Arch Pharm Res Vol 32, No 4, 465-480, 2009
DOI 10.1007/s12272-009-1400-1

Archives of Pharmacal Research
www.springer.com/12272

REVIEW

Innovative Vaccine Production Technologies: The Evolution and Value of Vaccine Production Technologies

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(Received February 1, 2009/Revised March 10, 2009/Accepted March 13, 2009)

This review paper provides an overview of innovative technologies designed to produce bacterial, viral, recombinant subunit, and polysaccharide vaccines, as well as combination vaccines. Advances in this field are illustrated by vaccines against DTP (diphtheria-tetanus-pertussis), influenza, hepatitis B (HepB) and typhoid fever. In addition, technological trends regarding antigens, adjuvants, and preservatives in vaccines are discussed. The progress achieved in vaccine production technologies is especially important for improving the protection of vulnerable populations against infectious diseases. These at-risk groups include infants, the elderly and immunocompromized individuals, as well as people living in developing countries or emerging economies.

Key words: Vaccine, Antigen, Adjuvant, CpG, Virosome, Ty21a

INTRODUCTION

Vaccination is regarded as one of the most beneficial biopharmaceutical interventions, due to its ability to induce protection against infectious diseases through targeted activation of the human immune system (Brusic et al., 2005). Nevertheless, millions of people continue to die each year from infectious diseases that are preventable using existing vaccines. This avoidable mortality occurs particularly in developing countries, where vaccine coverage is often limited by economic problems, lack of medical infrastructure and lack of cold chain facilities. At the same time, developed countries are increasingly demanding advanced vaccines that offer greater safety, efficacy, and convenience of use. Thus, innovative vaccine production technologies are believed to be important for satisfying the requirements of both developing and developed countries.

One example of such innovation is the development of improved adjuvants—including CpG motif, adenovirus or vaccinia virus vectors, and virosomal technology—that can enhance a vaccine's immunomodulatory properties, leading to an increase in efficacy (Hartman et al., 2008; Klinman, 2003; Ulaeto and Hruby, 1994). Another area where advances are being made is drug delivery. Innovative delivery systems such as sugarglass and modified dextran microparticles offer improved stability of vaccine antigens, while skin patches employing the immunogenic molecule LTB (subunit B of heat-labile enterotoxin) provide more convenient administration (Amorij et al., 2007; Kaistha et al., 1996; Moravec et al., 2007). Furthermore, improved host cell systems are efficient choices for the mass production of vaccines. These innovative production platforms include methylotrophic yeasts, which simplify fed-batch culture, and PER.C6® human cell lines, which endow antigens with human glycosylation patterns and efficiently support adenovirus propagation without the risk of generating replication-competent virus (Subramanian et al., 2007; Tsai et al., 2006).

Vaccines have long been classified as bacterial, viral, subunit antigen, or polysaccharide antigen vaccines. A relatively new addition to this armamentarium are combination vaccines comprising multiple

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antigens; these are in growing demand due to the advantages they offer, such as reduced vaccination frequency, lower cost and increased coverage (Marshall et al., 2007; Pichichero et al., 2007). This review describes trends in the technologies used for production of all these prophylactic vaccine types currently available in the market, illustrating each aspect of technological development with one or two specific examples. The recently emerging area, such as a DNA vaccine and a therapeutic vaccine intended to treat existing diseases (representatively, cancers), was not dealt with in this study even though many clinical researches on their development candidates have been done.

Review of the literature reveals that vaccine technologies employing recent advances in molecular biology, immunology, and biotechnology have enabled more efficient vaccine production and improved vaccine quality. The widespread application of these innovative approaches is expected to contribute to a significant increase in vaccine production and a decrease in the cost of goods (COGs) associated with vaccines, while enabling the production of vaccines with improved safety, efficacy and stability.

THE IMPORTANCE OF INNOVATIVE VACCINE PRODUCTION TECHNOLOGIES

Since Edward Jenner's pioneered the concept of vaccination with a smallpox vaccine in 1796, the suppression and control of a wide range of epidemic and pandemic infectious diseases has effectively been accomplished with vaccines (Bernstein, 2007; Heaton and Ciarlet, 2007; Matson, 2006; O'Ryan, 2007; Tovar and Bazaldua, 2008; Zimmerman, 2007). However, vaccines are not yet available for important deadly diseases such as malaria, AIDS and hepatitis C, and there is a clear need for a more efficacious vaccine against tuberculosis, a major cause of illness and death despite the widespread use of Bacillus Clamette-Guérin (BCG) vaccine. In addition, every year an estimated 2.5 million people worldwide die from vaccine-preventable diseases (VPDs), reflecting the underuse of available vaccines (Tovar and Bazaldua, 2008). Most deaths induced by VPDs occur in developing areas, which have low vaccine coverage rates due to low income, lack of medical infrastructure, lack of cold chain facilities, etc.

Innovations aimed at improving vaccine coverage in low-income regions include the development of high-yield production technologies to reduce COGs, the development of vaccines that only require one or two shots, the use of efficient adjuvant and/or drug delivery systems to reduce the number of clinic visits required, the development of heat-stabilized technology to preclude the need for cold chain systems, and the development of combination vaccines to increase the vaccination rate (Di Fabio and de Quadros, 2001; Pichichero and Stonehocker Quick, 2003; Tovar and Bazaldua, 2008). For developed countries, innovative vaccine technologies have enabled the development of safer, more effective vaccines that are easier to administer. Important advances include one-shot or two-shot vaccines, combination vaccines, vaccines with higher efficacy, vaccines that are effective for senior citizens and immunocompromized persons, and vaccines that are administered using prefilled syringes, skin patches, etc.

Taken together, these findings underline the importance of ongoing innovation in the area of vaccine production technologies.

VACCINE TYPES, AND THE ROLES OF THEIR ANTIGENS, ADJUVANTS AND PRESERVATIVES

Historically, a variety of vaccine types have been developed. Vaccines can generally be classified into those that include the whole organism, purified macromolecules, combined antigens, recombinant vectors, synthetic peptides or DNA. Whole-organism vaccines can be subclassified into attenuated microbial, attenuated viral, inactivated microbial and inactivated viral types. Purified macromolecule vaccines can be subcategorized into protein, recombinant protein, toxoid and polysaccharide antigen vaccine types (Myint and Gibbons, 2008; Safdar and Cox, 2007; Salehen and Stover, 2008; Tai, 2006; Taranger et al., 2001; Vitek, 2006). The present paper focuses exclusively on production technologies designed to develop vaccines comprising the whole organism, purified macromolecules or combined antigens, due to their prevalence in the current market.

Vaccines contain many components immediately after formulation, including essential ingredients such as antigens, adjuvants and preservatives. These components are all targets for technological development.

Antigens and adjuvants

In addition to inducing the targeted immune response, antigens can also induce unwanted immunogenic side-effects in the human body, depending on their characteristics (Evensen et al., 2005; Matheson

and Goa, 2000). Therefore, antigen improvement has recently generated great interest. Antigens used in vaccines must be equipped with the basic immunogenic properties, such as an immunodominant B cell epitope that can interact efficiently with a specific B cell receptor to produce an antibody capable of neutralizing pathogens or their virulence factors. For increased vaccine efficacy, antigens also need to possess immunodominant T cell epitopes that react efficiently with the major histocompatibility complex (MHC) antigen, as well as the broad T cell epitope spectrum, thus avoiding the nonresponsiveness associated with MHC restriction (Morrison et al., 1999; Prescott, 2004). For this reason, entire viral or microbial antigens are often regarded as the best immunogens due to their epitopic diversity, despite their high reactogenicity (Plotkin, 1997; Rappuoli, 1996; Wintermeyer et al., 1994).

Purified macromolecular antigens with relatively low reactogenicities generally require adjuvants to increase the immune response triggered in the human body. To date, alum has been the most commonly used adjuvant. However, alum is capable of promoting immunoglobulin E (IgE) antibody expression when administered together with antigens, and can therefore cause side-effects in atopic individuals (McElrath, 1995; Petrovsky and Aguilar, 2004; Thoelen et al., 2001). With the progress of immunology, the various roles that adjuvants play in relation to the human immune system have been elucidated.

For example, it is now known that the innate immune system detects highly conserved, relatively invariant structural motifs of viral and microbial pathogens. These pathogen-associated molecular patterns (PAMPs) can be regarded as natural adjuvants. PAMPs induce cytokine expression by binding to their specific ligands, which are Toll-like receptors (TLRs) on the plasma membrane of dendritic cells or macrophages. The expressed cytokines, in turn, determine the direction of Th cell differentiation by activating the appropriate transcription factors, such as NF-kB, IRF-3, and IRF-7 (Heeg and Dalpke, 2003).

Representative examples of synthetic PAMPs developed for vaccines (also known as TLR-dependent adjuvants) include a synthetic unmethylated CpG motif that mimics the unique DNA characteristics of pathogens, which bind to TLR9, and a synthetic poly (I:C) that imitates the double-stranded RNA of specific viruses, which binds to TLR3 (Ada and Ramshaw, 2003; O'Hagan et al., 1997; Ríhová, 2002; Yamamoto et al., 2002). The binding of TLR9 and TLR3 results in the stimulation of cellular immune responses, which occurs via the upregulation of interleukin (IL)-12 expression in dendritic cells (Kandimalla et al., 2005; Pawar et al., 2006). An interesting study involving an immunostimulatory sequence (ISS), which is a specific type of CpG motif developed by Dynavax Technologies Corp (USA), revealed that the CpG motif-conjugated protein antigen induces a Th-independent cytotoxic T lymphocyte (CTL) immune response, showing the avoidance of class II MHC restriction of antigen recognition, and also promotes a strong IgG1 immune response. This results in cellular and humoral immune responses at the same time, in contrast to the effects of CpG-unconjugated protein antigen (CpG + protein antigen), which only leads to Th1-dependent CTL immune responses (Barry and Cooper, 2007; Cho et al., 2000; Marshall et al., 2003).

Besides alum, currently available TLR-independent adjuvants include oil-in-water emulsions such as MF59 and AS03, which contain squalene as an oil component and have recently been developed for influenza and avian influenza vaccines, respectively. As mentioned earlier, alum is capable of improving the humoral immune response to antigens by enabling dendritic cells to express IL-4. Oil-in-water emulsions such as MF59 and AS03 also prompt a humoral immune response, by a mechanism that is still unclear (Faquim-Mauro and Macedo, 2000; Katayama et al., 1999).

Another approach to adjuvant innovation is to combine TLR-dependent and TLR-independent adjuvants. A specific example is the compound AS04, a combination of alum gel (which induces a Th2 immune response) and monophosphoryl lipid A (which binds to TLR4), which has been successfully prepared and applied to HPV and HepB vaccines (Desombere et al., 2002; Giannini et al., 2006).

Alternatively, adjuvants with a drug delivery effect have been produced using the virosomal technology developed by Crucell (the Netherlands). The virosomal adjuvant system is morphologically similar to a liposome and comprised of hemaglutinin and neuraminidase from influenza A, phospholipid, and antigen. The hemaglutinin from the influenza A virus can detect the sialic acid that is widely distributed on the cell membrane of dendritic cells and macrophages in humans. As a result, the immune response is enhanced. Moreover, the virosome can supply its antigen component to the cytosol of dendritic cells by fusing with their membranes. Accordingly, it is believed that the antigen embedded in the virosomal surface stimulates both the IgG and

CTL immune response in humans (Cusi et al., 2002; de Vries et al., 2008).

Preservatives

Preservatives are added during vaccine formulation to prevent any contaminating microorganisms from growing during storage. Thimerosal has been the most widely used vaccine preservative due to its effectiveness against a variety of pathogens. However, the fact that thimerosal contains intramercury has raised concerns about its safety, with several studies suggesting a link with autism. Although there is no clear evidence of a causal association, efforts to avoid thimerosal in vaccines are increasingly seen as prudent precautionary measures supporting the public health goal of reducing mercury exposure, especially in children. Therefore, many single-dose vaccines now contain reduced levels of thimerosal and some have eliminated it altogether.

Some vaccine manufacturers have started to use 2-phenoxyethanol in place of thimerosal to prevent the growth of gram-negative bacteria. Indeed, 2-phenoxyethanol is already used in single-dose vaccines such as DTaP *(diphtheria*, *tetanus and acellular pertussis*), DTaP-HepB, HepA, HepA-HepB, and inactivated polio vaccine (Heidary and Cohen, 2005; Meyer et al., 2007). In spite of this concerns, thimerosal is still used in multi-dose vaccines requiring a preservative worldwide (Clements and McIntyre, 2006; Talbot et al. 2008).

BACTERIAL VACCINES: TECHNOLOGICAL TRENDS ASSOCIATED WITH THE DTP VACCINE

The combined DTP vaccine designed to prevent diphtheria, tetanus and pertussis is considered to be one of the most important bacterial vaccines administered worldwide. This is because the causative agents of these diseases are ubiquitous and can cause serious illness and death in humans.

Currently, there are several types of DTP vaccines available. The DTwP vaccine, which contains killed whole *Bodertella pertussis,* was developed and introduced in the 1960s and can be regarded as the first-generation DTP vaccine (Milstien et al., 2003; Plotkin, 1997). Because *B. pertussis* is a gram-negative bacteria that contains outer-membrane lipopolysaccharide (LPS), DTwP has relatively high reactogenicity. To address this problem, a second-generation DTP vaccine with an acellular pertussis (aP) component was developed. The DTaP vaccine contains virulence factors of *B. pertussis*, including pertussis toxin (PT), filamentous hemaglutinin (FHA), pertactin (PRN) and fimbriae (FIM); (Casey and Pichichero, 2005; Gidengil et al., 2008; Granoff, 1999; Pichichero, 1996). These aP components of the second-generation DTaP vaccine are purified simultaneously, whereas the third-generation DTaP vaccine contains separately purified aP components.

The fourth-generation DTaP vaccine, which is based on its predecessor, contains genetically detoxified PT (PT-9K/129G), prepared by culturing genetically detoxified *B. pertussis*. In the fourth-generation DTaP, two essential amino acids that were found to enable the catalysis of intact PT were changed to Arg9Lys and Glu129Gly, leading to complete attenuation of the vaccine's toxicity without a loss of immunogenicity (Black et al., 1988; Burnette, 1997; Cyr et al., 2001; Gomez et al., 2007; Parton, 1999). This state-of-the-art vaccine (Triacelluvax, Chiron, Italy) was withdrawn from the market in 2002 for unknown commercial reasons (EMEA, 2002; Matheson and Goa, 2000).

The production of simultaneously purified aP components involves the culture and subsequent simultaneous purification of the aforementioned four virulence factors via a combination of salting-out, ultracentrifugation and calcium phosphate gel treatment to remove endotoxin (LPS), and detoxification of PT by formalin or formalin-lysine (Ju et al., 1997; Sato and Sato, 1999; Sheu et al., 2001; Tan et al., 1991). When the aP components are purified separately, the culture and purification processes are much more complicated and expensive because PT is generally expressed in relatively small amounts compared to FHA, but most DTaP formulations with separately purified aP components include similar amounts of PT and FHA (Table I).

The diphtheria and tetanus toxoids in the DTaP and DTwP vaccines are produced by the attenuation of toxins expressed by *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively. The diphtheria toxin plays a role in stopping protein synthesis via ADP-riboxylation of elongation factor 2, which is responsible for protein translation. The tetanus toxin leads to muscle contractions and spasms by interfering with neurotransmitter release in the central nervous system (Grumelli et al., 2005; Iglewski, 1994; Lubran, 1988; Perentesis et al., 1992; Zacks and Sheff, 1970).

A conventional method for attenuating these toxins is toxoiding immediately after culture, with subsequent purification, whereas a more advanced

Table I. Representative composition of DTaP vaccines comprising separately purified aP components

Company	Trade name	D (Lf/dose)	T (Lf/dose)	aP (μg/dose)			
				PT	FHA	PRN	FIM
Novartis	Triacelluvax	25	10	5*	2.5	2.5	-
Sanofi Aventis	Tripedia	6.7	5	23.4	23.4	-	-
	DAPTACEL	15	5	10	5	3	5
GSK	Infanrix	25	10	25	25	8	-

* Genetically detoxified PT: PT-9K/129G
Abbreviations - D: diphtheria toxoid; T: tetanus toxoid, aP: acellular pertussis; PT: pertussis toxoid; FHA: filamentous hemagglutinin; PRN: pertactin; FIM: fimbriae

method involves purification after culture, with subsequent toxoiding. The general process utilized in the former method consists of culture, detoxification by formalin, and toxoid purification by a combination of fractional salting-out and size exclusion chromatography (SEC). The latter process involves culture, followed by toxin purification using a combination of fractional salting-out, ion exchange chromatography (IEC) and SEC, and then toxoiding using formalin-lysine (Perentesis et al., 1992; Relyveld, 1980).

Using conventional toxoiding methods, the purity of toxoids is known to be approximately 2,000-2,500 Lf/mgPN (mg protein nitrogen), depending on the manufacturer, whereas the purity attained using the advanced method is reportedly greater than 3,000 Lf/mgPN (Frech et al., 2000; Perentesis et al., 1992). The relatively greater impurity of conventionally produced toxoids is due to the formation of a methylene bridge (-CH_2-) between toxins and impurities (mostly peptides and proteins) from bacterial components and remaining nutrients. Therefore, the toxoid content of vaccines produced using the conventional method should be significantly lower for adults than for infants because they can induce anaphylactic shock when used as a booster in adults (Ponvert and Scheinmann, 2003).

VIRAL VACCINES: TECHNOLOGICAL TRENDS ASSOCIATED WITH THE INFLUENZA VACCINE

In this review, the technological trends associated with the manufacturing of viral vaccines are illustrated by the evolution of vaccines against influenza virus. According to World Health Organization figures from 2007, one billion cases of influenza occur annually, including 3-5 million cases of severe illness leading to between 300,000 and 500,000 deaths a year.

The influenza virus is classified into three subtypes—A, B and C—based on antigenic differences in two of its structural proteins, the matrix protein (M1) and the nucleoprotein (NP). The influenza virus is an enveloped RNA virus with characteristic single-stranded, negative-sense RNA comprised of eight segments (Hampson and Mackenzie, 2006). It is important to note that influenza A virus has a high rate of natural mutation, which results in frequent antigenic drift (seasonal influenza) and/or potential antigenic shift (pandemic influenza) (Williams et al., 2002; Zambon, 1999).

The first influenza vaccine was developed in 1937 by culturing influenza virus in fertilized eggs. Viruses cultured in this way were later used in 1945 as a form of monovalent vaccine (A/H1N1). In the 1960s, a bivalent influenza vaccine (A/H2N2 and influenza B) became available, and in 1970, the current trivalent vaccine containing A/H1N1, A/H3N2 and the influenza B virus was introduced (Bachmayer and Wagner, 1973; Belshe, 2004). Technologically, the influenza vaccine has progressed from an inactivated whole-virus vaccine to a purified split vaccine, to a purified surface antigen vaccine, and finally to a live attenuated virus vaccine. Each of these vaccines has been cultured in fertilized chicken eggs.

Overall, the production of egg-based vaccines comprising inactivated whole viruses is relatively simple and cost-effective. However, such vaccines are seldom used due to their high reactogenicity caused by viral lipids (Cintra and Rey, 2006; Couch, 1993). Egg-based purified split vaccines represent a common alternative; these are produced by splitting purified viruses with ether, recovering the viral proteins by ultracentrifugation, and then inactivating them with formalin. Purified split vaccines include two major protective antigens, hemaglutinin and neuraminidase, as well as several viral proteins including transcriptase complex (PB1, PB2, and PA), nucleoprotein (NP), and matrix protein (M1).

To reduce impurities in purified split vaccines,

ether is often replaced with detergents such as sodium deoxycholate. This has led to the development of purified subunit antigen vaccines that primarily contain hemaglutinin and neuraminidase. Purified split vaccines and purified subunit antigen vaccines reportedly show much less reactogenicity than inactivated whole-virus vaccines, and are therefore widely used for vaccination against influenza virus (Couch, 1993; Rose and Cooper, 2006).

Another significant advance in the production of influenza vaccines was the introduction of a live trivalent attenuated virus vaccine (FluMist®, Medimmune, USA) in 2003. This vaccine can be administered intranasally to induce a mucosal immune response (influenza-specific IgA) in the nasal passages as well as a CTL immune response. FluMist contains live attenuated strains that are cold-adapted and temperature-sensitive, which enables them to grow at 25°C but causes inefficient growth at 37°C (Maassab and Bryant, 1999; Robert et al., 2000).

Innovative production platforms

Fertilized egg-based vaccines have long been used due to their proven safety record, but they have several disadvantages. These have to do with the limited supply of chicken eggs and the limited potential for mass production of egg-based vaccines, the relatively time-consuming production process, and the presence of residual antibiotics and residual egg proteins in such vaccines (Palache et al., 1997).

To overcome these drawbacks, several companies have recently developed influenza vaccines produced on the MDCK, Vero or PER.C6® mammalian cell lines (Table II). The production of mammalian cell-derived vaccines involves cell culture, viral infection, a combination of centrifugation, chromatography and concentration, disruption with detergent, and finally inactivation by formalin (Audsley and Tannock, 2008). This approach is not dependent on the availability of chicken eggs, is faster and more productive than egg-based manufacturing, and produces vaccines free of residual egg proteins. The fact that mammalian cell-based production methods enable large quantities of vaccine to be manufactured rapidly would obviously be of crucial benefit in the event of an influenza pandemic (Tree et al., 2001).

Pandemic preparedness

A number of vaccine companies worldwide have been directing their efforts towards developing prepandemic vaccines against several potentially pandemic influenza virus strains, including H5N1, H9N2, and H7N1. These programs involve a variety of production platforms—including fertilized eggs and MDCK, Vero and PER.C6® cell lines—and both whole-virus and purified split vaccines (Audsley and Tannock, 2004, Horimoto and Kawaoka, 2006; Soda et al., 2008; Stephenson and Nicholson, 2001; Swayne, 2004). Several prepandemic vaccines are currently in clinical trials and one has already reached the market: a fertilized egg-based H5N1 vaccine developed by Sanofi-Pasteur, which was approved in the USA in 2007 (Engin, 2007).

A limitation of this type of vaccine is that a variety of H5N1 strains (clades, subclades) have been found around the world, due to the high mutation rate of the H5N1 influenza virus genome, and there is little or no cross-protection among H5N1 clades in response to vaccination (Chen et al., 2008; Mahmood et al., 2008). Furthermore, an H5N1 vaccine would offer no protection against other possible pandemic strains of influenza such as H9N2, H7N1, and so on.

Several approaches are being explored to overcome the limitation of strain-based vaccines. One is to develop a universal vaccine using highly conserved M2 membrane protein, which is only found in influenza A. Such a vaccine can be expected to confer permanent immunity against all forms of influenza A, including potential pandemic strains, in humans. However, it cannot be used to treat people who have already been exposed to a potentially lethal influenza virus.

An alternative strategy involves treating patients with anti-HA2 monoclonal antibody, as the HA2 domain of hemaglutinin (HA protein) is highly conserved compared to the extremely variable HA1

Table II. Development status of mammalian cell-based influenza vaccines

Cell line	Company	Development stage	Remarks
MDCK	Solvay	Phase III finished (2006)	NDA submission to EU
	Norvatis Vaccines	Phase III (2008)	US
	GSK	Phase I (2007)	US
Vero	Baxter	Phase II (2007)	EU
PER.C6	Sanofi Aventis	Phase II (2007)	US

domain (binding region to sialic acid); (Fiers et al., 2004; Neirynck et al., 1999; Varecková et al., 1993; Varecková et al., 2003; Yamada et al., 2006). Importantly, novel monoclonal antibodies were recently identified that neutralize *in vitro* a broad range of influenza viruses, including those with pandemic potential (Throsby et al., 2008). These antibodies bind to an evolutionary conserved region in the HA2 domain and have been shown to protect mice from a lethal challenge with H5N1 influenza in both a prophylactic and therapeutic setting.

RECOMBINANT SUBUNIT VACCINES - TECHNOLOGICAL TRENDS ASSOCIATED WITH HepB VACCINE

Much research has been directed towards developing recombinant subunit vaccines since recombinant DNA technology was introduced in the 1970s. The first successful application of this approach to vaccine production was the development of a recombinant vaccine against hepatitis B virus (HBV), which was licensed in 1986. More recently, recombinant virus vaccines against human papillomavirus (HPV), which causes cervical cancer, have been produced using innovative technologies developed by Merck (a recombinant *S. cerevisiae* yeast expression system) and GlaxoSmithKline (an insect cell via the recombinant baculovirus); (Ljubojeviæ, 2006; McLemore, 2006).

In this review, advances in the development of recombinant subunit vaccines are illustrated by the technological trends associated with hepatitis B (HepB) vaccine. HBV is a partially double-stranded, enveloped DNA virus belonging to the *Hepadnaviridae* family. The virus replicates in the liver, causing either liver cirrhosis or hepatocellular carcinoma in approximately 25% of chronic HBV carriers (Colacino and Staschke, 1998; Yokosuka and Arai, 2006).

The first vaccine used to protect people against HBV infection was developed by Maurice R. Hilleman in 1975. It was a subunit vaccine utilizing hepatitis B surface antigen (HBsAg) particles (Blumberg, 1967) derived from the plasma of chronic HBV carriers (Fukuda et al., 1990; Hilleman, 2000). Although this vaccine represented a breakthrough in hepatitis B prevention, its use was limited by the drawbacks typical of plasma-derived subunit vaccines: a dependence on limited donor blood supplies, risk of viral contamination, and the high costs associated with blood procurement (Stephenne, 1990).

To overcome these limitations, a recombinant subunit vaccine based on the S gene, which encodes a small-sized HBsAg protein (S protein), was developed using *S. cerevisiae* as the expression platform in 1984. Later, in an attempt to significantly increase the HBsAg yield by bypassing the yeast crab-tree effect commonly encountered in high-density cultures of *S. cerevisiae* produced in fed-batch culture mode, host replacement with methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris* was applied to HepB vaccine production (Hardy et al., 2000; Kulkarni et al., 2006; Thierie, 2004). A recombinant subunit vaccine based on the M gene (preS2-S), which encodes a middle-sized protein (M protein), was also developed using Chinese Hamster Ovary (CHO) cells as the expression system. The protective efficacy of the M protein has been reported to be similar to that of the S protein (Bruss, 2004).

Besides being much less costly to produce than vaccines that are dependent on plasma procurement, recombinant subunit HepB vaccines offer the advantage of consistent quality and therefore easier quality control. In contrast to recombinant subunit vaccines, plasma-derived vaccines contain a variety of HBsAg subtypes (primarily adr, adw, ayr, and ayw) and genotypes, as well as differently sized HBsAg proteins (small, medium and large) in the HBsAg particles. For this reason, recombinant subunit vaccines with one subtype (primarily S protein) have become dominant in the market despite the fact that they have antigenic characteristics that may lead to nonresponsiveness in individuals with an MHC restriction (Hatae et al., 1992).

Recent studies report that this limitation has been overcome by using an S protein-CpG motif conjugate capable of inducing a Th-independent immune response against HBsAg, thereby enabling avoidance of class II MHC restriction. During clinical trials in which this innovative recombinant subunit HepB vaccine (Dynavax, USA) was administered to 400 seronegative adults aged 40-70 years,

Table III. Comparison of the efficacy of alum-adjuvanted and CpG motif-conjugated S proteins administered to adults

Randomization group	Alum-adjuvanted S protein	CpG motif-S protein conjugate
Subjects	400 seronegative persons aged 40 to 70 years	
Primary vaccination schedule	0, 1, 6 months	0, 1, 6 months
Seroprotection rate at 50 weeks after primary vaccination	68.6%	100%

a 100% seroprotection rate was observed, compared to a seroprotection rate of 68.6% among recipients of conventional alum-adjuvanted recombinant subunit HepB vaccine (Table III). These findings indicate that the CpG-conjugated HepB vaccine effectively protects nonresponders to alum-adjuvanted HepB vaccine and induces a better immune response in immunocompromized individuals.

POLYSACCHARIDE VACCINES - TECHNOLOGICAL TRENDS ASSOCIATED WITH TYPHOID VACCINE

Some bacteria, such as *Salmonella typhi*, *Neisseria meningitis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, have developed the ability to evade the human immune system by means of polysaccharide capsules on their surface. These polysaccharide capsules commonly prevent activation of the alternative pathway of the human complement system by interfering with the exposure of lipopolysaccharides (LPS, on gram-negative bacteria) or lipoteichoic acids (LTA, on gram-positive bacteria) to the complement system. This results in the inhibition of C3 convertase formation, thereby reducing the production of C3b and decreasing the efficiency of phagocytosis (Lindberg, 1999; Slaney and Curtis, 2008).

Polysaccharide capsules of these pathogenic bacteria have been used in the development of vaccines that neutralize the bacteria in the bloodstream and tissues by means of their anti-polysaccharide antibody (IgM), as well as inducing complement activation and antibody-dependent cellular cytotoxicity (ADCC) of macrophages.

Among these capsular bacteria, the gram-negative *S. typhi*, which causes enteric fever and subsequent death in humans, has distinct characteristics. Specifically, *S. typhi* possesses intracellular survival activity that enables it to infect phagocytic cells (primarily, macrophage and dendritic cells). In addition *S. typhi* is able to survive and multiply within infected phagocytic cells after penetrating specialized epithelial cells called M cells, which cover the Peyer's patches of the small intestine. Furthermore, *S. typhi* has a Vi polysaccharide capsule on its surface, which greatly reduces the efficiency of phagocytosis (Biedzka-Sarek and El Skurnik, 2006; Hamid and Jain, 2007). Therefore, both humoral and cellular immune responses are believed to be necessary to effectively eliminate *S. typhi* from humans (Hamid and Jain, 2007; Jones and Falkow, 1996).

Historically, vaccines against *S. typhi* infection have evolved from inactivated whole-cell vaccines, to attenuated whole-cell vaccines, to Vi polysaccharide vaccines, and recently to protein conjugated-Vi polysaccharide vaccines.

Inactivated whole-cell typhoid vaccines are manufactured by culturing a wild-type strain, *S. typhi* Ty2, harvesting the cells and killing them with acetone, alcohol, formalin or heat (Levine et al., 1989; Sarkar and Pal, 1990). Acetone-inactivated and heat-inactivated whole-cell vaccines confer a high level of protection for several years by stimulating serum antibody responses such as anti-H antibody against flagella, anti-O antibody against the O antigen of LPS, and anti-Vi antibody against Vi polysaccharide. However, they are known to be associated with high rates of systemic and local adverse reactions due to the production of LPS by *S. typhi*. This drawback has prompted the development of vaccines with a better safety profile, such as live-attenuated oral vaccine and Vi polysaccharide vaccine (Adachi et al., 2000; Sarkar and Pal, 1990).

The live-attenuated oral *S. typhi* vaccine was developed using attenuated *S. typhi* Ty21a derived from mutagenic adaptation of a wild-type strain by Rene Germanier and Emil Furer in the Netherlands in 1975 (Germanier and Furer, 1975; Germanier, 1976). Strain Ty21a is a galE mutant entirely deficient in uridine diphosphate-galactose-4-epimerase (UDP-Gal-4-epimerase) activity, which isomerizes UDP-galactose to UDP-glucose and vice versa. It exhibits reduced activity of galactose pathway-related enzymes and is therefore capable of synthesizing only a small amount of LPS with full length O-chains (smooth LPS), which leads to immunogenicity only in the presence of galactose. Furthermore, Ty21a lacks Vi polysaccharide. Moreover, due to the complete reduction of UDP-Gal-4-epimerase activity, intermediates like UDP-galactose and galactose-1-phosphate accumulate in the attenuated strain following the uptake of galactose, which results in bacterial death by lysis before the bacterium can revert into a virulent phenotype (Gilman et al., 1977; Hickman et al., 1982).

The attenuated Vi-negative *S. typhi* Ty21a containing a small amount of smooth LPS (Vivotif®, Crucell) is prepared by culturing *S. typhi* Ty21a in an appropriate medium that includes glucose and galactose as carbon sources. Bacterial lysis by galactose is inhibited in the presence of glucose in the medium, thereby inducing both humoral and cellular immune responses, especially to O and H anti-

gens, when its enteric-coated capsule formulation is administered orally to humans (Gentschev et al., 2007; Stubi et al., 2000).

Vi polysaccharide, which is a homopolymer of N-acetyl galacturonic acid, has the ability to enhance the virulence of wild-type *S. typhi* (Fraser et al., 2007; Zhang et al., 2008). For example, it can interfere with the host immune response by preventing LPS produced by *S. typhi* from being recognized by TLR4 (Raffatellu et al., 2005; Wilson et al., 2008). Therefore, a method for purifying undenatured Vi polysaccharide produced by *S. typhi* Ty2 was developed by Kwei-Hay Wong in 1974, and an injectable Vi polysaccharide vaccine was subsequently brought to market (Levin et al., 1975; Wong et al., 1974).

Extensive evaluation in clinical trials and many years of postmarketing surveillance have established the safety and efficacy of both the Ty21a and Vi vaccines (Ivanoff et al., 1994; Levine et al., 1988; Plotkin and Bouveret-Le Cam, 1995). Hence, both vaccines have been widely used worldwide. A disadvantage of the Vi polysaccharide vaccine is that it only induces an IgM immune response to the Vi antigen through the Th2-independent immune system, and therefore induces immunity against *S. typhi* for only a short period.

To address this limitation, Vi polysaccharide vaccines conjugated with proteins, such as tetanus or diphtheria toxoids, cholera toxin B subunit, or recombinant mutant *P. aeruginosa* exoprotein A (rEPA) vaccine, have been evaluated to determine whether they induce a long-lasting Th2-dependent immune response. The results of several clinical studies have shown that there is a high seroconversion rate of the Vi antigen in people of all ages, including toddlers that lack the IgM immune response to polysaccharides (Kossaczka et al., 1999; Levine and Noriega, 1995; Lin et al., 2001; Szu et al., 1994; Szu et al., 1994). Of these protein-conjugated Vi polysaccharide vaccine candidates, the one recognized as being most promising is a conjugate of Vi polysaccharide bound to rEPA (Vi-rEPA). Clinical trials have recently demonstrated that Vi-rEPA induces anti-Vi IgG antibody, is very safe and effectively prevents typhoid fever for at least 4 years in children aged 2-5 years (Lin et al., 2001).

COMBINATION VACCINES

A combination vaccine contains two or more immunogens within one formulation. Polyvalent vaccines offer many advantages over single-antigen vaccines, including decreased vaccination frequencies, reduced delivery and vaccination cost, increased vaccine coverage rate, and the immunization of populations that are difficult to access for geographic or cultural reasons. Hence, many combination vaccines have already been developed and they are expected to occupy more than 50% of the world market in the near future.

There are a number of special considerations when formulating combination vaccines, including the possibility of chemical and immunological interactions. Chemical interactions can cause incompatibility between the antigens and adjuvants, incompatibility between the antigens and preservatives, damage to the B cell epitope by impurities, and decreased antigen stability. For example, whole-cell pertussis antigen (wP) and alum hydroxide gel are an unsuitable combination for injectable vaccines, as they can form a strong aggregate due to their opposite electrostatic properties at regular formulation pH values. Therefore, combination vaccines with a wP component require the use of an alum phosphate gel instead of an alum hydroxide gel as an adjuvant (Bae et al., 2003). Thimerosal cannot be used in combination vaccines that contain inactivated poliovirus vaccine (IPV) because IPV is highly labile in the presence of thimerosal. For vaccines containing HBsAg proteins (such as DTP-HepB), a highly purified DT (diphtheria and tetanus toxoids) bulk is required, because any impurities in the DT bulk can result in instability of the HBsAg (Bae et al., 2002).

It is also important to consider immunological interactions when developing combination vaccines, because the coadministration of different antigens can induce a Th1/Th2 immune response that is unfavorable with respect to a specific antigen. For example, a subunit vaccine requiring a Th2-inducible adjuvant such as alum would significantly reduce the effectiveness of a live attenuated viral vaccine requiring a Th1-dominant immune response. There have been reports of a decrease in the immune response to HepB vaccination in people given a combined DTaP-HepB vaccine, and of a significantly reduced immune response to *Haemophilus influenzae* type b (Hib) and FHA in recipients of a combined DTwP-Hib (tetanus toxoid-conjugated PRP (polyribosylribitol phosphate)) vaccine. These decreased efficacies are believed to be due to immunological incompatibility between antigens, or between antigens and impurities of DT (Andre, 1999; Bae et al., 2003; Bae et al., 2002; Greenberg et al., 2000; Pines et al., 1999; Sesardic et al., 1999; Yoo et al., 2000).

Researchers have overcome such challenges in

order to develop the many combination vaccines now marketed around the world. Combination vaccines can generally be divided into four major classes: those based on DTaP, DTwP or HepB, and combinations of live attenuated viral vaccines. DTaP-based combination vaccines have primarily been used in developed countries, whereas the low-priced DTwP-based vaccines have generally been used in developing countries. Currently, the most advanced type of vaccine for developed countries is a DTaP-based hexavalent vaccine (DTaP-HepB-Hib-IPV, Hexavac®) developed by Sanofi-Aventis, while the most advanced vaccine used in developing areas is a DTwP-based pentavalent vaccine (DTwP-HepB-Hib, Quinvaxam®) developed by CrucellBerna Biotech Korea (Table IV). The next candidate for developing countries is generally expected to be a DTwP-HepB-Hib-IPV combination vaccine.

PERSPECTIVES

Many valuable new vaccine production technologies have been developed as a result of rapid progress in various areas, including microbial biology, cell culture technology, recombinant DNA technology and immunology. The value of these innovative approaches has been confirmed by their application to the production of a wide variety of vaccines, including bacterial, viral, recombinant subunit, polysaccharide and combination vaccines.

For bacterial vaccines that use toxoids, genetically detoxified vaccine production employing recombination technology is expected to become more popular as the mechanism of action of specific toxins is elucidated. In the field of viral vaccines, the host for viral cultures is likely to shift rapidly from conventional options, such as mouse brains and/or fertilized eggs, to mammalian cells, which offer greater productivity and avoid the potential for viral contamination and other impurities associated with conventional hosts. A detailed understanding of PAMPs and TLRs will enable the design of recombinant subunit vaccines with significantly greater efficacy and enhanced immunomodulatory properties.

The polyvalent vaccines of the future are likely to employ innovative PAMPs, such as CpG motif, to decrease chemical and immunological interactions in combination vaccines by reducing the quantities of the antigens they contain. Rapid advances are expected in the development of conjugation partners for polysaccharide vaccines. This strategy already shows promise for the development of a novel vaccine for prevention of typhoid fever in infants.

Overall, the innovative technologies reviewed in this paper are expected to be the focus of vaccine research and improvement in the future. The use of progressive vaccine production technologies can significantly reduce the cost of vaccines and therefore improve vaccine coverage in low-income populations. It will also enable the development of safer, more effective and more convenient vaccines that can be used in various age groups and in immunocompromized individuals, thereby bringing health benefits to many people worldwide.

Table IV. Commercialized vaccines listed by the WHO and US regulators as combination vaccines

Type	Combination vaccine		Manufacturing company
	WHO-prequalified	USA-approved	
DTaP-based	-	DTaP-IPV	GlaxoSmithKline (GSK)
		DTaP-HepB-IPV	GSK
		DTaP-HepB-Hib-IPV	Sanofi Aventis
DTwP-based	DTwP-HepB	-	Shantha, Bio Farma, GSK, Panacea, Serum Institute of India
	DTwP-Hib	-	Norvatis, Panacea, Sanofi Aventis
	DTwP-HepB + Hib	-	GSK
	DTwP-HepB-Hib	-	CrucellBerna Biotech Korea, Panacea, Shantha
HepB-based	-	HepB-Hib	Merck
		HepB-HepA	GSK
Combination viral vaccines	-	MMR-VZV	Merck

Abbreviations - DTaP: diphtheria-tetanus-acellular pertussis vaccine; DTwP: diphtheria-tetanus-whole cell pertussis vaccine; HepB: hepatitis B vaccine; HepA: hepatitis A vaccine; Hib: *haemophilus influenzae* type b vaccine; IPV: inactivated poliovirus vaccine; MMR: measles-mumps-rubella vaccine; VZV: varicella zoster virus vaccine

ACKNOWLEDGEMENTS

This work was supported by Crucell and Crucell-Berna Biotech Korea. And, we deeply thank Dr. Giuseppe Marzio and Dr. Andrea Dingemans of Crucell for their kind advice and professional discussion in this study.

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