Characterization of the major glycoproteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3 using monoclonal antibodies

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A panel of 14 monoclonal antibodies (MAbs) was used to characterize the high abundance glycoproteins of equine herpesviruses 4 (EHV-4) and 1 (EHV-1), and asinine herpesvirus 3 (AHV-3). The specificities of the MAbs, which had been determined previously for strains of EHV-4 and -1 from the U.S.A., in general were confirmed by ELISA for Australian strains of these viruses. Of the 14 MAbs seven were EHV-4 and -1 type-common and cross-reacted with AHV-3. Of the five MAbs that were EHV-1 type-specific, four cross-reacted with AHV-3, whereas neither of the EHV-4 type-specific MAbs reacted with AHV-3, providing further evidence for a closer evolutionary relationship between EHV-1 and AHV-3 than that between either of these viruses and EHV-4. By Western blot and immunoprecipitation analyses, the identity of the six major glycoproteins, gp2, gpl0, gpl3, gpl4, gpl8 and gp21/22a, of an Australian EHV-1 isolate was verified, and it was shown that AHV-3 had cross-reactive glycoproteins of very similar Mr to those of EHV-1; five homologous glycoproteins of EHV-4 were also identified. It was determined that the EHV-4 gp13 homologue had a much reduced Mr (67K) when the virus was grown in a continuous cell line than when grown in equine foetal kidney cells (95K). It is suggested that altered glycosylation by the cell line is responsible for this change in Mr. Those glycoproteins acting as major immunogens in the naturally infected host, at least in their ability to elicit antibody, were identified. It was found that gp2, gp13, gp14, gp18 and a glycoprotein at 120K (EHV-1) or 116K (EHV-4) were all important immunogens in mares following EHV-1-induced abortion, and in a specific pathogen-free foal experimentally infected with EHV-1 and later cross-challenged with EHV-4. Gp2, gp14 and gp18 were the major immunogens in the donkey in response to AHV-3 infection. The type specificity associated with these glycoproteins was also examined and it was found that although most if not all contain type-specific epitopes, gp2 and a glycoprotein at 120K, and to a lesser extent gp13 and gp18, were significantly type-specific in the serum from a mare following natural EHV-1 infection and abortion.

Introduction

Equine herpesvirus 1 (EHV-1; equine abortion virus) and EHV-4 (equine rhinopneumonitis virus) are alphaherpesviruses which are the major cause of abortion in mares and of respiratory disease in young horses, respectively (Allen & Bryans, 1986; Studdert et al., 1981), and are of considerable economic importance worldwide. Recently we isolated a virus from three latently infected donkeys and used it to produce respiratory disease in seronegative weanling donkeys; we have called this virus asinine herpesvirus 3 (AHV-3). By DNA–DNA hybridization, serum neutralization and immunoprecipitation analyses, EHV-1 and AHV-3 appear to be more closely related to each other than to EHV-4 (Browning et al., 1988; Crabb & Studdert, 1990).

The clear recognition that EHV-4 and EHV-1 are separate and distinct viruses, and not minor variants (subtypes) came in 1981 when it was shown that the restriction endonuclease DNA fingerprints of the two viruses were distinctly different (Studdert et al., 1981; Sabine et al., 1981). This finding required a complete reappraisal of our understanding of the clinical diseases, rhinopneumonitis and abortion, particularly their pathogenesis, immunity and epidemiology, and also focused attention on the evolutionary relationships between the two viruses. Although the genomic architectures of EHV-4 and EHV-1 are similar (Whalley et al., 1981; Cullinane et al., 1988) and they appear to be collinear, it has been reported that there is less than 20% DNA–DNA similarity between them (Allen & Turtinen, 1982), although comparison of the predicted amino acid
sequence of several individual proteins, namely thymidine kinase, gB (gp14), gC (gp13) and gH, has revealed identities of 89\%, 88\%, 79\% and 85\% respectively (Nicolson et al., 1990a, b; Riggio et al., 1989; Nicolson & Onions, 1990). Of considerable interest is the antigenic relationship between EHV-4 and EHV-1 virions, and individual proteins, particularly glycoproteins. It is apparent that EHV-4 and EHV-1 possess many shared as well as unique antigenic determinants (Yeargan et al., 1985; Allen et al., 1988; Crabb & Studdert, 1990). In one study it has been shown that antibody raised against EHV-4 in specific pathogen-free (SPF) foals and rabbits effectively neutralizes both viruses to equal titre, although some sera, somewhat anomalously, neutralize EHV-1 to higher titre, whereas antibody raised against EHV-1 essentially neutralizes only EHV-1, i.e. there was a one way cross-reaction between the two viruses (Fitzpatrick & Studdert, 1984).

The glycoproteins of herpesviruses play important roles in pathogenicity, particularly in adsorption, penetration and in the mediation of cell-to-cell spread of virus (Spear, 1984). These functional roles, together with their location in the virion envelope and expression on the surface of infected cells, make them principal targets for host immune responses (Norrild, 1985). At least seven glycoproteins, gB, gC, gD, gE, gG, gH and gI, of herpes simplex virus type 1 (HSV-1) have been identified, of which gD, gB and gC are highly immunogenic and appear to be the principal inducers of antibody during HSV-1 infection (Marsden, 1987; Longnecker et al., 1987; Vestergaard, 1980).

Six high abundance EHV-1 glycoproteins, designated gp2, gp10, gp13, gp14, gp17/18 (now gp18; G. P. Allen, unpublished results) and gp21/22a, have been identified and their gene locations mapped using a lgt11 expression system and screening with monoclonal antibodies (MAbs) (Allen & Yeargan, 1987). EHV-1 gp13 and gp14 have been identified as homologues of HSV gC and gB respectively (Allen & Coogle, 1988; Allen & Yeargan, 1987; Meredith et al., 1989; Whalley et al., 1989). Recently, DNA sequence analysis has revealed EHV-1 and EHV-4 gH homologues (Robertson et al., 1990; Nicolson et al., 1990b), although it is not yet known whether any of the EHV-1 glycoproteins described above correspond to gH. A detailed understanding of the glycoproteins of EHV-4, EHV-1 and AHV-3, particularly their immunogenicity in the naturally infected host, is of significance in the search for more appropriate vaccines and diagnostic reagents for EHV-4 and EHV-1. The antigenic relatedness of individual glycoproteins may also help unravel the evolutionary relationship between these viruses.

In this paper we report the reactivity of a panel of 14 MAbs, raised against the six major EHV-1 and/or EHV-4 glycoproteins, with high titre, cell line-grown strains of EHV-4, EHV-1 and AHV-3 in ELISA. The MAbs were also used in Western blotting and radioimmunoprecipitation analysis (RIPA) to identify the location of the major glycoproteins after electrophoretic separation of viral proteins on polyacrylamide gels, and describe a form of gp13 (the HSV gC homologue) of unusually low Mr. Those glycoproteins which are the major post-infection immunogens in the natural host, at least in their ability to elicit antibody, were identified and the degree of type-specificity associated with them is discussed.

**Methods**

**Cells and viruses.** Virus strains EHV-4.405/76, EHV-1.438/77 and AHV-3.804/87 were grown in equine foetal kidney (EFK) cells or adapted to growth in continuous cell lines, CL1 (EHV-4) or CL2 (EHV-1 and AHV-3), as described elsewhere (Crabb & Studdert, 1990).

**Antibodies.** A panel of 14 MAbs, directed against the major glycoproteins of EHV-4 and EHV-1, had been prepared previously (Yeargan et al., 1985; Allen & Bryans, 1986; G. P. Allen, unpublished results). The target glycoprotein and specificity of each MAb when tested against strains of EHV-4 and/or EHV-1 from the U.S.A. is shown in Table 1. Monospecific antisera to EHV-4 and EHV-1 were obtained from colostrum-deprived SPF foals after a series of immunizations and challenges with either EHV-4 or EHV-1 (Fitzpatrick & Studdert, 1984). Antisera were also obtained from a third SPF foal experimentally infected with EHV-1 and cross-challenged with EHV-4. An AHV-3 antiserum was obtained 40 days after experimental infection of a seronegative, weaning donkey with nasal washings from a second donkey that had received high doses of corticosteroids, which reactivated latent AHV-3 (Brownling et al., 1988). Sera were obtained from two thoroughbred mares (mares 1 and 2) 3 to 4 weeks after EHV-1-induced abortion. These sera were shown to have high antibody titres (> 10^6) when tested against EHV-1 in an ELISA (data not shown). Cross-absorption of the monospecific EHV-1 SPF foal antiserum and mare 1 antiserum with EHV-4-infected cells was carried out as described previously (Crabb & Studdert, 1990).

**Western blotting.** SDS-PAGE and electrophoretic transfer of viral proteins to polyvinylidene fluoride (PVDF) membranes were carried out as described previously (Crabb & Studdert, 1990). The PVDF membranes were probed with either MAbs diluted 1/800 or polyclonal sera diluted 1/100 for 1 h at room temperature using a 45-channelled probing apparatus (Miniblotter; Immunetics). Membranes were then washed for 15 min with PBS pH 7.5 containing 0.05\% (v/v) Tween 20 (PBST) by continuous flow through the Miniblottet. The primary antibody was detected with either a 1/1000 dilution of affinity-purified rabbit anti-mouse IgG (Dako Immunoglobulins) or a 1/500 dilution of an affinity-purified goat anti-horse IgG (Kirkegaard and Perry), both conjugated with horseradish peroxidase, where appropriate. The Miniblotter was used for the addition of conjugated second antibody in the case of the MAbs, but was not used in the case of the polyclonal sera. The blots were washed again with PBST and developed using 3,3'-diaminobenzidine (DAB; Sigma).

**RIPA.** Preparation of [14C]glucosamine-labelled viral antigens and their subsequent immunoprecipitation by antibody-saturated Protein G–Sepharose 4 Fast Flow beads (Pharmacia) was carried out as described previously (Crabb & Studdert, 1990). For the saturation of
beads with MAb the beads were incubated with 50 μl of a 1/20 dilution of ascitic fluid with 5 μl packed beads overnight at 4°C.

**ELISA.** ELISA was carried out in 96-well PVC microtitre plates (Nunc-Immunoplate Maxisorb). The plates were washed four times with PBST between each step and incubated in a humidified container at room temperature for 1 h unless otherwise stated. Wells were coated with approximately 10 μg/ml purified virus (100 μl/well) diluted in 0.05 M-carbonate-bicarbonate buffer pH 9-6 for 2 h at 37°C after which any unoccupied sites were blocked by incubation for 2 to 4 h with 10% goat serum (200 μl/well). Serial dilutions of MAb-containing ascitic fluid were then made in the wells using PBST containing 5 mg/ml bovine serum albumin (BSA) as diluent; the final volume in each well was 100 μl. Horseradish peroxidase-conjugated affinity-purified rabbit anti-mouse IgG (Dako Immunoglobulins), diluted 1/2000 in BSA-PBST, was added to each well (100 μl/well). Plates were developed using tetramethylbenzidine (Sigma) and read spectrophotometrically at 450 nm using a Titertek Multiskan MC3 (Flow Laboratories).

**Results**

**Reactivity of MAbs in ELISA**

An ELISA was developed to test the reactivity of the 14 MAbs with EHV-4, EHV-1 and AHV-3. The results are summarized in Table 1 and indicate that of the 14 MAbs, seven were directed against epitopes common to all three viruses, four to EHV-1/AHV-3 common epitopes, two to EHV-4-specific epitopes and one was directed against an EHV-1-specific epitope. The type specificity of all 14 MAbs and the target glycoproteins of 13 of the 14 MAbs had been determined previously using strains of EHV-4 (E4) and/or EHV-1 (E1) from the U.S.A.

**Identification of glycoproteins of EHV-4, EHV-1 and AHV-3 using MAbs**

Western blotting and RIPA identified six glycoprotein species of EHV-1 and AHV-3, and five of EHV-4 using MAbs against the six major glycoproteins, gp2, gp10, gp13, gp14, gp18 and gp21, of EHV-1 and/or EHV-4. Glycoproteins were identified by probing Western blots of purified virus where possible (Fig. 1), although some glycoproteins were not identified using this method; in these cases RIPA of [14C]glucosamine-labelled infected cell lysates was used (Fig. 2). The Mr values of the glycoproteins deduced from Fig. 1 and 2 are given in Table 2. Many of the individual glycoproteins, defined by a single MAb, comprise several species of differing Mr's or a large diffuse band, both of which are characteristic of other herpesvirus glycoproteins which have partially glycosylated precursor forms (Spear, 1984; van Drunen Littel-van den Hurk & Babuik, 1986). Only an approximate Mr value for the fully glycosylated,
mature forms of each glycoprotein, i.e. that showing the least migration in SDS-PAGE, is given in Table 2.

In Fig. 1 purified EHV-4 (a), EHV-1 (b) and AHV-3 (c) were run on 10% polyacrylamide gels and transferred to PVDF membranes. The membranes were cut and either stained with Coomassie blue, probed with MAbs or probed with monospecific, polyclonal sera to each of the three viruses. All 14 MAbs had been tested previously by Western blotting (data not shown) and only those reactive in this assay were used in the experiment shown in Fig. 1. All six glycoproteins of EHV-1 were detected by Western blotting (Fig. 1b) and had $M_r$ values which corresponded well with the major glycoproteins previously identified for this (Crabb & Studdert, 1990) and other strains (Turtinen & Allen, 1982; Allen & Yeargan, 1987; Meredith et al., 1989). Four or five glycoproteins of EHV-4 and AHV-3 respectively were identified by Western blotting; only gp13 and gp21/22a of EHV-4, and gp13 of AHV-3 were not identified using this method. RIPA revealed the presence and location of the gp13 homologues of EHV-4 and AHV-3 at 67K and 82K respectively (Fig. 2). Only one MAb specific for gp21/22a was available and this did not react with EHV-4 in any of the immunological assays used.

Of all the glycoprotein homologues identified, only gp13 of cell line-grown EHV-4, with an $M_r$ of 67K, differed significantly from those of EHV-1 and AHV-3, which have $M_r$s of 87K and 82K respectively. We have suggested previously that the EHV-4 glycoprotein at 67K is the probable homologue of gp13 and that the low $M_r$ observed is not an inherent property of the virus type, but was more likely the result of aberrant glycosylation by the particular cell type used to grow EHV-4 (Crabb & Studdert, 1990). To investigate this further, an MAb specific for gp13 (41D3) was used to immunoprecipitate [14C]glucosamine-labelled viral antigens prepared in EFK cells infected with low passage EHV-4 (Fig. 3a) or EHV-1 (Fig. 3b). When grown in EFK cells EHV-4 gp13 has an $M_r$ of 95K, which is in good agreement with that published for the probable gp13 homologue of the EHV-4. MD strain when grown in an equine dermal cell line (Meredith et al., 1989). An EHV-4 glycoprotein in
Identification of the immunodominant glycoproteins in naturally infected horses

To investigate the antibody response of naturally infected horses, sera from two mares after EHV-1-induced abortion and one from an SPF foal experimentally infected with EHV-1 were used to immunoprecipitate \(^{14}C\)glucosamine-labelled EHV-4 and EHV-1 antigens prepared in EFK cells (Fig. 4). This gel was run under non-reducing conditions to distinguish between gp18 and the smaller \(M_r\) species of the gB homologue (gp14 is the larger species), which migrate to similar positions under reducing conditions. MAbs
specific for the major glycoproteins were also used to immunoprecipitate these antigens to confirm the identity of bands, although these are not included in Fig. 4. These results show that gp2, gp13, gp14, gp18 and an unidentified glycoprotein at either 120K (EHV-1) or 116K (EHV-4) are important immunogens in both naturally infected mares post-EHV-1-induced abortion and the experimentally infected SPF foal. Two HSV-1 gB-related species, at approximately 200K and 127K, were identified (Fig. 4). These are likely to be the disulphide-linked heterodimer (200K) and the uncleaved form of the gB homologue respectively (127K).

To investigate the relative type specificity of these glycoproteins, two antisera, a monospecific anti-EHV-1 antiserum from a mare (mare 1) post-EHV-1-induced abortion were absorbed with EHV-4-infected cells (Fig. 5). The absorbed sera were used to immunoprecipitate [14C]glucosamine-labelled EHV-4, EHV-1 and AHV-3 antigens prepared in cell lines. MAbs were also used to immunoprecipitate the EHV-1 antigen to confirm the identity of each glycoprotein, although these lanes are not included in Fig. 5. The SPF foal antiserum absorbed with EHV-4 contained significant amounts of EHV-1-specific antibody directed against all the major immunogenic glycoproteins described above with the exception of gp2, although very little antibody against gp2 was present in the unabsorbed sera. The absorbed mare serum contained significant amounts of type-specific antibody against gp2 and gp120 and detectable amounts against gp13 and gp18. It is apparent that gp2 and gp120 were strongly labelled and they may not in fact be significantly more type-specific than gp13 and gp18, which were labelled weakly.

Discussion

Characterization of the envelope glycoproteins of herpesviruses and an understanding of their relative immunological significance in the naturally infected, natural host is a necessary prerequisite to the development of new effective vaccines for these important pathogens. The availability of a panel of MAbs against the six major EHV-1 and/or EHV-4 glycoproteins has allowed us to identify confidently the glycoproteins of Australian strains of EHV-4, EHV-1 and AHV-3 as well as those of major importance in eliciting antibody in the horse (EHV-4 and EHV-1) or the donkey (AHV-3).

The MAbs were tested by ELISA, RIPA and Western blotting to examine both their specificity and the nature of the epitopes to which they bind. All MAbs were reactive against EHV-4 and/or EHV-1 in ELISA, however only six of the 14 MAbs, 1G12, 13A9, 26A5, 3F6, 20C4 and 13B2, were strongly reactive in Western blotting (only these MAbs are shown in Fig. 1). These MAbs are most likely directed against continuous or linear epitopes that are not affected by SDS treatment. The remaining MAbs, which were reactive in ELISA and RIPA, are probably directed to discontinuous or conformational epitopes that require intact secondary and tertiary protein structure. The pattern of reactivities of the MAbs in ELISA (Table 1) shows that, apart from the seven MAbs that cross-reacted with all three virus types, four MAbs bind to both EHV-1 and AHV-3 but not EHV-4, and two other MAbs are EHV-4-specific. This is consistent with the view that EHV-1 and AHV-3 have a closer evolutionary relationship than they do with EHV-4 (Browning et al., 1988; Crabb & Studdert, 1990), and focuses attention on the origin and natural history of EHV-1 infection in the horse. One MAb (26A5) was reactive with EHV-1 only, indicating at least some difference in epitopes between EHV-1 and AHV-3. Significant epitope differences were not observed between the epidemiologically unrelated strains of EHV-1 and EHV-4 from the U.S.A. and Australia.

The Mr of each of the glycoproteins as determined by Western blotting and/or RIPA is summarized in Table 2. Of the six major glycoproteins, only EHV-4 gp21/22a was not identified by the panel of MAbs. Generally the homologous glycoproteins of cell line-grown EHV-4,
EHV-1 and AHV-3 had very similar Mr values, with the exception of EHV-4 gp13, which at 67K was 20K less than EHV-1 gp13; gp13 from the precursor EHV-4 grown in EFK cells was shown to have a much greater Mr, of 95K (Fig. 3). The large discrepancy in the Mr of gp13 of EFK cell- and cell line-grown viruses probably lies in differences in glycosylation of this molecule by the two cell types. Gp13 is a heavily glycosylated molecule as is highlighted by recent DNA sequence analysis which has shown it to have a primary translational product of only 51K (Allen & Coogle, 1988; Guo et al., 1989). HSV gC (the EHV-1 gp13 homologue) and bovine herpesvirus type 1 gIII (the HSV gC homologue) are also extensively glycosylated, containing N-linked complex-type and O-linked oligosaccharides (Spear, 1984; van Drunen Littel-van den Hurk & Babiuk, 1986). It is most likely that EHV-4 gp13 is normally similarly glycosylated, but when grown in the continuous cell line complete or partial inhibition occurs in the biosynthesis of one or both of the carbohydrate side-chains on gp13, resulting in a much reduced Mr. We are investigating this apparent aberration in glycosylation of the cell line-grown EHV-4 gp13 further using specific glycosylation inhibitors. Importantly, the antigenicity of this glycoprotein does not appear to be greatly affected by growth in the cell line, as assessed by its reactivity with the four EHV-4 gp13 MAb s and various polyclonal sera.

The major immunogenic glycoproteins of the three equid herpesviruses, at least those responsible for eliciting antibody production following natural infection of the natural host, were identified. From Fig. 4 it is clear that gp2, gp14, gp18 and an as yet unidentified glycoprotein of Mr, 120K (EHV-1) or 116K (EHV-4) all elicit significant levels of antibody in the naturally infected host. It is possible that the glycoprotein at 120K is the EHV-1 gH homologue described recently which is the EHV-1 antiserum or natural infection (mare 1 antiserum), which result in differences in both the epitopes presented to the horse immune system and possibly also in the relative immunodominance of a particular epitope. An example to illustrate this is the type-specific antibody to gp14 which is present in the absorbed monospecific EHV-1 antiserum but is absent from the absorbed mare 1 antiserum. Although only a single post-abortion mare's serum was examined, it seems that the type-specific epitope(s) of gp14 are either not exposed or not immunologically significant following natural infection. Nevertheless, all of the immunogenic glycoproteins were shown to contain some type-specific epitopes, with gp2 and gp120 (and to a lesser extent gp13 and gp18) appearing significantly type-specific in the naturally infected mare. Epitope analysis of gp13 carried out previously using a panel of 42 gp13-specific MAb s showed that 85% of an estimated 16 epitopes were type-specific (Allen et al., 1988). Our results certainly indicate that some type specificity is associated with gp13 although the decrease in the intensity of bands in either serum after absorption would suggest that either an overall figure of 85% of EHV-4 and -1 epitopes being different is an overestimate or, more likely, that type-common gp13 epitope(s) are more immunodominant than type-specific gp13 epitopes in the horse. An important direction for future work, in addition to vaccine development, is to express type-specific epitopes from some of these glycoproteins in Escherichia coli or other expression systems with a view to developing a much needed specific diagnostic test for EHV-1-infected horses.

We thank Nino Ficorilli for excellent technical assistance, and Dr H. S. Nagesha for helpful advice and discussion. Financial support was provided by Melbourne University Equine Research Fund and Ministry of Sport and Recreation, Victoria.

References


(Received 6 February 1991; Accepted 22 May 1991)