Development of an influenza virus vaccine using the baculovirus-insect cell expression system

Implications for pandemic preparedness

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Contents

Abstract

Influenza (or flu) is a highly contagious, acute viral respiratory disease that occurs seasonally in most parts of the world and is caused by influenza viruses. Influenza vaccination is an effective way to reduce the complications and the mortality rate following influenza infections. The currently available influenza vaccines are manufactured in embryonated chicken eggs, a 40-year old production technology. The research in this thesis was aimed at the design, validation and development of a production process for a recombinant hemagglutinin (rHA) influenza vaccine for the prevention of seasonal influenza. The viral surface protein HA is the key antigen in the host response to influenza virus since neutralizing antibodies directed against HA can mitigate or prevent infection. The baculovirus-insect cell system was selected for the synthesis of rHA molecules. The designed process was used to manufacture candidate trivalent rHA vaccines, which were tested in four clinical studies in a total of more than 3000 human subjects age 18 - 92 to support licensure of FluBlok under the "Accelerated Approval" procedure in the United States (U.S.). These studies demonstrated that the purified rHA protein was well tolerated and resulted in a strong and long lasting immune response. In addition, the novel vaccine provided cross protection against drifted influenza viruses. In response to the emergence of the new H1N1 A/California /04/2009 influenza strain, the outlined design was used to produce a rHA vaccine candidate and merely 6 weeks later, the first batches of vaccine were ready for human clinical testing. There are two especially important advantages to the use of this technology from a public health perspective: First, the insect cell-baculovirus system has demonstrated the potential to facilitate safe and expeditious responses to health care emergencies such as the one currently posed by the novel H1N1 virus pandemic and secondly, the rHA vaccine does not contain ovalbumin or other antigenic proteins that are present in eggs and may therefore be administered to people who are egg-allergic.

Key words

Influenza, rHA, vaccine, baculovirus, insect cells, production, pandemic preparedness

Chapter 1

General introduction

Influenza Disease

Influenza (or flu) is a highly contagious, acute viral respiratory disease caused by influenza viruses that occurs seasonally in most parts of the world. Epidemics occur annually and are the cause of significant morbidity and mortality worldwide [Glezen, 1982]. The symptoms of the flu are similar to those of the common cold but tend to be more severe. Fever, headache, fatigue, muscle weakness and pain, sore throat, dry cough, and a runny or stuffy nose are common and may develop rapidly. A number of complications, such as the onset of bronchitis and pneumonia, can occur in association with influenza and are especially common among the elderly, young children, and anyone with a suppressed immune system.

Influenza affects all age groups and in the United States (U.S.) alone, 25 to 50 million people contract influenza each year, and an annual average of 36,000 deaths in the years 1990 to 1999 [Thompson *et al.*, 2003] and 226,000 hospitalizations from 1979 to 2001 [Thompson *et al.*, 2004] have been associated with influenza epidemics. The majority of the influenza-related serious illness and deaths occur in people over age 65, children younger than 2 years and persons of any age who have medical conditions that place them at increased risk for influenza [MMWR, 2008].

Influenza vaccination is the most effective method for preventing influenza virus infection and its potential severe complications [LaMontagne *et al*., 1983; Quinnan *et al.*, 1983; Barker and Mullooly, 1980; Patriarca *et al.*, 1985; Nichol *et al.*, 1994; Nichol *et al*., 2003]. Antiviral drugs, including amantidines, Tamiflu and Relenza are also approved for use in influenza disease. Their use is recommended when vaccines are contra-indicated and/or in high risk populations. The disadvantage of these antiviral drugs is that they must be used within 24-48 hours after onset of the disease and the use is in general cautioned because of the potential side effects [MMWR, 2008].

Influenza Viruses

Influenza viruses have a segmented genome of linear single-stranded ribonucleic acid (RNA) molecules of negative polarity and are classified in the family *Orthomyxoviridae* [Lamb and Krug, 2001]. Influenza viruses are categorized in three main types, designated A, B, and C, determined by differences between their matrix and nucleoproteins (M and NP). These viruses further differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology [Lamb and Krug, 2001]. Influenza viruses belonging to type A and B can cause epidemic human disease.

The influenza virus particles, usually spherical or filamentous in shape (Fig. 1), are approximately 80-120 nm in diameter. They are surrounded by a lipid-containing envelope and contain ten (Influenza A) or nine (Influenza B) structural proteins. The three large proteins (PB1, PB2 and PA) represent subunits of the viral polymerase and are responsible for RNA transcription and replication of the virus. The M1 protein, which contributes about 40% of the viral protein, encloses the particle underneath the

lipid envelope and is considered to be important in virion morphogenesis. Two distinct surface glycoproteins - hemagglutinin (HA) and neuraminidase (NA) - project from the surface. HA, the major glycoprotein is responsible for the attachment of the virus to specific cell surface receptors and subsequent fusion between the virus and the cell. HA, is interposed irregularly by clusters of NA. The NA of influenza virus cleaves the glycoside bond of neuraminic acid, and is involved in the release of newly formed virions from the surface of infected cells. The ratio of HA to NA is about 4-5:1. Overall, HA represents about 25% of viral protein and NA about 5%. The M2 ion channel protein is only present on the envelope of influenza A viruses. This protein, however, is present in low quantities, at only a few copies per particle.

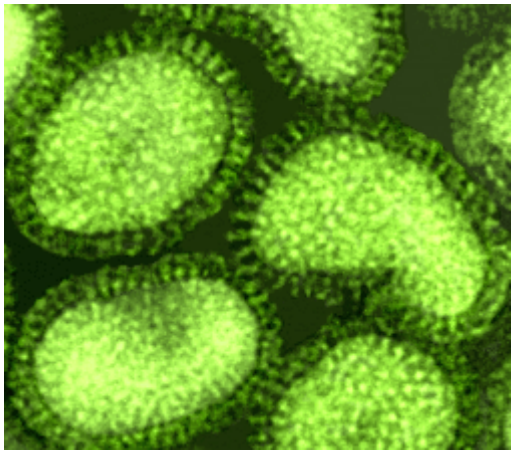

Figure 1a. Electron micrograph of influenza virus particles. The morphology of the influenza virus is somewhat variable, but the virion particles are usually spherical or ovoid in shape and 80 to 120 nm in diameter. On the surface of the virus the HA (≈80%) and NA (≈20%) glycoprotein spikes can be observed. Picture obtained from http://www.wales.nhs.uk/sites3/page.cfm?orgId=719&pid=23110.

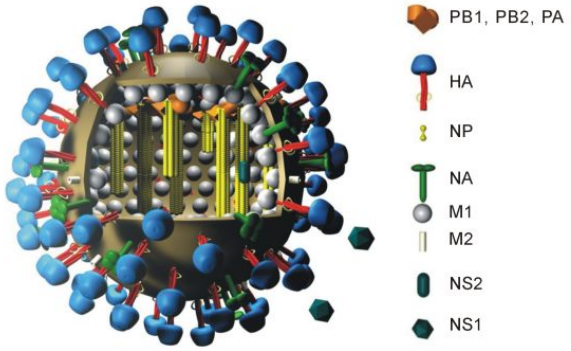

Figure 1b. Schematic view of an influenza A virus. The envelope carries two major glycoproteins, HA and NA, and the transmembrane ion channel protein M2. The matrix protein M1 underlies the bilayer. Within the core of the virus, the single stranded negative sense RNA is associated with the six other viral proteins expressed from its genome: the nucleoprotein (NP), three transcriptases (PB2, PB1, and PA) and two nonstructural proteins (NS1 and NS2).

http://en.wikipedia.org/wiki/File:Flu_und_legende_color_c.jpg

Influenza A and B virions contain eight (8) RNA segments and the total genome length varies between 12000 and 15000 nucleotides (nt) (Fig. 2). The largest genome segment is 2300-2500 nt long (note: the three largest segments encoding the polymerase proteins (P) are co-migrating in Fig. 2) and the smallest 800-900 nt. Most of these viral genome segments code for a single protein.

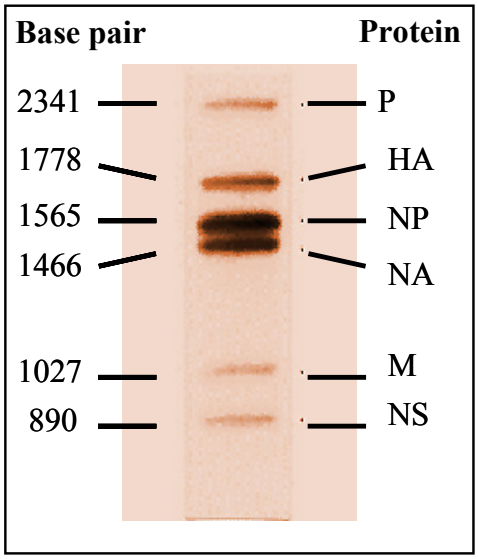

Figure 2. Agarose gel depicting cDNA products derived from influenza viral RNA (vRNA) using a universal primer (5'-AGCAAAAGCAGG-3') complementary to the conserved 3' end of influenza RNA segments. The right panel indicated which proteins are encoded by the respective cDNA fragments. P represents the comigrating cDNA fragments for subunits of the viral polymerase (PB1, PB2, and PA) encoded on segments 1–3. Segment 4 encodes HA, the hemagglutinin glycoprotein important for viral attachment to cell surface receptors and for fusion of the viral envelope to the host cell membrane. Nucleoprotein (NP) on segment five encapsulates vRNAs. Neuraminidase (NA) glycoprotein encoded on segment 6 is involved in the release of newly formed virions from the surface of infected cells using its ability to cleave sialic acid residues. Segment 7 produces a single transcript for two matrix (M) proteins, M1, and M2. M1 plays a structural role to the virus particle while M2 is an ion channel with a postulated role in virus assembly. Alternative splicing yields the nonstructural proteins (NS) of segment 8, NS1 and NS2, involved in vRNA processing.

HA and NA exhibit antigenic variation and their immunologic characterization forms the basis for classification of the influenza A virus subtypes. There are at least 16 different subtypes of the HA antigen, labeled H1 through H16. The first three HA serotypes, H1, H2, and H3, are found in human and swine influenza A viruses. Nine subtypes of influenza NA have been described of which only N1 and N2 have been linked to epidemics in man and swine. Birds are the natural hosts for all influenza viruses, whereas H3, H7, N7 and N8 have also been found in horses. H5, H7 and H9 viruses have been reported in human as well recently [Lamb and Krug, 2001] although human-to-human transmission does not appear to be efficient [Web link 1-1].

New influenza virus variants result from frequent antigenic changes (i.e. **antigenic drift**) resulting from accumulating point mutations that occur during viral replication [Cox NJ and Subbarao, 1999]. New influenza A viruses can also arise as a results of re-assortment of viral RNA (vRNA) molecules between viruses strains (**antigenic shift)**, resulting in a new subtype of the virus. This re-assortment whereby a "new" HA and/or NA is introduced in the circulating viral strains can result in a pandemic as discussed in greater detail below.

Influenza Laboratory Surveillance

The World Health Organization (WHO) and collaborating laboratories are actively involved in influenza surveillance. In this process human influenza viruses around the globe are isolated and characterized. For example, from September 2007 through May 2008, the U.S. WHO and collaborating laboratories characterized approximately 40,000 influenza viruses as influenza A (71%) or influenza B (29%) viruses. Among the influenza A viruses, that were subtyped; 26% were influenza A (H1) and the majority 74% were influenza A (H3) viruses [Web link 1-2] Antigenic characterization of circulating influenza viruses is crucial in determining the annual composition of the influenza vaccine. Viruses are characterized to be antigenically similar to / or distinct (a "drift" variant) from the vaccine component. This characterization is based on ferret serum hemagglutination-inhibition (HAI) antibody

cross-reactivity (i.e. red blood cells clump together [agglutinate] in the presence of hemagglutinin; this process is inhibited in the presence of hemagglutinin antibodies). Antigenic variants selected serologically are then tested for antibody cross-reactivity in human sera to evaluate the potential cross-protection against the antigenic variants provided by the current vaccines and to select vaccine strains for the next season. For influenza B viruses, two antigenically distinct lineages represented by B/Victoria/02/87 and B/Yamagata/16/88 viruses are currently circulating. In February of each year representatives from WHO and the reference laboratories convene in Geneva, review and evaluate the available surveillance data and make recommendations for the composition of the annual vaccine for the Northern hemisphere. Six months later a similar process results in the strain selection for the Southern hemisphere. Predictions have been difficult particularly for the B-lineage; for example, the selection of the B-variant has been problematic for the past many years resulting in a mismatch in the vaccine. As a result an active discussion is ongoing in the U.S. to include representative antigens from both lineages in the vaccine [Web link 1-3].

Influenza Vaccines

HA and NA have been recognized as key antigens in the host response to influenza virus in both natural infection and vaccination. Neutralizing antibodies that can prevent infection are directed against HA. Trivalent inactivated influenza vaccines (TIVs) have been developed to stimulate humoral and (to a lesser extent) cellular immunity to influenza. Live attenuated influenza virus vaccines (LAIVs) mimic a "mild" natural infection and induce high levels of local IgA antibodies in nasal washings, and local cellular immunity, but they induce lower serum antibody IgG titers than the inactivated vaccines. Inactivated influenza vaccines are immunogenic in healthy adults and induce increased levels of HAI antibodies in 70% to 90% of recipients [LaMontagne *et al*., 1983; Quinnan *et al.*, 1983]. In older age groups, the vaccines are less effective in preventing infection, but remain effective in preventing complications and death following influenza infection [Barker and Mullooly, 1980; Patriarca *et al.*, 1985]. Influenza vaccination can reduce hospitalization by about 50% and the risk of death in the elderly by about 75% [Nichol *et al.*, 1994]. Recent data also suggest that influenza vaccinations play a role in reduction of heart attacks and strokes in the elderly [Nichol *et al.*, 2003].

The effectiveness of these vaccines is, however, typically much lower during those seasons when a suboptimal match between vaccine strains and circulating strains is reported. For example, a vaccine efficacy of 50% against culture-confirmed influenza and no measurable efficacy in reduction of influenza-like illness was observed in a study reported by Bridges *et al.* (2000) during a year when a poorly matched strain was circulating, whereas the vaccine efficacy was 86% against laboratory confirmed influenza in the following year when there was a close match between the vaccine and circulating strains. Another study by Belongia *et al.* (2009) reported a vaccine efficacy of only 10% (95% Confidence Interval [CI] -36, 40) for 2004-05, 21% (95% CI -52, 59) for 2005-06 and 52% (95% CI 22, 70) for 2006-07 when percentages of antigenically matched viruses were only 5% during 2004-05 and 2005-06 and 91% in 2006-07. The estimate for the 2005-06 influenza season reported by Belongia *et al.* is very similar to the estimate obtained in a randomized, placebo-controlled trial conducted by GlaxoSmithKline (GSK) during the same influenza season (22.3% (95% CI -49.1, 58.5) [GSK, Study number 104438 (2009); NCT00197223] reported by Beran *et al*. (2009). In addition, a study conducted in

Canada [Skowronski *et al.*, 2009] reported an overall vaccine efficacy estimate of 47% (95% CI 18, 65) for the 2006-07 influenza season, with relatively high efficacy associated with well-matched strains (e.g., efficacy against A/H1N1 of 92% [95% CI 40, 91)]) versus a relatively poor efficacy against the B strains (19% [95% CI -112, 69]) all of which were a lineage-level mismatch to the vaccine. In addition, in the study reported by Wang *et al.* (2009) that compared the effectiveness of LAIV with TIV over three influenza seasons from 2004-2007 found that vaccine effectiveness for TIV ranged from 28-55% and from 10.7-20.8% for LAIV, depending primarily on the degree to which the vaccine strains were antigenically related to circulating wild-type strains. Unfortunately, none of these studies provides insight into the molecular, cellular and pathological basis of the vaccine failures.

Currently available licensed vaccines can be produced at low cost and are relatively effective in reducing the impact of influenza; therefore, the incentive to develop novel influenza vaccines has been relatively limited. The most widely available licensed influenza vaccines consist of inactivated whole or chemically split subunit preparations from two influenza A subtypes (H1N1 and H3N2) and one influenza B subtype. Production of influenza vaccines involves the adaptation of the selected variants for high yield in embryonated chicken eggs by serial passage or reassortment with other high-yield strains. Selected influenza viruses are grown in embryonated chicken eggs by infecting a 12-14 day-old chicken embryo with an influenza virus. Influenza virions are purified three days later from the allantoic fluid. Whole or split virus preparations are then inactivated for instances by treatment with the cross-linking agent formaldehyde [Chiron Vaccines, 2002].

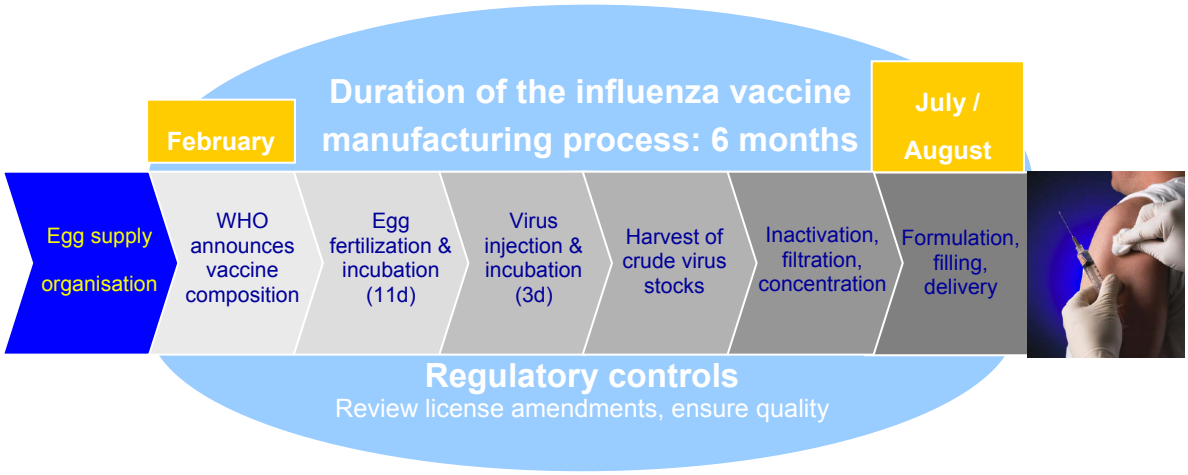

Figure 3. Schematic view of the annual manufacturing cycle for influenza vaccines *(Figure adapted from Datamonitor, 2007)*

Whole virion vaccines use the whole inactivated virus particle and, as a consequence, elicit more adverse effects [al-Mazrou *et al.*, 1991; Carle *et al.*, 1988]. Split virion vaccines are produced by splitting the virus particles by use of detergents or solvents. Split vaccines contain the surface antigens HA and NA, the NP and the M1 protein [Chiron Vaccines, 2002]. The trivalent inactivated subunit vaccines are further purified to remove the internal proteins, leaving mostly HA and NA.

Distinct roles have been attributed to the humoral response elicited by the influenza surface glycoproteins, HA and NA. The antibody against HA generally neutralizes viral infectivity, probably by interfering either with the viral attachment to host surface receptors or with the fusion between viral and endosomal membranes

[Kida *et al.*, 1983; Yoden *et al.*, 1986]. Anti-NA antibody, in contrast, does not prevent infection, but can reduce viral replication below a pathogenic threshold so that infection can occur without disease, presumably by inhibiting release of progeny virus from cells or by aggregating viral particles in the blood and thus preventing further spread of the virus [Beutner *et al.*, 1979; Couch *et al.*, 1974; Kilbourne *et al.*, 1968; Ogra *et al.*, 1997]. As discussed before, conventional vaccines are usually effective in preventing illness by preventing infection when the HA in the vaccine is antigenically similar to the expected wild-type HA strain [Couch *et al.*, 1974; Couch et al., 1971; Kilbourne, 1980; Bridges *et al.*, 2000; Skowronski *et al.*, 2009].
Limitations of the currently available influenza vaccines include:

(i) Reduced efficacy in the elderly. Studies have shown that inactivated influenza vaccine is effective in prevention of influenza in young adults, achieving levels of protection of 70 to 90% [Meiklejohn *et al.*, 1987; Ruben, 1987]. Among the elderly, the rate of protection against illness is lower, especially for those who are institutionalized [Clements, 1992]. Significant antibody responses to a trivalent subvirion influenza vaccine are observed in less than 30 percent of vaccinees of 65 years or older [Powers and Belshe, 1993];

(ii) Production in eggs. The manufacture of influenza vaccines is limited to influenza virus strains that replicate well in eggs and a large supply of eggs is required each year. Production is at risk each year because of the need to find a suitable virus combination. It would also not be feasible to rapidly increase production capacity, for example in the event of a pandemic outbreak – since eggs need to be ordered well in advance and the source (chicken) may be especially vulnerable to avian influenza viruses. In addition, on average one embryonated egg is needed to produce one vaccine dose;

(iii) Inability to respond to late appearing strains such as A/Sydney/5/97 in the late 1990s, or to respond to a potential pandemic strain such as the Hong Kong H5N1 virus that appeared in 1997 and killed the chicken embryos. Reverse genetics has recently been used to create less pathogenic H5N1 viruses that can be grown in chicken embryos; however, the efficacy of such vaccines remains to be proven [Wood and Robertson, 2004];

(iv) Antigenic variation. In response to immune selection, influenza viruses undergo antigenic drift through amino acid sequence changes in the hemagglutinin, and, to a lesser extent, the neuraminidase molecules. Protection with current whole or split influenza vaccines is short-lived, and its effectiveness wanes as genetic changes occur in the epidemic strains of influenza. Ideally, the vaccine strains should be matched to the influenza virus strains causing disease. Changes can occur in the hemagglutinin proteins of egg-grown influenza viruses when compared to primary isolates from infected individuals [Katz *et al.*, 1987; Rajakumar *et al.*, 1990; Wang *et al.*, 1989] resulting in a less effective vaccine;

(v) Adverse Reactions. Less than one-third of those who receive influenza vaccines experience some soreness at the vaccination site and 5% - 10% experience mild side effects such as headache or low-grade fever. The most serious side effect that can occur is an allergic reaction in people who have severe allergy to eggs. Therefore, people with egg allergy are advised not to take influenza vaccine; and

(vi) Inability to achieve the objective of vaccination of all people because of inadequate production capacity and egg-allergy.

Alternative Influenza Vaccines

Most pharmaceutical vaccine development efforts are aimed at producing influenza viruses in cell culture. Solvay [Brands *et al.*, 1999], Novartis, Nobilon and GSK [Percheson *et al.*, 1999] are developing vaccines that are manufactured using Madin Darby Canine Kidney (MDCK) cells, whereas Baxter has selected African Green Monkey Kidney (VERO) cells [Barrett *et al.*, 2009] and Sanofi Pasteur applies a human retina cell line (Per.C6) [Becker, 2004]. Production of influenza viruses in cell systems has proven to be challenging as demonstrated by the fact that none of these manufacturers has been successful in commercializing a cell-based vaccine to date. No such products are marketed despite the fact that two companies (Solvay and Novartis) have received regulatory approval in Europe.

A recombinant subunit vaccine based on HA would be a good choice as resistance to influenza infection correlates with anti-HA antibody levels in serum [Couch and Cate, 1983; Dowdle *et al.*, 1973], the antigenic structure of the HA is of primary importance in strain selection for inclusion in the influenza strain each year and TIV is standardized to contain 15µg HA of each strain represented in the vaccine. Resistance to disease can be correlated with local neutralizing antibody and secretary IgA antibody to HA as well as circulating serum IgG anti-HA antibody [Clements *et al.*, 1987].

Progress in recombinant DNA technology has allowed for the rapid cloning of influenza virus HA genes. Selection of an appropriate expression system that would enable high expression of correctly folded and biologically active HA is, however, critical. An influenza vaccine based on rHA could offer the following advantages:

(i) The influenza rHA antigens could be produced under safe, sterile and stringently controlled conditions using a scalable fermentation process;

(ii) rHA protein could be highly purified and would not contain contaminants from eggs, eliminating possible adverse reactions in individuals with severe egg allergies;

(iii) Since the ability to produce at high levels in eggs is no longer a criterion the vaccine strains with the best genetic match to the influenza virus strains causing disease can be chosen;

(iv) The procedure of cloning, expression, and manufacture of rHA influenza vaccine could be very rapid, allowing for vaccine strain selection later in the year when more reliable epidemiological data are available. Health officials would be better able to respond in the event of the emergence of a new epidemic or pandemic strain of influenza virus; and

(v) Purification procedures for rHA would not need to include virus inactivation or organic extraction procedures, thus avoiding possible denaturing effects and additional safety concerns due to residual toxic chemicals in the vaccine.

Selection of an appropriate expression system for the production of hemagglutinin

The selection of an appropriate expression system for the production of a protein can ultimately determine success or failure of a product. Factors such as time to market, cost of goods, the characteristics of the product, regulatory challenges and patents are all influenced by the expression system that is selected. For example, selecting a novel expression system can hugely affect the time that the regulatory authorities will need to review a biologics license application (BLA) or the biological function of a protein can be affected. Figure 4 below may provide some guidance for selection of a particular expression system for a particular protein.

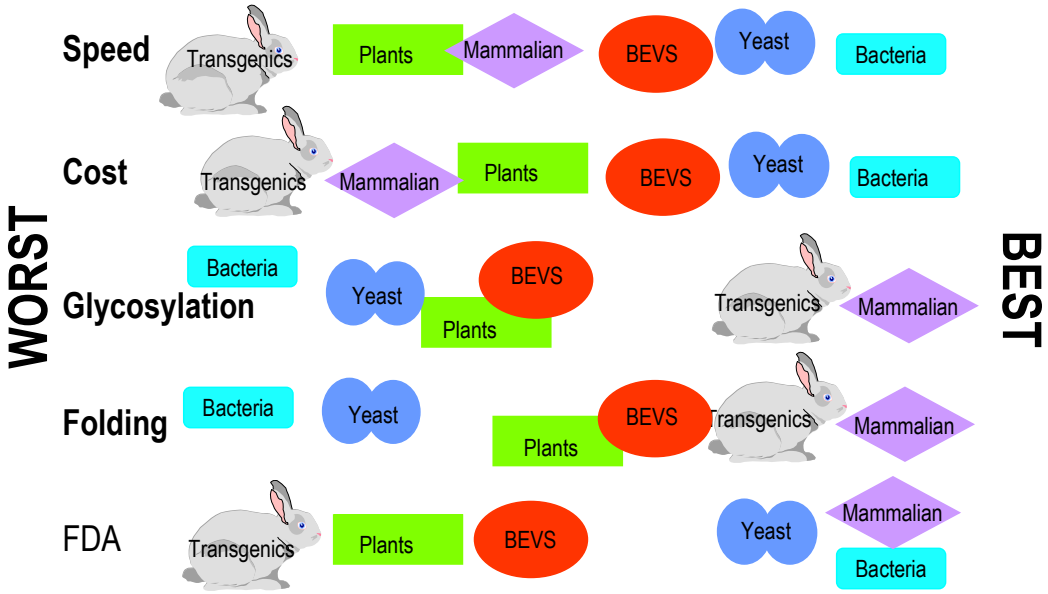

Figure 4. Qualitative ranking of six commonly used protein expression systems
Five important considerations for various expression systems are listed below with *least* attractive on the left and *most* attractive on the right. Bacteria and yeast are expression systems of choice for simple proteins since they are well accepted, cost effective protein production systems. Mammalian production systems are often systems of choice for the production of complex proteins, however development time lines may be long and production costs high. The baculovirus expression vector system (BEVS) provides an attractive "in between solution", while the Food and Drug Administration (FDA) to date has not approved a product made in BEVS the system is generally considered safe.

HA is a complex large protein requiring multiple post-translational modifications, including glycosylation and di-sulfide bond formation (Fig. 5) [Skehel and Wiley, 2000]. Influenza HA forms trimers and it has been reported that HA forms rosettes, which would have a length of twice a trimer [Sato *et al.*, 1983]. Sequence analysis of HA genes and serology data have identified the amino acids of the HA protein that are necessary for neutralizing the influenza virus [Skehel and Wiley, 2000]. These antigenic sites are predominantly located in the membrane-distal ectodomain known as HA1. Host cell endoproteases (e.g. bromelain) cleave HA into an active form consisting of two disulfide-linked fragments, the amino-terminal HA1 subunit and the carboxyterminal HA2 subunit. The HA2 fragment contains the transmembrane portion and the membrane fusion peptide, while the HA1 fragment has a number of glycosylation sites and the sialic acid receptor binding site, in addition to the antigenic determinants of the molecule [Skehel and Wiley, 2000]. Recently however, Sui *et al.*

(2009) identified a neutralizing antibody that bound into the conserved pocket in the stem region of the HA2 domain. The antibody further exhibited cross neutralizing potential, and it was suggested that this region is resistant to neutralization escape. Thus, the HA2 domain may be more important for protection against influenza disease than originally thought.

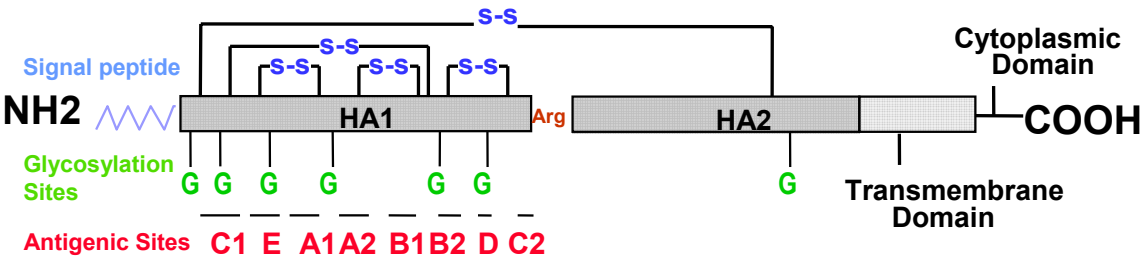

Figure 5. Schematic depicting the primary structure of the HA protein. HA monomers are cleaved by host endoproteases into two disulfide-linked fragments, an amino-terminal HA1 fragment and a carboxyl terminal HA2 fragment. The HA1 ectodomain has the five antigenic sites (A–E) on the surface of its globular headpiece. HA1 also contains many N-linked glycosylation sites (G) and the sialic acid receptor binding site. The HA2 fragment has a transmembrane domain and a hydrophobic peptide that penetrates the target membrane for viral entry. The considerable conformational change required for membrane fusion by the HA2 hydrophobic peptide necessitates the proteolytic cleavage event that releases it from the HA1 domain. The pathogenicity of viral strains has been correlated with the efficiency of this activation event.

The complexity of the HA protein and the modifications required for its biological function warrants the use of a higher eukaryotic expression system such as mammalian or insect cell systems. The baculovirus-insect cell expression system is such a system [Miller, 1981]. In particular the versatility and speed with which new recombinant baculoviruses can be generated make the latter system particularly attractive for the production of an influenza vaccine, which requires annual adjustments. A single, well characterized insect cell line can be used for the production of all influenza proteins, thereby eliminating the time-consuming process of preparing and qualifying a new cell line for each new protein as would be the case for e.g. a stable HA producing Chinese Hamster Ovary cell line [Knezevic et al., 2007]. Finally, manufacturing costs are important for vaccines, which usually do not carry high-profit margins, and also in this aspect the baculovirus – insect cell system is an attractive choice.

The baculovirus-insect cell expression system

The baculovirus-insect cell expression system is well known as tool for the production of complex proteins. Its reputation is one of providing quick access to biologically active proteins. It also has been extensively explored for the production of viral antigens [Oers, van 2006]. Recently, both veterinary and human vaccines manufactured using this production system have been commercialized such as Intervet's Porcilis Pesti (Classical swine fever) and CIRCUMVENT^{TM} (Porcine circovirus), Boehringer Ingelheim's Ingelvac® CircoFLEX^{TM} (Porcine circovirus) and Cervarix^{TM} GSK's bivalent Human Papilloma Virus (HPV 16/18) vaccine against cervical cancer.

Baculoviruses are insect pathogens and can cause fatal disease in lepidopteran, dipteran and hymenopteran larvae. They are primarily used as biocontrol agents of insect pests in agriculture and forestry. The name baculovirus is derived from the Latin word "baculum" (= rod) describing the shape of a baculovirus particle. These particles are present singly (in granuloviruses) or in multiples (in nucleopolyhedrosisviruses) in proteinaceous capsules, referred to as granula, polyhedra or occlusion bodies. Baculoviruses are characterized by their narrow host range [Tinsley and Harrap, 1978] and their inability to replicate in vertebrates including man. Baculoviruses are commonly found on green vegetables and, therefore, are part of the daily diet of healthy individuals. For example, a typical serving of coleslaw contains 112 million polyhedra, each containing multiple baculovirus virions [Heimpel *et al.*, 1973].

Although baculoviruses are not able to replicate in mammalian cells, the baculovirus *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV), the type species of the genus Alphabaculovirus, has .been shown to efficiently transduce a variety of mammalian cells [Kost *et al.*, 2005]. The baculovirus particles or virions contain a large double-stranded DNA genome, on average, depending on the virus species, 130 kilobase pairs in size, which can be easily characterized using genomic restriction digests, Southern blotting and sequencing techniques. In larvae the baculovirus virions are released from the polyhedra in the alkaline environment of the gut and are infecting midgut epithelial cells. After one round of replication a budded virus (BV) form is released into the hemolymph to initiate a systemic infection of the larvae. The BV is phenotypically different, but genetically identical to the occlusion-derived virus (ODV) and can be used to infect insect cell cultures. AcMNPV, for example, can be propagated in cell lines derived from a.o. the fall armyworm *Spodoptera frugiperda* (SF) or the cabbage looper *Trichoplusia ni* (T. ni) [Granados, 1994], which both grow well in suspension cultures [Jehle *et al.*, 2006].

Summers and Smith demonstrated in the 1980s that polyhedrin, the major capsule protein, was not essential for the propagation of the virus in a cell cultures and that its encoding open reading frame could be exchanged for sequences encoding proteins of medical importance such as β-interferon [Smith *et al.*, 1983]. This marked the beginning of the baculovirus-insect cell expression area and thousands of proteins have been produced since using the polyhedrin promoter or later also the p10 promoter to drive expression. A scheme depicting the baculovirus expression system is shown in Figure 6. Insect cells have the capability of performing many of the post-translational modifications such as glycosylation, disulfide bond formation, myristoylation and phosphorylation required for the biological activity of many complex proteins [Miller, 1981; Oers, van 2006]. However, complex glycosylation rarely occurs. Proteins can usually be produced in the baculovirus-insect cell system

in weeks rather than months or years because the virus used to infect the insect cells rather than the cell line is modified. After the baculovirus infects the insect cell, the cell is transformed into a baculovirus DNA and protein production facility until the cell finally dies. In the baculovirus expression system proteins are usually produced under the control of the polyhedrin gene promoter, one of the strongest promoters known in nature. Insect cell-produced proteins are generally biologically active [Oers, van 2006].

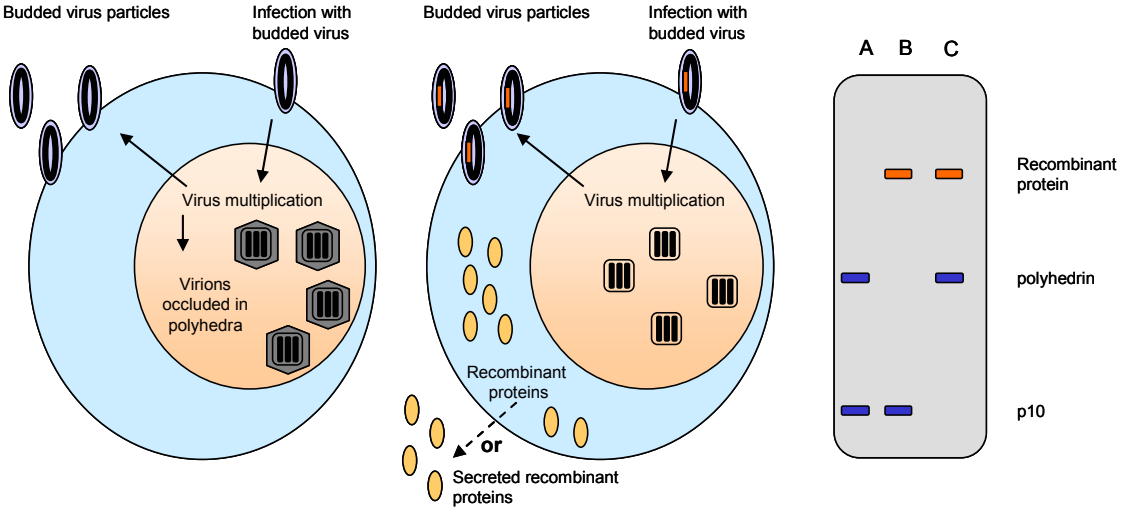

Figure 6. Baculovirus expression system. Left panel shows an insect cell infected with wild type baculovirus (A). The nucleus contains virions, occluded in polyhedra, mainly consisting of the polyhedrin protein. In the middle, the insect cell is infected with a recombinant baculovirus, now recombinant protein is expressed instead of polyhedrin (B) and on the right a SDS-PAGE protein analysis of cells infected with wild type baculovirus (A) or recombinant virus using the polyhedrin (B) or p10 (C) promoter is shown (*Figure was adapted from van Oers and Vlak, 2008)*.

Recombinant baculoviruses that express foreign genes are constructed in various ways and means. The 'classical' way of engineering baculovirus expression vectors is by homologous recombination in insect cells between wild type baculovirus genomic DNA and chimeric plasmids containing the open reading frame (ORF) of the gene of interest with flanking sequences matching the up and downstream regions of the polyhedrin ORF, including the 5' and 3'untranslated regions. Recombinant viruses can be detected by virtue of their distinct plaque morphology; plaques derived from viruses containing the polyhedrin gene have a cloudy appearance, and plaques derived from recombinant viruses in which the polyhedrin gene has been replaced by a foreign gene are clear. The presence of the inserted heterologous DNA in the baculovirus genome can easily be verified using genome digests, Southern blotting or polymerase chain reaction (PCR) techniques. Later on more sophisticated strategies have been used to generate recombinant baculoviruses in insect cells, e.g. by linearizing the baculovirus DNA rendering it non-infectious for insect cells except when recombination occurs between the linear baculovirus DNA and the chimeric plasmid [Kitts and Possee, 1993]

A general scheme for the construction of a recombinant baculovirus for expression of a foreign protein is shown in Figure 7. Coding sequences from a foreign gene are inserted into a plasmid known as a baculovirus transfer plasmid using standard cloning techniques. The transfer plasmid contains the polyhedrin promoter

upstream of a multiple cloning site, coupled to sequences naturally flanking the polyhedrin locus in AcMNPV and a portion of the essential gene ORF1629 located downstream of the polyhedrin locus. The transfer plasmid is co-transfected with baculovirus genomic DNA that has been linearized with an enzyme that removes the polyhedrin gene and part of ORF1629, rendering the non-recombined genomic DNA non-infectious [Kitts and Possee, 1993]. Homologous recombination between the transfer plasmid and the linearized genomic DNA rescues the virus. The efficiency of recovery of recombinant viruses versus non-recombinants is nearly 100%. This process results in plaques that are nearly homogeneous, eliminating the need for multiple rounds of plaque purification. These are undesirable because defective particles may be generated rendering the amplified baculovirus unstable [Kool *et al.*, 1991; Pijlman *et al.*, 2001] and interfering with the replication, and hence protein production, of the baculovirus-insect cell system. A further advance in the engineering of baculovirus expression vectors has been the generation of so-called 'bacmids' in *Escherichia coli* [Luckow *et al.*, 1993]. This system (also commercially available) allows for the production of recombinants via transposition of recombinant plasmids into the AcMNPV genome incorporated into a bacterial artificial chromosome (bacmid), allowing for manipulation of the whole AcMNPV genome in bacteria [Luckow *et al.*, 1993]. This not only speeds up the cloning process, but invariably results in genetically homogeneous baculovirus expression vectors. Unfortunately, viruses generated using bacmid tend to be highly unstable during scale-up and are therefore not suitable for commercial production purposes [Pijlman *et al.*, 2003].

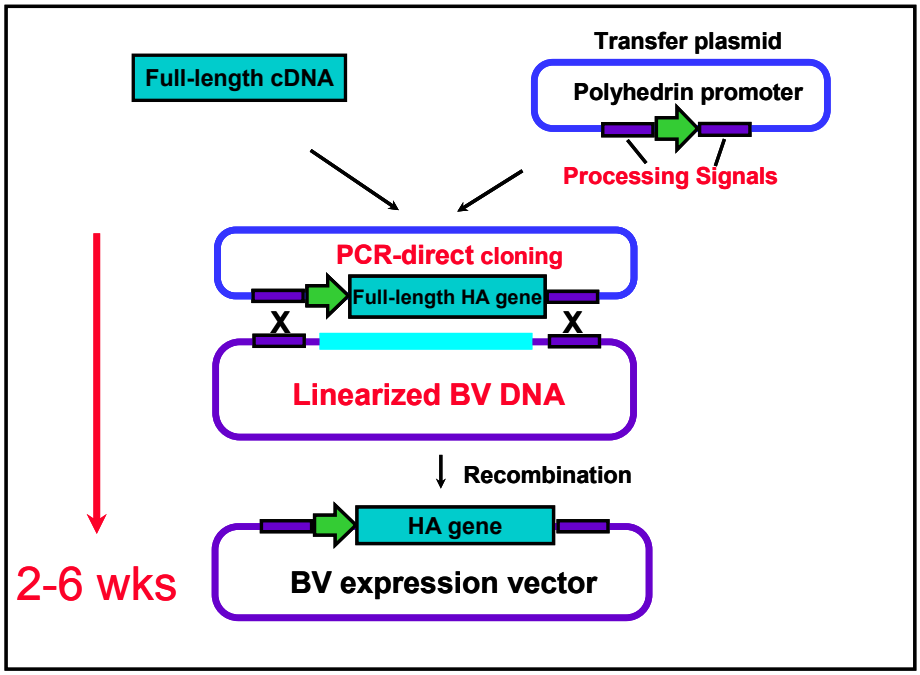

Figure 7. General scheme for construction of a recombinant baculovirus expression vector. The generation of a recombinant baculovirus takes approximately 1-2 weeks. Scale-up of the virus and characterization adds another 2-4 weeks.

Pandemic preparedness

An influenza pandemic is a global epidemic caused by an influenza virus and infects a large proportion of the human population. It is commonly believed that a pandemic occurs as a result of the earlier described "antigenic shift" when a new influenza virus emerges that has not previously, or not for a long period of time, circulated in humans. As a result no immunological memory exists in the human population for such a virus, or may be limited to older adults in the event the virus circulated previously. Three worldwide outbreaks (pandemics) of influenza occurred in the 20th century [Kilbourne, 2006]. The 1918 Spanish flu was the most serious one, killing somewhere between 20 and 40 million individuals. The causative agent was a H1N1 virus, a subtype that is today still actively circulating in humans and is included in the seasonal influenza vaccine. The other two milder pandemics were the Asian influenza in 1957, caused by a H2N2 virus and the Hong Kong influenza in 1968 caused by a H3N2 virus, the other subtype that is today still actively circulating in humans and included in the seasonal influenza vaccine. Pandemic influenza has become a high priority for all public health authorities, especially since the re-occurrence of avian H5N1 viruses that are capable of infecting and killing human beings. Reports from WHO as of March 2, 2009 indicate that the cumulative number of confirmed human cases of avian influenza caused by A/H5N1 is 409 with a case fatality of 56. The good news that we have seen a decline in cases since 2006 with 115 cases reported in 2006, 88 cases in 2007 and only 44 cases reported in 2008. Avian influenza in poultry remains, however, widespread. Most human cases of H5N1 are thought to have occurred as a result of direct contact with sick or infected poultry. Only in a few instances limited, inefficient and unsustained human-to-human transmission was suspected [Web link 1-1].

An effective vaccine will be needed to substantially reduce the impact of an influenza pandemic. Current influenza vaccine manufacturing technology is not adequate to support vaccine production in the event of an avian influenza outbreak and it is clear that new innovative production technology is required.

In 1997 health officials in Hong Kong were first alarmed by the death of a child following infection with a highly pathogenic avian H5N1 influenza strain. This virus had previously caused the death of 70-100% of the chickens in infected flocks in Hong Kong. Before year-end, six out of eighteen infected people died of the disease [Claas *et al.*, 1998]. Fortunately, the efficiency of transmission of this virus between humans was low, but the need for better vaccines became obvious [Belshe, 1998]. Particularly alarming was that the usual egg-based influenza vaccine manufacturing process was incapable of producing a vaccine for this kind of virus because the chicken embryos used for production of the vaccine were killed by this highly pathogenic virus.

The baculovirus-insect cell production technology offered a solution in this emergency situation. As a case, at Protein Sciences Corporation, the cDNA encoding the hemagglutinin gene from the avian H5N1 strain was used to produce a recombinant H5 HA (rHA) sub-unit vaccine in insect cells. Within a period of six weeks a near-authentic H5 rHA antigen was produced that was first tested in chickens. These tests confirmed the immunogenicity of the product and, more importantly, showed protection of chickens from a lethal viral challenge [Crawford *et al.*, 1999]. Four weeks later 1,700 doses were delivered to the National Institute of Health (NIH) for testing in humans and approval was received for compassionate use from the Food and Drug Administration (FDA). A clinical study was conducted using this material and the results suggested that the H5 rHA produced with the baculovirus

– insect cell expression system was able to induce functional antibodies in individuals who had no prior exposure to the H5 viruses [Treanor *et al.*, 2001]. Hence this expression system was shown to provide a powerful rHA manufacturing technology that can potentially provide healthcare solutions in pandemic, biodefense and emergency influenza situations.

Avian influenza has been a problem in the poultry industry for many years. Examples include the North American highly pathogenic outbreak in Pennsylvania in 1983 [Eckroade *et al.*, 1984] and in Central Mexico during 1994 -1995 [Garcia *et al.*, 1996]. Human cases of avian influenza have only been reported since 1997. Table I summarizes the occurrence of human cases and the disease outcome associated with concurrent poultry outbreaks in various geographic regions. The fact that human cases where first identified in Hong Kong, and subsequently in the US, the Netherlands, and Canada, suggests that the availability of improved diagnostic methods in these countries enabled the identification of these avian influenza viruses in humans. In other words, human infection with avian influenza viruses may have previously or in less well equipped countries, gone undiagnosed and may have been more commonly associated with outbreaks in poultry. The other important finding presented in Table I, is that besides H5N1 a wide variety of avian influenza viruses, including the H7 and H9 subtypes, are capable of infecting and causing disease in humans [Web link 1-1].

Table I: Human impact of avian influenza outbreaks in poultry between 1997 and February 2009.

Year	Strain	Impact*	Geographic Region
1997	**H5N1**	**18 (6)**	**Hong Kong**
1999	**H9N2**	**2**	**Hong Kong**
2002	**H7N2**	**1**	**US Virginia**
2003	**H5N1** **H7N7** **H9N2** **H7N2**	**4 (4)** **89 (1)** **1** **1**	**Vietnam/China** **Netherlands** **HongKong** **New York**
2004 – FEB09	**H5N1** **H7N3** **H7N2** **H9N2**	**409 (256)** **2** **4** **1**	**Asia/Africa** **Canada** **U.K.** **HongKong**

**Total number of subjects with disease. In brackets subjects with fatal outcome*

When Hong Kong in 1997 suffered from a severe H5N1 outbreak in poultry, the authorities undertook the following actions: 1.5 million chickens were culled, ducks and geese were removed, two cleaning days per month were introduced in the live bird markets and, finally, poultry flocks were vaccinated with an inactivated H5 vaccine [Webster, 2005]. Unfortunately, the above measures are not followed throughout Asia because they are too expensive. Bird culling is the most common and widespread approach to eradicate avian influenza in developed countries. Despite the

availability of poultry vaccines, countries often elect not to vaccinate their birds because of a potential negative impact on the ability to export the birds.

An influenza pandemic is believed to be imminent and scientists agree that it only is a matter of when, where and what will be the causative agent. Recently, most attention has been directed to human cases of avian influenza caused by a H5N1 avian influenza virus. When Dr. John La Montagne speculated that our strengthened surveillance systems to monitor disease spread and modern diagnostics tools would allow us to slowly see a disease unfold [Gellin, 2005], he was probably right. Currently the avian influenza viruses have not acquired the ability to transmit easily from human to human, but as we continue to monitor the disease and the genetic composition of the viruses as suggested by the work of Taubenberger et al. (2005) we may be able to make useful predictions as to when, where and what the next pandemic will be.

Developing and pre-vaccination with a safe, prophylactic vaccine containing, for example, H2, H5, H7 and/or H9 rHA proteins could stimulate a low level immune response against these viruses to which many people do not have pre-existing antibodies. This is because the viruses, H5, H7 and H9, do not efficiently infect humans or, like H2, have not circulated for the past 40 years. Such a vaccine may be the most effective proactive response to the threat of a potential pandemic. Various studies have suggested that memory against a specific HA subtype can stimulate a broad-spectrum immune response and may prevent from severe disease even in instance when there is not a close relatedness between strains. For example, a vaccine based on 1999 circulating H1N1 virus (A/New Caledonia) was able to cross-protect mice against a lethal challenge with the 1918 influenza virus [Tumpey *et al.*, 2004]. Also, preliminary data from a recently conducted re-vaccination study also suggest that vaccination with recombinant H5 can also prime for booster responses on revaccination with or exposure to drifted strains of H5 [Goji *et al.*, 2008].

The current reports of human cases caused by a novel swine-origin influenza A H1N1 variant in Mexico and the U.S. with high lethality in healthy adults are perhaps of greater concern [MMWR, 2009]. On April 29 2009, the WHO raised the pandemic influenza phase from 4 to 5. The emergence of this novel H1N1 virus has been reported to be the greatest pandemic threat since the emergence of the influenza A (H3N2) in 1968 [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009]. It is unsettling and surprising that an H1N1 virus could cause widespread lethal disease in humans, since H1N1 viruses have been actively circulating in humans during the past decades, even though immunological memory exists within the population.

Scope of this thesis

The main challenge in identifying a suitable manufacturing system for an influenza vaccine is that this system has to be sufficiently flexible to deliver the annual updates in a timely manner. The final determination for the composition of the influenza vaccine is made by the WHO in February and the vaccine needs to be available for administration preferably in September but not later than October, leaving only six months to complete the production of a vaccine that can have as many as three new components each year. In addition, the protein produced has to be immunogenic and exhibit the same characteristics as the licensed inactivated influenza vaccine, i.e. it has to be safe and able to prevent influenza illness.

The aim of this thesis is to evaluate whether the baculovirus-insect cell production system can be used to produce an influenza vaccine for both seasonal and pandemic emergency use. The work described in this thesis forms the basis for a biologics license application (BLA) for FluBlok.

In **Chapter 2**, the time needed to clone the influenza HA into baculovirus vectors is determined and the biochemical properties of rHA produced in the baculovirus-insect cell system are compared with those of viral HA. To this aim, various methods were developed to assess its biological activity. The greatest challenge is, however, to purify different rHA proteins within the short time available. A universal purification resin, lentil lectin was initially tried that specifically binds glycoproteins produced in insect cells. While this purification process is simple and can be implemented readily within the short development time available, it will not be suitable for commercialization of an insect cell vaccine. Residual lentil lectin resin causes safety concerns since many people are allergic to lectins. Nevertheless, the rHA produced using this method was analyzed in clinical studies for its immunogenicity.

In **Chapter 3** the focus is on developing a more scalable production process, methods to monitor rHA protein production and additional methods to characterize the rHA.

The questions whether rHA alone would be adequate to provide protection against influenza infection and whether the differences in glycosylation of rHA produced in insect cells compared to rHA synthesized in mammalian cells would impact the performance of the vaccine are addressed in **Chapter 4**.

Reduction in vaccine effectiveness of the licensed vaccine has been reported when the influenza vaccine was not well matched to the circulating strain [MMWR, 2004; Bridges *et al.*, 2000; Belongia *et al.*, 2009; GSK Study number 104438, 2009; Beran *et al.*, 2009; Skowronski *et al.*, 2009; Wang *et al.*, 2009]. To address the question whether more antigen can result in cross-protection of the vaccine against drifted viruses, ten H3N2 influenza viruses were isolated from six placebo recipients and four low dose vaccine recipients and characterized by sequence analysis in **Chapter 5.**

Questions and concerns that specifically relate to the safety of a novel cell substrate are addressed in **Chapter 6**. The specific concern regarding the potential presence of latent viruses in the cell line is addressed by transmission electron microscopy of stressed insect cells. Furthermore, PCR methods are developed for various adventitious agents that have been described for insects in general and for a specific virus that was described previously for a related insect cell line *T. ni* High Five [Li *et al.*, 2007]. Additionally, fluorescent PCR-based reverse transcriptase testing is performed at the end of the production procedure to rule out increased

presence of retrovirus-like particles. In addition, the safety and immunogenicity was assessed in clinical studies that are used to support licensure of the rHA-based subunit vaccines (FluBlok®) in the U.S. The total safety database for FluBlok includes 2497 subjects 18-49 years and 736 subjects older than 50 years who received the commercial formulation of FluBlok.

In **Chapter 7,** the general discussion, we assess the importance of this work for pandemic preparedness and public health in general. The recent reports of human cases in healthy adults of a novel swine influenza H1N1 variant in Mexico, the U.S. and its subsequent rapid spread around the globe are of major concern [MMWR, 2009], and demonstrate the need for a vaccine that can be produced rapidly and in large quantities. We further assess how FluBlok can be further improved both from a manufacturing (i.e. what can be done to improve production yields) and product quality (i.e. how can vaccine performance be improved) stand point. Also a critical assessment is provided of how FluBlok compares to other influenza vaccines in development (i.e. the baculovirus insect cell expression system versus alternative influenza vaccine production systems and the importance of inclusion of other influenza antigens [N and/or M])

Chapter 2

Production of a recombinant influenza vaccine using the baculovirus expression vector system

Summary

A method to produce recombinant influenza hemagglutinin (rHA) in the baculovirus-insect cell system aimed at seasonal vaccine development is described. Recombinant full length HA molecules from the three strains recommended by the World Health Organization for the 2003-2004 influenza season were cloned, expressed and purified. The production process for rHA isolated from the influenza virus A/Panama/2007/99 is described in detail. This process is adjusted slightly for rHAs derived from the other influenza strains. The biological activity of rHA expressed in insect cells was confirmed using a variety of methods. For example, a hemagglutinin assay demonstrated the ability to agglutinate red blood cells, a trypsin digest assay showed that the rHA was resistant to digestion and electron microscopy showed rosette-like structures. Therefore, we conclude that the baculovirus-insect cell system can support the production of full-length biologically active rHA proteins, which may potentially be used in a seasonal influenza vaccine.

This chapter is modified from: Holtz, KM, Anderson, DK and **Cox, MMJ** Production of a Recombinant Influenza Vaccine Using the Baculovirus Expression Vector System. *Bioprocessing Journal* 2003; 2: 25-32.

Introduction

Influenza is a highly contagious, acute viral respiratory disease that occurs seasonally in most parts of the world. The infection resides primarily in the respiratory tract (nose, throat and bronchi), but causes both local and systemic symptoms including fever, chills, cough, headache, myalgia, sore throat, and malaise. Influenza-related pneumonia is the main complication of infection. Annual epidemics cause significant morbidity and mortality worldwide [Glezen, 1982; MMWR 1993; MMWR 2000]. Each year, influenza infections result in an average of 110,000 hospitalizations, approximately 20,000 of which result in death [MMWR 2000]. These deaths are heavily concentrated (>90%) among persons who are at highest risk for influenza-related complications - elderly adults (over 65), children under age five, patients with pre-existing respiratory or cardiovascular disease, and women in the third trimester of pregnancy. Thus, the prevention of influenza virus infection is a major public health priority.

Influenza viruses are enveloped, negative-sense RNA viruses belonging to the family Orthomyxoviridae. The RNA genome is segmented into eight fragments that code for 10 proteins. The influenza viruses are divided into three types, A, B, and C, based on differences in nucleoproteins and matrix proteins. Although all cause disease in humans, influenza A also infects a wide variety of avian species and mammals. Influenza A causes the most serious respiratory illness in humans, whereas influenza C infections are of subclinical importance. The greater pathogenicity of influenza A, and to a lesser extent influenza B, has been attributed to the antigenic variability of the two major surface glycoproteins important in viral infection and immunity, hemagglutinin (HA) and neuraminidase (NA). HA mediates viral attachment by binding to sialic acid residues on host cell surface receptors and, following endocytosis, fuses the viral envelope to the cell membrane in a pH-dependent process; NA cleaves sialic acid residues from HA molecules and cell surface proteins, thus releasing budding progeny virions and preventing reinfection of the same cell. Differences in the HA and the NA surface antigens distinguish types A and B, as well as define subtypes of influenza A. Of the 16 recognized HA subtypes (H1-H16) and nine recognized NA subtypes (N1-N9), only three HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) are commonly isolated in humans [Zambon, 2001; Lamb and Krug, 2001].

Protection against influenza disease and infection is conferred primarily through HA, which stimulates production of anti-HA antibodies. In contrast, the anti-NA antibody response does not prevent disease but may slow its spread by reducing the release of progeny virions [Johansson, 1999]. Sequence analysis of HA genes and serology data have identified the amino acids of the HA protein targeted by neutralizing antibodies. These antigenic sites are predominantly located in the membrane-distal ectodomain known as HA1. Host cell endoproteases cleave HA into an active form consisting of two disulfide-linked fragments, the amino-terminal HA1 subunit and the carboxyterminal HA2 subunit. The HA2 fragment contains the transmembrane region and the membrane fusion peptide, while the HA1 fragment has a number of glycosylation sites and the sialic acid receptor binding site, in addition to the antigenic determinants of the molecule [Skehel and Wiley, 2000].

The protective efficacy of anti-HA neutralizing antibodies is continually challenged by the rapid mutation rate of the influenza genome. Replication errors introduced by influenza's low-fidelity RNA-dependent RNA polymerase can lead to amino acid substitutions [Wright and Webster, 2001]. In particular, point mutations

introduced to the antigenic sites HA may render preexisting, protective HA antibodies ineffective. 'Antigenic drift' refers to the progressive generation of molecular variants of existing influenza strains through replication and leads to annual epidemics [Zambon, 1999]. In contrast, 'antigenic shifts' have pandemic potential because novel viral strains of unpredictable pathogenicity are generated from the exchange of genomic segments from different influenza viruses (genetic reassortment) or from interspecies transmission of viruses [Zambon, 1999]. The direct transmission of a highly pathogenic avian influenza strain (H5N1) from chickens to humans occurred in the Hong Kong bird flu epidemic of 1997 that killed 70–100% of infected stocks and caused the death of six out of 18 infected people [Claas *et a*l., 1998]. The low transmission efficiency of this viral strain among the human population prevented a pandemic, however, the situation did underscore the need for a better vaccine to handle potential crises [Belshe, 1998].

Vaccination of high-risk persons each year before the flu season is the most effective measure for reducing the impact of influenza [MMWR, 1993]. The current licensed trivalent inactivated vaccine (TIV) consists of three chemically inactivated viruses (two A strains, H1 and H3, and one B strain) generated in embryonated hen's eggs [Treanor *et al.*, 1996; Lakey *et al.*, 1996]. These vaccines are immunogenic in healthy adults and induce an increase in hemagglutination inhibition (HAI) antibodies in 70% to 90% of recipients [Quinnan *et al.*, 1983; LaMontagne *et al.*, 1983]. In older age groups, TIV is less effective in preventing infection but can prevent complication and death following influenza infection [Barker and Mullooly, 1980; Patriarca *et al.*, 1985]. Adverse side effects including local soreness, erythema, and induration are commonly associated with this vaccine and may contribute to its poor acceptance among high-risk groups [Fedson, 1987; Govaert *et al.*, 1993]. In addition to the reduced efficacy in the elderly and the side effects, other drawbacks of TIV are related to the production process. TIV relies on a yearly supply of pathogen-free eggs for vaccine production. Also, the adaptation process typically required for high-yield production of the vaccine in eggs causes antigenic deviations in the vaccine strains compared to field isolates [Robertson *et al.*, 1987; Katz *et al.*, 1989]. Furthermore, the egg-based process is incapable of producing vaccines for highly pathogenic avian strains, such as H5N1, because they are lethal to chicken embryos [Wood *et al.*, 2002; Wuethrich *et al.*, 2003].

Several studies have shown that vaccines containing purified recombinant influenza HA produced in insect cells using the baculovirus expression vector system (BEVS) are safe, well-tolerated, and immunogenic in humans [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Johansson, 1999; Treanor *et al.*, 2001]. Based on randomized, double-blind, controlled clinical studies, these recombinant HA vaccines produce fewer side effects and yield enhanced immunogenicity at higher doses (up to 135 μg HA) in elderly and healthy adult populations when compared to TIV (15 μg HA/strain) [Keitel *et al.*, 1994; Keitel *et al.*, 1996]. This production system also permits selection of influenza strains later in the season for better genetic matching between circulating strains and vaccine strains, and avoids the dependence on an egg supply. The latter was extremely important in quickly responding to the outbreak in Hong Kong of the pathogenic H5N1 avian strain. Following tests in chickens confirming immunogenicity and protection from a lethal viral challenge, 1,700 doses of the BEVS/insect cell-derived H5 HA antigen were quickly produced for human clinical testing [Crawford *et al.*, 1999]. The H5 HA antigen was able to induce functional antibodies in individuals with no prior exposure to the H5 virus, thus demonstrating the utility of this system in a potential pandemic

crisis [Treanor *et al.*, 2001].

This paper describes the production and purification of recombinant HA molecules from the three strains recommended by the World Health Organization (WHO) for the 2003-2004 influenza season for use in a trivalent recombinant influenza vaccine. Using the antigen isolated from the influenza strain A/Panama/2007/99 as a representative example, recombinant HA produced using BEVS in insect cells is highly purified, properly folded, and biologically active.

Materials and Methods

Influenza vaccine strains and their propagation

Based on the recommendation of the WHO, the following influenza strains were obtained from the U.S. Centers for Disease Control and Prevention (CDC) as vaccine strains: A/New Caledonia/20/99 (H1N1, referred to as H1 HA), A/Panama/2007/99 (H3N2, referred to as H3 HA), and B/Hong Kong/330/2001 (referred to as B HA). Viral titers were determined in a standard hemagglutination assay using chicken red blood cells (RBCs) [Barrett and Inglis, 1995]. The influenza viral stocks were amplified in Madin Darby canine kidney (MDCK) cells (ATCC CCL34) by infection at a low multiplicity of infection (0.1 to 0.5). Infection was allowed to proceed at 37° C for 48 h while virus production was monitored in the media using the hemagglutination assay [Barrett and Inglis, 1995].

Cloning HA genes

Influenza virions for each of the strains were isolated from the MDCK culture and viral RNA was purified using the Qiagen RNeasy kit. The purified viral RNA served as a template in RT-PCR reactions (Titan One Tube RT-PCR System, Roche) to generate cDNA for each of the desired HA molecules (H1, H3, and B). For H3 HA, the RT-PCR was performed with primers designed to directly generate a cDNA gene fragment with 5' and 3' ends compatible for overlap extension (OE) PCR (Fig. 1). In OE PCR, the 5' end of the HA cDNA fragment anneals to the complementary sequence found on the 3' end of a PCR amplified fragment of the transfer plasmid pPSC12 (= MGS12 in Smith *et al.*, 1993) containing the polyhedrin promoter and the baculovirus chitinase signal sequence. Overlap occurs in the region of the baculovirus signal sequence and extension gives a full-length product for amplification in subsequent PCR cycles. The final OE product for each HA molecule includes a seamless fusion of the baculovirus chitinase signal sequence to the DNA sequence encoding the N-terminus of the mature protein and a *Kpn*I restriction site incorporated downstream of the stop codon. The OE-PCR products are then digested with *Kpn*I and *Ngo*MIV, gel purified, and cloned into the baculovirus transfer vector, pPSC12, similarly digested with *Kpn*I and *Ngo*MIV, Candidates for DNA sequencing were selected based on restriction digest analysis. The genes for H1 HA and B HA were cloned using a similar method.

DNA and amino acid sequence of the H3 HA gene

The DNA sequence of the cloned H3 HA gene was determined using primers that anneal to flanking sequences in the pPSC12 plasmid vector and using internal primers spaced roughly every 300 nucleotides. The sequencing reactions were performed by

MWG Biotech and the resulting sequence data assembled and analyzed using the SeqMan program (Lasergene, DNAstar, Inc.). The sequence of the cloned H3 HA gene is identical to the sequence of the HA1 region (N-terminal portion) published for this strain. No sequence for the HA2 region (C-terminal portion) is published for this HA. To analyze this region, the sequences of the HA clone was compared to known sequences of related HA strains from the H3 subtype. A/Panama/2007/99 H3 HA differs at only one position (R470K) over this region from its closest match among the H3 HA subtypes. Sequence comparison of various published H3 subtypes reveals variability in the amino acid sequence at position 470 (often a lysine substitution) (*unpublished data*). Thus, we conclude the Panama clone is correct.

Transfection and isolation of recombinant baculoviruses

Sf9 insect cells (ATCC CRL1771) were cotransfected with linearized AcMNPV baculovirus genomic DNA (PSC internal code AcB729.3) and the recombinant baculovirus transfer plasmid for H3 HA using the calcium phosphate precipitation method. During this process, the expression cassette was transferred from the transfer plasmid into the baculovirus genome via homologous recombination. The cotransfected cells were harvested by centrifugation, and the supernatants were used to grow isolated plaques on plates containing Sf9 cells. Recombinant plaques having a distinctive (clear) morphology were selected to generate virus stocks.

Generation of virus stocks

The isolated plaques were added to T-25 flasks containing Sf9 insect cells in 5 mls of TNM-FH medium with 5% fetal bovine serum (FBS) to generate passage one (P1) viral stocks. After incubation for five days at 28° C, the infected cells were harvested and removed from the culture medium by low-speed centrifugation. One milliliter of the supernatant containing the P1 virus stock was used to inoculate a 50 ml culture of *expres*SF+ (SF+) cells (ATCC CRL12579) in serum-free medium (Protein Sciences Fortified Medium, PSFM), at a density of 1.5×10^6 cells/ml, in a 100-ml spinner flask. Following incubation for 48 h at 28° C with stirring (100 rpm), the infected cells were removed from the culture supernatant (P2) by low-speed centrifugation. One milliliter of the P2 virus stocks was used to infect a 3-L spinner flask containing 500 ml of SF+ cells at a density of 1.5×10^6 cells/ml in serum-free PSFM cell medium. After incubating the culture at 28° C on a stir plate (100 rpm) for 72 h, the cells were removed by low-speed centrifugation, and the supernatant (P3) was titered by plaque assay.

Fermentation and harvesting of recombinant HA

Cultures of SF+ cells (10L to 45L) in serum-free PSFM medium at a density 1.5×10^6 cells/ml were infected with P3 virus stock at a multiplicity of infection (MOI) of 1. The reactors were maintained at 28° C with a stirring speed of 200 rpm and dissolved oxygen (DO_2) setting of 60%. The cultures were harvested by low-speed centrifugation at 72 hours post infection (hpi) and the supernatants discarded. The cell pellets containing membrane-bound HA were further processed.

Isolation of recombinant HA

The cell pellet was resuspended in 50 mM ethanolamine, 0.3 M NaCl, 0.1% ß-ME pH 9 followed by centrifugation at 6,000×g for 30 minutes at 4° C. The supernatant containing membrane proteins was discarded. The remaining pellet was then washed

with 50 mM ethanolamine, 0.1% ß-ME pH 9 and centrifuged as described above. The supernatant containing contaminating proteins was discarded. The remaining pellet was resuspended in extraction buffer containing 50 mM ethanolamine, 1% Triton X-100, 0.1% ß-ME pH 9. The resuspension was homogenized for five minutes followed by 15 min incubation at 4° C. The extracted cell pellet was centrifuged as before and the supernatant containing solubilized HA was stored on ice until further processing.

Chromatography

A generalized purification strategy for HA proteins produced in insect cells using BEVS has been established using a combination of ion exchange chromatography and affinity chromatography. The specific protocol used to purify A/Panama/2007/99 H3 HA was as follows. The H3 HA-containing supernatant was applied to an anion exchange (Q Sepharose) column equilibrated in 50 mM ethanolamine, 0.1% Triton X-100, 0.01% ß-ME pH 9. HA flows through this column while bound proteins elute with 0.2 M – 2.0 M NaCl.The Q flow-through (containing HA) is applied to a lentil lectin affinity column equilibrated in 0.5 M NaCl, 50 mM ethanolamine, 0.1% Triton X-100, 0.01% ß-ME pH 9. Lentil lectin is an affinity matrix that reversibly binds polysaccharides and glycoconjugates containing glucose and mannose type sugar groups. The column is washed with equilibration buffer containing 0.5 M NaCl to prevent non-specific protein interactions. The pH and conductivity of the column were adjusted with 20 mM Tris, 0.1% Triton X-100, 0.01% ß-ME pH 7.4 followed by HA elution with 0.5 M N-methyl-α-D-mannopyrannoside in 20 mM Tris, 0.1% Triton X-100, 0.01% ß-ME pH 7.4. The HA-containing material from the lentil lectin column is loaded onto a cation exchange (CM Sepharose) column equilibrated in 20 mM Tris, 0.1% Triton X-100, 0.01% ß-ME pH 7.4. After loading and washing the column with equilibration buffer, 10 mM sodium phosphate, 0.01% Tween-20 pH 7.4 is used to exchange the detergents and remove ß-ME. The purified HA is then eluted with 150 mM NaCl, 10 mM sodium phosphate, 0.01% Tween-20 pH 7.4.

Hemagglutination activity assay

This method was performed as described by Barrett and Inglis (1995). Briefly, chicken RBCs are washed with phosphate buffered saline (PBS) and suspended as a 0.5% solution in PBS. HA is serially diluted in PBS buffer in wells of the assay plate and an equal volume of RBCs is added. The plate is covered, incubated at 2–8° C for 30 minutes to one hour and then scored for agglutination. Agglutination is observed as a uniform cell suspension. In the absence of agglutination, the cells settle out and form compact pellets.

Trypsin digest assay

HA is incubated for 30 minutes at 0° C without or with 50 µg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. Denatured samples of HA were prepared by boiling the samples for 10 minutes prior to trypsin treatment.
The TPCK treatment inactivates any remaining chymotrypsin activity of trypsin. Modified trypsin cleaves Lys-Pro and Arg-Pro bonds at a much slower rate than bonds between other aminoacid residues (Perona, 1995).

Results

Three vaccine strains, A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Hong Kong/330/2001 were obtained from the CDC under WHO recommendations and used to infect MDCK cells in culture. mRNA purified from the virions was used to generate cDNA of the HAs from each strain. Using overlap extension PCR (Fig. 1), the HA genes were seamlessly fused to the baculovirus chitinase signal sequence behind the polyhedrin promoter and subsequently cloned into the PSC transfer plasmid pPSC12. Insect cells were cotransfected with the transfer plasmid and linearized AcMNPV baculovirus genomic DNA. The supernatants of the cotransfected insect cells were subjected to plaque assay, and stocks of selected recombinant baculoviruses were prepared. Virus stocks of the recombinant baculoviruses were scaled and used to infect SF+ insect cells in bioreactors. Recombinant HA was localized to the insect cell membrane and subsequently purified as shown in Figure 2 and 3 below.

Extraction of HA

The chitinase signal sequence present in the PSC vector directs the HA molecules to the secretory pathway for glycosylation. However, the hydrophobic C-terminal membrane anchor peptide results in protein docking in the insect cell membrane. A general extraction scheme was devised in order to simplify isolation of the three recombinant HA products during manufacturing. The first cell pellet wash containing 0.3 M NaCl in 50 mM ethanolamine, 0.3 M NaCl, 0.1% ß-ME pH 9 removes cytosolic and periphery membrane proteins in the supernatant (Fig. 2). The second wash without NaCl removes additional loosely bound proteins and lowers the conductivity for detergent extraction with 1% Triton X-100. Extractions carried out at pH 9 give improved efficiency relative to extractions performed at pH 7 (not shown). Typically, the efficiency of protein extraction is 60% or greater using 1% Triton X-100 at pH 9. However, the estimated 40% HA remaining bound to the membrane after solubilization is not extractable with additional detergent washes and most likely represents an improperly folded, inactive population of molecules that have not been fully processed.

HA Purification

Solubilized HA in 1% Triton X-100 flows through the Q-Sepharose anion exchange column at pH 9. As shown in Figure 3A, HA is found in the column flow-through and column wash while a significant amount of contaminating proteins and nucleic acids binds to the resin and elutes with sodium chloride. The Q-flow-through containing HA is applied directly onto a lentil lectin affinity column. This resin has a high specificity for non-reducing α-mannopyranosyl terminal residues that are typically produced in insect cell cultures. The HA binds tightly to the resin while contaminating proteins flow through and HA elutes with 0.5 M N-methyl-α-D-mannopyrannoside.

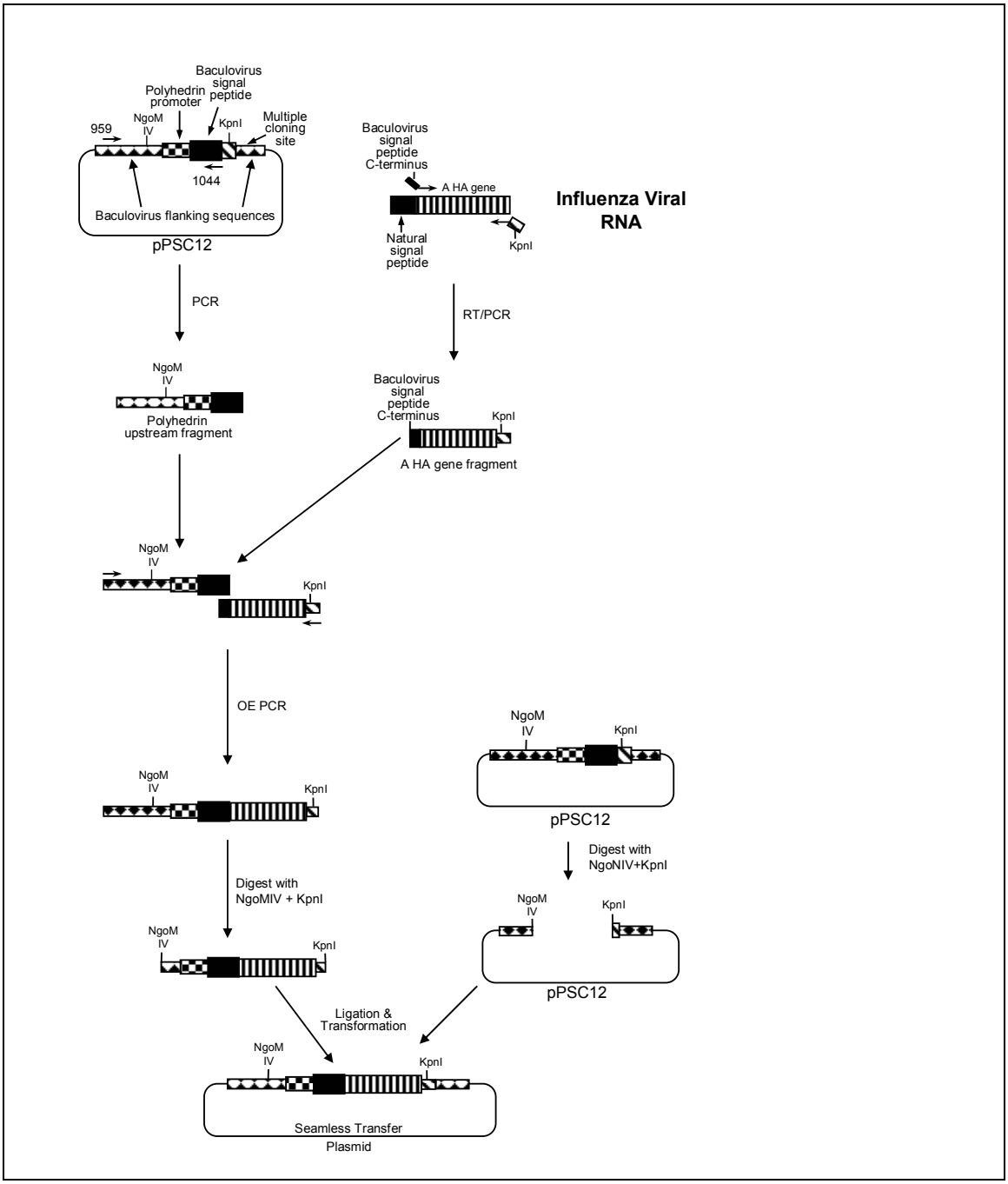

Figure 1. Cloning strategy for insertion of the influenza A virus H3 HA coding sequence into a PSC baculovirus transfer plasmid. The 5' end of the RT-PCR generated cDNA HA gene fragment has a nucleotide sequence that is complementary to the 3' end of a pPSC12 gene fragment generated by standard PCR. In OE-PCR, the complementary regions of these two fragments anneal and are extended. The resulting amplified product is digested and ligated into the pPSC12 vector. The final construct contains the H3 HA coding sequence seamlessly fused to the PSC signal sequence downstream of the polyhedrin promoter.

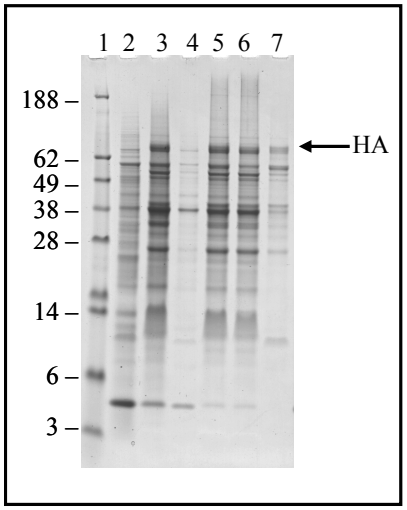

Figure 2. General procedure for extracting HA from the insect cell membrane. After washing cells in the presence and absence of NaCl (0.3M) using ethanolamine buffer, pH 9, to remove loosely associated proteins, the membrane proteins are solubilized with 1% Triton X-100 in ethanolamine buffer pH 9. The band corresponding to HA is denoted by the arrow. Based on the amino acid sequence, monomeric HA has a molecular mass of 64 kDa; however, each trimannose core unit added to the protein by insect cells is an additional 1.8 kDa. Lanes: 1, MW markers; 2, supernatant from 0.3 M NaCl wash; 3, pellet following the 0.3 M NaCl wash; 4, supernatant from ethanolamine wash; 5, pellet following the ethanolamine wash; 6, supernatant after membrane extraction with 1% Triton X-100 containing soluble HA; 7, pellet after 1% Triton X-100 extraction containing insoluble HA.

Final purity is achieved with lentil lectin chromatography (Fig. 3B). The highly pure HA is subsequently loaded onto a final CM column for concentration and exchanged into the final formulation buffer system. As shown in Figure 3B, the lentil lectin eluate is subsequently concentrated approximately 10-fold using CM column chromatography.

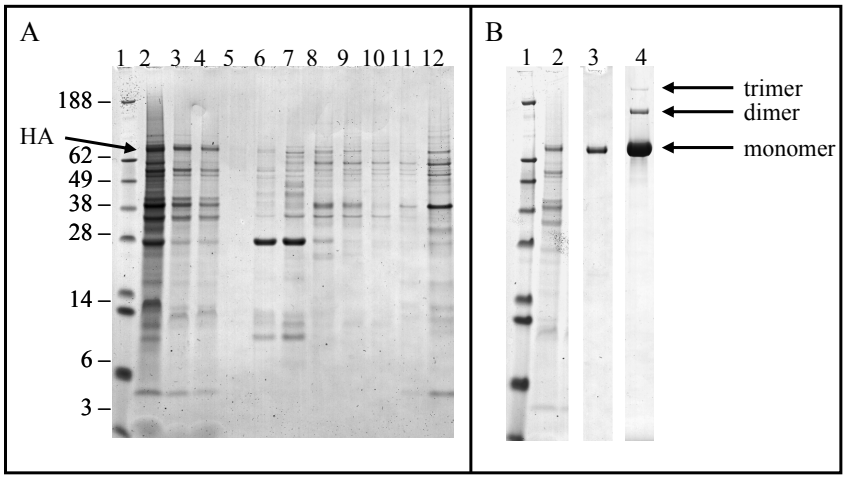

Figure 3A. Q column chromatography of Triton X-100 solubilized H3 HA. Solubilized HA is loaded directly onto the Q column. HA is collected in the Q flow-through and Q wash with minimal losses. Impurities and DNA remain bound to the column and elute with 0.2-2.0 M NaCl. HA is denoted by the arrow. Lanes: 1, MW markers; 2, pre-column solubilized H3 HA; 3, flow-through; 4–5, 50 mM ethanolamine, 0.1% Triton X-100, 0.01% ß-ME wash; 6–12, 0.2–2 M NaCl elution of bound material.
Figure 3B. Lentil lectin and CM column chromatography of pooled Q flow-through and Q wash containing H3 HA. Lentil lectin chromatography purifies HA to homogeneity while the CM column concentrates the material approximately 10-fold and exchanges the detergent for the final formulation (10 mM Na phosphate, 0.01% Tween-20, 0.15 M NaCl pH 7.4). Higher MW bands observed above and below the 188 kDa MW marker band represent higher molecular weight forms of HA (dimers and trimers). Lanes: 1, MW markers; 2, Q flow-through and Q wash combined; 3, lentil lectin eluate; 4, CM fraction containing highly concentrated HA.

HA Characterization

Based on the migration in SDS-PAGE gel and a comparison with the molecular weight markers (MW), the purified monomeric form of HA has a calculated molecular mass of 74 kDa. Based on the known theoretical mass from the amino acid sequence of the protein (64 kDa) and the molecular mass of a trimannosyl unit added by insect cells (1.8 kDa/unit), the purified protein has six out of the seven potential sites glycosylated. Higher bands observed in the SDS-PAGE gel of Figure 3B have calculated molecular masses of 143 and 219 kDa, values in strong agreement with dimeric and trimeric forms of the molecule. Based on bicinchonic acid (BCA) total protein determination and optical density measurements, average yields between 2-4 mgs pure material per liter of culture are obtained.

HA proteins can be tested for their ability to agglutinate chicken RBCs in a standard hemagglutination activity assay [Barrett and Inglis, 1995]. This activity assay is based on the ability of HA to bind erythrocytes through the sialic acid receptor binding site located on the globular headpiece of the properly folded molecule, causing the cells to aggregate. Figure 4 shows a typical activity assay performed in a 96-well plate. HA agglutination prevents cells from settling to the bottom of the wells and is observed as a uniform cell suspension. In the absence of agglutination, the cells settle out and form compact pellets as observed with PBS buffer serving as a negative control in lanes 1 and 5 (Fig. 4) The agglutination activity for H3 HA (lanes 2-4) spans three orders of magnitude and shows activity down to approximately 0.5 ng of pure protein (or 2,000 Units/mg of HA activity).

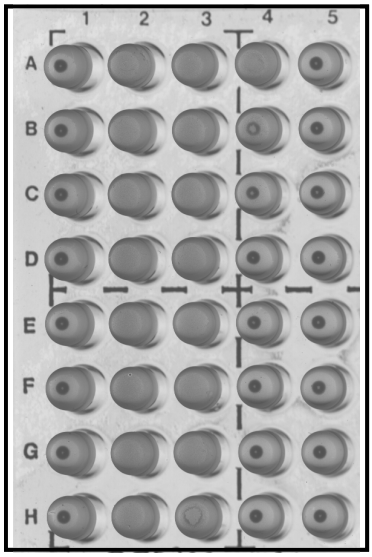

Figure 4. Hemagglutination assay for H3 HA. PBS buffer serves as a negative control in lanes 1 and 5. Purified H3 HA diluted in PBS buffer is loaded into the first well of lanes 2, 3, and 4 at concentrations of 100 μg/ml, 1 μg/ml, and 0.01 μg/ml, respectively. The protein aliquots are subsequently diluted twofold in a serial fashion down the plate using equal volumes of PBS buffer per row. Loose pellets are noticeable in lane 3, well H and lane 4, well A due to diminished activity as the protein concentrations reaches sub-nanogram levels (0.8–0.5 ng). One HA unit is defined as the amount of antigen at which 50% of the cells agglutinate (loose pellet).

The correct quaternary structure of recombinant HA is important for its stability and biological activity. The trypsin resistance assay treats the final product with the trypsin endoprotease to distinguish properly folded, trimeric HA from denatured and/or monomeric molecules. In this assay, full-length, intact HA molecules that have associated into trimers are converted into two smaller fragments, the amino-terminal HA1 fragment and the carboxy-terminal HA2 fragment, by proteolytic cleavage at an internal site (described in Chapter 1, Fig. 5). In the SDS-PAGE gel trypsin treatment of H3 HA

resulted in the characteristic HA1 and HA2 fragmentation with apparent molecular masses of ~50 kDa for HA1 and ~28 kDa for HA2 (Fig. 5, lane 3). The untreated samples show a major protein slightly above the 62 kDa marker for the HA monomer and two additional higher MW polypeptides above and below the 188 kDa marker that correspond to trimer and dimeric forms, respectively (lanes 2 and 4). No protein bands are observed for trypsin-treated HA that had been denatured by boiling prior to the assay, suggesting complete proteolytic degradation of the denatured HA protein (lane 5).

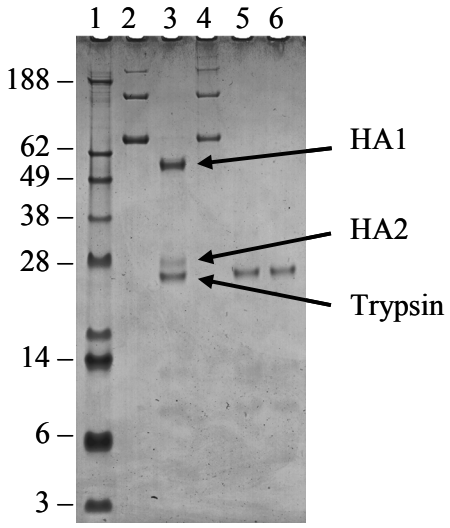

Figure 5. SDS-PAGE gel of the HA trypsin resistance assay. H3 HA samples (125 µgs) were left untreated (lanes 2 and 4) or treated with 50 µg/ml trypsin (lane 3 and 5) for 30 minutes on ice, and then analyzed by SDS-PAGE. The H3 samples in lanes 4 and 5 were boiled for 10 min prior to the assay and serve as controls for denatured protein. Lane 1 contains MW markers and lane 6 trypsin endoprotease (26 kDa).

Properly folded trimers of HA have been shown to join together end-to-end upon removal of detergent to form 'rosettes' visible by electron microscopy (EM) (Sato *et al.*, 1983). H3 HA specimens from this study have been prepared and examined by EM. As shown in Figure 6, a highly dense arrangement of rosette structures is observed containing six to eight trimers or spikes.

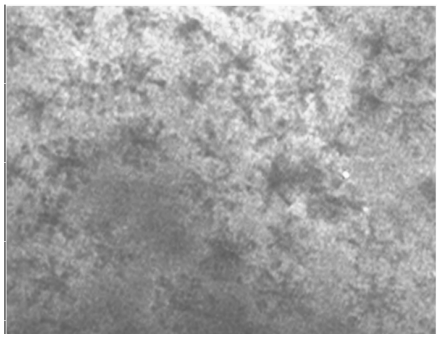

Figure 6. Electron micrograph of H3 HA. A specimen of purified H3 HA (600 µg/ml in 10 mM Na phosphate, 0.01% Tween-20, 0.15 M NaCl pH 7.4) was prepared on a carbon 300-mesh grid, negatively stained with 1% phosphotungstic acid, and viewed with a Zeiss EM10A transmission electron microscope at a magnification of 80,000×.

Discussion

The baculovirus-insect cell expression system supports the production of full-length HA molecules for a recombinant trivalent influenza vaccine. Recombinant baculovirus transfer plasmids for the expression of full-length HA molecules were constructed and cotransfected with AcMNPV baculovirus genomic DNA. Cell supernatants were subjected to plaque assay and the resultant isolated plaques containing recombinant baculoviruses were analyzed for expression of full-length, recombinant HA. The best candidates for protein expression as judged by SDS-PAGE and immunoblotting were selected and scaled to create virus stocks for infection of SF+ cells. Optimal cell culture conditions for production of the recombinant proteins were determined and 45-L fermentations were completed for each of the three HA antigens of the trivalent vaccine. The described process yields 2-4 mgs of highly pure HA per liter of culture. These yields are being improved through high-density cell culture, and improvements in the extraction of soluble HA from the insect cell membrane. The washing of the cell pellet prior to HA extraction from the cellular membrane results in HA losses (Fig. 2, lanes 2 and 4) and further development is needed to evaluate the necessity of this step.

Recombinant HA proteins produced in the BEVS system are full-length, and largely uncleaved, indicating that insect cells lack the proteases that convert the molecule into its mature form of HA1 and HA2 subunits covalently linked by a disulfide bond. Although different from the cleaved HA molecules of TIV, full-length baculovirus-derived HAs associate into homotrimers, a molecular form shown to induce immunogenicity in healthy young and elderly adult populations [Johansson, 1999; Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Treanor *et al.*, 2001].

Three assays based on hemagglutination activity, trypsin resistance, and electron microscopy are currently in place to verify the correct tertiary and quaternary structure of HA proteins that is essential for biological activity and immunogenicity. The amino terminal HA1 region of HA forms a globular headpiece containing five antigenic sites and a receptor binding site used in viral attachment during infection. Purified HA molecules having a correct structure and an intact receptor binding site will recognize sialic acid residues on the surface of RBCs in a standard hemagglutination assay. This assay has been useful in identifying purification conditions that potentially alter the structure and lead to permanent inactivation of the protein. For example, exposure to high ionic strength (> 1 M NaCl) media has been found to affect the protein structure and reduce agglutination activity even after its removal (*not shown*). Also, agglutination activity is irreversibly reduced at acidic pH (= 5.5). This phenomenon is in agreement with the intended biological function of HA as a membrane fusogen undergoing a dramatic pH-induced conformational change that results in the fusion of the viral envelope with the endosomal membrane of the host cell. As described above, in baculovirus-insect cell-derived HA, the HA2 fusion peptide is not released from the HA1 domain for fusion; nevertheless, a structural change is suspected to occur at low pH leading to the readily observed reduction in agglutination activity (*not shown*).

HA is unusually stable and biologically active when in its properly folded trimeric state. Trypsin sensitivity is useful as an assay for trimer formation. Each correctly folded full-length molecule of the trimer is cleaved by the protease trypsin at a single basic site to generate HA1 and HA2 as shown for H3 HA in Figure 5 (lane 3). The internally cleaved mature form of HA is resistant to further proteolysis despite

the presence of 54 potential digestion sites. In contrast, monomeric and denatured HA molecules are more susceptible to trypsin and are partially or completely degraded by the protease as shown in Figure 5 for heat denatured HA. Denaturation results in degradation of the protein into smaller peptides not resolvable by the SDS-PAGE analysis (Fig. 5, lane 5). Thus, the trypsin resistance assay is useful for confirming the correct quaternary structure of HA molecules as demonstrated here with H3 HA.

Properly folded HA trimers associate end to end through their hydrophobic transmembrane domains to form micellular structures called rosettes (Sato, 1983). As shown in Figure 6 for H3 HA, the rosettes are visible with EM. Each spike of the rosette is a trimer of full-length, uncleaved HA monomers. Approximately 6-8 trimers participate in a single rosette. The rosette structures formed from aggregation of trimers at their transmembrane domains closely resemble the surface morphology of infectious virions having HA trimeric spikes protruding from the viral envelope. This higher order aggregation state observed by EM may be an indicator of HA immunogenicity.

Previously, monovalent and bivalent HA molecules produced in the baculovirus- insect cell system were evaluated in six small human phase I/II clinical studies using both monovalent and bivalent preparations [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Johansson, 1999; Treanor *et al.*, 2001]. The results of these studies are promising and indicate that a monovalent or bivalent HA vaccines appear to be safe and well tolerated in both young adults (ages 18-45 years) and elderly adults (>65 years). The analysis of functional antibody titers (hemagglutinin-inhibition antibodies, HAI, and neutralizing antibodies) and binding antibody titers elicited by HA vaccines appears to comparable to that of TIV. Greater stimulatory effects have been observed for the recombinant HA vaccine using bivalent rather than monovalent preparations, and also with higher doses of HA. The higher purity of the HA vaccines has allowed the use of higher doses (up to 135 μgs/HA based on BCA determinations) without an increase in adverse side effects that are more frequently reported with the TIV using significantly less HA (15 μg HA/strain based on SRID assay) [Williams, 1993]. The addition of alum adjuvant has not been found to significantly improve antibody response. From these studies, it has been inferred that the uncleaved state of HA expressed in insect cells and the potential differences in glycosylation compared to mammalian derived proteins does not affect the immunogenicity of the product.

A clinical trial using the production and purification scheme discussed here for H3 HA will determine the efficacy of higher doses (15 μg, 45 μg and 135 μg) in a trivalent formulation in the elderly adult population and compare its performance with the current TIV (see Chapter 6). An enhanced stimulatory effect is expected from the inclusion of a third HA molecule and at the higher dosage (135 μg). The influenza vaccine based on recombinant HA produced in BEVS offers several potential advantages over TIV. The influenza HA antigens are produced under safe, sterile and stringently controlled conditions using a scaleable fermentation process in insect cells. Purification procedures for HA do not include virus inactivation or organic extraction procedures, thus avoiding possible denaturing effects and additional safety concerns due to residual toxic chemicals in the vaccine. The HA protein is highly purified and does not contain contaminants from eggs, eliminating possible adverse reactions in individuals with severe egg allergies. Selection or adaptation of influenza virus strains that produce at high levels in eggs is not required, making it possible to choose the best genetic match between the vaccine strains and the influenza virus strains that are causing disease. The cloning, expression, and manufacture of HA influenza vaccine

can be very rapid allowing for strain selection later in the year when more reliable epidemiological data are available.

Acknowledgements

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Chapter 3

Expression and Purification of an Influenza Hemagglutinin

One Step Closer to a Recombinant Protein-Based Influenza Vaccine

Abstract

Numerous human infections with avian influenza viruses in Asia in recent years have raised the concern that the next influenza pandemic is imminent. The most effective way to combat influenza is through the vaccination of the public. However, a minimum of 3-6 months is needed to develop an influenza vaccine using the traditional egg-based vaccine approach. The influenza hemagglutinin protein (HA), the active ingredient in the current vaccine, can be expressed in insect cells using the baculovirus expression vector system and purified rapidly. An influenza vaccine based on such a recombinant antigen allows a more timely response to a potential influenza pandemic. Here, we report an innovative monitoring assay for recombinant HA (rHA) expression and a rapid purification process. Various biochemical analyses indicate that the purified rHA is properly folded and biologically active.

This chapter was slightly modified from: Wang K, Holtz KM, Anderson K, Chubet R, Mahmoud W, **Cox MM** (2006): Expression and purification of an influenza hemagglutinin--one step closer to a recombinant protein-based influenza vaccine. *Vaccine* **24**: 2176-85.

1. Introduction

Influenza is a highly contagious, acute viral respiratory disease, which causes significant morbidity and mortality worldwide each year [Glezen, 1982; Cox and Subbarao, 1999; Hilleman, 2002]. Influenza viruses are single-stranded ribonucleic acid (RNA) viruses surrounded by a lipid containing envelope spiked with two glycoproteins: hemagglutinin (HA) and neuraminidase (NA). These glycoproteins, and HA in particular, have been recognized as key antigens in the host response to influenza virus in both natural infection and vaccination [Clements *et al.*, 1986; Skehel and Wiley, 2000]. The viruses are well known for their ability to mutate to circumvent immunity and reinfect the host. An antigenic shift, a major antigenic change of the virus due to the genetic re-assortment of two subtype strains that co-infected a host, can cause an influenza pandemic since the population may have no inherent immunity against the new strain [Wood and Robertson , 2004; Stephenson *et al.*, 2004]. The avian influenza A (H5N1) epizootic outbreak, numerous human infections with H5N1 in Asia in recent years, events such as the infection of two nurses attending to avian influenza patients in Vietnam (WHO, March 14, 2005) and a possible person-to-person transmission in a family cluster of the disease in Thailand [Ungchusak *et al.*, 2005], continue to raise concern that the next influenza pandemic is imminent.

A proven, effective way to combat influenza is through vaccination of the public using the trivalent vaccine produced in embryonated chicken eggs. In the current process, three influenza strains selected by WHO/CDC are propagated in chicken eggs, chemically inactivated, and semi-purified. The egg-based technology, however, is unable to respond to a pandemic crisis. Vaccine development and production takes several months following the identification of potential strains and typically requires the reassortment with a high yield strain to obtain adequate growth properties [Audsley and Tannock, 2004; Sheridan, 2004; Cox, 2005; Osterholm, 2005]. A minimum of 3-6 months is needed to develop an influenza vaccine using this approach. More importantly, the H5 avian influenza strains responsible for recent epizootic outbreaks involving numerous human infections are lethal to chicken eggs used for vaccine production and to the chickens that lay the eggs. If the 2003 severe acute respiratory syndrome (SARS)-coronavirus outbreak serves as a guide, the next influenza pandemic will likely have global consequences spreading within weeks, if not within days. Thus, a system that can rapidly produce new influenza vaccine is needed to prevent or to effectively reduce th; e impact of pandemic influenza.

Two new approaches have shown great promise to replace the egg-based technique Audsley and Tannock, 2004; Sheridan, 2004; [Cox, 2005]. One is cell culture-based, and the other is recombinant protein (antigen)-based. The cell culture-based approach involves production of influenza viruses in cell culture followed by the same virus inactivation and purification procedures currently used in the downstream processing of the egg-based virus. The advantages are: cell cultures are easier to handle and can be scaled up in a short period of time. The influenza vaccines produced with this approach have been tested in Phase I and Phase II clinical trials and were found to be safe and at least as effective as the vaccines produced in embryonated chicken eggs [Alymova *et al.*, 1998; Brands *et al.* 1999; Percheson *et al.*, 1999]. A limitation of the cell culture-based approach is that the process still requires the production of a high-yielding re-assorted virus. This process also may introduce cell line specific mutations in the viral genes that can lead to the selection of variants with antigenic and structural changes in the HA protein, potentially resulting

in less-efficacious vaccines [Schild *et al.*, 1983; Robertson *et al.*, 1985; Meiklejohn *et al.*, 1987]. Additional hurdles include: the production and handling of a dangerous virus requires the availability of high containment facilities; mammalian cells can harbor animal viruses that may lead to safety concerns; the residues from the expressing cells may cause some unknown side-effects since no thorough purification process has been introduced into the manufacturing process. On the other hand, the recombinant protein-based approach involves production of viral antigens such as HA and NA in cell culture with recombinant DNA technology and utilization of the purified antigens as the active ingredients in the vaccine. The rHA influenza vaccines developed using the baculovirus-insect cell expression system has been tested in several Phase I and Phase II human clinical trials involving over 1,200 subjects that demonstrated safety, immunogenicity and efficacy [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Treanor *et al.*, 2001]. In elderly adults, rHA vaccine is equally or more immunogenic than the egg-based vaccine [Treanor *et al.*, 2006]. Interestingly, two H1N1 rHA vaccines (derived from two strains of A/New Caledonia/20/99 or A/Texas/36/91) provided partial protection against the lethal challenge of a reconstructed highly lethal 1918 pandemic influenza virus (also a H1N1 strain) in mice, suggesting that cross protection against drifted strains is definitely feasible [Tumpey *et al.*, 2004]. To meet the challenge of a potential influenza pandemic, however, a reliable expression system and a quick, efficient downstream purification process are needed. In this chapter, a rapid process is reported capable to purify rHA (H1N1, A/New Caledonia/20/99) from an insect cell bioreactor to 95% purity within 6h with a 57% overall yield. Since all the chromatographic media used here are chemically stable and commercially available, the process can be easily scaled up in a GMP facility. Various biochemical analysis indicated that he purified rHA is properly folded and biologically active. In addition, we also developed a quick, simple analytical assay to monitor the expression of rHA in the insect cell fermentor to ensure the rHA production.

2. Materials and Methods

2.1. Cloning and expression of influenza HA

The influenza vaccine strain - A/New Caledonia/20/99 (H1N1) – was obtained from the CDC. The full-length HA gene (containing the HA1 and HA2 genes) from the influenza viruses was cloned using RT-PCR and inserted into a baculovirus transfer vector developed by Protein Sciences Corporation. This specialized vector contained the promoter from the baculovirus polyhedrin gene flanked by sequences naturally surrounding the polyhedrin locus. Next, the transfer vector was co-transfected into insect cells with the linearized baculovirus genomic DNA (*Autographa californica Multiple Nuclocapsid Polyhedrosis* Virus) depleted of the polyhedrin gene and part of an essential gene downstream of the polyhedrin locus. The homologous recombination between the transfer plasmid and the linearized viral DNA rescued the virus, resulting in recovery efficiencies of recombinant virus of nearly 100%. Recombinant viruses were then selected by plaque assay. The plaque-derived recombinant baculovirus was then used to create a virus stock by infecting increasingly larger cultures of the proprietary insect cells (*expres*SF+®, derived from Sf9 cells) in serum-free culture medium (Protein Sciences Fortified Medium). The virus stock was then used to infect insect cells (2.0 x 10^6 cells/ml) to produce rHA in a

15-liter Applikon bioreactor. The multiplicity of infection (MOI) was 1 (i.e. one plaque-forming unit [pfu]/cell was used) for this experiment.

To monitor the infection process and the expression of HA, 4 ml samples were taken from the bioreactor at various times. One milliliter was used for analyzing the changes in cell density, cell viability and cell size distribution. Two milliliters were centrifuged at 1600 rpm. The supernatant and pellet were stored separately at – 80^{o} C to be used the single radial immunodiffusion assay (SRID), gel and blot analysis. One milliliter was used for hemadsorption analysis. The remainder of the bioreactor content was used for protein purification.

2.2. Hemadsorption assay

To 0.5 ml fermentation samples (insect cells uninfected, infected with recombinant baculovirus containing HA genes, and infected with recombinant baculovirus containing a non-HA gene) in a 1.5 ml Eppendorf tube, 0.10 ml of 5% chicken red blood cells (RBCs [Charles River – Spafas, North Franklin, CT]) in PBS was added and shaken gently for 10 min at room temperature. At the end of the incubation, the tube was flipped gently for five times to get a homogenous suspension. Then, 10 µl of the suspension was pipetted on a glass plate and observed under a microscope (CK2, Olympus Optical Co., Japan) in three representative view fields. On average about 20-70 insect cells were counted in each field. To reduce the chance of false positives, only the insect cells attached by three or more RBCs were counted as RBC-bound insect cells. The percentage of RBC-bound insect cells against the total insect cells in each time point was calculated from three fields.

2.3. Cell analysis

At each time point, 1.0 ml of fermentation sample was analyzed with an automated cell analyzer (Cedex AS20, Innovatis GmbH, Germany) for cell density, cell viability and cell size distribution using the procedure described by the manufacturer.

2.4. Buffers and columns

Buffer A: 20 mM sodium phosphate, 1.0 mM EDTA, 0.01 % Tergitol-NP9, 5% Glycerol, pH 5.89. Buffer B: 20 mM sodium phosphate, 0.03 % Tergitol, 5% Glycerol, pH 7.02. Buffer C: 20 mM sodium phosphate, 150 mM NaCl, 0.03 % Tergitol, 5% Glycerol, pH 7.02. Buffer D: 40 mM sodium phosphate, 0.05 % Tween-20, 5% Glycerol, pH 7.20. Buffer E: 100 mM sodium phosphate, 0.05 % Tween-20, 5% Glycerol, pH 7.20. Buffer F: 500 mM sodium phosphate, 0.05 % Tween-20, 5% Glycerol, pH 7.20. Buffer G: 10 mM sodium phosphate, 150 mM NaCl, 0.01 % Tween-20, pH 7.22. Sanitation buffer: 1.0 M NaCl, 0.5 M NaOH.

UNOsphere-Q (Bio-Rad, Hercules, CA) column, ø1.6 cm x 10 cm, 20 ml; SP-Sepharose Fast Flow (GE/Amersham/Pharmacia, Piscataway, NJ) column, ø1.6 cm x 10 cm, 20 ml; Hydroxyapatite Type I column (HX-I, Bio-Rad, Hercules, CA), ø1.0 cm x 4.6 cm, 3.6 ml.

2.5. Purification procedure

The fermentations producing rHA were harvested by centrifugation at 56-65 h post infection. The cell pellet (approximately 6.4 g) was extracted with 225 ml of 1 % Tergitol NP-9 in buffer A by stirring on a magnetic stirrer at 4^{o} C for 30 min. The extract was clarified by centrifugation at 10,000 x g for 25 min. The supernatant was loaded on Q/SP columns (equilibrated with Buffer A) in tandem at a flow of 5 ml/min. After loading, the columns were washed with 140 ml of Buffer A. Then, the

columns were disconnected. HA was eluted from the SP column with 140 ml of buffer B and 80 ml of buffer C consecutively. The Q/SP columns were regenerated by washing with 5 column volumes (CV) of sanitation buffer and 5 CV of water and equilibrated with 5 CV of buffer A.

The HA fraction in buffer B (40 ml) was loaded on a HX-I column at 2 ml/min. The column was washed with 18 ml of buffer B. The HA was eluted from the HX-I column with increased phosphate concentration (buffers D, E and F). The HA preparation in buffer D was further purified and concentrated by ultrafiltration with a stir cell using a 100 kDa Molecular Weight Cut-off (MDCO) regenerated cellulose membrane with buffer G. The HX-I column was regenerated by washing with 10 CV of water, and equilibrated with 10 CV of buffer C.

2.6. Single radial immunodiffusion (SRID)

The rHA contents in all preparations were determined with SRID assay as described by Williams (1993) and Manchini *et al.* (1965). The assay is based on the diffusion of rHA into a 1% agarose gel containing antibodies against the HA. The interaction between antigen and antibody produces a precipitation ring of which the size is directly proportional to the amount of antigen applied. The diameters of the rings in the SRID assay were determined with a measuring magnifier (Baush/lomb, 81-34-38). The diameters of the precipitate ring were used to determine the actual concentrations based on standards provided by the Center for Biologics Evaluation and Research of FDA.

2.7. Deglycosylation

For complete deglycosylation, 20 μg of purified rHA was deglycosylated with 5,000 units of peptide-*N*-glycosidase F (PNGase F, New England BioLabs, Beverly, MA, USA) or Endoglycosidase H (Endo H, New England BioLabs, Beverly, MA, USA) at 37^{o}C for 60 min as described previously [Maley *et al.*, 1989]. For limited deglycosylation, 20 μg of rHA was treated with 0.2 μg of trypsin on ice for 30 min. The digestion was stopped by adding 10x denaturing buffer and boiling for 5 min. Then the trypsin treated rHAs were deglycosylated with 2, 20 or 200 units of PNGase F or Endo H on ice, at 25^{o}C or at 37^{o}C for 2-60 min. The reactions were stopped by adding 2x SDS sample buffer and boiling for 5 min. The protein species at various deglycosylation stages were resolved on SDS-PAGE.

2.8. Other biochemical analyses

Vaxigrip influenza vaccine was purchased from Canada Drug Delivery (Nanaimo BC, Canada). The purity of rHA was measured on SDS-polyacrylamide gels stained with Coomassie blue using scanning laser densitometry (model 710, Bio-Rad, Hercules, CA, USA) and peak integration analysis. The total amino acid analysis was carried out with a Beckman amino acid analyzer at Keck Facility of Yale University. The N-terminal amino acid sequence analysis was executed at the Protein Core Facility of Columbia University. The molecular size of the purified rHA was analyzed on size-exclusion columns (TSK-4000, 7.5 x 300 mm, TosaHaas, Japan) at a constant flow rate of 0.8 ml/min, using protein molecular mass markers as reference (Sigma, St. Louis, USA) as previously described [Wang and Spector, 1994]. Elution buffer: 50 mM sodium phosphate, 50 mM NaCl and 0.001% NaN_3. A trypsin resistance assay was carried out by incubating rHA for 30 min.at 0ºC without or with 50 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin as described by Copeland *et al.* (1986). For this assay, the denatured HA was produced by boiling

rHA for 5 min. Hemagglutination activity assays were done essentially as described by Barrett and Inglis (1991) with a 0.5% solution of fresh chicken red blood cells in a U-bottom 96-well microtiter plate.

3. Results

3.1. Monitoring the expression of rHA in insect cell

A critical issue in production of a therapeutic protein using recombinant DNA technology is determining when to harvest the fermentation [Molowa and Mazanet, 2003; Palomares *et al.*, 2004]. Too early, the yield may be suboptimal. Too late, the expressed protein may be degraded by a variety of proteases released during the lytic process of infected cells. Thus, a rapid and sensitive assay is needed to monitor protein expression and to choose the right harvest time. HA is well known for its ability to bind the sialic acid on the surfaces of red blood cells (RBCs) and agglutinate these cells [Barrett and Inglis, 1991]. This phenomenon has been successfully used to detect cells and tissues infected with influenza viruses [Flint *et al.*, 2004]. To determine whether the insect cells infected with the recombinant baculoviruses containing the HA gene can also agglutinate RBCs, both uninfected and infected insect cells were incubated with RBCs for 10 min. and observed under a microscope. In the uninfected insect cell sample, the RBCs (the smaller cells) were scattered around on the slide, and no specific binding of RBCs to the insect cells (the larger cells, about 16 μm in diameter) was observed as (Fig. 1a). On the other hand, most of RBCs were bound to the insect cells infected with baculovirus containing the HA gene derived from influenza strain A/New Caledonia/20/99 (H1N1) (Fig. 1b). This method also works for expression of the HAs derived from other strains such as B/Jiangsu/10/2003 (Fig. 1c) and A/New York/55/2004 (H3N2) (data not shown). To verify that the observed hemadsorption is due to the HA genes and not to the other genes expressed by the baculovirus, insect cells infected with recombinant baculovirus containing a non-HA gene were also incubated with RBCs. In this case, no binding of RBCs to insect cells was observed (Fig. 1d). These observations clearly demonstrate that the binding of RBCs to the surface of insect cells is HA expression dependent, not infection dependent. The data also support the conclusion that the HAs expressed in insect cells are displayed at the cell surface, are properly folded and biologically active.

This hemadsorption assay was further optimized to monitor the expression of HA during fermentation. Even though the infection of insect cells can be seen by observing morphological changes as early as 6 hours post infection (HPI), the first binding of RBCs to insect cells was only observed around 23 HPI, when about 20% of insect cells were bound by three to eight RBCs. Almost all insect cells were infected at 23 HPI according to cell morphology. The binding reached its peak around 45-55 HPI, when 70-80% of the insect cells were bound by 8-20 RBCs (in some fermentations, almost all insect cells were bound by RBCs during this time), as shown in Table 1. At the peak of hemadsorption, clusters of 20-200 insect cells agglutinated by RBCs have also been observed. Later around 70 HPI, the binding gradually reduced to 30-40% of the insect cells, most likely caused by the breakdown of some infected cells and the loss of cell membrane, as evidenced by the rapid decrease of cell viability. To determine the rHA levels in the fermentation at each sampling time, the single radial immunodiffusion (SRID) assay has been used, since it is a simple, reproducible technique, and relatively unaffected by other proteins in the crude extract

[Mancini *et al.*, 1965; Williams, 1993]. Consistently, the RBC binding to insect cells correlated well with the HA levels determined by SRID. Late HA expression is expected because the HA gene is regulated by the polyhedrin promoter, which is a late stage promoter in baculovirus infection. Similar results were also obtained for the expression of other HAs such as B/Jiangsu/10/2003 and A/New York/55/2004 (data not shown).

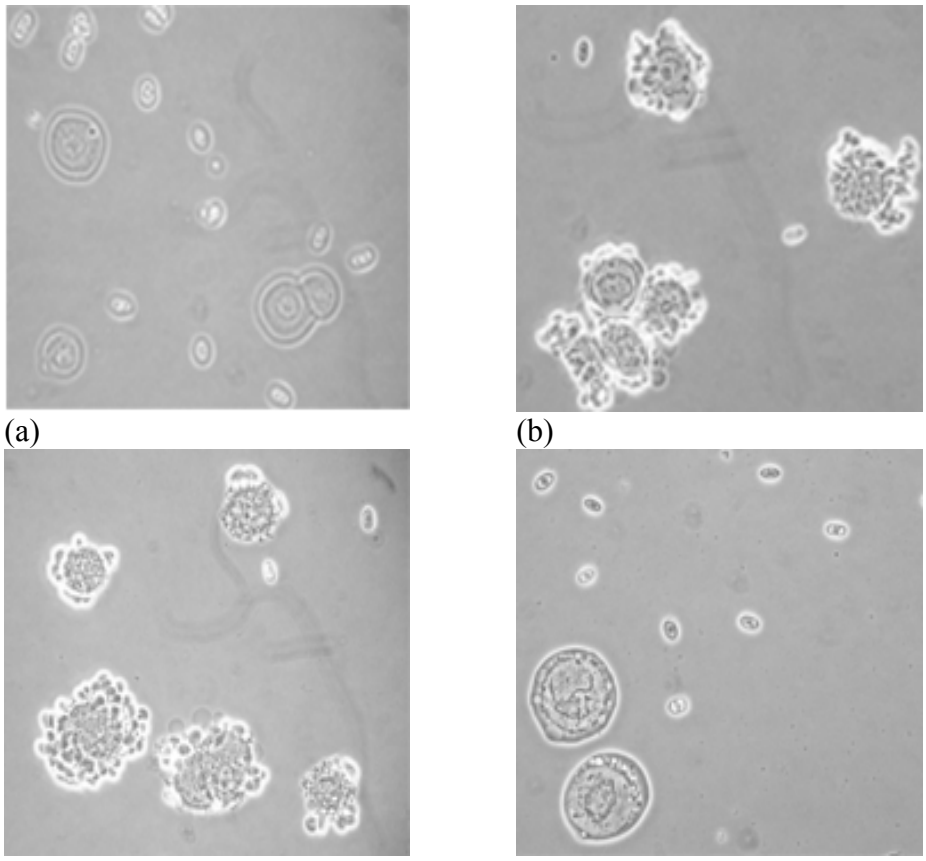

Figure 1. Monitoring rHA expression with hemadsorption. (a) Uninfected insect cells. (b) The insect cells infected with the baculovirus containing the HA gene from the A/New Caledonia/20/99 (H1N1) influenza virus. (c) The insect cells infected with the baculovirus containing the HA gene from the B/Jiangsu/10/2003 influenza virus. (d) The insect cells infected with the baculovirus containing a non-HA gene.

Table 1. Possible correlation among time post infection (HPI), hemadsorption, cell viability and HA yield[a]

HPI[b]	% Binding	Viability	HA (mg/L)
0	0	92.9	0
6	0	92.9	0
23	20	87.6	4
30	30	88.2	7
46	65	74.7	18
50	80	52.4	20
54	70	50.1	20
70	40	21.8	11
75	30	16.1	11

[a] HA yield was determined by SRID assay. The MOI using for the experiment was one.
[b] hpi = hours post infection

3.2. Designing and optimizing the rHA purification process

A major challenge in the biotechnology industry is purification of biologically active recombinant proteins [Ikonomou et al, 2003; Andersen and Krummen 2002]. An ideal purification process should be mild, efficient and capable of achieving high purity in a short period of time. Accordingly, each purification step has to be designed carefully to optimize the whole process. As demonstrated in the hemadsorption studies, rHAs are expressed, folded and transported to the cell membrane at a late stage. To extract rHA from the cell membranes, several non-ionic detergents at various concentrations were tested for their efficiency. The best result was obtained using 1% Tergitol NP-9. To get a relatively clean extraction, a magnetic stirrer was used to avoid the disruption of cell nuclei and other organelles. The extract was clarified by centrifugation to remove cell nuclei and other debris.

The HA monomer of the A/New Caledonia/20/99 influenza virus strain consists of 547 amino acids with a theoretical molecular mass of 63,156.43 kDa and a pI of 6.30. Therefore, this rHA has the potential to be bound on cation-exchange media like SP using a lower pH buffer and eluted with a higher pH buffer to achieve primary purification and concentration. The supernatant of the extract was loaded on UNO Sphere Q/SP columns in tandem. The anion-exchange Q column acts as a scavenger by binding the negative-charged impurities that may foul the SP column. After loading and washing, the columns were detached and eluted separately. As shown in the chromatogram (Fig. 2a), about 50% of the proteins flow through Q/SP columns, a small amount of protein elutes from the SP with the pH 7 buffer and the rest of the proteins bind tightly to either the SP or the Q column. As shown on the SDS-PAGE (Fig. 2b), the rHA captured on the SP column at pH 6 was selectively eluted by a shift to pH 7. The pH shift resulted in a 14-fold increase in HA purity based on densitometry of the SDS-PAGE gel.

To further purify rHA, use of hydroxyapatite type I (HX-I) media was explored. The binding preference of HX-I media is significantly different from the ion-exchange media, and yet the binding and eluting conditions are relatively mild so as to preserve the biological activity of the target protein [Schroder *et al.*, 2003; Karlsson and Winge, 2003]. Thus, the pH 7 SP column eluate was loaded on a HX-I column. After washing, rHA was eluted from the column by increasing the phosphate concentration. As shown in Fig. 2c, most rHA was eluted in 40 mM phosphate, and

there was a small loss in the wash and in the 100 mM phosphate elution. The purity was increased from 52% to 91% according to the densitometry of the SDS-PAGE.

However, there was still a protein contaminant of about 36 kDa in the preparation as revealed in lane 5 of Fig. 2c. Since the size of the rHA trimer is around 210 kDa, the difference in size between rHA and this impurity could be explored to remove this impurity. Thus, the 40 mM phosphate eluate was further purified with ultrafiltration using a stir cell (100 kDa MWCO). As demonstrated in Fig. 2d, the 36 kDa band (lane 5) was selectively removed from the retentate. The purified rHA migrated in SDS-PAGE gel as a single monomeric polypeptide (rHA) with an apparent molecular weight of approximately 70 kDa. On the blot, a small amount of rHA1 and rHA2 (the cleavage products of rHA) were also observed, with apparent molecular masses of ≈50 and ≈28 kDa, respectively (data not shown). Trace amounts of rHA dimers and trimers were also detectable, with apparent molecular weights of ≈ 140 and ≈ 220 kDa, respectively.

As summarized in Table 2, the process described here can purify rHA from the fermentation to 95% purity within 6 hours with a 57% overall yield. The largest single step loss (27%) is on the HX-1 column.

Table 2. Stepwise mass balance of A/New Caledonia rHA purification by SRID assay

	HA (μg/ml)	Volume (ml)	HA (mg)	Purity[a] (%)	Step Recovery (%)	Total Recovery (%)
Starting Material	53	225	12	3.6		
Q/SP	240	40	9.6	52	80	80
HX-1	280	25	7.0	91	73	58
Ultrafiltration	700	9.1	6.4	99	91	53

[a] Purity was determined by the densitometry of SDS-PAGE.

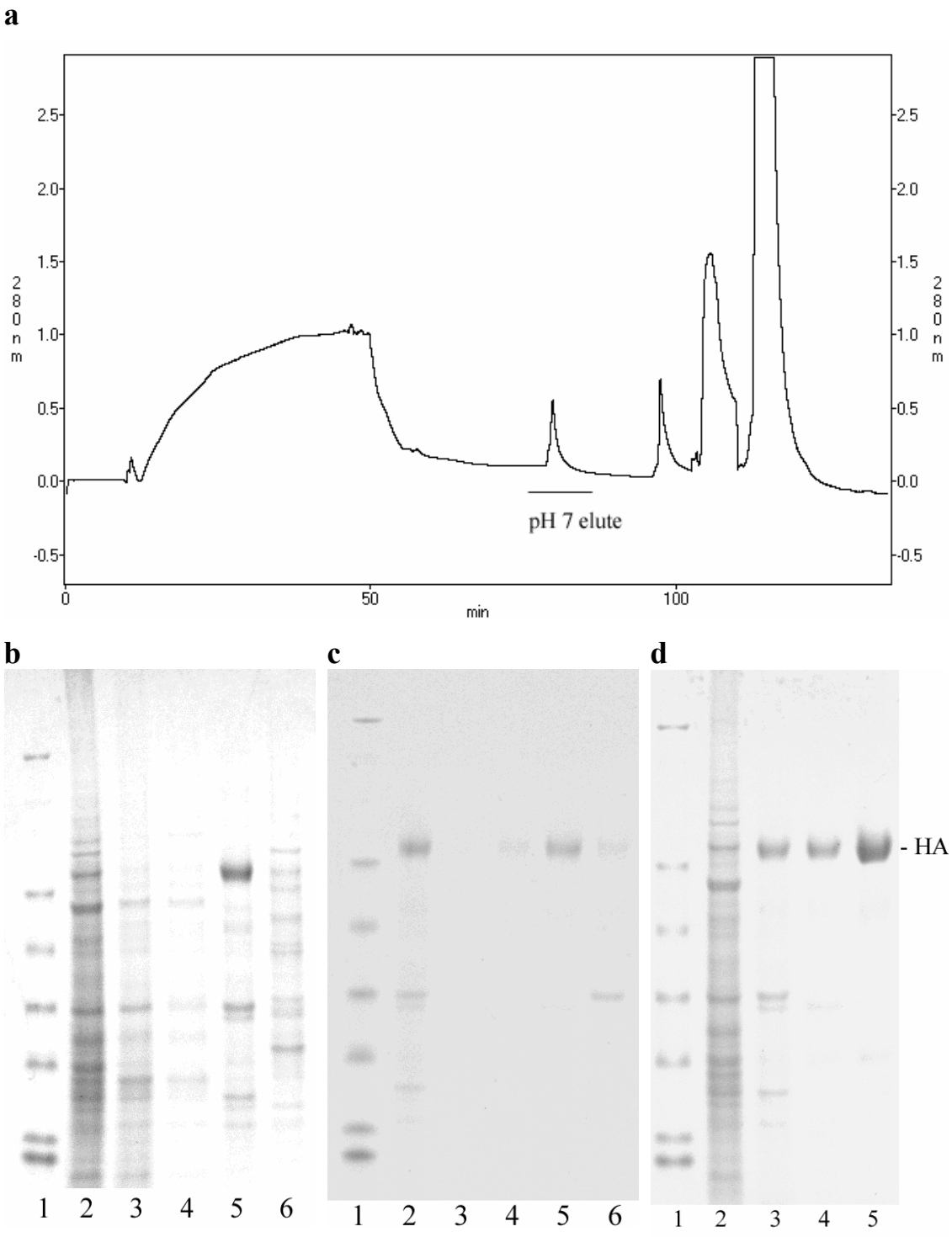

Figure 2. The purification process of rHA. (a) The chromatogram of primary purification and concentration on Q/SP columns. (b) The SDS-PAGE of Q/SP fractions, lanes 1-6 are molecular markers, applied sample, flow through, wash, pH 7 (buffer B) eluate, and buffer C eluate, respectively. (c) The SDS-PAGE of secondary purification on HX-I, lanes 1-6 are molecular markers, applied sample, flow through, wash, 40 mM phosphate eluate, and 100 mM phosphate eluate, respectively. (d) The SDS-PAGE of step-wise purification, lanes 1-5 are molecular markers, Q/SP applied sample, HA after the primary purification, HA after the secondary purification, HA after the final polish using ultrafiltration.

3.3. Characterizing rHA Protein

To confirm the authenticity of rHA, the purified protein was examined by N-terminal amino acid sequencing and total amino acid analysis (AAA). The N-terminal amino acid sequence matched the predicted one (10 cycles were used) and the chitinase signal sequence (as described in Chapter 2 [the first 17 amino acids]) of the full-length HA gene was absent in rHA. The measured amino acid composition of the purified rHA was consistent with the theoretical one (data not shown). The authenticity of rHA was further verified by Western blot analysis using A/New Caledonia/20/99 antibody provided by the Food and Drug Administration (FDA) (*not shown*)

The purified rHA in 0.005% Tween/PBS solution was analyzed using size-exclusion chromatography. It was eluted as a single peak at 9.1 min as shown in Fig. 3a, corresponding to a molecular mass around 800-1000 kDa, likely a complex of four to five HA trimers ((4-5) x 3 x 70 kDa). To test whether the purified rHA still retained its native structure, the purified protein was treated with trypsin on ice (as described Chapter 2). As shown in lane 3 of Fig. 3b, rHA was cleaved into only two bands, HA1-50.9 kDa and HA2-27.5 kDa. On the other hand, the heat-denatured rHA was digested into numerous small fragments. The trypsin-resistance data demonstrate that the rHA expressed in insect cells folded properly and retained its native structure after purification.

Since glycosylation may play an important role in the biological function of HA [Schulze, 1997; Klenk *et al.*, 2002; Helenius and Aebi, 2004], it is of interest to explore whether the rHA produced in insect cells is properly glycosylated. Thus, rHA was deglycosylated with peptide-*N*-glycosidase F (PNGase F) or endoglycosidase H (Endo H) and resolved on SDS-PAGE. As shown in Fig. 3c, the untreated HA migrated at 70.4 kDa, PNGase F deglycosylated HA at 57.9 kDa, and Endo H treated HA at 64.8 kDa, respectively. These data indicate that the rHA produced in insect cells is indeed glycosylated with N-linked oligosaccharide side chains. About 5.6 kDa of oligosaccharide chains have a high mannose content susceptible to Endo H, and 6.9 kDa of oligosaccharide chains have low mannose residues resistant to Endo H. To assess the number of N-linked oligosaccharide chains, the trypsin treated rHA was subjected to limited deglycosylation with PNGase F or Endo H under a variety of conditions. On the SDS-PAGE of PNGase F treated samples (Fig. 3d), there are seven distinguishable HA1 forms, 50.9, 49.0, 46.8, 45.2, 43.3, 42.0 and 39.8 kDa, and 2 HA2 polypeptides 27.1 and 25.4 kDa, respectively. On the SDS-PAGE of Endo H treated samples (Fig. 3e), there are three distinguishable types of HA1 molecules, 51.2, 49.4 and 46.5 kDa, and one HA2 of 27.1 kDa. The data suggest that there are six N-linked oligosaccharide chains in the HA1 region, and two of them have a high mannose content. There is only one N-linked oligosaccharide chain in the HA2 region, which is likely a hybrid or a complex oligosaccharide. Consistently, there are six predicted N-linked glycosylation sites in the HA1 region and one predicted N-linked glycosylation site in the HA2 region of A/New Caledonia/20/99 HA (schematically shown in Fig. 5, Chapter 1), according to the sequence analysis (NetNGlyc) at Technical University of Denmark [Web link 3-1].

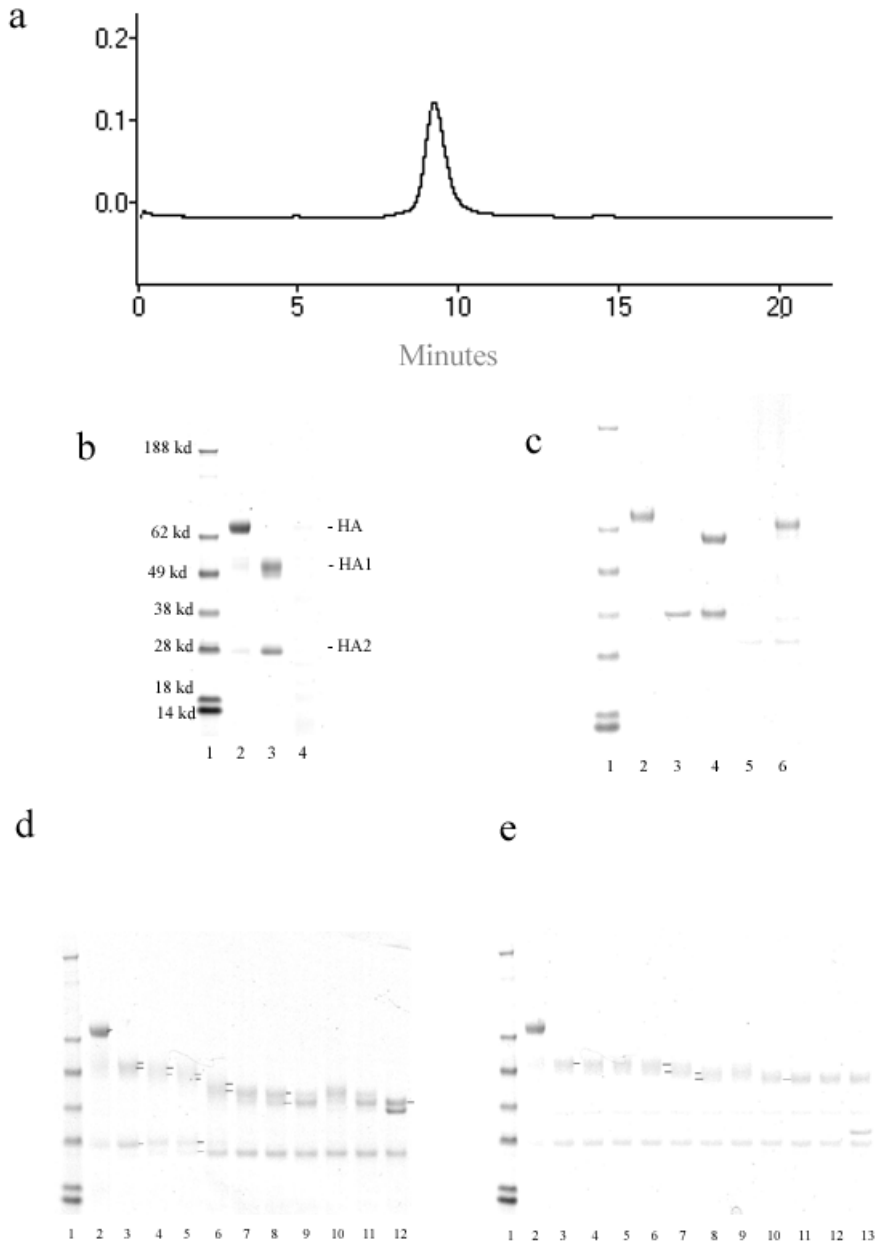

Figure 3. Characterization of rHA. (a) The SEC profile of the purified rHA. (b) The trypsin-resistant test analyzed on SDS-PAGE, lanes 1-4 are molecular weight markers, native rHA, native rHA treated with trypsin, and heat-denatured rHA treated with trypsin, respectively. (c) Deglycosylation of rHA with PNGase F and Endo H, lanes 1-6 are molecular weight markers, rHA, PNGase F, rHA treated with PNGase F, Endo H, and rHA treated with Endo H, respectively. (d) Limited deglycosylation of rHA with PNGase F. Lane 1, molecular weight markers; lane 2, rHA; lane 3, trypsin treated rHA; lanes 4-8, the trypsin treated rHAs were deglycosylated with 2 units of PNGase F on ice, at 25^{o}C or at 37^{o}C for 2-60 minutes; lanes 9-11, deglycosylated with 20 units of PNGase F; lane 12, deglycosylated with 200 units of PNGase F. (e) Limited deglycosylation of rHA with Endo H. Lane 1, molecular weight markers; lane 2, rHA; lane 3, trypsin treated rHA; lanes 4-8, the trypsin treated rHAs were deglycosylated with 2 units of Endo H on ice, at 25^{o}C or at 37^{o}C for 2-60 minutes; lanes 9-12, deglycosylated with 20 units of Endo H; lane 13, deglycosylated with 200 units of Endo H.

To directly compare with the A/New Caledonia antigen present in the egg-based vaccine, the purified rHA/A/New Caledonia was formulated either alone into a 15 µg/0.5 ml solution or with 15 µg rHA/B/Jiangsu and 45 µg rHA/A/Wyoming in a 0.5 ml dosage (FluBlok®, the expected trade name of Protein Sciences' rHA vaccine). As judged by the hemagglutination assay, FluBlok is as active as Vaxigrip (a licensed egg-based vaccine manufactured by Sanofi-Pasteur-Aventis) in agglutinating RBCs and preventing them from forming a tight pellet as shown in Fig. 4a. The antigen specific SRID assay is widely used to determine the concentration of active ingredients in a vaccine. On the SRID gel prepared with A/New Caledonia/20/99 antibody (Fig. 4b), the diffusion ring of FluBlok is slightly larger than that of Vaxigrip, suggesting that FluBlok is at least equivalent to Vaxigrip for the active ingredient of A/New Caledonia/20/99 strain. On the SDS-PAGE (Fig. 4c), the FluBlok lane shows only three major proteins, representing the HA, HA1 and HA2 of the 3 strains, respectively. The Vaxigrip lane is complicated with the major HA band around 59 kDa along with many minor bands. Similarly, numerous impurities have also been found in other egg-based vaccines Renfrey and Watts, 1994].

All these data demonstrate that the purified rHA expressed in insect cells is correctly translated, properly glycosylated and folded and biologically active.

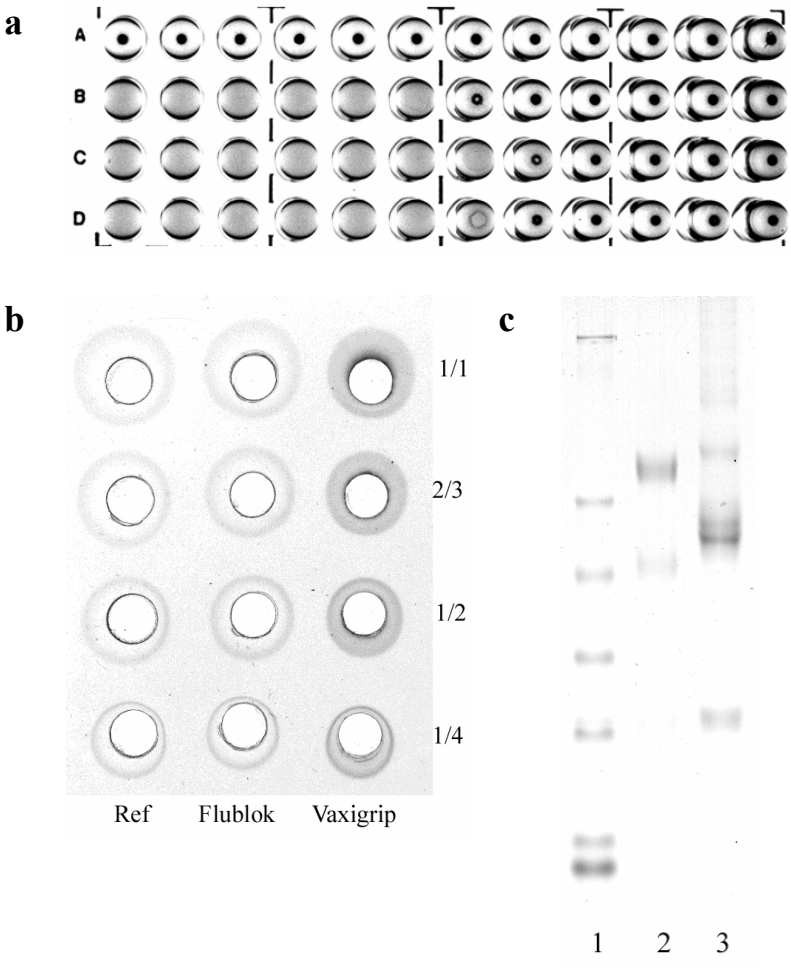

Figure 4. The comparison of FluBlok with Vaxigrip. (a) The hemagglutination activities of 1.0 µg/ml rHA or an equivalent amount from each of the vaccines. Row A, PBS as negative control; Row B, rHA; Row C, Vaxigrip; Row D, FluBlok. (b) Evaluation of the effective antigen concentration with SRID assay. (c) The SDS-PAGE of the vaccines. Lane 1, molecular markers; lane 2, FluBlok; lane 3, Vaxigrip.

4. Discussion

The traditional egg-based vaccines have been successfully used for more than 50 years to prevent influenza. They are reliable, effective (if there is a good match), and affordable. However, the production cycle of the egg-based vaccines is lengthy and heavily dependent on egg supply and unable to be developed quickly in response to the urgent need in an influenza pandemic [Audsley and Tannock, 2004; Sheridan, 2004; Cox, 2005; Osterholm, 2005]. To replace or supplement the egg-based vaccines, the new vaccine has to be equally effective, reliable, economical, and capable of being developed and delivered in a short period of time. The work reported here demonstrates important progress toward an alternative influenza vaccine - the recombinant protein-based vaccine.

A new analytical method based on hemadsorption has been developed to closely monitor the expression of HA in insect cells. This method plays a critical role in ensuring optimal HA production and in determining the right harvest time, in addition to other harvest parameters such as HPI, cell's morphology and viability. A purification process has been developed to quickly purify the recombinant HA from the bulk harvested from the bioreactor while retaining its biological activity. Previously, HA purifications were heavily dependent on affinity chromatography using specific monoclonal antibodies or various lectins as described in Chapter 2 [Mir-Shekari *et al.*, 1997; Holtz *et al.*, 2003]. Such methods are highly selective, but difficult to scale up to commercial levels due to a number of limiting factors: (1) some of the ligands will leach off the column during the purification and they must be removed from the final product; (2) it is difficult to regenerate an affinity column after use, and thus the performance declines after each use; (3) the batch to batch variations in the quality of affinity media make it almost impossible to have a robust purification process from time to time; (4) most affinity media are too expensive to be used at large scale. On the other hand, all chromatographic media used in the present process are chemically stable (can be regenerated repeatedly), commercially available and relatively inexpensive; thus more suitable to scale up in a GMP facility. If the reagents, columns and ancillary equipment are well prepared in advance, the whole purification can be completed in one full working day, avoiding the overnight storage of intermediate rHA preparation and possible inactivation of rHA. Several factors have made this rapid process possible. First, there is no sample manipulation between purification steps, which makes a quick, continuous purification process possible. Second, Q/SP columns are connected in tandem, combining two chromatographic processes into one. Third, a chemically different chromatographic media – HX-I is used to differentiate rHA from the remaining impurities. Unlike ion-exchange media, the adsorption of proteins to HX-I involves both anionic and cationic interaction. The calcium group can interact with carboxylate residues, whereas the phosphate group can bind the basic residues on the surface of the protein. The bound proteins can be eluted by an increasing phosphate gradient or a gradient of calcium, magnesium ions. It is worth to point out that the purification process described here needs to be further optimized for large scale production, for example, a tangential flow filter should be used to replace a stir cell at the final buffer exchange step. Nonetheless, we believe the strategies described here can also be used to develop a rapid purification process for other recombinant proteins.

Biologically, FluBlok is as active as the egg-based vaccine – Vaxigrip as determined by the hemagglutination assay. Based on the SRID assay, FluBlok is equivalent to Vaxigrip for the active ingredient of A/New Caledonia/20/99 strain of

influenza. In challenge studies, chickens were effectively protected against the H5N1 virus infection after inoculation with the rHA of the virus [Crawford *et al.*, 1999]. Moreover, two distantly related H1N1 rHA influenza vaccines using the baculovirus-insect cell expression system have also been demonstrated to partially protect mice against the lethal challenge of a recombinant 1918 pandemic influenza virus [Tumpey *et al.*, 2004]. In clinical trials, the trivalent rHA vaccine (FluBlok) stimulates anti-HA antibody production at least as well as, and in the case of H3 rHA, superior to the traditional egg-based vaccine [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Treanor *et al.*, 2001]. The 2004/05 influenza season Phase II/III field trial of FluBlok that enrolled 460 healthy subjects aged 18 to 49 further showed that the 45/45/45 dose was 100% efficacious in preventing culture positive influenza illness compared to placebo (as described in Chapter 4 and Press Release, Protein Sciences Corp., June 14, 2005).

Furthermore, a recombinant protein-based vaccine, such as the FluBlok described here, also has other advantages over the traditional egg-based vaccines. It consists solely of three antigens (proteins) stored in sterile phosphate buffered-saline and without preservatives such as thimerosal (a mercury derivative currently used in the egg-based vaccine), antibiotics or adjuvants. This may explain why FluBlok has shown lower side effects than the licensed vaccines in clinical trials [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Treanor *et al.*, 2001]. Unlike the egg-based vaccines, no live influenza viruses, high level biocontainment facilities or harsh chemicals such as formaldehyde are used in manufacturing.

Therefore, a reliable, effective, and affordable recombinant protein-based influenza vaccine can be and should be developed to meet the challenge of a potential influenza pandemic. For pandemic preparedness, developing and stockpiling rHA influenza vaccines against the present H5N1 strain may be a good option to provide some protection for the first response personnel and the population in the hard-hit areas in the case of a pandemic, and to win the precious time for manufacturing of a more specific influenza vaccine.

Chapter 4

Safety and Immunogenicity of a Baculovirus-Expressed Hemagglutinin Influenza Vaccine

A Randomized Controlled Trial

Abstract

A high priority in vaccine research is the development of influenza vaccines that do not use embryonated eggs as the substrate for vaccine production. A randomized, double-blind, placebo-controlled clinical trial was conducted at 3 US academic medical centers during the 2004-2005 influenza season among 460 healthy adults without high-risk indications for influenza vaccine to determine the dose-related safety, immunogenicity, and protective efficacy of an experimental trivalent influenza virus hemagglutinin (rHA) vaccine produced in insect cells using recombinant baculoviruses. Participants were randomly assigned to receive a single injection of saline placebo (n=154); 75 µg of an rHA vaccine containing 15 µg of hemagglutinin from influenza A/New Caledonia/20/99 (H1N1) and B/Jiangsu/10/03 virus and 45 µg of hemagglutinin from A/Wyoming/3/03 (H3N2) virus (n=153) or 135 µg of rHA containing 45 µg of hemagglutinin each from all 3 components (n=153). Serum samples were taken before and 30 days following immunization. Rates of local and systemic adverse effects were low, and the rates of systemic adverse effects were not different in either vaccine group from the placebo group. Hemagglutinin inhibition antibody responses to the H1 component were seen in 3% of placebo, 51% of 75-µg vaccine, and 67% of 135-µg vaccine recipients, while responses to B were seen in 4% of placebo, 65% of 75-µg vaccine and 92% of 135-µg vaccine recipients. Responses to the H3 component occurred in 11% of placebo, 81% of 75-µg vaccine and 77% of 135-µg vaccine recipients. Influenza infections in the study population were due to influenza B and A (H3N2), and influenza A infections were A/California/7/2004-like viruses, an antigenically drifted strain. Seven cases of culture-confirmed CDC-defined influenza-like illness occurred in 153 placebo recipients (4.6%) compared with 2 cases (1.3%) in 150 recipients of the 75µg of vaccine, and 0 cases in recipients of 135 µg of vaccine. In this study, a trivalent rHA vaccine was safe and immunogenic in a healthy adult population. Preliminary evidence of protection against a drifted influenza A (H3N2) virus was obtained, but the sample size was small. Inclusion of a neuraminidase component did not appear to be required for protection.

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Introduction

All currently licensed influenza vaccines in the United States are produced in embryonated hen's eggs. There are several well-recognized disadvantages to the use of eggs as the substrate for influenza vaccines. Eggs require specialized manufacturing facilities and could be difficult to scale up rapidly in response to an emerging need such as a pandemic. It is usually necessary to adapt candidate vaccine viruses for high yield-growth in eggs, a process that can be time consuming, is not always successful, and can select receptor variants that may have suboptimal immunogenicity [Katz *et al.*, 1990]. In addition, agricultural diseases that affect chicken flocks might be an important issue in a pandemic caused by an avian influenza virus strain and could easily disrupt the supply of eggs for vaccine manufacturing. Therefore, development of alternative substrates for influenza vaccine production [Cox, 2005] has been identified as a high-priority objective.

One potential alternative method for production of influenza vaccines is expression of the influenza virus hemagglutinin (HA) using recombinant DNA techniques. In this study, we evaluated an experimental influenza vaccine consisting of recombinant HA (rHA) expressed in insect cells by a recombinant baculovirus. This alternative avoids the dependence on eggs, and is very efficient because of the high levels of expression obtained under the control of the baculovirus polyhedrin promoter. Monovalent and bivalent baculovirus-derived influenza vaccines have been evaluated in other studies in young adults and in community-dwelling adults over the age of 65 [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997]. These studies found that the vaccines are well tolerated and immunogenic. Recently, doses of a trivalent vaccine ranging from 15 µg to 135 µg rHA per component were shown to be well tolerated in elderly persons and to induce antibody responses at rates comparable to or superior to the licensed trivalent vaccine [Treanor *et al.*, 2006]. While a clear dose-response relationship has been shown for the H3 component of the recombinant vaccine in both healthy adults and in elderly persons, relatively little difference has been observed in the immune response to rHA protein doses of the H1 and B components ranging from 15 µg to 135 µg. The comparative analysis between the H1 and B components of the recombinant vaccine and the trivalent inactivated vaccine (TIV) has been confounded by a poor correlation between the antigen content of the H1 and B components in the recombinant vaccine, as determined by measurement of total protein content (method previously used to standardize the recombinant vaccine), versus the antigen content determined by single radial immunodiffusion (SRID), the standard measurement used for the TIVs. Therefore, the primary objective of the current study was to evaluate the immunogenicity of the H1 and B components of the vaccine when formulated at either 15 µg or 45 µg per component, as determined by SRID. In addition, we used the opportunity to follow up participants throughout the influenza season to obtain preliminary evidence of protective efficacy in a healthy adult population.

Methods

Vaccine

The vaccine used in this study consisted of purified HA proteins produced in insect cells using a baculovirus expression system as previously described [Treanor *et al.*, 2006]. Genes encoding HA were amplified by RT-PCR using the same seed viruses used for the licensed TIV vaccine provided by the Centers for Disease Control and Prevention (CDC) as template and cloned. Insect cells were infected with the recombinant baculoviruses and the HAs were purified as described in Chapters 2 and 3. Because the HA produced in this system is not cleaved into an HA1 and HA2 component, the resulting product is also referred to as rHA. The vaccine was formulated as a trivalent preparation containing the purified rHA of the A/New Caledonia/20/99 (H1N1), A/Wyoming/3/03 (H3N2) and B/Jiangsu/10/03 influenza viruses in phosphate-buffer saline containing 0.005% detergent without preservative. Monovalent hemagglutinin preparations with purity of approximately 95% of the total protein as determined by SDS-PAGE) were mixed to prepare the trivalent vaccine.

The experimental vaccine was formulated at 2 different concentrations. The high-dose formulation contained 45 μg of each component, for a total dose of 135 μg of rHA per 0.5-mL dose based on the SRID values of the preblend bulk. After formulation, it was determined that the high dose contained 35 μg, rather than 45 μg of the H1 component. The low-dose formulation contained 45 μg of the H3 rHA and 15 μg each of the H1 and B rHA, for a total dose of 75 μg of rHA. The placebo consisted of normal saline for injection. Vaccine and placebo were supplied in coded, identical-appearing single-dose vials.

Clinical Study Design

The study was conducted as a randomized, double-blind, placebo controlled study at 3 medical centers (University of Rochester, Rochester NY; Cincinatti Children's Hospital, Cincinatti, OH; and University of Virginia, Charlottesville) during the 2004-2005 influenza season. Participants were healthy adults aged 18 to 49 years, who did not belong to high-priority target groups for influenza vaccination as defined by the Advisory Committee on Immunization Practice and who had not received previous influenza vaccination for the 2004-2005 season. Women of childbearing potential had to have a negative urine pregnancy test result at the time of randomization and be willing to use an adequate form of contraception during the course of the study. Participants were recruited by newspaper and radio advertisements, posters, and word of mouth. Race/ethnicity data for all participants were collected from self reports. Participants were compensated for each visit (≈$25).

After screening the medical history and a physical examination to determine eligibility of participants, 10 mL of blood was collected from an arm vein for serologic studies. Participants were randomly assigned to receive a single dose of either rHA at 135 μg, rHA at 75 μg, or saline placebo. Vaccine was administered as a single intramuscular injection in the upper deltoid.

Participants measured their oral temperature daily and maintained a diary card for 7 days after vaccination on which they recorded local and systemic reactions graded as mild (noticeable but not interfering with normal activities), moderate (some interference with normal activities), and severe (symptom prevented normal daily activities). Participants returned on day 2 and day 7 after vaccination for review of the diary card, change in concomitant medications and medical history and examination of the vaccination site. Participants returned approximately 28 days after vaccination

for review of interim change in medical history. In addition, 10 mL of venous blood was obtained from an arm vein for assessment of Day 28 antibody to influenza virus. A final study visit occurred at day 180 during which participants underwent a physical examination and review of change in medical history.

The study protocol was reviewed and approved by the investigational review boards at all 3 clinical sites, and written informed consent was obtained from all participants prior to study entry.

Surveillance for Influenza

Following the day 28 visit, participants started to complete a weekly diary to record influenza symptoms, and after influenza was recognized in the community, participants received weekly telephone calls to review the diary and ascertain presence or absence of respiratory illness symptoms. Participants were instructed to return to the clinic for illness evaluations if they observed any acute respiratory tract symptoms or fever. During these illness visits, symptoms were reviewed, a brief physical examination was conducted, and nasopharyngeal swabs for virus culture were obtained.

Laboratory Assays

Serum samples were assessed for antibodies titres to each of the 3 components of the vaccine by hemagglutination inhibition (HAI), using standard methods. Egg-grown influenza A/New Caledonia/20/99 and influenza A/Wyoming/03/2003 were obtained from CDC, while in assays against the influenza B/Jiangsu/10/03 antigen was used, which was prepared in Madin-Darby canine kidney cells from a seed virus also supplied by CDC. Serum samples were treated with neuraminidase (receptor-destroying enzyme, Denka Seiken, Tokyo, Japan) to remove non-specific inhibitors of hemagglutination prior to testing and were tested in serial 2-fold dilutions at an initial dilution of 1:4. Serum samples with no reactivity at 1:4 were assigned a value of 1:2. Assays were performed using chick red blood cells (Colorado Serum Company, Denver) for influenza A/New Caledonia/20/99 and B/Jiangsu/10/03 viruses and turkey red blood cells (Viromed Laboratories, Minnetonka, Minn) for the influenza A/Wyoming/03/2003 virus.

Nasopharyngeal swabs were stored at -70°C and shipped on dry ice to a central laboratory (Cincinnati Children's Hospital Medical Center), where virus amplification was performed in primary rhesus monkey kidney cells (Diagnostic Hybrids Inc., Athens, Ohio). The presence of influenza A or B viruses in the culture was determined by immunofluorescence using type-specific monoclonal antibodies (Diagnostic Hybrids Inc.). Influenza A isolates were further subtyped at Protein Sciences Corp. by sequence analysis of the entire HA1 region after RT-PCR amplification of viral RNA from Madin-Darby canine kidney cell-grown virus.

Definition of End Points

The primary safety end points for this study were the rates and severity of solicited and unsolicited adverse events. The primary immunogenicity end points were the rates of 4-fold or greater increases in serum HAI antibody to each of the 3 vaccine strains comparing prevaccination and 28-day post-vaccination samples. The prespecified primary efficacy end point was culture-documented influenza illness, defined as development of a CDC-defined influenza-like illness associated with recovery of influenza virus from a nasopharyngeal swab. A CDC-defined influenza-like illness was defined as the presence of documented fever with body temperature

higher than 37.7ºC (99.8ºF) plus either sore throat or cough.

Statistical Analyses

Rates of safety end points were based on the most severe response and were evaluated by a χ^2 test. Differences between the proportions of participants with at least a four-fold increase in HAI antibody for each pairwise treatment group comparison were also tested using a χ^2 test.

The sample size for this study was primarily based on the immunogenicity end point. Assuming that from 60% to 80% of the participants in an active treatment group would have a 4-fold or greater serum HAI antibody response to any specific strain, inclusion of 150 participants per group would have 80% power to detect an approximately 13% to 14% difference in response rates between study groups. Although not designed primarily as an efficacy trial, with a 5% attack rate in the placebo group, the study had the power ranging from 14%-53% to detect vaccine efficacy ranging from 40%-80%. A $P<.05$ level was considered to be statistical significant. The analyses were conducted using SAS software, version 9.1 (SAS Institute Inc, Cary, NC).

Results

A total of 460 participants were randomized, 458 were vaccinated, and 451 (98.5%) completed all study procedures. The disposition of the participants is shown in Figure 1. Of the 460 enrolled participants, 153 were randomized to 75 µg rHA vaccine, 153 were randomized to 135 µg rHA vaccine, and 154 were randomized to placebo. There were 9 participants who did not complete the study, 1 withdrew consent, 5 were lost to follow-up, 2 participants in the 75 µg rHA treatment group were randomized but not vaccinated, and 1 was incarcerated. The majority of participants were white (86%) and female (63%) (Table 1). The mean age was 31.7 years (range, 18 - 49 years). There were no differences with respect to age, sex or race/ethnicity between the groups.

Assessment of Vaccine Safety

The rates and severities of local and systemic symptoms reported on the diary card are shown in Table 2. Injection of rHA vaccine was associated with local pain at the site of injection that was significantly more frequent than after saline placebo and that was dose dependent ($P<0.001$ for pain, $P=0.04$ for tenderness). However, 97% of all reports of pain after rHA vaccine were rated as mild. Systemic symptoms following vaccination also did not occur at significantly different rates in vaccine and placebo recipients ($P>0.07$ for all comparisons). The most frequently reported systemic symptom following vaccination was headache. The majority (86%) of reports of headache were also rated as mild, and there was no difference in the frequency of headache between vaccine and placebo recipients ($P>0.07$). There were no reported fevers (oral temperature >37.7ºC [>99.8ºF]) following vaccination.

Two participants (1%) in the 135 µg group experienced serious adverse events that were considered to be unrelated to the vaccine (1 seizure related to hypoglycemia and 1 newly diagnosed lobular carcinoma *in situ*). Two additional participants (1 in the 75-µg group and 1 in the 135-µg group) experienced severe adverse events (1 infected nevus and 1 knee injury) that were also considered to be unrelated to vaccine. No participants discontinued the study due to adverse events and no participants died.

Table 1. Baseline Participant Characteristics

Characteristics	Placebo (n=154)	75µg Vaccine (n=151)	135µg Vaccine (n=153)	Overall (N=458)
Race/ethnicity, No. (%)				
White	139 (90)	126 (83)	130 (85)	395 (86)
Black/African American	9 (6)	12 (8)	9 (6)	30 (7)
Latino/Hispanic	1 (1)	2 (1)	5 (3)	8 (2)
Asian	4 (3)	10 (7)	4 (3)	18 (4)
American Indian/Alaskan Native	0	0	1 (1)	1 (<1)
Native Hawaiian/Pacific islander	0	1 (1)	1 (1)	2 (<1)
Other/not stated	1 (1)	0	3(2)	4 (1)
Male, No (%)	65 (42)	48 (32)	57 (37)	170 (37)
Age, median (range), y	32 (18-49)	32 (18-49)	30 (18-49)	31 (18-49)

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Figure 1. Flow of Participants Through the Trial

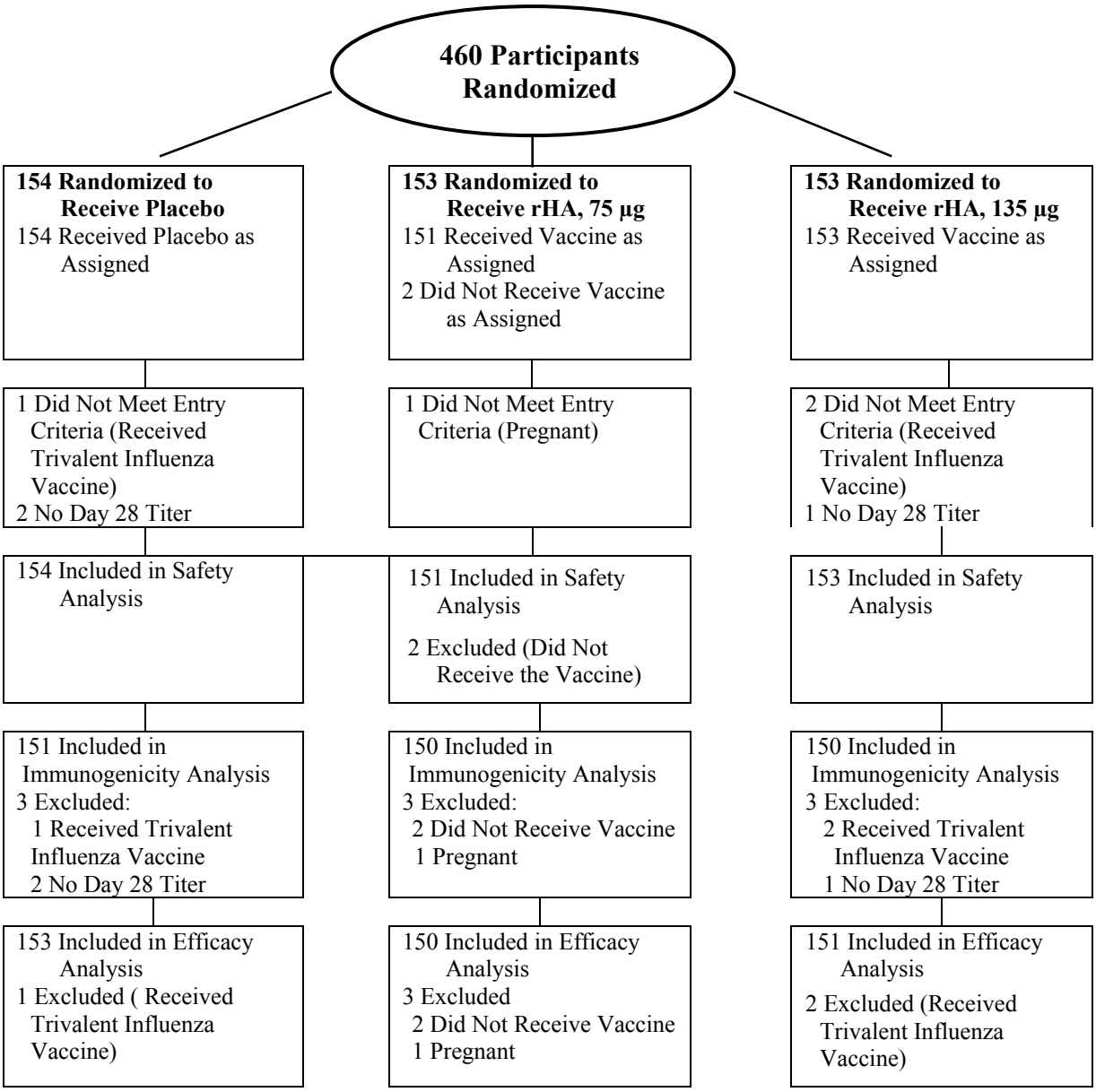

Table 2. Local and Systemic Symptoms Experienced in the 7 Days Following Vaccination*

Symptoms	Placebo (N = 154)			75-µg Vaccine (n=151)			135-µg Vaccine (n=153)		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
Arthralgias	7 (5)	1 (1)	0	7 (5)	2 (1)	0	7 (5)	1 (1)	0
Chills	3 (2)	0	0	2 (1)	2 (1)	0	2 (1)	2 (1)	0
Fatigue	21 (14)	7 (5)	0	21 (14)	7 (5)	0	22 (14)	3 (2)	0
Headache	48 (31)	15 (10)	0	48 (32)	4 (3)	0	53 (35)	12 (8)	0
Myalgias	16 (10)	3 (2)	0	24 (16)	2 (1)	0	28 (18)	3 (2)	0
Nausea	9 (6)	1 (1)	0	5 (3)	2 (1)	0	10 (7)	3 (2)	0
Pain	24 (16)	1 (1)	0	67 (44)	0	0	88 (58)	5 (3)	0
Sweats	6 (4)	1 (1)	0	6 (4)	1 (1)	0	2 (1)	2 (1)	1 (1)
Tenderness	3 (2)	0	0	12 (8)	0	0	9 (6)	1 (1)	0

* Data are expressed as No. (%) of patients experiencing local and systemic symptoms in the 7 days following vaccination based on the most severe responses as reported on the diary cards. Severity was graded as mild (no interference with daily activities), moderate (some limitation of activity due to the symptom), or severe (the symptom prevents normal daily activity).

Immunogenicity

The serum antibody responses to vaccination are summarized in Table 3. Overall, among participants vaccinated with the rHA vaccine, the frequencies of HAI antibody responses (≥4-fold increase comparing day 0 to day 28) to influenza A/New Caledonia, B/Jiangsu, and A/Wyoming were significantly greater (range, 51% to 92%) than was observed for participants vaccinated with placebo (range, 3 to 11%; $p<0.001$). Antibody responses were seen in most participants receiving either the 75-µg or the 135-µg dose of rHA vaccines. However, the frequency of responses to both the A/New Caledonia/99 and B/Jiangsu/03 influenza viruses were significantly higher in the group receiving the 135-µg dose (P = .003), consistent with the higher doses of H1 and B components in the 135-µg vaccine. The frequency of HAI antibody response to the A/Wyoming/03 (H3N2) influenza virus was not different between the 75-µg and 135-µg doses, which contained identical amounts of the H3 component. Similarly, there were significant differences in the day 28 geometric mean titer of HAI antibody between the 75-µg and 135-µg doses for both the H1 and B component, but not the H3 component.

Table 3. Serum Hemagglutination-Inhibiting Antibody Response to Vaccination

Influenza Subtype	Placebo (n=151)	75-µg Vaccine (n=150)	135-µg Vaccine (n=150)
A/New Caledonia/20/99 (H1N1) Prevaccination*	26.4 (19.9-35.0)	23.9 (18.0-31.7)	22.0 (16.6-29.2)
Postvaccination*	28.8 (22.8-36.4)	115.6 (91.5-146.2)	206.0 (163.0-260.5)
Response, %†	3	51	67
A/Wyoming/3/03 (H3N2) Prevaccination*	72.8 (56.4-93.9)	65.5 (50.7-84.6)	74.2 (57.5-95.8)
Postvaccination*	68.9(57.9-81.9)	933.6 (784.4-1111.2)	1028.7 (864.3-1224.5)
Response, %†	11	81	77
B/Jiangsu/10/03 Prevaccination*	6.1 (5.1-7.3)	6.4 (5.4-7.6)	5.5 (4.6-6.5)
Postvaccination*	5.7 (4.7-6.9)	33.4 (27.6-40.4)	74.9 (61.9-90.6)
Response, %†	4	65	92

*Day 0 (prevaccination) and day 28 (postvaccination) geometric mean titers (95% confidence intervals).
†Four-fold or greater increase

Protection Against Influenza Illness

Participants in this study were followed throughout the subsequent influenza season with weekly phone calls and instructed to return to the study clinics for acute respiratory illness, at which time a nasopharyngeal swab for viral culture was obtained. A total of 116 such cultures were obtained, 43 in the placebo group, 39 in the 75-µg vaccine group, and 34 in the 135-µg vaccine group. The primary efficacy end point for this study was the development of culture-confirmed influenza illness meeting the influenza-like ilness case definition of the CDC; ie, presence of fever higher than 37.7°C (99.8°F) and sore throat, cough, or both.

There were a total of 13 positive cultures for influenza in the study population, of which 10 were influenza A (all of which were confirmed as influenza H3) and 3 were influenza B. Sequence analysis of these viruses further revealed that all of the 10 H3N2 isolates were A/California/7/2004-like. Of these 13 cases, 9 (69%) occurred in individuals meeting the CDC-influenza illness case definition. The rates of culture positive influenza illness, the prespecified primary efficacy end point, were 7 (4.6%) of 153 placebo recipients, 2 (1.4%) of 150 recipients of the 75-µg vaccine dose, and 0 of 151 recipients of the 135-µg vaccine dose. Among the 4 positive cultures in individuals who did not meet the CDC case definition, 1 occurred in a placebo recipients, 2 occurred in recipients of the 75-µg vaccine dose, and 1 occurred in a recipient of the 135-µg vaccine dose, so the rates of participants with a positive culture associated with any acute respiratory illnesses were 8/153 (5.2%), 4/150 (2.7%), and 1/151 (0.7%) in placebo, 75-µg vaccine, and 135-µg vaccine recipients, respectively.

Discussion

We evaluated the safety, immunogenicity, and efficacy of a trivalent recombinant hemagglutinin (rHA) vaccine. We have shown that the rHA vaccine is well tolerated in healthy adults and immunogenic at both doses evaluated, and we obtained preliminary evidence of protection against influenza infection and disease. The safety data generated in this study are consistent with the safety profile observed in previous studies of the rHA vaccine [Powers *et al.*, 2005; Treanor *et al.*, 1996; Lakey *et al.*, 2006; Powers *et al.*, 1997; Treanor *et al.*, 2006 These vaccines have been well tolerated at all doses administered and are associated with low rates of local reactions. Because of wide-spread shortages of licensed TIV in the United States in 2004-2005, we were unable to perform a direct comparison of trivalent rHA vaccines and TIV. In other studies of TIV in healthy adult populations, pain at the injection site has been reported in 54% to 67% of recipients [Nichol *et al.*, 1996; Treanor *et al.*, 2005; Ohmit *et al.*, 2006] and systemic symptoms have been similar to placebo, suggesting that the trivalent rHA vaccine and TIV have a similar safety profile. This is consistent with previous direct comparisons of rHA and TIV suggesting similar rates of local reactions between the two vaccines [Powers *et al.*, 2005; Treanor *et al.*, 1996; Treanor *et al.*, 2006].

Both doses of the recombinant hemagglutinin vaccine evaluated induced serum HAI antibody responses to all three components (H1, H3, and B) in the majority of recipients. As expected, there were no differences in either the frequency of responses or the postvaccination geometric mean titer to influenza A/Wyoming (H3N2) between the 75-µg and 135-µg doses, since both doses contained the same 45 µg of the H3 component. Significant differences in both the frequency and the magnitude of the HAI response were demonstrated for both the H1 and B components. Although the responses to the 75-µg dose exceeded the European Union criteria for influenza vaccine licensure [Wood, 1998], the 135-µg dose (45 µg of each component) was more immunogenic and might be predicted to provide greater or longer-lasting protection. Therefore, further development of the rHA vaccine should use a formulation of 45 µg per component, and future studies should directly compare the safety and immunogenicity of this dose with that of TIV. A major advantage of the rHA approach is that these doses are well within the production capacity of the system at an economically and logistically feasible scale.

We also found that recipients of the rHA vaccine had reduced rates of culture-positive CDC-defined influenza-like illness compared with placebo recipients, although the numbers in this study were small. When considering both vaccine groups combined, the cumulative incidence of culture positive CDC-defined influenza-like illness was reduced by 86%. For comparison, in a recently reported study conducted in the same influenza season as was our study, the efficacy of TIV in healthy adults against culture confirmed influenza meeting a similar case definition was 77% (95% confidence interval, 37%-92%) [Ohmit *et al.*, 2006]. The majority of cases were due to influenza A, and all of the influenza A viruses isolated in this study that were further subtyped were of the H3N2 subtype, consistent with the report that 98.5% of all influenza A viruses typed in the United States during the 2004-2005 season were H3N2 [MMWR, 2005]. In addition, all of the influenza A (H3N2) viruses isolated from participants in this trial were genetically similar to A/California/7/2004, a significant antigenic variant which reacts poorly with antiserum samples from persons who received the 2004-2005 formulation of TIV [WHO, 2005]. This is consistent with reports from CDC that indicated that 75% of Influenza A (H3N2) isolates

throughout the United States were genetically similar to A/California/7/2004 [MMWR, 2005]. It has been suggested that the neuraminidase component of the TIV vaccine may be important for protection in situations where there is not a close antigenic match in the hemagglutinin [Brett and Johansson, 2005]. The current study suggests that it is possible to generate a substantial amount of protection in an immunologically primed population against influenza with a pure hemagglutinin vaccine, even in the presence of significant antigenic drift.

The development of functional antibody responses and the preliminary evidence of protective efficacy of the vaccine suggests that in adults, the differences in HA glycosylation seen in insect cells compared with mammalian cells, the presence of HA as an uncleaved precursor, and the lack of neuraminidase in the rHAvaccine do not have a major impact on the actual protection provided by the vaccine. Although this study and other studies in adults have shown excellent HAI and neutralizing antibody responses to the rHA vaccine [Powers *et al.*, 1995; Treanor *et al.*, 1996; Lakey *et al.*, 1996; Powers *et al.*, 1997; Treanor *et al.*, 2006] , further studies in immunologically naïve young children are clearly needed.

The use of recombinant DNA techniques to express proteins in cell culture has been a successful approach for generation of highly effective vaccines for the prevention of e.g. hepatitis B and human papillomavirus [Valenzuela *et al.*, 1982; Mao *et al.*, 2006]. Among the available expression technologies, recombinant baculovirus technology is especially well suited for the production of influenza vaccines because the rapidity with which genes can be cloned and inserted into the baculovirus vector facilitates updating of the vaccine at regular intervals. In addition, the extraordinarily high yields of protein possible in this eukaryotic system provide the opportunity to use higher and potentially more effective doses of vaccine. Expression of the HA protein in insect cells using recombinant baculovirus also avoids the need to work with large amounts of potentially pathogenic influenza viruses, and the attendant biocontainment issues that would be a particular concern for generation of pandemic vaccines [Treanor *et al.*, 2001]. The preliminary demonstration of protective efficacy in adults provides further support for the development of this promising approach for prevention of seasonal and pandemic influenza.

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Chapter 5

Production of a Novel Influenza Vaccine using Insect Cells: Protection Against Drifted Influenza Strains

Abstract

A recombinant trivalent hemagglutinin (rHA) vaccine produced in cell culture using the baculovirus insect cell expression system provides an attractive alternative to the current egg-based influenza vaccine (Trivalent Inactivated Influenza Vaccine = TIV) manufacturing process. The HA genes from the annual vaccine strains recommended by the World Health Organization were cloned, expressed and purified using a general purification process. The highly purified rHA vaccine was administered at a three times higher antigen content than TIV and resulted in stronger immunogenicity than TIV and a long-lasting immune response. In contrast to TIV, the baculovirus-derived rHA vaccine does not contain egg proteins, adjuvants, preservatives, endotoxins or antibiotics and can therefore be used in a broader human population. In this chapter the cross-protection of the rHA vaccine against a drifted variant of H3N2 influenza was also studied and shown to be effective.

This chapter was adapted from: Cox MMJ and Anderson DK. Production of a Novel Influenza Vaccine using Insect Cells: Protection Against Drifted Strains. Influenza and other Respiratory Viruses 2007; 1: 35-40.

Introduction

Influenza is a highly contagious, acute viral respiratory disease, occurring seasonally in most parts of the world. Epidemics occur annually and are the cause of significant morbidity and mortality worldwide [Glezen, 1982]. Influenza disease affects all age groups, with the highest hospitalization rates found in children and the elderly. Influenza causes an average of 110,000 hospitalizations and 20,000 to 50,000 deaths annually in the USA alone [Bridges et al., 2002]. Over 90% of influenza-related deaths occur in people over 65 years. Children under age 5 and women in the first and third trimester of pregnancy are also at higher risk for serious complications.

Influenza viruses are single-stranded ribonucleic acid viruses with a segmented genome encoding 10 proteins. The virus particles have a lipid containing envelope containing two major glycoproteins: hemagglutinin (HA) and neuraminidase. Both proteins have been recognized as key antigens in the host response to influenza virus in both natural infection and vaccination. Antibodies against HA have the ability to neutralize the virus [Kida et al., 1983; Yoden et al., 1986] and, for this reason, HA is generally considered to be the active ingredient in an influenza vaccine and the licensed inactivated vaccines are standardized to contain a least 15 μg HA from each of three influenza viruses selected to be contained in the vaccine. The HA protein consists of two subunits: HA1 and HA2. The HA1 domain contains all the structural epitopes and is connected with the HA2 domain through al disulfide bond. Both inactivated viral vaccines (Trivalent Inactivated Influenza Vaccine [TIV]) and a live-attenuated viral vaccine are approved for use to prevent influenza. The manufacturing of these vaccines involves the adaptation of the selected variants for high yield in eggs by serial passage or reassortment with other high-yield strains. Selected influenza viruses are grown in embryonated chicken eggs and the influenza virions purified from allantoic fluid. For the inactivated virus vaccines, the influenza virus preparations are then killed by treatment with an inactivating agent, such as formaldehyde [Chiron, 2002]. Split virion vaccines such as FluZone (Sanofi Pasteur, Swiftwater, PA, USA) are produced by splitting the virus particles using detergents or solvents. Subunit vaccines such as Fluvirin (Novartis Vaccines Ltd., Liverpool, UK) are further purified to remove the internal virion proteins, leaving mostly HA and neuraminidase in the vaccine.

As described in Chapter 1, the existing influenza vaccines are immunogenic in healthy adults and induce increased levels of HAI antibodies in 70% to 90% of recipients [LaMontagne et al., 1983; Quinnan et al., 1983]. However, the effectiveness of these vaccines is typically low during those seasons when a suboptimal match between vaccine strains and circulating strains is reported caused by the antigenic evolution (genetic drift) of the influenza viruses [Wright and Webster, 2001]. As a result, influenza vaccines require adjustment on an annual basis and vaccine manufacturing needs to be sufficiently flexible to support these annual updates to the vaccine.

The baculovirus-insect cell expression system is a suitable system for the production of viral antigens [van Oers, 2006] and has recently lead to the first vaccine registered for human use, against human papiloma virus causing cervical cancer (Gardesil^{R}, GlaxoSmithKine). The excellent safety profile makes this technology specifically suitable for influenza vaccines, which are generally given to healthy individuals. Furthermore, as shown in Chapters 1-3 [Holtz et al., 2003; Wang et al., 2006], a recombinant baculovirus encoding a new HA can be produced within weeks and a single well- characterized cell line is used in the manufacturing process, making

the annual adjustments required for influenza vaccines feasible in the limited time available.

Recombinant HA (rHA) produced using the baculovirus-insect cell expression system has been tested in multiple phase I/II human clinical trials conducted with the help of the National Institute of Allergy and Infectious Diseases (NIAID) and academic institutions. These studies involving over 600 subjects demonstrated safety, immunogenicity and efficacy as reported in four published studies [Powers et al., 1995; Treanor et al., 1996; Lakey et al., 1996; Powers et al., 1997]. In addition, a safety and immunogenicity study of a H5 rHA avian influenza vaccine as a potential pandemic influenza vaccine was performed by NIAID in response to the threat posed by the 1997-98 Hong Kong "Bird Flu". The vaccine candidate proved to be efficacious in chickens in a challenge study (100% prevention of illness, shedding of the virus and death) conducted by the United States Department of Agriculture in a high containment facility in Georgia [Crawford et al., 1999]. Subsequently, it was administered to over 200 healthcare workers and researchers and produced antibody responses that were believed to be protective in approximately 50% of the recipients who received two doses of the vaccine [Treanor et al., 2001]. In 2003-04, a clinical study was completed in 399 elderly subjects (average age 70 years). In this trial, the immune response to TIV was compared with three different doses of trivalent rHA vaccine (15, 45, or 135 μg of each HA) containing the same HA antigens, produced in insect cells [Treanor et al., 2006]. Recombinant HA was well tolerated and resulted in higher Hemagglutinin Agglutination Inhibition (HAI) antibody levels against the H3N2 influenza virus. This is an important observation because since 1968 the H3N2 influenza viruses cause the majority of the 30,000 - 40,000 excess influenza-related deaths each year in the USA. Of particular interest was a subset analysis of vaccine performance in a group of 100 subjects aged 75 and older where rHA vaccine performance was as good as in the group as a whole, whereas TIV performance was reduced. In addition, in 2003-04 Safdar et al. (2006) completed a study of the performance of a baculovirus-derived influenza rHA vaccine in immuno-compromised subjects and reported encouraging dose-dependent immunogenicity results. Recently, a proof of principle/field study was completed with a trivalent rHA vaccine in healthy adults (18-49 years) showing that the trivalent rHA vaccine was safe, immunogenic, and effective in the prevention of influenza disease (Chapter 4). The clinical studies conducted to date have been summarized in Table 1.

The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) reported that the majority of H3N2 influenza viruses circulating during the period October 2004 through January 2005 were similar to the A/California/7/04 strain [WHO, 2005; MMWR, 2005]. However, the rHA vaccine used in the trial contained the A/Wyoming/3/03 as H3N2 strain. This situation presented the opportunity to determine if the baculovirus-derived rHA vaccine protected against the circulating drifted H3N2 influenza strains.

Table 1. Overview of clinical studies using various prototypes of the hemagglutinin (HA) vaccine

Clinical Studies	n*	Influenza Strain	Dosage recombinant HA_0 (µg)	Reference
93A (young adults)	127	A/Beijing/32/92	15, 90; Fluzone;	[Powers *et al..,* 1995]
94A (young adults)	113	A/Beijing/32/92	15, 45, 135; FluShield	[Treanor *et al*., 1996]
94B (young adults)	153	A/Beijing/32/92 A/Texas/36/91 A/Beijing + B/Texas	45 15, 45, 135 2x 45†; Subvirion	[Lakey *et al..*, 1996]
94C (elderly adults)	109	A/Beijing/32/92	15, 45, 135; FluShield	[Treanor *et al*., 1996]
94D (young adults)	100	A/Beijing/32/92	45	[Powers *et al..*, 1995]
00 (healthy adults)	147	A/Hong Kong/156/97	25/25‡,45/45‡,90/10‡, 90/90‡	[Treanor *et al*., 2001]
03 (elderly)	399	A/New Caledonia/20/99 A/Panama/2007/99 + B/HongKong/330/01	3x15§,3x45§, 3x135§; FluZone	[Treanor *et al*., 2006]
03 (B-Cell lymphoma)	27	A/New Caledonia 20/99+ A/Panama/2007/99 + B/HongKong/330/01	3x15§,3x45§, 3x135§; FluZone	[Safdar et al., 2006]
04 (healthy adults)	460	A/New Caledonia/20/99 A/Wyoming/3/03 B/Jiangsu/10/03	Low 2x15 (H1,B)/45 (H3); High 3x45	[Chapter 4; [Treanor *et al*., 2007]

*Number of subjects included in each study.

† A single bivalent vaccine formulation.

‡ Subjects received two doses 21-28 days apart

§ A single trivalent vaccine formulation

Materials and Methods

Field efficacy study

Subjects in the study received various doses of rHA vaccine, produced in the baculovirus-insect cells system (Chapter 2 and 3), to establish the final commercial dose. The vaccine contained rHA from the influenza strains A/Wyoming/3/03 HA (45 µg), A/New Caledonia/20/99 (15 or 45 µg) and B/Jiangsu/10/03 (15 or 45 µg) (Table 1). The field efficacy study is described in detail in Chapter 4.

Virus Isolation and Sequence Analysis of the HA1 region

Virus isolation was carried out at the Cincinatti Children's Hospital test laboratory (Cincinatti, OH) using standard cell culture methods in primary rhesus monkey kidney (PRhMK) cells. Thirteen positive cultures were obtained from a total of 116 clinical samples (nasopharyngeal swabs). Briefly, clinical samples were adsorbed onto PRhMK monolayers for approximately 60 min, and then monitored daily for cytopathic effects up to 14 days. If a cytopathic effect score of 2+ was reached, the presence (or absence) of either influenza A or B was determined by fluorescent antibody testing with monoclonal antibodies (Diagnostic Hybrids, Inc). Ten isolates were characterized as influenza A and three were characterized as influenza B.

The ten influenza A isolates were further amplified on Madin-Darby canine kidney cells. Virions were disrupted in a buffer containing guanidine thiocyanat, to inactivate ribonucleases, thereby ensuring the purification of intact RNA. Viral RNA was purified over silicabased columns and stored at -70^{o}C. The purified influenza viral RNAs were then used as a template in a reverse transcriptase (RT) reaction to generate a single stranded cDNA for the required HA. The RT reactions were primed with H3 specific 3' oligonucleotides (Table 2). Subsequently, the HA1 region was amplified using high fidelity DNA Polymerase and and oligonucleotides specific for regions conserved in the H3 HA (Table 2). PCR-products were purified and subjected to sequence analysis. Primers used for sequence-analysis were spaced approximately every 300 nucleotides. The sequence reactions were carried out by MWG-Biotech, Inc. (Highpoint, NC). For each clone data from individual sequencing runs were compiled into a single contiguous sequence, which was then compared to the reference sequence obtained from the Influenza Sequence Database [ISD, web link: 5-1] or from GenBank [web link: 5-2]

Table 2. Oligonucleotides used for PCR amplification

Primer #	Description	Sequence*
A/Wyoming/03/03 (H3N2)		
2336	H3 strain 5' primer	gttagtaacgcgCAAAAGCTTCCCGGAAATG
2439	H3 strain 3' primer	ttaattaattacTTAAATGCAAATGTTGCACCTAATGTTG

Nucleotides in capital font match the HA sequence. Nucleotides in small type are added for cloning purpose.

Results

Clinical Results

Recently, a proof of principle/field study was completed with a trivalent rHA vaccine in 460 healthy adults (18-49 years) to establish the final commercial dose (Chapter 4). The trivalent rHA vaccine was safe, immunogenic, and effective in the prevention of influenza disease, with the higher dose containing 45 µg of each HA showing a 100% protective efficacy against cell culture confirmed influenza in subjects presenting with influenza-like illness (CDC-ILI). In addition, the number of subjects presenting with CDC-ILI in the group receiving the high dose formulation (45 µg of each antigen) was reduced by 54.4% when compared to the placebo group [Chapter 4; Treanor *et al.*, 2007]. Finally, protection appears long-lasting as the geometric mean titers for the H3 component still exceeded 500 after a period of 6 months. This higher dose containing 45 µg of each HA has been selected for further development and testing.

Characterization of Influenza Isolates

The WHO and CDC both reported that the majority of H3N2 influenza viruses circulating during the period October 2004 through January 2005 were similar to the A/California/7/04 strain [WHO, 2005; MMWR, 2005]. On the other hand, all commercial (TIV) vaccines and the experimental rHA vaccine for this influenza season contained A/Wyoming/3/03 as H3N2 strain. In addition, HA antibodies stimulated by the vaccine were lower in titer to A/California/7/04-like viruses than to the A/Wyoming/3/03 virus contained in the vaccine [WHO, 2005]. This suggests that the rHA vaccine used in the trial had the capacity to protect against the circulating drifted H3N2 strain.

To analyse this further, 10 influenza isolates from the 2004 to 2005 field efficacy study were characterized by sequence analysis of an RT-PCR cDNA product using primers for amplification of the variable HA1 gene region. The results are summarized in Table 3. The A/Wyoming/3/03 and A/California/7/04 strain H3 proteins differ from each other in 10 amino acid positions spread throughout the antigenic regions of the HA1 portion of the proteins. All of the influenza strains isolated from subjects (PS1-PS10) matched the A/California/7/04 strain H3 protein sequence in seven of the 10 positions. Isolates PS4 and PS5 also matched the A/California/7/04 strain H3 protein sequence at amino acid position 188. As shown in Table 4, many of the isolated influenza viruses contained additional mutation(s) in their HA amino acid sequences found in neither the A/Wyoming/3/03 nor the A/California/7/04 strain H3 proteins, further emphasizing their drifted nature.

Table 3. Comparison of HA amino acid sequence of field isolates (PS1 – PS10), A/Wyoming/3/03 and A/California/7/04

Wyoming ↕ California	**A 128 T**	**A 138 S**	**K 145 N**	**Y 159 F**	**V 186 G**	**D 188 N**	**S 189 N**	**A 196 T**	**Y 219 S**	**S 227 P**
PS1-PS3, PS6-P10	T	A	N	F	G	D	N	A	S	P
PS4, PS5	T	A	N	F	G	N	N	A	S	P

Grey area indicates similarities between field isolates and A/California/7/04

These results confirmed the WHO report that the majority of H3N2 influenza viruses circulating during this period were similar to the A/California/7/04 strain. Despite the changes in the HA sequence of the circulating compared to the vaccine strain, we were unable to culture H3N2 influenza viruses from any of the subjects in the study, who received the high dose of the rHA vaccine containing 45 µg of A/Wyoming/3/03 HA, 45 µg A/New Caledonia/20/99 and 45 µg B/Jiangsu/10/03. As shown in Table 4, the majority of subjects who were influenza culture positive had previously received placebo (six out of 10). Surprisingly, four individuals receiving the low dose vaccine containing 45 µg of A/Wyoming/3/03 HA as well, but only 15 µg A/New Caledonia/20/99 and 15 µg B/Jiangsu/10/03 were culture positive. Two of these subjects did not develop fever and were shown to be CDC-ILI negative, suggesting that the lower dose vaccine reduced the severity of the influenza infection in these subjects. One of the remaining two subjects (isolate PS10) showed an extremely poor antibody response to the A/Wyoming/3/03 strain, making cross protection very unlikely. Both the high-dose vaccine and the low-dose vaccine contained 45 µg A/Wyoming/3/03 HA, yet the protective efficacy against the circulating A/California/7/04 differs, suggesting that a higher content of the other HA antigens plays a role in providing protection against infection with drifted influenza H3N2 strains.

Table 4. Evaluation of Influenza H3N2 isolates (PS1 – PS 10)

Influenza H3N2 isolate ID	Treatment	CDC-ILI	HAI Titer Wyoming day 28	Additional mutations
PS8	Placebo	Positive	4	N206S; V213I; I216V
PS1	Placebo	Positive	8	F174Y
PS9	Placebo	Positive	16	A198S; R299K
PS5	Placebo	Positive	32	
PS7	Placebo	Positive	64	V88I
PS4	Placebo	Positive	256	
PS10	Low dose	Positive	32	G50E; L164M
PS6	Low dose	Positive	>1024	R150K
PS2	Low dose	Negative	512	N278K
PS3	Low dose	Negative	512	

CDC-ILI, Center for Disease Control Influenza-like illness, subjects presenting with fever plus at least one respiratory system.
Gray area indicates subjects receiving the low dose vaccine

Discussion

The formulation of the rHA influenza vaccine intended for commercialization contains 45 μg of each HA; this is three times more than the antigen content of existing inactivated influenza vaccines. The mechanism of action of this vaccine candidate is similar to TIV, namely the induction of HA antibodies to prevent influenza infection. The recombinant vaccine offers significant advantages over the traditional egg-based vaccines as it is highly purified, free of preservatives, endotoxins or adjuvants, and produced in cell culture. As a result the rHA vaccine is well tolerated by a wider patient population (e.g. those with egg allergies).

The higher HA content in the vaccine offers not only the possibility for the presence of circulating antibodies over an extended period of time, but also the potential to provide cross-protection for which the first evidence was presented here. The current results are consistent with the previously reported studies that showed that increased doses of purified HA and increased doses of subvirion vaccines result in an enhanced antibody response in both the elderly and the healthy adult population [Keitel *et al.*, 1994; Keitel *et al.*, 1996]. Characterization of the H3N2 influenza virus field isolates by sequence analysis clearly reveals the drifted nature of these viruses, with a minimum of 7 amino-acid changes from the HA represented in the vaccine. Even though the H3N2 HA content was 45 μg in both the low and high dose rHA vaccine, H3N2 influenza viruses were isolated from four low dose recipients (two of whom had only mild disease) whereas no influenza viruses were isolated from the high dose vaccine recipients. This result suggests that the other vaccine components (i.e. H1 and B rHA) may contribute to the overall vaccine performance.

The TIV provides an economical and effective means to reduce the impact of an influenza infection. It is effective in prevention of influenza in young adults with reported levels of protection as high as 70-90% [Meiklejohn *et al.*, 1987; Ruben, 1987]; however only 30% to 50% of vaccinated subjects older than 65 years achieve protective titers against the H3 strain [Powers and Belshe, 1993]. Therefore, there is need to develop a better vaccine for this at risk population. Furthermore, a reduction of 30% in the effectiveness of TIV was reported for the 2003-04 season when the influenza vaccine was not well matched to the dominant circulating strain [MMWR, 2004], suggesting that a vaccine that provides cross protection would also be beneficial.

The egg-based manufacturing technology used to produce all currently approved influenza vaccines has reached its limits, in terms of productivity, safety and production security and is likely to be replaced in the future with a modern cell-based production technology in the near future. Given the availability of a cheap and relatively effective vaccine with a well-defined mechanism of action, limited effort has been directed towards the development of protein-based vaccines. However, many new cell-based and protein vaccines are now in clinical development [Cox, 2005] and are expected to address some of the limitations of the current licensed vaccine, including egg-associated allergies and the need for surge capacity in a pandemic situation.

The recombinant trivalent HA vaccine (proposed trade name FluBlok; Protein Sciences Corporation, Meriden, CT, USA) is similar to TIV in that it contains antigens (HA proteins) that are derived from the three influenza virus strains that are selected for inclusion in the annual influenza vaccine by the WHO. The rHA antigens are developed using recombinant DNA and cell culture technology enabling the production of a perfect matching HA protein instead of introducing egg or cell culture

mutations that may result in a less effective vaccine [Schild *et al.*, 1983; Katz *et al.*, 1987; Rajakumar *et al.*, 1990]. Unlike the licensed egg grown vaccines and many cell culture vaccines in development, no live influenza viruses are used in manufacturing, which eliminates the need for biocontainment facilities or harsh chemical treatment such as formaldehyde. The vaccine is a pure protein preparation containing the three antigens (proteins) in sterile buffered salt water without preservatives such as thimerosal, a mercury derivative used in egg-production process, or adjuvants. The potential of a high dose influenza vaccine that can provide cross-protection would be extremely valuable especially since the influenza virus is constantly antigenically evolving both in time and in space (geographic regions). Finally, the recombinant, cell-based influenza vaccine offers an extremely rapid development cycle. This was demonstrated in making a vaccine for the 1997-98 Hong Kong "Bird" flu in only 8 weeks. In 2006 (more than 6 years later), similar clinical results were achieved with a "reverse genetics" vaccine candidate produced in embryonated chicken embryos by Sanofi Pasteur [Treanor *et al.*, 2006]. In addition to pandemic preparedness, a rapid development cycle would allow the inclusion of late appearing influenza virus strains in the annual vaccine preparations.

Chapter 6

Novel Cell Substrate: Safety and Immunogenicity of FluBlok, a Recombinant Influenza Vaccine Manufactured in Insect Cells

Abstract

FluBlok, a recombinant trivalent hemagglutinin (rHA) vaccine produced in insect cell culture using the baculovirus expression system, provides an attractive alternative to the current egg-based trivalent inactivated influenza vaccine (TIV). Its manufacturing process presents the possibility for safe and expeditious vaccine production. FluBlok contains three times more HA than TIV and does not contain egg-protein or preservatives. The high purity of the antigen enables administration at higher doses without a significant increase in side-effects in human subjects.

The insect cell - baculovirus production technology is particularly suitable for influenza where annual adjustment of the vaccine is required. The baculovirus-insect expression system is generally considered a safe production system, with limited growth potential for adventitious agents. Still regulators question and challenge the safety of this novel cell substrate as FluBlok continues to advance toward product approval. This chapter describes a study on cell substrate characterization for the *expres*SF cell line used for the manufacturing of FluBlok.

In addition, data from the four main clinical studies that were used to support licensure of FluBlok under the "Accelerated Approval" mechanism in the United States are presented. The highly purified protein vaccine, administered at three times higher antigen dose than TIV is well tolerated and results in stronger immunogenicity, a long lasting immune response and provides cross protection against drifted influenza viruses.

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1. Introduction

Most current influenza vaccines are generated in embryonated hen's eggs. Virions are harvested from the egg allantoic fluid, chemically inactivated and treated with detergent, and either a whole virion preparation is generated, or the hemagglutinin (HA) and neuraminidase (NA) proteins are partially purified to produce split-product, subvirion, or subunit vaccines [Wood, 1998]. Although this system has served well for over 50 years, there are several well-recognized disadvantages to the use of eggs as the substrate for vaccine production. The licensed egg-grown trivalent inactivated influenza vaccines (TIVs) are standardized to contain 15 μg of each of three HAs, derived from influenza A subtype H1N1, H3N2 and influenza B [Bridges *et al.,* 2002]. Thus, HA, the dominant surface glycoprotein on the influenza virus and recognized key antigen in the host response to influenza virus in both natural infection and vaccination, is a logical candidate for recombinant vaccine technology [Huber and McCullers, 2008].

FluBlok is a trivalent recombinant HA (rHA) vaccine, is under development, which contains rHA protein antigens that are derived from the three influenza virus strains, which have been selected for inclusion in the annual influenza vaccine by the World Health Organization (WHO) and are updated on an annual basis. As a result the composition of FluBlok and TIV (i.e. Fluzone) may change each influenza season and region.

The mechanism of action of FluBlok is the same as that of the licensed TIVs, thereby simplifying the regulatory pathway for product approval. FluBlok is formulated to contain 45 μg of each rHA, three times the amount of HA as is contained in TIV. The higher rHA content offers the potential to provide cross-protection for which preliminary evidence has been presented, but also the possibility for longer lasting and improved immunogenicity [Treanor *et al.,* 2006; Cox and Anderson, 2007; Treanor *et al.,* 2007] . Clinical results suggest that FluBlok may provide superior protection against influenza infection especially in at-risk populations (adults over 65 years, immuno-compromised individuals, etc.) as has been reported for increased antigen concentration of TIV [Keitel *et al.*, 1994; Keitel *et al.,* 1996]. This chapter describes the use of a novel cell substrate based on the baculovirus-insect cell system for the production of hemagglutinin and summarizes some key immunogenicity results from clinical studies that were used to support licensure of FluBlok under the "Accelerated Approval" mechanism in the United States. In addition, the correlation between post-vaccination titer (Day 28) and acquisition of influenza infection is discussed.

The three HA proteins in FluBlok are produced in a non-transformed, non-tumorigenic continuous cell line (*expres*SF+® insect cells) grown in serum-free medium. The cell line is derived from Sf9 cells of the fall armyworm, *Spodoptera frugiperda* [Smith *et al.*, 2000]. Each of the three recombinant HAs (rHAs) is expressed in this insect cell line using a viral vector (the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus). The HA antigens included are full length proteins containing the transmembrane domain and the HA1 and HA2 domains. The rHA proteins form trimeric structures visible by electron microscopy and are not cleaved in insect cells in the absence of exogenously added proteases (with the exception of HAs containing a highly cleavable sequence of basic amino acids at the cleavage site). Therefore, they are sometimes referred to as rHA0. Since the cleavage site is not known to be involved in the immune response, a significant difference between the immune response to cleaved or uncleaved HA is unlikely. The

individual rHAs are extracted from the cells with buffer and detergent and further purified by using a combination of filtration and column chromatography methods. Details on the production and characterization of rHA are described elsewhere [Chapter 2 and 3; Holtz *et al.*, 2003; Wang *et al.*, 2006]. The mechanism of action of this vaccine candidate is expected to be similar to TIV; namely, the induction of hemagglutination inhibition (HAI) antibodies to prevent influenza infection [Kida *et al.*, 1983; Yoden *et al.*, 1986].

Manufacturing in insect cells offers a number of advantages over currently licensed influenza vaccines that are produced in embryonated chicken eggs: (i) the influenza rHA antigens are produced using a scaleable, reproducible, and low bioburden fermentation process in insect cells, which results in a consistent, protein-based vaccine with low endotoxin content [Holtz *et al.*, 2003; Cox and Anderson, 2007]; (ii) selection or adaptation of influenza virus strains as for production at high levels in eggs is not required, enabling a good genetic match between the vaccine strains and the disease causing influenza virus strains [Holtz *et al.*, 2003; Cox and Anderson, 2007]; (iii) the cloning, expression and manufacture of FluBlok can be accomplished within a brief period of time, generally less than two months; and (iv) the manufacture of FluBlok does not require high-level bio-containment facilities, which may result in more rapid vaccine production at lower costs in the event of the emergence of a new epidemic or pandemic strain of influenza virus; and (v) purification procedures for rHA do not include influenza virus inactivation or organic extraction procedures, thus avoiding possible denaturing effects and additional safety concerns because of residual toxic chemicals in the vaccine [Holtz *et al.*, 2003; Cox and Anderson, 200]. Perhaps most importantly, from a clinical perspective, FluBlok is highly purified and does not contain antigenic proteins present in eggs [Holtz *et al.*, 2003; Cox and Anderson, 2007].

In addition to presenting advantages in manufacturing, as discussed above, insect cells provide safety advantages for the production of biologicals. Insect cells can be grown in the absence of fetal bovine serum and other animal derived ingredients, significantly reducing the chances of introducing an adventitious agent during manufacturing [Rohwer, 1996; Nims, 2006; Chen *et al.*, 2008]. The genetic distance between insects and vertebrates also reduces the likelihood of insect cells serving as a host for vertebrate viruses or the likelihood of vertebrates serving as a host for insect viruses. In fact, many insect viruses described to date exhibit a relatively narrow host range with only a small number of viruses capable of amplifying in both insects and vertebrates [Miller and Ball, 1998; Kuno and Chang, 2005]. These insect viruses along with some tick viruses that can also amplify in vertebrates are informally referred to as arboviruses reflecting their arthropod-borne origin, and have closely co-evolved with the hematophagous arthropods and the vertebrate hosts upon which they feed [Kuno and Chang, 2005]. The susceptibility of Sf9 cells to arbovirus infection is reported to be very low; with St. Louis encephalitis virus being the only arbovirus tested to date that could produce a persistent, productive and cytopathic infection [Zhang *et al.*, 1994]. In addition, Menzel and Rohrmann (2008) recently described the presence of errantivirus (retrovirus) sequences in two insect cell lines, including *Spodoptera frugiperda* (Sf) cells.

The recent progress in using novel animal cell lines as substrates for the production of biologicals has led to the re-evaluation of existing criteria used for evaluating the acceptability of such cell lines. Improvements to existing criteria for determining the acceptability of novel cell substrates as well as development of new criteria have recently been the focus of regulatory agencies [Knezevic *et al.*, 2007].

2. Insect cell substrate- *expres*SF+

The baculovirus expression vector system is used to produce recombinant proteins in the proprietary lepidopteran insect cell line *expres*SF+ (SF+). SF+ cells were derived from the Sf9 cell line, which was first cloned via dilution plating of the mixed population cell line IPLB-Sf-21AE by Cherry and Smith at Texas A&M University (unpublished, 1983). At the time the Sf9 line was developed, the IPLB-Sf-21AE cell line had been in continuous culture since its isolation in 1970 from primary cultures of normal ovarian tissues dissected from pupal ovaries of the fall armyworm, *Spodoptera frugiperda* [Vaughn *et al.*, 1977].

Several characteristics of SF+ cells make them well suited as cell substrates for the manufacture of biologicals using the baculovirus expression vector system: (i) SF+ cells are grown in suspension in an inexpensive animal product-free medium to high densities and without clumping; (ii) cultures of SF+ cells are routinely scaled to 450L under cGMP conditions and maintain a consistent cell doubling time of approximately 18-24 hours; (iii) significantly higher yields of recombinant proteins are obtained in SF+ cells as compared to the parental cell line Sf9; (iv) SF+ cells support robust growth of recombinant baculoviruses resulting in high titer stocks of virus, thus allowing for protein expression and virus stock production to be carried out using a single qualified cell line; and (v) these attributes are consistent for over 50 cell culture passages allowing for significant flexibility in maintenance of seed stocks during large scale cGMP production and have been described elsewhere [McPherson, 2008].

Qualification testing on the SF+ cell line has been previously described [Mc Pherson, 2008] and was performed according to the International Conference on Harmonization (ICH) document Q5A (2005), the Food and Drug Administration's (FDA) "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals," dated 1993, and the FDA's 2006 draft guidance "Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases." In addition to the testing described in McPherson (2008), additional subsequent testing has been performed based on product-specific comments from the FDA. A description of the testing undertaken and the results are described in sections 2.1 below.

2.1 Qualification testing

2.1.1 Transmission electron microscopic examination of stressed expresSF+ cells

Visual detection of virus particles in cells that harbor latent viral genomes or inefficiently replicating viruses is virtually impossible unless the virus can be induced to replicate, which sometimes occurs as a consequence of cellular stress. To address the potential that SF+ cells might harbor such an infection, cells from an SF+ Working Cell Bank (WCB) at the end of the production passage (>P50) were chemically treated or incubated at elevated temperatures to induce cellular stress prior to examination by transmission electron microscopy (TEM).

To induce stress by chemical treatment, SF+ cells were treated with the halogenated pyrimidine analog 5-iododeoxyuridine (IdU), which has been previously reported to potentiate viral replication in mammalian and insect cell cultures [Green and Baron, 1975; Carreno and Esparza, 1977; Patch *et al.*, 1981; Khan and Sears, 2001; Khan *et al.*, 2001]. Treatment with IdU was carried out by growing the cells in the presence of IdU for 1 day, followed by a 1 day recovery period. Analysis of the chemically treated cells by TEM revealed no virus-like particles or other biological contaminants in any of the 200 cells examined (Charles River Laboratories, MA).

A high temperature shock was used as a second independent method of inducing stress in SF+ cells. To ensure that the cells would genuinely be stressed by the temperature treatment, we chose conditions that have been previously reported to induce heat shock proteins in the related cell line Sf9 [Huhtala *et al.*, 2005]. In that study heat shock proteins were observed to be induced when cells were incubated for 15 minutes to 1 hour at temperatures above 37° C. Samples of heat shocked SF+ cells were prepared by incubating cells at 43° C for 45 minutes, followed by a 1-day recovery period and then analysis by TEM. No virus-like particles or other biological contaminants were observed in any of the 200 cells heat-treated cells examined.

It is important to note however, that a limitation of both analyses is a lack of an insect cell induction control. Even though there is a recent report on the presence of errantivirus (retrovirus) sequences in Sf cells [Menzel and Rohrmann, 2008], there are no retrovirus-like viruses reported to infect Sf cells, thus an adequate positive control for the treatments described above was not available. Treatment conditions were therefore developed based on assumptions using studies in mammalian cell lines as a guideline.

2.1.2 Adventitious virus detection

2.1.2.1 Approach.

Viruses that use insects as their primary natural host species are classified into 17 virus families and more than 30 genera, and utilize most known niches for genome coding. This great diversity among insect viruses coupled with large gaps in our scientific understanding of these viruses has created a challenging environment in which to develop viral assays to detect the presence of insect viruses in cell substrates. Ideally, a generic test that is capable of detecting all known insect virus members within a specific virus family is needed. Recently, PCR primers have been designed with the capability to detect both known and novel herpes- and papillomavirus species [Rose, 1995; Baines *et al.*, 2005; Jarrett *et al.*, 2006]. For these assays, PCR primers were designed according to the consensus-degenerate hybrid oligonucleotide primers (CODEHOPs) strategy [Rose *et al.*, 1998]. The CODEHOP approach is well suited to take advantage of short (3-4 amino acids) highly conserved amino acid motifs found in related proteins encoded by virus species within the same family. Based on the

success of others using this approach we have begun developing a PCR based assays utilizing the CODEHOP strategy to screen the SF+ cell line for the presence of contaminating insect viruses.

2.1.2.2 Virus family selection

To focus assay development towards insect virus species with the highest potential risks for contaminating SF+ cells, we assessed which families contained viruses known to infect lepidopteran insects. Table 1 lists the current virus families known to contain insect viruses excluding those families which only insect virus members are arboviruses, such as the *Flaviviridae*, *Bunyaviridae* and the *Togaviridae*. Based on this analysis we excluded the family *Dicistroviridae* due to the absence of a known lepidopteran infecting virus as well as the family *Birnaviridae*, which contains in the genus Entomobirnavirus so far only one insect-infecting virus species, *Drosophila X virus* [8th ICTV report, 2005]. Also excluded were the families *Reoviridae* and *Polydnaviridae,* both of which are comprised of viruses for which there is limited genetic information. Finally, assays for viruses belonging to the families *Metaviridae* and *Pseudoviridae* were also excluded for CODEHOP primer design due to their relatedness to retroelements, for which insect cells have been reported to be a rich source [Terzian *et al.*, 2001], The SF+ genome and members from the latter virus families most likely share DNA sequences encoding for conserved domains in proteins; Therefore, a positive result by PCR would not necessarily indicate an infectious agent was present.

Table 1. Virus families containing insect virus species [8th ICTV report, 2005]

Family	Genome Type	Genera	Type Species
Ascoviridae	dsDNA	*Ascovirus*	*Spodoptera frugiperda ascovirus 1a*
Baculoviridae	dsDNA	*Nuclear Polyhedrosis Virus*	*AcMNPV*
		Granulovirus	*Cydia pomonella granulovirus*
Birnaviridae	dsRNA	*Entomobirnavirus*	*Drosophila X virus*
Dicistroviridae	ssRNA(+)	*Cripavirus*	*Cricket paralysis virus*
Iridoviridae	dsDNA	*Iridovirus*	*Invertebrate iridescent virus 6*
		Chloriridovirus	*Invertebrate iridescent virus 3*
Metaviridae	ssRNA-RT	*Metavirus*	*Saccharomyces cervisiae Ty3 virus*
		Errantivirus	*Drosophila melanogaster gypsy virus*
		Semotivirus	*Ascaris lumbricoides tas virus*
Nodaviridae	ssRNA(+)	*Alphanodavirus*	*Nodamura virus* *Flockhouse virus*
Parvoviridae	ssDNA	*Densovirus*	*Junonia coenia densovirus*
		Iteravirus	*Bombyx mori densovirus*
		Brevidensovirus	*Aedes aegypti densovirus*
		Pefudensovirus	*Periplanta fuliginosa densovirus*
Polydnaviridae	dsDNA	*Ichnovirus*	*Campoletis sonorensis ichnovirus*
		Bracovirus	*Cotesia melanoscela bracovirus*
Poxviridae	dsDNA	*Entomopoxvirus A*	*Melolontha melolontha entomopoxvirus*
		Entomopoxvirus B	*Amsacta moorei entomopoxvirus*
		Entomopoxvirus C	*Chironomus luridus entomopoxvirus*
Pseudoviridae	ssRNA(+)	*Hemivirus*	*Drosophila melanogaster copia virus*
Reoviridae	dsRNA	*Cypovirus*	*Cypovirus 1*
		Idnoreovirus	*Idnoreovirus I*
Tetraviridae	ssRNA(+)	*Betatetravirus*	*Nudaurelia capensis β virus;*
		Omegatetravirus	*Nudaurelia capensis ω virus*

Note: *Iflaviridae* with the infectious *Bombyx mori flacherie virus* as the type species (Genera Iflavirus) has recently been identified as a family to infect insect cells as well.

2.1.2.3 Selection of the viral protein target

Viral proteins targeted for the design of CODEHOPs were selected by performing a systematic manual survey of the genetic information available for virus species in each family. Criteria for selecting a protein sequence as the target for primer design were based on: (i) the number of virus species within the same family for which a target protein sequence was available and (ii) the ability to identify conserved amino acids in alignments using the available sequences. The target proteins chosen from each virus family and an accession number corresponding to a representative sequence from the type species are listed in Table 2. After selection of the target sequence all available amino acid sequences from each virus family were used to generate multiple sequence alignments that were then formatted into blocks of aligned amino acids using a block multiple alignment processor [Web link 6-1]. The blocks of aligned amino acids were then imported into the CODEHOP analysis program located online [Web link 6-2] to generate recommended primer designs.

Table 2. Viral proteins targeted for CODEHOPs

Family	Target Protein	Accession #
Ascoviridae	Major Capsid Protein	CAF05815
Iridoviridae	Major Capsid Protein	Q05815
Nodaviridae	RNA dependant RNA Polymerase	ABS29339
Parvoviridae	Non-Structural Protein 1	NP051020
Poxviridae	DNA Polymerase/Spheroidin	AAA92858
Tetraviridae	Major Capsid Protein	AA073881

2.1.3 Specific screen for the Tn5 cell line (TNCL) virus

In a recent report by Li *et al.* (2007), Tn5 cells maintained in the investigator's laboratory or obtained fresh from a commercial source were found to be latently infected with a previously unknown nodavirus. Replication of the new nodavirus, Tn5 cell line (TNCL) virus, appears to be induced by infection with baculovirus, although genomic sequences were detected in uninfected Tn5 cells by PCR. Interestingly, Li and co-workers also tested Sf9 cells by PCR before and after infection with baculovirus and found no evidence of infection with TNCL virus. To address the potential that SF+ cells are latently infected with TNCL virus, we tested our SF+ WCB using PCR primers based on the published nucleotide sequence for the TNCL virus isolate. No amplification products were obtained with either uninfected or baculovirus infected SF+ cell samples, but a positive result was obtained when samples were spiked with TNCL virus control RNA. In addition to these findings, analysis using primers designed based on the CODEHOP strategy, as described above, also failed to produce a product except in the presence of spiked control RNA.

2.1.4 Quantitative PCR-Enhanced Reverse Transcriptase Assay

Cell-free supernatant samples from the SF+ Master Cell Bank (MCB) and end of production cells from a WCB (passage 60) were tested by a quantitative Fluorescent-PCR Based Reverse Transcription (PERT) assay in order to compare reverse transcriptase (RT) activity and rule out increased production of retroviral-like particles due to the manufacturing process. This assay can detect as few as 10 – 100 molecules of RT present in a sample. Samples of cell free supernatant were judged to contain RT activity in agreement with the presence of retro-elements in these cells. However, no increase in RT activity was observed between low passage cells from the master cell bank versus cells at passage 60 (end of production passage), ruling out an increased production of retroviral-like particles due to cell passaging. It should also be noted that end of production cell samples were determined not to contain infectious retrovirus particles when tested in a cellular co-cultivation assay [Mc Pherson, 2008].

3. Clinical Assessment

3.1 Clinical studies PSC01, PSC03, PSC04 and PSC06 used to support licensure

The studies described below were performed to support licensure of FluBlok, a recombinant hemagglutinin influenza vaccine indicated for active immunization of adults 18 years of age and older against influenza disease caused by influenza virus subtypes A and type B represented in the vaccine. FluBlok is a sterile liquid with no added preservatives or adjuvants for intramuscular injection. FluBlok is supplied as a single-dose vial (0.5 mL) and each dose contains three recombinant influenza hemagglutinin (rHA) proteins (45 µg of each of the subtype antigens).

The main goal of trial PSC01 was to determine safety and the optimal dose of rHA of the vaccine in healthy adults [Treanor *et al.*, 2007]. It was a randomized, prospective, double-blind, placebo-controlled multicenter study in which healthy adults 18-49 years of age were enrolled during the 2004-2005 influenza season. A total of 458 subjects were vaccinated with either a single dose of FluBlok at a total rHA dosage level of 135 µg (containing 45 µg of each antigen [153 subjects]) or 75µg (containing 45 µg of H3 rHA and 15 µg of B and H1 rHA [151 subjects]), or a saline placebo (154 subjects). The mean age of the subjects receiving FluBlok (135 µg) was 31 years and the majority was female (63%). Additionally, 85% had a Caucasian, 6% an African American, 3% a Latino/Hispanic, 3% an Asian, and 2% a Native American background The evaluable efficacy population consisted of 451 subjects (150 in the FluBlok 135 µg group, 150 in the FluBlok 75 µg group and 151 in the placebo group) with complete serological data as per protocol.

The main goal of the next study (PSC03) was to compare the safety and immunogenicity of FluBlok with a licensed vaccine, Fluzone, in elderly adults [NCT00395174]. PSC03 was a randomized, double-blind, active-controlled study in which 869 medically stable adults age 65-92 years (mean age 73 years) were enrolled during the 2006-2007 influenza season. Participants were randomly assigned to receive either a single dose of FluBlok (135 µg, 436 subjects) or commercially available trivalent influenza vaccine (Fluzone®, Sanofi Pasteur, Swiftwater, PA, USA; 433 subjects). The majority of subjects receiving FluBlok were female (52%). The majority was of Caucasian origin (99%). The evaluable efficacy population consisted of 431 FluBlok-treated subjects and 430 Fluzone-treated subjects. A total of 854 subjects completed all study procedures.

PSC04 was a randomized, double-blind, placebo-controlled clinical efficacy study in which 4648 healthy adults age 18-49 years (mean age 33 years) were enrolled during the 2007-2008 influenza season [NCT00539981]. The main goal of this study was to determine safety, immunogenicity and efficacy of the vaccine in healthy adults. Only data from the first 28 days of study are included herein. Participants were randomly assigned to receive either a single dose of FluBlok (135 µg, 2344 subjects) or placebo (2304 subjects). The majority of subjects receiving FluBlok were female (59%). Additionally, 67% were Caucasian, 18% were African American, 11% were Latino/Hispanic, 3% were Asian, and < 1% were Native American. A total of 4272 subjects completed all study procedures through Day 28. A subset of 391 subjects who received FluBlok served as the evaluable immunogenicity population. The efficacy data of this study were not included in the initial license application.

PSC06 was a randomized, double-blind, placebo-controlled study in which 602 healthy adults age 50-64 years (mean age 56 years) were enrolled during the 2007-2008 influenza season [NCT00539864]. Participants were randomly assigned to receive either a single dose of FluBlok (135 µg, 300 subjects) or commercially

available trivalent influenza vaccine (Fluzone®, 302 subjects). The majority of subjects receiving FluBlok were female (62%). Additionally, 73% were Caucasian, 4% were African American, 8% were Latino/Hispanic and 12% were Asian. A total of 602 subjects completed all study procedures through Day 28. There were 601 subjects in the evaluable population. Only data from the first 28 days of study are included herein. The main goal of this study was to compare the safety and immunogenicity of FluBlok with the licensed vaccine, Fluzone, in adults 50 -64 years of age.

In summary these four trials investigated the safety, optimal dosing and efficacy in adults in three age groups (18-49; 50-64, and 65 and older), and compared between the immunogenicity of FluBlok and the commercial trivalent vaccine in adults above 50 years of age.

3.2 Vaccine safety

The total population used for safety analysis performed with the data from these trials included 6577 adults 18 years of age and older. The four studies together included 5106 subjects age 18 - 49 years of age randomized to receive FluBlok (2497 subjects received 135 µg; 151 subjects received 75 µg) or placebo (2458 subjects), and 1471 subjects age 50 years and older who were randomized to receive FluBlok (736 subjects) or a US-licensed trivalent, inactivated influenza virus vaccine (Fluzone®) (735 subjects).

Collectively 59% of the volunteers were women; 73% of subjects were Caucasian, 8% Hispanic/Latino, 14% African-American, < 1% Native American, and 3% Asian. The mean age of subjects in the studies was 40 years (range 18-92 years); 9% of subjects were 50 to 64 years of age and 13% were 65 years of age and older.

In all studies, a series of symptoms and/or findings were specifically solicited by a memory aid used by subjects for the 7-day period following vaccination (see Table 3). In addition, in all 4 studies, spontaneous reports of adverse events were also collected for 28 days following vaccination (see below) and subjects were actively queried about changes in their health status 6 months after vaccination for studies PSC01 and PSC03.

PSC01 included 458 subjects for safety analysis, ages 18 - 49 years, randomized to receive FluBlok 75 µg (151 subjects), FluBlok 135 µg (153 subjects) or placebo (154 subjects). Serious adverse events (SAEs) reported from day 0 (day of vaccination) through 6 months were included in the safety analysis. Two subjects (1%) in the 135 µg FluBlok group experienced SAEs that were considered to be unrelated to treatment (one seizure related to hypoglycemia that occurred at 26 days post-vaccination and one lobular carcinoma *in situ* at day 55 and syncope at day 125). No subjects discontinued the study because of adverse events and no subjects died. Three female subjects became pregnant after vaccination with FluBlok. Two pregnancies ended in elective termination and one proceeded normally to full-term, resulting in the birth of a normal infant.

In the same age group of 18 - 49 years, PSC04 included 4648 subjects for safety analysis, randomized to receive FluBlok (2344 subjects) or placebo (2304 subjects). Results from an interim analysis are reported herein and include safety data reported from day 0 through day 28, when subjects were interviewed during a visit or phone call. A total of 24 possible SAEs were reported through the day 28 visit/phone call (eight in the FluBlok and 16 in the placebo treatment groups). Of these, five were pregnancies (1 FluBlok and 4 placebo). Only one SAE, "pericardial effusion," diagnosed 11 days post-vaccination in a FluBlok recipient, was judged to be possibly

related to treatment. None of the remaining six SAEs reported in the FluBlok treatment group were considered by the investigators to be related to study treatment.

In the age group of 50 - 64 years (PSC06) 602 subjects were included for safety analysis, randomized to receive FluBlok (300 subjects) or TIV (Fluzone) (302 subjects). Results from an interim analysis are reported herein and include safety data reported from day 0 through the day 28 visit/phone call. One subject receiving FluBlok reported a treatment-related SAE on the day of vaccination (syncope vasovagal) of moderate severity that resolved without sequelae. No subjects discontinued the study due to adverse events.

In the group of 65 years and older (PSC03) 869 subjects were available for safety analysis, randomized to receive FluBlok (436 subjects) or TIV (Fluzone) (433 subjects). SAEs reported herein include safety data reported from day 0 through 9 months (end of influenza season). A total of 70 (8%) SAEs were reported (36 [8%] for FluBlok and 34 [8%] for Fluzone). No SAEs were judged to be related to the study treatment by the investigators.

Across the four trials, there were no deaths that were considered as possibly or probably related to vaccination treatment. Table 3 shows the solicited adverse events reported by the subjects using a memory aid during the first 7 days post vaccination. In general, local and systemic reactogenicity events occurred with similar frequency across the four clinical studies except in PSC01, where most events tended to be reported more frequently. The only statistically significant difference between the FluBlok group (135 µg dose) and the placebo group was pain at the injection site in one study PSC01; 95% of these pain events were reported as mild.

Table 3. Solicited adverse events in the first 7 days after administration of FluBlok, placebo, or comparator influenza vaccine

	Study PSC01 Adults age 18-49 yrs		**Study PSC04 Adults age 18-49 yrs**		**Study PSC06 Adults age 50-64 yrs**		**Study PSC03 Adults age ≥ 65 yrs**	
	FluBlok*	**Placebo**	**FluBlok**	**Placebo**	**FluBlok**	**Fluzone**	**FluBlok**	**Fluzone**
Number of Subjects	**153**	**154**	**2344**	**2304**	**300**	**302**	**436**	**433**
Local Adverse Events (%)								
Pain	61	17	37	8	51	55	22	23
Redness	5	2	4	2	8	8	10	12
Swelling	10	3	3	2	8	10	11	13
Bruising	7	4	3	3	5	5	3	5
Systemic Adverse Events (%)								
Headache	42	41	15	15	20	21	11	9
Fatigue	16	18	15	14	13	21	9	10
Muscle Pain	20	12	10	7	13	14	7	9
Fever***	0	1	< 1	< 1	< 1	0	< 1	0
Joint pain	5	5	4	4	5	6	5	6
Nausea	8	6	6	5	4	5	4	3
Chills	3	2	3	3	4	5	4	4
Sweating	3	5	NA**	NA	NA	NA	3	2

NOTE: Data based on the most severe response reported by subjects on the memory aid. Results > 1% reported to nearest whole percent; results > 0 but < 1 reported as < 1%.

* Data restricted to 135 µg formulation; ** NA = data not available (not collected during the study);

***Fever defined as ≥ 99.6°F (37.6°C). In other studies fever was defined as > 100.4°F (38°C).

Table 4 summarizes the most common unsolicited adverse events reported during the four clinical studies during the 28 day post-vaccination period. These events were reported either spontaneously or in response to general queries about changes in health status. The most common events were headache and signs or symptoms of upper respiratory tract infection in the four studies. These, as well as diarrhea and muscle aches, were the only adverse events reported by > 1% of subjects. Older subjects were, in general, less likely to report adverse events, despite similar methods of ascertainment in PSC03 compared to the other three studies. The relatively high rates of reactogenicity in study PSC01 may have been due to an additional clinic visit on study day 2, along with the requirement of a third visit to the clinic on day 8.

Table 4. Adverse events reported by ≥ 1% of subjects in any group in the four clinical trials of FluBlok within 28 days of vaccination, irrespective of causality

	Study PSC01 Adults age 18-49 yrs		**Study PSC04 Adults age 18-49 yrs**		**Study PSC06 Adults age 50-64 yrs**		**Study PSC03 Adults age ≥ 65 yrs**	
	FluBlok	**Placebo**	**FluBlok**	**Placebo**	**FluBlok**	**Fluzone**	**FluBlok**	**Fluzone**
Number of Subjects	**153**	**154**	**2344**	**2304**	**300**	**302**	**436**	**433**
Any adverse events (%)	35	42	16	15	14	17	21	20
Diarrhea (%)	1	3	1	1	1	0	1	1
Nasophayngitis (%)	3	3	1	1	< 1	< 1	1	2
Upper respiratory tract infection (%)	6	5	1	1	1	< 1	1	1
Myalgia (%)	1	3	< 1	< 1	0	< 1	< 1	< 1
Headache (%)	8	8	2	2	< 1	< 1	< 1	1
Cough (%)	4	2	2	2	2	< 1	1	2
Nasal congestion (%)	3	4	2	1	1	< 1	1	1
Pharyngolaryngeal pain (%)	5	5	2	2	1	3	< 1	1
Rhinorrhea (%)	1	3	1	1	1	2	< 1	1
Fatigue (%)	1	2	1	1	0	< 1	< 1	< 1
Nausea (%)	2	0	1	1	0	0	< 1	1
Sinusitis (%)	1	1	1	1	0	< 1	1	< 1
Pyrexia (%)	1	0	< 1	< 1	0	0	< 1	<1
Back pain (%)	1	2	< 1	< 1	< 1	1	< 1	1
Injection site erythema (%)	0	0	0	0	2	< 1	2	< 1
Injection site hemorrhage (%)	1	0	< 1	< 1	0	< 1	1	1
Injection site swelling (%)	0	0	0	0	0	< 1	1	< 1
Pain in extremity (%)	0	0	< 1	< 1	0	< 1	1	< 1
Tooth abscess (%)	0	0	< 1	0	<1	0	< 1	1
Arthralgia (%)	1	2	< 1	< 1	< 1	< 1	< 1	< 1
Insomnia (%)	1	1	< 1	0	0	0	< 1	< 1
Sinus congestion (%)	1	1	< 1	< 1	0	0	< 1	< 1
Hyperhidrosis (%)	1	1	< 1	< 1	0	0	0	0

3.3 Immunogenicity results

In all four FluBlok studies, hemagglutination-inhibition (HAI) antibody titers to each virus strain represented in the vaccine were measured in sera obtained approximately 28 days after vaccination. Analysis of endpoints was performed for each HA contained in the vaccine, active control and/or placebo according to the criteria specified in the Food and Drug Administration (FDA) Guidance for Industry: "Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines" (May 2007). In studies PSC04 and PSC06, the following two pre-specified primary immunogenicity endpoints were assessed: (i) the lower bounds of the two-sided 95% confidence intervals (CI) for the proportion of subjects with HAI antibody titers of:40 or greater after vaccination (seroprotection rate), which should meet or

exceed 70% for each vaccine antigen strain; and (ii) the lower bounds of the two-sided 95% CI for rates of seroconversion (defined as a fourfold increase in post-vaccination HAI antibody titers from pre-vaccination titers of 10 or greater, or an increase in titers from less than 10 to 40 or greater), which should meet or exceed 40% for each vaccine antigen strain.

For study PSC03, these endpoints were pre-specified as secondary endpoints, except that criteria for subjects ≥ 65 years of age were applied (seroprotection rate should meet or exceed 60% for each vaccine antigen strain and the seroconversion rate should meet or exceed 30% for each vaccine antigen strain).

For study PSC01, the primary endpoints, as originally specified, were descriptive comparisons of immune response in the various study groups; therefore, a *post hoc* analysis of the endpoints, as using the same criteria as described earlier for study PSC04, was performed. For PSC01 only, seroprotection is defined (*post hoc*) as a post-vaccination (day 28) HAI titer of ≥64. Based on the serum dilution series used in the HAI antibody assay, 1:64 is the first dilution in which the antibody titer would be ≥40, the criterion specified in the Center for Biologics and Research (CBER) Guidance Document. Likewise, for PSC01 only, seroconversion is defined as a ≥4-fold increase in HAI titer on day 28 in subjects with a prevaccination titer of ≥4, with a minimum day 28 titer of 64; or an HAI titer of ≥64 on day 28 in subjects with a pre-vaccination titer <4 (Limit of detection or LOD) of the HAI assay used in PSC01).

As shown in Table 5, across all four studies, serum HAI antibody responses to FluBlok usually met the pre-specified seroconversion criteria for all three virus strains, and also the pre-specified criterion for the proportion of subjects with HAI titers ≥ 40 (seroprotection). In study PSC01 and PSC03, FluBlok did not meet the pre-specified seroprotection or seroconversion criterion, respectively, for the influenza B virus. The clinical relevance of these findings on vaccine-induced protection against illness caused by influenza type B strains is unknown, especially given the good responses against type B in young adults in study PSC04, and the lack of a head-to-head comparison for the B vaccine component in study PSC03 (see Table 5). In study PSC04 (subjects age 18-49 years), FluBlok met the pre-specified seroprotection and seroconversion criterion for all three strains. In study PSC06 (subjects age 50-64 years), FluBlok met the pre-specified seroprotection criterion for all three strains while Fluzone marginally passed the seroprotection criterion for the H3 strain (lower end of two-sided CI was rounded up to 70%). In addition, in PSC06, FluBlok met the seroconversion criterion for the H1 and H3 strains but not for the B strain, while Fluzone failed to meet the pre-specified seroconversion criterion for the H3 and B strains.

Table 5. Serum HAI antibody responses at post-vaccination day 28 in subjects age ≥ 18 years

	Study PSC01		**Study PSC04**	**Study PSC06**		**Study PSC03**	
	Adults age 18-49 yrs		Adults age 18-49 yrs	Adults age 50-64 yrs		Adults age ≥ 65 yrs	
	FluBlok[a]	Placebo	FluBlok[a]	FluBlok[a]	Fluzone	FluBlok[a]	Fluzone
Number of Subjects	150	151	391	299	302	431	430
A/H1N1	***A/New Caledonia***		***A/Solomon Islands***	***A/Solomon Islands***		***A/New Caledonia***	
% Seroprotected[b]	**87**	40	**98**	**96**	**96**	**95**	**95**
(95% CI)	**(81, 92)**	(33, 49)	**(97, 99)**	**(94, 98)**	**(93, 98)**	**(92, 97)**	**(92, 97)**
%Seroconversion[c]	**60**	0	**78**	**72**	**66**	**43**	33
(95% CI)	**(52, 68)**	(0, 2)	**(74, 82)**	**(67, 77)**	**(61, 72)**	**(39, 48)**	(28, 37)
A/H3N2	***A/Wyoming***		***A/Wisconsin***	***A/Wisconsin***		***A/Wisconsin***	
% Seroprotected[b]	**100**	66	**96**	**85**	**75**	**97**	**93**
(95% CI)	**(98, 100)**	(57, 73)	**(94, 98)**	**(81, 89)**	**(70, 80)**	**(94, 98)**	**(90, 95)**
%Seroconversion[c]	**77**	9	**81**	**61**	44	**78**	**58**
(95% CI)	**(69, 83)**	(5, 15)	**(76, 84)**	**(55, 67)**	(38, 50)	**(74, 82)**	**(53, 62)**
B	***B/Jiangsu***		***B/Malaysia***	***B/Malaysia***		***B/Ohio***	***B/Malaysia***
%Seroprotected[b]	65	7	**96**	**93**	**94**	**92**	**97**
(95% CI)	(57, 73)	(3, 12)	**(93, 98)**	**(90, 96)**	**(91, 97)**	**(89, 94)**	**(95, 99)**
%Seroconversion[c]	**63**	1	**53**	41	41	29	**39**
(95% CI)	**(55, 71)**	(0, 4)	**(48, 58)**	(35, 47)	(36, 47)	(25, 34)	**(34, 44)**

[a] Values shown for FluBlok are for those subjects who received the 135 μg dose. Numbers in bold meet the criteria listed in the FDA Guidance for Industry: Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines (May 2007) (see definitions below). These criteria were specified as secondary endpoints in study PSC03.

[b] Seroprotection rate (HAI titer ≥40) is defined as the proportion of subjects with a minimum post-vaccination HAI antibody titer of 40. The lower bound of the 2-sided 95% Confidence Interval (CI) for the seroprotection rate should be ≥ 70% for adults age 18-64 years, and ≥ 60% for adults age 65 years and older. However, for PSC01 only, seroprotection rate is defined (post-hoc) as the proportion of subjects with a minimum post-vaccination HI titer of 64. (Based on the serum dilution series used in the HAI antibody assay in PSC01, 1:64 is the first dilution in which the antibody titer would be ≥40.)

[c] Seroconversion rate is defined as a ≥ 4-fold increase in post-vaccination HAI antibody titer from the pre-vaccination titer ≥ 10 or an increase in titer from < 10 to ≥40. The lower bound of the 2-sided 95% CI for the seroconversion rate should be ≥ 40% for adults age 18-64 years, and ≥ 30% for adults age 65 years and older. However, for PSC01 only, seroconversion rate is defined (post-hoc) as a ≥4-fold increase in post-vaccination HAI titer from prevaccination titer ≥1:4, with a minimum Day 28 titer of 64; or an increase in titer from <4 (= limit of detection) of the HAI assay used in PSC01) to ≥64.

In study PSC03, the following co-primary endpoints were pre-specified for each HA contained in the vaccine and/or active control: (i) the upper bound of the two-sided 95% Confidence Interval (CI) on the ratio of Geometric mean Titer (GMTs) ($GMT_{US\ licensed\ vaccine}/GMT_{FluBlok}$) should not exceed 1.5; and (ii) the upper bound of the two-sided 95% CI on the difference between seroconversion rates ($Seroconversion_{US\ licensed\ vaccine} - Seroconversion_{FluBlok}$) should not exceed 10% points. These endpoints were specified as secondary in study PSC06. As shown in Table 6, for Study PSC03, non-inferiority of GMTs (in comparison to Fluzone) were met for all three strains, and non-inferiority of the difference in seroconversion rates was met for the two A strains. In PSC06, FluBlok, non-inferiority of both the GMTs and the difference in seroconversion rates was met for all three strains.

Table 6. Serum hemagglutination-inhibition responses following immunization with FluBlok (135 µg) or Fluzone in studies PSC03 (subjects ≥ 65 years of age) and PSC06 (subjects 50-64 years of age).

	PSC06		**PSC03**	
	FluBlok	**Fluzone**	**FluBlok**	**Fluzone**
Number of Subjects	**299**	**302**	**431**	**430**
A/H1N1	***A/Solomon Islands***		***A/New Caledonia***	
Pre-vaccination GMT*	28.7 (25.6, 32.2)	27.8 (25.1, 30.8)	69.0 (62.1, 76.6)	70.2 (62.8, 78.6)
Post-vaccination GMT*	181.3 (159.6, 206.0)	139.7 (124.6, 156.7)	176.8 (159.4, 196.0)	148.1 (134.2, 163.4)
Post-vax GMT ratio, Fluzone:FluBlok (two-sided 95% CI)	**0.77 (0.75, 0.79)**		**0.84 (0.81, 0.86)**	
No. (%) seroconverting** [two-sided 95% CI]	**216 (72) [67, 77]**	**200 (66) [61, 72]**	**187 (43) [39, 48]**	140 (33) [28, 37]
Difference in Seroconversion rate, Fluzone–FluBlok (two-sided 95% CI)	**-6% (-13, 1), p = 0.113**		**-11% (-17, -4), p = 0.001**	
A/H3N2	***A/Wisconsin***		***A/Wisconsin***	
Prevaccination GMT*	18.6 (16.4, 21.1)	18.2 (16.1, 20.6)	42.7 (37.6, 48.4)	44.7 (39.2, 51.0)
Postvaccination GMT*	105.4 (91.0, 122.1)	60.9 (53.6, 69.2)	338.5 (299.7, 382.5)	199.2 (176.8, 224.4)
Post-vax GMT ratio, Fluzone:FluBlok (two-sided 95% CI)	**0.58 (0.53, 0.62)**		**0.59 (0.57, 0.60)**	
No. (%) seroconverting** [two-sided 95% CI]	**183 (61) [55, 67]**	132 (44) [38, 50]	**335 (78) [74, 82]**	**248 (58) [53, 62]**
Difference in Seroconversion rate, Fluzone–FluBlok (two-sided 95% CI)	**-18% (-25, -10), p < 0.001**		**-20% (-26, -14), p < 0.001**	
B	***B/Malaysia***		***B/Ohio***	***B/Malaysia***
Prevaccination GMT*	48.5 (43.4, 54.2)	49.2 (43.8, 55.3)	79.9 (71.3, 89.5)	80.3 (72.0, 89.5)
Postvaccination GMT*	110.9 (100.1, 123.0)	116.0 (104.2, 129.3)	149.6 (134.5, 166.3)	194.8 (177.5, 213.7)
Post-vax GMT ratio, Fluzone:FluBlok (two-sided 95% CI)	**1.05 (1.01, 1.09)**		**1.30 (1.26, 1.34)**	
No. (%) seroconverting** [two-sided 95% CI]	122 (41) [35, 47]	124 (41) [36, 47]	126 (29) [25, 34]	**168 (39) [34, 44]**
Difference in Seroconversion rate, Fluzone–FluBlok (two-sided 95% CI)	**0.3% (-8, 8), p = 1.00**		10% (4, 16), p = 0.003	

NOTE: Numbers in bold meet the non-inferiority criteria listed in the FDA Guidance for Industry: Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines (May 2007) (see definitions below).

* Day 0 (pre-vaccination) and day 28 (post-vaccination) geometric mean titers (95% confidence intervals [CI]). The upper bound of the two-sided 95% CI on the ratio of GMTs ($GMT_{U.S.\ licensed\ vaccine}/GMT_{FluBlok}$) should not exceed 1.5; ** Seroconversion rate is defined as a ≥ 4-fold increase in post-vaccination HAI antibody titer from pre-vaccination titer ≥10 or an increase in titer from <10 to ≥40. The lower bound of the two-sided 95% CI for the seroconversion rate should be ≥ 40% for adults age 18-64 years, and ≥ 30% for adults age 65 years and older.

3.4 PSC01: correlation between post-vaccination titer and the acquisition of influenza infection

Study PSC01 also assessed the development of laboratory documented (culture-confirmed) influenza illness meeting the influenza-like illness case definition specified by the US Centers for Disease Control and Prevention (CDC-ILI), i.e., presence of fever ≥ 99.8°F (37.7°C) and either sore throat or cough, or both [Treanor *et al.*, 2007]. During the surveillance period in PSC01 in the 2004-2005 season, 10 influenza isolates were found to be genetically similar to A/California/7/04 (H3N2), based on complete cDNA sequencing of the HA1 region obtained from reverse transcriptase-polymerase chain reaction (RT-PCR) amplified Madin-Darby Canine Kidney cell-grown virus [Treanor *et al.*, 2007]. These strains were considered to represent significant drift from the H3N2 vaccine strain, first isolated in 2003, A/Wyoming/3/03. Table 7 shows the HAI titers on day 28 and day 180 in subjects with a positive H3N2 culture.

Table 7. HAI Titers on day 28 and day 180 in influenza A culture-positive (n=10) by study group

Lab Sample ID	Characterization	Date of swab collection	HAI Titer on Day 28	HAI Titer on Day 180	Study Group	Met CDC-ILI criteria?
Isolate No.						
38	H3	3 Jan 05	8	256	Placebo	Yes
301	H3	21 Dec 04	256	256	Placebo	Yes
356	H3	18 Feb 05	32	128	Placebo	Yes
484	H3	15 Feb 05	64	256	Placebo	Yes
606	H3	18 Mar 05	4	32	Placebo	Yes
617	H3	8 Feb 05	16	128	Placebo	No
54	H3	7 Feb 05	512	1024	75µg	No
146	H3	31 Jan 05	256	256	75µg	No
446	H3	6 Jan 05	>1024	512	75µg	Yes
653	H3	24 Jan 05	32	64	75µg	Yes

In study PSC01 13.9 % of the placebo recipients showed serological evidence of H3 influenza infection (i.e. four-fold increase in antibody titer between day 28 and day 180 for the H3 antigen) versus 5 % of the vaccinated subjects. This difference is not surprising since the day 28 titers of the vaccinated subjects are higher than the non-vaccinated subjects and therefore obtaining a four-fold increase in titer may be more difficult than among placebo recipients. As shown in Table 4, influenza infection in the placebo group as evidenced by culture is associated with low day 28 antibody titers. Five (83.3%) of the 6 placebo recipients who were culture-positive for A/H3N2 met the case definition for CDC-ILI. All but one of the 6 placebo recipients with laboratory-confirmed A/H3N2 infection associated with CDC-ILI had day 28 titers lower than 64. Only one had day 28 titers of 256. The results for the FluBlok group, in contrast, showed that cell culture confirmed infections occurred throughout a wide spectrum of post-vaccination titers (64 – 1024). Three of the 4 culture-confirmed

A/H3N2 infections occurred in subjects with day 28 titers of ≥256, including one in a subject with a post-vaccination titer of 4096; however, none of the culture-confirmed A/H3N2-infected individuals showed serological evidence of infection. In contrast to the experience in the placebo group, only 2 (50%) of the culture-confirmed A/H3N2 infections in the FluBlok group met the case definition for CDC-ILI. Although the numbers are small, the difference in outcome between placebo and FluBlok recipients, especially with regard to CDC-ILI, suggests a high degree of clinical efficacy.

3.5 PSC03: Immunogenicity in Subjects Ages 75 and older

In an exploratory analysis of PSC03, the proportions of subjects achieving seroconversion and seroprotection were examined in a subset of 322 subjects characterized by age 75 years and older. As shown in Table 8, vaccination with FluBlok yielded similar rates of seroprotection and seroconversion for this subpopulation when compared with the overall population of subjects 65 years of age and older. In addition, FluBlok met the CBER criterion for non-inferiority of geometric mean titers (GMTs) for all three strains in this subpopulation of older subjects.

Table 8. Serum HAI responses following immunization with FluBlok (135 µg) or Fluzone in subjects ≥ 65 yrs and ≥ 75 yrs from study PSC03

	PSC03			
	Adults age ≥ 65 yrs		Adults age ≥ 75 yrs	
Number of Subjects	**FluBlok 431**	**Fluzone 430**	**FluBlok 163**	**Fluzone 159**
A/(H1N1)	***A/New Caledonia***		***A/New Caledonia***	
Pre-vaccination GMT [a]	69.0 (62.1, 76.6)	70.2 (62.8, 78.6)	63.3 (53.6, 74.8)	65.5 (55.3, 77.6)
Post-vaccination GMT [a]	176.8 (159.4, 196.0)	148.1 (134.2, 163.4)	152.7 (128.1, 182.0)	125.3 (107.1, 146.7)
Post-vax GMT ratio, Fluzone:FluBlok (2-sided 95% CI)	**0.84 (0.81, 0.86)**		**0.82 (0.79, 0.85)**	
% Seroprotected[b] (95% CI)	**95 (92, 97)**	**95 (92, 97)**	**91 (87, 96)**	**94 (91, 98)**
%Seroconversion[c] (95% CI)	**43 (39, 48)**	33 (28, 37)	**39 (32, 47)**	30 (23, 37)
A/(H3N2)	***A/Wisconsin***		***A/Wisconsin***	
Prevaccination GMT [a]	42.7 (37.6, 48.4)	44.7 (39.2, 51.0)	39.7 (32.7, 48.1)	43.1 (35.2, 52.8)
Postvaccination GMT [a]	338.5 (299.7, 382.5)	199.2 (176.8, 224.4)	300.2 (244.7, 368.3)	178.4 (147.8, 215.3)
Post-vax GMT ratio, Fluzone:FluBlok (2-sided 95% CI)	**0.59 (0.57, 0.60)**		**0.59 (0.58, 0.61)**	
% Seroprotected[b] (95% CI)	**97 (94, 98)**	**93 (90, 95)**	**96 (93, 99)**	**93 (89, 97)**
%Seroconversion[c] (95% CI)	**78 (74, 82)**	**58 (53, 62)**	**79 (73, 85)**	**54 (46, 62)**
B	***B/Ohio***	***B/Malaysia***	***B/Ohio***	***B/Malaysia***
Prevaccination GMT [a]	79.9 (71.3, 89.5)	80.3 (72.0, 89.5)	101.9 (86.7, 119.9)	102.6 (86.1, 122.1)
Postvaccination GMT [a]	149.6 (134.5, 166.3)	194.8 (177.5, 213.7)	185.7 (160.8, 214.4)	224.8 (193.2, 261.5)
Post-vax GMT ratio, Fluzone:FluBlok (2-sided 95% CI)	**1.30 (1.26, 1.34)**		**1.21 (1.18, 1.24)**	
% Seroprotected[b] (95% CI)	**92 (89, 94)**	**97 (95, 99)**	**96 (93, 99)**	**99 (98, 100)**
%Seroconversion[c] (95% CI)	29 (25, 34)	**39 (34, 44)**	26 (20, 33)	35 (28, 43)

NOTE: Numbers shown in bold meet the non-inferiority criteria listed in the FDA Guidance for Industry: Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines (May 2007) (see definitions below).

[a.] Day 0 (prevaccination) and Day 28 (postvaccination) geometric mean titers (GMT) (95% confidence intervals [CI]). The upper bound of the 2-sided 95% CI on the ratio of GMTs ($GMT_{U.S.\ licensed\ vaccine}/GMT_{FluBlok}$) should not exceed 1.5.

[b.] Seroprotection rate (HI titer ≥40) is defined as the proportion of subjects with a minimum post-vaccination HI antibody titer of 40. The lower bound of the 2-sided 95% CI for the seroprotection rate should be ≥ 70% for adults age 18-64 years, and ≥ 60% for adults age 65 years and older.

[c.] Seroconversion rate is defined as a ≥ 4-fold increase in post-vaccination HI antibody titer from pre-vaccination titer ≥10 or an increase in titer from < 10 to ≥40. The lower bound of the 2-sided 95% CI for the seroconversion rate should be ≥ 40% for adults age 18-64 years, and ≥ 30% for adults age 65 years and older.

4. Conclusions

The baculovirus-insect cell expression system is particularly suitable for influenza vaccine production where annual adjustment of the vaccine is required. Extensive qualification testing performed on the SF+ cell line supports its application for this purpose. Additionally, the initial results from ongoing adventitious insect virus testing have failed to detect any contaminating viruses providing further safety assurances.

FluBlok is a trivalent rHA vaccine with a mechanism of action likely to be similar to that of the trivalent inactivated licensed influenza vaccine, namely the induction of HAI antibodies to prevent influenza infection [Kida *et al.*, 1983; Yoden *et al.*,1986] The commercial formulation of FluBlok contains three times the amount of HA of the inactivated influenza vaccines and consequently induces higher antibody titers (Tables 5, 6 and 8), which may be of particular importance to those most at risk for influenza (for example, the elderly [Keitel *et al.*, 1994; Keitel *et al.*, 1996; Treanor *et al.*, 2006] or immunologically compromised [Safdar *et al.*, 2006]. The immunogenicity results for the B/strain in the elderly study PSC03 must be interpreted cautiously in the context of a lack of a direct antigen comparison. Although the two strains (B/Ohio and B/Malaysia) were considered to be antigenically related by WHO Reference Laboratories, and therefore interchangeable for purposes of vaccine production, previous studies of influenza vaccines have shown that HAI titers achieved following vaccination with different influenza antigens of the same subtype typically differ from each other, often to variable degrees.

While FluBlok contains 135 µg HA per dose, the total amount of protein (HA plus host cell proteins) contained within one dose of FluBlok is roughly comparable to the total amount of protein contained in FluZone, which is approximately 115 µg total protein per dose (viral plus egg protein) [Renfrey and Watts, 1994; Hehme *et al.*, 2003]. The vaccine was shown to be well tolerated and immunogenic in more than 3000 adults older than 18 years. Importantly, this vaccine has demonstrated protective efficacy in a field efficacy trial against drifted influenza viruses [Chapter 5; Cox and Anderson, 2007; Treanor *et al.*, 2007].

Based on these studies FluBlok received Fast Track Designation from the FDA in December 2006 and a Biological License Application (BLA) has been filed in April 2008, and expects to receive FDA approval in 2010.

Chapter 7

General Discussion

1. Synopsis

The process to obtain licensure of a biologic product for human use is complicated and challenging. This thesis describes the path leading towards licensure of a novel influenza vaccine using a novel cell substrate. Specifically, the development of a safe, immunogenic and effective novel recombinant trivalent hemagglutinin (HA) influenza vaccine manufactured in insect cell cultures using the baculovirus-insect cell expression system is described.

The greatest challenge in the manufacturing of influenza vaccines results from the fact that the vaccine requires adjustments on an annual basis. The time available to make these adjustments is extremely short, a matter of months. Therefore it is important that a versatile, robust manufacturing process is established that can result in timely delivery of the new antigens. In **Chapter 2 and 3**, it was shown that cloning the influenza HA from the influenza virus into a baculovirus vector can be accomplished within a few weeks. The biochemical properties of recombinant HA (rHA) produced in the baculovirus-insect cell system appear to be similar to those of authentic viral HA as is demonstrated by various methods that were developed to assess the biological activity. Progress was also made towards scalable purification processes at the 500-L scale that enabled purification of sufficient amounts of diverse hemagglutinin molecules.

Preliminary evidence for the efficacy of a recombinant HA vaccine produced in the baculovirus-insect cell system was presented in **Chapter 4**. The fact that none of the high dose vaccine, containing 45µg rHA of each antigen, recipients developed cell culture confirmed influenza suggests that absence of influenza neuraminidase (NA) and differences in glycosylation in insect versus vertebrate cells do not interfere with vaccine performance. The H3N2 influenza viruses isolated from six placebo recipients and four low dose vaccine recipients were characterized by sequence analysis in **Chapter 5** and it was shown that they had high similarity to an A/California/7/04 virus - a drift variant from the Wyoming/3/03 virus, from which the HA in the vaccine originated. This experiment provided preliminary evidence that the use of more antigen per vaccine dose can result in cross-protection of the vaccine against drifted viruses.

Finally, questions and concerns were addressed specifically relating to the safety of this vaccine using this novel cell substrate in **Chapter 6**. Specifically, the potential presence of latent viruses in the insect cell line was studied using various techniques, including transmission electron microscopy of stressed insect cells and PCR methods to detect an array of viruses. Also, the safety and immunogenicity data from four clinical studies, that were used to support licensure of FluBlok® in the U.S., are included in this chapter. The total safety database for FluBlok included 2497 subjects 18-49 years and 736 subjects older than 50 years who received the commercial formulation of FluBlok. The vaccine was shown to be well tolerated and immunogenic in adults older than 18 years.

2. Implications of this work for public health and pandemic preparedness

All influenza vaccines currently licensed in the U.S. are manufactured in embryonated chicken eggs. FluBlok, manufactured using the baculovirus-insect cell production system eliminates the potential for anaphylaxis or other severe reactions in individuals with egg allergy. Although the number of persons who fall into this category is not precisely known, government and academic authorities have estimated that approximately 2-6% of children and 2-5% of adults in the U.S. have self-reported allergies to eggs [Kucukosmanoglu *et al*., 2008; Vierk *et al*., 2007]. This equates to up to approximately 2.4 million children age 10 and under and a minimum of approximately 5 million allergic adults aged 18 years and above. An estimated two-thirds of these egg allergic individuals fall into the Advisory Committee for Immunization Practices recommended target groups for annual influenza vaccination, that is, at least 1.5 million egg-allergic children and 3 million egg-allergic adults presently have no alternative other than to risk an anaphylactic shock or other severe allergic reactions upon receipt of an egg-derived trivalent inactivated influenza vaccine (TIV). Thus, there is a very substantial unmet medical need for an alternative influenza vaccine that is free of egg proteins. Such a need was specifically acknowledged by former Secretary of the Department of Health and Human Services (DHHS) Michael Leavitt in a press release, issued on April 1, 2005, as a key driver for the development of cell-derived influenza vaccines [web link: 7-1]

Clinical studies have shown that FluBlok, which contains three times more HA than TIV, is well tolerated and highly immunogenic, particularly in the elderly population [Treanor *et al*., 2006; Keitel *et al*., 2009; Chapter 6]. This is beneficial since the majority of the influenza related serious illness and death occur within the latter age group.

The baculovirus-insect cell production process does not include the production of large quantities of live influenza virus, followed by the inactivation of the virus, and therefore does not require a high bio-containment manufacturing environment. This has a number of clear advantages: manufacturing personnel is not exposed to live influenza viruses and escape of a live virus is impossible during production. Reports of a product contaminated with a residual live influenza virus H5N1 accidentally released from a facility where large quantities of live viruses are processed are alarming [web link: 7-2] and demonstrate that the risk of accidental release is far from zero.

Epidemics on a global scale also called pandemics may occur when a new virus capable of causing severe disease transmits easily among humans. Since there is no or little immunity to a newly emerging virus in the human population, it may spread quickly world wide. A pandemic was officially declared on June 11, 2009 when the World Health Organization (WHO) raised its influenza H1N1 alert to its highest level – phase 6 – for the first time in over 40 years. The events that let to this decision are described briefly. Responding to what some health officials feared could be the leading edge of a global pandemic emerging from a swine flu outbreak in Mexico, American health officials declared a public health emergency on Sunday April 26, 2009 as 20 cases of swine flu were confirmed in the U.S., including eight in New York City. At this time Mexico was already in a state of alarm with virtually all schools, restaurants and businesses closed in Mexico City, resulting in an estimated economic loss of around U.S. $400M per day. On April 29, 2009, WHO raised the

level of pandemic alert from Phase 4 to Phase 5, indicating that human-to-human spread of the virus had occurred in at least two countries in one WHO region. As of the end of May, over 15,000 cases of H1N1 had been confirmed in more than 53 countries, including 99 deaths, with 85 reported in Mexico alone. The case fatality rate in Mexico of H1N1 was estimated to be 1.5%, whereas in the U.S. it was only 0.1%. In addition, the virus seemed to be behaving similarly to seasonal influenza, although the transmission rate is relatively high (est. 22-33% and similar to the usual transmission rate reported in children). On May 19, WHO and the United Nations organized a meeting with influenza vaccine manufacturers to discuss the status of influenza vaccine supply. The conclusion was that influenza vaccine manufacturers are still relying on the growth of live influenza viruses, mostly using chicken eggs. Attendees were informed by WHO that if they were to ask manufacturers to switch production by June 30 to the new H1N1 strain, only 30% of the usual seasonal supply of TIV would be available since the manufacturers just started producing the B- (or third) component of the vaccine. By the end of July 2009, approx 65% of the usual supply would be available. In addition, no seed virus was available to enable manufacturers to begin production at this time.

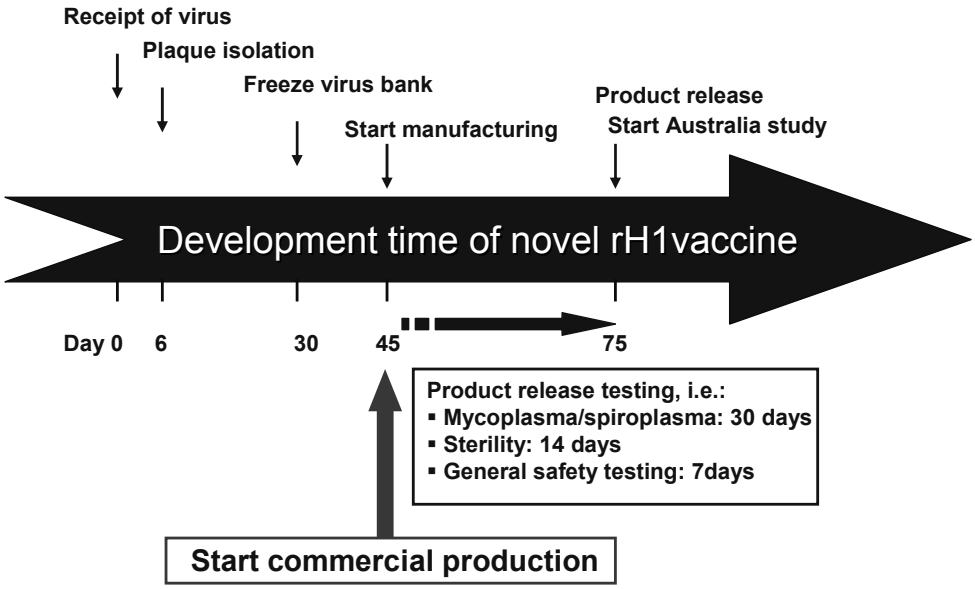
By the middle of August, 2009, the number of human cases of pandemic (H1N1) 2009 is still increasing substantially in many countries, particularly in the Southern hemisphere, which entered its winter flu season. WHO stopped reporting individual cases mid July, in part because many epidemiologists have pointed out that many millions of people have had mild forms of the novel H1N1 influenza [web link: 7-3].

The insect cell-baculovirus production technology is particularly suitable to address health care emergencies as currently posed by the H1N1 swine flu influenza virus. This manufacturing technology can be readily introduced into other countries, which would enable rapid expansion and availability of local vaccine production; for example, in Korea 50,000-L cell culture capacity exists that could supply millions of vaccine doses in a relatively short time. Assuming that yields obtained at the 500-L scale can also be achieved in a 10,000-L bioreactor, it is estimated that sufficient monovalent bulk protein for approximately 9 M doses containing 15µg of rHA can be produced in a 5-day cycle. As reported by Fedson and Dunnill (2007) 425 million doses of vaccine containing 10µg/dose could be produced within one month if 25% of the global bioreactor capacity (or 500,000-L) were to be allocated to rHA vaccine production. Furthermore, technology transfer would avoid the serious political impediments to export vaccine from manufacturing countries during a pandemic. For example, it has been described that the U.S. closed its borders in 1976 for vaccine export in anticipation of a potential swine flu outbreak [web link 7-4]. Shipment of a recombinant baculovirus that can be used to produce vaccine would generally not be limited by such regulations. It is essential that the above assumptions relating to yield and feasibility of technology transfer be tested.

The development of a rHA vaccine against the H1N1 A/California /04/2009 was started up on April 29, 2009 and by mid June, merely 6 weeks later, the first batches of vaccine were produced that could be used for clinical testing in human subjects. As shown in Figure 1 commercial production of a novel vaccine could start as early as 45 days after receipt of the virus. This timeline could be further improved by reducing the time needed to create a virus bank. Also shown in Figure 1 is the time required to release the product. Modern methods that are acceptable to the regulatory authorities are needed to release, for example the mycoplasma/spiroplasma cultivation test takes 30 days and could be replaced by a simple PCR test. It is unclear though at

this moment what vaccine formulation is needed to induce an acceptable immune response in various age groups, i.e. what potency is needed, will one dose be sufficient, and will an adjuvant be required? These questions need to be answered before an acceptable vaccination strategy can be formulated. Early June, the recombinant baculovirus was also shipped to various collaborators across the world and the University of Queensland reported successful vaccine production demonstrating a first step of the feasibility of technology transfer and local vaccine production [web link 7-5].

Figure 1. The development timeline of rH1 vaccine in insect cells. Commercial vaccine production could begin as early as 45 days after receipt of the virus.



Finally, as this technology matures it is likely to offer a powerful first line defense in combating the emergence of new viruses due to the increased contact between human and wildlife [Daszak *et al.*., 2001]. Vaccines against zoonotic diseases caused by for example Human Immunodeficiency Virus, West Nile Virus, Chikungunya Virus, Marburg Virus and Ebola Virus are desperately needed. It is important to acknowledge that it may not be easy or even feasible to identify the antigen that may offer protection as demonstrated by the failure to develop an effective vaccine against HIV over the past two decades. However, surface antigens offer a promising fast approach as exemplified by the virus neutralizing antibodies induced by the spike protein antigen derived from SARS coronavirus [Zhou *et al*., 2006]. The most attractive aspect of this technology is its versatility, i.e. it is perfectly suited as an emergency vaccine production system.

Also, many malaria vaccine candidates are being produced using the baculovirus-insect cell production system [van Oers, 2006]. Furthermore, baculovirus insect cell-derived recombinant proteins are used as vaccines against cancer [Neidhart *et al.*, 2004; Mao *et al.*, 2006; Betting *et al.*, 2009]. Thus, commercialization and further scale–up of this manufacturing technology beyond viral vaccines may have broad implications for disease control in general.

3. Other influenza vaccines in clinical development

Inactivated influenza vaccines and efforts to improve performance

It is widely recognized that there is a need to improve the efficacy of the inactivated egg-based influenza vaccines. Adjuvants, alternative antigen presentation forms, alternative routes of administration, or increasing the antigen content are the four key areas being pursued to improve the performance of existing vaccines (Table 1).

Table 1. Key areas for improving the efficacy of existing egg-based influenza vaccines and their stage of development.

Technology	Description	Product name /Manufacturer	Stage of development
Adjuvants	MF59 (oil-in water emulsion)	Fluad®/ Novartis	Approved for adults, age ≥60 by EMEA
	AS103 (oil-in water emulsion)	Pandemrix®/GSK	Approved for pandemic use by EMEA
	Alum	Pandemic vaccines (CSL/Solvay/Denken Seiken)	Approved for pandemic use by EMEA
Alternative presentation form	Virosomes	Inflexal® / Berna Biotech	Approved by EMEA
		Invivac® / Solvay	Approved
Alternative route of administration	Nasal: adjuvanted flu vaccine	Nasalflu® / Berna Biotech	Withdrawn
	Other vaccine for intranasal delivery include a variety of adjuvants, such as MF 59 lipid/polysaccharide ect.	Various	Early stage: Phase 1
	Intradermal	Inflexal® / Berna Biotech	Early stage: Phase 1
		Fluzone ®/ Sanofi Pasteur	BLA submitted to FDA
High antigen content	60 μg (4x)/ antigen	Fluzone HD®/ Sanofi Pasteur	BLA submitted to FDA

The role of an adjuvant such as Alum, MF59 or AS103 or AS104 is to enhance – or reduce the amount of antigen needed, accelerate or prolong the immune response. MF59 (oil in water emulsion) is included in Novartis seasonal influenza vaccine Fluad which has been approved in Europe for use in adults older than 60 years. GSK's AS103 (oil-in-water emulsion containing squalene) has been approved in Europe for use in its pandemic influenza vaccine candidate Pandemrix. To date Alum is the only approved adjuvant in the U.S., however, none of the currently used seasonal influenza vaccines contains Alum. The Alum adjuvant is used in various egg-based, pandemic vaccine products, including CSL, Denka-Seiken, and Solvay's vaccine candidates. While adjuvants hold promise, especially from a dose reduction standpoint, concerns have been raised relating to their long-term safety.

Alternative presentation forms such as virosomes are being pursued by Solvay and Berna Biotech. Virosomes are virus-like particles, resembling the native virus, but

lacking its genetic material. Virosomes are made using a process that includes solubilization of the viral envelope with detergents, centrifugation for removal of the nucleocapsid and reformation of virus-like particles lacking nucleic material [Stegmann *et al*., 1987]. Immunogenicity and safety data from two virosome based influenza vaccines currently on the market Inflexal V® (Berna Biotech) [Glück *et al*., 2004] and Invivac® (Solvay) [de Bruin *et al*., 2004] suggests that this is an attractive approach to improve immunogenicity without apparent safety drawbacks.

The standard influenza vaccine is administered by intramuscular injection. Alternative delivery methods include intradermal delivery or intranasal delivery. Various clinical studies have shown that alternative delivery methods can lead to dose reduction For example, Inflexal V® was shown to be overall highly immunogenic and well tolerated when given intradermal at reduced doses to healthy adults, eliciting an immune response similar to that observed with full dose intramuscular administration [Künzi *et al*., 2009]. Earlier Kenney *et al*. (2004) already demonstrated that in young adults an intradermal administration of one fifth of the standard intramuscular influenza vaccine dose elicited similar or better immune responses than a full dose intramuscular injection. Thus, suggesting that intradermal delivery can offer a promising antigen-saving strategy for influenza vaccination. Intranasal vaccine delivery has also been actively explored for delivery of inactivated influenza vaccines, however, due to the relatively low immunogenicity as measured by the induction of serum antibody responses, the combination with adjuvants was required. In 2001, the first adjuvanted, intranasal vaccine (Nasalflu®; Berna Biotech, Switzerland) was licensed in Switserland, however, it was taken off the market because there appeared to be an increased number of cases of Bell's palsy (acute paralysis of the facial nerve leading to inability to control facial muscles), in association with vaccination [Mutsch *et al*., 2004]. Other adjuvants for intranasal influenza vaccines in human clinical development include an MF59 adjuvanted subunit vaccine [Boyce *et al*., 2000], a lipid/polysaccharide molecule carrier [Halperin *et al*., 2005], a nontoxigenic *Escherichia coli* enterotoxin and a novel bioadhesive delivery vector as mucosal adjuvants [Stephenson *et al*., 2006], a chitosan carrier [Read *et al*., 2005] and a proteosome, consisting of the outer membrane proteins of *Neisseria meningitides* [Treanor *et al*., 2006]. In general these approaches show vaccine candidates to be well tolerated and modestly immunogenic, stimulating different parts of the immune system. The largest increase in circulating antibodies usually occurs in response to intramuscular vaccination; the largest mucosal immunoglobulin A (IgA) response occurred in response to mucosal vaccination. None of the alternative delivery methods has been approved yet for inactivated or subunit vaccines.

The fourth approach to improve immunogenicity is based on an increased antigen content of the vaccine. Human clinical studies conducted in the early nineties already demonstrated that increased doses of purified HA and subvirion vaccines produced an enhanced antibody response in both the elderly and healthy adult populations [Keitel, 1994; Keitel *et al*., 1996]. Recently, a large multicenter, randomized, double-blind controlled study in over 4000 adults 65 years and older compared a High Dose (FluZone HD vaccine - which contains 60 µg of hemagglutinin per strain) with the licensed standard-dose (SD) vaccine (FluZone - which contains 15 µg of hemagglutinin per strain) and showed FluZone HD to be well tolerated and more immunogenic than SD [Falsey, 2009]. Sanofi Pasteur has recently filed a biological license application with the FDA for this FluZone HD vaccine.

Live attenuated influenza vaccines (LAIV)

LAIV, delivered as an inhaled mist, closely mimics a natural influenza infection. While these vaccines have been used for decades in Russia, Flumist was approved in 2003 in the U.S. initially for healthy individuals between 5 – 49 years of age and more recently the label was expanded to include children between 2 – 5 years as well. The vaccine is made by re-assortment of a cold-adapted virus and gene segments from currently circulating influenza viruses. High levels of efficacy against influenza illness caused by both matched and mismatched strains in children and adults are reported in studies comparing LAIV and inactivated influenza vaccine in children, LAIV recipients experienced 35-53% fewer cases of culture-confirmed influenza illness caused by antigenically matched strains [Ambrose *et al.*, 2008]. In contrast, a recent study by Wang *et al*. (2009) which compared the effectiveness inactivated influenza vaccines and LAIV over three influenza seasons from 2004 – 2007, reported that vaccine effectiveness for inactivated influenza vaccines ranged from 28 – 55%, and from 10.7 – 20.8% for LAIV. The efficacy depended primarily on the degree to which the vaccine strains were antigenically related to circulating wild-type strains.

Alternative production methods to produce influenza vaccines

More than 95% of all available influenza vaccine is manufactured in embryonated chicken eggs. An urgent need for alternative production methods for influenza vaccines was identified more than a decade ago in a WHO report (1995) and as a consequence different cell culture systems are being explored. Production of influenza viruses in cell systems has proven to be challenging as demonstrated by the fact that none of the current manufacturers has been successful in commercializing a cell-based vaccine to date, even though both Solvay and Novartis received regulatory approval in Europe. Table 2 summarizes cell-culture based influenza vaccines in development.

Table 2. Different cell substrates used by manufacturers for the production of influenza various vaccines and their stage of development.

Cell Substrate	Vaccine Type	Product name /Manufacturer	Stage of development
MDCK	Sub-virion vaccine	Solvay	Approved by EMEA
	Sub-unit vaccine	Novartis	Approved by EMEA
	Live-attn vaccine	Pandemic vaccine (MedImmune)	Early stage: Phase 1
VERO	Whole-virus-vaccine	Baxter	Approved by EMEA
PER.C6©	Sub-virion vaccine	Fluzone ®/ Sanofi Pasteur	Early stage: Phase 1

The choice of a cell line for an industrial process is usually dictated by the yield obtained. When comparing three different cell lines (MDCK, VERO and BHK-21 C-13), Merten *et al*. (1999) concluded that the MDCK cells are most suitable as a substrate for the production of influenza viruses in serum-free media. VERO cells offer an advantage from a regulatory perspective since already licensed polio vaccines are produced using this cell substrate [Montagnon *et al*., 1983], however, yields of

influenza virus obtained with VERO cells were found to be approximately 10-fold lower than with other cell lines. This may explain why BAXTER decided to develop a whole-virus vaccine despite reports that whole-virus trivalent influenza vaccines, although more immunogenic than sub-virion vaccines, are also more prone to cause adverse reactions [Nicholson, 1976]. (Note: All currently licensed influenza vaccines are either sub-virion or sub-unit influenza vaccines).

Clinical evaluation of sub-virion influenza vaccines produced in MDCK cells has shown that these vaccines have a comparable immunogenicity and safety profile as egg-based vaccines [Palache *et al*., 1999; Halperin *et al*., 2002]. Cell culture offers significant advantages from a production perspective. Technologies described in Table 1 can also be used to improve the performance of cell-based vaccines.

Other recombinant influenza vaccine approaches in clinical development

Alternative recombinant influenza vaccine approaches in human clinical development are summarizes in Table 3.

Table 3. Alternative recombinant influenza vaccines in development

Technology	Description	Product name /Manufacturer	Stage of development
VLP Vaccines	VLP containing M1, HA and NA produced in the baculovirus-insect cell system	Novavax	Early stage: Phase 1/II
HA/flagellin Combination	HA fused to a toll-like receptor antagonist	Vaxinnate	Phase 1
HA Viral Vector Vaccine	HA is produced a non-replicating adenovirus using Per.C6© cells	Vaxin	Phase 1
NA subunit Vaccine	NA protein produced in the baculovirus-insect cell system	Protein Sciences Corporation	Phase 1/2
Universal Vaccine	M2e linked to the Hepatitis B core protein	ACAM-FLU-A/ Acambis	Phase 1
	Multi-epitope vaccine fused to a toll-like receptor antagonist	BiondVac	Abandoned
DNA vaccines	HA based	Powermed	Phase 1
	NP, M2, HA based	Vical	Phase 1

VLP vaccines

VLPs are produced using the baculovirus-insect cell system by co-expressing the M1 and various combinations of HA, NA, and/or M2 proteins of the influenza virus. All VLPs contain the M1 protein, presumably because this protein apparently possesses all the functions necessary for structure, budding and release of VLPs [Latham *et al*., 2001; Gómez-Puertas *et al*., 2000]. Novavax' s VLP vaccine candidate, contains M1, HA and NA and is currently being evaluated in Phase I/II clinical studies [Bright *et al*., 2007; Clinical Trial NCT00519389]. Human clinical data presented at various meetings suggest that the VLP vaccine induces comparable immune response to other inactivated influenza vaccines. The potential strength of VLP vaccines is that they may provide broader protection against heterologous viruses [Bright *et al*., 2007]. However, a challenge in the use of VLP vaccines is the production of particles of a consistent nature, since this is thought to be critically important in inducing strong immune responses [Quan *et al*., 2007].

HA/flagellin combination vaccine

Vaxinnate (www.vaxinnate.com) is developing a recombinant HA combination with the bacterial protein flagellin. In this vaccine the protective subunit of the HA molecule is genetically fused to the Toll-like receptor (TLR) agonist, flagellin. The recombinant HA-flagellin protein is produced in *E. coli.* Flagellin derived from *Salmonella typhimurium* is a protein that through pathogen-associated molecular patterns (PAMS) interacts with TLR-5 (TLRs) [Huleatt *et al*., 2007] and as such has the ability to enhance the cellular and humeral immunity of the vaccine. This vaccine - if proven to be safe and efficacious- could offer advantages from a manufacturing perspective, speed and cost-wise. VaxInnate's HA-flagellin vaccine for seasonal flu generated positive Phase I clinical results. Interestingly, BiondVax, decided to abandon clinical development when their "universal" influenza vaccine was citing mild side effects possibly attributed to the flagellin (see below).

HA Viral Vector Vaccines

The only viral vector vaccine that is currently in clinical development is a non-replicating adenovirus-vectored, nasal HA-based influenza vaccine candidate from Vaxin. The product has completed an initial Phase I clinical trial using a monovalent formulation. This study demonstrated safety and serological response in 48 healthy adults when a recombinant adenovirus vector was used to deliver the influenza hemagglutinin (HA) gene by nasal administration [van Kampen *et al.*, 2005]. The non-replicating adenovirus containing the HA gene is produced using the PER.C6© cell line (licensed from the Dutch biotechnology company Crucell©) which is also used by Sanofi-Pasteur for the production of an inactivated influenza vaccine. Insufficient information is available in the public domain to determine whether this vaccine approach may offer advantages compared to other vaccines in development.

Recombinant NA Influenza Vaccine

An NA vaccine produced using the baculovirus-insect cell system consisting of the NA protein from A/Johannesburg/33/94 (N2) was tested in human individuals for safety and immunogenicity [Matthews, 2000; Dr. G.E. Smith, Novavax, personal communication]. The vaccine candidate proved to be safe and immunogenic using doses ranging from 5 to 45 μg without the use of an adjuvant. In a follow-up clinical trial, individuals were vaccinated with TIV or with TIV supplemented with NA and then challenged with an attenuated influenza virus [Matthews, 2000]. Even though the results from this trial were not statistically significant, a trend toward reduction of severity of illness, reduced shedding and shorter duration of illness in those individuals vaccinated with the NA supplemented vaccine was apparent. This suggests that an NA vaccine may have potential as an additive to the licensed vaccine.

Universal Vaccine

Acambis is targeting a universal vaccine that targets all influenza A virus strains. The ACAM-FLU-A™ vaccine is based on the influenza M2e region, linked to the Hepatitis B core protein. M2e is a conserved region in the M2 ion-channel protein of all influenza A strains [Fiers *et al*., 2004; Neirynck *et al*., 1999]. M2 antibodies have been detected in humans following influenza virus infection; however, their role in the influenza-related disease process and viral clearance is not certain [Black *et al*., 1993]. It is clear that if this approach is successful this would offer a huge potential, offering the opportunity to vaccinate at any time of the year, to provide immunity for more than one influenza season and in that way to overcome the need for annual

vaccine reformulations. However, an M2-only based vaccine is limited because it would only protect against influenza A and not influenza B.

BiondVax was developing an intranasal peptide vaccine using a combination of conserved epitopes derived from the influenza virus as immunogens. The "conserved epitopes" are common to most human influenza A and B virus strains regardless of their antigenic drift and shifts [Ben-Yedidia and Arnon, 2007]. The safety of the first generation epitope-based vaccine within flagellin was evaluated in a Phase I clinical trial. At a safe dose, with a low concentration of influenza epitopes, cross-reactive immunological responses specific to the influenza viruses were found, demonstrating the protective potential of a vaccine based on these epitopes. Nevertheless, due to the appearance of mild side effects possibly attributed to the flagellin to which the epitopes were fused, the company decided to discontinue the trial.

DNA Vaccines

Formerly PowderMed (now Pfizer) and Vical are both exploring the development of a DNA vaccine. Historically DNA vaccines have been shown only to be immunogenic when using large quantities of DNA. As a result innovative delivery vehicles have been developed to further this field. The advantage of DNA vaccines is that the plasmids can be manufactured using uniform methods of fermentation and processing. This could result in faster development and production times than technologies that require development of product-specific manufacturing processes.

Powdermed recently reported results from a clinical study in healthy adults of a trivalent DNA vaccine for influenza consisting of three plasmids expressing HA from different seasonal influenza virus strains delivered using particle-mediated epidermal delivery (PMED) demonstrating an overall vaccine efficacy of 41%-53% [Jones *et al*., 2009]. The PMED requires 1 mg of gold/dose for efficient delivery, which appears to be expensive, but Powdermed claims that this represents only a fraction of the vaccine cost! It is unclear whether Pfizer is still pursuing the development of this vaccine.

Vical (www.vical.com) is developing a three component DNA plasmid encoding two highly-conserved influenza virus proteins—nucleoprotein (NP) and ion channel protein (M2)—plus the HA influenza virus surface protein with novel adjuvant: A dose of 0.03 mg VaXfectin and 1 mg DNA (not a typo) is needed to induce any kind of meaningful immune response. Preliminary human safety and immunogenicity data from a Phase 1 study conducted in 100 subjects generated preliminary safety and immunogenicity data and showed that Vaxfectin®-formulated H5N1 pandemic influenza DNA vaccines can induce antibody responses in up to 67% without significant safety issues.

4. What's Next: How can FluBlok be further improved?

Improvements in product quality and performance

FluBlok contains an increased antigen concentration compared to the standard TIV, i.e. 3x HA is included in the vaccine, which covers one of the four key areas discussed earlier to increase the performance of the licensed vaccine. So, the baculovirus insect-cell production system not only solves the qualitative, but foremost the quantitative constraints in producing a high number of doses of a protein-based vaccine, with adequate immunogenicity and in the shortest possible time. It meets all the requirements for a vaccine production system for both seasonal as well as emergency situations.

The immunogenicity of the recombinant HA vaccine needs to be improved both for pediatric [King *et al*., 2009] and pandemic use [Treanor *et al*., 2001]. A suitable adjuvant would not only improve the potential potency of recombinant influenza vaccines, but could also be antigen-saving thereby ensuring a faster and greater supply of vaccine doses in an influenza pandemic, assuming that antigen production is likely to be the major time-critical constraint in this event. Other advantages of adjuvant inclusion in recombinant influenza vaccines include potential for enhancement of T-cell based immunity, faster kinetics of protection, improved cross-protection, and longer-lasting immunity. The adjuvant that offers the lowest regulatory hurdle, since it has been frequently used as an adjuvant, is aluminum hydroxide (Alum). Alum enhances immunogenicity by converting soluble protein vaccines into particulate mass to make them more suitable for ingestion by antigen-presenting cells such as macrophages [Janeway *et al*. 2001]. Since Alum has provided mixed results in pandemic clinical studies [Cox, Review, 2007], we also plan to study inulin, a natural plant-derived polymer of fructose and glucose. Inulin exhibits many unique properties and has been used for many years in humans as an intravenous injection to measure renal function. When constituted into the appropriate delta isoform, inulin becomes an effective adjuvant that enhances both immunoglobulin and T-cell responses against many antigens including influenza HA (Vaxine Pty Ltd, Australia, *unpublished results*).

The addition of neuraminidase to FluBlok and other licensed vaccines may be another avenue to further improve any influenza vaccine. Neuraminidase antibodies may act in a similar fashion as the neuraminidase inhibitors, oseltamivir (Tamiflu) and zanamivir (Relenza), albeit in a prophylactic manner. Disadvantages of antiviral drugs include their required use within 24-48 hours after onset of the disease, potential side effects and perhaps most importantly antiviral drug resistance. Drug resistance of H5N1 has been reported for oseltamivir and zanamivir [Kiso *et al*., 2004]. The product label information for zanamivir states that a single point mutation in the NA gene can render the drug 1000-fold less efficacious. Also, a case study report by Le *et al*. (2005) suggests that drug resistance against oseltamivir can evolve within a two-week treatment period. Oseltamivir resistance has been reported frequently following treatment particularly against seasonal H1N1 influenza viruses [Stephenson, 2009]. Drug resistance of the novel H1N1 A/California/04/09 has now been reported on three occasions [Web link: 7-6] . Clearly, it would be desirable to analyze alternative delivery forms such as intra- or transdermal delivery for FluBlok or alternative presentation (virosomes). The preferred method would not require the use of needles or adjuvants because even the use of a well established adjuvant, such as aluminum hydroxide, can lead to discussions or questions regarding its safe use [Authier and Gherard, 2006]. Exploration of alternative delivery or presentation will

be initiated following licensure for the intramuscular FluBlok formulation.

Improvements in production cycle and yields

A universal process for seasonal influenza was established and only minor process changes to support the annual adjustment of the seasonal vaccine are evaluated and considered annually.

Due to the ever changing nature of the influenza viruses and the rapid emergence and spread of novel viruses as exemplified by the unprecedented spread of the novel H1N1 it would be desirable to even further reduce the development timelines and production cycles. Here we provide a brief overview of ongoing efforts to improve the manufacturing cycle and product yields.

Alternative baculovirus promoters, such as the p10 / p6.9 chimaeric promoter [Bonning *et al.*, 1994; Sun *et al*., 2004] are being evaluated from a yield improvement perspective using the Δ cathepsin- / chitinase-negative AcMNPV bacmid [Kaba *et al*., 2004].

Ongoing process improvement efforts include the development of a fed-batch fermentation process and the development of a defined growth medium. In the current influenza production process the insect cells are infected at a density of 2-2.5x10^6 cells/ml. The development of a fed-batch process for HA will be aimed at increasing the cells density at infection to 8-10x10^6 cells/ml as previously described [Bédard *et al*., 1997; Elias *et al*., 2000] without reducing specific productivity. A simple single shot feed-strategy resulted in a two-fold increase of HA production [Meghrous *et al*, *submitted*]. A semi-continuous fed-batch system was described by van Lier *et al*. (1995). Further improvements in cell culture will be aimed at establishing a continuous fed-batch process for which 40-fold improvements in antibody production in mammalian cells was reported [Birch and Racher, 2006].

Yield improvement has also frequently been reported as a result of changing the cell culture media. Additions of plant hydrolysates, other growth and production enhancing factors and control of proteolysis were reviewed by Ikonomou *et al*. (2003) and offer promising areas for yield improvement. Specifically, adding the plant hydrolysate, Hypep 1510, to an insect cell culture resulted in a doubling in expression of a reporter gene [Kwon *et al*., 2005], but also simple changes in pH may offer great benefit [Jakubowska *et al*., 2009].

It has further been shown that viral and host modifications can improve cell survival and production of heterologous proteins. Modifications to the host insect cell line for example by including the anti-apoptotic gene Bcl-2 may limit the cytopathic effects of the baculovirus and may result in enhancement of expression as well as was recently reported for Sindbis virus in a mammalian cell line [Nivitchanyong *et al*., 2009]. Co-expression of chaperones may also be a promising prospect for the efficient production of recombinant secretory proteins in insect cells as was recently reported by for instance Kato *et al*. (2005).

5. Concluding Remarks: Lessons learned …

Challenges of technical, regulatory and financial nature were encountered on the path to FluBlok product licensure. The technical challenges were mostly due to the required annual adjustment of the influenza vaccine within the limited time available, and the fact that insect cell manufacturing had yet to be established as a commercial manufacturing technology for human medicine. Some of these technical challenges were addressed in the previous chapters. The product approval of Cervarix, also manufactured in insect cells helped establishing this technology as a commercial manufacturing technology.

The complexity of product licensure is amplified exponentially with the number of novel elements included in the process. The mechanism of action of the FluBlok vaccine is similar to that of the licensed inactivated influenza vaccines, i.e. the induction of antibodies against hemagglutinin. FluBlok is also like TIV standardized to contain a certain amount of HA determined by the same potency method used for TIV. However, FluBlok is a novel vaccine, containing *only* three times greater quantity and is produced using a novel cell substrate. Progress in science leads to new ideas and new, potentially better products. However, a new product brings uncertainty. The outset was to develop a better influenza vaccine for the population that most needed it, those 65 or 75 years and older. Regulators cautioned along the way stating that an rHA vaccine had to perform equivalent to the currently licensed vaccines suggesting that further improvements to this type of vaccine could be delayed until after product approval. Perhaps the regulatory challenge would have been easier had we indeed developed a recombinant vaccine with the same antigen content as the licensed vaccines first (i.e. 15 µg per antigen instead of 45 µg per antigen) and saved the higher antigen concentration for a later stage. This would have changed the number of unknown factors in the product from two to just one.

FluBlok development nearly failed as a result of the challenge to secure adequate funding for development. The lesson taught in Business School that the next financing is always more difficult than the previous one and that you need to have sufficient cash to reach the next milestone (i.e. adequate revenue generation) is one that can not be underestimated. While there is no set recipe to entrepreneurship and establishing a successful business, it is key to success to have access to sufficient resources.

Protein Sciences holds a family of key patents to the production of rHA in the baculovirus-insect cell system [USP5762939, USP5858368; USP6245532 and foreign counterparts]. Therefore other companies interested in the development of an rHA vaccine produced using the baculovirus-insect cell expression technology would require a license for commercial use.

The baculovirus – insect cell technology addresses the need for new influenza vaccine production technology. Using this technology, influenza vaccines can be made available worldwide within a very short time, which is an absolute requirement in combating the next pandemic. The recent contract award from Health and Human Services [Web link 7-7] is not only a strong vote of confidence in FluBlok from the U.S. government, but also provide adequate financial support and other resources for the further development of a recombinant influenza vaccine. As a result, we expect to be able to provide a worldwide solution to pandemic preparedness within the next 5 years.

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Abbreviations and definitions of terms

Abbreviation	Definition
µg	microgram
ACIP	Advisory Committee on Immunization Practices; (refer to http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5306a1.htm).
AcMNPV	*Autographa californica* multiple nucleocapsid (multicapsid) nucleopolyhedrovirus
AE	Adverse event
BCA	Bicinchoninic acid
BLA	Biologics license application
BEVS	Baculovirus expression vector system
BV	Budded virus
°C	Degrees Celsius (Centigrade)
°F	Degrees Fahrenheit
CBER	Center for Biologics Evaluation and Research
CDC	Centers for Disease Control and Prevention
CDC-ILI	Influenza-like illness as defined by the Centers for Disease Control and Prevention: fever (temperature ≥100ºF oral) plus either cough or sore throat on the same day or on consecutive days
cGMP	current Good Manufacturing Practices
CI	Confidence interval
CODEHOP	Consensus-degenerate hybrid oligonucleotide primers
CV	Column volume
DHHS	Department of Health and Human Services
DO_2	Dissolved oxygen
EM	Electron microscopy
Endo H	Endoglycosidase H
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FluBlok®	rHA trivalent recombinant hemagglutinin influenza vaccine
GMT	Geometric mean titer
GSK	GlaxoSmithKline
HA	Hemagglutinin
HAI	Hemagglutination inhibition
hpi	Hours post infection
IgA	Immunoglobulin A (most important mucosal [secretory] immunoglobulin)
IgG	Immunoglobulin G (most abundant serum [circulating] immunoglobulin)
ICH	International Conference on Harmonization
IdU	Pyrimidine analog 5-iododeoxyuridine
kDa	kilo Dalton
LAIV	Live attenuated influenza vaccine
M	Matrix protein (M1 en M2)
MCB	Master cell bank

MDCK	Madin Darby canine kidney (cells)
MDCO	Molecular weight cut-off
mL	Milliliter
MOI	Multiplicity of infection
MW	Molecular weight markers
NA	Neuraminidase
NIAID	National Institute of Allergy & Infectious Diseases
NP	Nucleoprotein
NS	Nonstructural proteins
nt	Nucleotides
ODV	Occlusion-derived virus
ORF	Open reading frame
P	Viral polymerase proteins (PB1, PB2 and PA)
P1, P2, etc	Passage 1, Passage 2, etc.
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNGase F	Peptide-*N*-glycosidase F
Per.C6	Human retina cell line
PERT	Fluorescent- PCR based reverse transcription
PSC	Protein Sciences Corporation
PSFM	Protein Sciences fortified medium
OE-PCR	Overlap-extension polymerase chain reaction
RBC	Red blood cells
rHA	Recombinant hemagglutinin
RR	Relative risk
RNA	Ribonucleic acid
SAE	Serious adverse event / Serious adverse experience
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	*Spodoptera frugiperda*
SRID	Single radial immunodiffusion assay
TEM	Transmission electron microscopy
TIV	Trivalent inactivated influenza vaccine
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
T. ni	*Trichoplusia ni*
TNCL	*Trichoplusia ni* cell line
VERO	African green monkey kidney (cells)
VRBPAC	Vaccine and Related Biological Products Advisory Committee
vRNA	Viral RNA
WCB	Working cell bank
WHO	World Health Organization

Summary

Influenza (or flu) is a highly contagious, acute viral respiratory disease that occurs seasonally in most parts of the world and is caused by influenza viruses. Epidemics are seen annually and cause significant morbidity and mortality worldwide. The symptoms of the flu are similar to those of the common cold but tend to be more severe. Fever, headache, fatigue, muscle weakness and pain, sore throat, dry cough, and a runny or stuffy nose are common and may develop rapidly. A number of complications, such as the onset of bronchitis and pneumonia, can occur in association with influenza and are especially common among the elderly, young children, and anyone with a suppressed immune system. Influenza affects all age groups and in the United States (U.S.) alone, 25 to 50 million people contract influenza each year, and an annual average of 36,000 deaths and 226,000 hospitalizations has been associated with influenza epidemics. Over 90% of the deaths related to annual influenza epidemics occur in people over age 65. Epidemics on a global scale are called pandemics and may occur when a new influenza virus capable of causing severe disease transmits easily among humans. Since there is no or little residual immunity in the human population to such a newly emerging influenza virus, it may spread quickly world wide.

Influenza vaccination is an effective way to reduce the complications and the mortality rate following influenza infections. For example, studies have shown that influenza vaccination can reduce hospitalization rates by about 50% and the risk of death in the elderly by about 75%. The available inactivated and live attenuated virus influenza vaccines, that stimulate humoral and (to a lesser extent) cellular immunity, are cost-effective. Influenza vaccines currently licensed in the U.S. are manufactured in embryonated chicken eggs. The manufacture is therefore limited to influenza virus strains that replicate well in eggs but requires a large supply of eggs is required each year. Influenza viruses that are highly pathogenic to birds would therefore create a manufacturing problem since it could jeopardize the egg supply required for vaccine production. In addition, the current vaccines can not be administered to people with a severe allergy to eggs. Thus, a high priority in vaccine research is the development of novel influenza vaccines that do not use embryonated eggs as the substrate for production.

The research in this thesis was aimed at the design, validation and development of a production process for a recombinant hemagglutinin (rHA) influenza vaccine for the prevention of seasonal influenza. The viral surface protein HA has been recognized as a key antigen in the host response to influenza virus in both natural infection and vaccination since neutralizing antibodies directed against HA can mitigate or prevent infection. In the design, the baculovirus-insect cell system is used for the synthesis of rHA molecules. This expression system is generally considered as safe with unlikely growth potential for human pathogens. Extensive characterization of the novel cell substrate was performed, none of which revealed the presence of adventitious agents. In insect cells, full-length properly folded biologically active rHA was produced as concluded from its ability to efficiently agglutinate red blood cells, its resistance to trypsin, and the rosette-like structures revealed with electron microscopy. Hence the rHA produced in the baculovirus expression system could potentially be used in a seasonal influenza vaccine.

Influenza vaccines are adjusted annually and geographically based on surveillance data generated around the globe. This annual adjustment poses challenges for vaccine manufacturing as the time available to make adjustments is extremely short, a matter of months. Therefore, it is important that a versatile, robust manufacturing process is established that guarantees timely delivery of the new antigens. With the traditional egg-based approach a minimum of 3-6 months is needed to develop an influenza vaccine. As such, it is not feasible to surge capacity rapidly in case of a pandemic outbreak. The cloning of the HA from the influenza virus into a baculovirus vector and the expression of rHA in insect cell bioreactors, however, can readily be accomplished within the short time available. In addition, considerable progress was made towards establishing a scalable general purification processes for diverse rHA molecules within the available time frame.

The developed process was used to manufacture a candidate recombinant trivalent HA vaccine, which was tested in a randomized, double-blind, placebo-controlled clinical trial during the 2004-2005 influenza season among 460 healthy adults to determine the dose-related safety, immunogenicity, and protective efficacy. The study demonstrated that the vaccine was safe, well-tolerated and immunogenic. The fact that none of the recipients of the high dose vaccine, containing 45 μg of each rHA antigen, developed cell culture confirmed influenza whereas in unvaccinated individuals 4.6% developed influenza suggests that absence of the influenza neuraminidase (NA) in the vaccine and differences in glycosylation in insect versus vertebrate cells does not interfere with vaccine performance. Based on the success of this study a dose selection was made for the "45 μg" vaccine, which has a 3-fold greater rHA content than the licensed inactivated influenza HA vaccine. Subsequently, three additional clinical studies were performed in a total of more than 3000 human subjects to support licensure of FluBlok under the "Accelerated Approval" procedure in the United States (U.S.). These studies demonstrated that the highly purified rHA protein was well tolerated and resulted in a strong and long lasting immune response. In addition, the novel vaccine provided cross protection against drifted influenza viruses.

A pandemic was officially declared on June 11, 2009 when the World Health Organization (WHO) raised its influenza alert to the highest level – phase 6 – for the first time in over 40 years. The first initiative for a vaccine against the new H1N1 A/California /04/2009 strain, based on the design outlined in this thesis, was taken on April 29, 2009 and by mid June, merely 6 weeks later, the first batches of vaccine were produced, ready for clinical testing in human subjects. The commercial production of this novel vaccine could start as early as 45 days after receipt of the influenza virus. In principle, many millions of doses of vaccine can be produced within months if only a portion of the global bioreactor capacity were to be allocated to baculovirus-based rHA vaccine production.

The baculovirus-insect cell system tackles the need for new influenza vaccine production technology as it can be used for the expedited production of a safe and efficacious vaccine. Using this technology, influenza vaccines can be made available worldwide within a very short time, which is an absolute requirement in combating newly emerging influenza strains and in particular in the case of pandemics. As a result, it should be feasible to provide a worldwide solution to pandemic preparedness within the next five years using this technology.

Samenvatting

De griep is een zeer besmettelijke, acute virale ziekte, die jaarlijks heerst in grote delen van de wereld en veroorzaakt wordt door griepvirussen. Influenza-epidemieën zijn een jaarlijks voorkomend verschijnsel en leiden wereldwijd tot veel ernstig zieke patiënten en dodelijke slachtoffers. De symptomen van de griep zijn vergelijkbaar met die van een normale verkoudheid, echter meestal ernstiger van aard. Koorts, hoofdpijn, vermoeidheid, spierpijn en spierzwakte, keelpijn, droge hoest en een lopende of verstopte neus zijn normale griepverschijnselen, die zich snel kunnen ontwikkelen. Complicaties, zoals de ontwikkeling van bronchitis of longontsteking, komen vooral voor bij ouderen, jonge kinderen en andere mensen met een zwak immuunsysteem. De griep komt voor in alle leeftijdsgroepen en alleen al in de Verenigde Staten (VS) worden jaarlijks 25-50 miljoen mensen door de griep getroffen, met een jaarlijks gemiddelde van 36.000 doden en 226.000 ziekenhuisopnames. Meer dan 90% van de mensen die jaarlijks ten gevolge van een griepgerelateerde aandoening overlijden, is ouder dan 65 jaar. Een wereldwijde epidemie wordt een pandemie genoemd en kan ontstaan wanneer een nieuw griepvirus, dat in staat is ernstige ziekteverschijnselen te veroorzaken, gemakkelijk van persoon tot persoon overdraagbaar is. Omdat het immuunsysteem in dit geval grotendeels onbekend is met het nieuwe type influenzavirus, kan het virus zich snel over de hele wereld verspreiden.

Vaccinatie is een effectieve methode om de kans op complicaties te verkleinen en het dodental ten gevolge van griepinfecties te reduceren. Studies hebben bijvoorbeeld aangetoond dat antigriepvaccinatie het aantal ziekenhuisverblijven tot 50% kan terugbrengen en het overlijdensrisico met ongeveer 75% kan beperken. De beschikbare geïnactiveerde influenzavaccins en het verzwakte, levende vaccin zijn kosteneffectief en stimuleren antilichaamproductie en (in mindere mate) cellulaire immuniteit. Alle influenzavaccins, die op dit moment in de VS beschikbaar zijn, worden geproduceerd in bevruchte kippeneieren. Dit betekent dat de productie beperkt is tot griepvirussen die zich goed kunnen vermeerderen in kippenembryo's en dat er jaarlijks veel eieren nodig zijn voor vaccinproductie. Griepvirussen die (ook) ziekteverwekkend zijn voor vogels zorgen dus voor een probleem, daar zij de voorraad eieren die nodig is voor vaccinproductie in gevaar kunnen brengen. Bovendien moeten de virussen eerst nog verzwakt worden alvorens ze in kippenembryo's kunnen worden vermeerderd. Daarbij komt ook nog dat de huidige vaccins niet toegediend kunnen worden aan mensen, die allergisch zijn voor eieren. Dus de ontwikkeling van nieuwe griepvaccins, waarbij de productiemethode onafhankelijk is van eieren, heeft een hoge prioriteit.

Het beschreven promotieonderzoek was gericht op de ontwikkeling van een recombinant hemagglutinine (rHA) influenzavaccin ter preventie van de jaarlijkse griep. Het virale oppervlakte-eiwit HA is het sleutelantigeen voor het opwekken van antilichamen in de gastheer, zowel tijdens een natuurlijke infectie als na vaccinatie. De neutraliserende antilichamen, gericht tegen HA, kunnen infectie voorkomen. In het gepresenteerde procesontwerp worden de rHA-eiwitten gemaakt in het baculovirus-insectencelsysteem. Dit expressiesysteem wordt algemeen als veilig beschouwd, daar de groeimogelijkheden van ziekteverwekkers bij de mens zeer beperkt zijn. Omdat in het ontwerp een nieuwe cellijn gebruikt is, werd een

uitgebreide karakterisering van de cellijn uitgevoerd, waarbij geen pathogenen aangetoond konden worden. In de insectencellen wordt een volledig, goed gevouwen en biologisch actief HA-eiwit geproduceerd, wat geconcludeerd kan worden uit de eigenschappen van het rHA, dat rode bloedcellen laat samenklonteren, bestand is tegen trypsinebehandeling en dat roset-achtige structuren vormt, die met een elektronenmicroscoop waargenomen kunnen worden. Daarnaast wordt het rHA in grote hoeveelheden gemaakt in insectencellen. Dus het rHA, geproduceerd in het baculovirus-expressiesysteem, is een goede kandidaat voor een jaarlijks griepvaccin.

Het griepvaccin moet jaarlijks worden aangepast op basis van gegevens, die in een wereldwijd surveillancesysteem (WHO) gegenereerd worden. Deze jaarlijkse aanpassing vormt een uitdaging voor vaccinproducenten, die slechts enkele maanden de tijd hebben om deze aanpassing in hun productieproces door te voeren. Het is daarom belangrijk dat een robuust en breed toepasbaar productieproces beschikbaar is, dat tijdig nieuwe antigenen kan leveren. Met de bestaande productiemethode in eieren zijn 3 tot 6 maanden vereist om een influenzavaccin te ontwikkelen. Als gevolg daarvan is het niet mogelijk om de productiecapaciteit snel uit te breiden in het geval van een pandemische uitbraak. De klonering van HA in een baculovirusvector en de productie van rHA in bioreactoren kunnen zonder problemen op grote schaal en op diverse (internationale) locaties uitgevoerd worden binnen dit korte tijdsbestek. Voorts is bij deze studie een aanzienlijke vooruitgang geboekt in het ontwikkelen van een opschaalbaar zuiveringsproces, dat algemeen toepasbaar is voor diverse influenza-HA-eiwitten en dat binnen de beschikbare tijd kan worden gerealiseerd.

Het ontwikkelde proces werd vervolgens gebruikt voor de productie van een recombinant vaccin met rHA-eiwitten van drie verschillende griepstammen. Dit vaccin werd getest in een klinische studie gedurende het griepseizoen 2004-2005, waarbij zowel de 460 gezonde deelnemers als de onderzoekers, zolang de studie liep, niet wisten wie vaccin en wie fysiologisch zout toegediend had gekregen. Deze studie toonde aan dat het vaccin veilig en immunogeen was en goed getolereerd werd. Het feit dat geen enkele deelnemer, die de hoge antigendosering van 45 microgram van elk rHA-eiwit toegediend kreeg, aantoonbare griep ontwikkelde, suggereert dat de afwezigheid van neuraminidase in het vaccin en verschillen in eiwitglycosylering tussen insectencellen en cellen van gewervelde dieren de werking van het vaccin niet negatief beïnvloeden. In de controlegroep kreeg 4,6% van de proefpersonen griep. Op basis van het succes van deze studie werd de dosering vastgesteld op 45 microgram van elk rHA; driemaal meer dan de dosering van de op dit moment beschikbare geïnactiveerde vaccins. Vervolgens werden drie additionele studies met in totaal meer dan 3000 deelnemers uitgevoerd ten behoeve van productregistratie. Hierbij werd gebruik gemaakt van het “versnelde goedkeuring”-protocol in de VS. Deze studies hebben aangetoond dat een gezuiverd rHA-eiwit goed getolereerd wordt en dat een hoge en langdurige antilichaamproductie geïnduceerd wordt. Tevens bleek dat het nieuwe vaccin bescherming kan bieden tegen griepvirussen, die genetische veranderingen hebben ondergaan.

Op 11 juni 2009 verklaarde de Wereldgezondheidsorganisatie dat er een grieppandemie heerste en zij verhoogde voor het eerst in 40 jaar het alarmniveau naar fase 6.. Het eerste initiatief om een vaccin tegen dit nieuwe H1N1 A/California /04/2009-influenzavirus te ontwikkelen, gebaseerd op het ontwerp beschreven in dit proefschrift, werd genomen op 29 april 2009. Half juni, slechts zes weken later, werd

het eerste vaccin geproduceerd en getest in klinische studies. Dus commerciële productie van dit nieuwe vaccin was mogelijk binnen 45 dagen na ontvangst van het nieuwe influenzavirus. In principe zouden er binnen enkele maanden vele miljoenen doseringen entstof geproduceerd kunnen worden, als slechts een deel van de wereldwijde bioreactorcapaciteit gebruikt zou worden voor de productie van baculovirus-rHA-vaccin.

Het hier beschreven proces, dat gebruik maakt van het baculovirus-insectencelsysteem vervult de behoefte aan nieuwe technologie om influenzavaccins te produceren en kan ingezet worden voor de versnelde productie van een veilig en effectief griepvaccin. Met de ontwikkelde technologie is het ook mogelijk om in zeer korte tijd een griepvaccin wereldwijd beschikbaar te hebben, hetgeen absoluut noodzakelijk is om bescherming te bieden tegen nieuwe influenzastammen en in het bijzonder bij pandemieën. Het zou dan ook mogelijk moeten zijn om uitgaande van het hier ontwikkelde proces binnen vijf jaar tot een wereldwijde aanpak te komen ter voorbereiding en bestrijding van influenzapandemieën.

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The development of a process for the production of the recombinant hemagglutinin influenza vaccine and the path to licensure forms the basis of this thesis and has been a very exciting, exhausting and big learning experience. There are many people that have made different contributions along the way with teamwork being KEY to success!

To start with my story, it is a little different from the usual! Many moons ago, I started out as a graduate student exploring the usefulness of PCR-based human papilloma virus (HPV) screening for early detection of cervical cancer. However, when the opportunity arose to join Gist-brocades as a research scientist, the attraction to applied research made me decide to abandon that path.

During my six years in Research & Development at Gist-brocades, I had the opportunity to work with a fantastic team of scientists from whom I learned the importance of team-work and collaboration. I want to thank them, especially Gijs, Hesselien, Lydia, Jos, Ronald, Peter and Wim for everything they taught me. A special thanks to Volkert – my coach, paranimph, and our lipase project leader - while it was difficult to meet your demanding expectations, I enjoyed working with you and our friendship with all its challenges (keeping us on our toes) means a lot to me. My time in the Fast Track Development program brought new opportunities, coaches and friends. Auke: you were a great coach – I really started feeling smart; in Bruges (Belgium) – I learned all about manufacturing; and Cees showed us – me and my dearest funny and smart friend Annemiek – that you can be young, dynamic and successful. Annemiek: I will never forget our André van Duin connection, who as a grocery salesman praises many goods and every time a customer wants to buy, he tells them, sorry – I don't have that! Every time we landed a potential production contract after blood, sweat and tears, Simon informed us that we had no spare production capacity before sending us on a new assignment to identify partners for our spare production capacity! Finally, Jan-Willem: I enjoyed working with you on the "The Biopharmaceutical Adventure" and it shaped my future.

In between, there was Nijenrode – my core group was truly multidisciplinary, Ronald, a research director in physical science; Ricardo, an engineer and expert in logistics (we need you now at Protein Sciences!) and Adriaan, the lawyer. All this and the Rochester experience taught me even more about teamwork.

About 11 years ago I embarked on the adventure resulting in this thesis. I am grateful for everything I have learned from my wonderful colleagues. Let's start with Gale Smith – one of the "inventors" of the insect cell-baculovirus expression system. From being a long-distance admirer of his work I became his close co-worker – Gale: you were brilliant and it has been an honor to work with you for many years. At Protein Sciences I returned to science and I am greatly indebted to all my fellow scientists who participated and contributed pieces of the puzzle. Rick, Penny, Kathy, Trey, Keyang, Zhimin, Karl, Jason, Wafaa and Rob - while maybe not always notable - I tremendously enjoy working with you and continue to learn something new every day. We have made great progress and could still have the first cell-based influenza vaccine product to be approved in the U.S. despite all the last minute hurdles we are

encountering. The clinical development work allowed me to interact closely with some world-class internationally recognized influenza experts likes John Treanor, Wendy Keitel, Jim King and Peter Patriarca. Thank you, it has been and continues to be an honor. Marie-Paul Kieny and Robin Robinson, thank you for continuing to believe in this product! I am also very grateful to everyone who supported our work from the outside and provided constant encouragement during all these years; I would like to specifically acknowledge David Fedson. David: after I was finally able to convince you that FluBlok was IT – you became a true supporter. Your emails, introductions, papers and "facts of live" kept me upbeat and going: "Two steps forward, one step back" and "When you come to a fork in the road take it (Yogi Berra)". Success and a good product attracts predators and as a result 2008 became a very tough year. It often made me wonder whether the time had come to go home. Again, David's enlightening email: "Welcome to America's ways of conflict resolution: "pay attention and you will learn" puts a positive spin to the law-suits, deposition, and discovery.

The proposal from Monique and Just early 2009 to embark on the mission of compiling a thesis around the development of FluBlok was a welcome change in the destructive environment in which I was operating at the time. It was time for creation! Monique: I think we both felt like Truus, de Mier from "de Fabeltjes Krant" at times. You are amazing – the amount of work you accomplish in a short time. While annoying at times, I learned a lot from you: details, details, details, but it was a great experience. I admire you deeply. Just: You made it all sound so easy, but with Monique as co-promoter, it was certainly more work than I envisioned. I was so happy when I received your simple comment "I like it!" after reviewing the revision of Chapter 7. You are a great coach and made me realize once again that a few simple words can mean a lot. It was worth it. I am proud of what we accomplished. Thank you so much.

My family: Mom, thank you for ALWAYS believing in me no matter what! All the encouragement and support, you can never believe how much that means to me and Dad, finally, right? I hope you are proud. Remy and Manuela, sorry but thank you for giving up your Alaska vacation to help me finding the abbreviations in the document. My sister Aimée, thanks for being my paranimph and helping out.

Sandra, Eva, I wanted to have you both as my paranimphs but could only select two. Thank you for being friends, no matter what. And to all the other friends and colleagues, whom, I have failed to mention, the fact that you were able to listen, encourage, stimulate, has been tremendously important. I hope to have more time to spend with you.

Thank you, Jennifer Branch, for visualizing the cells producing the influenza vaccine in a global environment surrounded by emerging viruses. You are fabulous!

Finally, Dan: Thank you for believing in me and giving me the BEST opportunity I could have ever imagined. I hope we will be able to work on many more exiting projects together (Next: HIV?). Thanks to you we have Jennifer Branch as the artist (designer) of the front page: You taught me that I could buy original art work instead of posters and even introduced me to President Clinton. Not to mention all the other adventures. You bring out the best in me.

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Curriculum Vitae

Manon Cox was born on the 13th of August 1963 in Meerssen, Limburg, The Netherlands. She grew up in Nunspeet, and then returned to Limburg. She graduated from St. Maartens College in Maastricht and then studied Biology at the University of Nijmegen, specializing in Molecular Biology, Genetics and Biochemistry, and graduating in 1987. It was during her molecular biology internship that she became first interested in the baculovirus technology, writing a paper in 1986 about the promise this then newly invented technology held for the future of medicine. From 1990 until 1993 she studied Economics at the Open University. In 1997 she received a Master of Business Administration (MBA) degree (with distinction) from both Nijenrode and the University of Rochester, New York. Following graduation from the University of Nijmegen, she worked as a scientist at the University of Amsterdam from 1987–1988, where she participated in the development of a PCR-based screening for human papilloma virus (HPV). She joined Gist-brocades in 1988, initially as a scientist in the Department of Bacterial Genetics and later joined the fast track development team to explore various disciplines within the organization, including production and new business development, and a MBA in the U.S. This ultimately resulted in her opportunistic move to the U.S., the land of opportunity in 1998. Her roots in molecular biology and a strong interest in vaccine development landed her at Protein Sciences Corporation. The attraction of Protein Sciences was manifold: Daniel Adams, biotech pioneer - founder of Biogen and various other bioventures - was its Chief Executive Officer; Gale Smith, the inventor of the baculovirus technology, was its Chief Scientific Officer; and the product portfolio included influenza and HIV vaccines made using the baculovirus system. Originally Manon joined Protein Sciences Corporation as Director, Business Development, but soon became involved in and later responsible for its research program. Since 2002, she has been Chief Operating Officer and leads a team of process development scientists and oversees the manufacturing, quality and regulatory functions.

Manon travels the world discussing the importance of a break-through technology for the production of influenza vaccines. She speaks at international conferences for example conferences organized by WHO and Australian regulatory authorities, and she interacts with influenza industry leaders. In her free time, she enjoys dancing, running, reading, hanging out with friends, meeting interesting people, studying, the sea and the sun.

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