

# Production of a novel influenza vaccine using insect cells: protection against drifted strains

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A recombinant trivalent hemagglutinin (HA) vaccine produced in cell culture using the baculovirus 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 World Health Organization-recommended strains are cloned, expressed, and purified using a general purification process. Here, we provide an overview of the expression technology used to make the annual adjustment of the vaccine and the clinical studies completed to date with recombinant HA. The highly purified protein vaccine, administered at three times higher antigen content than TIV, results in stronger immunogenicity, a long-lasting immune

response and provides cross-protection against drift variant influenza viruses. Furthermore, the vaccine does not contain egg proteins, adjuvants, preservatives, endotoxins, or antibiotics and can therefore be used in a broader population.

**Keywords** Baculovirus, hemagglutinin, influenza, insect cells, vaccine.

**Conflict of Interests** MMJC is an employee of Protein Sciences Corporation and owns stock options and shares in the company. DKA is an employee of Protein Sciences Corporation and owns stock options in the company.

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## Introduction

### Influenza

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.<sup>1</sup> 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 20 000–50 000 deaths annually in the USA alone.<sup>2</sup> 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

Influenza viruses are single-stranded ribonucleic acid viruses with a segmented genome encoding 10 proteins. The viruses are surrounded by 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 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 15 µg of each of three HAs. 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 several disulfide bonds.

### Influenza vaccines

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.<sup>3</sup> Split virion vaccines such as FluZone (sanofi pasteur, Swiftwater, PA, USA) are produced by splitting

the virus particles using detergents or solvents. The subunit vaccines such as Fluvirin (Novartis Vaccines Ltd., Liverpool, UK) are further purified to remove the internal proteins, leaving mostly HA and neuraminidase.

### Baculovirus technology

Baculoviruses are found in nature commonly on green vegetables and, therefore, baculoviruses are part of the daily diet of healthy individuals. For example, a typical serving of coleslaw contains 112 million polyhedra (each polyhedra contains multiple baculoviruses).<sup>4</sup> The name of the virus is derived from the Latin 'Baculum' (rod) describing its shape. Baculoviruses are characterized by their narrow host range<sup>5</sup> and cause fatal disease in specific insect species. These viruses are not able to replicate in mammalian cells but have been shown to efficiently transduce a variety of mammalian cells.<sup>6</sup> The virus has a relatively small double-stranded DNA genome of approximately 130 kbp and can be easily characterized using genomic digests and Southern blotting techniques. The baculovirus *Autographa californica* nuclear polyhydrosis virus (AcNPV) can be propagated in cell lines derived from the fall armyworm *Spodoptera frugiperda* or from the cabbage looper *Trichoplusia ni*. These insect cell lines grow well in suspension cultures. In the 1980s, Summers and Smith demonstrated that the DNA encoding polyhedrin was unnecessary for the survival of the virus in a laboratory and could be exchanged for genes encoding proteins of medical importance, such as  $\beta$ -interferon.<sup>7</sup> Insect cells have the capability of performing many of the post-translational modifications such as glycosylation, disulfide bond formation and phosphorylation required for the biological activity of many complex proteins.<sup>8</sup> The protein of interest is usually produced under the control of the polyhedrin promoter, one of the strongest promoters known in nature. The transient nature of the Baculovirus Expression Vector System technology makes it particularly attractive for the production of an influenza vaccine, requiring annual adjustments because a single well-characterized cell line is used for the production of all HA proteins.

## Results

### Description and characterization of *expresSF+* cell line

The host cell line used by Protein Sciences for recombinant protein manufacturing is a serum-free cell line called *expresSF+*®. The *expresSF+* cell line was adapted from Sf9 cells, a *Spodoptera frugiperda* ovarian cell line. This cell line grows well in suspension in the absence of serum. It can be used for the production of both baculovirus AcNPV and recombinant proteins unlike the *T. ni*, which is not very efficient in AcNPV production. The *expresSF+* cells (lot number 081093) were characterized and tested for adventi-

tious agents. Identity testing included karyology, isoenzyme analysis, cell morphology, and growth characteristics such as growth, infectivity, and protein production. Freedom from microbial organisms was established by sterility, mycoplasma, and spiroplasma testing. The cell line was also tested for the absence of adventitious viruses using an *in vitro* method including MRC-5, Vero, BHK-21, and Sf9 cells and an *in vivo* method using guinea pigs, mice, and embryonated chicken eggs. The cell line tested negative in a 22-day tumorigenicity study when injecting nude mice with  $1 \times 10^7$  viable *expresSF+* cells subcutaneously. Furthermore, the cell line was also tested for the presence of retroviruses using electron microscopy and a reverse-transcriptase (RT) assay. In order to support commercial manufacturing, a larger cell bank was established and all the above testing was repeated this time with a 16-week tumorigenicity test. It is important to note that insect cells do not support replication of most types of viruses associated with human disease including retroviruses, which can infect mammalian cell culture. Only arboviruses have been sporadically reported to replicate in insect cells.<sup>9</sup>

### Production of a recombinant HA protein vaccine using insect cells

The HA genes are cloned using influenza viral RNA as a template in RT polymerase chain reaction to generate cDNA for each of the desired HA molecules (H1, H2, H3, H5, H7, H9, and B). The inserts are cloned in a baculovirus transfer vector, and clones are analyzed by DNA sequence analysis to ensure that the cloned HA is identical to the known sequence of the relevant HA gene. Alternatively, working from the known sequence, the DNA for a specific HA gene can simply be synthesized. *Spodoptera frugiperda* Sf9 insect cells are co-transfected with linearized AcNPV baculovirus genomic DNA and the baculovirus transfer plasmids containing the HA gene using calcium phosphate precipitation. Recombinant baculoviruses are formed by homologous recombination. Recombinant plaques having a distinctive (clear) morphology are used to generate virus stocks, which can be amplified by passage of the virus in fresh insect cell cultures. These virus stocks can then be used to produce the HA protein in larger culture volumes. The HA product is subsequently purified from the insect cells by first solubilizing the HA protein using mild detergent conditions, and purifying the protein using a combination of filtration and column chromatography methods. The process of producing a virus stock suitable for large-scale manufacturing takes approximately 1 month and there is no limit to the cell culture volume used for the actual manufacturing of the HA protein. The production cycle time is 5 days. The production process and purification of various HA proteins and their biochemical characterization has been described in detail elsewhere.<sup>10,11</sup>

## Clinical results

Recombinant HA (rHA) has been tested in multiple phase I/II human clinical trials conducted by 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.<sup>12–15</sup> 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.<sup>16</sup> 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.<sup>17</sup>

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.<sup>18</sup> Recombinant HA was well tolerated and resulted in higher Hemagglutinin Agglutination Inhibition (HAI) antibody levels against the H3N2 influenza virus. This is

important 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.*<sup>19</sup> completed a study of the rHA vaccine performance in immuno-compromised subjects and reported encouraging dose-dependent immunogenicity results.

Recently, Protein Sciences completed a proof of principle/field study with a trivalent rHA vaccine in 460 healthy adults (18–49 years) to establish the final commercial dose. The trivalent rHA vaccine was safe, immunogenic, and effective in the prevention of influenza disease, with the higher dose 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 was reduced by 54.4% when compared with placebo in this dose group (J.J. Treanor, G.M. Schiff, F.G. Hayden, R.C. Brady, C.M. Hay, A.L. Meyer, A. Gilbert, M. Cox, University of Rochester, Rochester, submitted for publication). 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 commercialization. The clinical studies conducted to date are summarized in Table 1.

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

Clinical studies	n*	Influenza strain	Dosage recombinant HA <sub>0</sub> (µg)	Reference
93A (young adults)	127	A/Beijing/32/92	15, 90; Fluzone	Powers <i>et al.</i> (1995) <sup>13</sup>
94A (young adults)	113	A/Beijing/32/92	15, 45, 135; FluShield	Treanor <i>et al.</i> (1996) <sup>15</sup>
94B (young adults)	153	A/Beijing/32/92 A/Texas/36/91	45 15, 45, 135	Lakey <i>et al.</i> (1996) <sup>12</sup>
94C (elderly adults)	109	A/Beijing + B/Texas	2 × 45 <sup>†</sup> ; Subvirion	Treanor <i>et al.</i> (1996) <sup>15</sup>
94D (young adults)	100	A/Beijing/32/92	15, 45, 135; FluShield	Powers (1997) <sup>14</sup>
00 (healthy adults)	147	A/Hong Kong/156/97	25/25 <sup>‡</sup> , 45/45 <sup>‡</sup> , 90/10 <sup>‡</sup> , 90/90 <sup>‡</sup>	Treanor <i>et al.</i> (2001) <sup>17</sup>
03 (elderly)	399	A/New Caledonia/20/99 A/Panama/2007/99 + B/HongKong/330/01	3 × 15 <sup>§</sup> , 3 × 45 <sup>§</sup> , 3 × 135 <sup>§</sup> ; FluZone	Treanor <i>et al.</i> (2006) <sup>18</sup>
03 (B-cell lymphoma)	27	A/New Caledonia 20/99 + A/Panama/2007/99 + B/HongKong/330/01	3 × 15 <sup>§</sup> , 3 × 45 <sup>§</sup> , 3 × 135 <sup>§</sup> ; FluZone	Safdar <i>et al.</i> (2006) <sup>19</sup>
04 (healthy adults)	460	A/New Caledonia/20/99 + A/Wisconsin/3/03 + B/Jiangsu/10/03	15 + 15 + 45 <sup>§</sup> , 3 × 45 <sup>§</sup>	Treanor (in press)

\*Number of subjects included in each study.

<sup>†</sup>A single bivalent vaccine formulation.

<sup>‡</sup>Subjects received two doses 21–28 days apart.

<sup>§</sup>A single trivalent vaccine formulation.

**Table 2.** Comparison of HA amino acid sequence of field isolates (PS1–PS10), A/Wyoming/3/03 and A/California/7/04

Wyoming ↓ California	A 128	A 138	K 145	Y 159	V 186	D 188	S 189	A 196	Y 219	S 227
California	T	S	N	F	G	N	N	T	S	P
PS1–PS3, PS6–PS10	T	A	N	F	G	D	N	A	S	P
PS4, PS5	T	A	N	F	G	N	N	A	S	P

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

### Characterization of influenza isolates

The World Health Organization (WHO) reported that the majority of H3N2 influenza viruses circulating during the period October 2004 to January 2005 were similar to the A/California/7/04 strain. However, all vaccines contained A/Wyoming/3/03. In addition, HA antibodies stimulated by the vaccine were lower in titer to A/California/7/04-like viruses than to A/Wyoming/3/03 virus contained in the vaccine.<sup>20</sup> This situation presented an opportunity to determine if the HA vaccine protected against the circulating drift strains influenza.

We characterized the 10 influenza H3N2 isolates from 2004 to 2005 field efficacy study by sequence analysis. The results are summarized in Table 2. 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 the influenza strains isolated from subjects (PS1–PS10) match the A/California/7/04 strain H3 protein sequence in seven of the 10 positions. Isolates PS4 and PS5 also match the A/California/7/04 strain H3 protein sequence at amino acid position 188. As shown in Table 3, 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.

These results confirm the WHO report that the majority of H3N2 influenza viruses circulating during this period were similar to A/California/7/04 strain. However, 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 3, the majority of subjects who were influenza culture positive had previously received placebo (six 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 therefore CDC-ILI negative, suggesting that the lower dose vaccine reduces 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

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, Centers for Disease Control Influenza-like Illness, subjects presenting with fever plus at least one respiratory system.

Gray area indicates low dose.

**Table 3.** Evaluation of influenza H3N2 isolates (PS1–PS10)

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 higher content of the other antigens plays a role in providing protection against infection with drift influenza H3N2 strains.

## Discussion

The formulation of the rHA influenza vaccine that we intend to commercialize 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 HAI 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 potential to provide cross-protection for which preliminary evidence was presented but also the possibility for longer lasting immunogenicity. These 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.<sup>21,22</sup>

The TIV provides an economical and effective means to reduce the impact of an influenza infection. It is effective in the prevention of influenza in young adults with reported levels of protection as high as 70–90%;<sup>23,24</sup> however, only 30–50% of subjects older than 65 years vaccinated achieve protective titers against the H3 strain.<sup>25</sup> 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,<sup>26</sup> suggesting that a vaccine that provides cross-protecting would also be beneficial.

The egg-based manufacturing technology used to produce all currently approved influenza vaccines is outdated and is likely to be replaced 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 toward the development of protein-based vaccines. However, many new cell-based and protein vaccines are now in clinical development<sup>27</sup> and are expected to address some of the limitations 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 FluBløk; Protein Sciences Corporation, Meriden, CT,

USA) is like 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 HA antigens are developed using recombinant DNA 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.<sup>28–30</sup> Unlike the licensed egg grown vaccines and many cell culture vaccines in development, no live influenza viruses are used in manufacturing eliminating the need for biocontainment facilities or harsh chemicals 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. Finally, the recombinant, cell-based influenza vaccine offers an extremely rapid development cycle. This was demonstrated by Protein Sciences' achievement 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.<sup>31</sup> In addition to pandemic preparedness, a rapid development cycle would allow the inclusion of late appearing influenza virus strains in the annual vaccine preparations.

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