Specificity of serum neutralizing antibodies induced by transient immune suppression of inapparent carrier ponies infected with a neutralization-resistant equine infectious anemia virus envelope strain

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INTRODUCTION

Equine infectious anemia virus (EIAV) is a lentivirus that causes a persistent infection and dynamic disease in equids. The dynamic nature of EIAV infection is in marked contrast to the slowly progressive degenerative diseases usually associated with the other members of the lentivirus family, such as HIV and SIV (Montelaro et al., 1993). Experimental EIAV infection in horses results in a characteristic disease progression. Within one month of infection, horses typically experience acute viraemia and disease characterized by fever, thrombocytopenia, diarrhoea, lethargy, oedema and other clinical signs. Following resolution of acute disease, infected horses typically progress to chronic disease characterized by recurring disease cycles at irregular intervals separated by weeks or months (Montelaro et al., 1993). The recurring disease cycles have been associated with the evolution of antigenic variants of the virus that are able to temporarily escape established immune surveillance, such that distinct populations of EIAV envelope quasispecies are present at each disease cycle (Leroux et al., 1997; Lichtenstein et al., 1996; Payne et al., 1987). By 8–12 months post-infection most horses become long-term asymptomatic carriers indefinitely, unless stressed or immune suppressed to cause a recrudescence of disease. The progression from chronic disease to inapparent carrier has been associated with a complex and lengthy development of enduring and broadly controlling host immunity that is able to suppress virus replication to subclinical levels, despite ongoing envelope antigenic variation (Craigio et al., 2002; Hammond et al., 2000; Harrold et al., 2000; Leroux et al., 2001; Montelaro et al., 1993). It is the unique ability of the equine immune system to maintain EIAV replication and disease under strict lifelong control that makes the EIAV system a useful model for the natural immune control of lentivirus infection and disease.

Various studies have demonstrated that the control of
EIAV replication and disease is directly related to host immune response, and not to attenuation of the infecting virus (Issel et al., 1982; Perryman et al., 1988). For example, dexamethasone-induced immune suppression of inapparent carriers results in the recrudescence of disease associated with markedly increased virus replication (Craigo et al., 2002; Kono et al., 1976; Mealey et al., 2001; Tumas et al., 1994). The evolution of humoral and cellular immune responses during the progression from chronic disease to inapparent carrier status has been examined and characterized in detail (Hammond et al., 1997; McGuire et al., 2002; Mealey et al., 2003; Rwambo et al., 1990a; Tschetter et al., 1997; Zhang et al., 1998). The results of these studies have indicated that a 8–10 month time period post-infection is required for the development of a mature steady-state immunity that can mediate effective and enduring control of EIAV replication and disease. Interestingly, a similar length of time post-infection with attenuated EIAV is required for the development of maximum immune protection from virus exposure (Hammond et al., 1999; Li et al., 2003; Montelaro et al., 1996, 1998).

The role of specific humoral and cellular immune responses in mediating enduring protective immunity remains to be defined, but a combination of these immune factors is likely to function in a synergistic manner. In this regard, there is contradictory data on the potential role of neutralizing antibody responses in protective immunity. Virus-specific neutralizing antibody is typically not detected in experimentally infected equids until about 3 months post-infection, apparently precluding a role for neutralizing antibody in resolving acute viraemia and disease. However, neutralizing antibodies steadily increase in titre and breadth of neutralization specificity during the first year post-infection. Steady-state levels are reached concomitant with the achievement of sustained immune control of EIAV replication and disease observed in long-term inapparent carriers (Hammond et al., 1997; Howe et al., 2002; Rwambo et al., 1990a). Finally, the envelope variation observed during sequential disease episodes results in alterations in serum neutralization sensitivity, suggesting escape from critical antibody control (Howe et al., 2002; Leroux et al., 1997; Montelaro et al., 1984; Payne et al., 1987; Rwambo et al., 1990b). These observations indicate a dynamic interaction between evolving virus populations and host immune responses in which neutralizing antibodies can be a determinant of control or escape.

Antigenic variation during persistent EIAV infection has been correlated with alterations in the surface (SU) gp90 and transmembrane (TM) gp45 envelope proteins, including amino acid substitutions and deletions, and frequent alterations in potential N-linked glycosylation sites (Hussain et al., 1987; Leroux et al., 2001; Payne et al., 1987; Rwambo et al., 1990b; Zheng et al., 1997). While variation may occur throughout the envelope sequence, variation is predominantly localized to the gp90 protein. Studies of EIAV envelope variation have identified eight conserved and eight variable regions within the heavy glycosylated gp90. In addition, a principal neutralizing domain (PND), located in the hypervariable V3 segment of the SU, has been suggested based on the presence of two adjacent neutralizing epitopes, E<sub>nt</sub> and D<sub>nt</sub>. Another neutralizing epitope, C<sub>ant</sub>, has been identified in the V5 region of gp90 (Ball et al., 1992; Grund et al., 1996; Hussain et al., 1987, 1988; Leroux et al., 1997, 2001). We recently reported on detailed neutralization epitope mapping studies using reciprocal domain substitutions between neutralization sensitive and resistant EIAV envelopes (Howe et al., 2002). The results of these studies indicated the V3 and V4 domains as the predominant determinants of gp90 sensitivity or resistance to neutralization by immune serum from experimentally infected equids.

The present investigation expands on a previous study of the in vivo neutralization characteristics of an experimental infection-derived, in vitro neutralization-resistant virus isolate, EIAVPV564APND (Craigo et al., 2002; Leroux et al., 1997). To determine the effect of this PND deletion on envelope immunogenic properties and host immune control, two ponies were experimentally infected with an EIAV proviral construct containing the ΔPND envelope, EIAV<sub>APND</sub> (Craigo et al., 2002). Both experimentally infected ponies remained asymptomatic for EIA and experienced relatively low levels of plasma viral RNA during the 14-month observation period. In addition, both ponies produced high steady-state levels of EIAV envelope-specific antibodies, but developed only minimal neutralizing antibodies to the infecting EIAV<sub>APND</sub>. Our initial interpretation of these data was that the PND domain gp90 was required for the production of neutralizing antibodies during persistent infection, indicating that the deletion in V3 affected envelope immunogenicity as well as antigenicity. To assess the role of host immune responses in control of virus replication, both ponies were transiently immune suppressed with a 10-day dexamethasone treatment that culminated with both animals developing EIA associated with a 4-log increase in virus loads. In characterizing the virus-specific host immunity in response to the dexamethasone treatment, we unexpectedly observed that high-titre, strain-specific, neutralizing antibodies against EIAV<sub>APND</sub> developed post-immune suppression concomitant with a 100-fold reduction in steady-state plasma virus loads in the absence of significant gp90 amino acid variation. Post-immune suppression serum did not neutralize the parental EIAVPV<sub>564</sub>, indicating highly type-specific serum neutralization. While differing markedly in antibody neutralization phenotypes, EIAV<sub>APND</sub> and EIAVPV envelope amino acid sequences differ by only 1.8%, including a 14 aa deletion in the V3 domain and a shift in an N-linked glycosylation site in the V4 domain the EIAV<sub>APND</sub> gp90. Thus, these studies demonstrate that transient immune suppression and increased viraemia resulted in a modification of steady-state host immunity to the infecting virus and production of neutralizing antibody responses to a neutralization ‘resistant’ ΔPND envelope.
This current study was designed to elucidate the mechanism behind the marked change in host immunity by differentiating between two possible routes to altering serum antibody neutralization properties. First, the transient immune suppression and increased viraemia may have induced antibodies to new envelope epitopes outside the previously defined V3 and V4 neutralization domains. Alternatively, the development of serum neutralization to the EIAV<sub>APND</sub> could be attributed to quantitative or qualitative changes in antibody responses to the defined V3 and V4 neutralization domains. In the current study, we have used the unique combination of EIAV<sub>APND</sub> and EIAV<sub>PV</sub> envelopes that differ in neutralization sensitivity to post-immune suppression serum to distinguish between these alternative mechanisms of immune modulation.

**METHODS**

**Experimental infections, clinical evaluations, and virus and serum isolations.** Two outbred ponies (animals #599 and #672) were intravenously inoculated with $1 \times 10^7$ TCID<sub>50</sub> EIAV<sub>APND</sub> as described previously in Craigo et al. (2002). The clinical progression, virus evolution, plasma virus load and host immune responses of these experimentally infected ponies have been described previously (Craigo et al., 2002) and are summarized in Fig. 1. Selected immune serum samples taken from the experimentally infected ponies at regular intervals pre- and post-immune suppression were used in this study to define the neutralization phenotypes of the EIAV envelope variants.

**Construction of chimeric envelopes between neutralization sensitive and resistant EIAV envelope variants.** Based on the observed *in vitro* neutralization phenotypes defined by post-immune suppression serum with our reference EIAV<sub>PV</sub> and the variant envelope EIAV<sub>APND</sub> (Craigo et al., 2002), these gp90 envelope proteins were chosen as reference neutralization resistant and sensitive envelopes, respectively. To elucidate the gp90 neutralization determinants, reciprocal chimeric envelopes exchanging defined variable domains were constructed and tested for their neutralization properties against the panel of reference immune serum from both ponies #599 and #672 on day 443 (post-immune suppression). To generate the desired mutations in the V3 and/or V4, and C6 regions, internal primers containing overlapping sequence mutations were used with external primers flanking the Blpl and BstXI restriction enzyme sites. The PCR was performed by using the Expand High Fidelity PCR system (Roche), 0.025 mM each deoxynucleoside triphosphate, 0.1 mM of each primer and 1–5 µl of original template or purified PCR fragments in a final volume of 100 µl. The following conditions were used: 4 min at 95°C, after which 0.4 µl of Expand High Fidelity enzyme was added; 1 min at 95°C, 1.5 min at 50°C and 1 min at 72°C for 35 cycles; 10 min at 72°C for one cycle. The

Fig. 1. Clinical and serological profiles of ponies experimentally infected with EIAV<sub>APND</sub>. Ponies were experimentally infected with $10^7$ TCID<sub>50</sub> of the molecular clone EIAV<sub>APND</sub>. The individual clinical (a and c) and envelope-specific antibody responses (b and d) for ponies #599 and #672 are shown from day 380 to day 470 post-infection. The period of immune suppression is indicated with a shaded box (DEX) and the resulting febrile episodes are identified with an arrow. (a and c) Summary of rectal temperature (---, right y axis) and platelet count (—, first left y axis), and virus load (copies of viral genomic RNA molecules per millilitre of plasma; †, second left y axis). (b and d) Summary of EIAV-specific end-point titres (■) (right y axis) and the 50% serum neutralization titres for either EIAV<sub>APND</sub> (▲) or EIAV<sub>PV</sub> (●) (left y axis). (a–b) Pony #599 and (c–d) pony #672. Adapted from Craigo et al. (2002).
resulting 1·6 kb env fragments were digested with BglI and BstXI prior to ligation with T4 ligase (NEB) into the EIAV<sub>UK</sub> genome (GenBank accession no. AF016316) with the corresponding 1·6 kb fragment removed. The ligation products were used to transform competent E. coli DH5α cells (Invitrogen). Clones from each of the new chimera constructs were screened by BamHI restriction enzyme digests for the presence of the insert and sequenced as described previously (Leroux et al., 1997) to confirm the correct envelope sequence. The in vitro replication properties of each of the variable region chimeras were then assessed by individually transfecting a 4 μg sample of purified plasmid DNA from each of the resulting env variant proviral clones into 10<sup>6</sup> fetal equine kidney (FEK) cells following the manufacturer’s directions for the GenePorter Transfection kit (GTS). Virus production was monitored every 5 days by measurements of the reverse transcriptase activity in the supernatants of the transfected cells using a standard micro-RT assay (Leroux et al., 1997). The TCID<sub>50</sub> values of supernatants from transfected FEK cell cultures were then determined in a standardized infectious centre assay in FEK cells that uses a cell-based ELISA detection system (Hammond et al., 1997).

**Serum antibody neutralization assays.** The level of neutralization activity of the panel of reference immune serum from the experimentally infected ponies against the variant and chimeric envelope proviruses was determined using a standard viral infectious centre assay, as described previously (Hammond et al., 1997). Briefly, 10<sup>5</sup> FEK cells were added into a 24-well tissue culture plate and allowed to adhere overnight at 37 °C. All immune serum samples were heat inactivated before use in the assay. Twofold serial dilutions of each of the serum samples were incubated in the presence of 100 infectious units of the selected chimeric virus at 37 °C for 1 h. The serum–virus mixture was then added to the cells and incubated overnight at 37 °C. An overlay of 0·8 % carboxymethylcellulose was added to the infected cultures and incubated for a further 7 days at 37 °C. The cells were then fixed and permeabilized. Reference immune serum from an EIAV-infected horse (Lady) was used as a primary antibody, followed by an affinity-purified, horseradish peroxidase-conjugated, goat anti-horse immunoglobulin G (Sigma). The peroxidase substrate 3-amino-9-ethyl-carbazole (Sigma) in a sodium acetate buffer (pH 5·5) supplemented with H<sub>2</sub>O<sub>2</sub>, was used to visualize the EIAV infectious centres. The number of infectious centres was counted, and the 50 % reciprocal neutralization titre of each serum sample was determined by linear regression analysis. Titres below 1 : 20 are considered background as determined with uninfected control sera. Each neutralization assay was repeated at least twice to determine standard error values. Neutralization titres were compared using paired <i>t</i>-test analyses to determine statistical significance.

**RESULTS**

**Clinical and serological profiles pre- and post-immune suppression**

The sources of virus and immune serum used in these studies were two mixed-breed ponies (#599 and #672) intravenously infected with the previously described replication competent, neutralization resistant EIAV<sub>PND</sub> as reported in detail by Craigo et al. (2002). Over an observation period of 410 days, both EIAV<sub>PND</sub>-infected animals remained asymptomatic for EIA, reflecting relatively low steady-state levels of plasma RNA (Fig. 1a, c). Beginning on day 420 post-infection, both ponies were transiently immune suppressed for 10 days with dexamethasone producing a febrile episode with corresponding drop in platelets and associated increase in viraemia (Fig. 1a, c). Clinical signs were resolved by day 434 post-infection, and plasma viral RNA copies declined to steady-state levels 100-fold lower than those observed before immune suppression (Fig. 1a, c).

Prior to immune suppression, both animals had developed a high EIAV envelope-specific antibody end-point titre averaging 1 × 10<sup>6</sup>, which increased to a titre of 1 × 10<sup>7</sup> post-immune suppression (Fig. 1b, d). EIAV<sub>PND</sub>-envelope-specific 50 % neutralizing antibody titres were around 1 : 50 in both animals prior to immune suppression. However, after immune suppression and resulting fever, envelope-specific neutralizing antibodies were detected against the infecting EIAV<sub>PND</sub> strain with titres of 1 : 275 in pony #599 and 1 : 550 in pony #672. Sequence analysis pre- and post-immune suppression revealed 3–4 % variation within the gp90 consistent with previously observed evolution rates and the retention of the V3 deletion. Interestingly, at no time before or after dexamethasone treatment did either animal develop serum antibodies capable of neutralizing the parental EIAV<sub>PV</sub> strain (Fig. 1b, d).

This lack of envelope-specific neutralizing antibody against EIAV<sub>PV</sub> and abundant neutralizing antibody against EIAV<sub>PND</sub> was unexpected due to the low level of gp90 variation observed between these two viruses (Fig. 2). The few areas of sequence variation between the two viral envelopes were localized to the V3, V4 and C6 regions of the envelope gp90. The V3 region contained the most extensive variation between the two viral envelopes including the 14 aa deletion of the PND En<sub>I</sub> epitope in the

![Fig. 2. Comparison of deduced amino acid gp90 variable region sequences of EIAV<sub>PV</sub> and EIAV<sub>PND</sub>. The variable regions (V1–V6) and conserved region C6 of the gp90 are summarised. Bold letters within the EIAV<sub>PND</sub> sequence indicate sequence variation from EIAV<sub>PV</sub>. Dashes (-) indicate amino acid deletions and underlined amino acids indicate potential N-glycosylation sites (NXS/T). Shaded bars indicate the previously defined neutralizing epitopes En<sub>I</sub>, D<sub>I</sub> and C<sub>I</sub>. Adapted from Craigo et al. (2002).](image-url)
EIAVAPND. Other minor variations observed in the EIAVAPND compared to the EIAVPV envelope involved the shifting and addition of potential N-linked glycosylation sites in the V4 and C6 domains, respectively. These variations in EIAVAPND from its parent EIAVPV were retained after immune suppression and resolution of disease (Craigo et al., 2002). Thus, these studies provided a novel panel of envelope variants and immune serum to examine the basis for the development of neutralizing antibodies to the EIAVAPND envelope after transient immune suppression of the inapparent carriers.

**EIAV gp90 V3 and V4 as determinants of neutralization sensitivity**

Previous studies with EIAV variant envelopes differing in neutralization phenotypes indicate that the V3 and V4 regions of the envelope can confer neutralization specificity either individually or in concert with each other (Howe et al., 2002). Thus, considering that two of the sites of variation between EIAVAPND and EIAVPV were within the V3 and V4 domains, we first sought to examine if the serum neutralizing antibodies induced by transient immune suppression were specific for the V3 or V4 domains of gp90. Towards this objective, a panel of chimeric viruses was constructed containing variable region exchanges of either the V3 domain, V4 domain or a combination of the V3 and V4 domains between the neutralization resistant EIAVPV envelope backbone (gp90PV) or in the neutralization sensitive EIAVAPND envelope backbone (gp90APND) (Fig. 3a). Characterization of the replication properties of the panel of chimeric envelope proviruses with substituted variable domains (Fig. 3b) demonstrated that all of the constructs containing substitutions of the EIAVAPND envelope V3 and V4 domains, singly or in combination, into the resistant EIAVPV backbone displayed replication properties similar to the parental viruses. In contrast, the reverse chimeric viruses containing the individual EIAVPV envelope V3 or V4 domains substituted into the EIAVAPND backbone were replication defective, whereas the double V3V4 chimera was replication competent. These results support previous findings indicating the requirement for a functional compatibility between the envelope V3 and V4 regions to support virus replication (Chen et al., 2001; Howe et al., 2002). Thus, the replication competent chimeric envelope proviruses were used to assess the sensitivity of the respective V3 and V4 domains as targets for serum neutralizing antibodies produced by transient immune suppression.

The neutralization phenotypes of the variable region exchange chimeras and the two parental envelope variants were examined with serum obtained from the two experimentally infected ponies after immune suppression and resolution of disease and suppression of viraemia (day 443). Using our standard in vitro neutralization assay, the reciprocal 50% neutralization titre of the immune serum to each envelope construct was determined (Fig. 4). Immune serum samples from both experimentally infected ponies displayed a similar pattern of virus neutralization activity against the respective variable region chimeric viruses, indicating a common effect of immune suppression on host antibody responses. As a reference, immune serum samples taken after transient immune suppression (day 443) of the two ponies were shown to have mean neutralization titres of about 1:20 against the parental resistant EIAVPV envelope provirus (gp90PV) (Fig. 4). In contrast, the same post-immune suppression serum samples displayed substantial neutralization activity levels against the EIAVAPND envelope provirus (gp90APND) with a mean neutralization titre of about 1:400 (Fig. 4).

Substitution of the V3 domain from the neutralization sensitive EIAVAPND envelope into the EIAVPV backbone

![Fig. 3. Schematic representation of the V3 and V4 variable region exchange chimeric envelope constructs and replication kinetics.](http://vir.sgmjournals.org)
The most dramatic increase in neutralization sensitivity occurred when both the V3 and V4 variable regions of the neutralization sensitive EIAPN were substituted into the neutralization resistant EIAPV backbone [gp90PV (V3V4)] (Fig. 4). Immune serum from both ponies demonstrated a marked increase in their serum neutralizing antibody titres against the gp90PV (V3V4) to a mean of 1:8600, compared with the 1:10 neutralization titre observed with the parental envelope gp90PV, post-immune suppression. These data indicated a highly additive effect of the V3 and V4 domain substitutions in conferring serum neutralization sensitivity to the parental neutralization resistant EIAPV.

As a complement to the preceding analyses, we next evaluated the neutralization sensitivity of the replication competent reciprocal chimeric envelope virus in which the V3 and V4 domains of the neutralization resistant EIAPV envelope were substituted into the neutralization sensitive EIAPN envelope backbone (Fig. 3a, b). Neutralization analysis of this gp90APNDV3V4 proviral construct demonstrated a 10-fold reduction in serum neutralization sensitivity compared with the parental gp90APND envelope provirus. Thus, the 50 % neutralizing antibody titres for the post-immune suppression serum from the two ponies was calculated to be a mean titre of 1:20 against the gp90APNDV3V4 envelope provirus, compared with a mean titre of about 1:400 against the parental envelope gp90APND (Fig. 4). These data demonstrate that the gp90 V3 and V4 domains are the predominant determinants of neutralization resistance to the post-immune suppression serum.

Taken together, the preceding combination of experiments evaluating changes in neutralization sensitivity and resistance, respectively, indicate that the V3 and V4 domains are the predominant determinants of neutralization sensitivity in post-immune suppression serum. These results suggest that the development of serum neutralizing antibodies by transient immune suppression was associated with changes in the qualitative or quantitative host antibody responses to the gp90 V3 and V4 domain and not to changes in the envelope determinants targeted by serum antibodies.

**Evaluation of C6 glycosylation variation on neutralization sensitivity**

The V4 region encompasses 7 aa that encode a single potential N-linked glycosylation site that is shifted only by two residues in the neutralization sensitive envelope gp90APND compared with the gp90PV (Fig. 2). Since we observed a significant change in neutralization sensitivity based on this defined glycosylation variation, we next evaluated the effect of other variations in potential N-linked glycosylation sites within the gp90 on neutralization specificity. One of the few envelope variations observed linked glycosylation sites within the gp90 on neutralization sensitivity, respectively, indicate that the V3 and V4 domains are the predominant determinants of neutralization sensitivity, as described in Methods. The 50 % neutralizing antibody titres were determined for day 443 (15 days post-immune suppression) (solid bars) post-infection for each of the replication competent variable region exchange chimeric viruses. Each experiment was run in duplicate and repeated twice. Asterisk (*) indicates the neutralizing antibody titres that are significantly different (P ≤ 0.05) from the parental backbone at that specific time point. (a) Pony #599, (b) pony #672.

![Fig. 4. Serum neutralization properties of the V3 and V4 variable region chimeric envelope proviral constructs. Post-immune suppression serum samples were selected from each pony to test the effect of exchanging specific variable regions on in vitro neutralization phenotypes as described in Methods. The 50 % neutralizing antibody titres were determined for day 443 (15 days post-immune suppression) (solid bars) post-infection for each of the replication competent variable region exchange chimeric viruses. Each experiment was run in duplicate and repeated twice. Asterisk (*) indicates the neutralizing antibody titres that are significantly different (P ≤ 0.05) from the parental backbone at that specific time point. (a) Pony #599, (b) pony #672.](image-url)
EIAV<sub>APND</sub> envelope that is not present in the C6 domain of the EIAV<sub>PV</sub> envelope (Fig. 2). To address the effect of the additional glycosylation site within the C6 domain on serum neutralization phenotype, a panel of conserved C6 domain exchange chimeras was constructed (Fig. 5a). The C6 region of the neutralization sensitive EIAV<sub>APND</sub> was substituted into the parental neutralization resistant EIAV<sub>PV</sub> [gp90<sub>PV</sub>(C6)] and into the highly neutralization sensitive gp90<sub>PV</sub> (V3V4) provirus construct [gp90<sub>PV</sub>(V3V4C6)]. Reciprocal C6 exchanges from the neutralization resistant EIAV<sub>PV</sub> were also substituted into the neutralization sensitive EIAV<sub>APND</sub> backbone [gp90<sub>APND</sub>-C6] and into the engineered resistant gp90<sub>APND</sub>(V3V4) construct [gp90<sub>APND</sub>(V3V4C6)]. All of the new C6 exchange chimeras were replication competent (Fig. 5b). In general, each of the chimeras had similar replication kinetics and similar RT levels by 30 days post-transfection.

The neutralization properties of the two parental envelope variants and each of the C6 chimeric envelope viruses were determined using the reference post-immune suppression serum samples from both ponies, as described above. Using our standard in vitro neutralization assay, the reciprocal 50% neutralization titre of each envelope construct was determined (Fig. 6). The data demonstrated that substitution of the neutralization sensitive EIAV<sub>APND</sub> C6 domain into the EIAV<sub>PV</sub> backbone did not increase the sensitivity of the latter envelope to neutralization by the post-immune suppression serum (Fig. 6a). Similarly, substitution of the C6, V3 and V4 domains from the neutralization sensitive EIAV<sub>APND</sub> into the resistant EIAV<sub>PV</sub> backbone, [gp90<sub>PV</sub>(V3V4C6)], failed to increase the neutralization sensitivity over the levels seen with V3V4 substitution alone (Fig. 6a). These observations indicate that the C6 is not a major determinant of envelope neutralization sensitivity to post-immune suppression serum antibodies.

![Fig. 5. Schematic representation of the C6 region exchange chimeric envelope constructs and replication kinetics.](http://vir.sgmjournals.org)

(a) Neutralization resistant envelope EIAV<sub>PV</sub> (open bars) and neutralization sensitive envelope EIAV<sub>APND</sub> (solid bars) were used as reference envelope species for the variable region exchanges. The names of the new variable region exchange chimeric viruses are listed at the left of each construct, starting with the name of the backbone followed by the exchanged variable region in parentheses. The gp90 envelope variable regions V1–V8 are schematically identified above the bars. (b) Equal amounts of DNA of each of the chimeric envelope constructs, depicted in Fig. 4, were individually transfected into FIK cells in duplicate. Supernatants from each transfected culture were collected at regular intervals over a 30-day period. The levels of RT present in the samples at each time point were measured in a micro-RT assay as described in Methods.

![Fig. 6. Serum neutralization properties of the C6 region chimeric envelope proviral constructs.](http://vir.sgmjournals.org)

(a) Pony #599. (b) Pony #672.
The exchange of the non-glycosylated C6 region from the neutralization resistant EIAV<sub>PV</sub> into the neutralization sensitive EIAV<sub>APND</sub> backbone [gp90<sub>APND(C6)</sub>] demonstrated less consistent results between animals than previously seen with the V3 and V4 region exchanges (Fig. 6b). The serum neutralization titres observed with pony #599 immune serum against gp90<sub>APND(C6)</sub> increased from 1 : 275 to 1 : 4000 post-immune suppression (Fig. 6a), indicating an unexpected increase in serum neutralization sensitivity. In contrast, the serum neutralization titres of pony #672 against gp90<sub>APND(C6)</sub> appeared to decrease slightly to 1 : 440 compared with the titre of 1 : 525 observed with the parental gp90<sub>APND</sub> (Fig. 6b). However, substitution of the same gp90<sub>APND</sub> C6 into the engineered gp90<sub>APND(V3V4)</sub> chimera failed to alter the neutralization properties of either pony serum relative to the parental gp90<sub>APND</sub> provirus; serum neutralization titres for both proviral envelopes were approximately 1 : 15 (Fig. 6a, b). Thus, the data presented in Fig. 6(a), excluding the pony #599 serum reactivity to gp90<sub>APND(C6)</sub> envelope, are consistent with the idea that the variant glycosylation in C6 domain is not a major determinant of envelope neutralization properties in this situation. The reason for the unexpected but unique serum neutralization reactivity of the serum from pony #599 to the C6 domain is unclear. However, this variation may reflect the outbred nature of these ponies and the intrinsic differences in host immunity.

**DISCUSSION**

The primary goal of this study was to determine the mechanisms leading to the production of highly type-specific neutralizing antibodies by transient immune suppression of ponies experimentally infected with a previously defined neutralization resistant EIAV<sub>APND</sub> strain. Two alternative models were considered for the induction of neutralizing antibodies by the transient immune suppression. The first model proposed that the transient immune suppression resulted in qualitative or quantitative changes in host antibody responses to previously defined predominant neutralization determinants in the V3 and V4 domains of the viral envelope. The second model proposed that the transient immune suppression resulted in a redirection of host antibody responses to new envelope determinants outside the principal neutralizing domain. The results presented here clearly indicate that the gp90 V3 and V4 domains are the predominant determinants for neutralization sensitivity by post-immune suppression serum. Thus, these observations support the concept that transient immune suppression and the associated wave of viraemia and antigenic stimulation can modify quantitative and qualitative properties of host antibody responses leading to an enhancement of serum neutralizing antibodies. In this regard, it is interesting to note that the transient immune suppression of these experimentally infected ponies resulted in a substantial reduction in steady-state virus replication levels as immune responses recovered from the dexamethasone treatment (cf. Fig. 1), perhaps in part due to the development of serum neutralizing antibodies.

Montefiori and colleagues have reported a similar enhancement of serum neutralizing antibodies to HIV-1 in patients subjected to structured interrupted antiviral drug therapy regimens (Montefiori et al., 2001; Ortiz et al., 1999, 2001), suggesting a general role for transient waves of virus replication in boosting host immune control of persistent virus infection. However, the basis for the increase in serum neutralization to HIV-1 was undefined. While most lentivirus infections are associated with progressively degenerative diseases, the episodic nature and eventual immune control of EIAV replication and disease are unique among lentiviruses. Based on the observations presented here it is interesting to speculate that the enduring natural immunologic control of virus replication and disease achieved in horses infected with EIAV may in part be due to the discrete waves of viraemia characteristic of this persistent infection and their ability to boost host immunity.

The exact mechanisms by which transient waves of viraemia, naturally or experimentally induced, can markedly modify the specificity of host antibody responses to lentivirus infections remains to be elucidated. However, this immune modulation appears to be related to a ‘boosting’ effect of the increased virus antigen presentation during the viraemia that cannot be accomplished by a steady-state virus-immune system status. Based on this model, one can postulate that the transient immune suppression and subsequent viraemia modify the existing viral envelope-specific antibody responses by boosting memory immune responses to critical viral envelope determinants. In the case of EIAV, the development of neutralizing antibody responses after transient immune suppression clearly correlates with changes in antibody targeted to the V3 and V4 domains of the gp90 envelope protein. It cannot be concluded from the current data whether this alteration is due only to quantitative increases in antibody levels to these principal neutralizing domains or also to qualitative changes in antibody population. Envelope-specific antibody titres increased only slightly in response to the dexamethasone treatment, while neutralizing antibody titres to the infecting EIAV<sub>APND</sub> increased from background levels (<1 : 20) to a mean of about 1 : 400. Thus, these data are consistent with the concept of substantial qualitative changes in envelope-specific antibodies in the absence of significant changes in the quantitative levels of envelope-specific antibodies.

The studies described here also elucidate further the antigenic architecture of the EIAV envelope glycoprotein and define in more detail envelope antibody neutralization determinants and the effects of natural variation on neutralization sensitivity. We previously identified the EIAV gp90 V3 domain as a principal neutralizing domain using synthetic peptide mapping of neutralizing monoclonal antibodies (Ball et al., 1992). These studies defined two adjacent neutralizing monoclonal antibody binding sites.
(D_{nt} and E_{nt}) in the V3 domain loop of gp90 (Fig. 2). Subsequent comparisons of natural variant EIAV envelopes differing in serum neutralization sensitivity confirmed the role of the V3 domain and identified the relatively small V4 domain as a second predominant neutralizing domain (Howe et al., 2002). Despite the small number of animals presented here, the current studies reconﬁrm the role of the gp90 V3 and V4 domains as principal neutralization determinants of the EIAV envelope and their ability individually or in combination to confer neutralization sensitivity when substituted into a resistant envelope (e.g. EIAV_{PV}). From the analyses of the EIAV_{APND} envelope neutralization determinants, it was demonstrated that the D_{nt} epitope of the V3 domain, in the absence of the adjacent E_{nt} epitope, can serve as an eﬀective target for serum neutralization. The characterization of the EIAV_{APND} envelope also highlighted the role of the potential N-linked glycosylation site in the 7-residue V4 domain in determining neutralization sensitivity, with absolute resistance or sensitivity being dictated by a shift in the glycosylation site by only 2 aa. While the V4 glycosylation site location was shown to be a major determinant of EIAV neutralization properties, the current data indicated that the diﬀerences in the number of N-linked glycosylation sites in the gp90 C6 domain in general did not aﬀect envelope neutralization sensitivity. While all variations in envelope glycosylation may not be related to antigenic variation, the role of lentivirus envelope glycosylation variation in deﬁning envelope antigenic and immunogenic properties is becoming increasingly evident (Back et al., 1994; Cheng-Mayer et al., 1999; Johnson & Desrosiers, 2002; Lue et al., 2002; Ly & Stamatos, 2000; Malenbaum et al., 2000; Polzer et al., 2002; Quinones-Koch et al., 2002). Our current working model to explain the interaction of the V3 and V4 domains as neutralization determinants is that the V3 domain is the actual target for neutralizing antibodies and that the V4 glycosylation site aﬀects the accessibility of these V3 sequences to antibody neutralization.

Taken together, this series of studies indicates that the V3 and V4 domains are predominant targets for neutralizing antibody responses, but that the ability of the immune system to respond to these targets is apparently inﬂuenced by the overall context of antigen presentation, particularly the V3–V4 domain interactions. For example, neutralizing antibodies are routinely produced in equids experimentally infected with EIAV_{PV}, but these neutralizing antibodies fail to inactivate the EIAV_{APND} envelope (Howe et al., 2002; Leroux et al., 1997). Conversely, the neutralizing antibodies elicited by transient immune suppression of ponies experimentally infected with EIAV_{APND} failed to inactivate the EIAV_{PV} envelope (Craigo et al., 2002). These observations suggest that the initial exposure of the EIAV envelope to the host immune system may restrict the speciﬁcity of neutralizing antibody responses to the highly variable envelope domains, similar to the original antigenic sin described for other virus and bacterial infections (Fazekas & Webster, 1966; Good et al., 1993; Klenerman & Zinkernagel, 1998; Mongkolsapaya et al., 2003; Tsuchiya et al., 2000). Studies in HIV have suggested that the initial antibody response to the immunodominant epitopes of the hypervariable V3 region may impede the future responses to emerging variants, thus provided the virus with a window of opportunity to escape immune surveillance (Kohler et al., 1994; Locher et al., 1999). Overcoming original antigenic sin for vaccine development is not a new problem and several other viral vaccines, such as dengue haemorrhagic fever, inﬂuenza, malaria and Lymphocytic choriomeningitis virus have been impeded by this immune restriction (Fazekas & Webster, 1966; Good et al., 1993; Klenerman & Zinkernagel, 1998; Mongkolsapaya et al., 2003; Tsuchiya et al., 2000) and continue to be an ongoing question for HIV vaccines (Kundu et al., 1998; Nara & Garrity, 1998; Singh et al., 2002; Verschoor et al., 1999).

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REFERENCES


