Antiviral, Anti-glycoprotein and Neutralizing Antibodies in Foals with Equine Infectious Anaemia Virus

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SUMMARY

Equine infectious anaemia virus is related by genome sequence homology to human immunodeficiency virus, caprine arthritis–encephalitis virus and visna virus. Failure of the host to mount a strong neutralizing response detectable in vitro or to eliminate persistent infection in vivo characterizes lentivirus infections in the natural host. In this study the specificities and neutralizing activity of antibodies induced during experimental infection with equine infectious anaemia virus were investigated using antiviral ELISA, radioimmunoprecipitation and neutralization assays. ELISA antibody titres of $10^5$ to $10^6$ were demonstrated in samples collected 30 and 60 days after infection. Immunoprecipitation titrations demonstrated that antibody titres to the glycoproteins gp90 and gp45 were 10 to 100 times higher than titres to the internal structural protein, p24. Low levels of neutralizing antibody appeared at 23 to 46 days post-infection. The presence of low levels of neutralizing activity in the presence of high levels of anti-glycoprotein activity suggests that the major immunogenic sites on the viral surface are not sensitive to neutralization.

INTRODUCTION

Equine infectious anaemia virus (EIAV) is a lentivirus causing debilitating disease in horses world-wide (McGuire & Crawford, 1979). Immune responses to EIAV antigens appear to cause much of the clinical disease, including immune complex-mediated glomerulitis (Banks et al., 1972), lymphoproliferative changes (Banks et al., 1978), mononuclear inflammatory cell infiltration in the liver (Henson & McGuire, 1971) and the haemolytic component of virus-induced anaemia (McGuire et al., 1969). Cell-free virus appears only transiently in the circulation (Kono, 1969), but horses are persistently infected and the disease is readily transmitted by transfer of whole blood to a susceptible host (Coggins, 1984). Genomic sequence homology between EIAV and other members of the lentivirus group, including human immunodeficiency virus (HIV; Chiu et al., 1985), caprine arthritis–encephalitis virus (CAEV) and visna virus (Stephens et al., 1986) has prompted comparative studies of these viral agents. In all four of these lentiviruses, the host immune response fails to eliminate persistent infection and may contribute to disease progression. Therefore, a better understanding of components of the antiviral immune response may help in developing control measures for lentivirus diseases.

The present study describes the relative titres of antiviral, anti-glycoprotein and neutralizing antibodies in foals infected with EIAV. The results demonstrate that high antibody titres to viral proteins and glycoproteins were detectable at 30 days post-infection (p.i.). Neutralizing antibody first appeared at day 23 p.i. and neutralization indices had risen only slightly by 60 days p.i. Neutralization titres ranged from 2 to 8. These data suggest that although the viral glycoproteins are highly immunogenic during early infection, most of the antibody is directed against epitopes not sensitive to neutralization.
**METHODS**

*Cell culture and virus production.* Virus was propagated in roller cultures of newborn equine kidney (EK) cells, maintained in MEM supplemented with 5-7 mM-sodium bicarbonate, 20 mM-HEPES and 3°/o to 5°/o calf serum (Hyclone, Logan, Utah, U.S.A.). Infectivity was monitored by direct fluorescent antibody assay on cells washed in phosphate-buffered saline (PBS) pH 7.4, fixed in 100°/o methanol, stained with fluorescein isothiocyanate conjugated to IgG purified from the serum of a horse chronically infected with wild-type virus (Crawford *et al.*, 1971): the cells were examined under a Zeiss IM35 inverted fluorescent microscope. Cultures were free of contamination by mycoplasma (Flow Laboratories kit) and bovine viral diarrhoea virus (direct fluorescent antibody assay). The medium was collected twice a week and the virus was concentrated by centrifugation at 16000 g for 6 to 16 h and stored at -70 °C in MEM. For some assays, virus was purified on linear 5°/o to 45°/o (w/w) sucrose density gradients.

*Virus isolates.* The tissue culture-adapted Wyoming strain of EIAV (Malmquist *et al.*, 1973) was serially passaged through three ponies, re-isolated from the plasma of the third pony during a febrile period 29 days p.i. and serially cloned three times by limiting dilution in EK cells. This was the fifth pony-passaged isolate cloned in this series and is identified as EIAV-WSU5 following the proposal for naming human immunodeficiency lentiviruses (Coffin *et al.*, 1986).

*Animals.* Six thirty-day-old Arabian foals were housed in isolation stalls and each was infected with 10°/o TCID$_{50}$ EIAV-WSU5 intravenously. Temperatures were monitored daily and blood was collected from the animals three times a week. Four of the animals were maintained for 60 days p.i.: one foal (A1913) died on day 36 p.i. and one foal (A1912) was killed on day 37 p.i. Virus was re-isolated from the plasma of two foals (numbers A1908 and A1924) on day 14 p.i. by incubation of 1 ml plasma, diluted 1:5 in MEM, with a subconfluent monolayer of EK cells and a 25 cm$^2$ tissue culture flask for 2 h at room temperature. Uncloned viral isolates, identified as EIAV-WSU6 and EIAV-WSU7, were propagated in roller cultures of EK cells and processed as described above.

*Blood sampling.* Plasma samples were used in this study because serum from acutely infected horses continues to clot. Small volumes of blood were collected by jugular venipuncture into heparinized tubes: larger volumes were collected into 500 ml bags containing 67.5 ml acid citrate dextrose (Fenwall, Deerfield, Ill., U.S.A.). The blood was centrifuged and plasma was stored at -70 °C for virus titrations and at -20 °C for plasma neutralizations. Before neutralization assays, the plasma was incubated at 56 °C for 60 min to inactivate circulating virus and complement, and then it was centrifuged to remove any clotted fibrin.

*Virus titrations.* Since EIAV causes neither cytopathic effect nor plaque formation in EK cells, titrations and neutralizations were done using fluorescent antibody determination of endpoints. Serially diluted virus was added to subconfluent monolayers of EK cells in 96-well tissue culture plates (Corning). At 9 to 11 days p.i., the cells were fixed and stained by direct immunofluorescence as described above. Six replicates of each dilution were made. TCID$_{50}$/ml endpoints were calculated by the method of Reed & Muench (1938). The reproducibility of this technique was demonstrated by titrating a standard pool of virus 15 times. The mean log$_{10}$ titre per ml was 6.84 [standard deviation (s.d.) 0.42]; the coefficient of variation was 6.14°/o.

*Plasma neutralizations.* The plasma neutralization assay was done by the virus dilution-constant plasma method. Serial threefold dilutions of virus beginning with approximately 10$^4$ TCID$_{50}$/ml were pretreated by incubation with an equal volume of either test plasma or MEM for 30 min at 37 °C. Six replicates of each pretreated virus dilution were added to subconfluent monolayers of EK cells and incubated at 4 °C for 2 h. Unadsorbed virus was aspirated and the cells were maintained for 9 to 11 days in MEM with 3°/o calf serum. The log$_{10}$ titre (TCID$_{50}$/ml) of each pretreated virus sample was determined by direct immunofluorescence. The neutralization index for a plasma sample was calculated as the difference in log$_{10}$ titre (TCID$_{50}$/ml) between virus pretreated with MEM and with the test plasma. Plasma from normal foals non-specifically inactivated virus slightly; the mean neutralization index for triplicate determinations of four different normal foal plasma samples was 0.46 (s.d. 0.23). Therefore, a neutralization index of at least 0.97 (95°/o confidence interval) was considered positive when evaluating neutralizing activity of plasma from infected foals.

*Antiviral ELISA.* ELISA plates (Immulon 1; Dynatech Laboratories, Alexandria, Va., U.S.A.) were coated with sucrose density gradient-purified EIAV-WSU5 in 0.05 M-sodium carbonate buffer pH 9.6, using 0.33 µg virus in 50 µl per well. The wells were incubated sequentially with plasma diluted serially 1:10, rabbit anti-equine immunoglobulin [reactive with equine IgG(T) and IgG], goat anti-rabbit IgG-horseradish peroxidase (Sigma) absorbed with normal horse serum to remove cross-reactive antibodies and the chromogenic substrate 5-aminosalicylic acid. Each incubation was 30 min at 37 °C and the plates were washed after each incubation with PBS containing 0.2% Tween 20. After approximately 2 h at room temperature, the absorbance of each well was determined with an automated ELISA reader (Dynatech Laboratories). A well was scored positive if the A$_{450}$ was two standard deviations above the mean of six replicates of the corresponding dilution of normal horse serum.
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Table 1. Comparison of antiviral and neutralizing titres of plasma from foals with EIAV

<table>
<thead>
<tr>
<th>Foal number</th>
<th>Days p.i.</th>
<th>Antiviral titre*</th>
<th>Neutralizing titre†</th>
<th>Titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p26</td>
</tr>
<tr>
<td>A1907</td>
<td>30</td>
<td>10^5</td>
<td>0</td>
<td>10^2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10^5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>A1908</td>
<td>30</td>
<td>10^6</td>
<td>0</td>
<td>10^1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10^6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A1924</td>
<td>30</td>
<td>10^6</td>
<td>0</td>
<td>10^2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10^6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A1925</td>
<td>30</td>
<td>10^6</td>
<td>0</td>
<td>10^1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10^6</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocal of the highest dilution of plasma giving positive results by ELISA using plates coated with whole virus, 300 ng per well.
† Reciprocal of the highest dilution of plasma causing neutralization.
‡ Reciprocal of the highest dilution of plasma which precipitated radiolabelled viral protein of the indicated Mr.

tested in the same assay. Six replicates of each dilution were tested and the last dilution with two or more positive wells was determined to be the endpoint. Plate-to-plate variability was assayed using a standard sample (1:100 dilution of serum from a chronically infected horse) on each plate; for 18 assays, the coefficient of variation was 9-95%.

Immune precipitation of solubilized viral proteins. EIAV-WSU5 was metabolically labelled with [35S]methionine using 750 cm^2 roller cultures of infected EK cells incubated with 1.0 mCi [35S]methionine (New England Nuclear) per roller bottle for 5 days in methionine-free MEM supplemented with 1% calf serum. Cells were incubated with methionine-free MEM (Flow Laboratories) for 4 to 8 h before labelling. Virus was concentrated from the media by centrifugation at 141000 g for 1.5 h and purified on 5~ to 45~ (w/w) linear sucrose density gradients in a Beckman SW50.1 rotor at 300000 g for 2 h. [35S]Methionine-labelled virus was solubilized in 0.5% sodium deoxycholate. Ten µl of serial 10-fold dilutions of each plasma was incubated sequentially with a fixed amount of labelled antigen (50000 acid-precipitable c.p.m, per dilution), goat anti-equine IgG/IgG(T), and Protein A bearing Staphylococcus aureus (Pansorbin, Calbiochem). Precipitated proteins were resolved on 7.5% to 17.5% gradient polyacrylamide gels, which were dried and exposed to Kodak X-OMAT film at -70 °C for 72 h. Endpoints were determined to be the last dilution giving a visible band.

RESULTS

Foal infections
Six foals were experimentally infected with 10^6 TCID_{50} of EIAV-WSU5. By 36 days p.i., two of the foals were depressed and anorexic: one of these (A1913) died on day 36 and the other (A1912) was killed on day 37. Three of the four remaining foals were febrile (rectal temperature ≥ 39 °C) by day 9 p.i.; the fourth was febrile on day 16 (Fig. 1). Packed red cell volumes in the six foals decreased from a pre-inoculation mean of 37% (S.D. 3) to values as low as 13% with a mean of 19% (S.D. 5) from days 23 to 32 p.i. The six foals had serum antibody to EIAV detectable by the agar gel immunodiffusion test on day 21 p.i.

Plasma viraemia
Virus was re-isolated from all six animals by day 7 p.i. The peak plasma log_{10} virus titres occurred on day 14 p.i. and ranged from 3.77 to 4.77. Plasma virus titres of samples collected three times per week from the four foals that survived until 60 days p.i. are shown in Fig. 1. Foal A1907 developed a second detectable viraemic period and foal A1925 had several viraemic episodes during the observation period.

Plasma neutralization indices
Neutralization indices were also determined. As seen in Fig. 1, neutralizing antibody (index > 0.97) was demonstrated at 42, 37, 23 and 46 days p.i. for foals A1907, A1908, A1924 and A1925, respectively. The maximum neutralization index observed in these four foals was 2.28. As shown in Table 1, neutralization titres ranged from 2 to 8.
Fig. 1. Viraemia, pyrexia and neutralizing antibody in foals infected with EIAV. Plasma viraemia titres expressed as log_{10} TCID_{50} per ml (●) or detectable but not titratable in a 1:10 dilution of plasma (×). A titre of 0 indicates undetectable viraemia at a 1:10 dilution of plasma. Plasma neutralization index (○) is expressed as the difference in log_{10} titre between control virus (EIAV-WSU5) incubated with media and with test plasma; values greater than 0.97 are indicative of neutralization. Shaded areas represent periods during which the foal’s rectal temperature was > 39 °C for at least 24 h. (a) Foal number A1907, (b) foal number A1908, (c) foal number A1924 and (d) foal number A1925.

To determine whether the low neutralizing capacity of the plasma was due to a poor immune response to viral antigens, plasma samples were titrated against whole virus by ELISA. At 30 days p.i. titres ranged from 10^5 to 10^6 although neutralizing activity could not be detected in these samples. By 60 days p.i., the samples neutralized virus infectivity in vitro although ELISA titres had not risen (Table 1).

In other retrovirus systems, the surface glycoproteins are the site of neutralization-sensitive epitopes (Schafer & Bolognesi, 1977; Scott et al., 1979; Ho et al., 1987). Therefore, we determined the relative antibody titres to the major viral proteins using radioimmunoprecipitation of solubilized virus with serially diluted plasma. Fig. 2 shows representative results from
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Fig. 2. Immunoprecipitation titrations with [35S]methionine-labelled EIAV-WSU5 using serially diluted plasma from foals A1907 (a), A1925 (b) and an uninfected normal foal (c). Each lane represents an immunoprecipitation using 50000 acid-precipitable c.p.m. radiolabelled virus, 10 μl plasma diluted 1:10 (lane 1), 1:100 (lane 2), 1:1000 (lane 3) or 1:10000 (lane 4), goat anti-equine IgG/IgG(T), and Protein-A bearing Staphylococcus aureus. The arrows refer to the migration of 14C-methylated protein standards of 200000 (myoglobin), 92500 (phosphorylase b), 69000 (bovine serum albumin), 46000 (ovalbumin), 30000 (carbonic anhydrase) and 14300 (lysozyme) Mr.

Table 2. Plasma neutralization index of EIAV isolates at 60 days p.i.

<table>
<thead>
<tr>
<th>Foal number</th>
<th>Virus isolate</th>
<th>WSU5*</th>
<th>WSU6†</th>
<th>WSU7‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1908</td>
<td></td>
<td>2.07</td>
<td>1.39</td>
<td>ND§</td>
</tr>
<tr>
<td>A1924</td>
<td></td>
<td>2.28</td>
<td>ND</td>
<td>1.32</td>
</tr>
</tbody>
</table>

* Foals were infected with 10⁶ TCID₅₀ EIAV-WSU5.
† Uncloned isolate from foal A1908.
‡ Uncloned isolate from foal A1924.
§ ND Not done.

two of the foals. The plasma samples reacted with major bands of Mr 24000, 39800 and 100000. The two larger proteins were glycosylated, as demonstrated by binding to and specific elution from lens lectin Sepharose 4B prior to immunoprecipitation (data not shown). EIAV is reported to have a 24000 to 26000 Mr internal protein (Charman et al., 1976; Cheevers et al., 1978; Parekh et al., 1980; Nishimura & Nakajima, 1984) and two glycosylated surface proteins referred to as gp90 and gp45 (Cheevers et al., 1978, 1980; Montelaro et al., 1982), although their electrophoretic mobility relative to Mr markers varies among isolates (Montelaro et al., 1984). The alteration in Mr of the glycoproteins in this study may be characteristic of EIAV-WSU5 or may be due to the purification and electrophoresis conditions used. Immunoprecipitating antibody to gp90 and gp45 was detected with a 1:1000 dilution of all four plasma samples collected 60 days p.i. Antibody to p24 was detectable at a 1:10 dilution in two foals and 1:100 dilution in the other two (Table 1, Fig. 2). These results indicate that a major portion of the antiviral antibody response is directed against the glycoproteins.

Antigenic variation of EIAV-WSU5 before the first viraemic period (Payne et al., 1987) might explain the low neutralizing activity of the plasma. Therefore, virus was isolated from the first viraemic episode of two of the foals. As seen in Table 2, the neutralization indices of the 60 day plasma samples were no higher against these isolates than against the infecting isolate, EIAV-WSU5.
DISCUSSION

Six foals were infected with tissue culture-adapted EIAV. All of them developed viraemia and clinical signs consistent with those reported following infection with wild-type virus (Kono, 1969). Immunological assays on plasma collected from four of the foals at 30 and 60 days p.i. demonstrate that the predominant targets of antiviral antibody are epitopes on viral glycoproteins. Neutralizing antibody was first detected at 23 to 46 days p.i.; neutralization titres, however, were less than 8.

The low neutralizing titres cannot be attributed to an impaired antibody response to the virus, as demonstrated by the high antiviral ELISA titres. In fact, the foal with the highest neutralizing titre had the lowest antiviral titre by ELISA. Assuming that the three major proteins label to approximately the same specific activity by metabolic incorporation of $^{35}$S]methionine, radioimmunoprecipitation data suggest that immunogenicity of the viral glycoproteins is not a limiting factor. Likewise, rapid antigenic variation of EIAV during the initial stage of infection could not be demonstrated. Thus the relatively low titres would not seem to be directly attributable to a neutralizing response to a variant different from the infecting isolate.

These observations are consistent with published data on two of the three major lentiviruses. Although gp130 is a major target of antibody in patients with HIV (Barin et al., 1985), only low levels of neutralizing antibody have been detected (Clavel et al., 1985; Weiss et al., 1985; Weber et al., 1987). CAEV gp135 is highly immunogenic in goats (Adams & Gorham, 1986) and high levels of anti-gp135 activity have been reported at 6 months p.i. (Johnson et al., 1983). However, neutralization of CAEV by sera from infected goats is not detectable (Klevjer-Anderson & McGuire, 1982; Narayan et al., 1984) or is low (unpublished data). The response of visna virus-infected sheep is highly variable; neutralizing antibody may be seen as early as 4 weeks p.i. or may be undetectable for 2 or more years (Narayan et al., 1981; Thormar et al., 1983). In contrast to the HIV and CAEV findings, however, neutralization titres of sera collected from sheep with visna virus are as high as 640 in samples collected within 1 year of infection (Narayan et al., 1978).

The results described in this report indicate that the early immune response in foals experimentally infected with EIAV is characterized by high levels of circulating antibody directed primarily against non-neutralizable epitopes on the viral glycoproteins. This finding supports our earlier observation that up to 99% of the circulating infectious virus in sera from some infected horses is complexed to antibody (McGuire et al., 1972). The biological relevance of our observations is not yet known. EIAV undergoes rapid antigenic variation in infected horses (Kono et al., 1973), presumably due to selection of naturally occurring variants by immune responses. This selective pressure may involve cell-mediated responses as well as neutralizing antibody. Also, assays in vitro using fibroblast indicator cells may not reflect accurately the virus–antibody interactions with the natural host cells, the monocyte/macrophage. Further, the protective effect of low levels of neutralizing antibody remains to be determined. Data from equine herpesvirus and equine arteritis virus vaccine trials suggest that pre-challenge serum neutralization titres are unreliable indicators of immunity on an individual basis. Different groups have reported that protective virus neutralization titres to equine herpesvirus I range from 25 to 100 (Bryans, 1969; Burrows & Goodridge, 1973; Mumford & Bates, 1984). If the titre of neutralizing antibody is critical to protection from infection with EIAV, vaccine design may depend on a better understanding of the factors controlling relative immunogenicity of neutralization-sensitive epitopes.

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