Glycoprotein 60 of equine herpesvirus type 1 is a homologue of herpes simplex virus glycoprotein D and plays a major role in penetration of cells

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Monoclonal antibodies (MAbs) specific for equine herpesvirus type 1 (EHV-1) glycoprotein 60 (gp60) and gp17/18 (F3132 and 5H6 respectively) were found to react with the same protein, which was identified as a homologue of herpes simplex virus type 1 gD. MAb F3132 strongly neutralized virus infectivity and inhibited the penetration of the virus into the cell. The effects on penetration were shared with three other MAbs against this protein (P68, F3116 and F3129), but no effect on virus penetration was found with any other anti-EHV-1 MAb tested. The level of glycosylation of gp60 was analysed using glycanase enzymes and glycosylation inhibitors, and consisted of mainly N-linked carbohydrate. The Mr of non-N-glycosylated gp60 was 50K.

Introduction

Equine herpesvirus type 1 (EHV-1) is a major infectious agent of the horse and is responsible for respiratory disease, abortion, paresis and encephalitis (Campbell & Studdert, 1983). EHV-1 glycoproteins have been studied by several groups (Turtinen & Allen, 1982; Allen & Bryans, 1986; Meredith et al., 1989), and between nine and 11 have been identified. Genes encoding six major glycoproteins have been mapped to the virus genome (Allen & Yee, 1987), but only one of these glycoproteins (gp17/18) maps to the short unique region. The region of the genome containing the gp17/18 gene has since been shown to contain three glycoprotein genes which are homologues of herpes simplex virus type 1 (HSV-1) genes encoding gE, gI and gD (Audonnet et al., 1990; Elton et al., 1991), but the identity of gp17/18 remains to be established. Little is known about gp17/18, although a monoclonal antibody (MAb) (5H6) directed against the protein passively protects hamsters against EHV-1 challenge (Stokes et al., 1989).

HSV gD is an essential glycoprotein (Ligas & Johnson, 1988), a potent inducer of neutralizing antibodies (Para et al., 1985) and has received particular attention as a component of a potential subunit vaccine (Long et al., 1984). Antibodies to HSV-1 gD block penetration of virus particles into the cell (Fuller & Spear, 1987; Highlander et al., 1987) and other evidence also implicates gD in the entry process, because entry of HSV-1 into gD-expressing cells is blocked at the level of penetration (Campadelli-Fiume et al., 1988, Johnson & Spear, 1989). Two explanations have been proposed to account for this effect. Either expressed gD competes for the binding of viral gD to a host cell receptor necessary for penetration of the virus (Campadelli-Fiume et al., 1988; Johnson et al., 1990), or recombinant (or viral) gD acts to prevent re-infection of cells by released virus (Campadelli-Fiume et al., 1990). Furthermore, a role in virus egress has been postulated (Johnson & Spear, 1989).

Genes homologous to gD have been found in other members of the alphaherpesvirinae. Whereas HSV-1 gD is both N- and O-glycosylated (Cohen et al., 1986, Serafini-Cessi et al., 1988), pseudorabies virus (PRV) gp50 is glycosylated by O-linked carbohydrate only (Petrovskis et al., 1986). This protein has also been shown to protect both mice and pigs against PRV infection (Marchioli et al., 1987). Bovine herpesvirus type 1 (BHV-1) gIV has also been identified as a gD homologue (Tikoo et al., 1990), and, when expressed in bovine cells, protects these cells against infection with BHV-1, PRV and HSV-1 (Chase et al., 1990).

We show here that the proteins described previously as gp17/18 and gp60 are the same glycoprotein, and that this glycoprotein shares homology with HSV-1 gD. In addition we show that this glycoprotein is mainly N-glycosylated, and that MAb raised against it neutralize virus infectivity and prevent the penetration of EHV-1 into cells.
Methods

Cell culture, virus strains and virus purification. EHV-1 (strain Ab-1) and EHV-4 (strain MD) were cultivated in RK-13 cells and NBL-6 cells respectively, and purified from the extracellular growth medium using standard techniques (Meredith et al., 1989). For transfection, COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 2 mM-glutamine, 0.1% (w/v) sodium bicarbonate, 0.1 mg/ml penicillin, 0.12 mg/ml streptomycin and 10% (v/v) foetal calf serum.

Transfection of cells. Subconfluent monolayers of COS-7 cells were grown on glass coverslips and transfected with DNA using a modified DEAE-dextran technique. Coverslips were rinsed twice with PBS and then 500 µl DEAE-dextran (1 mg/ml) containing 1 µg CsCl-purified plasmid DNA was added. Cells were incubated at 37 °C for 30 min, and 5 ml medium containing 100 µM-chloroquine in PBS (to inhibit lysosomal degradation of DNA) was added. Cells were then incubated at 37 °C for a further 4 h and washed three times with medium. Fresh medium was then added and cells were incubated at 37 °C for 48 h. Transfected cells were then analysed by immune fluorescence.

Immune fluorescence. Cells were rinsed in PBS, fixed with ice-cold methanol for 10 min at room temperature and washed with PBS. Cells were blocked with 10% (v/v) calf serum in PBS for 20 min and MAb ascitic fluid was added at a dilution of 1:50 in 10% calf serum. Coverslips were then washed three times in PBS pH 6.8 and fluorescein-conjugated horse anti-mouse IgG (Vector) was added for 30 min at a dilution of 1:50 in 10% calf serum. After washing three times in PBS, coverslips were mounted in glycerol and observed using a Zeiss Axioscope fluorescence microscope.

Iodination of purified virus. Glycoproteins were iodinated by resuspending freshly purified virus in 100 µl sodium borate buffer pH 8.6, 0.5% (w/v) Triton X-100, and mixed with 50 µCi 125I-labelled Bolton and Hunter reagent (2000 mCi/mmol; NEN) dissolved in anhydrous propanol. This was incubated on ice for 2 h and the reaction terminated by the addition of 100 µl 0.2 M-glycine. Iodinated material was then desalted using a 45 × 0.5 cm column of Bio-Gel P-6DG (Bio-Rad) equilibrated in PBS.

SDS–PAGE and Western blotting. These were carried out essentially as described previously (Meredith et al., 1989). Gels contained 9% (w/v) acrylamide and were cross-linked with N,N-diallyltartardiamide. Reduced samples contained 10 mM-DTT, whereas non-reduced samples contained no DTT. Western blots were blocked either with 10% (w/v) calf serum (for MAb P68) or with 1% (w/v) non-fat dried milk (for MAb F3132). Primary antibody was used at a dilution of 1:50 and secondary antibody was either peroxidase-linked rabbit anti-mouse immunoglobulin (Dako) or at a dilution of 1:2000. Bound material was then eluted with SDS–PAGE sample buffer at 100 °C for 5 min, and samples were subjected to SDS–PAGE and autoradiography.

Table 1. MAbs used in this study

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<tr>
<th>MAb</th>
<th>Reactive protein</th>
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<tr>
<td>5H6</td>
<td>gp17/18*</td>
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<tr>
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<td>92b</td>
<td>EHV-1 gC†</td>
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<td>P50</td>
<td>EHV-1 gB</td>
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* Data from Allen & Yeargan (1987).
† Data from Stokes et al. (1990).

MAbs. Murine MAbs were prepared by inoculation of BALB/c mice with purified EHV-1 as described previously (Killington et al., 1981). Positive clones were selected by screening tissue culture supernatants by either microneutralization or by an ELISA using purified virus as the antigen source, and these were then cloned by limiting dilution. The specificity of MAbs was then determined by Western blotting using purified virus as the antigen source.

Ascitic fluid containing MAb was produced in BALB/c mice pretreated with either pristane or Freund's incomplete adjuvant. MAb 5H6 was kindly provided as ascitic fluid by Dr G. Allen, University of Kentucky, Lexington, Ky., U.S.A. The MAbs used in this study are summarized in Table 1.

MAb ascitic fluid was purified by addition of 25% (w/v) sodium sulphate to a final concentration of 18% (w/v). This was mixed, incubated at room temperature for 30 min and centrifuged at 10000 g for 10 min. The pellet was then washed with 18% (w/v) sodium sulphate, recentrifuged at 10000 g for 5 min and the pellet was resuspended in PBS. The protein concentration of purified ascitic fluid was determined by using a commercial kit (Bio-Rad Protein Assay) and the concentration of MAb in unpurified ascitic fluid was determined by a comparative ELISA with purified antibody.

To label antibodies with biotin, purified MAb (1 to 10 mg/ml in PBS) was adjusted to pH 9.0 with 0·5 M-sodium carbonate (pH 9.0) and mixed with biotin-N-hydroxysuccinimide ester (Sigma) (50 mg/ml in anhydrous dimethyl formamide) at a ratio of 1:5 (weight of biotin ester:weight of antibody). This was incubated for 3 h at room temperature and the reaction stopped by the addition of ammonium chloride to a final concentration of 0·1 M. Free biotin ester was then removed by dialysis against PBS, and the sample was lyophilized and redissolved in PBS.

Treatment with glycosidases and glycosylation inhibitors. These were as described previously (Whittaker et al., 1990). Briefly, for enzymic deglycosylation, purified EHV-1 was treated with 1% (w/v) Triton X-100 and then with either N-glycosidase [0·5 units (U); Boehringer Mannheim], neuraminidase (0·2 U; Sigma), or with a combination of β-N-acetyl hexosaminidase (Sigma), α-L-fucosidase (Sigma) and β-galactosidase (Boehringer Mannheim) (0·01 U each).

For treatment with glycosylation inhibitors, RK cells were infected with 10 p.f.u. EHV-1. Cells were treated with 2 µg/ml tunicamycin (Sigma) or 0·2 µM-monomosin (Sigma) from the end of the adsorption period (1 h) until 21 h post-infection, or remained untreated.

Plaque reduction assay. EHV-1 (500 p.f.u.) was incubated at room temperature for 30 min with serial dilutions of MAb in serum-free medium containing either 10 µl of a 1:20 dilution of guinea-pig complement (167 CH50 U/ml; Sigma), or without complement. Complement was used at a concentration that had no effect on virus infectivity, but gave neutralization of infectivity with complement-
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dependent anti-EHV-1 antibodies (data not shown). The virus inoculum was allowed to adsorb onto a monolayer of RK cells at 37 °C for 45 min. Cells were overlaid with medium containing 0.45% (w/v) high viscosity carboxymethyl cellulose (CMC; Sigma) and incubated at 37 °C for 3 days. Plaques were then visualized by treatment with formal saline and gentian violet.

Assay of virus penetration. The procedure used was essentially that of Rosenthal et al. (1985). Confluent monolayers of RK cells in 24-well plates were incubated at 4 °C for 30 min before use, infected with EHV-1 and incubated at 4 °C for 30 min to allow virus adsorption, but not penetration, to take place. MAbs were then added at various dilutions, and cells incubated at 4 °C for a further 30 min. Cells were then shifted rapidly to 37 °C and incubated at this temperature for 20 min (to allow virus penetration to take place). Cells were washed three times with PBS and virus remaining on the cell surface was neutralized by incubation with 0.1 M-glycine, 0.1 M-NaCl, pH 3.0 for 1 min. Cells were then washed three times with PBS, overlaid with cell culture medium containing 0.45% (w/v) high viscosity CMC and incubated at 37 °C for 3 days. Plaques were visualized by fixing cells with formal saline and staining with gentian violet. A zero time control was obtained for each MAb by treating with pH 3.0 buffer before the temperature shift.

Results

Identification of EHV-1 gp17/18 as the homologue of HSV-1 gD

The DNA sequence of the EHV-1 homologue of the HSV-1 gD gene has been determined for EHV-1 strain Ab-1 and this has facilitated the cloning of the gene into plasmid expression vectors. This sequence was essentially the same as that of the Kentucky D strain (Audonnet et al., 1990), but differed at the C terminus from the Kentucky A sequence published by Flowers et al. (1991).

A 1242 bp HindIII fragment of the EHV-1 gD gene, corresponding to nucleotides 983 to 2221 of the Kentucky D sequence (Audonnet et al., 1990), was cloned into the HindIII site of pRSVori, a vector previously used for the expression of EHV-1 gB (Bonass et al., 1990), for expression under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter (Fig. 1). This fragment contains an open reading frame and three potential initiation codons corresponding to those at positions 989, 992 and 1016. These three potential initiation codons lie within a hydrophobic region predicted to be the signal sequence (Audonnet et al., 1990). This fragment would be expected to encode all the amino acids found in the predicted mature protein.

The gene was cloned in the correct orientation for expression, giving plasmid pDE402, which was used to transfect COS-7 cells. Fig. 2 shows immune fluorescence of COS-7 cells transfected with the EHV-1 gD gene and reacted with 5H6, the MAb originally used to map the gp17/18 gene (Allen & Yeargan, 1987). This shows that the EHV-1 gD gene product reacts with MAb 5H6 and confirms that gp17/18 is the homologue of HSV-1 gD.

Fig. 2 shows a representative field of view and demonstrates the presence of recombinant EHV-1 gD in both the perinuclear region and within membrane organelles, and also at the plasma membrane, suggesting that the gene product is being correctly transported to the cell surface.

Reaction of MAbs to gp60 and gp17/18 on Western blots and by immune precipitation

The protein identified as gp17/18 (Allen & Yeargan, 1987) and the protein described previously as gp60 (Meredith et al., 1989) have the same Mr on SDS-PAGE. Therefore, it was of interest to determine whether gp17/18 and gp60 were the same protein. This was achieved by performing an immune precipitation with the anti-gp17/18 MAb (5H6). This immune precipitate was then separated by SDS-PAGE using non-reducing conditions, transferred to nitrocellulose and reacted with the biotinylated anti-gp60 MAb F3132. This reaction is shown in Fig. 3. It is clear that the protein of approximately 60K that is immune-precipitated with MAb 5H6 also reacts with MAb F3132 on the Western blot, demonstrating that gp60 and gp17/18 are the same protein.

MAb P68 immune-precipitated an iodinated protein of 60K from 125I-labelled purified EHV-1 (Fig. 4) and a band of identical Mr, (60K) reacted when EHV-1 was subjected to SDS-PAGE and Western blotting, followed by reaction with MAb P68 (Fig. 5). When analysed under non-reducing conditions a shift in mobility from 60K to 53K was seen. This is probably due to the non-
reduced form of the protein having a more tightly folded structure than the reduced form, resulting in a lowered retardation of the non-reduced form during electrophoresis. MAb P68 also cross-reacted with EHV-4. With the EHV-4 strain MD bands of 58K (reduced) and 53K (non-reduced) were seen (Fig. 5). MAb F3132 gave essentially the same results, but reacted on Western blots only with the non-reduced form of the protein (data not shown) and showed no cross-reactivity with EHV-4.

**Carbohydrate content of gp60**

Purified virus was treated with both endo- and exoglycanases to study the amount and form of the glycosylation on gp60. Enzyme-treated material was subjected to SDS-PAGE and Western blots were developed with MAb P68, which is specific for gp60. The enzyme N-glycanase is an endoglycanase which has been shown to remove all forms of N-linked carbohydrate from glycoproteins (Tarentino et al., 1985); treatment of EHV-1 with this enzyme resulted in a shift in the $M_r$ of gp60 from 60K to 50K (Fig. 6). When EHV-1 was treated with the exoglycanase neuraminidase, or a combination of the exoglycanases hexosaminidase, fucosidase and $\beta$-galactosidase (which were chosen for their ability to cleave sugars present in O-linked glycosylated proteins), a slight alteration in mobility was seen in each case (Fig. 6a). This shift in mobility (equivalent to approximately 1K to 2K) may represent the cleavage of terminal N-linked sugars or sugars present in O-linked glycosylated proteins.

The glycosylation on gp60 was also studied by the use of the glycosylation inhibitors tunicamycin and monensin. Cell extracts were probed with MAb P68. In the absence of glycosylation inhibitor, gp60 was present as a
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set of closely spaced bands with an average $M_r$ of 60K (Fig. 6b). When EHV-1-infected cells were treated with tunicamycin, a shift in the $M_r$ of gp60 from 60K to 50K was found, but after treatment with monensin (Fig. 6b) the antibody failed to react.

Neutralization of EHV-1 infectivity

F3132 was originally isolated as an EHV-1-specific antibody by use of a microneutralization assay. To study the effects of this MAb on virus neutralization further, a plaque reduction assay was used and the results of this are shown in Fig. 7. MAb F3132 effectively neutralized virus infectivity with a 50% endpoint of 3 μg/ml. As a control an MAb (92b) specific for EHV-1 gC (Stokes et al., 1991) was used and this showed no neutralizing activity. The levels of infectivity appeared to be slightly higher with MAb 92b than with cells infected in the absence of antibody. The experiments were repeated in the presence of complement and were found to give essentially the same result (data not shown). The neutralization kinetics of F3132 show that the rate of loss of virus infectivity is proportional to the concentration of antibody, and is consistent with the 'one-hit' theory of virus neutralization, i.e. that the antibody is acting at a single site on the protein.

The experiments involving virus neutralization were repeated and modified so that the antibody was present only during the initial period of virus adsorption (data not shown). In this case MAb F3132 neutralized virus infectivity, again with a 50% endpoint of approximately 3 μg/ml. MAbs specific for other EHV-1 glycoproteins [F3123, anti-gp300; P50, anti-gp76/58 (EHV-1 gB); P28 and anti-gp88 (EHV-1 gC)] were also tested for their effects on virus neutralization during the adsorption period. In all cases these MAbs showed no neutralizing activity and resulted in an increase in overall infectivity.

Role of anti-EHV-1 antibodies in virus penetration

A range of MAbs specific for both gp60 and for other EHV-1 glycoproteins was tested for effect on the penetration of EHV-1 into RK cells. This assay relies on the adsorption of virus at low temperatures, such that membrane fluidity is lowered to the point where the virus envelope and the cell membrane cannot fuse, and
penetration does not occur. Penetration is then initiated by a shift up in temperature. MAbs can be tested for effects on penetration by treatment of adsorbed virus with antibody prior to the temperature shift, and any unpenetrated virus is neutralized by brief incubation with a low pH buffer. Penetrated virus is allowed to form plaques and these are counted as an assay of virus penetration. Initial experiments were carried out which showed that EHV-1 penetration of RK cells occurred within 20 min of temperature shift, and that treatment of cells with pH 3.0 buffer had no major effect on the viability of RK cells or of virus that had penetrated the cell (data not shown).

All of the anti-gp60 MAbs tested (F3132, F3116, F3129 and P68) blocked the penetration of the virus (Fig. 8a). F3132 was most effective and blocked penetration with a 50% endpoint of 3 μg/ml, and the other MAbs gave a 50% endpoint of around 10 μg/ml. MAbs F3123 (anti-gp300), P28 (anti-EHV-1 gC) and P50 (anti-EHV-1 gB) had no effect on virus penetration (Fig. 8b).

The experiments to study the penetration of EHV-1 in the presence or absence of MAb F3132 were repeated and samples studied by electron microscopy (data not shown). In the presence of F3132 the majority of virus was present either attached to the cell surface or detached from the cell. A small amount of virus could be observed as unenveloped capsid directly below the cell membrane. In the absence of antibody no virus was visible either on the cell surface or cell-free. It was difficult to demonstrate the presence of capsids within the cell, but in a few cases capsids were visible in the cytoplasm approximately equidistant between the cell surface and the nuclear membrane.

**Discussion**

The experiments reported here characterize the EHV-1 homologue of HSV-1 gD as a glycoprotein that plays a major role in the penetration of the virus into cells. The experiments described clearly show that the proteins previously described as gp17/18 (Allen & Yeargan, 1987) and gp60 (Meredith et al., 1989) are the same, and that an equivalent protein exists in EHV-4. The effects of this antibody on the infectivity of EHV-4 or any effects on EHV-4 penetration into cells have yet to be determined. Other major glycoproteins of EHV-1 are increasingly being known by the nomenclature of their HSV-1 counterparts, e.g. EHV-1 gB and gC. The availability of
DNA sequence data for EHV means that this type of nomenclature will become even more common, and it is therefore proposed that the proteins previously described as gp17/18 and gp60 be referred to as the EHV-1 gD homologue.

Expression of the EHV-1 gD gene showed that the protein localized to the cell surface in addition to internal membranes. The expressed gene product may lack the first six amino acids of the authentic signal sequence, if translation normally initiates at the preferred ATG codon at position 971 (Audonnet et al., 1990). It may be that this sequence is dispensable for the correct processing and localization of the protein, or that in vivo internal ATG residues operate to initiate translation, giving a mixed population of gD molecules. It is also possible that the correct initiation codon is included within the cloned fragment.

EHV-1 gD shows a great deal of similarity with its HSV-1 counterpart, as both glycoproteins are potent inducers of neutralizing antibodies and these can block the penetration of the virus into cells. Glycoprotein D has been studied extensively, and the domains on the protein that are involved in its biological and immunological activities have been mapped with a panel of gD-specific MAbs (Dietzschold et al., 1984; Cohen et al., 1984; Eisenberg et al., 1985). However, the residues of gD responsible for the penetration event remain to be determined. The neutralizing antibody F3132 that blocked EHV-1 penetration appeared to have no effect on virus adsorption, and this property is shared with anti-HSV-1 gD antibodies. A separate panel of weakly neutralizing anti-gD antibodies inhibit HSV-1 adsorption (Fuller & Spear, 1985). MAb F3132 was tested for its effects on EHV-1 adsorption by analysing the binding of [3H]thymidine-labelled stocks of virus to RK cells (data not shown), but no effect on the adsorption of the virus could be found.

The effects of neutralizing HSV-1 antibodies on penetration are not limited to gD; both gB and gH share this effect (Highlander et al., 1988; Fuller et al., 1989). Therefore it is possible that the process of virus penetration is either a multi-step event or can proceed by independent mechanisms. For EHV-1, genes homologous to gB and gH have been sequenced (Robertson & Whalley, 1988; Whalley et al., 1989), and the products of these genes identified (Meredith et al., 1989; Bonass et al., 1990; L. A. Taylor, unpublished results). The importance of these proteins for EHV-1 penetration remains to be determined. The MAb P50 (specific for EHV-1 gB) neutralizes virus infectivity in the presence of complement (L. A. Taylor, unpublished data) but has no effect on penetration. It will be of interest to test other EHV-1 gB-neutralizing antibodies (Shimizu et al., 1989) for their effects on virus penetration.

In addition to affecting penetration, MAbs to HSV-1 gD have been shown to affect cell–cell fusion (Noble et al., 1983; Minson et al., 1986). The EHV-1 antibodies used to block virus penetration were also tested for effects on cell–cell fusion. RK cells were infected at high multiplicity in the absence of antibodies; antibodies were then added and infection was allowed to proceed. None of the antibodies showed any effect on the cell–cell fusion typically seen with EHV-1-infected RK cells (data not shown). Anti-HSV-1 gD MAbs that neutralized infectivity in the absence of complement also showed no effect on cell–cell fusion (Minson et al., 1986), whereas other complement-dependent neutralizing antibodies affected the fusion processes.

HSV-1 gD is an essential glycoprotein (Ligas & Johnson, 1988) for penetration of virus into cells. It has yet to be determined whether EHV-1 gD is an essential glycoprotein, but this seems likely considering the pronounced effect that the protein appears to have on virus penetration. Other proposed functions of HSV-1 gD may indicate a role for the EHV-1 homologue in other processes such as cell–cell spread and virus egress.

The levels of glycosylation on EHV-1 gD are in general agreement with the predicted size (approximately 43K to 45K) of the primary translation product (Audonnet et al., 1990; Fuller et al., 1991). With approximately 10K of N-linked glycosylation and 1K to 2K of O-linked glycosylation, the M_r of the unglycosylated form of gp60 would be approximately 48K. The shift in mobility after neuraminidase treatment would indicate the presence of sialic acid residues, but a value for the M_r of these cannot be given because sialic acid has a pronounced effect on the charge of a protein and its subsequent migration on SDS–PAGE. Treatment of infected cells with tunicamycin gave a similar result to that obtained with N-glycanase treatment, confirming the presence of N-linked carbohydrate, but treatment with monensin resulted in a total lack of reaction with MAb P68. Monensin has a wide range of effects on the cell (Ledger & Tanzer, 1984), and so the lack of immunological reactivity may be due to factors other than glycosylation.

Much information on the role of HSV-1 gD has been generated by the use of cell lines that constitutively express the protein. We are currently designing a modified plasmid vector that will enable us to produce a cell line capable of stably expressing EHV-1 gD, to gain more insight into the role of this glycoprotein in the infectious process.

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