Comparative analysis of the immunoprotective abilities of glycosylated and deglycosylated parainfluenza virus type 3 surface glycoproteins

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The role of carbohydrate moieties on the immunoprotective ability of parainfluenza virus type 3 (PIV-3) haemagglutinin–neuraminidase (HN) and fusion (F) glycoproteins was tested in hamsters. HN and F proteins were purified from detergent-solubilized virus by lentil-lectin affinity chromatography and deglycosylated by treatment with endoglycosidase F (endo F). Immunization of hamsters with either 1 or 5 μg of mock-treated (glycosylated) affinity-purified proteins elicited strong haemagglutination inhibition and neutralizing antibody responses 4 weeks after the primary injection.

Parainfluenza virus type 3 (PIV-3) is one of the major viral pathogens responsible for severe respiratory tract infections in infants and young children (Murphy et al., 1989). Induction of neutralizing antibodies against the two major PIV-3 surface glycoproteins confers a protective response in immunized hamsters (Spriggs et al., 1987; Ray et al., 1988). The PIV-3 protective immunogens are the HN protein which possesses both haemagglutination and neuraminidase activities and the F (fusion) protein which is responsible for fusion of the virus to the host cell membrane and cell-to-cell spread of the virus (Chanock & McIntosh, 1990).

The carbohydrate moieties of several viral glycoproteins have been found to play a significant role in their functional (Gorman et al., 1991; Vidal et al., 1989; Lambert, 1988) and immunological properties (Benjouad et al., 1992; Sjöblom et al., 1987). Fully glycosylated, affinity-purified PIV-3 HN and F proteins elicited a protective response in cotton rats and hamsters (Ambrose et al., 1991; Ewasyshyn et al., 1992), whereas the immunoprotective ability of the deglycosylated antigens has not yet been reported. Thus, the objective of this study was to determine the role of the carbohydrate moieties in the immunoprotective ability of the PIV-3 HN and F proteins.

A clinical isolate of human PIV-3 (hPIV-3) was obtained from the Toronto Public Health Laboratory and propagated in MRC-5 cells (Connaught Laboratories) grown on Cytodex 1 microcarrier beads in 40 litre Bellco flasks. Haemagglutination and TCID_{50} titres were determined by standard procedures (Chanock, 1975). Vero cells were used in the TCID_{50} assay. Viral fluids were harvested and processed using the method outlined by Ewasyshyn et al. (1992). The HN and F proteins used in the deglycosylation studies were copurified from detergent-solubilized virus by lentil-lectin affinity chromatography (Ewasyshyn et al., 1992). The protein concentration of the copurified HN and F preparation was determined by a modified Lowry assay (Peterson, 1977).

Since the PIV-3 HN and F proteins contain only N-linked carbohydrates (Galinski et al., 1987; Morrison, 1988), endoglycosidase F (endo F) was selected to remove the high mannose and complex asparagine-linked glycans from the glycoproteins (Elder & Alexander, 1982). To deglycosylate the HN and F proteins, a 560 μg sample of lentil-lectin-purified HN and F proteins in 2.0 ml of 100 mM-sodium phosphate buffer pH 7.2 containing 0.02% (v/v) Triton X-100 was incubated for 16 h at 37 °C in 50 mM-sodium phosphate buffer pH 7.0, containing 0.06 mm-leupeptin (Boehringer Mannheim), 0.5 mM-α-2-macroglobulin (Boehringer Mannheim), 14 mM-aprotinin (Calbiochem), 1.0% (v/v) NP40 (Sigma) and 7.25 units of endo F (Boehringer Mannheim). Mock-treated control samples consisting of proteins incubated in the absence of enzyme were also included. After incubation, mock- and enzyme-treated proteins were tested for their ability to bind to a lentil-lectin affinity column. Mock- and endo F-treated
proteins present in the eluate and flowthrough fractions from the lentil-lectin affinity columns, respectively, were separated by electrophoresis on 10% SDS-polyacrylamide slab gels under reducing conditions according to Laemmli (1970). Gels were stained with Coomassie blue for scanning laser densitometry (LKB Ultrascan XL). The respective amounts of HN and F proteins were determined by integrating the HN and F peaks in the scan profile.

The immunoreactivity of mock- and enzyme-treated proteins was tested by Western blot analysis as previously described (Ewasyslyn et al., 1992) using guinea-pig monospecific anti-HN and anti-F1 peptide antisera. To prepare these antisera, guinea-pigs were immunized with three 50 lag doses of synthetic peptides corresponding to residues 430 to 448 (SKLQLGIIDITDYSDIRIK) and 496 to 522 (LDSQKSRVNPVTYSTSTERVNE) of the HN protein and residues 248 to 274 (VDKYDIYDLLFTESIKVRVIDVLDNDY) and 363 to 390 (CPRTVVTSIDVPRAYFVNGGVVANCIT) of the F1 protein emulsified in Freund’s adjuvant. Sera were collected 10 days after the final boost. Both the mock- and endo F-treated proteins reacted with monospecific anti-F1 (Fig. 1 a) and anti-HN (Fig. 1 b) peptide antisera on immunoblots. Treatment of the HN and F proteins with endo F abolished the haemagglutinating activity of the glycoproteins and significantly reduced the apparent $M_r$ of the glycoproteins by 4K for the HN protein and 3K for the F1 protein. These results are consistent with complete deglycosylation of the antigens since the apparent $M_r$ of the deglycosylated HN (65K) and F1 (47K) proteins corresponded to the predicted $M_r$ of their respective polypeptide backbones (Chanock & McIntosh, 1990). Complete removal of the carbohydrate moieties was further confirmed by the inability of the enzyme-treated proteins to rebind to a lentil-lectin affinity column in contrast to the mock-treated proteins which were retained on the column via their high-mannose glycans (Gerard, 1990).

To determine the contribution of the oligosaccharide moieties to the immunogenicity of the PIV-3 HN and F proteins, hamsters were immunized with two doses of either mock-treated antigens or endo F-treated HN and F proteins rechromatographed on a lentil-lectin column to ensure the removal of any residual glycosylated species. Groups of six pathogen-free hamsters (female, 4 weeks old, from Charles River) were injected intramuscularly with either PBS containing 0.02% (v/v) Triton X-100, 0.135% NP40 and 0.03 m-methyl-$\alpha$-D-mannopyranoside (buffer control) or 0.1, 1.0 or 5.0 lag of mock- or endo F-treated HN and F proteins adjuvanted in alum. Hamsters were bled 4 weeks after the primary injection and boosted with an equivalent dose of the antigen formulation. Sera were also collected 2 and 4 weeks after the booster dose. Haemagglutination inhibition (HAI) and neutralization titres were determined by standard assay procedures (Chanock, 1975). Reciprocal mean log$_2$ HAI and neutralizing titres are summarized in Table 1. Data from the bleed done at 4 weeks showed that animals immunized with either 1 or 5 lag of native HN and F proteins elicited a strong primary response. However, neutralizing and HAI antibody titres were significantly lower ($P < 0.001$) in animals immunized with an equivalent dose of the endo F-treated proteins. No immune response was observed with 0.1 lag of either protein preparation. These results demonstrated that carbohydrate removal affected the magnitude of the primary immune response.

Following the booster dose, no significant difference was observed in HAI or neutralizing antibody titres in the sera of animals immunized with 5 lag of either glycosylated or deglycosylated proteins. These titres were comparable to those obtained in hamsters intranasally instilled with $10^8$ TCID$_{50}$ units of live PIV-3 (Table 1). In contrast to the group immunized and boosted with 5 lag of protein, animals immunized and boosted with either 0.1 or 1.0 lag of deglycosylated antigens generated significantly lower serum neutralizing antibody titres than animals immunized with the same amounts of glycosylated proteins. However, in the 8 week sample, HAI titres were not significantly different in animals immunized with either 0.1 or 1.0 lag of deglycosylated antigens. Thus, enzymatic removal of the carbohydrate moieties from the PIV-3 HN and F proteins did not affect their ability to elicit a strong secondary antibody response when administered at a...
sufficient dose. The difference observed in the magnitudes of the primary responses obtained with native and deglycosylated antigens may be explained by the fact that these proteins are processed differently. Alternatively, the removal of carbohydrates may affect the conformation of B cell epitopes resulting in a decrease in the binding affinity of their cognate B cell receptors. However, after boosting of animals with at least 5 μg of antigen, selective expansion of B cell clones with high-affinity antigen receptors resulted in secondary antibody titres which were comparable for both forms of antigens.

The results of this study provide further evidence that the contribution of the carbohydrate moieties of a particular viral glycoprotein in inducing a neutralizing antibody response is unique for each viral protein. The relative contribution of oligosaccharides to the immunogenicity of a particular viral glycoprotein can be classified into three major categories. In the first category, enzymatic removal of the sugar moieties has no significant effect on the neutralizing antibody response. Indeed, the neutralization epitopes of bovine herpesvirus type 1 (BHV-1) glycoprotein gIV (van Drunen Littel-van den Hurk, 1990) and Rauscher leukaemia virus glycoprotein gp70 (Elder et al., 1986) are carbohydrate-independent. In the second category, carbohydrate removal significantly decreases the ability of the viral antigen to induce a neutralizing antibody response comparable to that elicited by its glycosylated form. This is the case for the glycoprotein gL of BHV-1 (van Drunen Littel-van den Hurk et al., 1990) and the recombinant gp160 envelope of human immunodeficiency virus (Benjouad et al., 1992). The PIV-3 HN and F glycoproteins fall into a third category where the elimination of oligosaccharides affects the magnitude of the primary immune response.

To compare the protective ability of the glycosylated and deglycosylated PIV-3 surface antigens, hamsters were challenged with $10^6$ TCID$_{50}$ units of live PIV-3 immediately after the bleed done after 8 weeks and sacrificed 3 days after challenge. Virus titres were determined in both the upper (nasal washes) and lower (lung homogenates) respiratory tracts. Lung homogenates and nasal washes were prepared for viral assay by the procedure outlined by Ewasyshyn et al. (1992). As shown in Table 2, there was no significant difference in the ability of mock- and endo F-treated proteins to protect either the upper or lower respiratory tracts of animals against live virus challenge. Although neither the mock- nor endo F-treated HN and F proteins were capable of completely protecting the upper respiratory tracts of hamsters against PIV-3 infection, virus titres in nasal washes of animals immunized with either form of the HN and F proteins were significantly lower than in control animals. In contrast, the lower respiratory tract of animals immunized with two 1 or 5 μg doses of either native or deglycosylated proteins were completely protected against live virus challenge. However, two 0·1 μg doses of either mock- or endo F-treated proteins failed to be fully protective. There was no significant difference between the lung virus titres of animals immunized with two 0·1 μg doses of either preparation, and titres were significantly lower than for control animals. It has been reported previously that the titre of infectious virus present in the lungs of hamsters (Ewasyshyn et al., 1992) and cotton rats (Ambrose et al., 1991) immunized with the glycosylated HN and F proteins correlated inversely with serum HAI and neutralizing antibody titres. Although serum neutralizing titres in the 8 week sample were significantly lower in animals immunized with 1·0 μg of deglycosylated proteins, antibody levels were still within the protective range. The possibility of in situ neutralization of the virus in lung homogenates during the extraction process was negated by the previously reported finding that, when lung homogenates from non-

### Table 1. Serum antibody response of hamsters immunized with various HN and F formulations

<table>
<thead>
<tr>
<th>Antigen formulation</th>
<th>Dose (μg)</th>
<th>4 week bleed</th>
<th>6 week bleed</th>
<th>8 week bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>-</td>
<td>≤10±00</td>
<td>≤10±00</td>
<td>≤10±00</td>
</tr>
<tr>
<td>HN + F Mock-treated</td>
<td>50</td>
<td>80±03</td>
<td>96±02</td>
<td>86±03</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>63±05</td>
<td>90±02</td>
<td>83±03</td>
</tr>
<tr>
<td></td>
<td>01</td>
<td>≤10±00</td>
<td>51±10</td>
<td>54±07</td>
</tr>
<tr>
<td>HN + F Endo</td>
<td>50</td>
<td>43±04†</td>
<td>95±03</td>
<td>85±04</td>
</tr>
<tr>
<td>F-treated</td>
<td>10</td>
<td>21±07‡</td>
<td>71±08‡</td>
<td>70±07</td>
</tr>
<tr>
<td>F-treated</td>
<td>01</td>
<td>≤10±00‡</td>
<td>≤10±00‡</td>
<td>32±10</td>
</tr>
<tr>
<td>Live PIV-3</td>
<td>$10^6$ TCID$_{50}$</td>
<td>7.3±0.4</td>
<td>7.3±0.3</td>
<td>7.7±0.2</td>
</tr>
</tbody>
</table>

* Reciprocal mean log$_2$ serum dilution ±S.E.M. that inhibits erythrocyte agglutination by 4 haemagglutinating units of PIV-3.
† Reciprocal mean log$_2$ serum dilution ±S.E.M. that blocks haemadsorption of 100 TCID$_{50}$ units of PIV-3.
‡ Denotes mean titres which are significantly different ($P<0.05$) from titres obtained with the corresponding dose of the mock-treated proteins.

Student’s t-test was used, with $P$ values > 0·05 considered to be non-significant.
challenged control or immunized animals with high titres of HAI and serum neutralizing antibodies (> log$_{10}$ 8) were spiked with 10 to 30 TCID$_{50}$ units of PIV-3, the added virus was neutralized by neither the lung homogenates of non-immunized controls nor by those of vaccinated animals (Ewasyshyn et al., 1992). The results of the current study not only substantiate previous findings that the copurified HN and F proteins are highly immunogenic and protective but also provide evidence that their immunoprotective ability does not appear to be carbohydrate-dependent.

The role that oligosaccharides may play in the immunogenicity of a viral glycoprotein has important implications in the choice of the expression system to be used if a recombinant approach is considered for producing the antigen. If glycosylation is important for preserving its immunoprotective ability, then the pertinent gene should be expressed in a system, such as mammalian cells (Dorner & Kaufman, 1990), which would preserve the glycosylation pattern of the native protein. Indeed, glycoproteins expressed in the baculovirus system are hypoglycosylated (Luckow & Summers, 1988), and proteins produced in yeast may be hyperglycosylated (Emr, 1990). However, it has been reported that the PIV-3 HN (van Wyke Coelingh et al., 1987) and F (Ray et al., 1989) glycoproteins produced in insect cells using the baculovirus expression system were capable of eliciting protective responses. These findings suggest that the glycosylation pattern of the native antigen does not necessarily have to be preserved to maintain its immunoprotective ability. The results of the current study provide additional evidence that PIV-3 HN and F oligosaccharides are not essential for the induction of a protective response thus allowing for the production of recombinant HN and F antigens in various systems. Further immunogenicity and protection studies with the HN and F proteins produced either in the presence of inhibitors of N-linked glycosylation such as tunicamycin (Takatsuki & Tamura, 1971; Nakamura et al., 1982) or in a mutant CHO cell line deficient in N-acetylglucosaminyl transferase (Stanley et al., 1975), are required to determine whether glycosylation contributes to the proper folding of their protective epitopes.

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