Comparison of soluble and secreted forms of human parainfluenza virus type 3 glycoproteins expressed from mammalian and insect cells as subunit vaccines

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Human parainfluenza virus type 3 (PIV-3) is one of the leading causes of paediatric viral respiratory disease. The PIV-3 genome encodes two envelope glycoproteins, F and HN, which are the major targets for the host antibody response. We have expressed secreted forms of the F and HN proteins and a novel chimeric FHN glycoprotein in insect cells using recombinant baculovirus vectors and secreted forms of the F and FHN glycoproteins in stably transformed Chinese hamster ovary (CHO) cells. Comparison of the mammalian cell- and insect cell-expressed F and FHN proteins by SDS-PAGE showed that the CHO cell-expressed proteins are several kilodaltons larger in size than the baculovirus-produced proteins. A partial characterization of the oligosaccharide structures of the F and FHN proteins revealed that the size difference is due to the different oligosaccharide structures added to these proteins by the two cell lines. The F, HN and FHN proteins were immunoaffinity-purified from the culture medium of baculovirus-infected SF9 cells and the F and FHN proteins were immunoaffinity-purified from the culture medium of CHO cells. A comparison of the immunogenicity and efficacy of the mammalian cell- and insect cell-produced FHN proteins was tested in cotton rats. The CHO cell- and baculovirus-produced FHN proteins were found to induce similar levels of PIV-3-specific ELISA-positive and neutralizing antibodies and both proteins provided near complete protection when animals were vaccinated with low doses of the FHN protein.

Introduction

Parainfluenza virus type 3 (PIV-3), a member of the family Paramyxoviridae, is second only to another virus in this family, respiratory syncytial virus (RSV), as a major agent of severe viral respiratory tract infections in infants (Chanock & McIntosh, 1990). The two envelope glycoproteins, the fusion (F) glycoprotein and the haemagglutinin–neuraminidase (HN) protein, are the primary immunogens of PIV-3 (Spriggs et al., 1987). F, a 60K glycoprotein, mediates viral penetration and facilitates syncytium formation (Choppin & Scheid, 1980; Scheid & Choppin, 1974, 1977; Spriggs et al., 1986). Proteolytic cleavage of these subunits plays an important role in virulence (Scheid & Choppin, 1974). HN, a 70K type 2 glycoprotein, is characterized by an inverted orientation in the membrane (Elango et al., 1986). An N-terminal hydrophobic stretch of amino acids serves as both signal and anchor sequence. The HN protein of PIV-3 appears as a disulphide-bonded homo-oligomeric tetramer on the surface of the viral envelope (Collins & Mottet, 1991). The HN protein is responsible for viral attachment and haemagglutination–neuraminidase activities (Scheid et al., 1972). Antibodies against the HN protein are capable of preventing spread of infection (Kasel et al., 1984; Spriggs et al., 1987; Ray et al., 1988). The majority of the neutralizing epitopes of the HN protein appear to be largely determined by conformation of the protein (Henrickson et al., 1991).

A subunit vaccine for RSV, termed FG, has been developed by our group (Wathen et al., 1989; Brideau et al., 1989). FG is a chimeric glycoprotein composed of the F and G glycoproteins of RSV. Cotton rats vaccinated with the chimeric FG glycoprotein were completely
protected against RSV challenge. In this report we describe the construction, expression and purification of an analogous PIV-3 subunit vaccine, termed FHN. Soluble forms of the PIV-3 F, HN and chimeric FHN glycoproteins were expressed in insect cells using recombinant baculovirus vectors, and soluble forms of the F and HN glycoproteins were also expressed from stably transformed Chinese hamster ovary (CHO) cells.

In the accompanying paper we evaluate the efficacy of the baculovirus-produced FHN glycoprotein compared to either F or HN proteins alone or to mixtures of the two glycoproteins (Brideau et al., 1993).

In the present study we examined the immunogenicity and efficacy of mammalian cell- compared to insect cell-expressed FHN glycoproteins. Several studies have reported that oligosaccharide structures synthesized by insect cells differ from those synthesized by mammalian cells (Butters & Hughes, 1981; Hsieh & Robbins, 1984; Weber et al., 1986; Wojchowski et al., 1987; Greenfield et al., 1988; Kuroda et al., 1990; Wathen et al., 1991). In particular, insect cells appear not to process high-mannose precursor oligosaccharides into mammalian-type complex structures. Work by Butters & Hughes (1981) suggests that insect cells lack the enzyme activities necessary for these processing reactions. Therefore, insect cells do not synthesize complex-type oligosaccharides but instead substitute truncated high-mannose structures on glycosylation sites that, when processed by mammalian cells, would contain complex-type structures (Hsieh & Robbins, 1984). Core fucosylation is often retained on the truncated structures made in insect cells (Kuroda et al., 1990; Wathen et al., 1991) but the outer N-acetylglucosamine, galactose and sialic acid residues are missing. To determine what role glycosylation has in immunogenicity, the oligosaccharide structures added to the insect cell- and mammalian cell-expressed F and FHN proteins were determined. To characterize the immunogenicity of the mammalian cell- and the insect cell-expressed FHN protein, the serum and neutralizing antibody responses of cotton rats immunized with the two proteins were compared. In addition, the ability of mammalian cell- and insect cell-produced FHN protein to protect cotton rats against PIV-3 challenge was studied.

Methods

Cell culture and RNA isolation. Human PIV-3 (ATCC strain C243) was grown in HEP-2 cell monolayers in Eagle’s MEM containing 10% fetal bovine serum (FBS). HEP-2 cell monolayers at a confluence of 60 to 80% were infected at a multiplicity of 2 with PIV-3 and total infected cell RNA was isolated at 24 h post-infection (p.i.) using RNAzol (Cinna Biotec) as described by the manufacturer. Dihydrofolate reductase (dhfr)-deficient CHO cells (DUXX-B11), were maintained in medium (high glucose DMEM plus 0.1 mM non-essential amino acids, 10 mM-HEPES, 100 units of penicillin, 100 μg/ml streptomycin and 2 mM-glutamine) containing 10% fetal calf serum. The baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) was grown in Spodoptera frugiperda (SF9) cells (ATCC CRL 1711). Conditions for growth of cells and virus have been described (Summers & Smith, 1987).

Synthesis of PIV-3 F and HN genes. cDNA copies of the PIV-3 F and HN genes were synthesized using PCR. Virion RNA for use as a template was obtained from HEP-2 cells infected with PIV-3. The F- and HN-specific oligonucleotides used for cDNA synthesis and PCR were derived from the published sequences of the F (Spriggs et al., 1986) and HN (Elnag et al., 1986) genes. A cDNA copy of the F gene was synthesized by reverse transcription of viral RNA with an oligonucleotide that is complementary to bases located at the 3’ end of the F mRNA (F primer 2 listed below). A cDNA copy of the HN gene was synthesized by reverse transcription of viral RNA using an oligo(dT) primer (Amersham cDNA synthesis kit).

PCR reactions were performed using the Perkin-Elmer Cetus GeneAmp kit according to the manufacturer’s directions. Approximately half of the F and HN cDNA synthesis reactions were used in each PCR reaction. PCR primers used in the reactions corresponded to regions flanking the F and HN protein coding regions (underlined sequences). The primers contain BamHI restriction sites for cloning of the PCR products. Written 5’ to 3’, these were: F primers (1) GCCACCCGGATCCTCTGATGATGCTGTAC (192 to 213) and (2) GCCACCCGGTACCTATGATTAACTGCAGCTTT (184 to 205); HN primers (1) GCCACCGGATCCTCTGATGATGCTGTAC (192 to 213) and (2) GCCACCCGGTACCTATGATTAACTGCAGCTTT (184 to 205).

PCR reactions were performed using a Perkin-Elmer Cetus thermocycler with the following cycles: 94 °C for 1 min, 37 °C for 2 min and 65 °C for 3 min. The final products were phenol-chloroform-extracted, precipitated with ethanol, digested with BamHI and ligated into the BamHI site of pUC18 to generate pUC-F and pUC-HN.

Construction of secreted forms of F, HN and FHN proteins. A secreted form of the F glycoprotein was constructed by PCR using the pUC-F cDNA clone as a template. A 36 nucleotide primer, CCGCGGATCCTCTGATGATGCTGTAC (192 to 213), that was complementary to bases 1567 to 1588 (underlined sequence) of the F gene was synthesized and used in the PCR reaction with F primer 1 described above. The 36 nucleotide primer was designed to introduce a stop codon prior to the F-terminus. The secreted form of the F protein was constructed by PCR using the pUC-F template and HN-specific PCR products. The secreted form of the HN protein was constructed by introducing the HN-terminus into the pUC-F template. The secreted form of the FHN protein was constructed by replacing sequences that encode the N-terminal signal/anchor sequence (amino acids 1 to 86) of HN with sequences that encode the signal sequence (amino acids 1 to 79) of the FIV-3 F protein. The secreted form of the HN protein was constructed as follows. First, pUC-HN was partially digested with BstI followed by ligation of a 10 bp BamHI linker (CCGGATCCCG). BstI cuts twice within the coding sequences of the HN gene; one of these sites is located just 5’ to the codon for amino acid 87 of the HN gene. Following digestion with BamHI, a 1400 bp BamHI fragment (containing sequences which encode amino acids 87 to 592 of the HN protein) was gel-purified. Second, pUC-F was digested with BstI and the vector fragment generated by this digest was gel-purified. BstI cuts the F gene twice and results in the deletion of sequences that encode amino acids 77 to 406 of the F protein from plasmid pUC-F. Third, the 1400 bp BamHI HN fragment was ligated
with the BcII-digested pUC-F vector fragment and the orientation of the inserted BamHI fragment was determined. Insertion of the 1400 bp HN fragment into the pUC-F vector results in an in-frame fusion of the F protein at amino acid 76 to the HN protein at amino acid 87 with the addition of a proline residue at the junction (Fig. 1).

The chimeric FHN glycoprotein was constructed as follows. pUC-HN was again partially digested with Xpol followed by ligation of an 8 bp BamHI linker (CGGATCCG). The resulting sample was digested with BamHI and a 1400 bp HN fragment (see above) was gel-purified and ligated into the unique BglII site of pUC-F. The addition of the BamHI linker to the HN gene and ligation into the BglII site of pUC-F results in an in-frame fusion of the F protein at amino acid 466 to the HN protein at amino acid 87 with the addition of a proline residue at the junction.

DNA sequencing was done by the dideoxynucleotide method using a commercial sequencing kit (Pharmacia). The F and HN genes of pUC-F and pUC-HN were sequenced in their entirety whereas in the other constructs the F and HN fusion junctions were sequenced.

Baculovirus and CHO cell expression of PIV-3 proteins. The secreted forms of the F, HN and FHN constructs were inserted into the BamHI site of the baculovirus transfer vector pAC737 (Fig. 1) and used to transform Escherichia coli strain DH5. Cotransfection of SF9 cells with wild-type AcNPV DNA and the PIV-3 recombinants was performed using calcium phosphate precipitation (Summers & Smith, 1987). Baculovirus recombinants were isolated by screening for ‘inclusion-negative’ plaques. The presence of the genes in the recombinant viruses was verified by dot blot hybridization using F and HN as probes. Recombinant baculovirus were plaque-purified a total of five times.

For expression in CHO cells the secreted forms of the F and FHN constructs were digested with BamHI, the ends were filled in using T4 polymerase and SalI linkers were then ligated to the blunted fragments. The linker F and FHN genes were inserted into the SalI site of the mammalian cell expression vectors p3CI-neo and p3CI-dhfr shown in Fig. 1. CHO cells were transfected with both the p3CI-neo and -dhfr shown in Fig. 1. CHO cells were transfected with both the p3CI-neo and -dhfr plasmids by the calcium phosphate method (Homa et al., 1986). Transfected cells were grown for 48 h in non-selective medium. The cells were trypsinized and split into 100 mm plates containing growth medium plus 400 µg/ml G418 (Gibco) and 10% dialysed calf serum. Surviving clones were trypsinized and transferred to 24-well dishes. Detection of F and HN proteins in the cell media was done by ELISA. Cells expressing either the F or HN proteins were amplified by progressive adaptation to increasing concentration of methotrexate (MTX) and subsequent limited dilution cloning.

Monoclonal antibodies (MAbs) and enzyme immunoassay. The enzyme immunoassay was performed as previously described (Wathen et al., 1989). Antibodies used in the enzyme immunoassay were a mixture of MAbs to F or HN proteins. MAb 13-5 (ATCC HB 8934) to the HN glycoprotein was purchased from ATCC. MAb B69 to the F glycoprotein was obtained from Brian Murphy (NIH).

Immunoprecipitation. SF9 cells were infected at an m.o.i. of 10 p.f.u./cell with recombinant baculovirus. At 24 h p.i. cells were labelled with 50 µCi/ml of [3H]methionine in Grace's methionine-free insect cell medium (Gibco) containing 5% FBS. At 48 h p.i. the medium was harvested and proteins were immunoprecipitated. CHO cells were labelled with 40 to 50 µCi/ml of [3H]methionine in methionine-free 199 medium (Gibco) containing 5 mM-sodium butyrate and 10% FBS for 24 h and the medium was harvested and proteins were immunoprecipitated. Immunoprecipitations were done as described previously (Wathen et al., 1989) using either the F (B69)- or HN (13-5)-specific MAbs.

Immunofinity purification of baculovirus- and CHO cell-expressed proteins. Shake flasks containing 10^6 SF9 cells per ml were infected at an m.o.i. of 5 p.f.u./cell and incubated with shaking (100 r.p.m.) at room temperature. Cells were removed from the medium by centrifugation at 1000 r.p.m. and the pH was adjusted to 8.0 followed by a low-speed centrifugation to remove precipitated material. The medium obtained from SF9 cells infected with baculovirus expressing the F protein was adjusted to a pH of 7.5 and then filtered through a 0.22 µm Millipak-20 filter (Millipore). The medium was diluted with water to the same conductivity as buffer A (50 mM-NaCl, 20 mM-Tris–HCl pH 7.5) and loaded on an S-Sepharose column (Pharmacia) equilibrated in buffer A. The medium obtained from SF9 cells infected with baculovirus expressing the HN or FHN genes was adjusted to pH 7.8 and filtered through a 0.22 µm Millipak-20 filter. The medium was diluted with water to the same conductivity as buffer B (50 mM-NaCl, 20 mM-MES pH 5.8) and loaded on an S-Sepharose column equilibrated in buffer B. Protein bound to the S-Sepharose columns was eluted with 1 M-NaCl in buffer A or B and the eluted fractions were analysed by ELISA. Column fractions containing F, HN or FHN proteins were pooled and applied to an immunofinity column (see below).

CHO cells were seeded in duplicate roller bottles (1700 cm², Corning) and grown to 90% confluence. Monolayers were washed with 1× PBS to remove residual serum, and fed with serum-free medium (1:1 high glucose DMEM-MCDN 301 containing: 5 µg/ml insulin, transferrin and selenium, 0.1 mM-non-essential amino acids, 10 mM-HEPES pH 7.3, 100 units of penicillin, 100 µg/ml streptomycin, 20 mM-glutamine, 1 µg/ml aprotinin, 10 mM-episonol amino caproic acid, 5 mM-sodium butyrate and 400 µg/ml G418). Cells were incubated for 48 to 72 h at which time the medium was harvested and replaced with a second 100 ml of serum-free medium. After an additional 48 to 72 h the medium was again harvested and replaced or the monolayer was rested for 72 h in medium containing 10% FBS. Harvested medium was cleared by centrifugation, filtered through a 0.22 µm filter and diluted 1:3 with deionized water to the same conductivity as buffer C (20 mM-Tris–HCl pH 8.0, 50 mM-NaCl) for the F or buffer A for the FHN protein and loaded on a Q-Sepharose column. Columns were eluted with 1 M-NaCl in buffer C or A and processed as described for the baculovirus samples.

Affinity columns for the purification of F, HN and FHN proteins were prepared by coupling MAb B69 or 13-5 to Affi-Gel HZ as described by the manufacturer (Bio-Rad). Prior to coupling, ascites fluid was concentrated by precipitation with saturated (NH₄)₂SO₄ and passage over a Protein A chromatography column (Clinitex) using Affi-Prep Protein A MAPS buffers (Bio-Rad). The S-Sepharose or Q-Sepharose eluates containing the recombinant PIV-3 glycoproteins were applied to the immunofinity column by looping the sample over the 10 ml column at a rate of 1 ml/min over 48 h. The column was washed with 10 column volumes of 0.5 M-NaCl in PBS, the proteins were eluted with 0.1 M-glycine pH 2.5 and neutralized with 1 M-Tris pH 8.0. The protein concentrations were determined using the Bio-Rad Protein (Bradford) Kit using BSA as a standard.

Oligosaccharide characterization. Analysis of N-linked oligosaccharides was done by labelling CHO cells or baculovirus-infected SF9 cells with [2-3H]mannose (18.5 Ci/mmol, Amersham) as described previously (Wathen et al., 1991). The F and FHN proteins were isolated by immunoprecipitation as described above. The proteins were separated by SDS-PAGE and the radiolabelled antigen bands were visualized by fluorography essentially as described by Wathen et al. (1991). Glycopeptides were prepared by pronase digestion of gel slices containing the labelled F and FHN proteins and fractionated on concanavalin A (Con A)-Sepharose essentially as described by Cummings et al. (1983).

Analysis of O-linked oligosaccharides was done by labelling CHO cells or baculovirus-infected SF9 cells with [6-3H]glucosamine (32 Ci/mmol, Amersham) as previously described (Wathen et al., 1991). The F and FHN proteins were isolated by immunoprecipitation...
as described above. The immunoprecipitate was either directly subjected to alkaline sodium borohydride treatment (FHN protein synthesized by SF9 cells), or separated by SDS-PAGE followed by release of the O-linked oligosaccharides by alkaline sodium borohydride treatment of gel slices containing the radiolabelled FHN band (FHN protein synthesized by CHO cells). The position of the FHN band on the gel was determined by fluorography as described above. Alkaline sodium borohydride treatment was performed as described by Carlson (1968) with minor modifications. The released O-linked oligosaccharides were separated by descending paper chromatography in pyridine-ethyl acetate-glacial acetic acid-water (5:5:1:3).

Gel electrophoresis. SDS-PAGE was performed according to the procedure of Laemmli (1970). The gel was silver-stained according to the procedure of Morrissey (1981).

Animal studies. Cotton rats were immunized twice at a 3 week interval, intramuscularly (i.m.), with different doses of insect cell- or mammalian cell-expressed FHN protein. Ten days following the second vaccination sera were prepared for antibody determinations as described previously (Brideau et al., 1989). Two weeks after the second vaccination the animals were challenged with approximately \(2 \times 10^6\) p.f.u. of PIV-3 intranasally. Four days after challenge lung tissues were removed and virus titres were determined on HEp-2 cells as described previously (Brideau et al., 1989). To exclude the possibility that the failure to isolate virus from the lungs of immunized rats (see Table 2) was not the result of neutralization of virus by antibody released during lung homogenization, we mixed immune cotton rat lungs (in which virus was not detected) with control cotton rat lungs and triturated the mixture. No evidence of in vitro neutralization was noted, as there was no reduction in virus titre (R. J. Brideau et al., unpublished data).

Results

Cloning and sequencing of the PIV-3 F and HN genes

The F and HN genes of PIV-3 were cloned from infected cell RNA by cDNA synthesis and PCR as described in Methods, using oligonucleotide primers that generated BamHI sites at the 5' and 3' ends of these genes. The F and HN genes were cloned into the BamHI site of pUC18 and the inserts were sequenced. Comparison with the published sequences of the F (Spriggs et al., 1986) and HN (Elango et al., 1986) genes revealed only minor differences with greater than 99% identity to the published sequence for both genes; the changes are listed in Table 1. The nucleotide changes resulted in six amino acid differences in the F protein and one amino acid difference in the HN protein when compared to the published sequence.

The full-length F and HN cDNAs were used as templates to construct secreted forms of the F and HN proteins by removing sequences that anchor these proteins in the plasma membrane (Fig. 1). A secreted form of the F protein was engineered by inserting a stop codon into the F gene to terminate translation prior to the C-terminal membrane anchor sequence. A secreted form of the HN protein was constructed by deleting sequences (first 86 amino acids of HN) that encode the N-terminal signal-anchor region of the HN protein and replacing the deleted region with sequences that encode the signal sequence (first 79 amino acids of F) of the PIV-3 F protein. A chimeric FHN glycoprotein gene was made by inserting sequences of the HN gene into the F gene such that the resulting clone generated an in-frame fusion of the F and HN genes. The chimeric protein consists of amino acids 1 to 466 of the F protein and amino acids 87 to 572 of the HN protein with the addition of a proline residue at the junction.

Expression of F, HN and FHN genes in SF9 cells from recombinant baculovirus vectors

The secreted protein forms of the F, HN and FHN genes were placed under the control of the baculovirus polyhedrin promoter by inserting these genes into the baculovirus transfer plasmid pAC373 (Fig. 1). Following cotransfection of SF9 cells with the pAC373 clones and wild-type AcNPV DNA, recombinant baculoviruses expressing these proteins were isolated as described in Methods. Analysis of the media from recombinant baculovirus-infected SF9 cells by ELISA demonstrated expression of F, HN and FHN genes from their respective recombinant viruses. The baculovirus-expressed proteins were further examined by immunoprecipitation with anti-F or anti-HN MAbs (Fig. 2a). Baculovirus-infected cells were labelled from 24 to 48 h p.i. with \[^{35}\text{S}\]methionine. Proteins in the medium were immunoprecipitated and examined by gel electrophoresis. Under reducing conditions, the F, HN and FHN proteins appear as 54K, 68K and 115K proteins, respectively. The stop codon engineered into the F gene removes the C-terminal 73 amino acids of the wild-type F protein (63K).

<table>
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* Nucleotide and predicted amino acid numbers are taken from published sequence of the F (Spriggs et al., 1986) and HN (Elango et al., 1986) genes.
resulting in a product that should be 8K to 9K smaller than the protein expressed from PIV-3-infected cells. Unlike the F protein from PIV-3-infected cells, in which the protein is cleaved into F1 and F2 subunits, no difference is seen in electrophoretic mobility for either the F or FHN baculovirus-expressed proteins when the proteins are compared under reducing and non-reducing conditions (Fig. 2b). Lack of cleavage of the full-length (membrane-bound) PIV-3 F protein expressed from a baculovirus recombinant has previously been reported (Ray et al., 1989). The amount of HN protein present in the Sf9 cell medium was lower than that found with the F and FHN proteins. Immunoprecipitation of HN gene-infected Sf9 cells showed that approximately 50% of the HN protein was cell-associated (data not shown).

**Expression of F and FHN genes in stably transformed CHO cells**

In order to evaluate the immunogenicity of mammalian cell-expressed PIV-3 proteins compared to those produced in insect cells we have constructed the two
mammalian cell expression plasmids, p3CI-neo and p3CI-dhfr, shown in Fig. 1. The two plasmids are identical except for the mammalian cell selectable marker gene that they contain. The p3CI-neo plasmid contains the neomycin (neo) gene under the control of the simian virus 40 (SV40) early promoter and poly(A) site whereas the p3CI-dhfr plasmid contains the mouse dhfr gene under the control of the SV40 early promoter and poly(A) site. Both vectors contain a DNA sequence composed of the promoter, enhancer and complete 5' untranslated region including the first intron of the major immediate early (IE) promoter of human cytomegalovirus (HCMV). The HCMV IE promoter was used to drive expression of the F and FHN genes in CHO cells. Dhfr-deficient CHO cells were cotransfected with p3CI-neo-F and p3CI-dhfr-F or with p3CI-neo-FHN and p3CI-dhfr-FHN. Transfected cells were simultaneously selected for neomycin and dhfr resistance and the media from clonal cell lines were assayed for expression of F or FHN proteins. The expression of F and FHN proteins from selected cell lines was amplified by progressive adaptation of the cells to increasing concentrations of MTX, up to 500 nM. Two cell lines that secreted high levels of the F and FHN glycoproteins, as measured by ELISA, were further examined by immunoprecipitation. The F and FHN proteins expressed by CHO cells were found to be 10K to 15K larger than the baculovirus-expressed proteins (Fig. 2b), with both proteins appearing as diffuse bands on SDS-PAGE. The diffuse nature and the increase in M_r of the CHO cell-expressed F and FHN proteins is indicative of a difference in glycosylation of the mammalian cell-expressed proteins compared to insect cell-expressed proteins (see below). Comparison of CHO cell-expressed F and FHN proteins on reducing and non-reducing gels (Fig. 2b) demonstrated that neither protein was cleaved.

Characterization of the oligosaccharide structures conjugated to the F and FHN proteins expressed in CHO and Sf9 cells

To investigate the N-linked oligosaccharide structures conjugated to the F and FHN proteins, CHO cells or baculovirus-infected Sf9 cells were labelled with [3H]mannose and radiolabelled glycopeptides were fractionated on Con A–Sepharose columns (Cummings
Baculovirus- and CHO-expressed PIV-3 proteins

Fig. 3. Separation of in vivo [3H]mannose-labelled baculovirus- or CHO cell-expressed F (BAC-F, CHO-F) or FHN (BAC-FHN, CHO-FHN) glycopeptides on Con A-Sepharose. Radiolabelled antigens from culture media were isolated by immunoprecipitation and separated by SDS-PAGE. The F and FHN glycoproteins were localized on the gel by fluorography, excised from the gel and digested extensively with pronase. The pronase-generated glycopeptides were chromatographed on Con A-Sepharose as described by Cummings et al. (1983). To the right is a summary of the oligosaccharide types recovered in the four major Con A fractions. Fraction I is composed of the flow-through portion of the Con A column, fraction II is composed of samples eluting with α-methylglucoside (αmGlc), fraction III is composed of samples eluted with α-methylmannoside (αmMan) and fraction II/III is the shoulder region of fraction III seen with the baculovirus glycopeptides. Symbols: (○) mannose, (●) galactose, (■) N-acetylglucosamine.

Fig. 4. Silver-stained 10% SDS-PAGE of immunoaffinity-purified baculovirus- (lanes 1, 3 and 5) and CHO cell-expressed (lanes 2 and 4) F (lanes 1 and 2), FHN (lanes 3 and 4) and HN (lane 5) glycoproteins. Twenty μg of samples was separated under reducing conditions. Lane M, Mr markers.

et al., 1983). Under the conditions used, this lectin will separate N-linked oligosaccharide structures in three fractions (Fig. 3). The first fraction (Con A fraction I) contains structures that do not interact with the lectin. Fraction I contains tri- and tetra-antennary complex-type structures. The α-methylglucoside eluate (Con A fraction II) contains primarily bi-antennary complex-type structures and the α-methylmannoside eluate (Con A fraction III) contains high-mannose and hybrid type structures. A summary of the typical structures eluting in the three Con A fractions is shown on the right-hand side of Fig. 3.

Separation of glycopeptides prepared from the CHO cell-expressed F protein on Con A–Sepharose resulted in the profile shown in Fig. 3. More than 90% of the sugar-linked radioactivity eluted in Con A fractions I and II, suggesting that the majority of the oligosaccharides on this molecule are of the complex type. The proportion of high-mannose/hybrid structures is probably even lower than it appears on the elution profile since these structures contain more (radioactivity) mannose residues per structure (six to nine) than the complex structures (three, plus often one fucose residue). Fractionation of the baculovirus-expressed F protein on the Con A–Sepharose resulted in a significantly different elution profile (Fig. 3). Essentially all the radioactivity bound to the column and elution resulted in a pattern different from what is typically seen for structures synthesized by mammalian cells; no radioactivity eluted from the column until Con A fraction II and then tailed over into fraction III, resulting in a fraction III elution peak with a shoulder extending into the position of fraction II. We refer to this shoulder fraction as fraction II/III (Fig. 3). The SF9-produced molecules therefore contain a large portion of high-mannose-type structures (44% of the radioactivity). The majority of the radioactivity (54%), however, is incorporated into the oligosaccharides
eluting late in Con A fraction II. Analysis of Con A-Sepharose-fractionated glycopeptides prepared from other glycoproteins has shown that this is the elution characteristic of the truncated (Man₃) high-mannose-type oligosaccharides often found on SF9-produced-glycoproteins (P. A. Aeed & A. P. Elhammer, unpublished data); these structures frequently contain a core fucose residue. Thus, consistent with reported data these structures more frequently contain a characteristic of the truncated (Man₃) high-mannose-type structures.

Fractionation of the glycopeptides prepared from FHN protein showed a higher proportion of high-mannose/hybrid type structures (32% of the radioactivity compared to 8% for the F protein) for the CHO cell-produced molecule (Fig. 3), suggesting differences in the distribution of N-linked oligosaccharide types on the F and HN molecules. The FHN protein synthesized by insect cells again demonstrated a complete lack of complex-type structures (Fig. 3) and, similar to the CHO cell-produced molecule, a higher proportion of high-mannose-type structures.

Labelling experiments using [³H]glucosamine showed that only a minor portion of the oligosaccharide structures on the FHN molecule are linked via an O-glycosidic linkage to the molecule. Preliminary studies indicated the presence of sialylated Galβ1-3GalNAc on the CHO cell-produced molecule, whereas the SF9-synthesized FHN protein contained both GalNAc and Galβ1-3GalNAc none of which contained any terminal sialic acid residues (data not shown).

The considerably smaller size of the oligosaccharide structures on the SF9-produced molecules and the lack of terminal sialic acid residues on these structures provide a very likely explanation for the lower $M_c$ of the SF9-produced F and FHN proteins on SDS-PAGE (Fig. 2b). The diffuse appearance of the proteins synthesized by CHO cells is also consistent with a more heterogeneous population of oligosaccharides on these molecules.

**Immunoaffinity purification of insect cell- and mammalian cell-expressed PIV-3**

Purification of insect cell- and mammalian cell-expressed PIV-3 proteins was done by two-step column chromatography. In the first step the proteins were concentrated from the cell media by ion-exchange column chromatography. Following elution the concentrated proteins were loaded onto either an F or HN MAb column and the column was washed extensively. The PIV-3 proteins were eluted from the immunoaffinity column in a low pH buffer and analysed on silver-stained SDS–PAGE gels (Fig. 4). With the exception of the baculovirus-expressed F protein the other purified proteins appear as near homogeneous bands on the silver-stained gel. Minor bands are apparent in all the samples but the major staining bands for the baculovirus-expressed FHN and HN, and CHO cell-expressed F and FHN proteins migrate with similar $M_s$ as found by immunoprecipitation (Fig. 4). The affinity-purified baculovirus F protein sample contains a second major silver staining band. This protein, which is approximately 10K to 15K smaller than the F protein, appears to be a proteolytic product of F based on its reactivity with PIV-3 MAbs on a Western blot (data not shown). The smaller F protein is not seen in the other purified proteins. The purity of the proteins was determined by densitometry of the silver-stained gels and was estimated to be > 95% for all five proteins.

**Comparison of the immunogenicity and efficacy of insect cell- and mammalian cell-expressed FHN protein**

The relative protective effect of the FHN glycoprotein produced in insect cells and in CHO cells was compared in the cotton rat animal model (Table 2). Analysis of sera from animals vaccinated with either the baculovirus- or

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>Dose (μg)</th>
<th>ELISA (lgG</th>
<th>Neutralizing titre</th>
<th>No.</th>
<th>Average</th>
<th>$P$ value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac-FHN</td>
<td>10</td>
<td>&lt; 400</td>
<td>&lt; 40</td>
<td>7/7</td>
<td>3.17±0.21</td>
<td>0.008</td>
</tr>
<tr>
<td>50</td>
<td>1300</td>
<td>80</td>
<td>1/6</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1800</td>
<td>160</td>
<td>0/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO-FHN</td>
<td>10</td>
<td>1200</td>
<td>40</td>
<td>5/7</td>
<td>3.96±0.24</td>
<td>0.008</td>
</tr>
<tr>
<td>50</td>
<td>1700</td>
<td>80</td>
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<tr>
<td>200</td>
<td>2300</td>
<td>160</td>
<td>0/7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alhydrogel</td>
<td>–</td>
<td>&lt; 400</td>
<td>&lt; 40</td>
<td>7/7</td>
<td>4.94±0.18</td>
<td></td>
</tr>
</tbody>
</table>

* Animals received two i.m. vaccinations and were bled 10 days following the second vaccination.
† Each of the glycoproteins was > 95% pure and was formulated with 200 μg/dose of alhydrogel.
‡ ELISA titre expressed as the reciprocal of the serum dilution giving 50% maximum binding against PIV-3-infected target cells.
§ Neutralizing titre expressed as the reciprocal of the dilution that reduced viral c.p.e. by 50% relative to non-neutralized controls (alhydrogel) against PIV-3.
¶ Number of animals with detectable virus (> 10¹⁷) in their lungs at removal.
* Average lung titre per gram tissue of infected animals in each group. The s.e.m. is indicated for groups with more than one infected animal.
** $P$ value comparisons of each group to alhydrogel-infected animals.
CHO cell-expressed FHN protein revealed that similar levels of serum antibodies reactive with PIV-3-infected target cells were induced by the two proteins. Increasing the amount of protein used to vaccinate animals resulted in a dose-dependent increase in the amount of PIV-3-specific antibodies. Antisera to both proteins also showed significant virus-neutralizing activity when compared to controls and this activity increased for both groups with increasing doses of protein. The protective role of the two recombinant FHN proteins was tested by challenge of immunized animals with live PIV-3. Little if any difference was found between the two proteins since vaccinations with as low as 50 ng of FHN completely protected animals in the CHO cell group whereas only one of six animals was infected in the baculovirus group. These results clearly demonstrate that in terms of immunogenicity and efficacy there is no difference between the baculovirus- and CHO cell-expressed FHN protein.

Discussion

As an initial step in developing a recombinant subunit vaccine against human PIV-3, we cloned the genes for the two major surface glycoproteins of PIV-3 from viral RNA. The cloned genes were completely sequenced. Several differences in the nucleotide and amino acid sequence were found when compared to the published sequences for these genes (Table 1). Identical nucleotide and amino acid differences have been found in clinical isolates and appear to be the result of strain variations of different PIV-3 isolates (van Wyke Coelingh et al., 1983; van Wyke Coelingh & Winter, 1990). The cloned genes were used to construct secreted forms of the F and HN genes and to construct a secreted, chimeric FHN gene. The secreted F, HN and FHN glycoproteins were cloned into baculovirus and animal cell expression vectors and recombinant baculoviruses and stable CHO cell lines that expressed these proteins were isolated. Expression of membrane-associated forms of the F and HN proteins from baculovirus vectors has been reported (van Wyke Coelingh et al., 1981; Ray et al., 1989; Hall et al., 1991). The absence of the membrane anchor sequence from all of the constructs described in this paper allowed simple purification of these proteins from cell culture media. Greater than 90% of the baculovirus F and FHN proteins were detected in the cell media but approximately half of the baculovirus-expressed HN protein remained cell-associated. Proper folding and oligomerization of newly synthesized HN have been shown to be important for efficient transport of the HN protein through the secretory pathway (Parks & Lamb, 1990; Spriggs & Collins, 1991; Collins & Mottet, 1991). Parks & Lamb (1990) showed that the simian paramyxovirus 5 (SV5) HN protein could be converted into a soluble and secreted form by replacing the HN signal/anchor domain with the fusion-related domain from the SV5 F protein but that only 40% of the synthesized protein was secreted. The extracellular SV5 HN molecules were identified as disulphide-linked dimers whereas the intracellular HN molecules were mainly monomers. We converted the PIV-3 HN protein to a secreted form by replacing the signal/anchor with the signal sequence of the PIV-3 F protein and found that levels of the secreted HN protein were similar to those reported by Parks & Lamb (1990). Taken together these data indicate that a type II membrane protein such as HN can be converted to a secreted protein by replacing its signal/anchor sequence with a cleavable signal sequence and that this form of the protein can be utilized as a means to isolate large quantities of soluble protein.

We expressed the secreted F and FHN glycoproteins from stably transformed CHO cells and characterized the oligosaccharide structures added to both the insect cell- and mammalian cell-expressed proteins. To obtain CHO cell lines that express high levels of the secreted F and FHN proteins we utilized a method for direct isolation of high producing cell lines based on direct screening following the simultaneous transfer of two selectable marker genes (Wirth et al., 1988; Zettlmeissl et al., 1988). CHO cells were transfected with two plasmids (p3CI-neo or p3CI-dhfr) each containing the gene to be expressed under control of the HCMV IE promoter containing a 5′ intron. The transfected cells were then selected for both G418 and MTX resistance. The clonal cell lines isolated following double selection express at significantly higher levels than if they were selected for just G418 or MTX resistance (Wirth et al., 1988). The presence of the dhfr gene also allows for amplification of the expression level by selecting for cells that are resistant to increasing concentrations of MTX. Using this technique we isolated two CHO cell lines that express the secreted F and FHN glycoproteins at levels similar to the baculovirus-expressed proteins. It is interesting to note that we failed to detect expression of either the F or FHN genes from CHO cells when an HCMV IE promoter was used that lacked the 5′ intron. We have found that it is difficult to express RSV or PIV-3 proteins in animal cells and assume that this is due to internal RNA processing sequences [splice acceptor/donor sites and internal poly(A) sites] on these genes. These sequences would not normally be a problem since paramyxovirus mRNA is made in the cytoplasm of infected cells. The CMV 5′ intron may function to override the internal RNA processing signals.

The general types of N-linked oligosaccharides found on the Sf9- and CHO cell-produced F and FHN proteins appear to be similar to structures found on many other
recombinant proteins expressed in these cells (Hsieh & Robbins, 1984; Weber et al., 1986; Kuroda et al., 1990; Wathen et al., 1991; Aeed et al., 1992). The vast majority of structures found on the CHO cell-expressed proteins are of the complex type whereas the insect cell-produced proteins contain only high-mannose and truncated high-mannose structures (Fig. 3). Only a minor portion of the oligosaccharide structures found on the CHO cell- and baculovirus-expressed F and FHN proteins are O-linked oligosaccharides.

The oligosaccharide structures added to glycoproteins by insect cells differ from those found on proteins made in mammalian cells (Hsieh & Robbins, 1984; Weber et al., 1986; Kuroda et al., 1990; Wathen et al., 1991; Aeed et al., 1992) and carbohydrate structure has been shown to contribute to protein antigenicity (Weber et al., 1987; Moore et al., 1990). In one such study Moore et al. (1990) showed that a significant proportion of the anti-gp120 antibodies found in human immunodeficiency virus-positive human sera recognize epitopes that are dependent on the glycosylation pattern. Comparison of the CHO cell- and baculovirus-expressed FHN proteins as subunit vaccines demonstrated that both proteins induced similar levels of neutralizing antibodies and protected cotton rats against live virus challenge at low doses of antigen (Table 2). The results of these experiments demonstrate that, in terms of immunogenicity and efficacy, the baculovirus- and CHO cell-expressed FHN proteins are equivalent.

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both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. *Virology* 50, 640-652.


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