Comparative studies of the proteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3: antibody response of the natural hosts

Brendan S. Crabb and Michael J. Studdert*

School of Veterinary Science, The University of Melbourne, Parkville, Victoria 3052, Australia

Proteins of purified virions of equine herpesvirus 4 (EHV-4; equine rhinopneumonitis), EHV-1 (equine abortion virus) and asinine herpesvirus 3 (AHV-3) were compared by metabolic labelling with [35S]methionine or [14C]glucosamine during growth of low passage virus in natural host cells (horse or donkey) and high passage virus in an appropriate cell line and analysis by SDS-PAGE. Approximately 25 different proteins (Mr 300K to 21K) were clearly resolved for each virus. The three viruses had similar profiles although significant differences were found. The proteins of the cell line-grown viruses were similar to their precursor viruses grown in natural host cells although some small differences, probably related to differences in glycosylation by the various cell types, were noted. Six or seven high abundance glycoproteins were identified for EHV-4, EHV-1 and AHV-3. The profile of seven glycoproteins of AHV-3 was more similar to EHV-1 than to EHV-4. Antigenic relationships of the proteins of the three viruses were examined using radioimmunoprecipitation (RIP) and Western blot analyses and a series of polyclonal sera raised in colostrum-deprived, specific pathogen-free (SPF) foals which were immunized with inactivated EHV-4 (foal 3) or EHV-1 (foal 1), challenged and cross-challenged; a polyclonal donkey serum to AHV-3 was also used. The ontogeny of the antibody response in the SPF foals was studied and the major immunogenic proteins, as determined by RIP, were correlated with previously determined serum neutralizing antibody titres. Antibodies were first detected 14 days after primary immunization and were directed to EHV-4 proteins of Mr 113K, 75K and 56K or EHV-1 proteins of 110K, 78K, 60K and 58K. Antibodies to these same three (EHV-4) or four (EHV-1) proteins, together with antibodies to the major capsid protein and proteins of 67K (EHV-4) and 87K (EHV-1) were detected in response to primary infection (control foal 2) and these sera had high neutralizing antibody titres. The antigens of the three viruses were extensively cross-reactive with immunodominant proteins in the Mr ranges 150K to 110K and 62K to 56K. However, cross-absorption of EHV-4 and EHV-1 SPF foal antisera indicated the presence of significant amounts of type-specific antibody.

Introduction

Equine herpesviruses 4 and 1 (EHV-4 and EHV-1) and asinine herpesvirus 3 (AHV-3) are related alphaherpesviruses infecting members of the family Equidae. EHV-1 (equine abortion virus) is a major cause of abortion and the recognized cause of abortion ‘storms’. EHV-1 is also considered a cause of respiratory disease and some strains cause central nervous system disease (Campbell & Studdert, 1983). EHV-4 (equine rhinopneumonitis) is a common respiratory pathogen of horses although it has been occasionally isolated from sporadic abortions (Sabine et al., 1981; Studdert et al., 1981). Both viruses occur world-wide and are of considerable economic importance to the equine industry (Allen & Bryans, 1986). AHV-3 was recently described as a respiratory pathogen of donkeys (Browning et al., 1988). There is evidence based on serological and DNA analyses that AHV-3 is more closely related to EHV-1 than is EHV-4 (Browning et al., 1988).

Comparative studies of the proteins of EHV-4 and EHV-1 reveal similar but not identical overall electrophoretic profiles (Meredith et al., 1989; Turtinen et al., 1981). Significant and constant differences in electrophoretic mobility of eight of the viral proteins were recognized when several strains of each virus were compared (Turtinen et al., 1981). Despite considerable differences at the genomic level, i.e. distinct restriction endonuclease DNA fingerprints and low base sequence similarity, it is clear that EHV-4 and EHV-1 are genetically collinear and are antigenically related, possessing both shared and unique antigenic determinants (Studdert et al., 1981; Turtinen et al., 1981; Allen & Turtinen, 1982; Fitzpatrick & Studdert, 1984; Yeargan et al., 1985; Cullinane et al., 1988). The proteins of AHV-3 have not been described.
The degree of cross-protection between EHV-4 and EHV-1 remains unclear. Allen & Bryans (1986) described some degree of cross-protection of horses against EHV-1 challenge after repeated infections with EHV-4. Using sera from three colostrum-deprived specific pathogen-free (SPF) foals that were immunized, challenged, and cross-challenged with various combinations of EHV-4 and EHV-1, Fitzpatrick & Studdert (1984) showed that serum virus-neutralizing antibody responses were type-specific for foals given EHV-1 but cross-reactive after receiving EHV-4. While these results imply some degree of cross-protection (at least in one direction) a lack of clinical respiratory disease following challenge did not allow these investigators to conclude that adequate protection against EHV-1 challenge had been achieved by immunization with EHV-4. Natural infection of horses with EHV-4 does not appear to render the horse totally immune to EHV-1 infection at least in providing protection against abortion although a prevalence of respiratory disease has been implicated in reduced occurrences of abortion (EHV-1) outbreaks (Doll & Bryans, 1963). These studies suggest that further work is necessary to define better the antigenic relationships between EHV-4 and EHV-1 particularly at the level of individual proteins and that their role in protective immunity requires further study.

The glycoproteins of herpesviruses are present in the virion envelope and have important functional roles in infection particularly in mediating entry of the virion into the host cell via adsorption and penetration. It has been shown that the immune response to herpes simplex virus type 1 (HSV-1) is largely directed against the structural glycoproteins of the virus (Norrild, 1985). Seven glycoproteins are described for HSV-1: gB, gC, gD, gE, gG, gH and gI of which gD, gB and gC are highly immunogenic and appear to be the principal inducers of virus-specific antibody during HSV-1 infections (Marsden, 1987; Vestergaard, 1980). Allen & Yeargan (1987) showed that the EHV-1 glycoproteins designated gp14 and gp13 were the HSV gB and gC homologues respectively and proposed, on the basis of genomic collinearity, that EHV-1 gp17/18 was the homologue of HSV gE (Allen & Coogle, 1988; Allen & Yeargan, 1987). EHV-4 and EHV-1 homologues of HSV gB have since been identified using a gB-specific monoclonal antibody and shown to be disulphide-linked heterodimers with polypeptide chains of Mr 76K and 58K (EHV-1) and 112K and 80K respectively. EHV-4 was adapted to a different cell line and used as a representative of EHV-4 and EHV-1 respectively. These viruses were representatives of EHV-4 and EHV-1 respectively. These viruses were grown in equine foetal kidney (EFK) cells and used at passage 11 (EHV-4) and 18 (EHV-1) as described elsewhere (Studdert, 1983; Studdert & Blackney, 1979). An AHV-3 isolate, designated strain 804/87 (Browning et al., 1988), was used after four passages in donkey foetal kidney (DFK) cells. Each virus strain was adapted to growth in a heterologous cell line. Monolayer cell cultures used in the adaptation and passaging of virus were infected initially with approximately 0-1 TCID50 per cell and in later passages with 1 to 5 TCID50 per cell. EHV-1 and AHV-3 were grown in the same cell line and used at passage 45 and 11 respectively. EHV-4 was adapted to a different cell line and used at passage 65. Both cell lines were grown in MEM (Gibco-BRL) containing Earle’s salts, 1-glutamine and non-essential amino acids and supplemented with 5% foetal bovine serum (Gibco-BRL) and 0-08 m-NaHCO3. Infected cells were maintained in MEM supplemented with 1% foetal bovine serum, 40 µg/ml gentamicin, 4 µg/ml trimethoprim, 0-13 m-NaHCO3, and 0-015 m-HEPES (Sigma) at pH 7-4.

Methods

Cells and viruses. Virus strains 405/76 and 438/77 were used as representatives of EHV-4 and EHV-1 respectively. These viruses were grown in equine foetal kidney (EFK) cells and used at passage 11 (EHV-4) and 18 (EHV-1) as described elsewhere (Studdert, 1983; Studdert & Blackney, 1979). An AHV-3 isolate, designated strain 804/87 (Browning et al., 1988), was used after four passages in donkey foetal kidney (DFK) cells. Each virus strain was adapted to growth in a heterologous cell line. Monolayer cell cultures used in the adaptation and passaging of virus were infected initially with approximately 0.1 TCID50 per cell and in later passages with 1 to 5 TCID50 per cell. EHV-1 and AHV-3 were grown in the same cell line and used at passage 45 and 11 respectively. EHV-4 was adapted to a different cell line and used at passage 65. Both cell lines were grown in MEM (Gibco-BRL) containing Earle’s salts, 1-glutamine and non-essential amino acids and supplemented with 5% foetal bovine serum (Gibco-BRL) and 0.08 m-NaHCO3. Infected cells were maintained in MEM supplemented with 1% foetal bovine serum, 40 µg/ml gentamicin, 4 µg/ml trimethoprim, 0.13 m-NaHCO3, and 0.015 m-HEPES (Sigma) at pH 7.4.

Infection and radiolabelling. Monolayer cell cultures were infected in 25 cm2 cell culture flasks with 5 to 20 TCID50 per cell. After adsorption for 1 h at 37°C the inoculum was removed and replaced with maintenance medium containing one-fifth the normal concentration of methionine. At 6 h post-infection (p.i.) 5 μCi/ml of [35S]methionine (Amersham) was added to each flask. For labelling of glycoproteins, infected cell cultures were maintained in maintenance medium until 5 h p.i. at which time the medium was replaced with maintenance medium containing half the normal concentration of glucose and 1 μCi/ml [14C]glucosamine (Amersham). At 22 to 36 h p.i. virus was purified from the cell culture medium (see below). Infected cell proteins were prepared for RIP by scraping the cells into the cell culture medium and centrifuging the virus-infected cell mixture at 100000 x g for 1 h (SW60 rotor). The resulting pellet was disrupted in RIP lysis buffer (0.05 M-Tris–HCl pH 7.5, 2% Triton X-100, 0.6 mM-EDTA) sonicated briefly (1 to 2 s) at a low setting and left on ice for 30 min. The disrupted pellet was centrifuged again at 100000 x g for 1 h at 4°C and the supernatant used in immunoprecipitations.

Virus purification. Purification of virus from infected cell culture fluid was based on the method of Schrag et al. (1989). Briefly, the medium was clarified at 500 g for 15 min followed by a second clarification at
M-NaCl and 0.001 M-EDTA (TNE) buffer and layered onto preformed fivefold in TNE, pelleted again, resuspended in a small volume of TNE (20 μl) and stored at -20 °C.

Antiserum: Fitzpatrick & Studdert (1984) carried out experiments in which three colostrom-deprived SPF foals were immunized intramuscularly with inactivated EHV-1, cell lysate or inactivated EHV-4 (foals 1, 2 and 3 respectively) and challenged and cross-challenged with combinations of EHV-1 and EHV-4. Procedures for preparing and inactivating inocula and for immunizing the foals were described previously (Fitzpatrick & Studdert, 1984); the immunization and challenge protocols are also incorporated in Fig. 3. Sera were obtained twice weekly from all foals over the time-course of the experiment (approx. 120 days) and serum neutralizing antibody titres determined for each serum. For comparison these data are incorporated in Fig. 3.

For the further ontogeny studies carried out in this study a selection of the SPF foal sera were used in RIP experiments. For Western blot and absorption studies selected sera used were those taken just prior to challenge and/or sera taken just prior to infection of a seronegative, weanling donkey with nasal washings from a second donkey that had received high doses of corticosteroids that inactivated latent AHV-3 (Browning et al., 1988).

Radioimmunoprecipitations: Protein G Sepharose 4 Fast Flow (Pharmacia) beads (PG) were prepared for RIP by washing three times in 0.05 M-Tris-HCl pH 8.0 containing 1 mM-EDTA, 0.15 M-NaCl, 0.25% bovine serum albumin (BSA) and 1% Triton X-100 (Sigma, RIP buffer). Antigen was preclarified by incubating day 0 SPF foal sera with radiolabelled antigen (25 μl serum per ml of solubilized antigen) for 1 h on ice. The antigen was then used to twice resuspend a washed pellet of PG beads (25 μl PG packed beads per ml antigen) incubating the mixture each time for 30 min on ice. The beads and any non-specifically bound material were removed by brief centrifugation at 12000 g for 5 s. Aliquots of preclarified antigen (approx. 50000 c.p.m.) were incubated with 5 μl of PG beads that had been previously saturated with 50 μl of 1/10 dilution of antiserum. The mixture was shaken for 30 min at 4 °C after which time the beads were washed twice in RIP buffer followed by a further wash in the absence of BSA. The beads were then resuspended in Laemmli sample buffer comprising 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycylglycine and 380 mM-glycine and 0.1% SDS at 30 V for 18 h. The gel and the membrane were equilibrated in transfer buffer for 15 min prior to transfer. After transfer unoccupied sites on the membrane were blocked by soaking in 20% normal goat serum in phosphate-buffered saline containing 0.3% Tween-20 (PBS) for 2 h at room temperature (RT). Membranes were then probed with virus-specific antibody diluted 1/50 in PBS using a multi-channeled antibody probing apparatus (Immunetics) for 1 h at RT. The blots were washed four times with PBS and soaked in horseradish peroxidase-conjugated goat anti-horse antibody (Kirkegaard and Perry Laboratories) diluted 1/200 in PBS, for a further 1 h at RT. Blots were washed again and developed using a tetramethylbenzidine (TMB) substrate system (Kirkegaard and Perry Laboratories). The identity of glycoproteins was independently confirmed using a Glycan Detection Kit (Boehringer Mannheim) which is based on the method of O'Shannessy et al. (1987).

Absorption of SPF foal sera. Confluent monolayers of the appropriate cell line in 890 cm² roller bottles were infected with EHV-4 or EHV-1 respectively. At 18 h p.i. infected cells were scraped into the medium, washed twice in cold PBS, divided into tubes containing approximately 2 x 10⁵ cells per tube and stored at 4 °C. Five-hundred μl of SPF foal 1 serum, diluted 1/10 in PBS, was used to resuspend cells infected with heterologous virus. Suspensions were incubated with intermittent vortexing for 1 h at 37 °C. The tubes were then centrifuged and the supernatant was removed and added to the next tube completing one absorption step. The sera were tested for reactive antibody in RIP analysis after four and six absorption steps.

Results

Comparisons of the structural proteins of EHV-4, EHV-1 and AHV-3

Virions of EHV-1, EHV-4 and AHV-3 were radiolabelled with [35S]methionine (Fig. 1 and 2) or with [14C]glucosamine (Fig. 2), purified on 5 to 15% Ficoll-400 gradients and analysed by SDS-PAGE. Fig. 1 shows fluorographic images of the protein profiles of the three viruses and examines changes that occur in these profiles when low passage viruses, grown in natural host cells (EFK cells for EHV-4 and EHV-1 or DFK cells for AHV-3), were adapted to growth in cell lines. Overall the protein profiles of EHV-4 and EHV-1 in Fig. 1 are very similar to those previously reported for other strains of these two viruses (Allen & Bryans, 1986; Meredith et al., 1989; Turtinen et al., 1981). Approximately 25 different polypeptides in a Mr range of 300K to 21.5K were clearly resolved for each virus. The profile of AHV-3 is very similar to those of the same virus after adaptation to a cell line. Some small differences are apparent however and these usually appear as small Mr shifts such as in the 92.5K to 97K region of AHV-3. A 67K EHV-4 protein (shown to be a glycoprotein, Fig. 2) appears in the profile of the cell line-grown virus. The cell line-grown viruses were used in all subsequent experiments.
Fig. 1. SDS-PAGE of the virion proteins of purified EHV-4 (E4), EHV-1 (E1) and AHV-3 (A3). Proteins were labelled metabolically with $[^{35}\text{S}]$methionine from 6 to 24 h p.i. The profiles of the three viruses when grown in low passage, natural host cells (EFK or DFK) or an appropriate cell line (CL1 for EHV-4 and CL2 for EHV-1 and AHV-3) were compared. Viruses were purified on 5 to 15% Ficoll 400 gradients and analysed by SDS-PAGE under reducing conditions. All samples were run on the same 10% polyacrylamide gel and photographed from a single fluorographic image. The position of the major capsid protein (MCP, $M_r$ 150K) is indicated. The $M_r$ standards used are myosin (200K), phosphorylase b (92.5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K) and trypsin inhibitor (21.5K).

Fig. 2 shows $[^{14}\text{C}]$glucosamine-labelled glycoproteins of the three purified cell line-grown viruses next to a corresponding $[^{35}\text{S}]$methionine-labelled virion profile. The EHV-1 profile shows glycoproteins of $M_r$ 250K, 110K, 87K, 78K, 60K, 58K and 49K which are in good agreement with those previously published for EHV-1 strain A183 (Allen & Yeargan, 1987; Turtinen, 1983) and strain Abl (Meredith et al., 1989) and appear to correspond to the six high abundance glycoproteins described by Allen & Yeargan (1987) and Allen et al. (1987), namely gp2, gp10, gp13, gp14, gp17/18 and gp21/22a. The EHV-4 glycoprotein profile shows major glycoproteins of $M_r$ 270K, 113K, 77K, 75K, 67K, 62K and 56K. The glycoprotein profile of AHV-3 shows similarities to the EHV-1 profile with proteins of $M_r$ 250K, 150K, 115K, 82K, 78K, 60K and 57K. A second method for detecting glycoproteins involving electrophoretic separation and immobilization of viral proteins on PVDF membranes (Glycan Detection Kit, Boehringer Mannheim) confirmed the above results as well as allowing better resolution of the 77K/75K bands of EHV-4 and detecting a AHV-3 glycoprotein of 44K (data not shown).

Ontogeny of the antibody response in SPF foals
Antisera were obtained from three colostrum-deprived, SPF foals following a series of immunizations, challenges and cross-challenges with combinations of EHV-4 and EHV-1. These sera were previously tested for serum neutralizing antibodies (Fitzpatrick & Studdert, 1984) and the results for each foal are shown in Fig. 3 (a) (foal 1), Fig. 3 (b) (foal 2) and Fig. 3 (c) (foal 3). A selection of these sera were used to immunoprecipitate $[^{35}\text{S}]$methionine-labelled EHV-4 or EHV-1 proteins to investigate the ontogeny of the antibody response in SPF foals to individual viral proteins.

None of the foals had EHV antibody before primary immunization (foals 1 and 3) or experimental infection (foal 2). A low antibody response was observed for both foals 1 and 3 (Fig. 3a and 3c) 14 days after primary immunization with inactivated EHV-1 and EHV-4 respectively. Three (foal 3) or four (foal 1) immunoreac-
Proteins of EHV-4, EHV-1 and AHV-3

Fig. 3. Immunoprecipitation of [35S]methionine-labelled, detergent-solubilized preparations of EHV-4 (E4) and EHV-1 (E1) by sera from three colostrum-deprived, SPF foals that were immunized with inactivated EHV vaccines, challenged and cross-challenged with EHV-4 and EHV-1. The days of immunization (I) or challenge (C) with either EHV-4 or EHV-1 (I) or immunization (foal 2) with mock-infected cell lysate (L) are indicated by the arrows and the appropriate code (I1, I4, C1, C4, or IL) immediately beneath each gel. Foals 1 (a) and 3 (c) sera were used to immunoprecipitate proteins of the virus with which they were immunized, i.e. EHV-1 for foal 1 and EHV-4 for foal 3; foal 2 (b) sera were used to immunoprecipitate the proteins of both EHV-4 (left side) and EHV-1 (right side). Proteins of [35S]methionine-labelled purified virus are shown in the left-hand lane of each serum set. Mr values of some of the major immunodominant proteins are indicated to the right of gels (a) ~md (c). Serum neutralizing antibody titres (SN Ab titre) to EHV-4 ( ) and EHV-1 (---) for all sera, derived from earlier studies (Fitzpatrick & Studdert, 1984), are reproduced beneath each gel. The SN Ab titre is the reciprocal of the highest dilution of each serum that neutralized approximately 100 TCID₅₀.

Responsive proteins were observed at this time with Mr values corresponding to apparently comparable EHV-4 and EHV-1 proteins of 113K/110K, 75K/78K and 56K/60 to 58K. Sera from foals 1 and 3 precipitated considerably more viral antigens after secondary immunizations (day 28 onwards). Maximal antibody responses in foals 1 and 3 were recognized after challenge with live, homologous virus as seen by the increase in intensity of the bands at day 49. This did not correspond to maximum serum neutralizing antibody titres. Although the intensity of bands generally decreased in foals 1 and 3 for sera tested after day 49, the amount of neutralizing antibody either increased (foal 1) or remained relatively constant (foal 3). Cross-challenge did not result in antibodies being produced that were capable of precipitating any new viral antigens in either foal 1 or 3; however, the increase in intensity of the bands at day 118 for foal 1 and day 98 for foal 3 shows an increase in antibody response after the second cross-challenge in both foals. The control, foal 2, had detectable antibody after primary infection with live EHV-1 (day 60) (Fig. 3h). The same three or four immunoreactive EHV-4 and EHV-1 proteins as described above were detected by these sera. The antibody response of foal 2 was boosted significantly after EHV-4 challenge as seen by the increase in the number and intensity of EHV-4 and EHV-1 proteins on day 107. The serum neutralizing antibody titres of the control foal 2 first indicated the presence of EHV-1 or EHV-4 antibody after day 64 or day 88 respectively; however, the antibody titres of foal 2 sera were very high, especially against EHV-1, after about day 95, although only a relatively small number of immunoreactive proteins of low intensity were detectable by RIP at day 107.

Immunogenicity and cross-reactivity of EHV-4, EHV-1 and AHV-3 antigens

Fig. 4 and 5 show the extent of cross-reactivity of EHV-1, EHV-4 and AHV-3 antigens using selected polyclonal sera to each virus. Fig. 4 shows Western blots of purified viral preparations of EHV-1, EHV-4 and AHV-3 probed with each of the three viruses. No reactivity was seen when day 0 sera were used to probe these Western blots. Antibody to eight to 12 structural proteins of each virus was detected and there was extensive cross-reactivity between the three viruses. Comparisons of the Mr, values
from SDS-PAGE of [14C]glucosamine-labelled virus proteins show that antibody was present to proteins having $M_r$ values comparable to those of the major glycoproteins of each virus. Some proteins appear to be significantly more immunodominant than others, i.e. 113K/110K/115K, 62K/60K/60K and 56K/58K/57K proteins of EHV-4, EHV-1 and AHV-3 respectively. Other non-glycosylated proteins such as a 150K protein, probably the major capsid protein (MCP), are also strongly immunogenic. The EHV-1 serum possesses antibodies to a 64K EHV-1 protein that does not react with either EHV-4 or AHV-3 sera; there was also a 33K EHV-1 protein that reacted with EHV-1 and AHV-3 sera but not with EHV-4 serum. Otherwise differences in reactivity between the sera and a particular protein were usually seen as variations in intensity rather than the absolute presence or absence of a band.

A method of cross-absorbing EHV-4 and EHV-1 SPF foal sera with cells infected with heterologous virus was developed to investigate the relative amounts of typespecific antibody present in the sera. EHV-4 (Fig. 5a) and EHV-1 (Fig. 5b) antisera were tested, both before absorption and following four and six absorption steps, for their ability to immunoprecipitate [35S]methionine-labelled EHV-4, EHV-1 and AHV-3 antigens. There was a progressive reduction in cross-reactive antibody in the EHV-4 and -1 antisera from absorption steps 1 to 6. By six absorptions all cross-reactive antibody was removed as indicated by the failure of these absorbed antisera to immunoprecipitate proteins of the virus used for their absorption. The remaining antibody, reactive with proteins from the virus against which it was originally raised, is considered type-specific for that virus. The detection of all the structural proteins eliciting type-specific antibody is difficult, however; EHV-4 and EHV-1 proteins corresponding to gp10 (113K and 110K respectively) and EHV-1 proteins corresponding to gp17/18 (60K/58K) are precipitated by their respective absorbed sera. Some AHV-3 antigens are precipitated by both absorbed sera.

**Discussion**

Our desire to work with high titre, cell line-grown viruses led us to compare the protein profiles of these viruses with those of the precursor viruses grown at low passage in natural host cells and to examine the possibility of significant changes occurring as a result of the adaptation process. The number of passages in the cell lines, 65 for EHV-4, 45 for EHV-1 and 11 for AHV-3, were low when compared with EHV-1 strains KyA-ha or KyA-LM which were adapted from the KyA foetal strain and passaged extensively in Syrian hamsters or mouse L-M cells respectively over a period of more than 10 years (Perdue et al., 1974). O’Callaghan & Randall (1976) reported only slight differences in the protein profiles between these extensively passaged host range mutant strains, primarily in some minor envelope glycoproteins. Our findings show small differences in the profiles after adaptation and somewhat limited passage. The proposal by Turtinen et al. (1981) that different cell types may glycosylate viral proteins differently because they may possess distinct glycosyl transferases would explain the small shifts in $M_r$ exhibited by some viral proteins after adaptation to cell lines.

The total protein profiles for the three viruses are very similar and they are obviously closely related herpesviruses. The glycoprotein profile of EHV-4, however, was clearly different to those of EHV-1 and AHV-3. There are two main possibilities to explain this: (i) the changes are the result of differences at the level of DNA that result in carbohydrate modifications that are specific for a particular virus type; (ii) the cell lines used differently glycosylate protein backbones that are more similar than the relatively large migrational differences exhibited by some glycoproteins might suggest. The first point implies that AHV-3 is indeed closer in evolutionary
Proteins of EHV-4, EHV-1 and AHV-3

Fig. 5. Immunoprecipitations of [35S]methionine-labelled EHV-4 (E4), EHV-1 (E1) and AHV-3 (A3) proteins using an EHV-4 antiserum (a) or an EHV-1 antiserum (b) from SPF foals. Each serum was used to immunoprecipitate proteins of each of the three viruses either unabsorbed or after four or six absorption steps (× 4 or × 6 abs steps) EHV-4 antiserum was absorbed with EHV-1-infected cells and EHV-1 antiserum was absorbed with EHV-4-infected cells. The positions of EHV-1 glycoproteins gp10 (110K) and gp17/18 (60K/58K) are indicated. The Mr standards are as in Fig. 1.

Distance to EHV-1 than is EHV-4 as previously suggested by Browning et al. (1988). However, the slight difference in the profiles of EFK-grown and cell line-grown EHV-4, such as the appearance of a glycoprotein at 67K, and the fact that EHV-1 and AHV-3 were both adapted to growth in the same cell line, suggests that the second point may at least be a contributing factor to the observed differences.

Although the glycoprotein profile of EHV-1 (and AHV-3) was very similar to those reported for other EHV-1 strains the EHV-4 profile possesses both similarities and differences to the MD strain of EHV-4 described by Meredith et al. (1989). The most notable differences are the absence of a detectable glycoprotein in the 92K region and the presence of 77K and 67K glycoproteins in the cell line-grown EHV4.405/76 used in this study. The 92K protein of the EHV4.MD is heavily glycosylated (as seen by the amounts of [14C]glucosamine incorporated) in comparison to other glycoproteins whereas the EHV-4 strain used in the present study does not have any such dominant glycoproteins. It is possible, therefore, that the gp92 equivalent is less glycosylated in this particular strain and therefore appears as a protein of lower Mr, i.e. at 77K or 67K. Monoclonal antibodies are required to identify definitively the structurally homologous proteins.

In the study of the ontogeny of the antibody responses to EHV-4 and EHV-1 in the SPF foals, antibodies to viral proteins were first detected at day 14 after primary immunization with either EHV-1 or EHV-4. This initial antibody response was directed to four (EHV-1) or three (EHV-4) proteins in foals 1 and 3 respectively that correspond to EHV-1 proteins of 110K, 78K, 60K and 58K and EHV-4 proteins of 113K, 75K and 56K all of which are likely to be glycoproteins. A minor nonglycosylated protein has been shown to have an Mr similar to the 110K EHV-1 glycoprotein and although unlikely it cannot be discounted as the major immunogen seen at 113K/110K for EHV-4/EHV-1 respectively (Turtinen, 1983). The four EHV-1 proteins have Mr values corresponding to gp10, gp14 and gp17/18 of Allen & Yeargan (1987). It is possible that these are not all different species but are some of the three Mr forms of the gB homologue described by Meredith et al. (1989).
and subsequently by Sullivan et al. (1989). This is as yet unclear as the latter authors did not attempt to reconcile the earlier view of Allen & Yeargan (1987) that gp17/18 maps to the Uα component of the EHV-1 genome despite the smaller component of gB having a similar Mr to gp17/18, a view incompatible with the single gene location of gB in U1. One must assume that they subscribe to the suggestion by Meredith et al. (1989) that two comigrating but distinct glycoproteins are present. Nevertheless, gp14 (the large component of the HSV gB homologue) appears important in the early antibody response to EHV immunization.

The control foal 2, which received mock-infected cell lysate and was experimentally infected at day 38 with EHV-1, produced antibodies that were directed to the same three or four proteins (probably glycoproteins) as observed in the post-inactivated virus vaccine responses of foals 3 and 1. Serum from foal 2 after cross-challenge with EHV-4 precipitated additional EHV-1 proteins of 150K and 87K which correspond to the MCP and gp13 (the HSV gC homologue) and EHV-4 proteins of 150K (MCP) and 67K which may be a lower Mr form of gp13. The serum neutralizing antibody titres of foal 2 sera after EHV-4 cross-challenge were high, especially against EHV-1 (day 107). It would appear that some or all of the MCP and EHV-1 glycoproteins corresponding to gp10, gp13, gp14 and gp17/18 and their EHV-4 counterparts are important immunogens in the naturally infected host, eliciting antibody in response to live virus that is strongly neutralizing. It would be of interest, particularly in the context of subunit vaccine development, to determine the relative immunological importance of these glycoproteins. Further identification of EHV-4 and EHV-1 glycoproteins structurally homologous to glycoproteins of HSV or other alphaherpesviruses would be useful for this process.

In the case of foals 1 and 3, immunized with inactivated EHV-1 and EHV-4 respectively, a maximal antibody response was detected by RIP following first challenge with live virus (day 49) that was significantly stronger, in terms of the number and intensity of precipitating proteins, than the antibody response observed at any time for foal 2. The serum neutralizing antibody titres were not high at this time. In short, the absorption experiments by the ability of both EHV-1- and EHV-4-absorbed sera to immunoprecipitate some antigens of each virus are largely cross-reactive. The immunodominant viral proteins are found in the region of 150K to 110K and 62K to 56K and it is likely that the proteins include the MCP, gp10 and gp17/18. Proteins having Mr values comparable to all the major glycoproteins were shown to elicit some antibody (although this is not clear from the Western blot chosen for Fig. 4) as were several other non-glycosylated proteins. Absorption experiments (Fig. 5) revealed the presence of type-specific epitopes on several infected cell/viral proteins including the immunodominant proteins that correspond to gp10 and gp17/18. Though non-quantitative it appears that as many as 20% to 50% of the epitopes of both EHV-4 and EHV-1 are type-specific. The degree of type-specificity presumably depends on the conserved nature of a particular protein; i.e. between EHV-4 and EHV-1, gp14 is thought to be highly conserved whereas gp13 possesses predominantly type-specific epitopes (Sullivan et al., 1989; Allen & Coogle, 1988). Our findings echo those of Yeargan et al. (1985) who found that about 30% of monoclonal antibodies to EHV-4 and EHV-1 (termed subtypes 2 and 1 respectively) were type-specific. It is likely that most (all) of the glycoproteins possess type-specific epitopes, but the technique of RIP was not a sufficiently sensitive immunological detection method to detect antibody to these proteins.

Analyses of the structural proteins/glycoproteins of AHV-3 reveal remarkable similarities to both EHV-4 and, especially, EHV-1. The extensive antigenic cross-reactivity between the three viruses shows that AHV-3 shares a large number of epitopes with both equine viruses although epitope differences are highlighted in the absorption experiments by the ability of both EHV-1- and EHV-4-absorbed sera to immunoprecipitate some AHV-3 antigens (Fig. 5). These findings lend support to those of Browning et al. (1988) who suggested that AHV-3 is more closely related to EHV-1 than EHV-4 and focus attention on the origin and natural history of EHV-1 infection in the horse.

We thank Nino Ficorilli for excellent technical assistance. Financial support was provided by Melbourne University Equine Research Fund and Ministry of Sport and Recreation, Victoria.
Note added in proof. Since preparing the manuscript we have obtained monoclonal antibodies specific for the high abundance glycoproteins of EHV-4 and EHV-1 from Dr G. P. Allen, University of Kentucky, Lexington, U.S.A. Using Western blot and radioimmunoprecipitation analyses the identity of the structural glycoproteins and immunoreactive proteins as designated in our paper was confirmed. Individual monoclonal antibodies specifically identified gp2, gp10, gp13, gp14, gp17/18 and gp21/22 as described for EHV-1 by Allen & Yeargan (1987). EHV-4 gp67 was confirmed as a low $M_1$ form of gp13, a possibility discussed in our paper.

References


(Received 25 February 1990; Accepted 9 May 1990)