholera, and Crucell's Vivotif® is an oral, live bacterial vaccine for typhoid. The bacteria are grown using standard fermentation technology. They are then lyophilized and formulated into a solid dosage form to resist the low pH environment in the stomach. The bioprocessing for the manufacture of BCG vaccine is also quite simple and involves propagation of the chosen strain, and then harvest, which is followed by formulation for lyophilization. In contrast to the vaccines previously mentioned, BCG is administered intradermally. Advances in molecular biology have enabled the development of nonvirulent and nonreverting strains of these and other pathogenic bacteria as delivery vehicles of heterologous antigens, although the bioprocess aspects are not much different than these two traditional vaccines.

An important parenteral whole-bacterial vaccine is the one against pertussis. *Bordetella pertussis* is a small gram-negative bacterium that causes whooping cough. The vaccine, first licensed in 1918, was a suspension of whole-killed bacteria. The fermentation culture is harvested, inactivated by heat, and the suspension is formulated with formaldehyde (Aunins et al., 2010; Plotkin and Ornstein, 1999). The whole-cell pertussis vaccine was combined with the subunit vaccines for diphtheria and tetanus in the combination vaccine – DTwP. The whole-cell

pertussis vaccine was eventually replaced in the 1990s with the acellular subunit vaccine in many developed countries to address reactogenicity of the whole-cell vaccine (more on acellular subunit vaccine to be discussed in the following section). The production for both vaccines has now reached large industrial scale fermentors up to 15,000 l (FDA, 1992; Njamkepo et al., 2002). In addition, there have been significant advances in the understanding and control of the toxin production during fermentation (Bogdan et al., 2001; van de Waterbeemd et al., 2009; Streefland et al., 2009). Techniques for enhanced process control such as process analytical technology (PAT) and Quality by Design (QbD) (Streefland et al., 2007; Streefland et al., 2009) are now employed. PAT is "*a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality*," as defined by the FDA (http://www.fda.gov). This is now employed across most modern biomanufacturing operations.

It is important to mention a few words on lyophilization, particularly within the realm of live vaccines. Lyophilization was introduced as a process step as early as in the 1930s and represented a true bioprocess advance (Adams, 2003) enabling the delivery of live bacteria and viruses in a stable form for administration. Lyophilization also allows some vaccines such as typhoid vaccine to be delivered in a solid dosage form. Lyophilization needs to be carefully considered, since it can cause loss of potency during processing and in extreme cases, can render the vaccine ineffective (Levine et al, 1976). This technology continues to be a key focus of process development for the enhancement of thermostability of vaccines (Rexroad et al., 2002a; Rexroad et al., 2002b).

1.5 CLASSICAL SUBUNIT VACCINES

Classical subunit vaccines are a natural evolution to the killed bacterial vaccines analogous to the pertussis vaccine described previously. Diphtheria and tetanus vaccines were the first subunit vaccines to be developed in the 1930s (Plotkin and Plotkin, 1999). The diphtheria toxin is also the first protein purified for active immunization. The toxin is produced by the bacterium that causes the disease *Corynebacterium diphtheriae*. The early fermentations were performed using a beef digest medium and the toxin was secreted into the medium. The medium was harvested via centrifugation and/or filtration, and the toxin was inactivated using formaldehyde (Rappouli and Pizza, 1989). The toxoiding process, namely the conversion of the toxin into an inactive vaccine antigen using formalin was a result of a serendipitous bioprocess experiment (Glenny and Hopkins, 1923) where residual formalin in "cleaned" containers was found to have weakened the toxin. Contemporary processes for the production of diphtheria vaccine include more extensive protein purification steps such as ammonium sulfate precipitation and chromatography and membrane filtration to isolate the toxin from cellular impurities (Rappouli and Pizza, 1989; Relyveld et al., 1998). The fermentation process has also evolved in a manner similar to that described

previously for pertussis. Beef digest medium has been replaced by a semisynthetic medium made up of an alternate protein source, such as casein (Tchorbanov, et al., 2004; WHO, 2012).

Analogous to diphtheria and pertussis, the tetanus vaccine is produced by the fermentation of *Clostridium tetani*. Its process evolution is also similar where early fermentations were performed with media containing animal components such as brain–heart infusion broth, followed by a shift to dairy sources such as casein digest. More recently, the complete removal of animal-derived components has been demonstrated (Demain et al., 2005; Fratelli et al., 2005; Demain et al., 2007). Similar to diphtheria toxin purification, tetanus toxoid purification has advanced from employing precipitation and low resolution chromatography (Surian and Richter, 1954; Schwick et al., 1967) to using modern tangential flow membranes, facilitating large scale manufacturing (Vancetto et al., 1997; Ravetkar et al., 2001).

Besides the diphtheria and tetanus protein vaccines, another important class of subunit vaccines is the capsular polysaccharide vaccines. For example, an alternative to the live oral typhoid vaccine is a capsular polysaccharide subunit vaccine, Typhim Vi®. This involves a lot more bioprocessing, including purification of the bacterial capsular polysaccharide via precipitation using CTAB (Sanofi-Pasteur, 2005). The most prominent vaccines in this class are the pneumococcal and meningococcal polysaccharide vaccines. The pneumococcal polysaccharide vaccine, was first licensed as a 14-valent vaccine in the 1970s but then grew to a – 23-valent vaccine in the 1980s to form the broadest valency vaccine to-date (e.g., Pneumovax®23). The fermentation processes used for these capsular polysaccharide vaccine have evolved similarly to the processes mentioned previously – transitioning away from animal-derived components. Furthermore, as with the aforementioned microbial fermentations, the application of fermentation process design principles evolved significantly (Baart et al., 2007). Fermentation processes for these pathogenic microorganisms are generally of the batch type owing to the desire to minimize intrusions into the fermentor, and are conducted in specially designed facilities to address biocontainment. The biodefense vaccine for anthrax such as BioThrax® also falls in this classical subunit vaccines category, where the strain of *Bacillus anthracis* used is avirulent and nonencapsulated (FDA, 2012).

All of these subunit vaccines undergo downstream processing designed to remove cellular components and process residuals. The extent of purification depends on the particular vaccine, although most modern vaccines are designed to be highly purified. Downstream processing is discussed further below.

Most inactivated and subunit vaccines are formulated with adjuvants to enhance the immunogenicity of the vaccines either in magnitude of the immune response or in its persistence. The field of adjuvants is quite vast and is getting more attention with the recent advances in the understanding of innate immunity. Vaccines that contain adjuvants make the manufacturing process more complex. Although adjuvants such as aluminum salts were relegated to being simply an added excipient in the early days of their use, greater understanding of the physicochemical properties of the adjuvant (Hem and HogenEsch, 2007) and the impact of the interactions between the antigen and the adjuvant on immunogenicity is emerging. For example, the strength of

adsorption of hepatitis B surface antigen on aluminum adjuvant has an impact on the immune response (Hansen et al., 2009). As stated previously, the area of adjuvants in a rich one and a more in-depth discussion is beyond the scope of this chapter.

1.6 RECOMBINANT SUBUNIT VACCINES

The classical examples described in the previous section, where the cell substrate is a strain of the microorganism that is closely related to the one that causes disease, constitute the vast majority of "cell substrates" used for producing subunit vaccines. Recombivax HB®, a hepatitis B vaccine, was the first recombinant vaccine for human use licensed in the United States in 1986. Cloning of the hepatitis B surface antigen into Saccharomyces cerevisia commenced the use of rDNA technology in the vaccine industry and has been followed by significant activity in heterologous production of vaccine antigens over the last 30 years (Burnette, 1991; Burnette, 1992). Many reviews on the production of recombinant subunit vaccines exist (e.g., Liljeqvist and Stahl, 1999; Hansson et al., 2000; Clark and Cassidy-Hanley, 2005; Soler and Houdebine, 2007). Furthermore, owing to the significant growth in the therapeutic protein and monoclonal antibody industry, there have been significant technological advancements in the production of recombinant proteins that extend to vaccine antigen production. These advances have been cataloged extensively for *E. coli* (Chou 2007, Ni and Chen, 2009, Kolai et al., 2009, de Marco, 2009), CHO (Butler, 2005, Butler, 2006, Butler, 2007, Jayapal et al., 2007, Kwaks and Otte, 2006, Durocher and Butler, 2009, Grillberger et al., 2009, Kantardjieff et al., 2019), and yeast (Galao et al., 2007, Hamilton and Gerngross, 2007, Marasugi, 2008, Takegawa et al., 2009, Curran and Bugeja, 2009, Graf et al., 2009, Potgieter et al., 2009).

Needless to say, the etiology of disease, specifically the correct identification and cloning of a protective immunogen, is critical to such an approach. At the same time, the pitfalls of pushing the envelope of reductionism are noted (Van Regenmortel, 2001). Generally, antigen presentation – either in the form of virus-like particles (VLPs) (in the case of viral subunit vaccines) and/or with the use of adjuvants – is an important attribute owing to the lower inherent immunogenicity of these kinds of vaccines relative to their classical counterparts (Perrie et al., 2008; Reed et al., 2009). Recombivax HB is a 22-nm VLP consisting of the recombinant Hepatitis B surface antigen (rHBsAg) associated with lipids and was first produced in *Saccharomyces cervesiae* (Elliott et al., 1994; Dekleva et al., 1999; Zhou et al., 2006). Since then, the same host has been used to produce Engerix®, another hepatitis B vaccine, and Gardasil®, human papillomavirus (HPV) vaccine. Another HPV vaccine, Cerverix®, is made using a recombinant baculovirus system in Hi-5 cells derived from Trichoplusia ni (a type of moth). In addition, a seasonal influenza vaccine made in insect cells, Flublok®, was recently approved. *E. coli* was used to produce Lymerix®, although the vaccine was later withdrawn. Besides these host systems, Chinese hamster ovary cells, *Pichia pastoris* and *Hensenula polymorpha*, have also been used, mostly for the production of Hepatitis B vaccine. Lysogenic *Corynebacterium diphtheria* (Rappuoli, 1983; Srivastava and Deb, 2005) has been used for the production of the carrier

protein CRM197, used in polysaccharide conjugate vaccines. *E. coli* is also used as the recombinant host for the production of three of the four antigens in Bexsero® (Serruto et al., 2012), the only meningococcal serogroup B vaccine to be approved to date.

For vaccines in development, a variety of expression systems are being used (or have been used), including the ones described previously. In addition to the workhorse *E. coli*, novel microbial hosts being evaluated in vaccine development include *Pseudomonas fluorescens* and *Lactococcus lactis* (Bahey-El-Din, 2012; Chen, 2012; Unnikrishnan et al., 2012). Baculovirus expression in insect cells is increasingly being used for the production of glycoprotein antigens (Hu et al 2008; Dalemans, 2006; van Oers, 2006). Mammalian cell lines have also been used for the development of glycoprotein-based subunit vaccines. The HIV vaccine candidate gp120 protein was produced in CHO cell line (Billich, 2004). Other examples include HCV vaccine candidates from Chiron (Choo et al., 1994), HSV-2 (Langenberg et al., 1995, Corey et al., 1999), EBV subunit vaccines (Jackman et al., 1999), CMV (Spaete, 1991), and HSV (Lasky, 1990). Finally, plants have garnered a lot of interest recently as recombinant hosts for vaccines (Yusibov et al, 2011). Plants had seen a similar surge in interest in the past decade for the production of monoclonal antibodies, until improvements in more conventional mammalian methods made their use less attractive.

Principles developed for choosing an expression host for therapeutic proteins naturally translate to the production of vaccine antigens. There are many resources for providing guidance to choose a host (Andersen and Krummen, 2002; Makrides and Prentice, 2003, Graumann and Premstaller, 2006, Giuliani et al 2007, Choi et al., 2005, Demain and Vaishnav, 2009, Ferrer-Miralles et al., 2009, Chen, 2012). In general, however, the antigen productivity burden on the manufacturing process for vaccines is far less than that for therapeutic proteins, particularly monoclonal antibodies, because of the low dosage, although cost of goods pressure on vaccines is much more severe than for therapeutic proteins. Finally, as with the classical subunit vaccines, downstream purification is critical for the production of consistent and characterized recombinant vaccines, which is discussed further below.

1.7 CONJUGATE VACCINES

Conjugate vaccines are another interesting class of vaccines. They are complex vaccines, because they are comprised of two subunit components—a hapten and a carrier protein, covalently joined by chemical conjugation. Conjugation to the carrier protein to the hapten helps to elicit a T-cell-dependent immune response, which is particularly important in humans with a less developed immune system, such as infants. As such, conjugate vaccines have become an integral part of infant immunization. The first conjugate vaccine was a *Haemophilus influenzae* type b (Hib) conjugate vaccine, ProHibit®, licensed in the United States in 1987. ProHibit was a PRP-diphtheria toxoid conjugate vaccine, but was quickly superceded by more improved Hib vaccines conjugated to CRM197 (nontoxic mutant diphtheria toxin),

OMPC (the outer membrane protein carrier from *Neisseria meningitidis*) and tetanus toxoid. Menjugate®, a meningococcal C conjugate vaccine, was licensed in 2000 as was Prevnar®, a pneumococcal conjugate vaccine. Prevnar®, consists of seven different polysaccharide-CRM197 conjugates and has had a significant impact on the burden of pneumococcal disease in the pediatric population. The vaccine won the Discovers Award from PhRMA in 2005. The 7-valent vaccine has now been exceeded by Prevnar-13®, where six additional polysaccharide-CRM197 conjugates have been added, making it the world's most complex biological product ever produced (Frasch, 2009; Emini, 2010). This tour de force vaccine won the Prix Galien award in 2011. Analogous to the pneumococcal conjugate vaccine, a quadrivalent meninogococcal conjugate vaccine, Meactra®, was first licensed in 2005. Menafrivac®, a meningococcal A conjugate vaccine, was licensed for the developing world in 2010 in a unique collaboration between the developed and developing world partners (Frasch et al., 2012), with vaccine manufacturing expertise at large scale and low production cost supplied by Serum Institute of India.

In order to facilitate conjugation, chemistry is performed on one or both of the biomolecules to prepare them suitable for the conjugation reaction. It is critical to maintain antigenic fidelity of the hapten, while also optimizing the extent of reaction and process yields for both the antigen and the carrier protein, because both are high value intermediates. Minimizing the many side reactions including degradation of one or more of the components involved is also important. Several different chemistries have been used, each with its own pros and cons (Frasch, 2009). Process control and scale-up of such conjugation operations are more akin to chemical processing, where control of the relative rates of reaction and fluid transport is very relevant. The bioprocess steps used subsequent to conjugation, are similar to those of subunit vaccine purification, where the unreacted components are removed from the conjugate. Removal of the unreacted polysaccharide from the conjugate is one separation challenge, especially if only size-based methods are employed (Meacle et al., 1999; Wen et al., 2005).

Until recently, conjugate vaccines were manufactured at a relatively modest scale owing to the small number of doses sold. However, with the advent of the GAVI Alliance, Hib vaccines and now pneumococcal vaccines are slated for near-universal global infant immunization, thus needing large-scale production of these conjugate vaccines (http://www.gavialliance.org/funding/pneumococcal-amc/). Scale-up of these vaccines is nontrivial because of the issues listed previously and is further complicated by the multivalent composition of these vaccines.

1.8 DOWNSTREAM PROCESSING

Downstream processing has received relatively less attention in vaccine bioprocessing relative to upstream production. This is probably due to the dominance of whole-organism vaccines for most of vaccine history. Owing to the lack of complete knowledge of the etiology of disease and the molecular basis of immune protection, the conservative approach had been to keep the preparation rather crude.

As illustrated by the pertussis vaccine (La Montagne, 1997), reactogenicity concerns with whole-organism vaccines have led to the development and proliferation of subunit vaccines (Ellis 1996; Lattanzi et al., 2004), thus increasing the need and relevance of downstream processing. Even for whole-viral vaccines, the removal of residual DNA is of particular concern when produced in continuous cell lines (Sheng-Fowler et al., 2009). The manufacturing requirements of subunit vaccines today rivals those of therapeutic proteins. Although the dose of a vaccine is much lower relative to monoclonal antibodies, the safety requirements for vaccines are much greater, since they are administered prophylactically to a healthy population including infants.

The first recombinant vaccine—Recombivax-HB® used many contemporary bioseparation techniques. The vaccine consists of a highly hydrophobic protein–lipid particles of 22 nm. As a result, the manufacture of this vaccine is quite challenging, analogous to the expression and purification of membrane proteins. In fact this is a common feature of many subunit vaccine candidates because many are bacterial or viral surface protein antigens. The downstream process of Recombivax-HB® also involves a thiocyanate treatment step which results in reforming the disulfide bonds in the virus-like particle (VLP), thus adding to the complexity. Purification of the HBSAg was initially performed using density gradient ultracentrifugation. This is a common feature of historical processes where the scale-up methodology had been to scale-up laboratory techniques, or simply scale-out. Modern processes use unit operations that are designed to be scaleable. Density gradient ultracentrifugation has been replaced by chromatographic adsorption steps (Sitrin and Kubek, 1992), which have further evolved in sophistication with regard to the configuration of the binding surface to enable adsorption and transport of the large macromolecular sizes of these particles, such as with the use of membrane chromatography or monoliths. The Hepatitis A vaccine, Vaqta® is an excellent example of the use of modern bioseparation techniques for the manufacture of a highly purified complete inactivated virus (Junker et al., 1994; Hagen et al., 1996; Hennessey et al., 1999). The manufacturing process for Vaqta® was awarded the 1998 ACS Industrial Biotechnology Award. The same award was garnered in 2006 for the process to manufacture an HPV vaccine (Gardasil®), which incorporates not only modern bioseparation techniques, but also a highly sophisticated well-controlled virus-like particle disassembly/re-assembly step. The structural protein of the virus when expressed in *Saccharomyces cerevesiae* spontaneously assembles into VLPs inside the cell. After purification of the VLPs, they are disassembled and then reassembled in a controlled manner to produce highly uniform and stable VLPs (Shi et al., 2007). Gardasil® also received the Prix Galien Award in 2007 and the PhRMA Discovers Award in 2009. Purification processes for the capsular polysaccharide vaccines include the use of selective precipitation steps, facilitated by the development of newer analytical methods to analyze and quantify the product, host cell impurities, and process residuals (Abeygunawardana et al., 2000; Pujar et al., 2005). Conjugate vaccines have additional purification challenges that were outlined above. Two other examples, although for vaccine development candidates which were not commercialized, have also contributed to the state of the art of vaccine purification.

An industrial-scale purification of plasmid DNA vaccines was developed using controlled and continuous heat lysis of cell paste, and nonchromatographic methods to overcome the low binding capacity of plasmid DNA to conventional wide-pore chromatographic resins (Lee and Sagar 2001; Murphy et al., 2006). Large scale live adenovirus suitable for human use was accomplished by developing a novel selective precipitation step for cellular DNA among other steps (Goerke et al., 2005; Konz et al, 2008). Overall the downstream purification processes for such diverse entities such as a fully intact virus, a protein–lipid complex virus-like particles, multimeric protein complex, and plasmid DNA represent significant bioseparation process technology advances, significantly enabling the development of modern vaccines and vaccine candidates.

Key issues for downstream processing can be summed up as follows: removal of cellular impurities including proteins, DNA, RNA, and lipids; removal of process residuals; preservation of the structure of the antigen; and formulation of the antigen in an environment that keeps it stable for at least 2 years in a refrigerated state. Vaccines are diverse, complex biomolecules, often multivalent, and, consequently, the bioseparation challenges are manifold and vaccine specific. This can be contrasted with the purification of monoclonal antibodies where a platform process using protein A chromatography followed by typically two additional chromatography steps is commonly used across the industrial landscape. Despite these challenges, modern analytical techniques enable a deep understanding and control of both upstream and downstream operations, increasing yielding well-characterized vaccines, a feature nicely illustrated by Gardasil®.

1.9 VACCINES FOR THE DEVELOPING WORLD: LARGE VOLUME, LOW COST, AND THERMOSTABLE

Vaccine technology that has largely originated in the United States and Europe is fast proliferating to the developing world because of the combined efforts of developing country vaccine manufacturers, nongovernmental organizations such as GAVI and Bill and Melinda Gates Foundation, and technology developers in the developed world (Jadhav et al., 2008; Pagliusi et al., 2013). The vaccines that are of most relevance in this case are the pediatric vaccines, where the large birth cohort in countries such as India and China dominates vaccine needs. Hence, large volume production at low cost, with sufficient thermostablilty to address cold chain issues, is very important (Chen and Zehrung, 2012). The biomanufacturing challenges of accomplishing these goals have been well articulated (Rexroad et al., 2002a; Rexroad et al., 2002b; Milstien et al., 2009). Bioprocess advances in making vaccine manufacturing processes robust, productive, and portable will be necessary for achieving greater access to a much larger population; this need is particularly acute for live viral vaccines.

1.10 SUMMARY

Vaccine manufacturing processes have come a long way since Edward Jenner's cow pox vaccine. The evolution in the biomanufacturing of influenza and JE vaccines

truly captures the transformation of upstream processes technology—from production *in vivo* to *in ovo*, to microcarriers, and finally to suspension cell culture, as well as the evolution from a whole virus to a subunit vaccine. Downstream processing has evolved significantly as well, from simple filtration or lyophilization of the upstream feedstock to highly selective purification unit operations as well as operations involving chemical reactions. This is uniquely illustrated by Prevnar-13®, which consists of 14 distinct fermentation and purification processes as well as 39 different chemical reactions. The modern vaccine manufacturing processes of today are already producing consistent, well-defined and highly purified antigens, with a high level understanding of the vaccine critical quality attributes. However opportunities exist to extend these advances for the more complex vaccines, as well as in the production of all vaccines at desired quantities and cost. Only then would bioprocess science and engineering fully deliver on its promise.

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