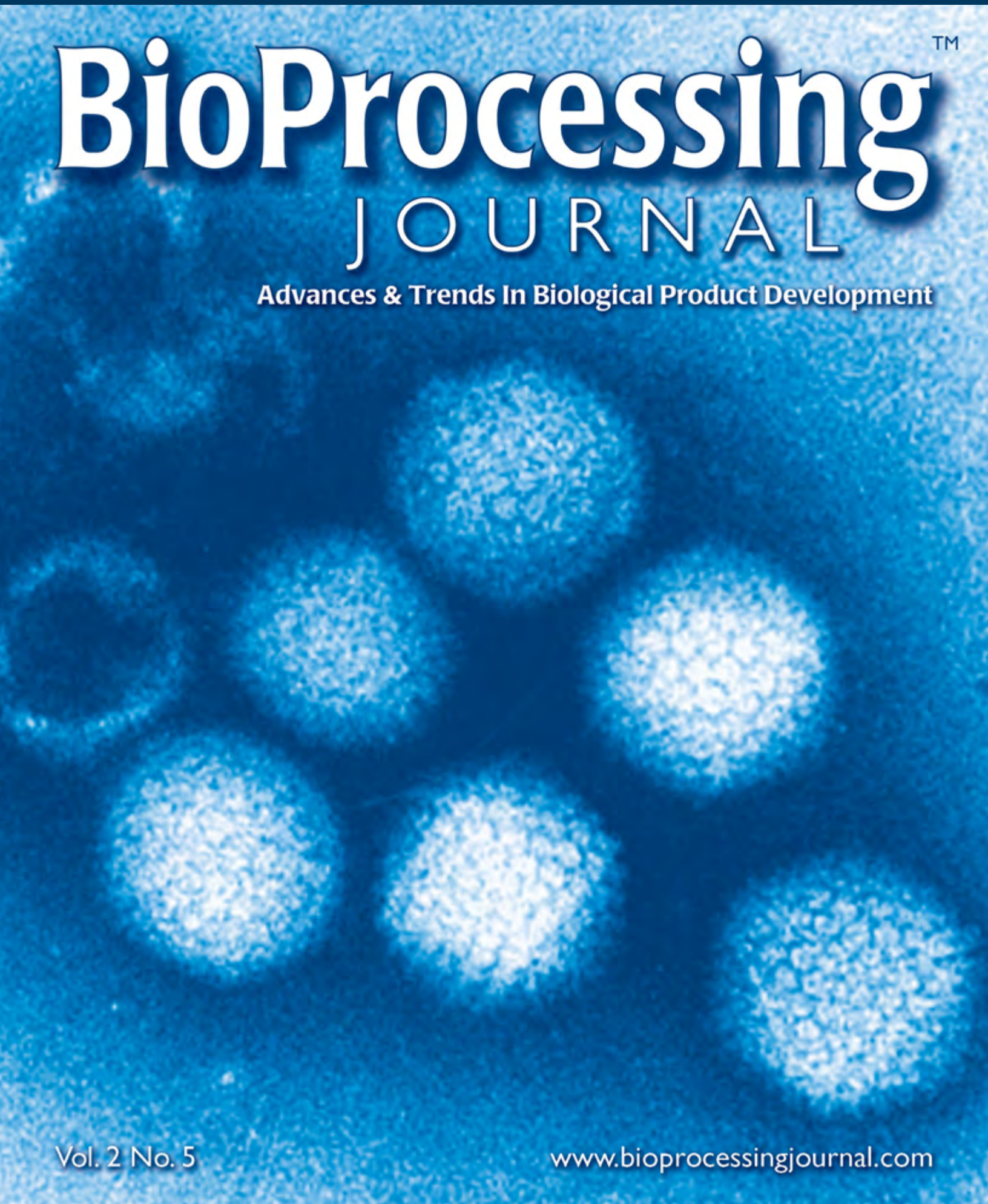
A publication of
The Williamsburg BioProcessing Foundation

September/October 2003

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development



Vol. 2 No. 5

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Production of a Recombinant Influenza Vaccine Using the Baculovirus Expression Vector System

BY KATHLEEN M. HOLTZ, D. KARL ANDERSON, and MANON M.J. COX

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.[1] Each year, influenza infections result in an average of 110,000 hospitalizations, approximately 20,000 of which result in death.[2] 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.[2] 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 (Fig. 1). Recently an additional novel influenza protein implicated in disease virulence has been identified.[3] The influenza viruses are divided into three types: A, B, and C, based on differences in internal 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 NA surface antigens distinguish types A and B, as well as define subtypes of influenza A. Of the 15 recognized HA subtypes (H1–H15) and nine recognized NA subtypes (N1–N9), only four HA subtypes (H1, H2, H3, and H5) and two NA subtypes (N1 and N2) have been isolated in humans.[4]

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.[5] Sequence analysis of HA genes and serological data has identified the amino acids of the HA protein necessary for neutralizing the influenza virus. These antigenic sites are predominantly located in the membrane-distal ectodomain known as HA1 (Fig. 2). Host cell endoproteases cleave HA into an active form consisting of two disulfide-linked fragments, the amino-terminal HA1 subunit and the carboxy-terminal 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.[6]

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. In particular, point mutations introduced to the antigenic sites of 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.[7] In contrast, 'antigenic shifts' have pandemic potential because novel viral strains of unpre-

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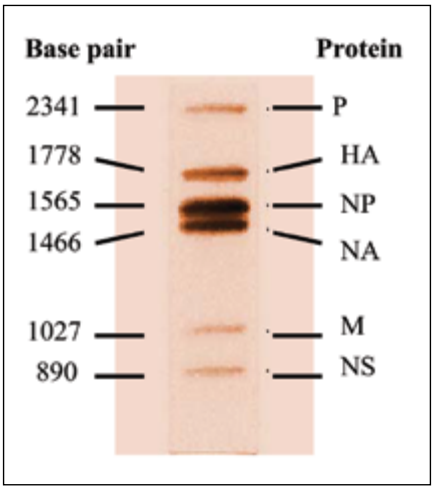

Figure 1. Agarose gel depicting cDNA products derived from influenza viral RNA (vRNA) using a universal primer (5'-AGCAAAAGCAGG-3') complementary to the conserved 3' end of influenza RNA segments. P represents the comigrating cDNA fragments for subunits of the viral polymerase (PB1, PB2, and PA) encoded 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 5 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 proteins represented by M, 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 the eighth segment, NS1 and NS2, involved in vRNA processing.

dictable pathogenicity are generated from the exchange of genomic segments from different influenza viruses (genetic reassortment) or from interspecies transmission of viruses.[7] 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 6 out of 18 infected people.[8] 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.[9]

Vaccination of high-risk persons each year before the flu season is the most effective measure for reducing the impact of influenza.[10] 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 eggs.[11,12] These vaccines are immunogenic in healthy adults and induce an increase in hemagglutination inhibition (HAI) antibodies in 70% to 90% of recipients.[13,14] In older age groups, TIV is less effective in preventing infection but can prevent complications and death following influenza infection.[15,16] 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.[17,18] 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.[19,20] 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.[21,22]

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.[5,11,12,23–25] 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).[26,27] 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 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.[28] 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.[23]

This paper describes the production and purification of recombinant HA molecules from three WHO/CDC recommended strains for the 2003–2004 influenza season for use in a trivalent recombinant influenza vaccine currently in clinical trials. Using the antigen isolated from the influenza strain

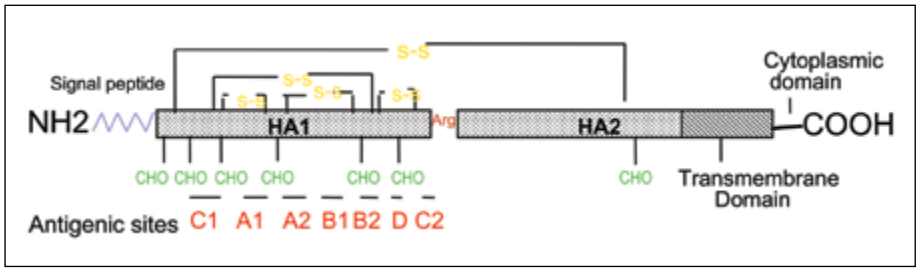

Figure 2. Schematic depicting the HA protein. HA monomers are cleaved by host endoproteases into two disulfide-linked fragments, an amino-terminal HA1 fragment, and a carboxyl terminal HA2 fragment. The HA1 ectodomain has the four antigenic sites (A–D) on the surface of its globular headpiece. HA1 also contains many N-linked glycosylation sites 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.

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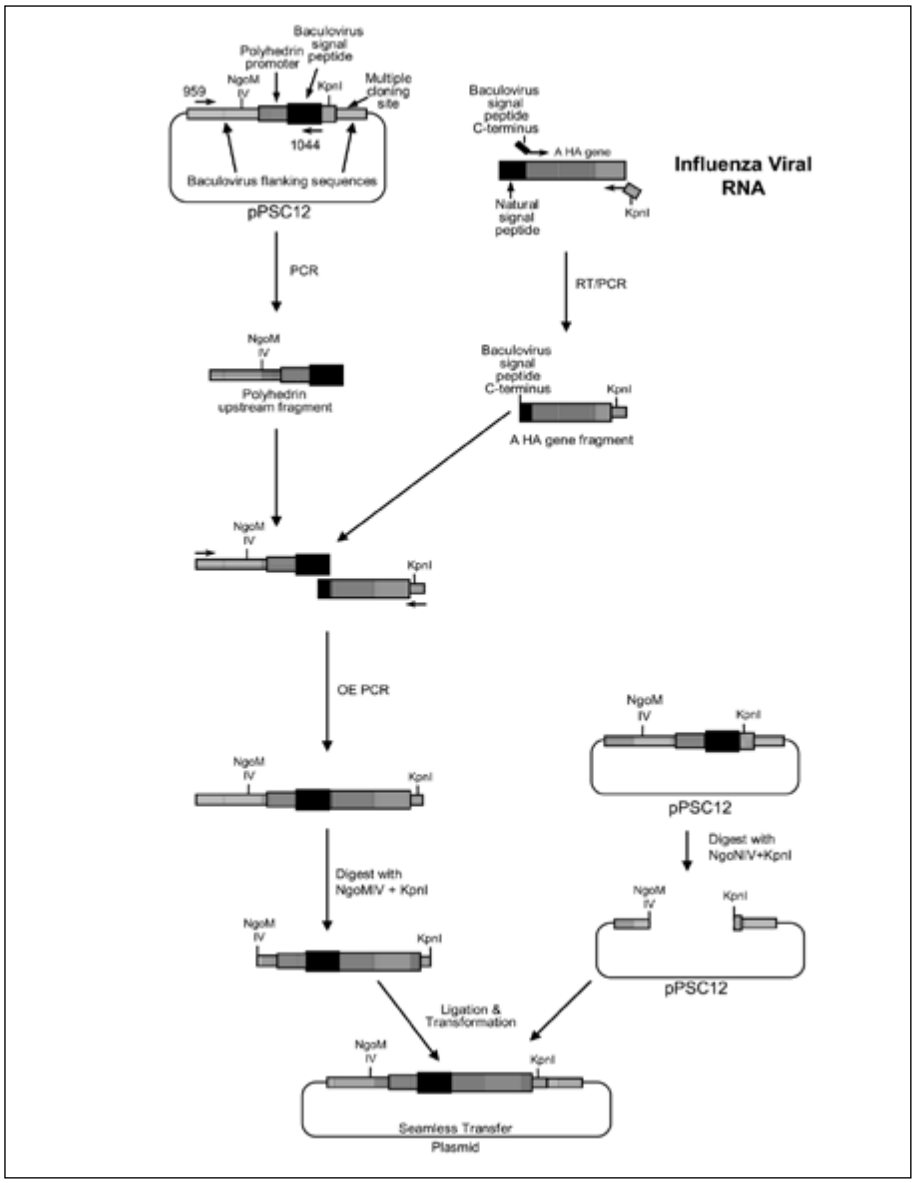

Figure 3. Cloning strategy for insertion of the influenza A virus H3 HA coding sequence into a PSC baculovirus transfer plasmid. The 5' end of the RT-PCR generated cDNA HA gene fragment has a nucleotide sequence that is complementary to the 3' end of a pPSC12 gene fragment generated by standard PCR. 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.

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 World Health Organization (WHO), the following influenza strains were obtained from the U.S. Centers for Disease Control and Prevention (CDC) as vaccine strains: A/Panama/2007/99 (H3N2, referred to as H3 HA), A/New Caledonia/20/99 (H1N1, referred to as H1 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).[29] The influenza viral stocks were then used to infect Madin Darby canine kidney (MDCK) cells (ATCC CCL34) at a low multiplicity of infection (0.1 to 0.5). Infection was allowed to proceed at 37° C for 48 hours while virus production was monitored in the media using the hemagglutination assay.[29]

Cloning HA Genes

Influenza virions for each strain 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 desired HA molecule (H1, H3, and B).

For H3 HA, 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. 3). In OE PCR, the 5' end of the HA gene fragment anneals to the complementary sequence found on the 3' end of a PCR-amplified fragment of the transfer plasmid pPSC12 containing the polyhedrin promoter and the baculovirus 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 NgoM *IV*, gel purified, and cloned into the baculovirus transfer vector, pPSC12, and digested with Kpn *I* and NgoM *IV*. 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 was 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 data for the HA2 region (C-terminal portion) is published for this HA. To analyze this region, the sequence 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. Variability in the amino acid identity at position 470 (often a lysine substitution) is observed among the H3 subtypes. Thus, we conclude the Panama clone is correct.

Transfection and Isolation of Recombinant Baculoviruses

Sf9 insect cells were cotransfected with linearized AcNPV baculovirus genomic DNA (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 ml 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 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 hours 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 hours, 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 (10 L to 45 L) 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 a dissolved oxygen (DO_2) setting of 60%. The cultures were harvested by low-speed centrifugation at 72 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% β-ME pH 9 followed by centrifugation at 6,000×g for 30 minutes at 4° C. The supernatant containing cytosolic and periphery membrane proteins was discarded (Fig. 4). The remaining pellet was then washed with 50 mM ethanolamine, 0.1% β-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% β-ME pH 9. The resuspension was homogenized for five minutes followed by a 15 minute 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.

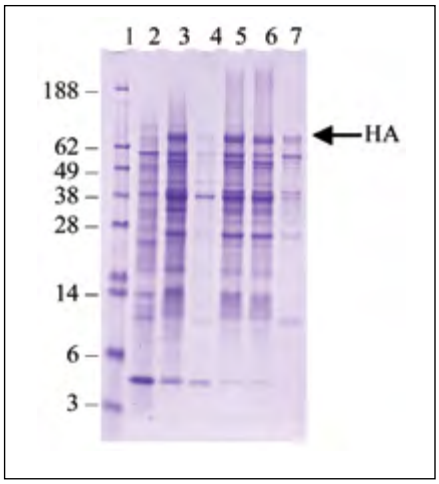

Figure 4. General procedure for extracting HA from the insect cell membrane. After washing cells in the presence and absence of NaCl (0.3M) using ethanolamine buffer, pH 9, to remove loosely associated proteins, the membrane proteins are solubilized with 1% Triton X-100 in ethanolamine buffer pH 9. The band corresponding to HA is denoted by the arrow. Based on the amino acid sequence, monomeric HA has a molecular weight of 64 kD; however, each trimannose core unit added to the protein by insect cells is an additional 1.8 kD. 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.

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 purification protocol for A/Panama/2007/99 H3 HA will be discussed here in detail.

The H3 HA-containing supernatant is applied to an anion exchange (Q Sepharose) column equilibrated in 50 mM ethanolamine, 0.1% Triton X-100, 0.01% β-ME pH 9. HA flows through the column while bound proteins elute with 0.2 M–2.0 M NaCl (Fig. 5A). 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% β-ME pH 9. Lentil lectin is an affinity matrix that reversibly binds polysaccharides and glycoconjugates

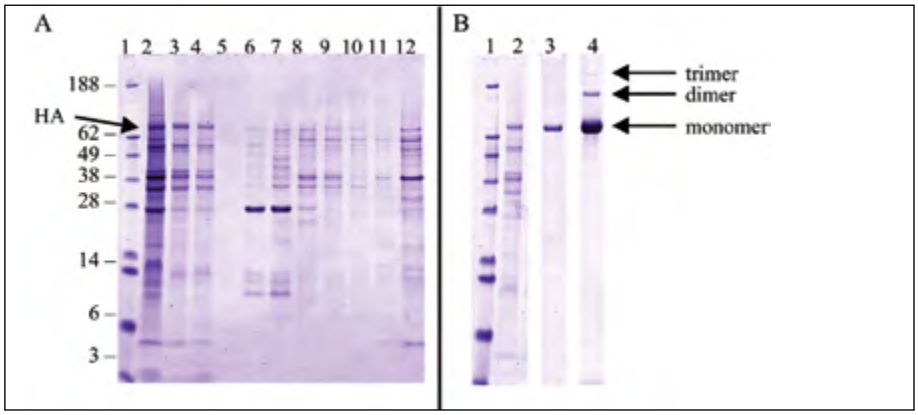

Figure 5A. Q column chromatography of Triton X-100 solubilized H3 HA. Solubilized HA is loaded directly onto the Q column. HA is collected in the Q flow-through and Q wash with minimal losses. Impurities and DNA remain bound to the column and elute with 0.2-2.0 M NaCl. HA is denoted by the arrow. *Lanes*: 1, MW markers; 2, pre-column solubilized H3 HA; 3, flow-through; 4–5, 50 mM ethanolamine, 0.1% Triton X-100, 0.01% β-ME wash; 6–12, 0.2–2 M NaCl elution of bound material. Figure 5B. 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 kD 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.

containing glucose and mannose type sugar groups. HA binds tightly to the resin while contaminating proteins flow through. The column is washed with equilibration buffer containing 0.5 M NaCl to prevent non-specific protein interactions. The column pH and conductivity is adjusted with 20 mM Tris, 0.1% Triton X-100, 0.01% β-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% β-ME pH 7.4 (Fig. 5B). 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% β-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 β-ME. The purified HA is then eluted with 150 mM NaCl, 10 mM sodium phosphate, 0.01% Tween-20 pH 7.4 (Fig. 5B).

Results

Three vaccine strains, A/Panama/2007/99 (H3N2), A/New Caledonia/ 20/99 (H1N1), and B/Hong Kong/330/2001 were obtained from the CDC under WHO recommendations and used to infect MDCK cells in culture. Messenger RNA purified from the virions was used to generate cDNA of the HAs from each strain. Using overlap extension PCR (Fig. 3), the HA genes were seamlessly fused to the baculovirus chitinase signal sequence behind the polyhedron promoter and subsequently cloned into the PSC transfer plasmid pPSC12. Insect cells were cotransfected with the transfer plasmid and linearized AcNPV 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.

Extraction of HA

The PSC signal sequence 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 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% β-ME pH 9 removes cytosolic and periphery membrane proteins in the supernatant (Fig. 4). The second wash without NaCl removes additional loosely bound proteins and lowers the conductivity prior to detergent extraction with 1% Triton X-100. Extractions carried out at pH 9 result in improved efficiency relative to extractions performed at pH 7. 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 5A, 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 and elutes with 0.5 M N-methyl-α-D-mannopyrannoside while the remaining protein impurities flow through. Final purity is achieved with lentil lectin chromatography (Fig. 5B). The highly pure HA is loaded onto a final CM column for concentration and exchanged into the final formulation buffer system. As shown in Figure 5B, the lentil lectin eluate is subsequently concentrated approximately 10-fold using CM column chromatography.

HA Characterization

Based on the migration in SDS-PAGE gel and a comparison with the

molecular weight markers, the purified monomeric form of HA has a molecular weight of 74 kD. Based on the known theoretical weight from the amino acid sequence of the protein (64 kD) and the molecular weight of a trimannosyl unit added by insect cells (1.8 kD/unit), the purified protein has six out of the seven potential sites glycosylated. Higher molecular weight bands observed in the SDS-PAGE gel of Figure 5B have calculated molecular weights of 143 and 219 kD, 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 mg 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.[29] 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 6 shows a typical activity assay performed in a 96-well plate. 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 as observed with PBS buffer serving as a negative control in lanes 1 and 5 (Fig. 6). The agglutination activity in Figure 6 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).

The correct quaternary structure of recombinant HA is important for stability and biological activity. The trypsin resistance assay treats the final product with the 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 (Fig. 2). HA is incubated for 30 minutes at 0° C without or with 50 μg/ml TPCK-treated trypsin. Denatured samples of HA are prepared by boiling the samples for 10 minutes prior to trypsin treatment. In the SDS-PAGE gel of Figure 7, trypsin treatment of H3 HA results in the characteristic HA1 and HA2 fragmentation with apparent molecular weights of ~50 kD for HA1 and ~28 kD for HA2. No bands are observed for trypsin-treated HA that had been boiled prior to the assay, suggesting complete proteolytic degradation of the denatured protein.

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). H3 HA specimens from this study have been prepared and examined by EM. As shown in Figure 8, a highly dense arrangement of rosette structures is observed with rosettes containing six to eight trimers or spikes.

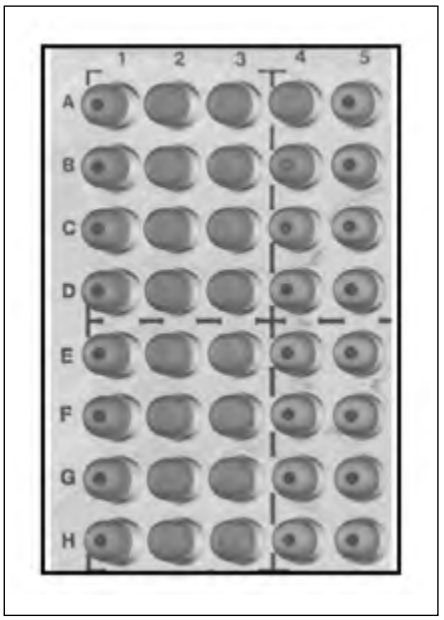

Figure 6. Hemagglutination assay for H3 HA. PBS buffer serves as a negative control in lanes 1 and 5. Purified H3 HA diluted in PBS buffer is loaded into the first well of lanes 2, 3, and 4 at concentrations of 100 μg/ml, 1 μg/ml, and 0.01 μg/ml, respectively. The protein aliquots are subsequently diluted two-fold 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 cell agglutinate (loose pellet). Roughly 2,000 units of activity is measured for H3 HA.

Discussion

The baculovirus expression vector system in insect cells 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 AcNPV 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 mg 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; these methods have previously resulted in significant improvements. Procedures to prevent the losses incurred in the washing steps of the cell pellet prior to HA extraction from the cellular membrane (Fig. 4, lanes 2 and 4) are also being developed.

Recombinant HA glycoproteins produced in the BEVS system are full-length and uncleaved because insect cells lack the proteases to 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 molecu-

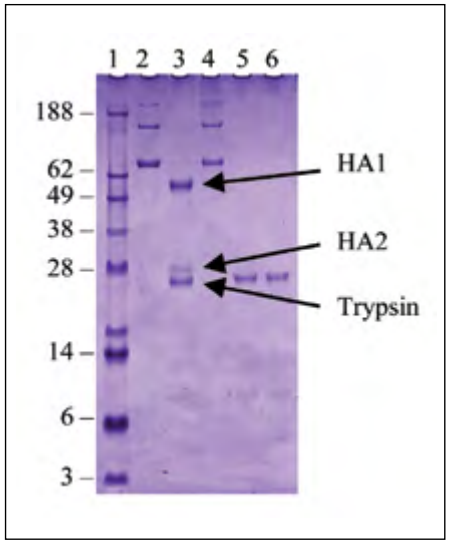

Figure 7. SDS-PAGE gel of the HA trypsin resistance assay. H3 HA samples (125 µg) were left untreated (lanes 2 and 4) or treated with 50 µg/ml trypsin (lane 3 and 5) for 30 minutes on ice, and then analyzed by SDS-PAGE. The H3 samples in lanes 4 and 5 were boiled for 10 minutes prior to the assay and serve as controls for denatured protein. The untreated samples show a major band slightly above the 62 kD marker for the HA monomer and two additional higher MW bands above and below the 188 kD marker that correspond to trimer and dimeric forms, respectively (lanes 2 and 4). The boiled sample of HA treated with trypsin is digested into protein fragments that are not resolvable on the SDS-PAGE gel (lane 5). In contrast the undenatured, trypsin-treated sample of HA is digested into two fragments having MWs of ~50 kD and ~28 kD (lane 3). *Lanes*: 1, MW markers; 2, untreated H3 HA; 3, trypsin treated H3 HA; 4, boiled, untreated H3 HA; 5 boiled, treated H3 HA; 6, trypsin endoprotease (MW 26 kD).

lar form shown to induce immunogenicity in healthy young and elderly adult populations.[5,11,12,23–25] 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 correct structure and an intact receptor binding site will recognize sialic acid residues on the surface of RBCs in a standard hemagglutinatin assay. HA agglutination prevents cells from settling to the bottom of the wells. Figure 6 shows the assay performed with H3 HA in a 96-well plate format. This assay has been useful in identifying purification conditions that potentially alter the structure, leading to a loss of activity or permanent inactivation of the protein. For example, exposure to high ionic strength (> 1 M NaCl) has been found to affect the protein structure and reduce agglutination activity even after its removal. 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. In BEVS/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.

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 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 7 (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 7 for heat-denatured HA. Denaturation results in degradation of the protein into smaller peptides not resolvable by the SDS-PAGE analysis (Fig. 7, 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. As shown in Figure 8 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 morphology of infectious virions having HA trimeric spikes protruding from the viral envelope. Thus, this higher order aggregation state observed by EM has become a reliable indicator of HA immunogenicity.

Correctly folded, trimerized, biologically active HA molecules produced in BEVS using insect cells have been evaluated in six human Phase I/II clinical studies using both monovalent and bivalent preparations.[5,11,12,23-25] The results of these studies indicate that HA vaccines are safe and well tolerated in both young adults (ages 18–45) 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 is 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

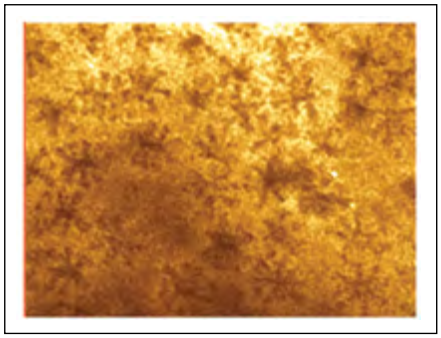

Figure 8. Electron micrograph of H3 HA. A specimen of purified H3 HA (600 µg/ml in 10 mM Na phosphate, 0.01% Tween-20, 0.15 M NaCl pH 7.4) was prepared on a carbon coated 300-mesh grid, negatively stained with 1% phosphotungstic acid, and viewed with a Zeiss EM10A transmission electron microscope at a magnification of 80,000×.

(up to 135 µg/HA based on BCA determinations) without an increase in the adverse side effects that are more frequently reported with the TIV using significantly less HA (15 µg HA/strain based on SRID assay).[30] The addition of alum adjuvant has not been found to 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.

The ongoing 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 format in the elderly adult population, and compare its performance with the current TIV. An enhanced stimulatory effect is expected from the inclusion of all three HA molecules and using a higher dosage (135 µg). We believe an 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. Finally, health officials would be better able to respond in the event of the emergence of a new epidemic or pandemic strain of influenza virus with a recombinant trivalent HA vaccine.

ACKNOWLEDGEMENTS

Based on a presentation given by Keith Canada at the Williamsburg BioProcessing Foundation's 6th annual Baculovirus and Insect Cell Culture Meeting, February 24–27, 2003 in Santa Fe, NM.

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