The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type 1 glycoproteins gI and gIV

S. van Drunen Littel-van den Hurk,1 G. Hughes1 and L. A. Babiuk1,2

1Veterinary Infectious Disease Organization and 2Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type 1 (BHV-1) glycoproteins gI and gIV was investigated. Deglycosylated proteins induced a significantly lower antibody response in rabbits than native glycoproteins suggesting that the immunogenicity of several epitopes on gI and gIV is carbohydrate-dependent. Loss of carbohydrate from gI also resulted in a significantly decreased ability to induce a serum neutralizing antibody response to BHV-1, due to modifications in three distinct carbohydrate-containing continuous epitopes. Similarly, in vitro lysis of BHV-1-infected cells was significantly reduced when antibodies raised against deglycosylated gI were employed; this was attributed to changes in two of the three carbohydrate-dependent neutralizing epitopes on gI. The oligosaccharides may be directly involved as actual components of these continuous epitopes, rather than in stabilization of the conformation of the protein. In contrast, carbohydrate removal from gIV did not have a significant effect on the capacity to stimulate a neutralizing antibody response. Accordingly, none of the neutralizing epitopes on gIV appeared to be carbohydrate-dependent. Similarly, lysis of virus-infected cells was not significantly reduced when antibodies specific for deglycosylated rather than native gIV were used. In contrast to the humoral response, the delayed-type hypersensitivity response was stronger in rabbits immunized with deglycosylated proteins than in those inoculated with native glycoproteins gI or gIV. Consequently, the carbohydrates on gI and gIV may play a dual role in the host's immune recognition and response by contributing to certain epitopes, but masking others. The implications for the development of a subunit vaccine against BHV-1 are discussed.

Introduction

Bovine herpesvirus type 1 (BHV-1), a member of the alphaherpesvirinae subfamily, is an economically important pathogen in cattle. It is the causal agent of a variety of disease conditions, including respiratory infections, conjunctivitis, vulvovaginitis, abortions, encephalitis and generalized systemic infections (Gibbs & Rwemamur, 1977). Recovery from most herpesvirus infections, including BHV-1 infection, is mediated by a variety of cellular and humoral immune responses (Rouse & Babiuk, 1978).

Viral glycoproteins are the major structural components present in the envelope of herpesviruses. They play an important role in the virus–host relationship as they are involved in the recognition, attachment (Little et al., 1981; Fuller & Spear, 1985) and penetration (Sarmiento et al., 1979; Fuller & Spear, 1987) of the virus into susceptible cells, as well as in viral neutralization (Vesterberg & Norrild, 1979; Glorioso et al., 1984). During infection the viral glycoproteins are inserted into the plasma membrane of the host cell and are able to mediate cell fusion (Manservigi et al., 1977; Noble et al., 1983), complement factor C3b (Friedman et al., 1984) and immunoglobulin G (Johnson & Feenstra, 1987) binding and immune destruction of virus-infected cells (Norrild et al., 1980; Carter et al., 1981; Bishop et al., 1984; Rouse & Horohov, 1984).

Four (sets of) glycoproteins have been identified in BHV-1: gI, a 130K disulphide-linked 74K/55K heterodimer; gII, a 108K glycoprotein; gIII, a 180K/91K dimeric glycoprotein; and gIV, a 140K/71K dimeric glycoprotein (van Drunen Littel-van den Hurk et al., 1984; Collins et al., 1984; Marshall et al., 1986; Hughes et al., 1988). Glycoproteins gI, gIII and gIV all stimulate the production of neutralizing antibodies in mice, rabbits and cattle, and they all serve as targets for antibody-dependent, complement-mediated lysis of virus-infected cells (Collins et al., 1984; van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk & Babiuk, 1985b; Marshall et al., 1986; Okazaki et al., 1986; Trepanier et al., 1986; Israel et al., 1988). Several
studies have shown that the ability to mediate virus neutralization as well as destruction of virus-infected cells is localized in a number of different epitopes on each of these three glycoproteins (Collins et al., 1984; van Drunen Littel-van de Hurk et al., 1985; Okazaki et al., 1986; Marshall et al., 1988; Hughes et al., 1988). Glycoproteins gI, gIII and gIV have also been identified as the major immunogens recognized by cattle infected with BHV-1 (Collins et al., 1985; van Drunen Littel-van den Hurk & Babiuk, 1986a). Furthermore, each of glycoproteins gI, gIII and gIV, individually or in combination, was able to induce protective immunity in cattle against challenge with BHV-1 (Babiuk et al., 1987).

Oligosaccharides are integral components of many viral envelope and eukaryotic cell surface proteins. Although the precise function(s) of the carbohydrate moieties of these proteins remains unknown, they appear to play significant roles in specific recognition phenomena, as well as protein folding and conformation (Berger et al., 1982; Sharon & Lis, 1982). Evidence for the glycoprotein nature of BHV-1 glycoproteins gI, gII, gIII and gIV has been obtained by the incorporation of radiolabelled sugars (van Drunen Littel-van den Hurk et al., 1984) and their reactivity with a variety of lectins (personal observation). Treatment of these glycoproteins with endoglycosidases (van Drunen Littel-van den Hurk & Babiuk, 1986b) and incorporation of glycosylation inhibitors in the tissue culture medium during BHV-1 infection (van Drunen Littel-van den Hurk & Babiuk, 1985a) have shown that glycoproteins gI and gII contain N-linked sugars, whereas gIII and gIV have both N-linked and O-linked carbohydrates. Cloning and sequencing data have indicated the presence of six potential asparagine-linked glycosylation sites on gI, four on gIII and three on gIV (Whitbeck et al., 1988; T. J. Zamb, unpublished results).

Glycoproteins gI and gIV are primary targets for neutralizing and protective antibodies (Babiuk et al., 1987) and potentially for cellular immune responses as well (unpublished observations). Studies on several viruses like influenza virus, respiratory syncytial virus and Rauscher leukaemia virus, have indicated that the carbohydrate moiety of viral glycoproteins can have a strong influence on functional (Basak & Comps, 1983; Lambert, 1988) as well as antigenic and immunogenic activities (Alexander & Elder, 1984; Elder et al., 1986). In contrast, the carbohydrates did not appear to play a major role in the antigenic structure of the E protein of flaviviruses, a lightly glycosylated protein (Winkler et al., 1987).

The objective of this study was to analyse the contribution of the carbohydrate portion of glycoproteins gI and gIV to their ability to induce an immune response to BHV-1. Our experiments indicate that loss of carbohydrate results in a reduced neutralizing antibody response to BHV-1. In the case of gI, this was due to a change in three defined carbohydrate-dependent neutralizing epitopes. Similarly, lysis of BHV-1-infected cells appeared to be at least partially mediated by carbohydrate-dependent epitopes. In contrast, the cellular immune response as measured by a delayed-type hypersensitivity (DTH) reaction was stronger following immunization with deglycosylated protein than after inoculation with native glycoprotein, suggesting that carbohydrate may mask at least some epitopes involved in T cell recognition or induction.

Methods

Cells, virus and glycoproteins. Madin–Darby bovine kidney (MDBK) cells were grown as monolayers in Eagle's MEM (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco). Strain P8-2 of BHV-1 was propagated in MDBK cells and quantified by plaque titration in microtitre plates with an antibody overlay as previously described (Rouse & Babiuk, 1974). Glycoproteins gI and gIV were purified from virus-infected cell lysate as previously described (van Drunen Littel-van den Hurk & Babiuk, 1985b).

Glycosidase digestion of glycoproteins. Glycoprotein gI was deglycosylated by treatment with peptide:N-glycosidase F (N-glycanase; Genzyme) or with endo-β-N-acetylglucosaminidase F (endo F from Flavobacterium meningosepticum; New England Nuclear) whereas gIV was treated with N-glycanase or endo F, followed by neuraminidase (from Clostridium perfringens; Sigma) and endo-β-N-acetylglucosaminidase (O-glycanase; Genzyme). Incubation with N-glycanase was carried out in 0.2 m-sodium phosphate buffer pH 8·6, 50 mm-EDTA, whereas digestion with endo F was performed in 0·1 m-sodium phosphate buffer pH 6·1, 50 mm-EDTA. Usually, 10 µg of glycoprotein at a concentration of 0·5 mg/ml was digested with 1 unit (U) of enzyme for 20 h at 37 °C. Neuraminidase digestion was performed in 0.02 m-Tris-maleate, 1 m-calcium acetate, 10 mm-D-galactos-gamma-lactone for 1 to 3 h at 37 °C. Ten mg of purified glycoprotein was incubated with 0·1 U of neuraminidase. Subsequently, O-glycanase treatment was carried out for 6 h at 37 °C under the same buffer conditions, using 10 ml of endo F for 10 µg of glycoprotein. For analysis by discontinuous SDS–PAGE the proteins were precipitated with ice-cold acetone, resuspended in electrophoresis sample buffer (0·625 m-Tris–HCl pH 6·8, 1·25% SDS, 12·5% glycerol, 0·15 m-mercaptoethanol, 0·00125% bromophenol blue) and boiled for 1 min. For rabbit immunizations the proteins were dialysed against PBS.

Polyacrylamide gel electrophoresis and immunoblot analysis. SDS–PAGE was carried out in 7·5% discontinuous slab gels (Laemmli, 1970) under reducing conditions as described previously (van Drunen Littel-van den Hurk et al., 1984). The Western blotting technique of Burnette (1981) was used as previously described (van Drunen Littel-van den Hurk et al., 1984). After electrophoresis, affinity-purified gI was electroblot transblotted to nitrocellulose sheets, using 25 mm-sodium phosphate buffer pH 6·8 as the electrode solution. Subsequently, the instructions for use of the Bio-Rad immunoblot assay kit were followed.

Lectin binding assay. Polystyrene microtitre plates (Immulon 2, Dynatech Laboratories) were coated overnight at room temperature with 200 µl per well of purified glycoprotein or deglycosylated protein.
(0.5 μg/ml) in coating buffer (0.05 M-NaHCO3, pH 9.6). After coating, the plates were washed four times in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Two hundred μl of biotinylated lectin (1 mg/ml, Vector Laboratories) serially diluted in PBST was added to each well and incubated for 2 h at 37°C. Alternatively, purified glycoprotein and deglycosylated protein (0.5 μg/ml) were serially diluted and incubated with 200 μl of biotinylated lectin (10 μg/ml). The plates were washed four times and the wells were incubated with avidin DH-biotinylated alkaline phosphatase H complex (Vector Laboratories) according to the manufacturer's instructions. The reaction was allowed to continue for 30 min at room temperature, whereafter the absorbance at 450 nm was determined on a Dynatech Microelisa reader (Model MR580).

Immunization of rabbits. The glycoproteins and deglycosylated proteins were diluted in PBS and emulsified in an equal volume of Freund's adjuvant (Gibco). Groups of two New Zealand white rabbits were given one immunization with each of the glycoproteins or deglycosylated proteins at different concentrations in Freund's complete adjuvant, followed by a booster immunization in Freund's incomplete adjuvant 4 weeks later. They were bled 3 weeks after each immunization.

ELISA. The ELISA was performed essentially as described previously (van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk & Babiuk, 1985b). To determine the antibody responses of rabbits immunized with glycoproteins or deglycosylated proteins, micortitre plates were coated with 0.05 μg purified glycoprotein or 1 μg purified BHV-1 per well. Affinity-purified, horseradish peroxidase (HRPO)-conjugated goat anti-rabbit Ig (Boehringer Mannheim) was used at a dilution of 1:1000 for detection.

Competitive antibody binding assay (CBA). The CBA was based on the ELISA modified as previously described (van Drunen Littel-van den Hurk et al., 1985). The percentage of competition was calculated using the formula 100 × (A − B)/A, where A is absorbance in the absence of competitor antibody and B is absorbance in the presence of competitor monospecific antibody, a modification of the formula described by Kimura-Kuroda & Yasui (1983).

Serum neutralization (SN) test and antibody and complement (C) -mediated (Ab/C) cytolysis. The neutralization titres of the rabbit sera and the ability of the rabbit sera to mediate complement-dependent cell lysis were determined as previously described (Babiuk & Rouse, 1975). The SN titres were expressed as the reciprocal of the highest serum dilution causing a 50% reduction of plaques relative to the virus control. Specific release was calculated as 100 × (release by Ab + C − release by control without Ab or C)/(total releasable 51Cr − release by control).

Delayed-type hypersensitivity (DTH) assay. Seven weeks after the second immunization the rabbits were inoculated subcutaneously with 1 μg of homologous antigen (gl or gIV) in one ear and with 1 μg of heterologous antigen (gII) in the other ear. The DTH response of the rabbits was followed by measuring ear thickness with a dial thickness gauge (Mitutoyo) at the time of inoculation and for 4 days afterwards. The increase in thickness of the test ear, inoculated with homologous antigen, was corrected for naturally occurring variation by subtracting increase or decrease in thickness of the control ear, inoculated with heterologous antigen, and expressed in μm.

Results

Deglycosylation of glycoproteins gl and gIV

As glycoprotein gl contains only N-linked carbohydrates, whereas gIV has both N- and O-linked sugars (van Drunen Littel-van den Hurk & Babiuk, 1985a, 1986b), endoglycosidases used to generate deglycosylated proteins dgI and dgIV were chosen accordingly. Incubation with both endo F and N-glycanase, which remove N-linked carbohydrates, decreased the apparent Mr of gl from 130K/74K/55K to 103K/55K/50K (Fig. 1). Because the size of the deglycosylated species corresponded well with the apparent Mr, of 105K reported for the polypeptide backbone of gl (van Drunen Littel-van den Hurk & Babiuk, 1986b), this suggests complete removal of carbohydrate from gl. After sequential treatment with endo F and N-glycanase, neuraminidase and O-glycanase, which remove O-linked oligosaccharides, the apparent Mr of gIV decreased from 71K to 60K and accordingly the size of the dimer changed from 140K to 120K (Fig. 1). This is consistent with the removal of virtually all carbohydrates as the polypeptide backbone of gIV has an apparent Mr of 58K (van Drunen Littel-van den Hurk & Babiuk, 1986b).

![Fig. 1. Deglycosylation of gl and gIV. Affinity-purified glycoproteins gl (a) and gIV (b) were fractionated by SDS-PAGE (7.5%) before (−) and after (+) endoglycosidase treatment and visualized by staining with Coomassie blue. The positions of glycoproteins gIa, gIb, glc, gIV and gIV dimer are indicated in the right margins. Mr shifts, occurring as a result of deglycosylation, are shown with arrows. Positions of Mr markers are indicated in the left margins.](https://www.microbiologyresearch.org/content/2055/1/2)
The removal of carbohydrate to yield the more rapidly migrating species after endoglycosidase treatment was verified by a lectin-binding assay in which the ability of a panel of lectins to bind to the native and deglycosylated proteins was assessed. Table 1 shows that lectins specific for α-mannose (α-Man), galactose (Gal) and sialic acid (NANA) bound to gI, but not to dgI. Similarly, lectins specific for α-Man, α-Gal, N-acetylgalactosamine (GalNAc), α-N-acetylgalactosamine (GalNAc) and α-fucose (α-Fuc) bound to gIV, but not to dgIV. Binding of these lectins to dgI and dgIV was at a level comparable to that of binding to an unglycosylated control antigen, BHV-1 VP8, whereas their reactivity with gI and gIV was at least 10-fold higher. In a similar assay, the limit of antigen detection by this panel of lectins was determined. The lectins that were reactive in the previous assay (Table 1) detected as little as 3.1 to 12.5 ng of gI or gIV whereas they did not react with >~ 100 ng of dgI, dgIV or VP8.

**Effect of carbohydrate removal on the antibody response to gI and gIV**

In order to examine the role of carbohydrate in the humoral antiviral response, we tested the relative efficacy of gI and gIV and their deglycosylated counterparts to elicit an antibody response in rabbits. The sera of these rabbits were analysed for the presence of total antibody, virus-neutralizing antibody and antibody mediating lysis of BHV-1-infected cells.

As shown in Fig. 2(a), one injection of at least 1 μg of native or deglycosylated protein induced a detectable serum antibody titre as measured by ELISA. After the booster immunization 28 days later there was a further increase in the antibody levels. By contrast, a dose of 0.1 μg induced a detectable antibody response only after two immunizations, which was at least 100-fold lower than that obtained with two doses of at least 1 μg of immunogen. Fig. 2(a) also indicates that the total antibody response was significantly lower (P < 0.01) in rabbits immunized with deglycosylated proteins than in those vaccinated with native glycoproteins gI and gIV. The mean difference between the antibody responses to gI and dgI was 4.5-fold, ranging from two- to 10-fold depending on the concentration of immunogen. The difference between the gIV and dgIV antibody responses ranged from two- to 8.5-fold, with a mean of 3.5-fold.

In the absence of complement, two doses of at least 1 μg of gI, gIV or dgIV were sufficient to induce reasonable serum neutralizing antibody titres, whereas two doses of 50 μg of dgI were required to obtain a detectable neutralizing antibody response (Fig. 2b). In the presence of guinea-pig complement the titres increased two- to fourfold. The neutralizing antibody response of the rabbits immunized with dgIV was fourfold (ranging from two- to 10-fold) lower than the response of the gIV-vaccinated animals, which is in good agreement with the difference in total antibody response. However, animals immunized with dgI had 10-fold (ranging from two- to 16-fold, P < 0.05) lower neutralizing antibody titres than gI-immunized animals, indicating that carbohydrate removal has a relatively strong impact on the neutralizing epitopes of gI.

In Fig. 2(c) the percentage specific 51Cr release as a result of AbC lysis of BHV-1-infected MDBK cells is shown. It is apparent that the gIV/dgIV-specific antibodies mediate cell lysis more efficiently than the gI/dgI-specific antibodies. A similar pattern was observed for
Fig. 2. Antibody responses to glycoproteins gI and gIV before and after deglycosylation. Rabbits were immunized with increasing concentrations of (glyco)protein and boosted 4 weeks later. They were bled 3 weeks after each immunization. (a) The total antibody response was determined in an ELISA using gI or gIV as the antigen. Lower (white) bars show the ELISA titres after the first immunization and the upper (shaded) bars those after the second immunization. The ELISA titres were expressed as the highest dilution that still gave a reading of 0.1 at 450 nm. The difference between gI and dgI-specific antibody titres, as well as between gIV- and dglV-specific antibody titres was significant (\( P < 0.01 \); analysis of variance analysis). (b) The serum neutralizing antibody titres between gI/dgI- and gIV/dglV-specific sera (Fig. 2b). The capacity to lyse virus-infected cells was fivefold (ranging from two- to 12-fold) lower in dglI-specific sera than in gI-specific sera, which appeared to be a significant difference (\( P < 0.02 \)) and is in accordance with the observed reduction in total antibody titre following deglycosylation. The ability to mediate cell lysis was reduced threefold (ranging from 1.5- to fourfold) in dglV-specific sera as compared to gIV-specific sera, which corresponds to the decrease in total and neutralizing antibody titres.

**Analysis of epitopes affected by deglycosylation**

Previously, we developed a panel of monoclonal antibodies specific for seven different epitopes on gI and eight different epitopes on gIV (van Drunen Littel-van den Hurk et al., 1985; Hughes et al., 1988). The properties of these monoclonal antibodies are summarized in Table 2. In order to analyse the influence of carbohydrate on the immunogenicity of specific epitopes on glycoproteins gI and gIV, the reactivity of the rabbit sera with these epitopes was determined in CBAs. Both gI- and dgI-specific rabbit antibodies recognized epitopes I, II, III and V, as they both competed successfully and at the same level with monoclonal antibody conjugates 1B10, 3F3, 1E11 and 1F10, respectively. However, dgI-specific antibodies competed with monoclonal antibodies 1F8, 5G2, 3G11 and 6G11 at much lower levels than gI-specific antibodies, indicating that the immunogenicity of epitopes IVa, IVb and IVc, respectively, is dependent upon the presence of carbohydrate (Fig. 3).

No difference between the maximum level of competition reached by the gIV- and dglV-specific rabbit antibodies was observed with any of the monoclonal antibodies, indicating that none of the epitopes which have been defined to date, Ia, Ib, II, III\(a\), III\(b\), III\(c\), III\(d\) and IV, are carbohydrate-dependent (Fig. 4).

**Effect of deglycosylation on the antigenicity of gI and gIV**

The ability of specific monoclonal antibodies to bind to deglycosylated gI and gIV was analysed in an ELISA, in

neutralizing antibody titres were determined for the second bleed in the absence (lower, white bars) and presence (shaded bars) of guinea-pig complement. The titres were expressed as a 50% endpoint using 100 p.f.u. of BHV-1. The difference between gI- and dgI-specific antibody titres was significant (\( P < 0.05 \); paired t-test). (c) The AbC lysis was determined at a 1:10 dilution of the second bleed and a 1:40 dilution of guinea-pig complement and expressed as percentage specific \( ^{51} \)Cr release. The difference in percentage specific \( ^{51} \)Cr release between gI- and dgI-specific sera was significant (\( P < 0.02 \); paired t-test).
Table 2. Properties of monoclonal antibodies to glycoproteins gI and gIV

<table>
<thead>
<tr>
<th>Antibody designation</th>
<th>Antigen specificity*</th>
<th>Epitope specificity†</th>
<th>Isotype</th>
<th>Conformation dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B10</td>
<td>gI</td>
<td>I</td>
<td>IgG1</td>
<td>-</td>
</tr>
<tr>
<td>3F3</td>
<td>II</td>
<td>IgG2b</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1E11</td>
<td>III</td>
<td>IgG2a</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1F8</td>
<td>IVa</td>
<td>IgG2a</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5G2</td>
<td>IVb</td>
<td>IgG2b</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3G11</td>
<td>IVb</td>
<td>IgG2a</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5G11</td>
<td>IVc</td>
<td>IgG1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6G11</td>
<td>IVc</td>
<td>IgM</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1F10</td>
<td>V</td>
<td>IgG2a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2C5</td>
<td>V</td>
<td>IgG2a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>GlV</td>
<td>Ia</td>
<td>IgG1</td>
<td>+</td>
</tr>
<tr>
<td>9D6</td>
<td>Ib</td>
<td>IgG1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3F7</td>
<td>II</td>
<td>IgG1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1OC2</td>
<td>IIIa</td>
<td>IgG1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4C1</td>
<td>IIIb</td>
<td>IgG1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>IIIc</td>
<td>IgG1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3C1</td>
<td>IIIId</td>
<td>IgG1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3D9S</td>
<td>IV</td>
<td>IgG1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Antigen specificities were determined by immunoprecipitation, immunoblot analysis and ELISA (van Drunen Littel-van den Hurk et al., 1984; Hughes et al., 1988).
† Epitope specificities were determined by competition binding assays (van Drunen Littel-van den Hurk et al., 1985; Hughes et al., 1988) and the epitopes of gI have been mapped using deletion mutants (Fitzpatrick et al., 1990).

Table 3. Reactivity of gI-specific monoclonal antibodies with native and deglycosylated gI

<table>
<thead>
<tr>
<th>Antibody designation</th>
<th>Epitope specificity†</th>
<th>ELISA* titre against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gI</td>
</tr>
<tr>
<td>1B10</td>
<td>I</td>
<td>2.5 x 10^4</td>
</tr>
<tr>
<td>3F3</td>
<td>II</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>1E11</td>
<td>III</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>1F8</td>
<td>IVa</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>5G2</td>
<td>IVb</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>3G11</td>
<td>IVb</td>
<td>1 x 10^3</td>
</tr>
<tr>
<td>5G11</td>
<td>IVc</td>
<td>4 x 10^3</td>
</tr>
<tr>
<td>6G11</td>
<td>IVc</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>1F10</td>
<td>V</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>2C5</td>
<td>V</td>
<td>1.6 x 10^6</td>
</tr>
</tbody>
</table>

* ELISA titres were expressed as the reciprocal of the highest dilution that still gave a reading of at least 0.1. The titre of gI-specific rabbit serum was 3 x 10^5 both against gI and dgI.
† Epitope specificity of the monoclonal antibodies was determined using competition binding assays (van Drunen Littel-van den Hurk et al., 1985).

an attempt to identify changes in antigenicity induced by carbohydrate removal. Table 3 shows that binding of monoclonal antibodies 1E11 (epitope III), 1F10 and 2C5 (epitope V) to gI is independent of or perhaps even slightly enhanced after deglycosylation. In contrast, removal of carbohydrates resulted in strongly reduced binding of monoclonal antibodies 1F8 (epitope IVa),
Carbohydrate role in BHV-1 glycoproteins

Fig. 4. Competition binding assays of sera from rabbits immunized with 50 µg of glV (○) or with 50 µg of dglV (●) with HRPO-conjugated glV-specific monoclonal antibodies 136 (epitope Ia), 9D6 (epitope Ib), 3E7 (epitope II), 10C2 (epitope IIIa), 4C1 (epitope IIIb), 2C8 (epitope IIIc), 3C1 (epitope IIIId) and 3D9S (epitope IV).

Fig. 5. Immunoblot analysis of gl-specific monoclonal antibodies. Affinity-purified gl was electrophoretically separated into gla, pgla, glb and glc, transferred to nitrocellulose and incubated with monoclonal antibodies 1B10, 1E11 and 1F8. Monoclonal antibodies 3F3 and 1F10 reacted like 1E11, whereas 5G2, 3G11, 5G11 and 6G11 reacted like 1F8. Positions of M, markers are shown in the right margin.

5G2, 3G11 (epitope IVb), 5G11 and 6G11 (epitope IVc), which is in agreement with the reduced ability of epitopes IVa, IVb and IVc to induce an immune response in rabbits. Monoclonal antibodies 1B10 (epitope I) and 3F3 (epitope II) bound with slightly reduced efficiency to the deglycosylated protein as compared to the native glycoprotein, which, however, was not reflected in their capacity to induce an antibody response in rabbits.

In order to investigate the extent of carbohydrate dependence, the reactivity of gl-specific monoclonal antibodies with gla and pgla (high-mannose intermediate form of gl) was determined in a Western blot assay. Fig. 5 shows that monoclonal antibodies 1B10 and 1E11 reacted with pgla, whereas monoclonal antibody 1F8 did not recognize the high-mannose intermediate
precursor. Monoclonal antibodies 3F3, 1F10 and 2C5 showed the same reactivity pattern as 1E11, whereas monoclonal antibodies 5G2, 3G11, 5G11 and 6G11 reacted like 1F8. This not only confirms the data from the ELISA, but also suggests that the carbohydrates in epitopes IVa, IVb and IVc need to be processed for maximum antigenicity and immunogenicity.

No changes in antigenicity were observed after carbohydrate removal from gIV (ELISA data not shown), which again suggests that epitopes Ia, Ib, II, IIIa to d and IV are carbohydrate-independent.

**The role of carbohydrate in the DTH response**

The effect of carbohydrate removal on the cell-mediated immune response was determined by measuring the DTH response following ear challenge in rabbits that had been immunized with 5 μg of native or deglycosylated glycoprotein. Fig. 6 indicates that the DTH response was 1.5-fold higher in animals immunized with dgI than in animals that received gI. The difference in DTH response between gIV- and dgIV-immunized animals was twofold, which appeared to be significant ($P < 0.05$; analysis of variance analysis).

Discussion

It has been shown for a number of viruses that the carbohydrate moiety of the viral glycoproteins can have a strong influence on functional as well as antigenic activities (Basak & Compans, 1983; Lambert, 1988; Alexander & Elder, 1984; Elder et al., 1986). Since the major glycoproteins of BHV-1 are heavily glycosylated (van Drunen Littel-van den Hurk & Babiuk, 1985a, 1986b), we were interested in exploring the contribution of the carbohydrate portion of these glycoproteins in the induction of an immune response to BHV-1, both at the humoral and at the cellular level.

Following treatment of gI and gIV with the appropriate endoglycosidases, the $M_r$ values of the resulting deglycosylated proteins corresponded well with those previously determined (van Drunen Littel-van den Hurk & Babiuk, 1986b) for the polypeptide backbones of these glycoproteins, indicating that complete or virtually complete deglycosylation had been achieved. This was confirmed by the absence of any reactivity of the deglycosylated proteins with a series of lectins, which did show a strong binding affinity for native gI and gIV.

The humoral immune response to the deglycosylated proteins appeared to be significantly lower than that against the native glycoproteins, suggesting that the immunogenicity of at least some epitopes on gI and gIV is carbohydrate-dependent. However, in the case of gIV, the neutralizing antibody response and the ability of the antibodies to mediate cell lysis were not significantly reduced, indicating that most functional epitopes on this glycoprotein were unaffected by deglycosylation. In the case of gI, the capacity to mediate cell lysis and the neutralizing antibody response was significantly reduced by loss of carbohydrate. Consequently, the immunogenicity of the neutralizing epitopes on gI appeared to be preferably affected by deglycosylation, or the response to the most strongly neutralizing epitopes may have been modified. Alternatively, the decreased neutralizing capacity of antibodies to dgI may be due to the induction of a different immunoglobulin subclass by the deglycosylated protein. This possibility is presently under investigation.

Seven different epitopes on gI, as well as eight epitopes on gIV, have been mapped in our laboratory, using two panels of monoclonal antibodies in competition binding assays (van Drunen Littel-van den Hurk et al., 1985; Hughes et al., 1988). However, the decreased induction of gIV-specific antibodies after carbohydrate removal from this glycoprotein could not be attributed to any of these epitopes. The reactivity of monoclonal antibodies to these epitopes also remained unchanged after deglycosylation of gIV, suggesting that both the antigenic and the immunogenic structure of the eight epitopes on gIV, seven of which are neutralizing, is carbohydrate-independent.

In contrast, three of the carbohydrate-dependent epitopes on gI were shown to correspond to neutralizing
Carbohydrate role in BHV-1 glycoproteins

epitopes IVa, IVb and IVc (van Drunen Littel-van den Hurk et al., 1985). Two of these epitopes, IVa and IVb, are also involved in infected cell lysis. Epitope IVc either does not mediate cell lysis, or the IVc-specific monoclonal antibodies 5G11 and 6G11 are of the wrong epitopes IVa, IVb and IVc are carbohydrate-dependent, in contrast with the carbohydrate-independence of epitopes I, II, III and V. Epitope IV has been mapped to the amino terminus of gI between residues 68 and 119 (Fitzpatrick et al., 1990). This region contains a potential N-linked glycosylation site at position 105 of the gI sequence, further suggesting carbohydrate dependence of this epitope. As these epitopes appear to be continuous, as is evident from their reactivity in a Western blot assay under denaturing conditions, the carbohydrate is probably involved as a component of the epitopes, rather than in stabilization of this glycoprotein. This is supported by the observation that the carbohydrate side-chains of these epitopes have to be processed in order to be fully recognized by the monoclonal antibodies specific for epitopes IVa, IVb and IVc. Alternatively, these epitopes may seem to be continuous because they are protected from complete denaturation by the presence of the carbohydrates. In this case, protection is only completely achieved by the processed oligosaccharides. Removal or partial absence of carbohydrates would then result in loss of protection, as well as of antigenic and immunogenic structure.

In contrast to the humoral immune response, the cell-mediated immune response, as measured by a DTH reaction, was higher in rabbits immunized with deglycosylated proteins than in those inoculated with native glycoproteins gI and gIV. This indicates that the carbohydrates may mask the induction or recognition of certain T cell epitopes, particularly on gIV, as the DTH response between gIV and dgIV immunized animals was significantly different.

The presence of carbohydrate can influence the antigenicity and immunogenicity of viral glycoproteins in a number of ways. The immunological reactivity of avian myoblastosis virus gp85 appears to depend on an intact carbohydrate side-chain (van Eldik et al., 1978). In addition, the reactivity of polyclonal and monoclonal antibodies to the influenza virus haemagglutinin and the gp70 of Rauscher murine leukaemia virus was dramatically decreased following endo F treatment (Alexander & Elder, 1984). In these cases, carbohydrate may either be directly involved as part of an epitope or it may stabilize a certain conformation of the protein. However, the carbohydrate on gp70 may not be needed to elicit a neutralizing antibody response and in fact may even protect the virus from immune surveillance during infection (Elder et al., 1986). Similar observations were reported for the gp51 of bovine leukaemia virus (Portetelle et al., 1980, Bruck et al., 1982). By contrast, the reactivity of polyclonal and monoclonal antibodies defining eight epitopes on the E protein of a flavivirus was not affected by endo F treatment of this glycoprotein (Winkler et al., 1987).

In the case of BHV-1, the effect of carbohydrate was studied at both the humoral and the cellular level of the immune response. Our data suggest that the carbohydrate moieties of glycoproteins gI and gIV do play a role in the host's immune recognition and response. Deglycosylation of these glycoproteins resulted in a decreased ability to elicit antibodies involved in virus neutralization and killing of virus-infected cells and an increased capacity to induce a DTH response in rabbits. These results indicate that the carbohydrates on gI and gIV may play a dual role in the host's immune recognition and response to these glycoproteins by contributing to certain epitopes required for the B cell response, but masking other epitopes involved in T cell recognition. In the mouse system, two types of helper T cell clones have been identified (Mossmann et al., 1986; Kurt-Jones et al., 1987). The first type of helper T cell, designated Th1, produces interferon γ and interleukin-2 (IL-2), which stimulate growth of T cells, and the second type, Th2, synthesizes IL-4 and IL-5, which stimulate B cell growth and antibody responses. The potential existence of such types of helper cells in the rabbit system would explain the different effects of deglycosylation of gI and gIV on the antibody and DTH responses. Th1 analogues might recognize epitopes that are masked by the carbohydrates, whereas Th2 analogues might recognize epitopes in conjunction with carbohydrates.

Both glycoproteins gI and gIV are good candidates for a subunit vaccine to BHV-1 (Babiuk et al., 1987). In order to be produced efficiently they will have to be expressed in a system employing a vector such as vaccinia virus, baculovirus or others. We have shown that vaccinia virus recombinant glycoproteins and authentic BHV-1 glycoproteins are glycosylated in a very similar manner (van Drunen Littel-van den Hurk et al., 1989). However, glycoproteins expressed in a baculovirus system have been reported to lack terminal carbohydrates (Luckow & Summers, 1988). The observation that the carbohydrates may be needed as part of at least three neutralizing epitopes on gI, whereas most neutralizing epitopes of gIV appear to be carbohydrate-independent, may have important implications for choosing appropriate expression systems for the production of these glycoproteins.
We thank Melanie Elliott and Terry Beskorwayne for their technical assistance, the animal support staff at VIDO for care and handling of the animals, Richard Harland for assistance with the statistical analyses, and Marilee Hagen for typing his manuscript. This work was supported by grants from the Medical Research Council and the National Science and Engineering Council of Canada, Farming for the Future and the Saskatchewan Agricultural Research Foundation.

References


Carbohydrate role in BHV-1 glycoproteins


(Received 28 November 1989; Accepted 23 April 1990)